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PRECLINICAL STUDY

# Both t-Darpp and DARPP-32 can cause resistance to trastuzumab in breast cancer cells and are frequently expressed in primary breast cancers

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**Abstract** The clinical use of trastuzumab (Herceptin$^{TM}$), a humanized antibody against the HER2 growth factor receptor, has improved survival in patients with breast tumors with ERBB2 amplification and/or over-expression. However, most patients with advanced ERBB2 amplified breast cancers whose tumors initially respond to trastuzumab develop resistance to the drug, leading to tumor progression. To identify factors responsible for acquired resistance to trastuzumab, gene expression profiling was performed on subclones of an ERBB2 amplified breast cancer cell line, BT474, which had acquired resistance to trastuzumab. The most overexpressed gene in these subclones was PPP1R1B, encoding the DARPP-32 phosphatase inhibitor. Western analysis revealed that only the truncated isoform of the DARPP-32 protein, t-Darpp, was overexpressed in the trastuzumab resistant cells. Using gene silencing experiments, we confirmed that t-Darpp over-expression was required for trastuzumab resistance in these cells. Furthermore, transfecting t-Darpp in parental BT-474 cells conferred resistance to trastuzumab, suggesting that t-Darpp expression was sufficient for trastuzumab resistance. We also found that t-Darpp over-expression was associated with Akt activation and that the T75 residue in t-Darpp was required for both Akt activation and trastuzumab resistance. Finally, we found that full-length DARPP-32 and t-Darpp are expressed in a majority of primary breast tumors. Over-expression of full-length DARPP-32 can also confer resistance to trastuzumab and, moreover, is associated with a poor prognostic value in breast cancers. Thus, t-Darpp and DARPP-32 expression are novel prognostic and predictive biomarkers in breast cancer.

**Keywords** t-Darpp · DARPP-32 · Trastuzumab · Resistance · Breast cancer

## Introduction

Breast cancer is by far the most common cancer in women, with a lifetime risk of about 1 in 8. Mortality from breast cancer has been falling over the last decades, in part due to advances in drug therapy [1]. Recently, the development of targeted therapies has ushered in a new era of "smart drugs" with promising rates of breast tumor response. These targeted therapies were made possible by the discovery of the molecular characteristics of breast tumors. One of the more important of these involves the epidermal growth factor receptor (EGFR) family, especially EGFR itself and human epidermal growth factor receptor 2 (HER2/ERBB2), which play critical roles in regulating breast tumor growth and apoptosis [2]. The ERBB2 gene is amplified and the receptor over expressed in ~20–30% of breast tumors [3]. The first monoclonal antibody approved for the treatment of

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a solid tumor, trastuzumab (Herceptin$^{TM}$), is a humanized antibody against the HER2 growth factor receptor [4]. Recent clinical studies showed that the administration of trastuzumab in both early [4] and metastatic [5] breast cancer patients has remarkable anti-tumor activity. However, about 15% of breast cancer patients with early, micrometastatic breast cancer [4] develop recurrence after taking trastuzumab, and almost all advanced breast tumors eventually progress while receiving trastuzumab, i.e., the duration of response of growth inhibition is limited to months. Resistant tumors are also more refractory to other breast cancer treatments.

Several studies have begun to investigate the mechanism of resistance to trastuzumab. Molecular factors responsible for insensitivity to trastuzumab include down-regulation of p27 [6], loss of phosphatase and tensin homolog (PTEN) activity [7], activation of insulin-like growth factor I receptor (IGF-1R) [8], upregulation of transforming growth factor alpha (TGF$\alpha$) [9] and increase in EGF receptor and ligands [10]. Most of these studies focused primarily on the factors responsible for intrinsic resistance to trastuzumab. Chan et al. [11] have described a model for acquired resistance to trastuzumab in a breast cancer cell line that contains ERBB2 gene amplification, BT-474. BT-474 cells are known to be sensitive to trastuzumab and to express the estrogen receptor (ER) [12]. They do not harbor mutations in PTEN, Ras or B-raf [12]. BT-474 cells were grown in the presence of 1.0 μM trastuzumab for 6 months until several resistant subclones grew out. In the initial characterization of these BT/Her$^{R}$ clones, Chan et al. [11] showed that HER2 receptor expression and phosphorylation levels were unchanged, but phosphorylated AKT and Akt kinase activity were constitutively high and insensitive to the presence of trastuzumab.

We now report the further analysis of these clones and show that t-Darpp, the truncated form of the DARPP-32 (Dopamine and cAMP regulated phosphoprotein of 32 kDa) protein encoded by the PPP1R1B gene, is necessary for trastuzumab resistance in at least some BT/Her$^{R}$ clones and sufficient for conferring resistance to trastuzumab in two different Her2-positive breast cancer cell lines. These results independently support a recent report by Blekhiri et al. [13], which also implicated t-Darpp in resistance to trastuzumab in a mouse xenograft model of breast cancer. However, we further demonstrate that the T75 phosphorylation site appears to be necessary for t-Darpp's function in conferring resistance to trastuzumab. We also found that full-length DARPP-32 and t-Darpp are expressed in a majority of primary breast tumors, and over expressed especially in HER2 amplified breast tumors. Like t-Darpp, DARPP-32 can confer resistance to trastuzumab in breast cancer cells [14]. The current work further validates the role of t-Darpp/DARPP-32 in anti-cancer drug resistance and the potential use of t-Darpp and DARPP-32 as predictive and prognostic markers in human breast cancer.

## Materials and methods

### Cell culture

BT-474 and SKBR3 cells were cultured in RPMI 1640 (R8758; Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT). The isolation of two trastuzumab-resistant clones (BT/Her$^{R}$ 1F and 1B) has been previously reported by Chan et al. [11], and these clones were obtained as a kind gift from Susan Kane. The BT/Her$^{R}$ cells were maintained at the same trastuzumab concentration in which they were initially selected (1 μM).

### Gene expression profiling

Total RNA was isolated from cells using TriZol (Invitrogen Invitrogen Life Technologies, Carlsbad, CA) followed by purification on RNeasy columns (Qiagen, Germantown, MD). RNA quality was assessed on a 2100 bioanalyzer with an RNA 600 LabChip kit (Agilent Technologies, Palo Alto, CA). We performed expression profiling on the Whole Human Genome Oligo Microarray 44A slides (Agilent Technologies), which contain ~41,000 60-mer oligonucleotide probes covering over 33,000 human genes and transcripts. For each hybridization, 10 μg of total RNA was reverse transcribed, labeled, and purified using the FairplayII microarray labelling kit (Stratagene, La Jolla, CA) and Cy5-monoreactive dye for the experimental sample or Cy3-monoreactive dye for the reference (GE Healthcare, Piscataway, NJ). For each microarray experiment, the reference was the same (reference pool of total RNAs from 16 different human cell lines to ensure a broad coverage of genes on human arrays) so as to allow direct comparison between cell lines in the different experimental models. Labeled cDNAs were denatured, added to the hybridization buffer (Agilent Technologies) and hybridized to microarray chips for 18 h at 65°C in a rotating hybridization oven. Slides were then washed at room temperature using recommended buffers and scanned using an Agilent 2565AA DNA microarray scanner. Image analysis was performed using Agilent's Feature Extraction software, version 8.5. Normalization of the microarray data was performed by the Feature Extraction software using locally weighted linear regression, preceded by linear scaling in each dye channel. Values were normalized using median centering to enable comparison from microarray to microarray. Filtering was performed by removing probes for which the mean green or red signal was not significantly higher than the respective background signal. For

each experimental replicate, less than 10% data was filtered out.

### Array CGH

We used the Human Genome CGH Microarray 244A platform (Agilent Technologies, Palo Alto, CA), which contains ~236,000 60-mer oligonucleotide probes that span the human genome, including coding and noncoding sequences, and enriching for cancer related genes. Genomic DNA was extracted using the QIAamp DNA mini kit as indicated by the manufacturer (QIAGEN). 3.0 μg of genomic DNA from the experimental sample and from normal diploid DNA (Promega, Madison, WI) were digested with *Alu*I and *Rsa*I (New England Biolabs, Ipswich, MA). Labeling reactions were performed using random primers and the exo-Klenow from the Agilent Genomic DNA labelling kit PLUS according to the manufacturer's instructions. Labeled experimental and reference DNAs were pooled and hybridization carried out for 40 h at 65°C, followed by washing according to the manufacturer's protocol (Agilent).

### Plasmid transfer and generation of stable cell lines

t-Darpp and FLAG-DARPP-32 plasmids were obtained as a generous gift from Wael El-Rifai. Plasmid transfection was performed by plating $1 \times 10^6$ BT-474 or SKBR3 cells in a 6 well/plate using 10 μl of lipofectamine 2000 (#111668-019; Invitrogen) and 1 μg of DNA. Stably transfected BT-474 and SKBR3 cells expressing pcDNA3.1 zeo empty vector, t-Darpp or FLAG-DARPP-32 were selected using 0.8 mg/ml (BT-474) and 1 mg/ml (SKBR3) zeocin (ant-zn-5; Invivogen, CA). After a month of selection, several colonies were isolated and transferred to fresh flasks in RPMI 1640, 10% FBS and 0.5 mg/ml zeocin. The expression of t-Darpp/DARPP-32 protein in the transfectants was confirmed using Western analysis. The mutant t-Darpp.T75A plasmid, also a generous gift from Wael El-Rifai, described in reference [13] was transfected in BT-474 cells using the same protocol as above. The selection for stable transfectants was performed under constant treatment with neomycin (G418, Invitrogen). Again, protein expression in the transfectants was confirmed by Western analysis.

### shRNA transfection

$1 \times 10^6$ BT/Her$^R$.1F clones were transfected with 1 μg of either SHC002 MISSION$^{TM}$ non-target shRNA control vector or MISSION$^{TM}$ TRC shRNA target set TRCN00000 52878, TRCN0000052881 and TRCN0000052882 (Sigma, St. Louis, MO) using 10 μl lipofectamine 2000 (Invitrogen). The stably transfected clones were selected by the addition of 1 μg/ml puromycin. Stable transfectants grew out after 3 weeks of selection.

### Western analysis

For Western analysis, cell lysates were obtained from subconfluent parental SKBR3, BT-474, transfectant and BT/Her$^R$ resistant cells maintained with or without 1 μM trastuzumab for 48 h. Protein concentrations were determined using the Micro BCA$^{TM}$ protein assay kit (Pierce). To detect t-Darpp/DARPP-32 expression, 10 μg of total protein was resolved on a 12.5% SDS–PAGE and electroblotted onto a PVDF membrane (Immobilon-P; Millipore, Billerica, MA). The membrane was probed overnight with the designated primary antibody at a dilution of 1:1,000 in each case, unless specified otherwise. The primary antibodies were: rabbit anti-DARPP-32 (H-62, raised against amino acids 134–195; Santa Cruz Biotechnology, CA), rabbit anti-DARPP-32 (NB300-304, raised against the N-terminus region of DARPP-32, Novus Biologicals, CO), rabbit anti-phospho-DARPP-32 Thr75 (NB300-234; Novus Biologicals, CO), rabbit anti-phospho-AKT Ser473 (#9271; Cell Signalling Technology, Danvers, MA), and rabbit anti-AKT (#9272; Cell Signalling Technology, Danvers, MA). Secondary antibodies were horseradish peroxidase conjugated goat anti-mouse or anti-rabbit (#115-035-003 and 111-035-003, respectively; 0.08 μg/ml, Jackson Immunoresearch Laboratories, Inc.). To control for loading, the membranes were either reprobed with mouse anti-lamin A/C (#05-714; 1:500 dilution, Upstate, NY) or tubulin (Ab7291-100, 1:5,000 dilution, Abcam, Cambridge, MA) or GAPDH (14C10, Cell Signalling Technology, MA), or the membranes were coloured with amido black. All membranes were developed with the enhanced chemiluminescence method (ECLplus; GE Healthcare, Buckinghamshire, UK). For Western analysis of tumors, tumor lysates were obtained from homogenized freshly frozen breast cancer specimens that are part of a large institutional tumor bank of consecutive breast tumors collected from 2000 to 2003. Cores of paraffin-embedded formalin-fixed samples of these same tumors were used to build a breast cancer tissue microarray, and used for immunohistochemistry. Patients signed informed consent to participate in this tumor bank. Western analysis was performed as above for DARPP-32 protein expression using the same antibodies.

### 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay

The MTT test was used to determine the effect of trastuzumab on the proliferation of BT-474 and SKBR3 cells. Cells were plated without drug in 96-well plates, in triplicate, at a confluence of $1 \times 10^4$ (BT-474) and $0.75 \times 10^4$

(SKBR3) cells/well. The cells were allowed to attach for 24 h. Time 0 cell counts were measured 24 h post-plating, after which medium with 1 μM trastuzumab or without drug was added to the cells. Medium was replenished on the third day. At each time point (3, 4, and 5 days), the plates were subjected to MTT assay according to the manufacturer's instructions. The absorbance of the converted dye was measured by a spectrophotometer.

### Immunohistochemistry

Immunohistochemical staining of DARPP-32/t-Darpp was done on a tissue microarray (TMA) constructed using Manual Tissue Arrayer I (Beecher Instruments, Sun Prairie, WI) in the Basik laboratory containing biopsies from 230 breast adenocarcinomas in duplicate (two 6 mm cores/sample). Patients signed informed consent as part of an institutional tumor bank. Median follow-up for the entire cohort is 3.3 years. Tissue microarray slides were deparaffinized in xylene, rehydrated in graded ethanol and heated in a steamer with antigen unmasking solution (Vector Laboratories, Burlingame, CA). Endogenous peroxidase activity was then blocked by incubation in 3% $H_2O_2$ for 10 min at room temperature. After blocking, the sections were incubated overnight at 4°C with either 1 μg/ml of rabbit polyclonal anti-DARPP-32 antibody which recognizes the C-terminal end of DARPP-32 (clone H-62, Santa-Cruz Biotechnology), or rabbit polyclonal anti-DARPP-32 antibody which recognizes the N-terminal end of DARPP-32 (NB300-224, Novus Biologicals). Bound antibody was detected with an anti-rabbit biotin-conjugated secondary antibody (2.2 μg/ml, Jackson ImmunoResearch Laboratories, West Grove, PA). After the addition of streptavidin-horseradish peroxidase (HRP) conjugate (100 ng/ml, Pierce Biotechnology, Rockford, IL), the enzyme complex was visualized with 3,3′-diaminobenzidine tetrachloride solution (Vector Laboratories, Burlingame, CA). Slides were read blinded by two independent observers (LQ and MB). Only epithelial cells were evaluated and each core was recorded separately. The intensity (high (3+) versus medium (2+) versus low (1+) versus negative (0)) as well as the percentage of maximally stained tumor/epithelial cells in each core biopsy was recorded. All statistical analysis used the product score whereby the staining intensity was multiplied by the percentage of positive cells to obtain a score ranging from 0 to 300.

Patients were divided into three groups, low, medium, and high, based on the cytoplasmic DARPP-32 product score, using X-tile software [15]. Survival intervals were measured from the time of surgery to the time of death due to breast cancer. Kaplan Meier survival curves were constructed for $n = 230$. Multivariate analysis was performed using a Cox proportional hazards regression model, and adjustment was performed for following covariates: age, tumor size, lymph nodes, HER2 status, hormonal receptor status, and chemotherapy or hormonal therapy. About 35 patients were excluded in multivariate analysis due to incomplete information about clinico-pathological characteristics.

## Results

### BT/Her$^R$ trastuzumab resistant cells overexpress the truncated isoform of DARPP-32, t-Darpp

To identify molecular effectors of trastuzumab resistance in BT/Her$^R$ resistant cells, we performed gene expression profiling comparing resistant to parental BT-474 cell lines. RNA levels of putative trastuzumab resistance genes such as EGFR, p27, and TGFα were not significantly changed in the resistant BT/Her$^R$ clones (data not shown). The most highly upregulated transcript in the two resistant clones was PPP1R1B (Protein phosphatase 1, regulatory (inhibitor) subunit 1B), coding for the DARPP-32 (Dopamine and cAMP regulated phosphoprotein) protein. Levels of this transcript showed a remarkable increase of over 50-fold in the resistant BT/Her$^R$ clones compared to parental cells (data not shown). To confirm protein over-expression in these clones, we carried out Western analysis on parental and resistant cells. As there are two reported isoforms of DARPP-32, full-length DARPP-32 and the N-terminal truncated form known as t-Darpp [14], two different antibodies were used, one recognizing the C-terminus, common to both DARPP-32 and t-Darpp, and the other recognizing the N-terminus, present only in full-length DARPP-32. The antibody detecting the common C-terminus showed no expression in parental BT-474 cells, but very high expression in the BT/Her$^R$ resistant clones. However, the antibody against the N-terminus did not detect full-length DARPP-32 in the resistant clones, suggesting that it was the truncated t-Darpp protein that was induced in BT/Her$^R$ (Fig. 1a, b). We confirmed through RT-PCR that the mRNA expressed in BT/Her$^R$ cells was indeed the alternative transcript encoding t-Darpp (data not shown). Next, we assessed if t-Darpp expression was stable even after removal of trastuzumab. Resistant clones grown in the absence of the drug for over 1 month retained t-Darpp expression (Fig. 1c). These results suggest that these BT/Her$^R$ resistant cells have stably acquired t-Darpp over-expression. Moreover, when re-challenged with trastuzumab, they remained resistant compared to the parental BT-474 cell line (data not shown).

Interestingly, the DARPP-32/PPP1R1B gene is located very close to the trastuzumab target ERBB2 gene on chromosome 17q12-21, 64 Kb from the ERBB2 gene. Indeed, our own array comparative genomic hybridization (array CGH) data from 4 ERBB2 amplified breast cancer

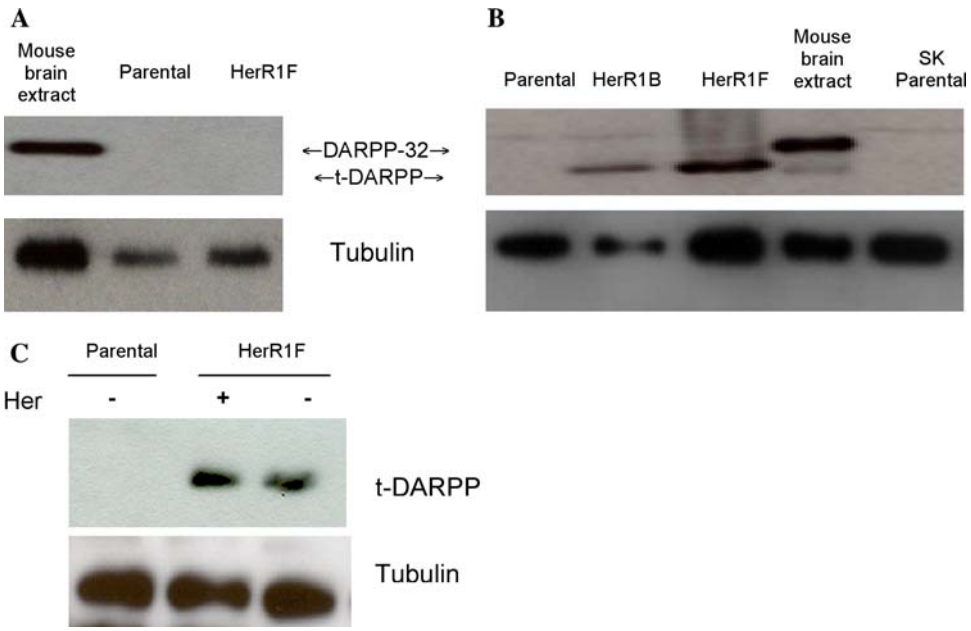


**Fig. 1** t-Darpp is overexpressed in trastuzumab-resistant BT-474 cells. **a** Western analysis of parental BT-474 cells and the BT/Her$^R$.1F resistant clone probed with an antibody recognizing the N-terminal end of the DARPP-32 protein, which is missing from t-Darpp. **b** Western analysis of BT-474 parental, SKBR3 parental and the BT/Her$^R$.1F resistant clone probed with an antibody recognizing the C-terminal end of DARPP-32, which is present in t-Darpp. Mouse brain extract is the positive control for full-length DARPP-32 protein. Note a shorter isoform of DARPP-32, recognized only by the C-terminal antibody, is observed in the resistant cells. **c** trastuzumab resistant BT-474 cells were grown in the absence or presence of 1 μM trastuzumab for 1 month. Western analysis of these cell lines probed with the C-terminal DARPP-32 antibody to illustrate the stability of t-Darpp expression. Tubulin was used as loading control

cell lines and 4 primary tumors reveals that ERBB2 and PPP1R1B are always co-amplified (unpublished observations). We performed array CGH microarray analysis on both parental BT-474 and resistant BT/Her$^R$ cells to determine whether relative differences in DNA copy number of PPP1R1B could explain the observed differences in gene expression. We found that the amplitude and length of the 17q12-21 amplification in BT/Her$^R$ resistant clones was comparable to that of their parental counterparts (supplemental Fig. 1), suggesting that a mechanism other than DNA copy number change is promoting t-Darpp expression in our resistant cells.

## t-Darpp over-expression is responsible for trastuzumab resistance in BT/Her$^R$.1F cells

To evaluate the role of t-Darpp in acquired resistance to trastuzumab, we performed stable shRNA transfections to silence DARPP-32 gene expression in the resistant cells (clone BT/Her$^R$.1F, unless otherwise indicated). Western analysis confirmed that t-Darpp expression was silenced in shRNA-transfected BT/Her$^R$ cells (Fig. 2a). These cells regained their trastuzumab sensitivity to a level comparable to the parental cells (Fig. 2b). These results indicate that t-Darpp expression plays an essential role in acquired resistance to trastuzumab in our resistant clones, at least in the case of BT/Her$^R$.1F.

To determine if t-Darpp over-expression was sufficient to confer trastuzumab resistance, we transfected parental BT-474 cells with a t-Darpp expression plasmid, selected for stable over-expression, and then measured sensitivity to trastuzumab. As shown in Fig. 2c and d, BT/t-Darpp cells expressed t-Darpp and were resistant to trastuzumab, relative to cells transfected with empty vector control. To determine if t-Darpp-mediated trastuzumab resistance could be generalized to other breast cancer models, we established stable transfectants of SKBR3 cells, which also show high level ERBB2 amplification but differ from BT-474 cells by not expressing the estrogen receptor. Like BT-474 cells, parental and mock-transfected SKBR3 cells showed no detectable DARPP-32 or t-Darpp expression, by Western analysis (Supplemental Fig. 2), and they were sensitive to trastuzumab (Fig. 3a, b). SKBR3 cells stably transfected with t-Darpp, on the other hand, were resistant to trastuzumab (Fig. 3c). Thus, t-Darpp over-expression is sufficient for conferring resistance to trastuzumab in two different cellular contexts, the BT-474 and SKBR3 cell lines.

## Phosphorylation at T75 is necessary for t-Darpp-dependent trastuzumab resistance

Previous reports have shown that an important function of full-length DARPP-32 is to inhibit protein phosphatase-1 and protein kinase A activity, depending on the phosphorylation

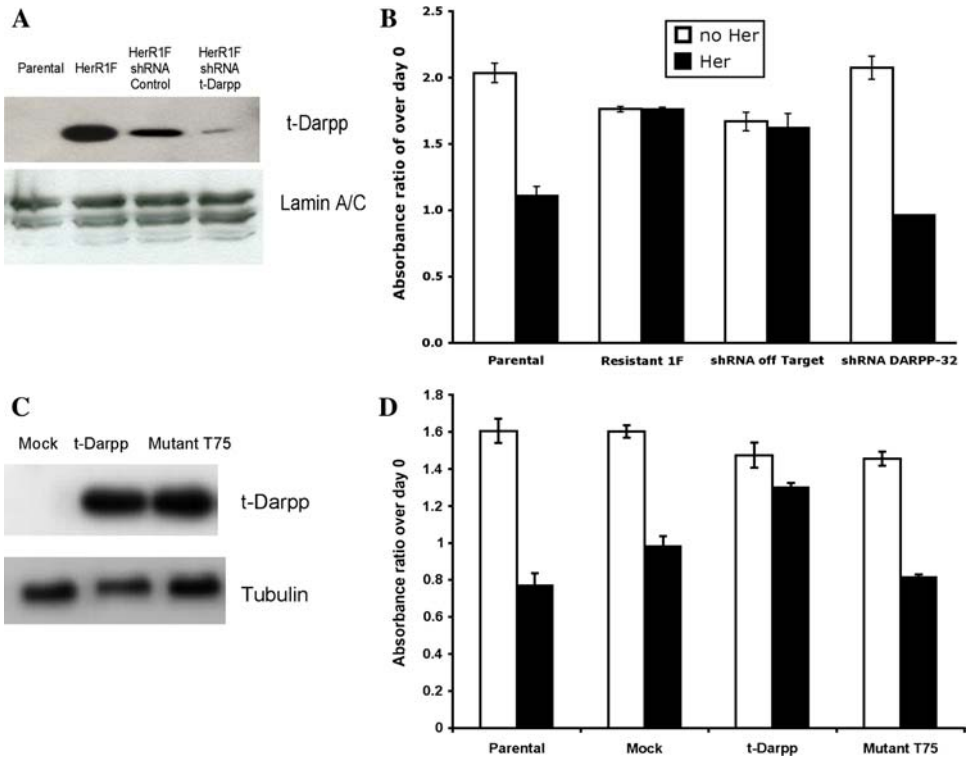


**Fig. 2** t-Darpp overexpression is required and sufficient for trastuzumab resistance in our model. **a** Western analysis of t-Darpp expression using the C-terminal DARPP-32 antibody in BT/Her$^R$.1F cells transfected with specific shRNA against DARPP-32 or off-target shRNA (Sigma Inc.). **b** results of proliferation assay of BT/Her$^R$.1F cells transfected with specific shRNA against DARPP-32 or off-target shRNA cells, treated or not with 1 μM trastuzumab (Her) cells. Y-axis: cell count (MTT absorbance value) at day 5 of growth, relative to number of cells plated at day 0. **c** Western analysis of t-Darpp expression using the C-terminal DARPP-32 antibody of mock-transfected BT-474 cells and BT-474 cells stably transfected with t-Darpp or t-Darpp.75A. **d** Results of proliferation assays of parental BT-474 (WT), mock-transfected BT-474 cells, or BT-474 cells stably transfected with t-Darpp or t-Darpp.T75A, treated with 1 μM trastuzumab for 5 days and then harvested for MTT assay. Y-axis: cell count (MTT absorbance value) at day 5 of growth. All experiments were performed in triplicate, with standard deviation shown as error bars

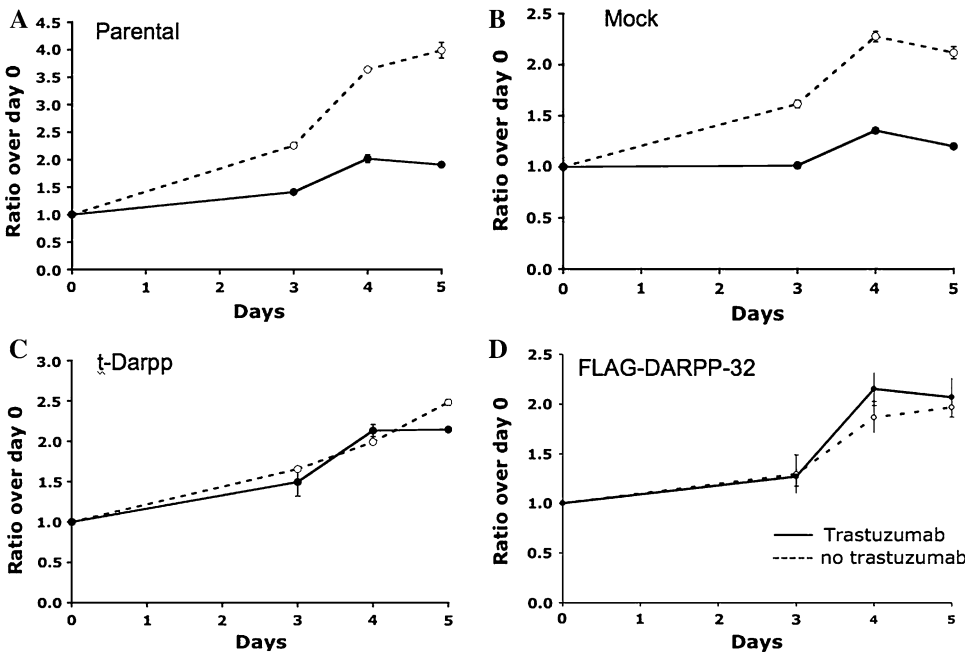


**Fig. 3** t-Darpp and DARPP-32 are sufficient to confer resistance to trastuzumab in SKBR3 cells. **a** Growth curves of parental cells treated or not with 1.0 μM trastuzumab. **b** Growth curves of SKBR3 cells transfected with empty plasmid, treated or not with 1.0 μM trastuzumab. **c** Growth curves of SKBR3 cells transfected with t-Darpp, treated or not with 1.0 μM trastuzumab. **d** Growth curves of SKBR3 cells transfected with FLAG-DARPP-32, treated or not with 1.0 μM trastuzumab. Growth was measured by MTT assay at days 3, 4, and 5, shown as absorbance ratio over day 0 count. All experiments were performed in triplicate (±SD)

status of its T34 and T75 sites, respectively [16]. t-Darpp is missing the first 36 amino acids from the N-terminus of the full-length DARPP-32 protein, leaving the T75 phosphorylation site, but not the T34 site, intact. To evaluate the relevance of the T75 phosphorylation site in t-Darpp-mediated trastuzumab resistance, we first measured the state of T75 phosphorylation in BT/Her$^R$ resistant cells using a DARPP-32 T75 phospho-specific antibody. The t-Darpp expressed by trastuzumab-resistant BT/Her$^R$ cells was phosphorylated at T75 in both the presence and absence of trastuzumab (Fig. 4a), suggesting that the high expression of t-Darpp in these resistant cells is accompanied by sustained phosphorylation at the T75 site. Similarly, t-Darpp expressed in transfected SKBR3 and BT-474 cells was also phosphorylated at T75 (Fig. 4b, c).

To determine if the T75 phosphorylation site is required for resistance to trastuzumab, we used the t-Darpp.T75A mutant encoding a threonine-to-alanine substitution, which abolishes the T75 phosphorylation site, as reported previously [14]. BT-474 parental cells stably transfected with t-Darpp or t-Darpp.T75A expressed recombinant protein, as detected with antibody against the C-terminus (Fig. 2c). As expected, the phospho-T75-specific antibody detected protein in the cells transfected with t-Darpp but not in cells transfected with t-Darpp.T75A (Fig. 4b). Parental BT-474 cells transfected with t-Darpp.T75A were sensitive to trastuzumab (Fig. 2d) despite expression of the mutant protein. This indicates that the T75 residue is required for trastuzumab resistance imparted by t-Darpp over-expression.

## t-Darpp-mediated trastuzumab resistance is associated with sustained AKT phosphorylation

Trastuzumab treatment of ERBB2 amplified cells results in a decrease in AKT phosphorylation and activity [17]. Moreover, Belkhiri et al. [18] recently showed that t-Darpp inhibits apoptosis in part through a stimulation of Akt kinase activity. Chan et al. [11] previously showed that AKT phosphorylation is sustained in trastuzumab-resistant clones upon treatment with trastuzumab [11], so we wanted to determine if t-Darpp over-expression and phosphorylation were responsible for this facet of trastuzumab resistance. We first measured the state of AKT phosphorylation in BT/Her$^R$.1F resistant cells and in t-Darpp-transfected BT-474 cells. We found that, like the trastuzumab-resistant BT/Her$^R$ clones, the t-Darpp transfected cells had high levels of phospho-AKT in the absence of trastuzumab and sustained AKT phosphorylation in the presence of trastuzumab (Fig. 4d). This sustained AKT phosphorylation required T75 phosphorylation, as cells transfected with the t-Darpp.T75A mutant exhibited AKT dephosphorylation in response to trastuzumab, similar to parental and mock-transfected cells (Fig. 4d).

## DARPP-32/t-Darpp expression in clinical breast cancer samples

To determine the clinical relevance of our findings, we analyzed the expression of DARPP-32/t-Darpp protein in

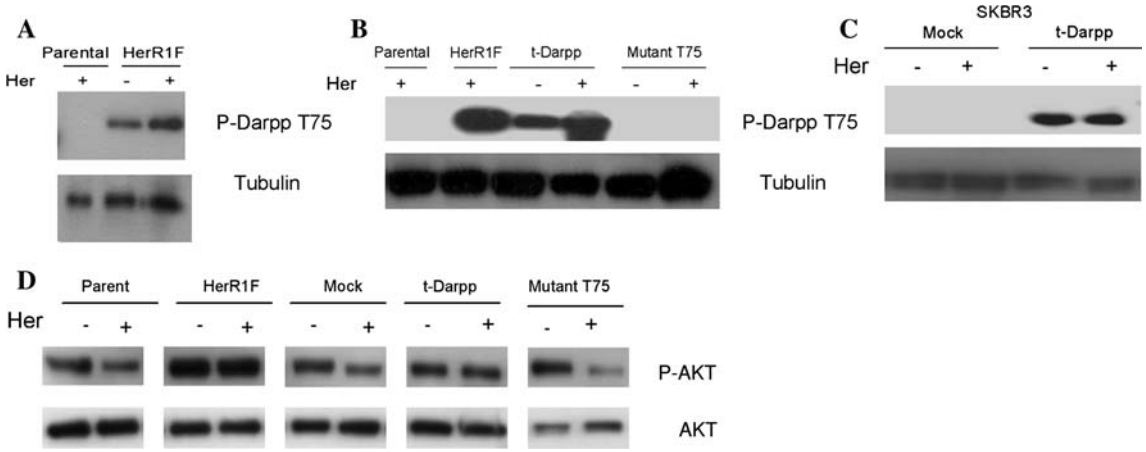


**Fig. 4** t-Darpp is phosphorylated at the T75 site leading to sustained AKT phosphorylation in trastuzumab resistant clones. **a** Western analysis with an antibody specific for phosphorylated T75 in DARPP-32. Parent BT-474 (WT) and BT/Her$^R$.1F were treated or not with 1.0 μM trastuzumab for 48 h. The bottom panels in **a**, **b** and **c** show tubulin control of protein loading. **b** Western analysis with an antibody specific for phosphorylated T75. Parent BT-474 (WT), BT/Her$^R$.1F, and BT-474 cells transfected with the t-Darpp or t-Darpp.T75A mutant, incubated with and without with 1.0 μM trastuzumab for 48 h. **c** Western analysis with an antibody specific for phosphorylated T75 in DARPP-32 in mock-transfected SKBR3 and t-Darpp transfected SKBR3 cells, incubated with or without 1.0 μM trastuzumab for 48 h. **d** Western analysis of phospho-Akt and Akt levels in the wild type BT-474 cells, BT/Her$^R$.1F clone, and BT-474 transfected with empty vector (mock), t-Darpp or t-Darpp.T75 mutant incubated with or without 1.0 μM trastuzumab for 48 h. Phospho-Akt levels were measured with an antibody specific for Ser473 phospho-AKT. Note that trastuzumab decreases Akt phosphorylation in the T75 mutant transfectants, unlike in the wild type t-Darpp transfectants

clinical samples of breast carcinomas. We first used a tissue microarray built in our laboratory containing a cohort of 230 consecutively banked breast tumors. Since there is no antibody unique to the t-Darpp form of the protein, we first used an antibody against the C-terminus of DARPP-32 that detected both t-Darpp and full-length DARPP-32. Moderate to high cytoplasmic expression of C-DARPP-32 was observed in 46% of breast carcinomas. Cytoplasmic C-DARPP-32 expression was more frequently detected in Her2-positive cases than Her2-negative cases ($P = 0.001$; Fig. 5a). Indeed, 14 of 17 (82%) Her2-positive breast tumors expressed moderate to high levels of C-DARPP-32, as opposed to 44% of the Her2-negative tumors. Of these 17 HER2 positive tumors, 7 (41%) expressed high levels of the protein.

To determine which of the proteins, the full-length or the truncated form, is expressed in the primary breast tumors, we used two approaches. First we obtained an antibody against the N-terminal end of DARPP-32, which does not recognize t-Darpp (Fig. 1a). Expression of N-terminal DARPP-32 was determined in a cohort of patients using the same TMA. About 31% of patients had medium to high expression of N-terminal DARPP-32 (Supplemental Fig. 3). As with the C-terminal antibody, we found that twice as many patients with Her2 positive breast tumors over expressed cytoplasmic N-DARPP-32 compared to patients without Her2 over-expression ($P = 0.02$). Expression data for both N-terminus and C-terminus DARPP-32 was available for 226 patients. 21% of these patients expressed medium or high levels of both N-DARPP-32 and of C-DARPP-32, while 25% of medium/high level C-DARPP-32 expressors did not express high levels of N-DARPP-32 (Fig. 6), suggesting that about half of breast tumors expressing DARPP-32 (by C-terminal antibody) express significant levels of the full-length form of the protein, and that in about 25% of primary breast tumors, shorter forms of DARPP-32 may be expressed at higher levels than the full-length protein. The correlation of the product score of the two antibodies was statistically significant ($\rho = 0.32$, $P < 0.0001$).

Second, we performed Western Blots on 10 breast tumors for which tumor samples were available for protein extraction (Fig. 5c). Eight tumors showed expression of both full-length DARPP-32 and t-Darpp with DARPP-32 being the predominant form of DARPP-32 in 7 of these tumors. Full-length DARPP-32 and t-Darpp were the only form of the proteins in one tumor each. Thus, full-length DARPP-32 is the more common form of the protein in primary breast tumors, although t-Darpp is frequently co-expressed. T-Darpp is the predominant form of the protein in about 20% of the primary breast tumors, compatible with the results obtained with IHC. To determine if full-length DARPP-32 also confers resistance to trastuzumab, we transfected parental SK-BR-3 with a plasmid containing the full-length protein, FLAG-DARPP-32. We found that FLAG-DARPP-32 confers resistance to trastuzumab at levels equivalent to that of t-Darpp (Fig. 3d). Only one of the women in the entire 230-cohort study received

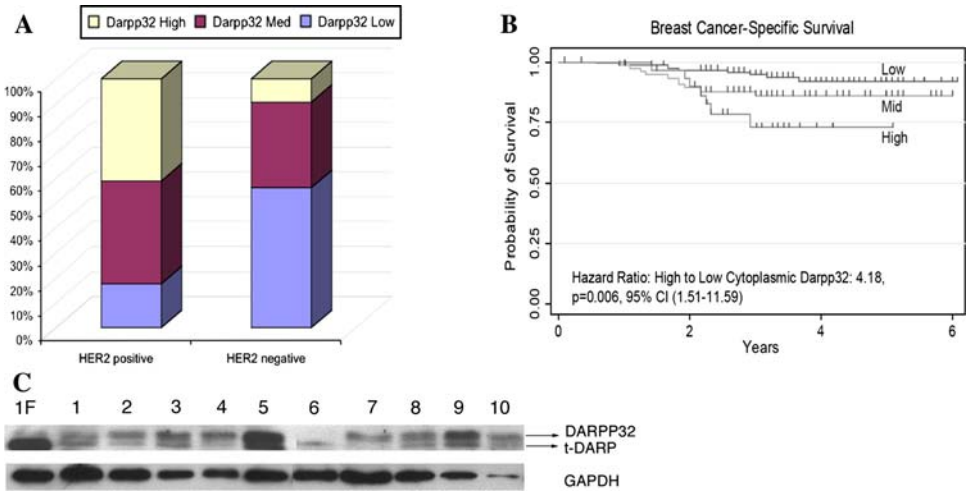


**Fig. 5** DARPP-32/t-Darpp is overexpressed in human breast cancer and associated with poor prognosis. **a** Bar graph illustrating relative proportions of low/medium/high expression of DARPP-32 by immunohistochemical analysis in 230 breast tumors on a tissue microarray. Slides were stained with an antibody recognizing the C-terminal end of DARPP-32, also present in t-Darpp. Results for Her2-positive ($n = 17$) and Her2-negative ($n = 213$) are shown, distinguished according to DARPP-32 staining intensity (low, medium, high). **b** Breast cancer specific survival for 230 breast cancers (low, medium, and high DARPP-32 expressors), with median follow-up of 3.5 years. The same antibody recognizing C-terminal DARPP-32 was used. Y-axis: fraction of patients not having died of breast cancer. **c** Western blot of 10 breast tumors using the antibody recognizing C-terminus of DARPP-32, with GAPDH antibody as loading control. The first lane on the left is lysate from the trastuzumab resistant cells, BT/Her$^R$ 1F

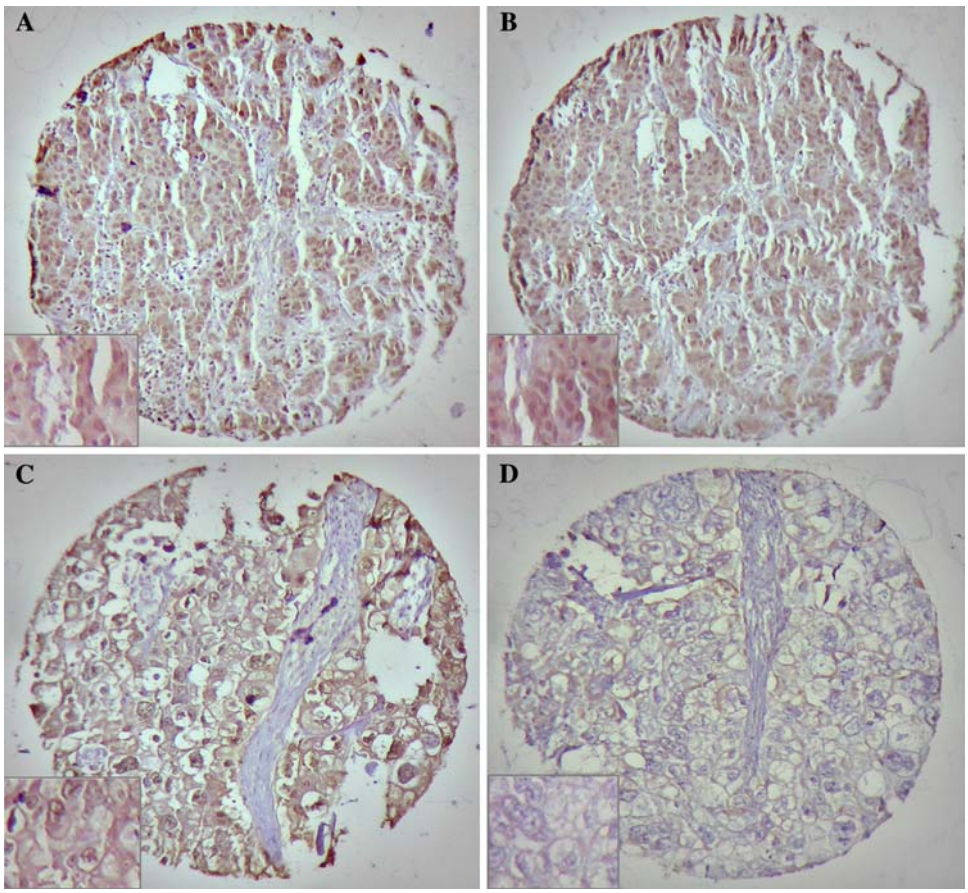


**Fig. 6** DARPP-32 expression by immunohistochemistry in breast tumors. **a**, **b**, Example of a breast tumor stained with antibody specific for the C-terminal end of DARPP-32 (**a**) and the N-terminal end of DARPP-32 (**b**). This tumor expresses full-length DARPP-32 as both antibodies stain the tumor strongly. **c**, **d,** Example of a second breast tumor stained with antibody specific for the C-terminal end of DARPP-32 (**c**) and the N-terminal end of DARPP-32 (**d**). This tumor may express t-Darpp but not full-length DARPP-32, as only the C-terminal antibody stains the tumor

trastuzumab in the clinic following her surgery and therefore no statement regarding trastuzumab responsiveness can be made in our cohort of breast cancer patients.

Finally, since Belkhiri et al. [18] reported a broader drug resistance phenotype for t-Darpp expression due to its anti-apoptotic effects, we assessed the prognostic value of t-Darpp/DARPP-32 in our cohort of primary breast cancer patients. We found that the cytoplasmic expression of DARPP-32/t-Darpp (using the C-terminal antibody) was statistically significantly associated with poor prognosis with a hazard ratio of 4.18 for breast cancer-specific survival, ($P = 0.006$, 95% confidence interval 1.51–11.59 (Fig. 5b). In multivariate analysis, DARPP-32/t-Darpp expression showed a weak association with breast cancer-specific survival ($P = 0.07$). Patients with high expression of N-terminal DARPP-32 also demonstrated a poorer prognosis due to breast cancer related causes (Hazard ratio, 3.55; 95% confidence interval, 1.31–9.60, $P = 0.01$), suggesting that expression of full-length DARPP-32 is associated with poor prognosis in primary breast cancers (Supplemental Fig. 3). When DARPP-32/t-Darpp expression was adjusted for Her2 alone, the prognostic significance and rate of mortality remained (data not shown), showing that DARPP-32/t-Darpp over-expression is an indicator of poor prognosis in breast cancer, independent of Her2 expression.

## Discussion

With the clinical success of trastuzumab in the treatment of early and late breast cancers, medical oncologists will increasingly be faced with the occurrence of acquired resistance to the therapy. Most metastatic breast tumors do not respond to trastuzumab alone and even those that initially respond, either to trastuzumab monotherapy or to combination therapy with chemotherapy drugs, eventually progress. Although various mechanisms have been found to account for intrinsic resistance to trastuzumab, fewer studies have examined the factors responsible for the development of acquired resistance. The trastuzumab resistant BT-474 clones developed by Chan et al. [11] present a suitable model to study the etiology of acquired resistance to trastuzumab. Although resistance to antibody therapy in human cancers may theoretically involve the host immune system, clinical evidence to date suggests that tumor cell factors play a critical role, as for instance, K-ras mutations do in resistance to cetuximab in colorectal

cancers. In the current report, we show that trastuzumab resistant clones exhibit marked over-expression of PPP1R1B, a gene coding for the phosphatase regulator, DARPP-32. We also found that it is the truncated form of DARPP-32, t-Darpp, that is overexpressed in BT/Her$^R$ cells and that its expression is necessary for resistance to trastuzumab in BT/Her$^R$.1F cells (Fig. 2). These results confirm the results from the recent Belkhiri et al. [13] paper, which used a mouse xenograft model, and thus validates the use of in vitro models to study acquired resistance to antibody therapy in human cancers. In addition, our analysis of clinical data suggests that the over-expression of full-length DARPP-32 in primary breast cancers is more prevalent than that of t-Darpp. In fact, we found that the full-length form of the protein is also sufficient to confer trastuzumab resistance in breast cancer cells. We thus speculate that high expression of DARPP-32/t-Darpp in over 40% of Her2-positive breast tumors might also implicate it in intrinsic resistance to trastuzumab. Indeed, results from clinical trials of single-agent trastuzumab administered to patients with metastatic, Her2-positive breast cancer indicate a rate of clinical intrinsic resistance of ~66–88% of patients [5].

Our results confirm that the ectopic over-expression of t-Darpp is sufficient for conferring trastuzumab resistance (Figs. 2, 3) and show further that the T75 residue is required for this resistance (Figs. 2, 4). Moreover, resistance is associated with sustained AKT activation, which also requires T75 phosphorylation (Fig. 4). The association with PI3 K/AKT signalling is consistent with t-Darpp activation of Akt originally observed by Belkhiri et al. [17] in gastric cancer cells, and confirmed in their model of trastuzumab resistance [13]. Bibb et al. [16] have demonstrated that the T75 residue of DARPP-32 is responsible for inhibiting the activity of the catalytic subunit of protein kinase A (PKA). PKA can either stimulate or inhibit the activity of PI3 K, depending on cell type [19]. If PKA were inhibitory in our cells, then suppression of PKA activity by t-Darpp could prevent this inhibition, leading to sustained PI3 K signalling and AKT activity, thereby mediating trastuzumab resistance. Although this model is consistent with our data, further experiments will be required to determine if the PI3 K/AKT effects of t-Darpp over-expression are mediated via PKA inhibition. It is also possible that t-Darpp functions by some other mechanism, independent of PKA and different from full-length DARPP-32.

Interestingly, PPP1R1B is located very close to the ERBB2 gene on the same amplicon. Indeed, it appears always to be co-amplified in breast tumors containing this amplicon [20], consistent with our clinical data of very frequent co-expression of both genes. However, BT-474 cells, which have amplified ERBB2 and PPP1R1B genes, do not express PPP1R1B mRNA at any appreciable level. In addition, we did not find any difference between parental and resistant cell lines with respect to DNA copy number of the gene (Supplemental Fig. 1). Other possible explanations for PPP1R1B expression in BT/Her$^R$ resistant cells involve alterations at the promoter level, specifically, changes in the methylation status of the promoter. Indeed, treating parental BT-474 cells with the DNA methyltransferase inhibitor 5′azacytosine induced the expression of t-Darpp mRNA and protein to a level comparable to that in some of our resistant cells (data not shown), suggesting that demethylation of the PPP1R1B promoter might enable selection of trastuzumab resistance.

The involvement of t-Darpp in trastuzumab resistance is not limited to a single cell model. Over-expression resulted in resistance to trastuzumab in the estrogen receptor negative SKBR3 breast cancer cell line as well as in the estrogen receptor positive BT-474 cell line (Figs. 2, 3). We also found that t-Darpp/DARPP-32 over-expression was associated with poor prognosis, independently of Her2 over-expression, in human breast cancer. This might be due to the anti-apoptotic function of t-Darpp described by Belkhiri et al. [14], which could affect prognosis and response to a variety of anti-cancer therapies, regardless of Her2 expression.

As already noted, our results are compatible with a recently published report associating trastuzumab resistance with t-Darpp expression in a xenograft model of acquired resistance to trastuzumab using BT-474 cells [13]. Those results suggest that t-Darpp expression may play a role even in the in vivo context, where other factors such as antibody-mediated cytotoxicity may be partly responsible for the efficacy of trastuzumab in Her2-amplified breast tumors. Indeed, our report lends support to the use of cell line models to investigate mechanisms of resistance to anti-cancer antibodies.

Although highly successful as therapy in both early and late breast cancers, many patients recur or relapse shortly after or during trastuzumab therapy. Understanding the biology of trastuzumab resistance will allow for the rational selection of this expensive drug, avoiding unnecessary toxicity and cost in those patients in whom it is not likely to be effective. We propose that DARPP-32/t-Darpp expression is a putative negative predictor for trastuzumab response. Targeting this protein's over-expression is a rational avenue for the treatment of patients with Her2-amplified breast tumors whose tumors relapse either shortly after or during trastuzumab treatment as a result of t-Darpp over-expression.

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