PTK 7 Is a Transforming Gene and Prognostic Marker for Breast Cancer and Nodal Metastasis Involvement

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Abstract

Protein Tyrosin Kinase 7 (PTK7) is upregulated in several human cancers; however, its clinical implication in breast cancer (BC) and lymph node (LN) is still unclear. In order to investigate the function of PTK7 in mediating BC cell motility and invasivity, PTK7 expression in BC cell lines was determined. PTK7 signaling in highly invasive breast cancer cells was inhibited by a dominant-negative PTK7 mutant, an antibody against the extracellular domain of PTK7, and siRNA knockdown of PTK7. This resulted in decreased motility and invasivity of BC cells. We further examined PTK7 expression in BC and LN tissue of 128 BC patients by RT-PCR and its correlation with BC related genes like HER2, HER3, PAI1, MMP1, K19, and CD44. Expression profiling in BC cell lines and primary tumors showed association of PTK7 with ER/PR/HER2-negative (TNBC-triple negative BC) cancer. Oncomine data analysis confirmed this observation and classified PTK7 in a cluster with genes associated with agressive behavior of primary BC. Furthermore PTK7 expression was significantly different with respect to tumor size (ANOVA, p = 0.033) in BC and nodal involvement (ANOVA, p = 0.007) in LN. PTK7 expression in metastatic LN was related to shorter DFS (Cox Regression, p = 0.041). Our observations confirmed the transforming potential of PTK7, as well as its involvement in motility and invasivity of BC cells. PTK7 is highly expressed in TNBC cell lines. It represents a novel prognostic marker for BC patients and has potential therapeutic significance.

Introduction

Breast cancer (BC) is the most commonly diagnosed malignoma and the leading cause of cancer related death in women worldwide [1]. The utilization of clinicopathological and tumor molecular characteristics to determine patient prognosis and response to treatment are important features in the current management of BC. Recognized prognostic factors predicting disease outcome include tumor grade and size, hormone receptor status, HER2 expression, lymph node status, and patient age [2]. Although these classical prognostic markers are reliable in general, more specific prognostic and predictive markers are needed. This requires that we have a better understanding of the molecular mechanism of BC development and metastazation.

The receptor protein tyrosine kinase PTK7, also known as CCK4, was discovered as a gene overexpressed in colon cancer cell lines [3]. PTK7 is characterized as a transmembrane glycoprotein of 1071 amino acids containing an extracellular domain with seven immunoglobulin (Ig)-like loops and a catalytically inactive tyrosine kinase domain [4,5]. The human PTK7 gene is located on chromosome 6 (6p21.1–p12.2) and consists of 20 exons [6]. It was recently shown that the orphan receptor PTK7 plays a major role in non-canonical Wnt/planar cell polarity (PCP) signaling during vertebrate neural crest movement and establishment of inner ear hair cell polarity [7]. Perhaps the function of PTK7 is not only limited to PCP. An interaction with factors in canonical Wnt signaling e.g. β-catenin is discussed, but the proper role of PTK7 is still controversial. Puppo et al [8] suggested that PTK7 seems to favour β-catenin stabilization and canonical Wnt signaling, whereas Peradziryi et al [9] propose an inhibition of these pathways through PTK7. Furthermore, PTK7 has been identified as a protein with an important role not only in embryogenetic tube formation, but also migration and invasion of endothelial and cancer cells in vitro [10,11]. While PTK7 expression is upregulated in various cancers including colon, lung, gastric, breast cancer, and acute myeloid leukemia, PTK7 is also considered as a gene involved in the initiation of tumorigenesis [4,12–17]. Therefore, PTK7 plays an important role in the motility and invasivity of cancer cells. However, the biological significance of PTK7 in human BC and lymph node (LN) involvement has not been investigated so far.
In this study we determined the transforming potential of PTK7, and investigated its role in mediating BC cell motility and invasivity. We also analyzed mRNA expression of PTK7 in human BC and ipsilateral axillary LN by RT-PCR. PTK7 expression was also compared with some important BC related genes, like HER2, HER3, PAI1, K19, MMP1, and CD44 to understand the role of PTK7 in BC progression and metastasis.

Materials and Methods

We obtained ethics approval for this project from the ethics committee of the LMU (Ludwig-Maximilians-University) Munich. Patients gave written informed consent for the use of biological materials and relevant clinical data.

Cell Lines

Cell lines Hs578T and MDA-MB-157 (basal like) were obtained from ECACC; MDA-MB-453, SK-BR-3, BT-474, T-74D, ZR-75-1 and MDA-MB-175 VII (luminal), BT-20, MDA-MB-468, MDA-MB-231, MDA-MB-435S, and BT-549 (basal like), NIH3T3, HEK293 from ATCC, MDA-MB-361, BT-483, and ZR-75-50 (luminal) from Sugen; MCF7 (luminal), MCF7 (basal like), and MDA-MB-436 (basal like) and MDA-MB-415 (luminal) from DFKZ. MCF10A (basal like) were a generous gift by B. Gillies [18], SUM-1498 (basal like) and SUM-149PT (basal like) by H. Heremeking [19].

All cell lines were authenticated in 2012 using the StemElite ID System (Promega, Madison, WI).

Antibodies

PTK7-specific antibody was generated by immunization of rabbits with recombinant GST-PTK7 extracellular domain containing amino acid residues 1-703 (PTK7-XCD). For antibody treatment of cells, PTK7-XCD polyclonal antibodies were purified on protein A Sepharose-affinity columns. Anti-α Tubulin antibody was obtained from Sigma (St. Louis, MO).

Immunoblot Analysis

For immunoblotting, cells were washed with ice-cold PBS and then lysed on ice in Triton X-100 lysis buffer (50 mM HEPES, 150 mM NaCl, 10% Glycerol, 1 mM EDTA, 1% Triton X-100, 10 mM Na3P2O7, 150 mM NaCl, 10% Glycerol, 1 mM EDTA, 1% Triton X-100, 10 mM Na3P2O7, 10 mM NaF, 1 mM Na3VO4). Clarified whole-cell lysates were separated by SDS/PAGE and transferred to Protran Membrane (Whatman). For assessing the expression level of PTK7, the membranes were probed with anti-PTK7-XCD antiserum (1:20000 dilution) and reprobed with anti-α Tubulin for the loading control.

Generation of Expression Constructs

The 3.2-kbp cDNA sequence coding for PTK7 wild type (PTK7wt) were inserted into the EcoRI/XbaI restriction sites of pcDNA3 (Invitrogen) or the retroviral vector pLXSN. The dominant-negative kinase domain deletion-mutant (PTK7DN) was generated by subcloning the 2.3-kbp EcoRI/XbaI fragment coding for AA1-736 into the same vectors. The extracellular domain-containing construct PTK7XCD was generated by subcloning the 2.1 kbp fragment coding for AA1-703 into a GST-expressing pcDNA3 vector.

Construction of Stable Cell Lines

Hs578T cells were stably infected with PTK7DN or empty vector using supernatants of the amphotropic retrovirus producing cell line PhoenixA which had been transiently transfected with pLXSN constructs. After selection with 1 mg/ml G418 for 14 days cell lines were selected for high expression of PTK7DN, as monitored by Western blot analysis.

HEK293 cells were stably transfected with pcDNA3 constructs containing PTK7XCD using Lipofectamine 2000 (Invitrogen).

RNA Interference

Transfection of a pool of four 19-nucleotide siRNA duplexes (ON-TARGETplus, Thermo scientific) was carried out using Lipofectamine RNAiMAX (Invitrogen) and OPTI-MEM medium (GIBCO) without FBS. Cells were seeded for Oris cell migration assay 24 hrs and for in-vitro migration assay 48 hrs post-transfection.

Focus Formation Assay

For Focus Formation Assays NIH3T3 cells were infected with PTK7wt, PTK7DN, empty vector, or v-src as a positive control using supernatants of the ecotropic retrovirus-producing cell line PhoenixE which had been transiently transfected with pLXSN constructs. 2×10^4 cells were seeded into 6 cm-dishes 4 days after infection and left to grow in DMEM containing 4% FBS for 11 days with changing of media every two to three days. Cells were then stained and fixed with 0.5% Cristal Violet/20% Methanol.

Soft-Agar Colony Formation Assay

NIH3T3 cells were infected as for Focus Formation Assay. 3.9×10^4 cells in a top layer of 0.2% Agar Gel (Invitrogen) were seeded onto a bottom layer of 0.7% Agar Gel and left to grow for 16 days. Colony formation was visualised with a Zeiss AxioObserver.A1 microscope.

Matrigel Assay

For Matrigel outgrowth assays, cells were seeded at 2.5×10^4 cells per well into 96 well dishes which had previously been coated with 3% Matrigel (BD Biosciences, Bedford, MA). Colony outgrowth was visualized after three days with a Zeiss Axiovert S100 microscope.

Matrigel Invasion Chamber Assay

Cells were plated at 2×10^4 cells per well into Matrigel Invasion Chambers (BD Biosciences, Bedford, MA) using DMEM +0.1% FBS +1 μg/ml Mitomycin. The lower compartments contained DMEM +1% FBS +1 μg/ml Mitomycin. After 20 hours incubation, cells were fixed and stained with 20% methanol, 1% crystal violet and cotton swabs were used to remove the cells from the upper side of the membrane. An Axio Observer.A1 microscope (Carl Zeiss, Jena, Germany) was used to record 5 micrographs per well. Photoshop CS5 (Adobe, San Jose, CA) and a proprietary script were used for automated quantification of migration.

Oris™ Cell Migration Assay

Oris™ Cell Seeding Stoppers (Platypus Technologies, Madison, WI) were inserted into the wells of a 96 well dish to create the migration zone. Cells were then seeded at 9×10^3 cells per well and incubated for 18 hours to allow cell adherence. Stoppers were then removed and Mitomycin was added to the growth medium at a concentration of 1 μg/ml to inhibit cell proliferation. Cells were permitted to migrate into the wound area for 32 hours and were fixed and stained with 20% methanol, 1% crystal violet. Micrographs of the wound area were recorded with a Zeiss Axio Observer.A1 microscope and the size of the wound area was measured using the MetaVue imaging software (Molecular Devices).
In vitro Migration Assay
Cells were plated at $2.5 \times 10^4$ cells per well into transwell migration inserts (BD Biosciences, Bedford, MA) using DMEM +0.1% FBS+1 μg/ml Mitomycin. The lower compartments contained DMEM +1% FBS+1 μg/ml Mitomycin. After 4.5 hours incubation, cells were fixed and stained with 20% methanol, 1% crystal violet and cotton swabs were used to remove the cells from the upper side of the membrane. An Axios Observer.A1 microscope (Carl Zeiss, Jena, Germany) was used to record 5 micrographs per well. Photoshop CS5 (Adobe, San Jose, CA) and a proprietary script were used for automated quantification of migration.

Analysis of Oncomine Data
The Oncomine database tool [20] was used to analyze mRNA expression microarray data from several BC studies (meta-analysis of gene expression of PTK7 as a cancer target). Briefly, PTK7 gene was queried in the database and the results were filtered by selecting BC (Reporter ID: NM_002821). The data from study classes of benign vs. cancer were used for heat-maps. P-values for each group were calculated using student t-test. Standardized normalization techniques and statistical calculations are provided on the Oncomine website and published [21,22].

Patients and Tissue Collection
As shown in Table 1, 128 breast cancer patients (median age 59 years, range 27–87 years) with 83 (69%) infiltrated LN, as well as 38 (31%) non-infiltrated LN were included at the Red Cross Women's Hospital Munich in the time period of 2006-2010. Patients with neoadjuvant systemic therapy were excluded. Surgery on breast and axillary was performed stage adapted. BC tissue and LN were collected and stored immediately at −80°C since preparation. To ensure the histological status of harvested LN, they were bisected, with one half submitted for routine histology and the other half taken for examination by RT-PCR. Tumor grade, TNM-classification, and the histopathological tumor subtype were recorded. Hormonal receptor (ER/PR)- and HER2-status was assessed by immunohistochemistry (IHC). Thereby 17 (13%) patients were identified with ER-, PR- and HER2-status, classified as triple negative breast cancer (TNBC).

Total RNA Isolation
Total RNA was isolated using the acid guanidine thiocyanate-phenol-chloroform extraction method [23]. After Na-acetate treatment the total RNA was extracted with phenol-chloroform, precipitated with isopropanol, washed with 80% ethanol, and finally dissolved in diethyl pyrocarbonate treated H2O. The total RNA concentration was determined by absorbance measurement (260/280 nm) and the quality of total RNA was verified by 1% agarose gel electrophoresis. Only samples with no evidence of DNA contamination and RNA degradation were used for cDNA synthesis.

cDNA-Synthesis
cDNA synthesis was done using 5 μg of total RNA in an oligo(dT) primer mix (MWG Biotech; total volume 10 μL), which was heated at 70°C for 3 min and then cooled on ice. A mastermix containing 1x reverse transcriptase buffer, 1 mmol/L deoxynucleotide triphosphates, 10 mmol/L DTT, 40 unitsRNase inhibitor (40 u/μL, Fermentas), and 50 units avian myeloblastosis virus reverse transcriptase (25 u/μL Molecular Diagnostics, Roche) was added and subsequently incubated at 42°C for 2 h. A stop reaction was done with 80 μL Tris-EDTA 10/0.1 followed by heating at 72°C for 7 min. The quality of the cDNA was verified using 1.5% agarose gel electrophoresis. Afterwards, the cDNA probe was denatured with 10 μL 1 N NaOH, pH neutralized with 5 μL 2 N HCl and 5 μL 2NTris-HCl (pH 7.5), and purified using the QAquick PCR Purification kit (Qiagen) according to the manufacturer’s instructions.

RT-PCR
The PCR method was used to determine mRNA expression levels of PTK7, HER2, HER3, MMP1, K19, CD44, and α-Tubulin as a housekeeping-gene in fresh-frozen BC and LN samples. The following primer sequences were applied: PTK7-ex7fwd5'- GGA AGC CAC ACT TCA CCT AGC AG -3', PTK7-ex11rev 5'-CTG CCA CAG TGA GCT GGA CAT GG -3', HER2a fwd 5'- CAC ATG ACC CCA GCC CTC TAC AGC -3', HER2a rev 5'- CAC GGC ACC CCG AAA GGC AAA AAC -3', HER3 fwd 5'- CTC CGG CCT CAG CCT ACC AGT T-3',

| Table 1. Clinico-pathological features of patients. |
|-----------------|-----------------|
| Variable        | Number (%)      |
| Age             |                 |
| ≤50 years       | 42 (33)         |
| >50 years       | 86 (67)         |
| Sex             |                 |
| Female          | 124 (97)        |
| Male            | 4 (3)           |
| Menopausal status|               |
| Pre             | 42 (33)         |
| Post            | 82 (67)         |
| Histopathological subtype |             |
| Invasive ductal | 104 (81)        |
| Invasive lobular| 17 (13)         |
| Other           | 7 (6)           |
| Tumorstatus     |                 |
| pT1             | 40 (31)         |
| pT2             | 65 (51)         |
| pT3             | 12 (9)          |
| pT4             | 11 (9)          |
| Lymph node status|               |
| pN0             | 33 (26)         |
| pN1             | 47 (37)         |
| pN2             | 29 (23)         |
| pN3             | 19 (15)         |
| Distant metastasis|               |
| M0              | 120 (94)        |
| M1              | 8 (6)           |
| Grading         |                 |
| Grade 1         | 10 (8)          |
| Grade 2         | 75 (59)         |
| Grade 3         | 42 (33)         |
| Unknown         | 1 (1)           |
| Estrogen receptor status |         |
| ER-             | 32 (25)         |
| ER+             | 96 (75)         |
| Progesterone receptor status |     |
| PR-             | 48 (37)         |
| PR+             | 80 (63)         |
| Her2/neu status |                 |
| Her2/neu-       | 103 (80)        |
| Her2/neu+       | 23 (18)         |
| Unknown         | 2 (2)           |

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HER3 rev 5’-TGC TCC GGC TTC TAC ACA ACA-3’, MMP1 fwd 5’- CGA CTC TAG AAA CAC AAG AGC AAG A-3’, MMP1 rev 5’- AAG GTT AGC TTA CTG TCA CAG GCT T-3’, PAI-1 fwd 5’- GCT GAA TTC CTG GAG CTG AG-3’, PAI-1 rev 5’- CTG CGC CAC CTG CAT AAA CA-3’, CD44 fwd 5’- GAT CCA CCC CAA TTC CAT CTG GCC CCT-3’, CD44 rev 5’- AAC CGC GAG AAT CAA AGC CAA GGC-3’, K19 fwd 5’- GAG GTG TAT TCC GCT CCG CGG GAA -3’, K19 rev 5’- ATC TTC CTG TCC TTC CAT CAG GAC-3’, α-TUB fwd 5’- AAG TGA CAA GAC CAT TGG GGG AGG-3’, α-TUB rev 5’- GGG CAT AGT TAT TGG CAG CAT-3’. All primers were synthesized by MWG Biotech. PCR was done using 1 μL cDNA as template in a mastermix containing 1 × PCR buffer, 1 mmol/L deoxynucleotide triphosphates, 1 pmol/μL of each specific primer, and 2.5 μL Taq DNA polymerase (Sigma Inc., USA) for each sample and carried out in a thermal cycler (Eppendorf). PCR cycling conditions began with an initial DNA denaturation step at 94°C for 2 min followed by 32 cycles with denaturation at 94°C for 30 s, primer annealing at the appropriate annealing temperature for 30 s, extension at 72°C for 1 min and followed by a final extension step at 72°C for 5 min. The amplified PCR products were analyzed by separation in a 2% agarose gel at 80 V for 30 min. The mRNA expression of the PCR products, with a size of approximately 100 bp- 1 kbp, was evaluated semiquantitatively by using Aida Image Software (Raytest, Germany).

qRT-PCR for cell lines

Quantitative real time-PCR assays of breast cancer cell lines were performed using the Applied Biosystems StepOnePlus® detection system. The PTK7 specific primer sequences were fwd 5’- GAG TTC CTG AGG ATT TTC CAC AAG AG-3’ and rev 5’- TGC ATA GGG CCA CCT TC-3’. Amplification of α-Tubulin as an endogenous reference was performed to standardize the amount of sample mRNA. α-Tubulin primers (fwd 5’- CGG GGC AGT GTT TGT AGA C-3’, rev 5’- GTG CCA GTC GGA ACT TC-3’) and probes were purchased from Thermo Fisher Scientific. The PCR was carried out with Solaris qPCR Master Mix using 1 μL of cDNA, diluted 1:20 in H2O and 1 μL 20x Solaris primer set in a final volume of 10 μL. The reactions were incubated in a 96-well optical plate at 95°C for 15 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Experiments were performed in duplicate for each sample and Ct data of each sample was determined using default threshold settings. Relative quantification of mRNA expression was calculated with the 2-ΔΔCt method. Results were reported as average expression ± SEM.

Statistical analysis

Statistical analysis was done using R 2.13.2 (R Foundation for Statistical Computing, Vienna, Austria). Considering the PTK7 expression in BC cell lines, groups were compared using exact Mann-Whitney U (MW) tests. For clinicopathological evaluation of PTK7 expression the Mann-Whitney U test was used. Survival analysis end points were overall survival (OS) and disease free survival (DFS). DFS and OS were determined in months from the date of surgery. DFS end points corresponded with the detection of a recurrent tumor or distant metastasis or death. Overall survival was measured from the time of surgery until death or last follow-up. Survival data are presented using the Kaplan-Meier life-table method; differences between groups were tested by long-rank tests. Hazard ratios were calculated using the Cox proportional hazards model. To illustrate the effect of PTK7 expression on DFS the PTK7 expression was divided in low and high according to the median of PTK7 expression. A significance level of 5% was used. All tests were performed two sided.

Results

Transforming potential of PTK7

The ability of cells to grow independently of their attachment to tissue culture surfaces and to overcome contact inhibition is a strong indicator of transforming potential.

After transient infection of NIH3T3 cells with pLXS/N/PTK7wt, pLXS/N/PTK7DN, or pLXS/N/α-src (positive control) focus formation and soft agar colony formation assays were performed in order to determine the transforming potential of PTK7. Focus formation was detectable in the case of pLXS/N/PTK7wt and pLXS/N/α-src infection, but not after infection with pLXS/N/PTK7DN. Similar results were observed in soft agar assays with a high number of colonies in pLXS/N/PTK7wt infected NIH3T3 cells in contrast to pLXS/N/PTK7DN (lower colony number) and mock infected cells (no colonies) (Fig.1), indicating that PTK7 has strong transforming potential.

Expression of PTK7 in Breast Cancer Cell Lines

To identify the role of PTK7 in BC, we determined mRNA expression levels in 1 non tumorigenic mammary epithelial and 20 BC cell lines by qRT-PCR. We grouped them in estrogen receptor (ER) positive (luminal) and ER negative (luminal, basal-like) and examined PTK7 expression. PTK7 expression was increased in ER negative BC cell lines (n = 12) (median: 20.7 (range: 0.0 to 86.9)) compared to ER positive cell lines (n = 9) (median: 5.9 (range: 0.9 to 16.7)). This high numerical difference was not statistically significant (p = 0.082) which can be due to small sample sizes. PTK7 overexpression was detected in basal like tumors.

For the comparison of not infiltrated LN and LN metastasis in respect of gene expression the Mann-Whitney U test was used. Survival analysis end points were overall survival (OS) and disease free survival (DFS). DFS and OS were determined in months from the date of surgery. DFS end points corresponded with the detection of a recurrent tumor or distant metastasis or death. Overall survival was measured from the time of surgery until death or last follow-up. Survival data are presented using the Kaplan-Meier life-table method; differences between groups were tested by long-rank tests. Hazard ratios were calculated using the Cox proportional hazards model. To illustrate the effect of PTK7 expression on DFS the PTK7 expression was divided in low and high according to the median of PTK7 expression. A significance level of 5% was used. All tests were performed two sided.

Relevance for PTK 7 in Breast Cancer
Suppression of cancer cell invasivity by an anti-PTK7 polyclonal antibody

To evaluate the efficacy in vitro of a more therapy-like intervention strategy, we generated a polyclonal antiserum against the extracellular domain of PTK7 and studied its effect on the invasivity of Hs578T cells in Matrigel assay. PTK7 antibody treated cells grew only as spherical clusters on the surface without potential of penetration into the Matrigel in contrast to cells which were treated by control antibody (Figure 3G).

PTK7 expression and clino-pathological parameters in primary breast cancer tissue

We investigated whether PTK7 overexpression in ER-negative BC cell lines also reflects the expression status in ER-negative human breast tumors and LN metastasis. Therefore, we evaluated the hormonal receptor (HR)- and HER2 receptor-status in relation to PTK7 expression. Whereas there was no statistically significant difference in BC (mean difference: −0.18, 95% CI: −0.89 to 0.52, p = 0.602), PTK7 expression in LN metastasis was strongly linked to ER-status (mean difference: 1.26, 95% CI: 0.46 to 2.06, p = 0.003), PR-status (mean difference: 0.87, 95% CI: 0.09 to 1.66, p = 0.030) and HER2-status (mean difference: −1.35, 95% CI: −2.39 to −0.65, p = 0.001). In grouped analysis for PTK7 expression in ER/PR/HER2-negative (TNBC) vs. ER/PR/HER2-positive BC there was a tendency for higher expression in TNBC, but was not statistically significant due to a small sample size of TNBC (n = 14) and ER/PR/Her2-positive (n = 6) cohort (mean difference: −1.35, 95% CI: −3.36 to 0.25, p = 0.083, t-test). In univariate analysis significant differences in PTK7 expression for tumor size (ANOVA, p = 0.033) in BC and nodal status (ANOVA, p = 0.007) in LN (higher expression level with number of LN metastasis) was seen (Table 2).

In order to validate these qPCR data, PTK7 immunohistochemistry was performed with 35 BC and 35 LN tissue samples of breast cancer patients. The results are shown as supporting information.

Co-expression of Breast Cancer Related Genes and PTK7

To assess the tumorigenic potential of PTK7 in BC and LN we compared the PTK7 expression level and co-expression with other breast cancer related genes (HER2, HER3, PAI1, MMP1, CK19, CD44). In primary BC we found significant correlation between PTK7 and PAI1 (r = 0.494, p < 0.001), MMP1 (r = 0.286, p = 0.002) and CD44 (r = 0.247, p = 0.014). For LN metastasis PTK7 co-expression was seen with HER2 (r = 0.448, p < 0.001), PAI1 (r = 0.349, p < 0.001) and MMP1 (r = 0.433, p < 0.001) (Table 3). When gene expression levels of non-involved LN and LN metastasis were examined, we saw significantly higher expression in LN metastasis for all genes except CD44 (PTK7, HER2, HER3, PAI1, MMP1, CK19) (Fig. 4A–G). Taken together, PTK7 seems to be linked to tumor progression and metastasis like the other genes.
Figure 3. Expression of full length PTK7 correlates with motility and invasivity of breast cancer cells. (A) Expression of full length and dominant negative PTK7 protein in wild type or PTK7DN overexpressing Hs578T cells which were subsequently used in cellular assays. Whole cell lysates were used to detect the protein level of PTK7 by immunoblot analysis (top) with α-Tubulin as loading control (bottom). (B) Expression of PTK7 protein 48 and 72 hrs after siRNA transfection. Whole cell lysates were used to detect the protein level of PTK7 by immunoblot analysis (top) with α-Tubulin as loading control (bottom). (C) The motility of cells was analysed by Oris™ cell migration assay. Cells were allowed to migrate into the wound area for 32 hrs. Cell migration was visualized at 4× magnification, representative micrographs are shown (scale bar = 500 μm). Error bars
Prognostic role of PTK7 expression

In order to determine the prognostic impact of PTK7 we correlated expression levels with patient survival. According to our hypothesis we further grouped patients in TNBC and non-TNBC cohorts. 118 breast cancer patients were followed-up for a median period of 29 months (9 patients died and 21 relapsed). For OS no correlation was detected with PTK7 expression most likely due to short follow-up time. Patients with higher PTK7 expression in LN metastasis had a significantly shorter DFS with a HR of 1.25 (95% CI: 1.04–1.50, p = 0.016) for every increase of one unit in the PTK7 expression. The estimated 24-months rates for DFS were 96% (95% CI: (0.91–1.00) in the low expression group, as compared with 80% (95% CI: (0.69–0.92); p = 0.041) in the high expression group, as detected. However, this may be due to limited sample size of TNBC.

Discussion

In our study we identify PTK7 (originally CCK4) as being differentially expressed together with certain other genes in TNBC compared to ER-positive and normal breast cell lines. The pseudokinase PTK7 was originally identified as a protein overexpressed in several cancer cell lines, including melanoma [32] and colon [4].

There have been several reports about the upregulation of PTK7 expression in different types of cancer such as pulmonary adenocarcinoma [12], gastric [13] and breast carcinoma [17] but none about its oncogenic potential. In this work we could show that PTK7, like most RTKs including HER3, is indeed able to transform NIH3T3 cells, as shown in Focus Formation and Soft Agar Colony Formation Assays (Figure 1).

Furthermore we could identify PTK7 as a potential mediator of motility and invasivity of BC cells. We found that the expression of PTK7 was correlated with the invasive properties of BC. Inhibition of endogenous PTK7 signaling in highly invasive BC cells with TNBC characteristics by a dominant negative mutant or siRNA silencing results in loss of the capacity to invade the surrounding matrix and to migrate into the wound area. The dominant negative mutant might function here as a competitor of endogenous PTK7 for a yet to be identified ligand or other binding partner, as has been described for a soluble form of PTK7 [11].

Antibodies, by virtue of the fact that they are highly specific, represent an ideal approach for selectively interfering with a specific target molecule. In this study we performed experimental

Table 2. Association of PTK7 expression and patient’s clinico-pathological variables in primary tumors and lymph nodes of 128 breast cancer patients.

| Clinicopathological parameters | BC | LN |
|-------------------------------|----|----|
| **PTK7 Expression**          |    |    |
| Tumorsize                    |    |    |
| pT1                          | 35 | 20 | 0.033 |
| pT2                          | 58 | 44 | 0.158 |
| pT3                          | 9  | 10 | 0.228 |
| pT4                          | 10 | 9  | 0.228 |
| Nodal status                 |    |    |
| pN0                          | 28 | 2  | 0.007 |
| pN1                          | 44 | 40 | 0.188 |
| pN2                          | 25 | 23 | 0.148 |
| pN3                          | 15 | 18 | 0.168 |
| Grading                      |    |    |
| G1                           | 9  | 5  | 0.094 |
| G2                           | 64 | 50 | 0.178 |
| G3                           | 38 | 27 | 0.003 |
| ER status                    |    |    |
| ER−                          | 28 | 22 | 0.003 |
| ER+                          | 84 | 61 | 0.003 |
| PR status                    |    |    |
| PR−                          | 43 | 32 | 0.030 |
| PR+                          | 69 | 51 | 0.178 |
| Her2/neu                     |    |    |
| Her2/neu−                    | 88 | 64 | 0.001 |
| Her2/neu+                    | 22 | 18 | 0.168 |
| Grouped receptor status      |    |    |
| ER, PR, Her2/neu−            | 13 | 9  | 0.884 |
| ER, PR, Her2/neu+            | 6  | 5  | 0.348 |

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therapy against BC in vitro by using a polyclonal PTK7 antibody, and showed that the antibody successfully inhibited the invasivity of Hs578T breast cancer cells, which proved that developing a therapeutic monoclonal anti-PTK7 extracellular domain antibody is a promising strategy for the treatment of invasive BC.

Treatment decisions for BC are mainly based on clinicopathological variables that are prognostic, such as tumor size, presence of lymph node metastasis, grading, and three predictive markers (ER, PR and HER2). However, current markers do not allow accurate prediction of the likelihood of recurrence, and improvements are needed to clearly identify which women are at sufficiently low risk to be able to safely avoid the use of chemotherapy and its accompanying adverse effects. Present first generation prognostic signatures are clinically useful only in patients with ER positive disease, but not for ER negative patients. The identification of receptor tyrosine kinases as promising targets for directed cancer therapy approaches has further increased the

**Table 3.** Co-expression of BC related genes and PTK7 expression in BC and LN metastases as measured by RT-PCR.

| Genes       | Pearson’s r | p-value | Pearson’s r | p-value |
|-------------|-------------|---------|-------------|---------|
| Her2/neu    | 0.171       | 0.077   | 0.448       | <0.001  |
| Her3        | 0.013       | 0.897   | 0.549       | <0.001  |
| Pai1        | 0.494       | <0.001  | 0.433       | <0.001  |
| MMP1        | 0.266       | 0.002   | 0.433       | <0.001  |
| CK19        | 0.139       | 0.144   | 0.179       | 0.116   |
| CD44        | 0.247       | 0.014   | 0.184       | 0.124   |

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PTK7 expression is associated with poor prognosis in various cancers as well as in breast cancer patients. AML cases with high PTK7 expression achieved significantly lower DFS compared to patients with low expression levels. This is in accordance with the results of Oncomine analysis that PTK7 expression is prognostic in BC patients. We were not able to prove our hypothesis generated by Oncomine analysis that PTK7 expression is prognostic in TNBC patients, but in our opinion, this was due to small sample size. If so, PTK7 could offer a target in this poor prognostic cohort, where an effective treatment is urgently needed.

Supporting Information

Figure S1 Immunoblot Analysis of PTK7 Expression in BC cell lines. (B) Whole cell lysates were used to detect the protein level of PTK7 in 21 BC cell lines. (A) Band intensity was determined using the AIDA Advanced Image Data Analyzer Software (Raytest, Straubenhardt, Germany).

Figure S2 Representative cases demonstrating the relation of PTK7 expression in breast cancer by immunohistochemical staining and rtPCR value. (A) Weak (+) IHC scoring and low PTK7 rt-PCR (0.356) in invasiv-ductal TNBC (upper panel: magnification 200×, scale bar: 100 μm; lower panel: magnification 400×, scale bar: 50 μm). (B) Strong (+++) IHC scoring and high PTK7 rtPCR (2.496) in invasiv-ductal TNBC (upper panel: magnification 200×, lower panel: magnification 400×), (C) Weak (+) IHC scoring in normal breast tissue (upper panel: magnification 200×, lower panel: magnification 400×).

Table S1 Association of PTK7 expression and patient’s clinicopathological variables in primary tumors (BC) and lymph nodes (LN) by immunohistochemistry (IHC).

Text S1 Immunohistochemistry.

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Figure S5 PTK7 serves as a prognostic marker in lymph nodes involvement. Kaplan-Meier curve showing disease-free survival (DFS) in breast cancer patients with lymph node metastasis classified into PTK7 low and high groups according to RT-PCR expression. PTK7 expression is associated with poor survival in BC patients with lymph node metastasis expressing high PTK7. P-values were calculated by the log-rank test. doi:10.1371/journal.pone.0084472.g005

PTK7 overexpression in LN metastasis was associated with a higher amount of involved LN and significantly inferior DFS in BC patients. We were not able to prove our hypothesis generated by Oncomine analysis that PTK7 expression is prognostic in TNBC patients, but in our opinion, this was due to small sample size. If so, PTK7 could offer a target in this poor prognostic cohort, where an effective treatment is urgently needed.
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Author Contributions

Conceived and designed the experiments: PK AU. Performed the experiments: SG AG BA BH. Analyzed the data: SG AG BA PK WE BH PW AU. Contributed reagents/materials/analysis tools: BA TK SG AU PW. Wrote the paper: SG AG BA PK AU.