Regulation of ERBB2 Receptor by t-DARPP Mediates Trastuzumab Resistance in Human Esophageal Adenocarcinoma

Jun Hong1, Ahmed Katsha1, Pengcheng Lu2, Yu Shyr2,4, Abbes Belkhir1, and Wael El-Rifai1,3

Abstract

Esophageal adenocarcinoma (EAC) is an aggressive malignancy with a poor outcome. Although targeting ERBB2 with trastuzumab has been evaluated in clinical trials, the molecular mechanisms of trastuzumab resistance remain uncharacterized in EAC. The dopamine and cyclic AMP-regulated phosphoprotein of MR 32000 (DARPP-32), also known as PPP1R1B, is located together with ERBB2 at the 17q12-q21 amplicon. We evaluated the expression of a transcript variant of DARPP-32 (t-DARPP) and ERBB2 in 141 primary tumors and investigated the role of t-DARPP in trastuzumab resistance using OE19 and OE33 EAC cell models. Overexpression of t-DARPP mRNA was detected in two-thirds of tumors with a correlation between ERBB2 and t-DARPP overexpression levels (r = 0.58, P = 0.003). Cell viability and clonogenic survival assays showed that t-DARPP increased survival by 40% in response to trastuzumab (P < 0.01). The Annexin-V staining and Western blot analysis indicated that t-DARPP effectively abrogated trastuzumab-induced apoptosis, blocked the trastuzumab-induced dephosphorylation of ERBB2 and AKT proteins. The knockdown of endogenous t-DARPP reversed these effects and sensitized cells to trastuzumab (P < 0.01). The cycloheximide-based protein degradation analysis indicated that t-DARPP extended the half-life of ERBB2, explaining the increase in the basal levels of ERBB2, p-ERBB2(Y1248), and p-AKT(S473). Coimmunoprecipitation and Western blot analysis showed that t-DARPP associated with ERBB2 in a protein complex, and interfered with trastuzumab binding to the ERBB2 receptor. Using EAC-xenografted mouse model, t-DARPP enhanced tumor growth and rendered tumors unresponsive to trastuzumab. This study establishes t-DARPP as a mediator of trastuzumab resistance and underscores its potential importance in clinical trials of EAC. Cancer Res. 72(17): 4504–14. ©2012 AACR.

Introduction

Esophageal carcinoma is the sixth most common cause of cancer-related death worldwide (1). Histologically, esophageal cancer can be divided into adenocarcinoma and squamous cell carcinoma. The incidence of esophageal adenocarcinoma (EAC) has been rising rapidly in the past 3 decades (2, 3). There are approximately 14,000 cases of esophageal cancer per year in the United States, of which most are EAC (4, 5). The overall 5-year survival rate of EAC is less than 15% (4–7), indicating that current treatment regimens are ineffective.

Chromosomal amplification at the 17q21 region is a frequent finding in adenocarcinomas of the stomach and esophagus (8, 9). This region is a gene-rich area that contains several candidate cancer genes (9). The ERBB2 locus, within the 17q12-q21 amplicon, has been heavily implicated in several malignancies. ERBB2 is amplified and overexpressed in approximately 15% to 25% of EAC tumor specimens and has been implicated in the pathogenesis of EAC (10–12). In addition to ERBB2, the amplicon region contains several other genes such as DARPP-32 [also known as protein phosphatase 1 regulatory subunit 1B (PPP1R1B)], GRB7, and TOP2A. Recently, DARPP-32 and its cancer-specific truncated variant (t-DARPP) have been mapped to the ERBB2 amplicon (8), t-DARPP is overexpressed in several malignancies, such as those of the stomach, colon, breast, and prostate (13–15). We and other researchers have shown that the t-DARPP protein promotes cell growth, survival, and drug resistance through activation of AKT signaling in cancer cells (14, 16–18).

The ERBB2 gene-targeted therapy continues to be applied in several clinical trials; Trastuzumab (Herceptin), a humanized monoclonal anti-ERBB2 antibody, was first used for the treatment of ERBB2-overexpressing advanced metastatic breast cancers (19). To date, most of our understanding of ERBB2-targeted therapy comes from studies in breast cancer. Although ERBB2-positive tumors initially respond to trastuzumab treatment, the majority of responders progress within 12 months of initiating therapy as a result of acquired trastuzumab resistance (20). Several studies have elucidated some of the
mechanisms of trastuzumab resistance using in vitro cell models. This resistance has been attributed to disruption of interaction between ERBB2 and trastuzumab by MUC4 expression (21), compensatory signaling by other ERBB receptor members (22), compensatory signaling from other types of receptors such as insulin-like growth factor type 1 (IGF-IR; ref. 23), increased circulating ERBB2 ECD (24), and altered downstream signaling, including PTEN deficiency (25), increased AKT activity (26), and downregulation of P27 (CDKN1B; ref. 27).

Trastuzumab, in combination with cisplatin, has been recently used in clinical trials to treat patients with ERBB2-positive metastatic gastric or gastroesophageal junction adenocarcinoma (28). Notably, a phase 3 clinical trial (RTOG 1010 protocol) is currently ongoing to evaluate the addition of trastuzumab to increase disease-free survival when combined with tridomality treatment (radiation plus chemotherapy followed by surgery) for EAC patients. Therefore, it is crucial to characterize novel mechanisms of trastuzumab resistance in EAC because our capabilities to identify, overcome, or clinically manage this resistant phenotype in EAC are currently limited.

In this study, we elucidated a novel mechanism by which t-DARPP binds and stabilizes the ERBB2 protein, thereby activating the AKT signaling and promoting trastuzumab resistance by interfering with trastuzumab interaction with the ERBB2 receptor.

Materials and Methods

Cell lines and reagents

The human esophageal adenocarcinoma cancer cell lines, OE19 and OE33, were obtained from the European Collection of Animal Cell Cultures (Sigma-Aldrich) and the American Type Culture Collection, respectively. To generate trastuzumab-resistant clones, OE19 cells were cultured with increasing concentrations of trastuzumab for more than 6 months in vitro, and the resistant cells were maintained with 20 μg/mL trastuzumab in culture. Cycloheximide was purchased from Sigma-Aldrich. ERBB2, AKT, p-AKT(S473), caspase-3, cleaved caspase-3, and β-actin antibodies were obtained from Cell Signaling Technology. DARPP-32 antibody was purchased from Santa Cruz Biotechnology, and the p-ERBB2(Y1248) antibody was obtained from Cell Signaling Technology. t-DARPP coding sequence was amplified and cloned into pcDNA3 mammalian expression vector (Invitrogen). OE19 cells stably expressing t-DARPP or pcDNA3 empty vector were generated in accordance with standard protocols as described previously (16). Flag-tagged t-DARPP coding sequence was amplified and cloned into the adenoviral shuttle vector (pAACC MV), and the recombinant adenovirus was generated by cotransfecting HEK-293 cells with the shuttle and backbone adenoviral (pPM17) plasmids using the Calcium Phosphate Transfection Kit (Applied Biological Materials Inc., Richmond, BC). Control siRNA (sc-37007) and t-DARPP siRNA (sc-35173; a cocktail of 3 different oligonucleotides) was obtained from Santa Cruz Biotechnology.

Cell viability assays

The CellTiter-Glo Luminescent Cell Viability Assay (Promega) was conducted according to supplier instructions. Briefly, cells (5 × 10^4 well) were seeded onto a 96-well plate. Approximately 18 hours after seeding, cells were treated with trastuzumab (20 μg/mL) for 48 hours. The luminescence was read on a Multilabel Reader (FLUOstar OPTIMA). For trypsin blue dye exclusion assay, viable cells for each concentration were counted on a hemocytometer after trypsinization. All experiments were carried out in triplicate and repeated 3 times.

Clonogenic survival assay

Cells were trypsinized and harvested in single-cell suspension. Cells were plated at low-density (2 × 10^3 cells/well) in 6-well plates. The following day, cells were treated with vehicle or 20 μg/mL trastuzumab. Culture media were replaced every 3 days with the addition of vehicle or fresh trastuzumab. After culturing for 2 weeks, cells were fixed with methanol:acetic acid (3:1, vol:vol) and stained with 1% crystal violet. Colonies with ≥50 cells were counted.

Apoptosis assay

OE19 cells infected with control (10 MOI) or t-DARPP (10 MOI) recombinant adenoviruses, and parental or trastuzumab-resistant OE19 cells were seeded onto 60 mm culture plates. The next day, cells were treated with trastuzumab (20 μg/mL) or vehicle for 48 hours. Cells were then collected and stained with Annexin-V fluorescein isothiocyanate and propidium iodide (PL R&D Systems). The samples were washed with PBS and re-suspended in binding buffer (2 mL HEPES buffered saline solution supplemented with 2.5 mmol/L CaCl2), and then subjected to fluorescence-activated cell sorting (FACS) analysis (Becton Dickinson).

Cycloheximide-based ERBB2 protein stability assay

Cells (2 × 10^5 cells/well) were seeded into 12-well plates. The next day, cells were treated with 80 μg/mL of cycloheximide (CHX) and harvested at different time points. Proteins were extracted and analyzed by Western blotting to assess ERBB2 protein stability. The intensities of protein bands were semiquantitatively analyzed by densitometry using ImageJ software (NIH Image). ERBB2 band intensities for each treatment condition were normalized to β-actin. The protein degradation curve was generated by plotting band intensities ratios as a function of the time period of CHX treatment. Linear regression was carried out, and the protein half-life (t1/2), which is expressed as the time for degradation of 50% of the protein, was calculated from the fitted line equation (29).

Quantitative real-time reverse transcriptase PCR

Frozen de-identified human tissue samples were obtained from the archives of pathology at Vanderbilt University and the National Cancer Institute Cooperative Human Tissue Network.
The use of coded specimens was approved by the Institutional Review Board at the Vanderbilt University. The samples included 141 adenocarcinomas of the esophagus and stomach and 51 nontumor normal mucosal samples. Histopathological diagnosis was verified based on hematoxylin and eosin (H&E)-stained sections. The adenocarcinomas ranged from well-differentiated to poorly differentiated, stages II to IV, with a mix of intestinal- and diffuse-type tumors. Total RNA was isolated from cells using the RNeasy Mini Kit (Qiagen). Single-stranded cDNA was synthesized from 1 μg total RNA by an iScript cDNA synthesis kit (Bio-Rad). Primers for ERBB2, t-DARPP, and HPRT1 were designed, and the results were normalized to HPRT1 as a stable reference gene for quantitative real-time reverse transcriptase PCR (RT-PCR). All primers were purchased from IDT (Integrated DNA Technologies, Inc.). The quantitative reverse transcription polymerase chain reaction was carried out in an iCycler (Bio-Rad), with the threshold cycle number was determined by iCycler software version 3.0. The relative mRNA expression levels were calculated according to the formula $2^{([\text{RT}]/[\text{Input}])}$, as described previously (30).

Western blot analysis

Cells were washed with PBS and lysed in RIPA buffer (50 mmol/L Tris–HCl buffer, pH 7.4, 150 mmol/L NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS) supplemented with 1× Halt Protease Inhibitor Cocktail and 1× Halt Phosphatase Inhibitor Cocktail (Pierce). Protein concentrations were determined with the Bio-Rad Protein Assay (Bio-Rad). Total protein (200 μg) was incubated with 50 μL Dynabeads Protein G (Invitrogen) in an Eppendorf tube for 1 hour at 4°C on a rotating platform to pull down specifically trastuzumab-bound ERBB2 protein. The tubes were placed in a magnetic stand and supernatants were discarded. The beads were then processed and the bound protein was eluted as described above. Protein bands intensities were semiquantitatively analyzed by densitometry using ImageJ software (NIH Image). Pulled-down ERBB2 protein bands intensities were depicted as ratios relative to their corresponding input ERBB2 proteins.

Nude mice xenograft experiments

Five-week-old female athymic nude-Foxn1 nu/nu mice (Harlan Laboratories Inc.) were purchased and maintained under specific pathogen-free conditions. The mice were randomized into 2 groups (10 xenografts per group). OE19 cells stably expressing t-DARPP or pcDNA3 empty vector were injected subcutaneously (s.c.; $4 \times 10^6$ cells, suspended in 200 μL growth-factor-reduced Matrigel per site) into the flanks. After the xenografted tumors reached a volume of 200 mm$^3$ or more, mice were treated with 20 mg/kg trastuzumab diluted in sterile PBS by intraperitoneal (i.p.) injection twice weekly. To determine tumor volume, the greatest longitudinal diameter (length) and the greatest transverse diameter (width) were serially measured by external caliper. Tumor volume was calculated by the formula: Tumor volume $= \frac{1}{2} (l \times w^2)$. The Vanderbilt Institutional Animal Care and Use Committee approved all animal work.

Statistical analysis

Data are presented as means ± standard error of mean. All in vitro experiments were carried out in triplicate. Statistical significance of the in vitro studies was evaluated by the parametric unpaired Student $t$ test. The $t$ test, Wilcoxon Rank Sum test, and Spearman correlation test were used for analyses of primary tumors. Differences with $P$ values ≤0.05 are considered significant.

Results t-DARPP overexpression is associated with tumor stage and directly correlates with ERBB2 mRNA levels

Using the $t$-test, t-DARPP and ERBB2 were found to be significantly overexpressed in tumors as compared with normal samples ($P < 0.001$; Fig. 1A). The Wilcoxon Rank Sum test showed a similar result. The expression of t-DARPP and ERBB2 showed a similar pattern in esophageal and gastric adenocarcinomas. Overexpression (log$_2$ fold expression ≥2.0) of ERBB2 was detected in 57 tumors (40%) whereas t-DARPP was overexpressed in two-thirds of the tumors. The use of a cutoff log$_2$ fold expression ≥4.0 showed overexpression of ERBB2 and t-DARPP in 22% and 48% of the tumors, respectively. Statistical analysis using Spearman’s correlation coefficient and correlation test, where the cutoff for gene expression is ≥log(5,2) = 2.32, indicated that ERBB2
overexpression values in tumors were significantly correlated with those of t-DARPP \((r = 0.58, P = 0.003; \text{Fig. 1B})\). The multivariate regression model analysis indicated that tumor stage has a significant effect on t-DARPP mRNA gene expression levels \((P = 0.02)\). D, left, cell viability of OE19 and OE33 cells in response to trastuzumab treatment was evaluated by Trypan blue staining. OE19 cells were 2-fold more sensitive to trastuzumab than OE33 cells \((P < 0.001)\). D, right, Western blot analysis shows higher protein expression of ERBB2 in OE19 cells than OE33 cells. In contrast, t-DARPP expression was undetectable in OE19 cells but highly expressed in OE33 cells.

Figure 1. t-DARPP and ERBB2 are significantly overexpressed in adenocarcinomas. A, significant mRNA overexpression of t-DARPP and ERBB2 in adenocarcinomas of the esophagus and stomach \((141 \text{ tumors and 51 normal tissue samples}; P < 0.001)\). B, Spearman correlation coefficient and correlation test in which the cutoff gene expression is log(5,2) = 2.32 or more show that t-DARPP and ERBB2 overexpression levels are significantly correlated in tumors \((r = 0.58, P = 0.003)\). C, the multivariate regression model analysis indicates that tumor stage has a significant effect on t-DARPP mRNA gene expression levels \((P = 0.02)\). D, left, cell viability of OE19 and OE33 cells in response to trastuzumab treatment was evaluated by Trypan blue staining. OE19 cells were 2-fold more sensitive to trastuzumab than OE33 cells \((P < 0.001)\). D, right, Western blot analysis shows higher protein expression of ERBB2 in OE19 cells than OE33 cells. In contrast, t-DARPP expression was undetectable in OE19 cells but highly expressed in OE33 cells.

To establish cell models for investigating the role of t-DARPP in trastuzumab resistance in esophageal adenocarcinoma, we evaluated the survival of 2 esophageal adenocarcinoma cell lines, OE19 and OE33, in response to trastuzumab. The results of the trypan blue dye exclusion assay indicated that OE33 cells were more resistant to trastuzumab than OE19 cells \((P < 0.001)\), as treatment with 20 or 60 \(\mu\text{g/mL}\) trastuzumab decreased the survival by 70% in OE19 cells as opposed to 40% in OE33 cells (Fig. 1D, left). In addition, the results showed that 20 \(\mu\text{g/mL}\) trastuzumab was a saturation concentration, as a higher drug concentration \((60 \mu\text{g/mL})\) had a similar effect on survival in the 2 cell lines. The Western blot analysis data showed that both OE19 and OE33 cells have high protein expression levels of ERBB2, which is in line with the reported increase in copy numbers of ERBB2 gene in these 2 cell lines (Fig. 1D, right). Although the t-DARPP protein was highly expressed in OE33 cells, it was undetectable in OE19 cells (Fig. 1D, right), indicating that t-DARPP expression was associated with increased trastuzumab resistance in esophageal cancer cells.

**t-DARPP promotes cell survival in esophageal adenocarcinoma**

The CellTiter-Glo viability assay results showed that transiently expressed t-DARPP increased cell survival by 40% relative to control cells in response to trastuzumab \((P < 0.001; \text{Fig. 2A})\). Similarly, stably expressed t-DARPP in OE19 cells enhanced cell survival by approximately 40% relative to control cells after treatment with trastuzumab \((P < 0.01; \text{Fig. 2B})\). As an additional cell model in our study, we generated trastuzumab-resistant OE19 cells through culturing sensitive parental cells in increasing concentrations of trastuzumab for 6 months. Interestingly, unlike parental cells, the resistant cells acquired expression of endogenous t-DARPP (Fig. 2C, bottom). Indeed, following treatment with trastuzumab, cell survival was significantly higher in resistant cells than parental cells \((P < 0.01)\) (Fig. 2C, top).
To confirm the short-term assay results, we conducted a long-term clonogenic survival assay using the 2 OE19 cell models. The results indicated that stable expression of t-DARPP doubled the cell survival relative to control (P < 0.001) in response to trastuzumab (Fig. 2D). Moreover, endogenous expression of t-DARPP in resistant cells tripled the cell survival relative to parental cells (P < 0.01) in response to trastuzumab (20 μg/mL) for 48 hours. These results show that t-DARPP significantly promoted cell survival in response to trastuzumab in OE19 cells.

Figure 2. t-DARPP enhances survival of esophageal cancer cells. A, cell viability of OE19 cells infected with control adenovirus (10 MOI) or t-DARPP adenovirus (10 MOI) in response to treatment with vehicle or trastuzumab (20 μg/mL) for 48 hours was assessed by CellTiter-Glo Luminescent Cell Viability Assay. B, cell viability of OE19 cells stably expressing pcDNA3 or t-DARPP treated with vehicle or trastuzumab (20 μg/mL) for 48 hours and determined as in A. C, cell viability of OE19 cells treated with vehicle or trastuzumab (20 μg/mL) for 48 hours and evaluated as in A. D, OE19 cells stably expressing pcDNA3 or t-DARPP were subjected to clonogenic survival assay after treatment with vehicle or trastuzumab (20 μg/mL) for 48 hours. Quantitative data are shown on the right. E, parental and trastuzumab-resistant OE19 cells were subjected to clonogenic survival assay after treatment with vehicle or trastuzumab (20 μg/mL) as in D. Quantitative data are shown on the right. These results show that exogenous and endogenous t-DARPP significantly promoted cell survival in response to trastuzumab in OE19 cells.

t-DARPP inhibits trastuzumab-dependent apoptosis and activation of caspase-3

The Annexin-V staining and FACS analysis results showed that adenoviral transient expression of t-DARPP suppressed early apoptosis events by approximately 25% relative to control (P < 0.02; Fig. 3A). In accordance with this, Western blot analysis data revealed the cleaved caspase-3 form in control cells, but not in t-DARPP-expressing cells following treatment with trastuzumab (Fig. 3B). Similarly, endogenous expression of t-DARPP in the resistant cells did
not lead to an increase in apoptosis as compared with non-treated cells, whereas parental cells showed an approximate doubling in apoptosis levels after treatment with trastuzumab ($P < 0.01$; Fig. 3C). In line with this result, Western blotting showed cleavage of caspase-3 in parental cells, but not in resistant cells after treatment with trastuzumab (Fig. 3D).

Together, these data indicated that t-DARPP expression counteracted trastuzumab-induced apoptosis in esophageal adenocarcinoma cells.

**t-DARPP stabilizes ERBB2 protein and activates downstream signaling in response to trastuzumab**

The CHX-based chase assay results indicated that stable expression of t-DARPP increased the half-life of ERBB2 protein to 42.2 hours as opposed to 30.8 hours in control cells (Fig. 4A). Similarly, the protein half-life of ERBB2 was 31.3 and 60.8 hours in parental cells and resistant cells, respectively (Fig. 4B). These results showed that t-DARPP expression significantly enhanced ERBB2 protein stability in esophageal adenocarcinoma cells. In accordance with these data, we used data from immunofluorescence analysis to show that ERBB2 expression on the cell surface was approximately 2-fold higher in t-DARPP-expressing cells than in control cells ($P < 0.01$; Supplementary Fig. S1).

We further investigated the role of t-DARPP in regulating the AKT signaling pathway downstream of ERBB2 following trastuzumab treatment. Western blot analysis data showed that trastuzumab treatment induced significant dephosphorylation of ERBB2 and AKT in control OE19 cells, but this effect was suppressed in OE19 cells transiently expressing t-DARPP (Fig. 4C). Similarly, stably expressed t-DARPP blocked trastuzumab-induced dephosphorylation of ERBB2 and AKT in OE19 cells (Fig. 4D). Further, these results were confirmed in an endogenous t-DARPP expression–resistant cell model (Fig. 4E). These data clearly showed that t-DARPP expression maintained ERBB2 phosphorylation and activated the downstream AKT survival pathway in response to trastuzumab treatment. Notably, t-DARPP-mediated ERBB2 protein stability was associated with increased levels of ERBB2, p-ERBB2 (Y1248), and p-AKT(S473) proteins in OE19 cells without treatment with trastuzumab (Figs. 4C–4E).

**Knockdown of endogenous t-DARPP sensitizes cells to trastuzumab**

Western blot analysis data indicated that knockdown of t-DARPP and treatment with trastuzumab significantly decreased p-ERBB2(Y1248) and p-AKT(S473) protein levels relative to controls (Fig. 5A). Knocking down t-DARPP alone, without treatment with trastuzumab, decreased the p-AKT (S473) protein level in OE33 cells (Fig. 5A). The CellTiter-Glo viability assay results revealed that knockdown of t-DARPP in combination with trastuzumab treatment decreased cell survival by 30% relative to control ($P < 0.01$; Fig. 5B). These results clearly showed that knocking down endogenous t-DARPP in OE33 cells significantly sensitized cells to trastuzumab.

**t-DARPP/ERBB2 protein interaction interferes with trastuzumab binding to ERBB2 receptor**

We hypothesized that t-DARPP interacts with ERBB2, thus interfering with trastuzumab binding to ERBB2
receptor, and subsequently affecting downstream signaling. The results of 2-way reciprocal immunoprecipitation assay showed that t-DARPP and ERBB2 coimmunoprecipitated, indicating their association in the same protein complex (Fig. 6A). When we investigated the effect of t-DARPP expression on trastuzumab binding to the ERBB2 receptor. Data from the immunoprecipitation assay showed that transiently expressed t-DARPP in OE19 cells decreased the trastuzumab binding to ERBB2 receptor by 2.8-fold relative to control cells (Fig. 6B). Similarly, endogenous t-DARPP expression in trastuzumab-resistant OE19 cells was associated with a 2.5-fold decrease in trastuzumab binding to ERBB2 relative to control cells (Fig. 6C). To further confirm these data, we treated OE19 cells stably expressing t-DARPP or control vector with trastuzumab and a BS3 cross-linking reagent. The immunofluorescence staining results showed that the trastuzumab-bound ERBB2 protein level was approximately 5-fold lower in t-DARPP-expressing cells than control cells (P < 0.01; Supplementary Fig. S2). In line with this finding, the immunofluorescence data showed strong cytosolic expression of t-DARPP that also colocalized with the membranous ERBB2 signal. Notably, cells expressing

Figure 4. t-DARPP promotes ERBB2 protein stability, inhibits trastuzumab-dependent ERBB2 dephosphorylation, and activates downstream signaling. A, ERBB2 protein stability in OE19 cells stably expressing t-DARPP or pcDNA3 empty vector was evaluated by Western blot analysis after treatment with 80 μg/mL CHX to block new protein synthesis for the indicated times. The protein degradation data indicate that t-DARPP expression extended the protein half-life of ERBB2 from 30.8 to 42.2 hours relative to control (bottom). B, ERBB2 protein stability in parental and trastuzumab-resistant OE19 cells was assessed by Western blot analysis after treatment with CHX (80 μg/mL) for the indicated times. The protein degradation data show that endogenous t-DARPP expression in resistant cells was associated with increased ERBB2 protein half-life (60.8 hours) relative to parental cells (31.3 hours; bottom). C, Western blot analysis of p-ERBB2 (Y1248), ERBB2, p-AKT (S473), AKT, and t-DARPP proteins in OE19 cells infected with control (10 MOI) or t-DARPP (10 MOI) adenoviruses after treatment with vehicle or trastuzumab (20 μg/mL) for 24 hours. These data indicate that transient expression of t-DARPP increased p-ERBB2(Y1248) and p-AKT(S473) basal protein levels, and blocked trastuzumab-dependent dephosphorylation of ERBB2 and AKT proteins. D, Western blot analysis of p-ERBB2 (Y1248), ERBB2, p-AKT (S473), AKT, and t-DARPP proteins in OE19 cells stably expressing t-DARPP or pcDNA3 vector after treatment with vehicle or trastuzumab (20 μg/mL) for 24 hours. The results show that stable expression of t-DARPP increased basal levels of p-ERBB2(Y1248) and p-AKT(S473), and inhibited trastuzumab-dependent dephosphorylation of ERBB2 and AKT proteins. E, Western blot analysis of p-ERBB2(Y1248), ERBB2, p-AKT(S473), AKT, and t-DARPP proteins in parental or trastuzumab-resistant OE19 cells following treatment with vehicle or trastuzumab (20 μg/mL) for 24 hours. The results indicate that endogenous t-DARPP expression was associated with increased basal levels of p-ERBB2(Y1248) and p-AKT(S473), and suppression of trastuzumab-dependent dephosphorylation of ERBB2 and AKT proteins.
higher levels of t-DARPP showed stronger ERBB2 signal (Supplementary Fig. S2C).

t-DARPP enhances tumor growth and inhibits response to trastuzumab

The results showed that tumors derived from OE19 cells stably expressing t-DARPP failed to respond to trastuzumab treatment (Fig. 7A). In contrast, trastuzumab effectively inhibited growth of control tumors (Fig. 7B). Notably, t-DARPP expression significantly increased tumor growth rate ($P < 0.01$) compared with the control (Fig. 7C, left). The control tumors grew significantly slower than t-DARPP tumors in response to treatment with trastuzumab ($P < 0.001$; Fig. 7C, right). The results from H&E staining indicated that trastuzumab effectively killed control tumors leaving fibrotic and necrotic areas with no obvious tumor cells (Fig. 7D, left), whereas this treatment failed to affect growth of t-DARPP tumors as manifested by the presence of moderately to poorly differentiated tumors (Fig. 7D, right).

Discussion

Although the incidence of EAC has increased rapidly in the last decade, especially among Caucasian men, limited progress in the treatment of EAC has been achieved (31). The prognosis of patients diagnosed with EAC remains poor with a 5-year relative survival rate of 10% to 20%. Over the past 15 years, targeted therapy approaches have made significant advances due to the rapid development of new drugs that aim treatment at specific molecular targets that are critical for cancer cell survival (32). Amplification and overexpression of ERBB2 has been identified in 15% to 20% of primary EAC tumor specimens and their corresponding metastases (33, 34). Therefore, in addition to breast cancer, ERBB2 has been proposed as a plausible target for treatment in esophageal cancers. Trastuzumab, a recombinant humanized monoclonal anti-ERBB2 antibody, was initially approved by the U.S. Food and Drug Administration for the treatment of ERBB2 metastatic breast carcinoma (19). With its successful application in breast
cancer, trastuzumab antitumor activity was investigated in patients with ERBB2-positive metastatic cancer of gastroesophageal junction in combination with chemotherapy (28). Notably, a phase III clinical trial (RTOG 1010 protocol) is currently ongoing to evaluate the addition of trastuzumab to increase disease-free survival when combined with trimodality treatment for patients with ERBB2-positive esophageal adenocarcinoma. However, previous studies have shown that cancer patients that initially respond well to trastuzumab develop acquired trastuzumab resistance within a year of treatment (20). Our current study provides important preclinical evidence indicating that t-DARPP could mediate trastuzumab resistance in EAC.

Our results indicate that both t-DARPP and ERBB2 were significantly overexpressed in a subset of EAC tumors. Moreover, overexpression of t-DARPP was significantly associated with advanced tumor stage. Notably, both ERBB2 and t-DARPP are located inside the 17q21 chromosomal region, a commonly amplified region in adenocarcinomas of the stomach and esophagus (33, 35). Several studies have shown that ERBB2 plays an important role in activation of the prosurvival phosphoinositide-3 kinase (PI3K)/AKT signaling pathway (36). Similarly, t-DARPP can mediate activation of the PI3K/AKT pathway (14). On the basis of these data, we hypothesized that t-DARPP and ERBB2 may have a functional relationship where t-DARPP-mediated activation of AKT could lead to resistance to trastuzumab. The results indicated that endogenous and exogenous t-DARPP expression can significantly enhance cell survival and block apoptosis in response to trastuzumab in esophageal adenocarcinoma cell models. The xenografted esophageal adenocarcinoma mouse model results confirmed the in vitro data and t-DARPP overexpressing tumors were unaffected by trastuzumab treatment and continued to grow. These results confirmed the role of t-DARPP in mediating resistance to trastuzumab in EAC cells. Our finding that trastuzumab-resistant OE19 cells, generated by trastuzumab selection, expressed a significantly higher endogenous level of t-DARPP than parental cells provides additional evidence supporting the role of t-DARPP in mediating resistance to trastuzumab. Interestingly, a breast cancer trastuzumab-resistant cell model expressed higher levels of t-DARPP (37). Taken together, these data indicate the possible presence of a small subpopulation of cells overexpressing t-DARPP among cancer cells where treatment with trastuzumab provides the appropriate selection advantage for this subpopulation to continue to grow and replace trastuzumab-sensitive cell populations.

Figure 7. t-DARPP overexpression promotes tumor growth and blocks response to trastuzumab treatment in vivo. OE19 cells stably expressing t-DARPP or pcDNA3 control vector were injected subcutaneously (4 × 10⁶ cells per site) into nude mice. When tumor volume reached 200 mm³, the mice were treated with 20 mg/kg trastuzumab twice weekly for 20 days. A, a representative sacrificed control mouse with xenograft tumors (left) and resected xenograft tumors (right) at the end of the experiment. B, a representative sacrificed t-DARPP mouse with xenograft tumors (left) and resected xenograft tumors (right) at the end of experiment. C, tumor growth curve from OE19 cells stably expressing t-DARPP or control vector before the treatment (left) and after the treatment (right). Each data point represents the mean ± SD. The data show that t-DARPP significantly enhanced tumor growth rate (P < 0.01), and inhibited response to treatment with trastuzumab (P < 0.001) as compared with control. D, the H&E staining at the end of trastuzumab treatment shows effectively diminished control tumors leaving necrotic and fibrotic lesions (left), whereas t-DARPP tumors were unaffected (right).

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Although we as well as other researchers have previously shown that t-DARPP can lead to activation of AKT and resistance to trastuzumab in breast cancer cells in vitro (14, 37–39), the mechanism by which t-DARPP activates the AKT survival pathway was not fully identified. In the current study, our data clearly indicated that t-DARPP expression, without treatment with trastuzumab, significantly increased ERBB2, p-ERBB2(Y1248), and p-AKT(S473) protein levels relative to control cells. Notably, the trastuzumab-resistant OE19 cells showed, in addition to t-DARPP overexpression, increased p-ERBB2(Y1248) and p-AKT(S473) protein levels as compared with parental cells, consistent with our findings from transient and stable expression of t-DARPP. Taken together, these results indicate that t-DARPP can significantly enhance ERBB2 protein stability thereby enhancing the AKT pathway. In addition, the possibility that t-DARPP could enhance the protein stability of other members of the ERBB family remains to be investigated in EAC.

Previous studies have shown that failure to suppress the AKT pathway plays central role in resistance to trastuzumab in breast cancer. Several mechanisms have been proposed, such as activating mutations in the PI3KCA, deletions of PTEN, overexpression of cMET, and overexpression of ERBB3 (40, 41). In an attempt to elucidate the mechanistic role of t-DARPP in trastuzumab resistance in EAC, we found that t-DARPP was associated with ERBB2 in a protein complex. On the basis of this finding, we hypothesized that t-DARPP interaction with ERBB2 prevents trastuzumab binding to ERBB2 receptor, thus blocking trastuzumab-induced downregulation of ERBB2/AKT signaling. Indeed, our results clearly showed that t-DARPP significantly decreased trastuzumab binding to the ERBB2 receptor in OE19 cells. The immunofluorescence data clearly indicated that t-DARPP expression was cytosolic (Supplementary Fig. S2C), strongly indicating that t-DARPP interaction with ERBB2 cytosolic domain could alter ERBB2 protein folding and conformation, thereby interfering with trastuzumab binding to the ERBB2 extracellular domain. Nagy and colleagues (42) uncovered a similar mechanism of trastuzumab resistance in JIMT-1 breast cancer cells and showed that the expression of MUC4, a transmembrane glycoprotein, promoted resistance to trastuzumab through masking of ERBB2, which leads to diminished binding of trastuzumab. These data indicate that molecular mechanisms that lead to the impairment of binding of trastuzumab to its target, ERBB2, are important upstream determinants of therapeutic response.

In conclusion, our findings indicate that frequent overexpression of t-DARPP in ERBB2-positive EAC forms the basis for a trastuzumab resistance phenotype. Therefore, our data indicate that t-DARPP expression status could potentially be exploited for more effective clinical management of patients with ERBB2-positive esophageal adenocarcinoma who qualify for treatment with trastuzumab.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: J. Hong, A. Belkhiri, W. El-Rifai
Development of methodology: J. Hong, A. Belkhiri, W. El-Rifai
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Hong, A. Katsha, W. El-Rifai
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Hong, P. Lu, Y. Shyr, W. El-Rifai
Writing, review, and/or revision of the manuscript: J. Hong, A. Belkhiri, W. El-Rifai
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): W. El-Rifai
Study supervision: A. Belkhiri, W. El-Rifai

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Jun Hong, Ahmed Katsha, Pengcheng Lu, et al.


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