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Role of thioredoxin reductase 1 and thioredoxin interacting protein in prognosis of breast cancer

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Abstract

Introduction: The purpose of this work was to study the prognostic influence in breast cancer of thioredoxin reductase 1 (TXNRD1) and thioredoxin interacting protein (TXNIP), key players in oxidative stress control that are currently evaluated as possible therapeutic targets.

Methods: Analysis of the association of *TXNRD1* and *TXNIP* RNA expression with metastasis free interval (MFI) was performed in 788 patients with node-negative breast cancer, consisting of three individual cohorts (Mainz, Rotterdam and Transbig). Correlation with metagenes and conventional clinical parameters (age, pT stage, grading, hormone and ERBB2 status) was explored. MCF-7 cells with a doxycycline-inducible expression of an oncogenic ERBB2 were used to investigate the influence of ERBB2 on *TXNRD1* and *TXNIP* transcription.

Results: TXNRD1 was associated with worse MFI in the combined cohort (HR = 1.955; $P < 0.001$) as well as in all three individual cohorts. In contrast, TXNIP was associated with better prognosis (HR = 0.642; $P < 0.001$) and similar results were obtained in all three subcohorts. Interestingly, patients with ERBB2 status positive tumors expressed higher levels of *TXNRD1*. Induction of ERBB2 in MCF-7 cells did not only cause an immediate increase in *TXNRD1* but also a strong decrease in *TXNIP*. A subsequent upregulation of *TXNIP* as cells undergo senescence was accompanied by a strong increase in levels of reactive oxygen species.

Conclusions: *TXNRD1* and *TXNIP* are associated with prognosis in breast cancer and ERBB2 seems to be one of the factors shifting balances of both factors of the redox control system in a prognostic unfavourable manner.

Introduction

Control mechanisms of reactive oxygen species (ROS) play a crucial role in tumor development. Transformed cells are known to generate more ROS than normal cells [1, 2]. Importantly, ROS does not only contribute to tumor progression by amplifying genomic instability but transformed cells use ROS signals to drive proliferation [1]. On the other hand ROS addiction may render tumor cells more vulnerable to apoptosis or senescence [3, 4] because they depend on constantly increased basal levels of ROS and an additional increase may exceed toxic thresholds.

Thioredoxin reductase 1 (TXNRD1) and thioredoxin interacting protein (TXNIP, also called thioredoxin binding protein 2, TBP2 or vitamin D3-upregulated protein 1, VDUP1) [5], are key players in oxidative stress control. TXNRD1 reduces and activates thioredoxin, an oxidoreductase containing a dithiol-disulfide active site, which in turn reduces oxidised cysteine residues on cellular proteins. Importantly, a reducing environment mediated by thioredoxin is required for effective DNA binding of redox-sensitive transcription factors, including p53 and nuclear factor-kappa B [6, 7]. Thioredoxin binds ROS before they can harm cells and thus protects cells against oxidative stress. In addition to its critical role in the regulation of cellular redox homeostasis, thioredoxin has multiple actions in the cell, such as activation of ribonucleotide reductase (RR), inhibition of apoptosis signal regulating kinase 1 (ASK-1) and induction of HIF-1 and VEGF, that contribute to many hallmarks of cancer, like increased proliferation, inhibited apoptosis and angiogenesis [8]. In contrast to TXNRD1 that supports thioredoxin function, TXNIP binds to and inhibits the reduced form of thioredoxin [9-11], blocking its activity as well as its interaction with other factors, including ASK-1. Therefore, TXNIP functions as a pro-apoptotic

protein [12]. As for thioredoxin, multifunctional roles of TXNIP are known [13, 14] that point out the crucial role of TXNIP as a link between pathways of redox regulation, antioxidant defence, energy metabolism and cell growth and survival [15, 16]. A summary of the roles of these key players of the thioredoxin system is provided in Figure 1.

Recently, the thioredoxin redox system has been suggested as a therapeutic target for cancer therapy [17, 18] based on the observation that thioredoxin is overexpressed in many aggressive tumors and siRNA mediated knockdown of TXNRD1 decreased tumor progression and metastasis in mice [19]. Several inhibitors of the thioredoxin pathway have been shown to have antitumor activity in mice bearing breast, colon and renal xenografts [20-22]. In addition, high thioredoxin expression in prechemotherapy tumor samples has been reported to be associated with resistance to docetaxel in primary breast cancer [23, 24].

Despite of the ongoing evaluation of the thioredoxin system as a therapeutic target and the central role of TXNRD1 and TXNIP in oxidative stress control little is known about their prognostic relevance. Increased expression of thioredoxin in human colorectal cancer is associated with decreased patient survival [25] whereas absence of thioredoxin expression in non-small cell lung carcinoma is associated with a better outcome [26]. Decreased TXNIP expression in patients with diffuse large B-cell lymphoma (DLBCL) has been shown to correlate with a poor prognosis [27]. However to our knowledge, TXNRD1 as well as TXNIP expression has not yet been analysed in relation to prognosis in breast cancer. In the present study we observed that high expression of TXNRD1 and low expression of TXNIP are associated with worse prognosis in breast cancer. Since both, higher TXNRD1 and lower TXNIP

expression were observed in ERBB2 status positive tumors we analysed whether ERBB2 can influence these factors using a MCF-7 cell line that allows conditional expression of an oncogenic version of ERBB2.

Materials and methods

Cultivation of MCF-7/NeuT cells and analysis of RNA expression patterns

The MCF-7/NeuT breast carcinoma cell line was obtained from ATCC, cultured at 37°C in a humidified 5% CO_2 air atmosphere and transfected with pINSpBI-NeuT/EGFP and pcDNA3Neo/rtTA2 as described in ref. [3]. Expression of NeuT, an oncogenic version of ERBB2, was induced by doxycycline (obtained as the hydrochloride salt from Sigma, Munich, Germany) at a final concentration of 1 µg/mL in all experiments. MCF-7/NeuT cells were exposed to doxycycline for periods of 0, 6, 12 and 24 h as well as 3 and 14 days. Three independent repeat experiments were performed. Between each repeat experiment the MCF-7/NeuT cells were cultivated for at least 2 passages. Cells were harvested using TRItidy G-Reagent (AppliChem GmbH, Darmstadt, Germany) and subsequently RNA was isolated according to the manufacturer's protocols and stored at -80°C. Before microarray analysis RNA integrity and concentration were examined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) using the RNA 6000 LabChip Kit (Agilent Technologies) according to the manufacturers instructions. Microarray analysis was conducted at the microarray core facility of the Interdisziplinäres Zentrum für klinische Forschung (IZKF) Leipzig, Germany (Faculty of Medicine, University of Leipzig). To create cDNA the High Capacity cDNA Reverse Transcription Kit was used (Applied Biosystems, Darmstadt, Germany). TaqMan technique was used for further gene expression analysis by quantitative real-time PCR and confirmation of microarray data. Ubiquitin C (UBC) was chosen as the reference gene and the untreated cells were taken as the controls. The expression assays used were Hs00824723_m1 (*UBC*), Hs00197750_m1 (*TXNIP*) and Hs00917067_m1 (*TXNRD1*) (Applied Biosystems, Darmstadt, Germany). The PCR conditions followed the standard

specifications recommended by Applied Biosystems. For calculations of relative gene expression we used the $2^{-\Delta\Delta Ct}$ method as described by Schug *et al.* [28].

Immunoblotting

Immunoblot analysis was performed as described by [3, 29]. The Neu antibody (sc-284, Santa Cruz Biotechnology, Heidelberg, Germany) and the p44/42 MAP kinase antibody (Cell Signaling #9102, MA, USA) were diluted at 1:1000 and incubated for 2 hours at room temperature. The phospho-p44/p42 MAP Kinase Tyr202/Thr204 antibody (Cell Signaling #9101) and the phospho-Akt Ser473 (Cell Signaling #9271) were diluted at 1:1000 and incubated overnight at 4°C. The phospho-p38 MAPK Thr180/Tyr182 (3D7) (Cell Signaling #9215) was diluted at 1:500 and incubated over night at 4°C. The Txnip (Vdup1) (Santa Cruz, sc-166234) antibody was diluted 1:250 and the Txnrd1 antibody (Abcam, ab 16840) was diluted 1:1000. Both were applied over night at 4°C. The β-actin antibody (Sigma Aldrich, Germany, clone AC-74) was diluted 1:5000 and incubated 30 min at room temperature. Horseradish peroxidase-conjugated secondary antibodies were obtained from Cell Signaling (anti-rabbit-HRP, #7074) or Sigma (anti-mouse-HRP, A9044) and diluted 1:10000 in 5%BSA/TBS Tween 20. Protein signals were detected by enhanced chemiluminiscence (PerkinElmer LAS, Rodgau-Jügesheim, Germany).

Detection of Malondialdehyde (MDA) as a thiobarbituric acid (TBA) or a N-methyl-2-phenylindole (NMPI) complex

Malondialdehyde (MDA) is a major degradation product of lipid hydroperoxides and widely used as a marker of lipid peroxidation. To estimate the content of MDA in a sample we performed two analytical methods: First we measured thiobarbituric acid reactive substances (TBARS) via HPLC according to the procedure described at [30].

Aqueous trichloroacetic acid in presence of hexane and butylated hydroxytoluene was used to homogenize the sample. Within this reaction a complex of MDA and 1,3 diethyl-2-thiobarbituric acid (DETBA) is formed. The chromophore of the MDA-DETBA adduct was then chromatographed on a HPLC column and quantified by fluorescence emission at 553 nm with an excitation at 532 nm. Second, we used a spectrophotometric assay based on a reaction of MDA with N-methyl-2-phenylindole (NMPI) (Bioxytech MDA-586, Oxis Research, CA, USA). One molecule of MDA reacts with 2 molecules of NMPI. The resulting stable carbocyanine dye shows a maximum absorption at 586 nm and was detected spectrophotometrically. For MDA estimation eight measurements of three independent experiments were performed.

Patient characteristics, tissue specimens and gene array analysis

Three recently described cohorts of 788 node-negative breast cancer patients who did not receive chemotherapy were analysed [31]. The combined cohort consisted of three individual cohorts, the Mainz (n=200), the Rotterdam (n=286) and the Transbig (n=302) cohorts. The Mainz study cohort [31] consists of 200 lymph node-negative breast cancer patients treated at the Department of Obstetrics and Gynecology of the Johannes Gutenberg University Mainz between 1988 and 1998 [31]. Patients of the Mainz cohort were all treated with surgery and did not receive any systemic therapy in the adjuvant setting. The established prognostic factors (histological grade, tumor size, age at diagnosis, and steroid receptor status) were collected from the original pathology reports of the gynecologic pathology division within our department. Patients' characteristics have been published by Schmidt et al. [31] and are described in Additional file 1. For all tumors, samples were snap frozen and stored at -80°C. Gene expression profiling of the patients' RNA was performed using the Affymetrix HG-U133A array and the GeneChip System as described [31]. These data

have been deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) and are accessible through GEO series accession number [GEO:GSE11121]. Results obtained from the Mainz cohort were validated in two previously published microarray data sets. Two breast cancer Affymetrix HG-U133A microarray data sets including patient outcome information were downloaded from the NCBI GEO data repository. The first data set, the Rotterdam cohort [32] represents 180 lymph node-negative relapse free patients [GEO:GSE2034] and 106 lymph node-negative patients that developed a distant metastasis. None of these patients had received systemic neoadjuvant or adjuvant therapy (Rotterdam cohort). The original data were recalculated to a TGT of 500. The second data set, the Transbig cohort consists of 302 samples from breast cancer patients that remained untreated in the adjuvant setting after surgery [33, 34]. GSM numbers of samples (from [GEO:GSE6532] and [GEO:GSE7390]) used for analysis are listed in the Supplementary Tables previously published by Schmidt et al. [31]. Raw .cel file data were processed by MAS 5.0 using a TGT of 500. Ethical approval for the analysis of RNA levels was required and granted by the local ethical committee (Landesärztekammer Rheinland-Pfalz, ref. 837.139.05(4797)).

Statistical analysis

Univariate and multivariate Cox models were applied to analyse a possible association between RNA expression (log2 transformed) of TXNRD1 and TXNIP and prognosis. Disease free survival (DFS) was computed from the date of diagnosis to the date of local recurrence of disease, or distant metastasis, or cancer of the contra lateral breast, or death from cancer. The metastasis free survival interval (MFI) was computed from the date of diagnosis to the date of diagnosis of distant metastasis. Patients who died of an unrelated cause were censored at the date of death. Survival

times were compared using Kaplan Meier plots and the Log-rank test. Since the frequency distributions of TXNRD1 and TXNIP did not perfectly match a normal distribution (Additional file 2) we used non-parametric tests for comparison of groups, such as the Mann-Whitney test for unpaired data. Dichotomization was performed as described by Schmidt et al. [35, 36]. Briefly, the cutpoints differentiating between high and low expression were identified in the combined cohort of patients from Mainz, Rotterdam and the Transbig group (Additional file 3). Similar bimodal distributions and cutpoints were observed in each individual cohort (Additional file 4). Therefore, the cutpoints of 10 (estrogen receptor), 12.6 (ERBB2) and 4.9 (progesterone receptor) could be used for all cohorts. Dichotomization was performed using these cutpoints as follows: ERBB2 status, estrogen receptor status, and progesterone receptor status in positive (ER and/or PR positive) and negative (both ER and PR negative). Concerning histological grading the patients were dichotomized in GIII *versus* GI and GII, pT stage in pT_2 and pT_3 (>2 cm) *versus* pT_1 (≤2 cm) (patient characteristics: Additional file 1). Correlations were analysed using the Spearman correlation test. All p-values are two-sided. As no correction for multiple testing was performed they should be regarded as descriptive measures. All analyses were performed using SPSS17.0.

Results

TXNDR1 and TXNIP are associated with prognosis in breast cancer

To study a possible association with metastasis free interval we analysed our cohort (Mainz cohort) of patients and validated the results in two previously published cohorts (Rotterdam and Transbig cohorts). All patients were node-negative and did not receive chemotherapy. In the Mainz cohort high *TXNRD1* RNA expression was associated with a higher hazard ratio (HR = 1.920; P = 0.032) of metastasis (Table 1). In contrast, *TXNIP* was associated with a decreased HR (HR = 0.598; P = 0.017) (Table 1). Similar results were obtained in the Rotterdam ,, the Transbig and the combined cohorts (Table 1).

Besides MFI, which captures the time between surgery and occurrence of a distant metastasis, disease free survival (DFS) and overall survival (OS) were documented in the Mainz cohort. DFS comprises metastasis as well as local recurrence or carcinomas at the contralateral breast, whereas OS represents the time interval until breast cancer specific death. Also when analyzing DFS and OS (Table 2) significant associations with TXNRD1 and TXNIP were obtained. Kaplan Meier analysis was used to visualize the association between metastasis free survival time and *TXNDR1* as well as *TXNIP* (Figure 2). The time interval until occurrence of metastasis was shorter for patients with *TXNRD1* expression higher than the median compared to patients with relatively low expression levels (Figure 2a). In contrast, patients with high *TXNIP* had longer MFI compared to patients with low *TXNIP* (Figure 2b). In conclusion, the influence of TXNRD1 and TXNIP could be confirmed in three independent cohorts of node-negative breast cancer patients.

TXNDR1 and TXNIP in relation to known biological motives in breast cancer

In recent years the genome wide search for markers predicting prognosis in breast cancer has led to a global picture in which three coordinates representing important biological processes have outstanding prognostic consequences [31, 35, 36]: (i) the proliferation metagene, consisting of a group of genes indicating transition from slow to fast proliferation, (ii) the B- and T-cell metagenes as markers for immune cell infiltration and (iii) estrogen receptor dependent genes. In order to understand the role of TXNRD1 and TXNIP we determined their relation to these three coordinates according to [37]. *TXNRD1* showed a positive (R = 0.465; P<0.001) (Figure 3a) and *TXNIP* an inverse (R = -0.367; P<0.001) correlation with the proliferation metagene (Figure 3b). In addition, *TXNRD1* showed an inverse correlation with the estrogen receptor metagene (R = -0.432; P<0.001) and a weakly positive correlation with the T-cell metagene (R = 0.230; P<0.001). TXNIP showed also a weakly positive correlation with the B-cell metagene (R = 0.278; P<0.001).

TXNRD1 is associated with ERBB2 status

In order to understand, whether TXNRD1 and TXNIP are independent from conventional clinical parameters, we performed a multivariate Cox analysis (Table 3). For this purpose the regression model is usually adjusted to age, pT stage, grading, hormone (estrogene and progesterone) receptor as well as ERBB2 status [38]. In multivariate analysis only *TXNIP* was independent from the clinical parameters (P = 0.037; HR = 0.654), whereas *TXNRD1* was not (Table 3). The reason for a lack of significance of *TXNRD1* in the multivariate regression model was its association with

higher pT stage (P<0.001), higher grading (P<0.001), negative hormone receptor status (P<0.001) and positive ERBB2 status (P<0.001) (Additional file 5).

Because of the high clinical relevance of ERBB2 its correlation with TXNRD1 seemed particularly interesting and was analysed in detail (Figure 4a). Higher *TXNRD1* expression in ERBB2 status positive compared to ERBB2 status negative patients was observed in the Mainz cohort. This result was confirmed in the Rotterdam and in the Transbig cohorts (Figure 4a). In contrast to TXNRD1 the association with ERBB2 status was much weaker for *TXNIP* (Figure 4b). A trend towards lower *TXNIP* expression in ERBB2 status positive patients was obtained that amounted to statistical significance only in the combined cohort with 788 patients (Figure 4b). In conclusion, *TXNRD1* showed a positive and *TXNIP* a trend towards an inverse association with ERBB2 status.

Other members of the thioredoxin pathway are also associated with prognosis in breast cancer

The findings above prompted us to investigate if other members of the thioredoxin pathway and downstream factors are also associated with prognosis of breast cancer. For this purpose we focused on the most prominent members of the thioredoxin pathway as summarized in Figure 1. The results demonstrate that not only expression of *TXNIP and TXNRD1*, the two factors influencing the thioredoxin pathway, but also downstream effectors show prognostic relevance in breast cancer (Table 4): Thioredoxin (*TXN*), the M2 subunit of ribonucleotide reductase (*RRM2*), peroxiredoxin 2 (*PRDX2*), hypoxia inducible factor 1 alpha (*HIF-1α*) and vascular endothelial growth factor (*VEGF*) were significantly associated with worse prognosis

in the combined cohort as well as in at least one of the studied cohort. The most convincing association was shown by *RRM2* with similar results in all three individual cohorts. RR depends on activation by thioredoxin in order to reduce ribonucleotides to deoxyribonucleotides and therefore supports DNA synthesis.

TXNRD1 and TXNIP depend on ERBB2 expression in MCF-7 cells

In order to analyse whether ERBB2 influences the levels of *TXNRD1* and *TXNIP* expression, we used an MCF-7 cell line that allows doxycycline dependent expression of an oncogenic version of ERBB2 (NeuT) by the Tet-on system [3, 4]. Addition of doxycycline to the culture medium resulted in overexpression of oncogenic ERBB2 to levels seen in overexpressing breast carcinomas (Figure 5a). Induced ERBB2 overexpression was accompanied by increased phosphorylation of ERK1/2 and AKT/PKB, illustrating that doxycycline induced ERBB2 is functional (Figure 5a). As previously observed [3, 4] overexpression of oncogenic ERBB2 also induced cell cycle arrest and premature senescence in MCF-7 cells, which is accompanied by several phenotypical changes as can be observed in Additional file 6. ERBB2-triggered premature senescence has been found to be mediated by the P21 protein [3] and *P21* mRNA levels were increased as early as 6h after onset of NeuT expression (Additional file 7). Interestingly, induction of ERBB2 influenced the expression of TXNIP and TXNRD1 in a way that was reproducible in three independent experiments: (i) the *TXNIP* transcript was immediately downregulated during the first 24 h after addition of doxycycline but recovered to initial levels at later time intervals (Figure 5b). (ii) The *TXNRD1* transcript was immediately upregulated after doxycycline addition and fluctuated during the observed period with levels remaining clearly high above the initial (Figure 5b). (iii) The TXNIP and TXNRD1

proteins showed the same trend than the corresponding transcripts, as assayed by immunoblotting. The onset of the up and downregulation appeared somewhat delayed compared to that of the transcripts (Figure 5c). In addition to this, reactive oxygen species (ROS), as evidenced by analysis of reaction of malonaldehyde (MDA) with 1,3 diethyl-2-thiobarbituric acid (DETBA) (TBARS assay) and with N-methyl-phenylindole (NPMI) (Figure 5d) as well as by a lipid peroxidation assay (LPO) (Additional file 8), were not increased at day 2 but clearly increased at day 14 after onset of NeuT expression. In conclusion, induced overexpression of ERBB2 caused alterations of *TXNRD1* and *TXNIP* expression, whereby *TXNRD1* was up and *TXNIP* downregulated. Both redox factors were influenced by ERBB2 in a way that in our patient cohorts was associated with worse prognosis.

Discussion

Recently, targeting thioredoxin reductase and thioredoxin has been suggested as a basis for cancer therapy, including breast cancer [22, 39, 40]. However, the prognostic role of TXNRD1 and TXNIP, key players of the thioredoxin system, in node-negative breast cancer has not yet been analysed. Therefore, we studied three cohorts of patients who have not been treated by chemotherapy. In order to understand the role of TXNRD1 and TXNIP for the natural history of breast cancer it seemed relevant to focus on untreated patients, because the thioredoxin system has been reported to influence sensitivity of tumor cells to chemotherapy [23, 24, 41]. We observed that high expression of *TXNRD1* was associated with worse prognosis, whereas high *TXNIP* expression was associated with better prognosis. These results obtained in our cohort, the Mainz cohort [31], could be confirmed in two independent, previously published cohorts, the Rotterdam [32] and the Transbig [33, 34] cohorts.

The inverse association of *TXNRD1* and *TXNIP* with MFI found in this study supports the hypothesis that the maintenance of an active thioredoxin system is advantageous to the tumor cells because it limits oxidative damage and enables them to survive. On the other hand the important ROS-independent roles of TXNIP and thioredoxin might as well account for the promotion of tumorigenesis. It is noteworthy that both factors have opposite effects on HIF-1α in a way that thioredoxin favors its increase and TXNIP its destabilization [42, 43]. Additional associations with MFI found for thioredoxin and downstream factors of the thioredoxin pathway, including *PRDX2*, *RRM2*, *HIF1A* and *VEGF*, confirm the importance of the thioredoxin system in breast cancer regardless of its ROS-related or unrelated roles.

To better understand the interrelation of TXNRD1 and TXNIP with biologically relevant processes in breast cancer, we compared their expression levels to previously published metagenes [31, 35, 36, 38]. The most striking correlations were observed with the proliferation metagene showing that *TXNDR1* is expressed strongly in fast proliferating tumors, whereas *TXNIP* is expressed at low levels, which is in accordance with published data [44-47]. This constellation fits to the negative prognostic influence of TXNRD1 and the favourable effect of TXNIP. In addition, the estrogen receptor metagene was inversely correlated with *TXNRD1*. *TXNRD1* also correlated with the T-cell metagene, which was surprising, because the latter was associated with better prognosis in our previous studies [31]. Thioredoxin is known to be secreted by leukocytes and may exhibit cytokine-like properties in the extracellular environment [48]. It has been a long controversial debate, whether immune cell infiltration leads to a better prognosis of breast cancer by an attack on the tumor cells or whether secreted cytokines may cause on adverse effect creating a microenvironment that favours tumor cell proliferation [31].

Multivariate analysis adjusted for the conventional clinical parameters serves to identify whether a new factor adds some independent prognostic information to the already established parameters. In the Transbig cohort, *TXNIP* was independent from the established clinical parameters, whereas *TXNRD1* was not. Of course a lack of influence in the multivariate Cox model does not exclude biological relevance. If several genes are responsible for progression of tumors from pT1 to pT4 it is likely that tumor stage and not one of the many genes influencing tumor stage will be influential in the multivariate regression model. In the case of *TXNRD1* associations were observed with tumor grade and pT stage, hormone receptor status and ERBB2 status. We found the latter association particularly interesting, because it might be

explained by an influence of overexpressed ERBB2 on *TXNRD1* expression levels. When we studied *TXNRD1* expression in ERBB2 status positive versus negative carcinomas, significantly higher levels were obtained for ERBB2 status positive tumors in all three individual study cohorts. On the other hand, the difference in *TXNRD1* expression levels was relatively small. However, this is not surprising, since an influence of ERBB2 will probably represent only one of several factors influencing *TXNRD1* expression.

Inducible expression systems with erbB2 in mice or cell lines have contributed a lot to our understanding of erbB2 associated mechanisms [49, 50]. Therefore, in order to understand whether ERBB2 causes the increase in *TXNRD1* expression or whether their association represents only an epiphenomenon of other primary events, we applied the MCF-7/NeuT cell line, which allows inducible expression of an oncogenic version of ERBB2 (NeuT) [3, 4]. Interestingly, switching on ERBB2 caused a clear increase in TXNRD1 mRNA and protein levels. A link between ERBB2 and *TXNRD1* expression has been established in rat cardiomyocytes [51], in which neuregulin 1β, a ligand of the ERBB receptor tyrosine kinase, upregulated the expression of thioredoxin and TXNRD1, among that of other genes involved in protection against oxidative stress, both at the mRNA and protein level. This supports the possibility that the immediate early increase in *TXNRD1* mRNA levels observed after NeuT overexpression in our MCF-7/NeuT cell system is a direct response to ERBB2 signalling. The high *TXNRD1* levels observed at relatively long periods (14 days) after NeuT induction could be due to the accumulation of reactive oxygen species at this later stage. Since it is known that 4-hydroxynonenal, one of the major products of lipid peroxidation, can increase *TXNRD1* mRNA levels *via* transcriptional activation of the NF-E2 related factor 2 (Nrf-2) signalling pathway and that TXNRD1 promoter

contains an ARE (antioxidant response element) [52] it is plausible that the observed accumulation of ROS in our cells at day 14 is contributing to the higher *TXNRD1* expression at this time point.

Surprisingly, TXNIP was also influenced by NeuT but followed a completely different kinetic with an initial downregulation of *TXNIP* transcript levels within 24 h and a later recovery at 14 days. The immediate decrease of *TXNIP* mRNA upon ERBB2 expression suggests again a direct link between both factors. To our knowledge, nothing has been reported so far concerning a direct influence of ERBB2 signalling on *TXNIP* mRNA expression. The later increase of *TXNIP* mRNA levels begins concomitant with the the G1 arrest of the MCF-7 cells as they undergo senescence [3]. Although the molecular mechanisms leading to the *TXNIP* biphasic behaviour remain unknown, it fits the inverse correlation of *TXNIP* with the proliferation metagene observed in our study and it agrees with the known function of the TXNIP gene as a tumor supressor [42].

The observed initial activation of *TXNRD1* and repression of *TXNIP* transcription by ERBB2 signalling independent of ROS in our MCF-7/NeuT cell model could be interpreted as part of an ERBB2-triggered survival program that intends to prevent later accumulation of ROS. Further, it suggests that TXNIP and TXNRD1 are ERBB2 effectors whose multiple cellular functions contribute to proliferation, apoptosis resistance, metabolic reprogramming and finally, to the hallmarks of ERBB2 positive breast tumors (Figure 6).

Accumulation of ROS in senescent MCF-7/NeuT cells (14 d) is accompanied by the recovery of *TXNIP* mRNA to initial levels , which is expected to negatively influence

the ROS-scavenging activity of thioredoxin . This could therefore partially explain increased ROS at this stage. Interestingly, mRNA expression of thioredoxin itself decreases slightly in MCF-7/NeuT cells at 14 days (Additional file 9). However many other factors are also likely to contribute to the late accumulation of ROS in senescent cells. This is under current investigation.

Conclusions

In conclusion, we have shown that high *TXNRD1* and low *TXNIP* expression are associated with worse prognosis in node-negative breast cancer. ERBB2 can influence expression levels of *TXNRD1* and *TXNIP* to shift balances in a prognostic unfavourable manner.

Abbreviations

TNXRD1: thioredoxin reductase 1; TXNIP: thioredoxin interacting protein; RR: ribonucleotide reductase; ASK-1: apoptosis signal regulating kinase 1; HIF-1: hypoxia inducible factor 1; VEGF: vascular endothelial growth factor; MFI: metastasis free interval; HR: hazard ratio; ROS: reactive oxygen species; MDA: malondialdehyde; TBARS: thiobarbituric acid reactive substance; DETBA: 1,3 diethyl-2-thiobarbituric acid; NMPI: N-methyl-2-phenylindole; LPO: lipid peroxidation assay; ARE: antioxidant response element; DFS: disease free survival; OS: overall survival; DLBCL: diffuse large B-cell lymphoma.

Competing interests

M. Gehrmann is employed by Siemens Healthcare Diagnostics Products GmbH, which is in the business of commercializing diagnostic products. The other authors declare that they have no competing interests.

Authors' contributions

CC and JGH contributed to study conception and design, analysis and interpretation of collected data and drafted and revised the manuscript critically for important intellectual content. DF, MS and LJM carried out the real time PCR experiments in MCF-7 cells. MS collected patient related tissue and data and contributed to the design of the study. MG carried out the gene array expression analysis of patient tissue and contributed to the design of the study. MH carried out the gene array expression analysis of MCF-7/NeuT cells. BG and WS characterized the MCF-7/NeuT cell system and performed the immunoblot assays. AS and CW carried out the lipid peroxidation assays. EF, KI and JR performed the statistical analysis. JIB and AS participated in study´s design and coordination and revised the manuscript for important intellectual content. All authors read and approved the final manuscript.

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Figure legends

Figure 1. A scheme of cellular functions of the cytoplasmic thioredoxin system. Thioredoxin reductase 1 (TXNRD1) reduces thioredoxin 1 (TXN) which in turn reduces oxidized cysteins in cellular proteins and scavenges peroxides by peroxiredoxins (PRDX) thus protecting cells against oxidative stress. TXN stimulates ribonucleotide reductase (RR) activity and supports DNA synthesis. TXN stimulates the transcriptional activity of NF-κB, AP-1 and SP-1 and elevates hypoxia inducible factor 1α (HIF-1α) protein levels, which in turn influence VEGF. TXN also binds to and inhibits the pro-apoptotic proteins apoptosis signal regulating kinase 1 (ASK-1) and PTEN. All these events promote cell growth, inhibit apoptosis and increase angiogenesis in tumors as reviewed in [8]. TXNIP binds and inhibits reduced TXN. TXNIP also interacts with several transcriptional co-repressors, like promyelocytic leukemia zinc-finger (PLZF), Fanconi anemia zinc-finger (FAZF) and histone deacetylase 1 (HDAC1) and inhibits transcription of cyclin A2, leading to reduced cell growth. TXNIP increases p16 and p27KIP1 protein levels leading to cell cycle arrest. TXNIP also associates with the von Hippel-Landau protein (pVHL) enhancing the interaction between pVHL and HIF1α to promote nuclear export and degradation of HIF1α. The roles of TXNIP in the immune system and in human metabolism are not yet fully understood. For reviews see [13] and [14].

Figure 2. Association of TXNRD1 and TXNIP with metastasis free survival time. High thioredoxin reductase 1 (TXNDR1) expression is associated with shorter metastasis free survival time **(a)**, whereas high thioredoxin interacting protein (TXNIP) expression is associated with longer metastasis free survival time **(b)**. The analysis included 788 patients with node-negative breast cancer who have not been treated by chemotherapy. Both, TXNDR1 and TXNIP were dichotomized at the

median. The log-rank test was used to assess statistical significance of the Kaplan-Meier plots.

Figure 3. Correlation of TXNRD1 and TXNIP with the proliferation metagene. Thioredoxin reductase 1 (TXNDR1) shows a positive correlation with the proliferation metagene **(a)**, whereas thioredoxin interacting protein (TXNIP) inversely correlates with the proliferation metagene **(b)**. The analysis includes carcinomas of 788 patients with node-negative breast cancer.

Figure 4. Association of ERBB2 status with TXNRD1 and TXNIP expression. Positive ERBB2 status is associated with significantly higher levels of thioredoxin reductase 1 (TXNDR1) expression **(a)**. A significant association was obtained in all three individual cohorts and in the combined cohort including 788 patients with node-negative breast cancer. In contrast to TXNDR1, a trend towards lower thioredoxin interacting protein (TXNIP) expression was obtained in ERBB2 status positive carcinomas **(b)**. However, this trend amounted to statistical significance only in the combined cohort.

Figure 5. Induction of ERBB2 (NeuT) by the Tet-on system in MCF-7/NeuT cells influences TXNRD1 and TXNIP. Oncogenic ERBB2 (NeuT) overexpression in MCF-7 cells, induced by exposure to doxycycline, triggers phosphorylation of ERK1/2 and AKT (PKB) as shown by immunoblotting **(a)**. ERBB2-mediated changes in the transcription of *TXNRD1* and *TXNIP* are demonstrated both by Affymetrix Gene Array data and qRT-PCR **(b)**. The gene array graphs display the mean values (± SD) of the Affymetrix probesets. The qRT-PCR graphs show a representative experiment of three with the mean value (± SD) of a triplicate amplification. TXNRD1 and TXNIP

protein levels were analyzed by inmmunoblotting. Bands of ~55 kDa for TXNRD1 and ~50 kDa for TXNIP were observed **(c)**. An increase in accumulation of ROS 14 days after onset of ERBB2 (NeuT) expression was shown by detection of MDA by a TBARS assay and a NMPI assay **(d)**. The results represent the mean (± SD) of three experiments, each measured eight times.

Figure 6. ERBB2-mediated altered expression of *TXNRD1* and *TXNIP* and its association with prognosis. ERBB2 mediates upregulation of *TXNRD1* expression and downregulation of *TXNIP* expression, which in turn are associated with bad prognosis in breast cancer.

Tables

Table 1. Association of *TXNRD1* and *TXNIP* RNA expression with metastasis free survival

	Prognostic factor	P-value	HR	95%-CI
Mainz cohort (n=200)	*TXNRD1* RNA expression	0.032	1.920	1.058-3.486
	TXNIP RNA expression	0.017	0.598	0.392-0.912
Rotterdam cohort (n=286)	*TXNRD1* RNA expression	0.004	1.755	1.196-2.576
	TXNIP RNA expression	<0.001	0.654	0.525-0.815
Transbig cohort (n=302)	*TXNRD1* RNA expression	0.021	1.692	1.084-2.642
	TXNIP RNA expression	0.013	0.639	0.448-0.910
Combined cohort (n=788)	*TXNRD1* RNA expression	<0.001	1.955	1.519-2.518
	TXNIP RNA expression	<0.001	0.642	0.554-0.743

TXNRD1 (thioredoxin reductase 1) RNA expression is associated with worse metastasis free survival (MFI) in patients with node-negative breast cancer in the univariate Cox analysis. In contrast, *TXNIP* (thioredoxin interacting protein) is associated with better MFI. Results obtained in the Mainz cohort could be validated in the Rotterdam and in the Transbig cohorts of node-negative breast cancer patients. Also in the combined cohort of 788 patients significant associations of TXNRD1 and TXNIP with MFI was obtained. HR: hazard ratio, 95%-CI: 95% confidence interval.

Table 2. Association of TXNRD1 and TXNIP with disease free survival and overall survival

	Prognostic factor	P-value	HR	95%-CI
Disease free survival	*TXNRD1* RNA expression	0.010	2.025	1.180-3.476
	TXNIP RNA expression	0.012	0.617	0.423-0.899
Overall survival	*TXNRD1* RNA expression	0.032	1.838	1.054-3.204
	TXNIP RNA expression	0.192	0.774	0.526-1.137

In patients from the Mainz cohort (n=200) of node-negative breast cancer in the univariate Cox analysis an association of *TXNRD1* and *TXNIP* RNA with disease free survival (DFS) and overall survival (OS) was observed. HR: hazard ratio, 95%-CI: 95% confidence interval.

Table 3. Multivariate analysis of the association between TXNRD1 and TXNIP RNA expression with metastasis free survival

Prognostic factors	P-value	HR	95%-CI
Age (<50 *vs* ≥50 years)	0.511	1.176	0.725-1.910
pT stage (≤2cm vs >2cm)	<0.001	3.526	1.866-6.661
Histological grade (Grade 3 vs. grade 1 and 2)	0.223	0.708	0.406-1.234
HR[1] (ER or PR) (negative *vs.* Positive)	0.865	0.951	0.529-1.707
ERBB2-2 status (positive *vs* negative)	0.449	1.276	0.679-2.399
TXNRD1 RNA expression	0.162	1.445	0.862-2.420
TXNIP RNA expression	0.037	0.654	0.439-0.974

The analysis was carried out in patients with node-negative breast cancer in the univariate Cox analysis (Transbig cohort n= 302). HR: hazard ratio, 95%-CI: 95% confidence interval.

[1]The hormone receptor status (HR) is positive as soon as one of both, the estrogen (ER) or the progesterone receptor status (PR), is positive.

Table 4. Association of *TXN*, *RRM2*, *PRDX2*, *HIF1A*, *VEGF* and *PTEN* RNA expression with metastasis free survival (MFI) in patients with node-negative breast cancer in the univariate Cox analysis

	Prognostic factor	P-value	HR	95%-CI
Mainz cohort (n=200)	*TXN* RNA expression	0.001	3.029	1.599- 5.738
	RRM2 RNA expression	0.001	1.598	1.205- 2.120
	PRDX2 RNA expression	0.066	1.950	0.957-3.975
	HIF1A RNA expression	0.568	0.870	0.540-1.403
	VEGFA RNA expression	0.485	1.206	0.713-2.042
	PTEN RNA expression	0.313	0.774	0.470-1.273
Rotterdam cohort (n=286)	*TXN* RNA expression	0.067	1.428	0.976-2.091
	RRM2 RNA expression	< 0.001	1.440	1.181-1.756
	PRDX2 RNA expression	0.234	0.767	0.496-1.187
	HIF1A RNA expression	0.003	1.545	1.160-2.058
	VEGFA RNA expression	0.907	0.982	0.724-1.332
	PTEN RNA expression	0.339	0.851	0.612-1.184
Transbig cohort (n=302)	*TXN* RNA expression	0.069	1.411	0.974-2.043
	RRM2 RNA expression	0.003	1.354	1.107-1.655
	PRDX2 RNA expression	0.687	1.129	0.627-2.033
	HIF1A RNA expression	0.024	1.464	1.052-2.038
	VEGFA RNA expression	0.045	1.393	1.007-1.926
	PTEN RNA expression	0.197	0.802	0.573-1.121
Combined cohort (n=788)	*TXN* RNA expression	< 0.001	1.755	1.436-2.145
	RRM2 RNA expression	< 0.001	1.469	1.298-1.663
	PRDX2 RNA expression	0.004	1.423	1.120-1.807
	HIF1A RNA expression	< 0.001	1.440	1.184-1.750
	VEGFA RNA expression	0.033	1.239	1.018-1.508
	PTEN RNA expression	0.243	0.885	0.720-1.087

In the Mainz cohort *TXN* and *RRM2* RNA expressions are associated with worse metastasis free survival (MFI). In the Rotterdam cohort *RRM2* and *HIF1A* are associated with MFI. In the Transbig cohort significant associations were found for *RRM2*, *HIF1A* and *VEGFA*. In the combined cohort of 788 patients a significant association of all factors with MFI, except PTEN, was obtained. HR: hazard ratio, 95%-CI: 95% confidence interval.

Additional files

Additional file 1

A pdf file containing a table that displays the clinicopathological characteristics of node-negative breast cancer patients from the Mainz, the Rotterdam and the Transbig cohorts.

Additional file 2

A pdf file showing frequency distribution of *TXNRD1* and *TXNIP* RNA expression in the combined cohort of 788 patients with node-negative breast cancer.

Additional file 3

A pdf file showing the frequency distributions of estrogen receptor, ERBB2 and progesterone receptor RNA expression in the combined cohort, allowing the identification of the cutpoints for dichotomization.

Additional file 4

A pdf file showing the frequency distributions of estrogen receptor, ERBB2 and progesterone receptor RNA in the three individual cohorts Mainz, Rotterdam and Transbig.

Additional file 5

A pdf file showing the association of *TXNRD1* and *TXNIP* RNA expression with the established clinical parameters age, pT stage, grading, hormone receptor as well as ERBB2 status in the combined cohort of 788 patients with node-negative breast cancer.

Additional file 6

A videoclip showing phenotypical alterations of MCF-/NeuT cells in their first three days after NeuT induction. The transition from the proliferating into the senescent state is evident.

Additional file 7

A pdf file showing upregulation of *P21* in doxycycline-induced, ERBB2 (NeuT)-overexpressing MCF-7 cells as assayed by qRT-PCR.

Additional file 8

A pdf file showing increase in the accumulation of ROS 14 days after onset of ERBB2 (NeuT) overexpression in doxycycline-induced MCF-7 cells, as determined by the lipid peroxidation assay LPO-586, Oxis research.

Additional file 9

A pdf file showing RNA levels of thioredoxin, assayed by Affymetrix gene arrays in MCF-7/NeuT cells.

Figure 1

Target Protein	Output
PRDX	ROS scavenging
RR	DNA synthesis
NF-κB, AP-1, SP-1	Activation of transcription factors
HIF-1α, VEGF	Angiogenesis
ASK-1, PTEN	Inhibition of apoptosis

Target Protein	Output
TXN	Inactivation
PLZF, FAZF, HDAC1	Modulation of transcription
P16, CDKs	Regulation of cell cycle
pVHL	Degradation of HIF-1α
?	Glucose and FA metabolism
?	Immune cell response

(a)
Metastasis free survival rate
1.0
0.8
0.6
0.4
0.2
0.0
TXNDR1 ≤ median
TXNDR1
> median
Combined cohort
N=788
P<0.001
0
2
4
6
8
10
12
14
Metastasis free survival time (years)

(b)
Metastasis free survival rate
1.0
0.8
0.6
0.4
0.2
0.0
TXNIP > median
TXNIP ≤ median
Combined cohort
N=788
P<0.001
0
2
4
6
8
10
12
14
Metastasis free survival time (years)

Figure 2

(a)
Proliferation metagene
2
1
0
-1
-2
R = 0.465
P<0.001
N = 788
9
10
10
11
11
12
TXNRD1 RNA expression
(b)
Proliferation metagene
2
1
0
-1
-2
R = -0.367
P<0.001
N = 788
10
11
12
13
14
TXNIP RNA expression

Figure 3

(a)
Mainz cohort (n=200)
Rotterdam cohort (n=286)
Transbig cohort (n=302)
Combined cohort (n=788)
TXNRD1 RNA expression
P=0.002
P=0.032
P<0.001
P<0.001
negative
positive
ERBB2 status
(b)
TXNIP RNA expression
P=0.320
P=0.241
P=0.059
P=0.031

Figure 4

(a)
MCF7/NeuT + Doxycycline
0h 6h 1d 3d 7d
NeuT
50 kDa
P-P44 (ERK-1)
P-P42 (ERK-2)
40 kDa
β-Actin
MCF7/NeuT + Doxycycline
0h 6h 1d 3d 7d
220 kDa
NeuT
60 kDa
P-AKT
60 kDa
AKT
42 kDa
β-Actin
(b)
TXNRD1 Gene Array
Fold Change Relative to t=0h
0h 6h 12h 24h 3d 14d
Time after onset of NeuT expression
TXNIP Gene Array
Fold Change Relative to t=0h
0h 6h 12h 24h 3d 14d
Time after onset of NeuT expression
TXNRD1 qRT-PCR
Fold Change Relative to t=0h
0h 6h 12h 1d 3d 7d 14d
Time after onset of NeuT expression
TXNIP qRT-PCR
Fold Change Relative to t=0h
0h 6h 12h 1d 3d 7d 14d
Time after onset of NeuT expression
(c)
MCF-7/NeuT + Doxycycline
0h 6h 12h 1d 3d 7d 10d 14d
60 kDa
50 kDa
TXNRD1
β-actin
40 kDa
MCF-7/NeuT + Doxycycline
0h 6h 12h 1d 3d 7d 10d 14d
60 kDa
50 kDa
TXNIP
β-Actin
40 kDa
(d)
TBARS (DETBA)
MDA [nmol/mg protein]
0 2 14
Time after onset of NeuT expression [d]
NPMI
MDA [nmol/mg protein]
0 2 14
Time after onset of NeuT expression [d]

Figure 5

ERBB2
P
P
TXN
S
S
TXNRD1
Reduced
thioredoxin
TXN
SH
SH
TXNIP
Poor prognosis

Figure 6

Additional files provided with this submission:

Additional file 1: Additional file 1_revised.pdf, 62K
http://breast-cancer-research.com/imedia/1559741928405535/supp1.pdf
Additional file 2: Additional file 2_revised.pdf, 41K
http://breast-cancer-research.com/imedia/4529311064055354/supp2.pdf
Additional file 3: Additional file 3_revised.pdf, 40K
http://breast-cancer-research.com/imedia/1436081024405535/supp3.pdf
Additional file 4: Additional file 4_revised.pdf, 93K
http://breast-cancer-research.com/imedia/9347618644055359/supp4.pdf
Additional file 5: Additional file 5.pdf, 107K
http://breast-cancer-research.com/imedia/1657243322405536/supp5.pdf
Additional file 6: Additional file 6_NeuT48(2)_0h_3d dox_3 divx.avi, 6905K
http://breast-cancer-research.com/imedia/1067346068405536/supp6.avi
Additional file 7: Additional file 7.pdf, 12K
http://breast-cancer-research.com/imedia/1837278784055363/supp7.pdf
Additional file 8: Additional file 8.pdf, 11K
http://breast-cancer-research.com/imedia/9628475594055372/supp8.pdf
Additional file 9: Additional file 9.pdf, 11K
http://breast-cancer-research.com/imedia/7080630141426139/supp9.pdf