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Loss of protein tyrosine phosphatase, non-receptor type 2 is associated with activation of AKT and tamoxifen resistance in breast cancer

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Abstract

Breast cancer is a heterogeneous disease and new clinical markers are needed to individualise disease management and therapy further. Alterations in the PI3K/AKT pathway, mainly *PIK3CA* mutations, have been shown frequently especially in the luminal breast cancer subtypes, suggesting a cross-talk between ER and PI3K/AKT. Aberrant PI3K/AKT signalling has been connected to poor response to anti-oestrogen therapies. *In vitro* studies have shown protein tyrosine phosphatase, non-receptor type 2 (PTPN2) as a previously unknown negative regulator of the PI3K/AKT pathway. Here, we evaluate possible genomic alterations in the *PTPN2* gene and its potential as a new prognostic and treatment predictive marker for endocrine therapy benefit in breast cancer.

PTPN2 gene copy number was assessed by real-time PCR in 215 tumour samples from a treatment randomised study consisting of postmenopausal patients diagnosed with stage II breast cancer 1976-1990. Corresponding mRNA expression levels of PTPN2 were evaluated in 86 available samples by the same methodology.

Gene copy loss of *PTPN2* was detected in 16% (34/215) of the tumours and this was significantly correlated with lower levels of PTPN2 mRNA. *PTPN2* gene loss and lower mRNA levels were associated with activation of AKT and a poor prognosis. Furthermore, *PTPN2* gene loss was a significant predictive marker of poor benefit from tamoxifen treatment.

In conclusion, *g*enomic loss of PTPN2 may be a previously unknown mechanism of PI3K/AKT upregulation in breast cancer. PTPN2 status is a potential new clinical marker of endocrine treatment benefit which could guide further individualised therapies in breast cancer.

Keywords: 18p, AKT, breast cancer, endocrine resistance, phosphatases, PTPN2

Abbreviations

v-AKT murine thymoma viral oncogene homologue (AKT), amyloid precursor protein (APP), breast cancer specific survival (BCS), cyclophosphamide-methotrexate-5-fluorouracil (CMF), distant recurrence-free survival (DRFS), epidermal growth factor receptor (EGFR), oestrogen-receptor (ER), human epidermal growth factor receptor 2 (HER2), insulin receptor (IR), Janus kinase/signal transducers and activators of transcription (JAK/STAT), phosphatidyl inositol 3-kinase (PI3K), phosphatase and tensin homologue (PTEN), protein tyrosine phosphatase, non-receptor type 2 (PTPN2), radiotherapy (RT)

Introduction

The survival rate of breast cancer is continuously increasing as a result of earlier detection and improved treatment strategies. However, in the light of a constant increase in disease incidence, breast cancer still constitutes one of the most common causes of pre-term death among women in developed countries. The high heterogeneity of breast tumours results in a need for identification of further clinically useful markers of prognosis and treatment prediction, in order to develop more individualised therapies.

The phosphatidyl inositol 3-kinase/protein kinase B/v-AKT murine thymoma viral oncogene homologue (PI3K/AKT) growth signalling pathway is essential for normal cellular homeostasis, regulating cell survival, proliferation and differentiation in response to growth factors and hormones [1]. In malignant cells, this pathway is commonly overstimulated and genetic and epigenetic alterations of the PI3K/AKT pathway are suggested as one driving cause of tumour development and progression [1,2]. In breast cancer, common aberrations include mutations in the *PIK3CA* and *AKT* genes, overexpression or activation of several upstream growth factors and receptor tyrosine kinases as well as loss of function of negative regulators, e.g., the phosphatase and tensin homologue (PTEN) [2,3]. Enhanced activation of the PI3K/AKT pathway has been suggested as a useful prognostic and treatment predictive factor in breast cancer. Cross-talk between PI3K/AKT and oestrogen-receptor (ER) signalling pathways has been associated with poor response to anti-oestrogen therapy [4].

The protein tyrosine phosphatase, non-receptor type 2 (PTPN2) regulates receptor tyrosine kinase signalling, through dephosphorylation of tyrosine residues thereby preventing downstream activation of intracellular pathways, among others the PI3K/AKT pathway [5,6]. The gene encoding PTPN2 is located in the chromosomal region 18p. Loss of 18p in breast tumours has previously been related to poor outcome [7] and an association between 18p deletion and decreased levels of *PTPN2* mRNA has been reported [8].It may be hypothesised that loss of 18p in breast cancer could lead to decreased levels of PTPN2 and be one previously unexplored mechanism behind PI3K/AKT pathway overstimulation in breast tumours. Consequently, the aim of this study was to evaluate *PTPN2* loss in a large breast cancer patient material, its possible associations to AKT-activation and its potential as a new marker of prognosis and therapy resistance in breast cancer.

Materials and methods

Patients

The cohort used in the present study was derived from a large, randomised trial conducted by the Stockholm breast cancer group between November 1976 and April 1990, mainly aiming to evaluate the benefit from adjuvant tamoxifen treatment. The patients were randomised using a 2 x 2 factorial study design to receive tamoxifen for two years, or no endocrine treatment and furthermore to post-operative radiotherapy (RT) or cyclophosphamide-methotrexate-5-fluorouracil (CMF)-based chemotherapy. Most of the patients in the tamoxifen arm, if disease-free after two years, were then randomised to receive tamoxifen for three more years, or no further adjuvant treatment. Detailed information about study design and long-term follow-up data have been previously reported in detail [9,10]. Median follow-up time was 11 years. All patients were post-menopausal and had positive lymph nodes and/or a tumour diameter exceeding 30 mm. Patient flow through the studies is presented in **Figure 1**. For *PTPN2* DNA analysis, 215 tumour samples were available, whereas *PTPN2* mRNA levels could be estimated in 86 samples. Retrospective studies of biomarkers were approved by the local ethics board at the Karolinska Institute, Stockholm, Sweden, and at the time, no further consent from the patients was needed. The present study was designed and presented with regard to the reporting recommendations for tumor marker prognostic studies (REMARK) guidelines [11].

DNA and mRNA extraction

DNA and total RNA were extracted from fresh-frozen tumour tissue estimated to contain >50% cancer cells, as well as from cell lines. DNA preparation has been described previously [12]. Purified DNA was dissolved in sterile water and DNA concentration was measured using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). For subsequent analyses, the DNA was long term stored in -70°C.

For RNA preparations, fresh-frozen tumour tissue was homogenised with a microdismembrator (B. Braun Biotech, Germany) or a TissueLyser (Qiagen, Hilden, Germany) and total RNA was isolated with the mirVana^{TM} miRNA isolation kit (Ambion, Austin, TX, USA) according to instructions provided by the manufacturer. Purified RNA was dissolved in nuclease-free water with addition of RNAsin ribonuclease inhibitor (Promega, Madison, WI, USA) and stored at -70°C. RNA integrity numbers (RIN) and concentrations were assessed

with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Only samples with RIN values ≥ 5 were included in the analysis.

Real-time PCR and microarray

PTPN2 DNA or mRNA quantity of each sample was estimated by fast real-time PCR using an ABI Prism 7900ht (Applied Biosystems, Foster City, CA, USA). The default thermal conditions: 95°C for 20 s; 40 cycles of 95°C for 1 s, and 60°C for 20 s were used for all reactions. For gene quantification, 20 ng total DNA was added to a 10 µL reaction with 1x Taq Man Fast Universal PCR master mix (Applied Biosystems, Foster City, CA, USA) and 0.1 µM primer and probe for PTPN2 or the endogenous control Amyloid Precursor Protein *(APP)*. *APP* served as endogenous control since no amplifications or deletions have been reported in breast cancer [13]. Primers and probes were designed using the software Primer Express version 1.5a (Applied Biosystems, Foster City, CA, USA). *PTPN2* probes were attached to FAM and a non-fluorescent quencher (Applied Biosystems, Foster City, CA, USA) and the *APP* probe was coupled to the dye FAM and the quencher TAMRA (Sigma-Aldrich, St.Louis, MO, USA). Primers and probes sequences were as follows:

PTPN2 forward primer: 5´-AAGCCCACTCCGGAAACTAAA- 3´, reverse primer: 5´-AAACAAACAACTGTGAGGCAATCTA-3´, probe: 5´-TGAGGCTCGCTAACC-3´,

APP forward primer: 5´-TTTGTGTGCTCTCCCAGGTCT-3´, reverse prime: 5´-TGGTCACTGGTTGGTTGGC-3´, probe 5´-CCCTGAACTGCAGATCACCAATGTGGTAG-3´

PTPN2 gene quantification was performed with the Comparative Ct method using DNA from the cell line MCF7 as the calibrator sample on each plate.

Reverse transcription of RNA was performed using the high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) with 200 ng total RNA in reactions of 20 µL according to manufacturer's instructions. TaqMan assays (Applied Biosystems, Foster City, CA, USA) for PTPN2 (Hs00959886_g1), and the endogenous controls MRLP19 (Hs00608519_m1) and PPIA (Hs99999901_m1) were used according to the manufacturer's instructions. Quantitative PCR was performed in duplicates with 10 µL reaction volume, in 1x TaqMan fast universal master mix (Applied Biosystems Foster City, CA, USA). To confirm specificity, reactions without reverse transcriptase (-RT), as well as no template

controls (NTC) were included on each plate. Relative PTPN2 mRNA expression levels were calculated with the Comparative Ct method, using BT-474 cDNA as the calibrator. The mean value was taken from the duplicates and for the two endogenous controls, an average value for each sample was used. For correlation analyses, expression levels of the genes were divided into four groups based on the quartiles. In the survival analyses, quartiles 1-3 (Q1-Q3) were considered as low expression and the upper quartile (Q4) as high expression.

Deletions at 18p were estimated in 25 of the samples using Affymetrix SNP Gene Copy number arrays as previously described [14].

Evaluation of ER, HER2, PIK3CA and pAKT

ER expression was determined at the time of diagnosis, before 1988 this was done using isoelectric focusing and after this year, quantitative enzyme immunoassay (EIA) was used [9,10]. Cut-off level for ER positivity was 0.05 fmol/µg DNA. Isoelectric focusing/EIA and IHC data have been shown to be comparable [15]. Human epidermal growth factor receptor 2 (HER2) protein was quantified retrospectively by flow cytometry as described previously [16] and HER2 amplification was determined with quantitative Real-Time PCR [17]. S-phase fraction (SPF) was previously determined by flow cytometry [18], and mutations in *PIK3CA* exon 9 or 20 were assessed by single-strand conformational polymorphism (SSCP) and sequencing [19]. Levels of AKT phosphorylated at S473 (pAKT) were estimated by immunohistochemistry [20]. Breast cancer subtypes were defined according to St Gallen 2011 [21] as follows: Luminal A (ER+, HER2-, SPF<12%), Luminal B1 (ER+, HER2-, SPF≥12%), Luminal B2 (ER+, HER2+), HER2-like (ER-, HER2+), and Triple-negative (ER-, HER2-), where HER2 status was defined by its gene copy number.

Statistical analysis

For estimation of relationships between different variables in two groups, Chi2 tests were used. The Kaplan-Meier product limit method was used to estimate the cumulative probabilities of distant recurrence-free survival (DRFS) or breast cancer specific survival (BCS). Differences between the curves were evaluated with the Log-rank test. For univariate and multivariate analysis of event rates, as well as interaction analysis, Cox proportional hazard regression was

used. All statistical analyses were performed with Statistica 12.0 (Statsoft, Inc, Tulsa, OK, USA) and $p<0.05$ was considered statistically significant.

Results

PTPN2 gene loss in breast cancer and decreased expression of corresponding mRNA.

PTPN2 gene copy number was assessed with real-time PCR in 215 breast tumours. Loss of the *PTPN2* gene could be detected in 16% (34/215) of the tumours. In 13% (28/215) of the samples, gain of the *PTPN2* gene, defined as ≥3 gene copies, was found. *PTPN2* gene deletion could be verified by whole genome arrays were data was available in 25 of the 215 tumours (p=0.026).

Real-time PCR was used to study PTPN2 mRNA expression levels in 86 available tumours. There was a significant correlation between *PTPN2* gene copy number loss and low mRNA levels (p=0.038, **Table 1**).

PTPN2 loss and associations with clinicopathological data and pAKT.

For *PTPN2* gene copy number, no significant correlations were found to any clinicopathological data (**Table 1**). Since PTPN2 has been shown as a negative regulator of PI3K/AKT signalling *in vitro*, loss of *PTPN2* was tested against pAKT levels. No significant correlation could be found in the total cohort, however in the Luminal A subgroup *PTPN2* gene copy number loss was associated with strong pAKT (P=0.040, **Table 1**).

Similarly, no correlations between PTPN2 mRNA and clinicopathological data were found (**Table 2**). However, low levels of PTPN2 mRNA were associated with strong pAKT (P=0.023) and absence of mutations in *PIK3CA* (P=0.013) (**Table 2**). The correlation to pAKT was even more apparent in the group of patients with tumours having upstream pathway activation defined by HER2+ and/or *PIK3CA* mutation **(Table 2** and **Figure 2).**

PTPN2 gene loss and low mRNA-levels correlate to poor outcome

Gene copy loss of *PTPN2* was associated with a poor outcome in terms of distant recurrence-free survival as well as breast cancer survival **(Figure 3 a, b).** This was especially evident in the Luminal A subgroup **(Figure 3 c, d).** The prognostic value of *PTPN2* loss could also be confirmed in a multivariate analysis including common clinicopathological factors **(Figure 3).**

Accordingly, low PTPN2 mRNA expression levels were associated with a poor prognosis and this was significant in a multivariate analysis **(Figure 4 a).** This was especially pronounced in the group of patients whose tumours harboured *HER2* amplification or *PIK3CA* mutations compared with tumours without these aberrations **(Figure 4 b, c).**

The patients with tumours showing gene copy gain of *PTPN2* had a similar clinical outcome as those with 2 gene copies (P=0.74 (DRFS), P=0.82 (BCS)).

Loss of PTPN2 is associated with tamoxifen resistance

Overactivation of the PI3K/AKT pathway has previously been connected with cross-talk to ER signalling and subsequent endocrine resistance [4]. In the present study, patients with ER-positive breast cancer and whose tumours had deletion of the *PTPN2* gene had poor benefit from tamoxifen (HR=1.90, 95% CI 0.73-4.96) **(Figure 5a)** compared with the group with normal *PTPN2* gene copy number (HR=0.48, 0.28-0.84) **(Figure 5b).** The interaction between tamoxifen treatment benefit and *PTPN2* genomic loss was significant (p=0.011).

Discussion

The present study suggests the phosphatase PTPN2 as a tumour suppressor gene and a new potential clinical marker in breast cancer. Loss of *PTPN2* may be a previously unexplored mechanism for upregulation of the PI3K/AKT pathway *in vivo*.

The post-translational modifications mediated by the balance between protein tyrosine kinases and protein tyrosine phosphatases (PTPs), regulate the activity of numerous essential biological processes. Consequently, an imbalance between these factors is suggested to be involved in several human diseases [22]. The protein phosphatase PTPN2 was first isolated in T-cells by Cool *et al.* in 1989 [23], thereby referred to as T-cell protein tyrosine phosphatase (TCPTP). Later, the gene was mapped to the chromosomal region 18p11.3-p11.2 [24,25]. Three splicing

variants of the gene have been reported, resulting in three different isoforms of the protein, differing in their C-termini [26]. The difference is assumed to determine substrate specificity as well as cellular locations of the proteins. PTPN2 is ubiquitously expressed and has been shown able to regulate the activity of several receptor tyrosine kinases, including the insulin receptor (IR), the epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptor (VEGFR) and the hepatocyte growth factor receptor (HGFR/Met) [6,27-31]. In normal intestinal epithelial cells, PTPN2 has been shown to regulate cytokine and chloride secretion through EGFR and TNFα-signalling [32,33]. Upregulation of PTPN2 and the 72% homologues phosphatase PTP1B has been associated with altered glucose homeostasis and development of diabetes through inhibition of IR signalling [34].

Loss of the 18p chromosomal region has been shown as an early event in breast cancer progression [35] and has been associated with a poor outcome [7]. Addou-Klouche *et al.* identified *PTPN2* as one candidate tumour suppressor gene in this region and showed a correlation between *PTPN2* gene loss and low mRNA expression levels [8]. In the present study, we confirm the correlation between PTPN2 gene deletion and low mRNA expression levels in a breast cancer material. In addition, this was associated with a poor outcome. Downregulation of PTPN2 has been previously shown in different types of cancer. In hepatocellular carcinoma, loss of PTPN2 was correlated with presence of lymph node metastasis [36]. PTPN2 loss through gene deletions or mutations have been reported in T-cell acute lymphoblastic leukaemia (T-ALL) and peripheral T-cell lymphoma not otherwise specified, and has been coupled to increased cytokine sensitivity and proliferation through stimulation of the Janus kinase/signal transducers and activators of transcription (JAK/STAT) -signalling pathways [37-39]. There are few functional studies conducted on PTPN2 in breast cancer cells, however, in triple-negative breast cancers PTPN2 downregulation and subsequent upregulation of SFK (Src family of protein tyrosine kinases) and STAT3-signalling was detected [40,41]. *In vitro* studies on other cell types, including glioblastoma cells, have shown PTPN2 as a negative regulator of receptor tyrosine kinases (RTKs), by dephosphorylating tyrosine residues [5,6,29]. Aberrant activation of RTKs is assumed to drive proliferation and growth in a large proportion of breast cancers. Therefore, a role for PTPN2 in this context is plausible.

In non-small cell lung cancer cells, inhibition of PTPN2 was shown to promote increased phosphorylation of AKT in the presence, but not in the absence of RAS-mutations [30]. Here, we show a correlation between low PTPN2 mRNA levels and high expression of pAKT, in

particular in the presence of upstream pathway stimulation by *HER2* amplification and/or *PIK3CA* mutations. In addition, *PTPN2* loss was associated with pAKT in the Luminal A subtype. Tumours of this subtype are in general hormone responsive with low proliferation rate and associated with a relatively good prognosis. Alterations in the PI3K/AKT pathway, mainly *PIK3CA* mutations, have been shown most frequent in the luminal subtypes, suggesting a cross-talk between ER and PI3K/AKT [3]. Aberrant PI3K/AKT signalling has been connected to poor response to anti-oestrogen therapies in several studies [20,42-50]. In the present study, we showed *PTPN2* gene loss as a new potential marker of endocrine resistance. This finding needs to be confirmed in independent cohorts and furthermore it would be interesting to investigate if it is also relevant for treatment with aromatase inhibitors. In the light of its role as a tumour suppressor, agonists activating PTPN2 are under development and have been shown to inhibit RTK signalling effectively *in vitro* [51]. One may suggest that PTPN2 agonists could be useful in combination with endocrine therapies in the group of patients described above.

Although a significant correlation was found between *PTPN2* gene loss and mRNA expression levels, the prognostic value of these aberrations was shown mainly in separate tumour subtypes. The involvement of several co-deleted genes in the 18p region may be one explanation. The presence of several PTPN2 isoforms could also obstruct the interpretation. Previous studies have suggested the nuclear PTPN2 isoform referred to as TC45 as mostly involved in tumourigenesis [29,34,41,52]. The expression assays used in the present study detect all known splicing variants and do not distinguish between them. In a future study, isoform-specific analysis of PTPN2 would be valuable to give further insight in their possible different oncogenic roles.

In conclusion, this study demonstrates genomic and transcriptomic loss of PTPN2 as a new possible mechanism for PI3K/AKT upregulation in breast cancer. PTPN2 may be a potential clinical marker of prognosis and endocrine treatment prediction, thus allowing further tailored patient therapies.

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Ethical standards

The experiments comply with the current laws of Sweden

Conflict of interest

The authors declare that they have no conflict of interest

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Table 1. Correlations between *PTPN2* gene copy loss and clinicopathological data.

	***PTPN2* gene copies**		
	Deletion	*Two or more copies*	*P-value*
Tamoxifen treated			
No	16 (14.6)	94 (85.5)	P=0.60
Yes	18 (17.1)	87 (82.9)	
RT/CMF			
RT	15 (15.5)	82(84.5)	P=0.90
CMF	19 (16.1)	99 (83.9)	
Lymph node status			
Negative	3 (13.6)	19 (86.4)	P=0.77
Positive	31 (16.1)	162 (83.9)	
Tumour Size			
≤20 mm	14 (16.3)	72 (83.7)	P=0.88
>20 mm	20 (15.5)	109 (84.5)	
S-phase fraction			
<12%	20 (13.9)	124 (86.1)	P=0.18
≥12%	11 (22.0)	39 (78.0)	
ER			
Negative	10 (16.4)	51(83.6)	P=0.93
Positive	24 (15.9)	127 (84.1)	
***HER2* gene amplification**			
Negative	24 (15.2)	134 (84.8)	P=0.80
Positive	6 (13.6)	38(86.4)	
HER2 protein			
Negative	23 (15.2)	128(84.8)	P=0.96
Positive	9 (15.5)	49 (84.5)	
***PIK3CA* mutation**			
-	25 (15.6)	135 (84.4)	P=0.78
+	9 (17.3)	43 (82.7)	
pAKT (All patients)			
≤10%	22 (14.2)	133 (85.8)	P=0.21
>10%	12 (21.4)	44 (78.6)	
pAKT (Luminal A)			
≤10%	7 (9.9)	64 (90.1)	**P=0.040**
>10%	6 (27.3)	16 (72.7)	
PTPN2 mRNA levels			
Low	10 (20.4)	39 (79.6)	**P=0.038***
High	0 (0)	18 (100)	
*) P=0.036 with *PTPN2* gene copy no in three groups (<2 copies, 2-3 copies and, >3 copies).			

Table 2. Correlations between PTPN2 mRNA levels and clinicopathological data.

	PTPN2 mRNA expression n (%)		
	Low (Q1-Q3)	*High (Q4)*	*p-value*
Tamoxifen treated			
No	23 (67.7)	11 (32.4)	p = 0.17
Yes	42 (80.8)	10 (19.2)	
RT/CMF			
RT	27 (71.1)	11 (29.0)	p = 0.38
CMF	38 (79.2)	10 (20.8)	
Lymph node status			
Negative	6 (100)	0 (0)	p = 0.15
Positive	59 (73.8)	21 (26.3)	
Tumor Size			
≤ 20 mm	29 (78.4)	8 (21.6)	p = 0.60
> 20 mm	36 (73.5)	13 (26.5)	
S-phase fraction			
< 12%	37 (69.8)	16 (30.2)	p = 0.30
≥ 12%	21 (80.8)	5 (19.2)	
ER			
negative	16 (80.0)	4 (20.0)	p = 0.60
positive	49 (74.2)	17 (25.8)	
***HER2* gene amplification**			
negative	50 (76.9)	15 (23.1)	p = 0.50
positive	11 (68.8)	5 (31.3)	
HER2 protein			
negative	50 (76.9)	15 (23.1)	p = 0.53
positive	14 (70.0)	6 (30.0)	
***PIK3CA* mutation**			
-	54 (80.6)	13 (19.4)	**p = 0.019**
+	9 (52.9)	8 (47.1)	
pAKT (All patients)			
≤ 10%	38 (67.9)	18 (32.1)	**p = 0.023**
> 10%	27 (90.0)	3 (10.0)	
pAKT (*HER2*+and/or *PIK3CA*+)			
≤ 10%	8 (42.1)	11 (57.9)	**p = 0.0058**
> 10%	11 (91.7)	1 (8.3)	

Figure legends

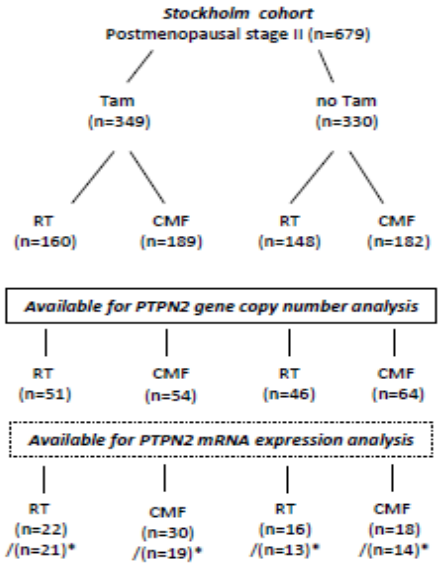

Figure 1

Figure 1. Patient flow through the study. (Tam: tamoxifen, RT: radiotherapy, CMF: cyclophosphamide, metotrexate, 5-fluorouracil chemotherapy, TMA: tissue microarray, IHC: immunohistochemistry, *data available for both DNA and mRNA).

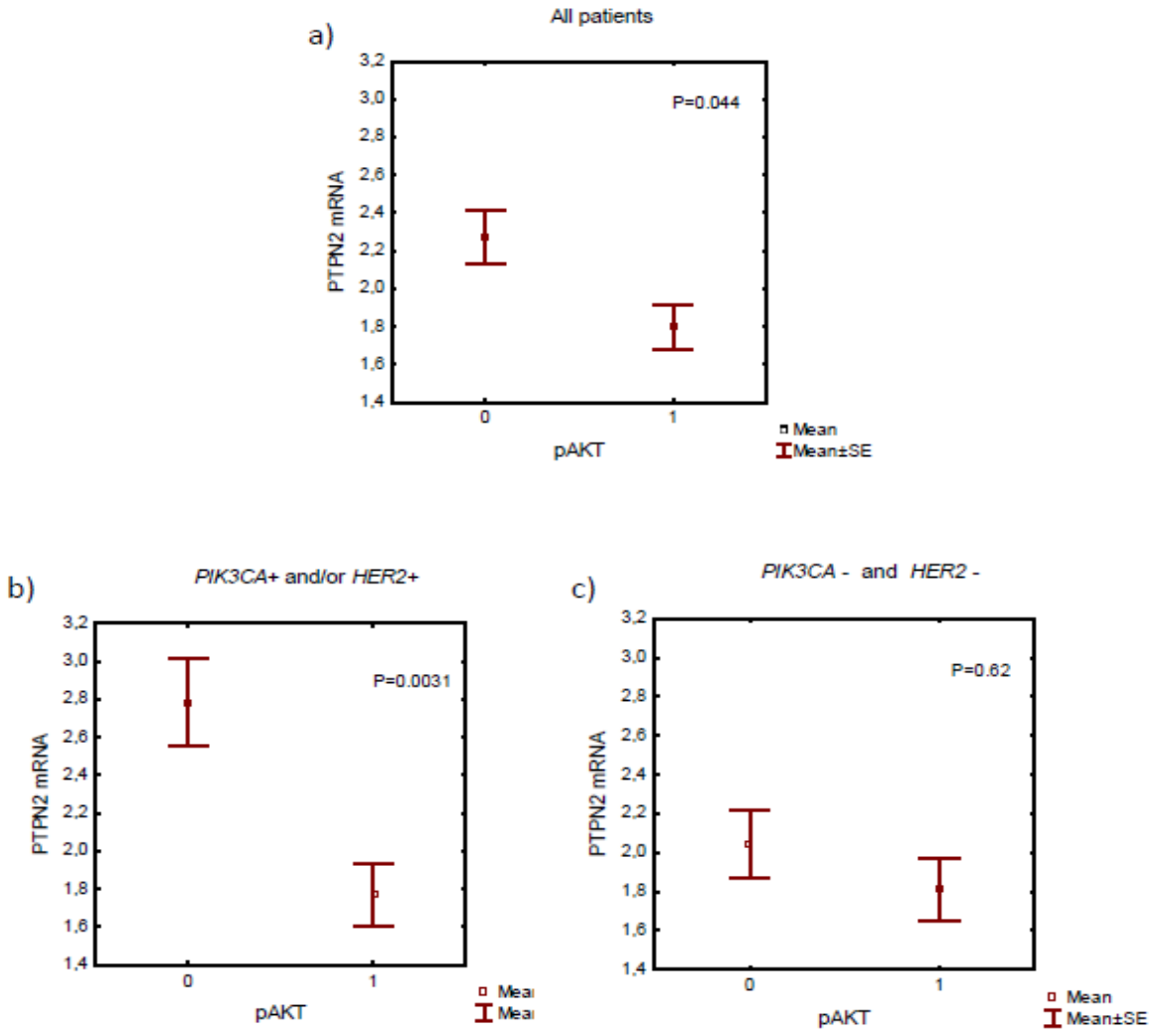

Figure 2

Figure 2. Mean plot of PTPN2 mRNA (continuous values) in relation to pAKT_S473 protein expression status in all patients (a), the subgroup of patients with tumours harboring

PI3K mutation and/or HER2 amplification (b) or no PI3K mutation or HER2 amplification (c). P-values refer to the Kruska-Wallis test.

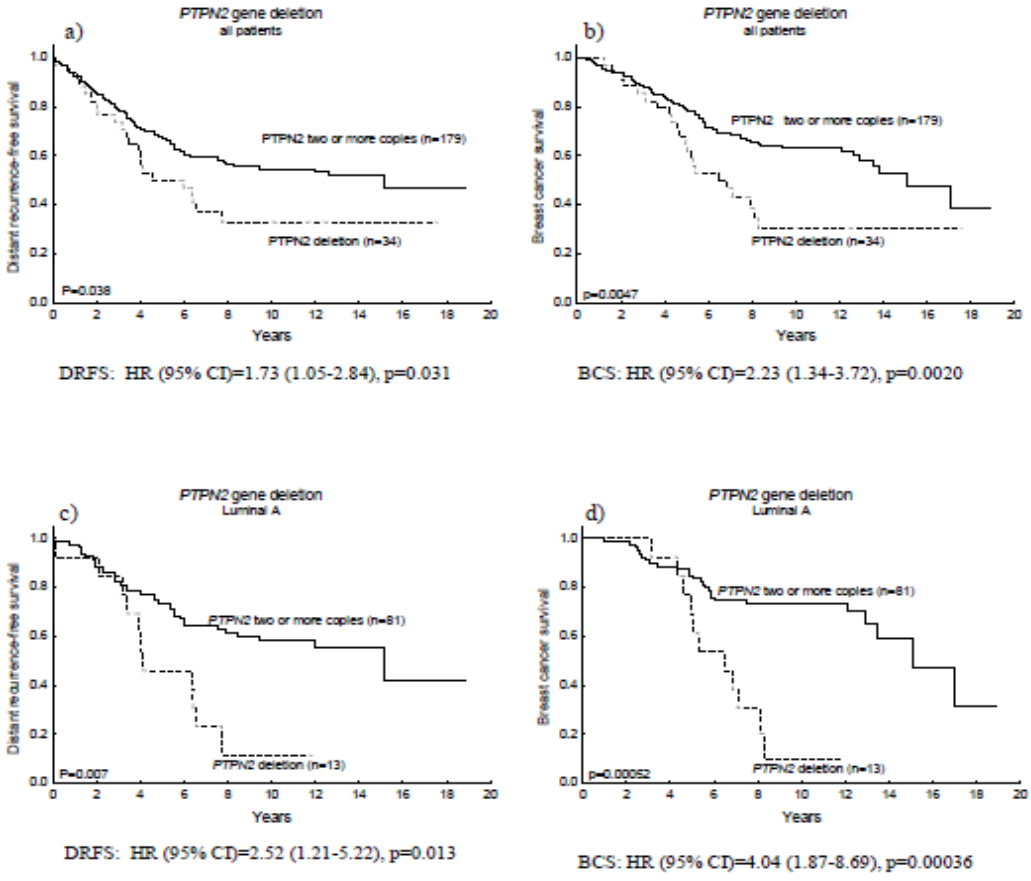

Figure 3

Figure 3. Univariate and multivariate analysis of distant recurrence-free survival (DRFS) (a, c) and breast cancer survival (BCS) (b, d) in relation to *PTPN2* gene copy number loss in the Stockholm cohort. All patients (a, b), Luminal A subtype (c, d). The Cox analysis included the following variables: adjuvant chemotherapy treatment, endocrine treatment, lymph node status, tumour size, and when all patients were included (a,b) also HER2 and ER status.

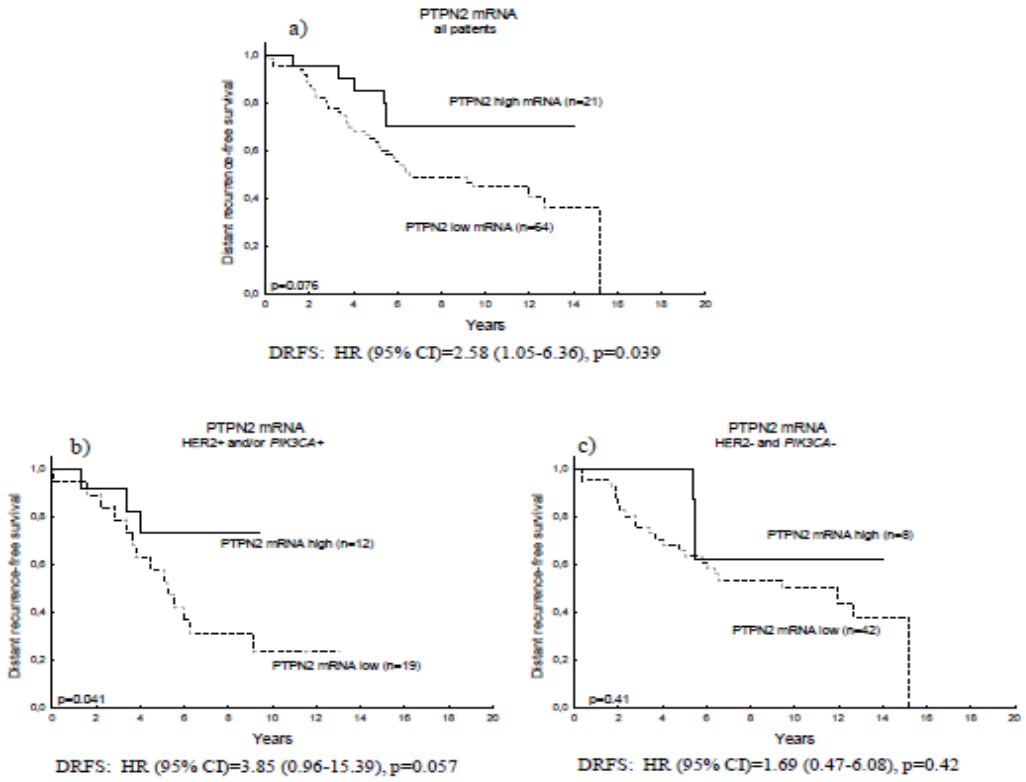

Figure 4

Figure 4. Univariate and multivariate analysis of distant recurrence-free survival (DRFS) in relation to PTPN2 mRNA expression levels in the Stockholm cohort, all patients (a), HER2+ and/or *PIK3CA*+ (b) and HER2- and *PIK3CA*- (c). The Cox analysis included the following variables: adjuvant chemotherapy treatment, endocrine treatment, lymph node status, tumour size, HER2 (a) and ER status.

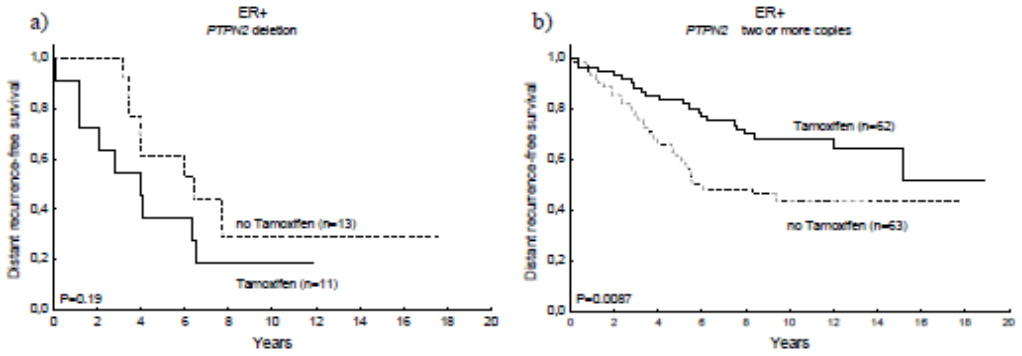

Figure 5

Figure 5. Distant recurrence-free survival (DRFS) in relation to tamoxifen treatment in the group with *PTPN2* gene deletion (a) and two or more *PTPN2* gene copies (b) in the Stockholm cohort.