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Reverse engineering of triple-negative breast cancer cells for targeted treatment

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ABSTRACT

Objective: Targeting the human epidermal growth factor receptor HER2 has increased survival in HER2-positive breast cancer patients. In the contrast, for triple-negative breast cancer (TNBC) patients, no targeted agents are available. We hypothesized that artificial overexpression of HER2 in TNBC cells might induce sensitivity to anti-HER2 agents in these cells.
Methods: TNBC cell lines were transduced using lentiviral HER2 overexpression particles. Functionality of HER2 was determined by protein analysis and localization studies. The tumorigenic potential of HER2 overexpressing cells was assessed by analysis of proliferation, migration and invasion capacity. Response to chemotherapeutic agents and anti-HER2 agents was determined by cell viability assays.
Results: We demonstrated functional overexpression of HER2 in TNBC cell lines of different subtypes. Whereas in cell types with more pronounced epithelial features (e.g. MDA-MB-468) HER2 overexpression increases proliferation and migration, in mesenchymal cell lines (MDA-MB-231 and BT-549) HER2 was able to further increase invasive potential. No changes were found in cancer stem cell characteristics or in response to chemotherapy, a trait of TNBC. When treated with anti-HER2 agents, however, HER2 overexpressing TNBC cells showed increased sensitivity to these agents.
Conclusion: This proof-of-principle study demonstrates that reverse engineering of TNBC cells might offer a novel targeted treatment strategy for this most aggressive subtype of breast cancer.

1. Introduction

The HER2 positive breast cancer subtype is associated with aggressive behavioral traits, including enhanced growth and proliferation, increased invasive and metastatic capability, and stimulation of angiogenesis [1]. This subtype correlates with poor outcome [2]. The development of targeted therapies, however, significantly improved the prognosis of this aggressive subtype of breast cancer. Mainly, development of HER2 targeting drugs, e.g. the HER2 blocking antibody trastuzumab, which binds to the extracellular domain (ECD) of HER2, improved the prognosis of this subtype [3]. Upon binding of trastuzumab, either homodimerization or heterodimerization to EGFR, HER3 or HER4 is blocked. Thus, intracellular phosphorylation of the tyrosine kinase domain is inhibited, thereby blocking both MAPK and PI3K signaling pathways [4]. In 1998, trastuzumab has become the first anti-HER2 therapy for HER2 positive breast cancer [5]. Since then, even more treatment strategies for HER2 positive breast cancer have been developed; some of them, which are HER2:HER3 heterodimerization blocking antibodies (pertuzumab), antibody drug conjugates (trastuzumab emtansine, T-DM1) or intracellular inhibitors of the tyrosine kinase domain (lapatinib). All these drugs rely on binding to domains of the functional HER2 protein, either extracellularly (trastuzumab, pertuzumab) or intracellularly (lapatinib).

In triple negative breast cancer (TNBC), tumor cells express neither growth factor receptor (HER2) nor the hormone receptors estrogen receptor (ER) or progesterone receptor (PR)). Therefore, this cancer subtype cannot be targeted by anti-HER2 treatment or endocrine therapies. Consequently, chemotherapeutic treatment is the most common therapeutic option for this breast cancer subtype. Thus, there is an urgent need for development of novel treatment strategies for this aggressive cancer subtype.

As targeted treatment of HER2 protein has significantly improved prognosis and survival of HER2 positive breast cancer patients, we hypothesized that reverse engineering of TNBC cells to HER2 positive cells might positively influence the treatment potential for TNBC. Therefore, we reprogrammed TNBC cells to HER2 positive cells and analysed both molecular changes of the HER2 signaling axis as well as the potential of anti-HER2 treatment in these reverse engineered tumor

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cells.

2. Material and methods

2.1. Cloning of pCDH-EF1-HER2-T2A-Puro

For cloning, HER2 cDNA was PCR amplified from HER2 positive BT474 breast cancer cell line cDNA using primers containing restriction sites for XbaI at 5′ end and NotI at 3′ end. PCR primers did not amplify the TGA stop codon, since HER2 cds was cloned in frame to a T2A sequence in the cloning vector pCDH-EF1-MCS-T2A-Puro (System Biosciences, USA). The PCR product was ligated into the cloning vector and correct product size was determined by sequencing analysis. Primers are listed in Table S1.

2.2. Cell culture, lentiviral production and transduction

All cell lines (MDA-MB-468, MDA-MB-231, BT-549 and BT474) were purchased from ATCC (USA) and cultured under recommended conditions. Medium, trypsin-EDTA, PBS, fetal calf serum and horse serum were received from PAA Laboratories (Germany). STR authentification analysis of cell lines was performed twice a year at DSMZ (Braunschweig, Germany). For lentiviral production, HEK293 cells were transiently transfected with pCDH-EF1-HER2-T2A-Puro along with 2 packaging plasmids. After 72 h, lentiviral particles were sterile filtered from the HEK293 supernatant and subsequently incubated with breast cancer cell lines for 24 h. After 5 washing cycles, HER2 positive breast cancer cell lines were further incubated in the presence of puromycin (Sigma-Aldrich, Germany).

2.3. Western blot analysis

Cells were incubated with RIPA buffer (10 mM NaF, 1 mM Na3VO4, 10 mM β-Glycerophosphate, 7.6 mM Tris pH 7.4, 52 mM NaCl, 0.4% Triton X-100, 0.8 mM EDTA, proteinase inhibitor (Sigma-Aldrich, Germany). Protein quantification was performed via Pierce BCA assay (Thermo Scientific, Germany) according to the manufacturer's protocol. SDS page electrophoresis and blotting were performed using standard protocols. Detection was performed using primary antibodies against HER2 (#2242), phospho-HER2 (Tyr1248, #2247), p44/42 MAPK (#9102), phospho-p44/42 MAPK (Thr202/Tyr204, #9101), AKT (#9272) and phospho-AKT (Ser473, #9271) (all Cell Signaling, Netherlands), tubulin (#T5168, Sigma-Aldrich, Germany) and Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific, Germany). Bands were visualized with AGFA developer and fixer (AGFA, Belgium).

2.4. Quantitative real-time PCR

RNA isolation was performed using NucleoSpin RNA Kits (Macherey-Nagel, Germany) with on-column DNase digestion. Reverse transcription for real-time quantitative polymerase chain reaction (RT-qPCR) was performed using M-MLV reverse transcriptase (USB (Affymetrix), USA) and Oligo-dT15 priming at 42 °C for 1 h and at 60 °C for 10 min. A cDNA equivalent of 50 ng total RNA was used as template in a total reaction volume of 20 μl with Power SYBR Green PCR mix (Applied Biosystems, Germany) on a Step One Plus cycler (Applied Biosystems, Germany). Primers were added at 0.375 μM each. Calculations were based on the ΔΔCt method using two housekeeping genes for normalization. Real-time primer sequences are listed in Table S1.

2.5. Cell migration/invasion assay

Migration and invasion assays were performed as described elsewhere [6]. Briefly, cells were seeded without serum onto either 8 μm

membranes (Corning, Germany) or BioCoat Matrigel invasion chambers (BD Biosciences, Germany). Membranes were cultured in 24-well plates containing medium with serum. After 48–96 h, cells that passed the membrane were fixed, stained and analysed.

2.6. Immunocytochemistry

For immunocytochemistry, cells were fixed with phosphate buffered formalin and blocked with 10% Aurion (Dako, USA) in PBS for 1 h. Cells were then washed and incubated with primary antibody (HER2, #2242, Cell Signaling, Netherlands) diluted with Dako REALTM Antibody Diluent (overnight at 4 °C). Fluorescent visualization was carried out using suitable Alexa Fluor-conjugated secondary antibody (1:600) together with 4′,6-diamidino-2-phenylindole (1:400) in Dako REALTM Antibody Diluent for 1 h at RT.

2.7. Chemotherapy sensitivity assay/Dose dependent response to anti-HER2 drugs

For analysis of chemotherapy sensitivity, cells were incubated with cytotoxic agents (paclitaxel and doxorubicin hydrochloride) using decreasing concentrations. After 72 h, cell viability was determined via MTT (Thiazolyl Blue Tetrazolium Bromide) (all substances were received from Sigma-Aldrich, Germany) according to the manufacturer's protocol. For analyses of dose dependent response to anti-HER2 drugs, cells were incubated with decreasing concentrations of trastuzumab, pertuzumab, lapatinib and T-DM1. After 72 h, cell viability was determined via MTT according to the manufacturer's protocol. Measurements were performed in triplicates. Significance was calculated via one-side Welch's *t*-test.

2.8. Flow cytometry

Flow cytometric cell analyses were performed on a FACS Aria (BD FACSAriaTM, BD Biosciences, Germany) as described previously [7].

3. Results

3.1. Reverse engineering of TNBC cells to HER2 positive cells

In order to examine the potential of reverse engineering as a novel therapeutic strategy for TNBC, we stably overexpressed HER2 in the TNBC cell lines MDA-MB-468, MDA-MB-231 and BT-549 using lentiviral particles. Upon lentiviral transduction, all cell lines showed overexpression of HER2 mRNA as well as HER2 protein determined by RT-qPCR and Western Blot analysis using HER2 antibodies against both the extracellular domain and the intracellular phosphorylation domain of HER2 (Fig. 1A, B). Besides, immunocytochemistry analysis revealed strong signals for HER2 protein on the cell membrane (Fig. 1C), suggesting membrane bound localization. These results confirm the correct translation as well as cellular localization of HER2 protein.

3.2. HER2 activates MAPK but not PI3K pathway in TNBC cells

Next, we analysed the functionality of overexpressed HER2. For this purpose we determined the influence of HER2 on MAPK and PI3K signaling pathways, two known downstream pathways of HER2 [8], by using phospho-specific antibodies for AKT and p42/44 MAPK. No changes were found in levels of phosphorylated AKT, which was present in HER2 positive cells as well as in control cells (Fig. 2A). While levels of MAPK were similar in HER2 positive and control cells, we found induction of phosphorylated MAPK in HER2 positive BT-549 as well as MDA-MB-231 cells, but not in MDA-MB-468 cells (Fig. 2B). These results suggest specific induction of MAPK signaling pathway but not of PI3K signaling pathway by HER2 in MDA-MB-231 and BT-549 cells.