PTK7 Expression in Triple-negative Breast Cancer

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Abstract. Background: Protein tyrosine kinase-7 (PTK7) plays an important role in cancer. Our aim was to evaluate PTK7 in triple-negative breast cancer (TNBC). Materials and Methods: PTK7 Expression was assessed by immunohistochemistry (IHC) in 133 patients with TNBC. Expression levels were correlated with clinicopathological features and survival, taking chemotherapy into account. Results: Positive PTK7 expression was detected in 28.6% of tumors. In the total population, no significant difference was detected in disease-free survival (DFS) or overall survival (OS) related to PTK7 status. However, in patients treated by chemotherapy, patients with PTK7-negative tumors seemed to have better DFS than those with PTK7-positive tumors, particularly for patients treated with only anthracycline-therapy drugs. In patients receiving other chemotherapy regimens, PTK7 status had no significant impact on DFS or OS. Conclusion: Our results support earlier findings that PTK7 may be associated with resistance to anthracycline-based chemotherapy.

Protein tyrosine kinase-7 (PTK7), a pseudokinase, also known as colon carcinoma kinase (CCK4), was originally identified as a protein overexpressed in colon cancer cell lines (1). Structurally, it contains an extracellular domain with seven immunoglobulin-like loops, a transmembrane domain and an inactive catalytic tyrosine kinase domain (2, 3). PTK7 seems to be highly involved in the WNT (named after the Drosophilia Wingless (Wg) and the mouse Int-1 genes)-pathways (4), which again represent key pathways for epithelial mesenchymal transition (EMT) and play important roles in cancer (5-8). A potential impact of PTK7 expression has been studied in several malignancies, including colon, lung, gastric and breast cancer, acute myeloid leukemia and liposarcoma (9-15). The observed impact of PTK7 overexpression on patient outcome (prognosis) varies among malignancies. Interestingly, patients with AML with PTK7 overexpression seemed to be resistant to anthracycline-based chemotherapy with a reduced leukemia-free survival compared to those with PTK7-negative AML (11). Triple-negative breast cancer (TNBC) is a clinically defined subtype of breast cancer with lack of expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor-2 (HER2). In comparison to patients with receptor-positive tumors, TNBC is associated with younger age, poorer differentiation, larger tumor size, and worse outcome (e.g. shorter DFS and OS, higher incidence of visceral and brain metastasis, shorter time to death after recurrence) (16). In terms of chemotherapy, anthracyclines are central components of standard neo- and adjuvant regimes. However, there are controversial data addressing the question of anthracycline benefit in breast cancer (17). Despite this controversial discussion, anthracyclines are an integral component of chemotherapy in TNBC in current guidelines. There have not been any prospective trials defining the role of anthracyclines specifically in TNBC. The biological significance of PTK7 in TNBC and its clinical impact on anthracycline-based chemotherapy have, therefore, not yet been investigated. Due to a lack of targeted-therapy options and its...
unfavorable biology, TNBC continues to be associated with poor outcome. Novel biomarkers and potential new therapy approaches are therefore urgently needed. The aim of our study was for the first time (i) to investigate PTK7 expression in TNBC by IHC, (ii) to correlate PTK7 expression with clinicopathological features, (iii) to evaluate the impact of PTK7 expression according to (neo-)adjuvant therapy and (iv) to identify a possible predictive role of PTK7 regarding resistance to anthracycline-based chemotherapy.

Materials and Methods

Patient population and data collection. For this retrospective study, we searched consecutive patient records (1999 to 2010) at our institution (Red Cross Women’s Hospital, Munich, Germany); 133 patients with primary TNBC were included. This study was approved by the local Ethics Committee (aapproval number 422-11). TNBC was defined as negative ER and PR status, as assessed by an immunoreactive score (IRS) (18) and negative HER2 (according to American ASCOCAP Guidelines) (19). Stage-adapted breast/axillary surgery was performed. Patients were treated by radiotherapy according to current national guidelines. Clinicopathological features, type of chemotherapy and follow-up were recovered from patient records. The chemotherapy regimen was determined according to guidelines at the time of diagnosis. Taxanes were more frequently included in recent years: Out of the 133 patients, 101 (75.9%) received chemotherapy [16 neoadjuvant (NACT), 85 adjuvant]; 32 (24.1%) patients did not receive chemotherapy based either on patient preferences or due to old age or early stage of disease. Typical chemotherapy regimes were either anthracycline-based (Epirubicin/Cyclophosphamide, Doxorubicin/ Cyclophosphamide, 5-FU/Epirubicin/Cyclophosphamide, Fluorouracil/Doxorubicin/ Cyclophosphamide, 4-6 cycles), or taxane-based (Docetaxel/ Doxorubicin/Cyclophosphamide, Docetaxel/Carboplatin, 4-6 cycles), or 6 cycles of Cyclophosphamide/Methotrexate/Fluorouracil (CMF). In patients receiving NACT, PTK7 expression was measured both before and after NACT. In 18 cases, PTK7 expression was obtained in primary tumors and recurrent disease. In order to identify potential interactions between PTK7-expression and resistance to anthracyclines, we defined cohort A as patients receiving exclusively anthracycline-based chemotherapy (n= 62, 46.6%); cohort B as those receiving chemotherapy including agents other than anthracyclines (e.g. taxane-based and others; n=39, 29.3%); and cohort C as patients receiving no chemotherapy (n=32, 24.1%).

Immunohistochemistry. For our standardized PTK7 antibody staining procedure, we obtained 2-3 μm slices of formalin-fixed, paraffine-embedded tumor tissue. These sections were deparaffinized in xylene and rehydrated through graded concentrations of ethanol to distilled water. Endogenous peroxidase activity was blocked by incubating the sections in 3% hydrogen peroxide for 10 min at room temperature. Immunodetection of PTK7 was performed using the primary rabbit polyclonal antibody against PTK7 (Atlas Antibodies, Stockholm, Sweden) at a dilution of 1:200 and heat antigen retrieval (100°C, 6 min) in 10 mmol/l citrate buffer (pH 6.0) as a pre-treatment. Sections were then incubated with the horseradish peroxidase (HRP) one-step polymer (Zytochem, Berlin, Germany) and were visualized using 3,3’-diaminobenzidine (DAB, Biogenex, Fremont CA, USA) as chromogen, resulting in brown staining. Finally, the sections were counterstained with hematoxylin, dehydrated and mounted in Eukitt (VWR, Darmstadt, Germany). Immunohistochemical staining of PTK7 was independently evaluated by two pathologists in a double-blinded setting with negative controls. The level of expression was scored according to the criteria for IHC HER2 assessment. We defined a binary score from HER2 DAKO IRS by classifying DAKO-negative and borderline staining (0, 1+ and 2+) as PTK7-negative, and highly positive staining (3+) as PTK7-positive.

Statistics. For associations of categorical parameters, Chi-square and Fisher’s exact test were performed. Continuous variables were compared in two groups by the t-test or Mann-Whitney test where appropriate. Survival estimates (OS and DFS) were obtained using the Kaplan Meier method and compared by the log-rank test. Cox regression and time-varying survival analysis (non-proportional hazards) were performed. Significance is reported for two-sided p-values below 0.05 DFS and OS were defined in months from the date of initial surgery until progression (local/distant) and death from any cause, respectively. Patients lost to follow-up or without documented events were censored at last follow-up. Calculations were performed using SPSS (version 20, IBM Corporation, New York, USA).

Results

Patients’ characteristics and outcome. Clinical and histopathological patients’ characteristics are summarized in Table I. The median age at diagnosis was 56.5 years (range=27-87 years). At a median follow-up of 48.1 months, there were 29 deaths and 17 recurrences. Cohort C patients who did not receive any chemotherapy were older (median age=68.0 years) than patients in cohort A (median age=54.9 years) and cohort B (median age=49.5 years). Regarding tumor size, there were no significant differences between the cohorts. Comparing nodal status, cohort B patients more frequently had node-positive disease than did cohort A and C patients (p<0.001). In cohort C, significantly fewer patients had grade 3 tumors than in cohorts A and B (p=0.003). Estimated 5-year DFS and OS in patients with pT1 tumors was higher than in those with larger tumors (DFS 71.7% vs. 51.7%, p=0.031 and OS 91.3% vs. 57.0%, p<0.001), respectively. 5-Year DFS/OS rates in patients treated with chemotherapy were 66.6 %/75.3% versus 50.3%/74.4% without chemotherapy (p=0.184/0.854), respectively.

PTK7 expression and association with clinicopathological features. PTK7-positive expression, as defined above, was detected in 28.6% (38/133) of tumors. Expression of PTK7 was predominantly located in the cytoplasm. Membranous staining was also observed, particularly at the transition between normal and malignant tissue, and in tumors with high expression (Figure 1).

Comparing PTK7 expression with clinicopathological features, higher levels of PTK7 occurred more frequently in smaller tumors (≤2 cm); no further significant associations were detected (Table I).
PTK7 Expression and outcome. In the collective as a whole, no significant impact of PTK7 expression on DFS or OS was seen. To evaluate potential interactions of PTK7 with chemotherapy, we first compared DFS in cohort A vs. B (systemically-treated patients). As illustrated in Figure 2A, in time-varying survival analysis, DFS was significantly better in cohort A than B (p=0.025) within the first two years after initial surgery. These findings suggest a particular benefit from anthracyclines for prevention of early relapse in our TNBC cohort. DFS in patients receiving chemotherapy was investigated according to PTK7 status: A non-significant trend towards better DFS for patients with PTK7-negative tumors was observed (p=0.452) (Figure 2B). To evaluate the hypothesis of anthracycline resistance in patients with PTK7-positive tumors, we assessed survival times of patients treated by exclusively anthracycline-based chemotherapy (n=62) according to PTK7 expression. A substantial though non-significant difference in 5-year DFS between PTK7-positive (n=18) and PTK7-negative cases (n=44) (53.8% vs. 76.0%) was observed (Figure 2C).

Discussion

PTK7 is up-regulated in various malignancies including gastric cancer (12), lung cancer (15), and AML. PTK7 knockdown or silencing assays in different cancer cell-lines resulted in growth inhibition, reduced cell migration, reduced proliferation and invasiveness, and increased apoptosis (9, 11, 14). These data offer a rationale for targeted therapy in PTK7-overexpressing cancer. In our study, IHC PTK7 expression in TNBC was evaluated for the first time. We correlated PTK7 expression with clinicopathological parameters and patient outcome and investigated whether PTK expression was related to anthracycline resistance. The only significant association between PTK7 expression and clinicopathological parameters was more frequent PTK7-positivity in patients with smaller tumors (≤2 cm). Whereas no impact of PTK7

Table 1. Clinicopathological parameters of patients and Protein tyrosine kinase 7 (PTK7) expression.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>N</th>
<th>Negative (%)</th>
<th>Positive (%)</th>
<th>Chi-square (2-sided)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age ≤50 years</td>
<td>43</td>
<td>28 (65.1)</td>
<td>15 (34.9)</td>
<td></td>
</tr>
<tr>
<td>Age &gt;50 years</td>
<td>90</td>
<td>67 (74.4)</td>
<td>23 (25.6)</td>
<td>1.241 0.265</td>
</tr>
<tr>
<td>Tumor size pT ≤2cm (T1)a</td>
<td>71</td>
<td>49 (69.0)</td>
<td>22 (31.0)</td>
<td></td>
</tr>
<tr>
<td>pT &gt;2cm (T2-4)a</td>
<td>46</td>
<td>40 (87.0)</td>
<td>6 (13.0)</td>
<td>4.936 0.026</td>
</tr>
<tr>
<td>Grade pG 1a</td>
<td>3</td>
<td>3 (100.0)</td>
<td>0 (0.0)</td>
<td></td>
</tr>
<tr>
<td>pG 2a</td>
<td>35</td>
<td>25 (71.4)</td>
<td>10 (28.6)</td>
<td></td>
</tr>
<tr>
<td>pG 3a</td>
<td>79</td>
<td>61 (77.2)</td>
<td>18 (22.8)</td>
<td>1.415 0.493</td>
</tr>
<tr>
<td>Invasive ductal</td>
<td>101</td>
<td>73 (72.3)</td>
<td>28 (27.7)</td>
<td></td>
</tr>
<tr>
<td>Invasive medullary</td>
<td>23</td>
<td>18 (78.3)</td>
<td>5 (21.7)</td>
<td></td>
</tr>
<tr>
<td>Other histological type</td>
<td>9</td>
<td>4 (44.4)</td>
<td>5 (55.6)</td>
<td>3.773 0.152</td>
</tr>
<tr>
<td>Node-negative (pN)a</td>
<td>81</td>
<td>64 (79.0)</td>
<td>17 (21.0)</td>
<td></td>
</tr>
<tr>
<td>Node-positive (pN)a</td>
<td>36</td>
<td>25 (69.4)</td>
<td>11 (30.6)</td>
<td>1.253 0.263</td>
</tr>
<tr>
<td>Patients in Cohort A</td>
<td>62</td>
<td>44 (71.0)</td>
<td>18 (29.0)</td>
<td>n.a.</td>
</tr>
<tr>
<td>Cohort B</td>
<td>39</td>
<td>28 (71.8)</td>
<td>11 (28.1)</td>
<td>n.a.</td>
</tr>
<tr>
<td>Cohort C</td>
<td>32</td>
<td>23 (71.9)</td>
<td>9 (28.1)</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

*pPatients treated with preoperative chemotherapy (n=16) were excluded from statistical analyses; n.a. Not applicable.

PTK7 expression differences before and after NACT and in recurrent disease. In 16 patients, we assessed PTK7 expression before and after NACT. In four patients after NACT, pathological complete remission (pCR) was achieved; out of these, two were primarily PTK7-positive. In the remaining cases, PTK7 expression after NACT was either comparable to the pre-therapeutic staining or weaker. No increase in staining was seen (data not shown). In the 18 cases with primary tumor and recurrence, no systematic trend of PTK7 expression change between primary tumor and recurrence was seen (PTK7 expression: 13 cases with no difference; one case with weaker PTK7 expression; 4 cases with stronger staining).
expression was found in the TNBC collective as a whole, we detected a trend towards poorer survival in patients with PTK7-positive disease treated by chemotherapy.

In the literature, a multifaceted picture has emerged regarding the clinical relevance of PTK7. For example, PTK7 expression was associated with good prognosis in gastric and pulmonary adenocarcinoma. In gastric cancer, PTK7 expression assessed by IHC was an independent prognostic factor for favorable DFS and OS (12). Endoh et al. (15) calculated a prognostic risk index in pulmonary adenocarcinoma as a weighted combination of gene expression levels. Interestingly, the PTK7 coefficient was negative, i.e. high PTK7 gene expression was associated with good prognosis. In liposarcoma, high PTK7 expression was related to shorter 3-year distant relapse-free survival (14). In AML, PTK7 was also associated with poorer leukemia-free survival and OS (11) in univariate and multivariate analysis. Moreover, since leukemia-free survival in anthracycline-treated AML patients was significantly reduced in cases of PTK7 overexpression, resistance induction was hypothesized for PTK7. In PTK7-positive cell lines, this clinical discovery was substantiated showing higher cell survival and resistance to apoptosis induced by doxorubicin (11). We observed a (non-significant) trend towards poorer survival in patients with PTK7-positive tumors treated with anthracycline-based chemotherapy, i.e. consistent with this hypothesis. If PTK7 was indeed able to induce anthracycline resistance, this would have major clinical consequences, because anthracyclines currently represent the backbone of breast cancer chemotherapy, especially in TNBC. Even though a significant difference was not identified, our results provide the first evidence for PTK7 in breast cancer, particularly TNBC. However, as existing literature shows, an important role of PTK7 in breast cancer, especially TNBC, is likely and therefore further investigations are warranted. Identification of anthracycline resistance in breast cancer will require either larger sample sizes or longer follow-up. In particular, future studies should be powered to examine subgroups, due to the heterogeneous nature of TNBC. The optimal determination method and scoring for PTK7 expression also deserve further investigation. To our knowledge, this is the

Figure 2. A: Comparing disease-free survival (DFS) with respect to applied chemotherapy regimen. Exclusively anthracycline-based scheme (cohort A) versus taxane-based or other schemes (cohort B). In time-varying survival analysis, DFS was significantly better in Cohort A than B (p=0.025) within the first two years after initial surgery. These findings suggest a particular benefit from anthracyclines for prevention of early relapse in our triple negative breast cancer cohort. B: Comparing DFS with respect to Protein tyrosine kinase 7 (PTK7) expression in patients treated with chemotherapy (cohort A+B). Patients with PTK7-negative tumor tended to have better DFS (p=0.452). C: In patients treated exclusively with anthracycline-based therapy (cohort A), PTK7 expression seemed to be associated with worse DFS, although not significantly (p=0.203).
first study reporting on PTK7-expression in breast cancer focusing on TNBC as well as the clinical implications of PTK7. Due to having a small collective of only 133 TNBC cases, our findings can only be regarded as hypothesis-generated. Yet, considering the multi-faceted role of PTK7 in WNT signaling, our results underline the fact that further studies elucidating the clinical impact of PTK7 are needed.

Conflicts of Interest

P. Knyazev is listed as inventor on the European Patent Application No. 10075169.2 with the title "PTK-7 protein involved in breast cancer". Much of this work was conducted at our hospital in collaboration with the Ludwig Maximilian University Munich; some of the results reported here are included in the doctoral thesis of Regina Angerer (LMU).

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References


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