Hypoxia

TRIB3 protein denotes a good prognosis in breast cancer patients and is associated with hypoxia sensitivity

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ABSTRACT

Background: Tribbles homolog 3 (TRIB3) is a pseudokinase involved in the regulation of several signaling pathways involved in cell survival and/or cell stress. Here, we determined the correlation between breast cancer prognosis and TRIB3 protein levels and established the role of TRIB3 in cell survival after hypoxia and/or radiotherapy.

Material and methods: TRIB3 mRNA and protein were quantified in a new independent breast cancer patient cohort using QPCR and a new specific avian antibody against TRIB3. In addition, we used siRNA-mediated knockdown of TRIB3 in a colony-forming assay after hypoxia and radiotherapy.

Results: TRIB3 mRNA and protein levels did not correlate in breast cancer cell lines or human breast cancer material. We validated our earlier finding that high TRIB3 mRNA denotes a poor prognosis, but found that high TRIB3 protein levels were associated with a good prognosis in breast cancer patients. We also show that knockdown of TRIB3 resulted in an increased survival under hypoxic conditions.

Conclusion: Whereas mRNA levels of TRIB3 are related with a poor prognosis, TRIB3 protein is associated with a good prognosis in human breast cancer patients, possibly due to the fact that TRIB3 is involved in hypoxia tolerance.

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TRIB3 is one of three human homologs of the Drosophila cell cycle inhibitor tribbles [1,2]. TRIB3 is induced by ER stress [3], hypoxia [4], and nutrient starvation [5], all of which are potentially related to treatment resistance. This cell stress induced upregulation of TRIB3 is mediated via the unfolded protein response (UPR), more specifically via the transcriptional regulators ATF4 and CHOP [6]. The UPR is likely to be involved in therapy resistance of tumor cells, especially under hypoxic conditions [7–11]. TRIB3 functions as a negative feedback regulator of the ATF4/CHOP pathway [6,12,13]. TRIB3 is also known to inhibit phosphorylation of AKT/Protein Kinase B (AKT) [14]. AKT is a phosphoinositide-dependent serine/threonine protein kinase that plays a critical role in the signal transduction of growth factor receptors. There is abundant evidence that the PI3-kinase/AKT pathway is central in determining the sensitivity of solid tumors to ionizing radiation, chemotherapy and possibly to endocrine therapy [15–19]. In addition, all three human homologs of tribbles bind mitogen-activated protein kinase kinases (MAPKKs) and regulate the activation of MAP-kinase pathways by serving as scaffold proteins [20]. These intracellular signaling pathways are relevant for tumor growth and treatment resistance [21,22]. However, how TRIB3 is involved in tumor progression and/or treatment resistance is unclear, as both cytoprotective [13] as well as pro-apoptotic roles of TRIB3 [6] have been described.

Recently, we found that TRIB3 mRNA denoted a poor prognosis in breast cancer patients (Wennemers et al., submitted), suggesting that the involvement of TRIB3 in the UPR and/or growth factor receptor signaling results in an association with disease progression and/or treatment resistance. Here, we set out to validate these results in an independent patient cohort, and to extent the data by specific immunohistochemical staining of TRIB3 protein and by siRNA mediated knockdown in radiation experiments.

Material and methods

Patients samples

Breast cancer patients were selected from a cohort surgically treated between January 1991 and December 1996 that had no axillary lymph node invasion, received no adjuvant systemic treatment, and had at least 5 years follow up or a recurrence before that. The selection criteria led to a patient cohort with 25% ER positive, 13% PR positive, 30% HER2-positive, and 44% triple negative patients. As approved by the institutional review board and according to national law, coded tumor tissues were used.
TRIB3 mRNA measurement

Total RNA was isolated and reverse transcriptase was performed as described earlier (Wennemers et al., submitted). For patient material an adjacent slice was H&E stained to determine tumor percentage. cDNAs were amplified with TRIB3-specific primers (forward: act cag cct cgc cgg a; reverse: agt atg cgg cga tgg tgg a) using SYBR Green Master Mix (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) on an ABI Prism 7700 Sequence detection system (Applied Biosystems). All samples were normalized for levels of hypoxanthine–guanine phosphoribosyltransferase (HPRT) expression.

TRIB3 antibody development

Chickens and rabbits were immunized with three different LPH conjugated peptides from different parts of the human TRIB3 protein sequence obtained from Biogenes GmbH (Berlin, Germany). Peptide sequences were peptide 1: DNLDTERPQKRARSGPQ, peptide 2: DNLDTERPQKRARSGPQ, and peptide 3: and DGLLDEAREEEGDEV. The experimental design was approved by the Animal Ethics Committee of the Radboud University Nijmegen and described earlier [23]. Polyclonal antibodies isolated from chicken egg yolks and citrate plasmas from rabbits were purified by affinity chromatography against the peptides in question. Further characterization was done with one side ELISA and Western blotting techniques.

TRIB3 Western blot analysis

Breast cancer cells (LGC Promochem, London, UK) were harvested in RIPA buffer with phosphatase and protease inhibitors (Roche, Indianapolis, IN). Thirty microgram of protein was fractionated on 12% Criterion XT Bis–Tris gels (Bio-Rad Laboratories B.V., Veenendaal, The Netherlands). Samples were transferred to PVDF membranes (Millipore Immobilon, Millipore, Bedford, MA), and incubated with chicken anti-TRIB3 peptide 3 or rabbit-anti-β-actin IgG (#4970, Cell Signaling Technology, Beverly, MA). Proteins were detected using chemiluminescent peroxidase substrate (Sigma–Aldrich, St. Louis, MO, USA) and visualized with a ChemiDoc XRS + imaging system in combination with Quantity One® 1-D Analysis Software (Bio–Rad Laboratories B.V.).

Tissue microarray

Hematoxylin and eosin (HE) stained 4 μm sections of formalin-fixed paraffin-embedded tumor sections were used to identify tumor areas by an experienced pathologist. The tumor areas were outlined on the glass slides and used to select the appropriate area in the tissue blocks to punch out tissue (2 mm ø) for the tissue microarrays (TMA) using a Tissue-Tek Quick Ray puncher (Sakura Finetek Europe B.V., Leiden, The Netherlands). Four μm thick sections of the TMA were incubated with chicken anti-TRIB3, and donkey anti-chicken biotin labeled secondary antibody (Jackson ImmunoResearch Europe Ltd., Suffolk, UK), followed by incubation with ABC reagent and visualization with DAB reagent (Vector Laboratories Ltd., Peterborough, UK). Sections were counterstained with Mayer’s hematoxylin solution. Negative control included substitution of the primary antibody with primary antibody diluent (PAD, AbdSerotec, Oxford, UK).

Colony forming assay

A colony forming assay was performed to determine cell survival [24]. Knockdown of TRIB3 was performed in MDA-MB-231 cells using siRNA transfection reagent SAINT-RED (Synvolux Therapeutics B.V., Groningen, The Netherlands), siRNA’s MISSION® si-NA Universal Negative Control #1 (SASL_Hs01_00197511) and TRIB3 (2) (SASL_Hs01_00197513) were acquired from Sigma–Aldrich. After transfection the cells were seeded in 6-well plates and normoxic or hypoxic incubation (0.1% O2, H35 hypoxystation, Don Whitley Scientific Ltd., West Yorkshire, UK) started 24 h before radiation with 4 Gy. Before irradiation, cells were sealed under hypoxia, therefore, the hypoxic status of the cells was unchanged during irradiation. Directly after irradiation cells were transferred back into the hypoxic chamber. Hypoxic incubation continued for 24 h, after which all plates were cultured under standard culture conditions for 1 week to divide and form colonies. After fixation with 70% EtOH the cells were stained with 0.5% w/v Crystal Violet and colonies were counted using a ChemiDoc XRS + imaging system in combination with Quantity One® 1-D Analysis Software (Bio–Rad Laboratories B.V.).

Statistical analysis

Statistical analyses were carried out using SPSS 16.0 software (SPSS Benelux BV, Gorinchem, The Netherlands). Normality of distribution of variables was tested using Kolmogorov–Smirnov testing. Equality of distribution of patient characteristic, biological markers and treatment among different TRIB3 groups was tested using the χ² test. Non-parametric correlations were established using Spearman Rank correlation testing. Disease free survival (DFS) time (defined as the time from surgery until diagnosis of recurrent or metastatic disease) and overall survival (OS) time (defined as the time between date of surgery and death by any cause) were used as follow-up endpoints. Survival curves were generated using the method of Kaplan and Meier. Equality of survival distributions was tested using log-rank testing and using Cox univariate regression analyses. Two-sided P-values below 0.05 were considered statistically significant.

Results

TRIB3 mRNA correlates with breast cancer prognosis

To validate our earlier results, TRIB3 mRNA expression levels were measured in an independent patient cohort. In these patients (n = 95), TRIB3 mRNA levels were significantly higher in tumors with a triple negative phenotype (P = 0.004) compared to the other breast cancer subtypes. After dichotomizing patients based on the median expression level of TRIB3 mRNA in the tumor, the group with low TRIB3 mRNA expression had a DFS of 133 (CI = 121–144) months whereas the group with high TRIB3 mRNA expression had a DFS of 109 (CI = 92–125) months (P = 0.016) (Supplementary Fig. 1).

Development of TRIB3 antibody

Commercially available antibodies for TRIB3 showed multiple, non-specific, bands on Western blot of both cell lines and tumor tissue (Supplementary Fig. 2A). Therefore, we developed our own TRIB3 antibodies. Three pairs of chickens and three pairs of rabbits were immunized with three different peptides. Primary characterization of all antibodies was done using one-sided ELISA with plates coated with original unconjugated peptides and none of the peptides showed a cross reaction with the other peptides used (Supplementary Fig. 2B). Antibodies against peptide 2 gave lower titers than peptides 1 and 3 in all animals. After affinity purification, the antibodies against peptides 1 and 3 were tested according to a previously described two-sided ELISA format [25]. A commercially available TRIB3 lysate was used as a standard. These ELISA’s generated clear dose–response curves (Supplementary Fig. 2C). Further specificity testing was performed using Western blotting
techniques. The antibody from the chicken immunized with peptide 3 gave a clear and predominant band at 48 kDa (Supplementary Fig. 2D). This antibody was further used for immunohistochemical staining and Western blot analyses to determine TRIB3 protein levels.

Expression levels of protein and mRNA of TRIB3 do not correlate

With the availability of an antibody for TRIB3 we tested the correlation between TRIB3 mRNA and protein expression in six human breast cancer cell lines using QPCR, and Western blotting (Fig. 1A). No correlation was found between RNA and protein levels of TRIB3 (Fig. 1B). Next, we quantified TRIB3 protein expression levels in our cohort of human breast cancer material on the TMA and compared this with TRIB3 mRNA expression levels. TRIB3 protein expression was scored as negative (n = 45), weakly positive (n = 58) or strongly positive (n = 18) (Fig. 2). Here, TRIB3 protein levels did also not correlate with TRIB3 mRNA levels (Fig. 1C, correlation coefficient −0.042, P = 0.684).

TRIB3 protein expression is correlated with breast cancer prognosis

Considering the poor correlation between RNA and protein levels, we assessed whether TRIB3 protein levels were of relevance in breast cancer patients. Patient characteristics, biological markers and treatment distribution among the three different groups of TRIB3 protein expression, negative, weakly positive, or strongly positive, were similar for most parameters tested (Supplementary Table 1). Only a relation of TRIB3 protein with smaller tumor size was found (X² P = 0.018). Surprisingly, TRIB3 protein was related to a good prognosis, which was in direct contrast to the RNA data we found earlier and have validated above; patients with negative TRIB3 protein scoring had a mean DFS of 94 (CI = 81–108) months, with weakly positive scoring had a mean DFS of 108 (CI = 92–123), whereas the group with a strongly positive expression had a mean DFS of 159 months (CI = 135–182, P = 0.014, Fig. 3A). Similarly, patients with high TRIB3 protein staining tended to exhibit a better overall survival (P = 0.058, Fig. 3B). These results were independent of tumor size, as the prognostic value of TRIB3 protein remained significant after correction for tumor size in a multivariate Cox regression (not shown).

Knockdown of TRIB3 results in improved hypoxia survival of breast cancer cells

To explore the role of TRIB3 in radiotherapy and/or hypoxia resistance we performed a colony forming assay using human breast cancer cells after siRNA mediated TRIB3 knockdown. Surviving fractions were corrected for plating efficiencies of the normoxic non-irradiated cells (control siRNA 45%, TRIB3 siRNA 1 36% and TRIB3 siRNA 55%). In MDA-MB-231 cells 4 Gy of X-rays lead to a surviving fraction of 29% under normoxic conditions. In addition, 48 h of hypoxia lead to a surviving fraction of 6%, which was further reduced to 1% when combined with a single fraction of 4 Gy. After transfection with two different siRNA’s against TRIB3 the survival rates exhibited a drastic increase, most notably during hypoxia (Fig. 4). After TRIB3 knockdown, the survival fractions were 43% and 30% after 4 Gy under normoxia, whereas under hypoxia 70% and 87% cell survival was observed, respectively. Survival of cells with TRIB3 knockdown after 4 Gy under hypoxic conditions increased to 24% and 37%, respectively.

Discussion

Here, we validated our earlier results that TRIB3 mRNA expression denotes a poor prognosis, but unexpectedly also found that TRIB3 protein – as quantified with a self-developed, highly specific antibody – denoted a good prognosis in human breast cancer patients. siRNA mediated knockdown experiments show that this is
due to a role of TRIB3 in hypoxia sensitivity. Thus, tumors that express high levels of TRIB3 protein will be more sensitive to hypoxia, whereas low TRIB3 expressing tumors are more hypoxia tolerant. Hypoxia tolerance is an important aspect of tumor progression and treatment efficacy as hypoxia tolerant tumors are less sensitive to different treatments in breast cancer such as radiotherapy, which is at least partly under control of the (TRIB3 inducing) UPR [7].
The protein levels of TRIB3 are regulated by both transcriptional and post-transcriptional mechanisms. TRIB3 mRNA is induced by the UPR and involves the transcription factor ATF4 [6,26]. In addition, the NFKβ and PI3K pathways induce transcription of TRIB3 [5,26]. Of note, TRIB3 exerts a strong negative feedback loop on the ATF4, NFKβ and PI3K pathways [12–14,27]. Further, mRNA levels are stabilized by hypoxia-regulated HuR [26]. Interestingly, during anoxia in vitro protein levels of TRIB3 increase earlier than mRNA levels [4,27]. This indicates that not only transcriptional but also additional hypoxia-associated translational mechanisms regulate TRIB3 protein levels on a relatively short time scale. Thus the TRIB3 protein levels, as found in human breast cancer tissue, are the result of a number of regulatory feedback loops and temporally distinct events. The discrepancy in results considering the described cytoprotective [13] as well as pro-apoptotic roles of TRIB3 [6], might, consequently, be caused by the fundamentally different correlations of TRIB3 mRNA and protein with tumor phenotype. Furthermore, the fact that TRIB3 is responsible for the set point in several regulatory feedback loops could also contribute to these discrepancies.

A great number of studies have used QPCR and/or expression profiling to investigate the role of particular genes in prognosis or treatment sensitivity of human breast cancer, most notably the Oncotype DX and Mammaprint assays, respectively [27]. Our results presented here indicate that these data on mRNA levels do not necessarily translate to protein, nor to function, of all genes. Our mRNA data results, with TRIB3 mRNA being correlated with poor prognosis, should be considered indicative of a process that both stimulates TRIB3 mRNA transcription and/or mRNA stability, and is associated itself with a poor prognosis. However, caution should be given that the association of gene ontologies with prognosis or treatment sensitivity cannot function as proof of a functional relation. As such, much more credence can be given to studies into the association of (phospho)proteins with prognosis [28].

In conclusion, whereas mRNA levels of TRIB3 are related with a poor prognosis, TRIB3 protein is associated with a good prognosis in human breast cancer patients, apparently due to the fact that TRIB3 is involved in hypoxia and treatment tolerance. Our results on TRIB3 described here suggest it to be an interesting target for sensitizing cancers to cancer therapy, because modifying the set point of different intracellular signaling pathways could drastically change the survival balance of the tumor cells under stressful microenvironmental circumstances or during treatment.

Conflict of interest statement

The authors declare no conflict of interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.radonc.2011.05.057.

References