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An Induced CD44v6 – EGFR scaffold kinase interaction predisposes a subpopulation of chemotherapy tolerant breast cancer cells and serves as a bi-valent target of resistance

Abstract

The failure of chemotherapeutic agents to completely ablate tumor cells and the resulting relapse remain key obstacles of effective cancer therapy. Despite the initial success of most cancer treatments, failure over time is accompanied by the acquisition of chemoresistance. A subpopulation of cancer cells is inherently tolerant to chemotherapy. These cells exert a non-mutational and deterministic alteration to confer a survival advantage during chemotherapy treatment. Although P13K/Akt signaling pathway does help cancer cells to survive lethal doses of chemotherapy agents, elucidating the mechanism to target these cells is required. In-vitro and in-vivo models revealed that the transmembrane glycoprotein, CD44v6 is involved in recruiting a complex scaffold with Ezrin/Radixin/Moesin (ERM) and Akt. Epidermal growth factor receptor (EGFR) activates this cell cortex scaffold in a kinase dependent mechanism along with physical interaction with CD44v6 which renders a subpopulation of chemotherapy tolerant cells sensitive to treatment. A novel bi-specific targeting of CD44v6-EGFR scaffold kinase interaction leads a innovative treatment for relapse in breast cancer.

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1. Introduction

A major obstacle to successful cancer therapy is the acquisition of resistance to chemotherapy typically characterized by relapse, tumor progression and aggressive metastasis. Though the established dogma stipulates stochastic events and mutational alterations as a reason for the refractoriness, emerging evidence also implicates non-mutational and acute mechanisms underlying the failure of chemotherapy. Recent studies have demonstrated the epigenetic mechanisms underlie the survival of a population of drug tolerant cancel cells. Similarly another study has demonstrated a chromatin-mediated drug tolerant state in a subset of cancer cells. Thus, Understanding the heterogeneity of breast tumors and the mechanisms underlying drug-tolerance therefore is important in the quest for successful cures for breast cancer.

Highly malignant triple-negative breast cancer cells are associated with resistance and relapse after drug treatments. Elucidating the role of survival signaling would be necessary in identifying the nature of drug tolerance. Recent evidences indicates that active epidermal growth factor receptor (EGFR) occurs simultaneously with active Akt survival signaling in tumor cells. Ezrin/Radixin/Moesin (ERM) is implicated in survival signaling to act downstream of EGFR. Acting as an important scaffold, CD44 functions as complex membrane recruiting the proteins leading to the active survival and tolerance.

Our study particularly focusses on the sophisticated scaffold interactions responsible for the transient survival signaling induced by chemotherapy. CD44, a cancer stem cell marker (CSC) does not alone predict malignancy but its role to induce tolerance by forming a complex scaffolding interaction with EGFR and downstream ERM. Also, chemical recombination of the individual monoclonal antibodies of CD44v6 and EGFR targets a subpopulation of chemotherapy tolerant breast cancer and renders them sensitive and synergize along with anititubulins.

2. Methods

2.1 Cell culture and gene knockdown with siRNA

MDA-MB-231 (ATCC) were cultured in DMEM containing 10% Fetal Bovine Serum (FBS), MDA-MB-468 MDA-MB-435 and 4T-1 mammary carcinoma cells (ATCC) were cultured in RPMI containing 10%FBS (Invitrogen, Carlsbad CA) at 37C and 5%CO_2. During treatments with chemotherapeutics, cells were grown to semi-confluence and treated with indicated concentrations of chemotherapy in serum-containing medium for indicated time points. When small molecule

inhibitors were included in treatments, they were added simultaneously with chemotherapy. For siRNA gene knockdown, cells were plated at a concentration of $5x10^4$ cells/ml. Pre-validated Silencer Select siRNA targeting pan-CD44 (5'UAUUCCACGUGGAGAAAAAtt3') or Ezrin (5'CGUGGGAUGCUCAAAGAUAtt3') were purchased from Ambion (Invitrogen, Grand Island, NY). siRNA plasmids were transfected using lipofectamine 2000 (Invitrogen, Carlsbad CA) following manufacturer protocol and cultured for 72 hours prior to treatment. Scrambled siRNA was used as a control.

2.2 Generating a subpopulation of chemotherapy-tolerant breast cancer cells (DTC)

Cells were plated in 100mm Plates at a density of $1x10^6$ cells/ml and cultured for 48 hours. Cells were then treated with indicated concentrations of chemotherapy and cultured for 48 hours, DTC were treated with 100nM docetaxel which is a concentration >20 times the IC50. After 48 hours of treatment floating cells were washed with PBS, adherent cells were trypsinized and re-plated at a density of $1x10^6$ cells/ml and cultured in serum-containing medium. After 24 hours incubation, floating cells were removed and remaining cells were washed with 1X PBS. The remaining adherent cells are considered a subpopulation of chemotherapy-tolerant cells. A separate plate of control cells were maintained concurrently in serum containing medium and harvested at the same time as chemotherapy tolerant cells.

2.3 Generating EGFR-CD44v6 Bispecific antibodies (BsAb)

Neutralizing antibodies targeting the extracellular domain of EGFR (Millipore, LA1) or CD44v6 (R&D, 2F10) were chemically recombined following an established procedure {Brennan, 1985 #989}. Briefly, following Ficin digestion to reveal two $F(ab)^2$ fragments (Thermo Fisher, IgG1 preparation kit), antibodies were independently incubated with mercaptoethylamine, EDTA and sodium arsonite to reduce the hinge region revealing F(ab) fragments and subsequently subjected to buffer exchange (Thermo Fisher, Zeba desalt column). Protection of the free thiol was performed using Ellman's reagent followed by buffer exchange, thiol regeneration with mercaptoethylamine and finally recombination of the two F(ab) fragments. Unreacted thiols were cleared by Ellman's reagent and then purified by several rounds of centrifugal size exclusion (Millipore, Billerica MA). Dual specificity was confirmed by western blot of MDA-MB-468 lysate using biotinylated BsAb (Thermo Fisher, Rockford IL) and HRP-streptavidin chemiluminescence. Fluorescent conjugation was performed using a microprotein Fluor-488 conjugation kit (Anaspec, Fremont CA).

2.4 Immunoprecipitation and Immunoblotting

Laemli sample buffer was prepared as a 5X solution containing beta-mercaptoethanol as a reducing agent. Immunoprecipitaion was performed using both classic and direct IP kits purchased from Pierce following manufacturer protocols (Thermo Fisher inc. Rockford, IL). Briefly, cell lysates were prepared using IP/Lysis Buffer (Thermo Fisher inc. Rockford, IL) in the presence of 2X HALT protease/phosphatase inhibitor cocktail (Thermo Fisher inc. Rockford, IL). For classic Immunoprecipitation, lysates were combined with indicated antibodies for 48 hours at 4C and combined with protein A/G agarose beads for 4 hours prior to elution with 2X Laemli Buffer at 100C. Direct immunoprecipitation was performed following manufacturer protocol. Briefly, antibodies were covalently attached to agarose beads, lysate was combined with antibody-agarose bead conjugates for 24 hours prior to washes and elution with provided Elution Buffer.

Protein samples were resolved by SDS-PAGE and transferred to PVDF membranes prior to incubation at 4C with indicated primary antibodies. PVDF membranes with primary antibody were incubated at room temperature with HRP conjugated secondary antibodies (BD bioscience) and resolved by chemiluminescence using the G-Box and Syngene software (Syngene Cambridge, UK). Optical densities were measured using ImageJ open source software (National Institutes of Health).

3. Results

3.1 A subpopulation of breast cancer cells reveals a broad chemotherapy tolerance

A subpopulation of cancer cells exists within heterogeneous tumors which are tolerant to chemotherapy. Cytotoxicity studies of antitubulins on triple-negative breast cancer cell lines MDA-MB-468 (468) and MDA-MB-231 (231) shows a population of breast cancer cells persist after prolonged exposure (Fig. 1A). This plateauing effect is consistent with even drugs circumventing efflux related concerns. We sought to isolate the population of cells which survive treatment of docetaxel on the basis of substrate reattachment in spite of treatment with 100nM docetaxel, a concentration 20 times greater than the published IC-50 values. These cells were termed as Docetaxel Tolerant Cells (DTC) (Fig. 1B).

This subpopulation of cells displayed low active apoptosis. This was indicated by the activated cleaved caspase 3, compared to the chemotherapy treated parent cells (Fig. 1C). This subpopulation of docetaxel tolerant cells exhibit tolerance over a broad range of chemotherapy agents.

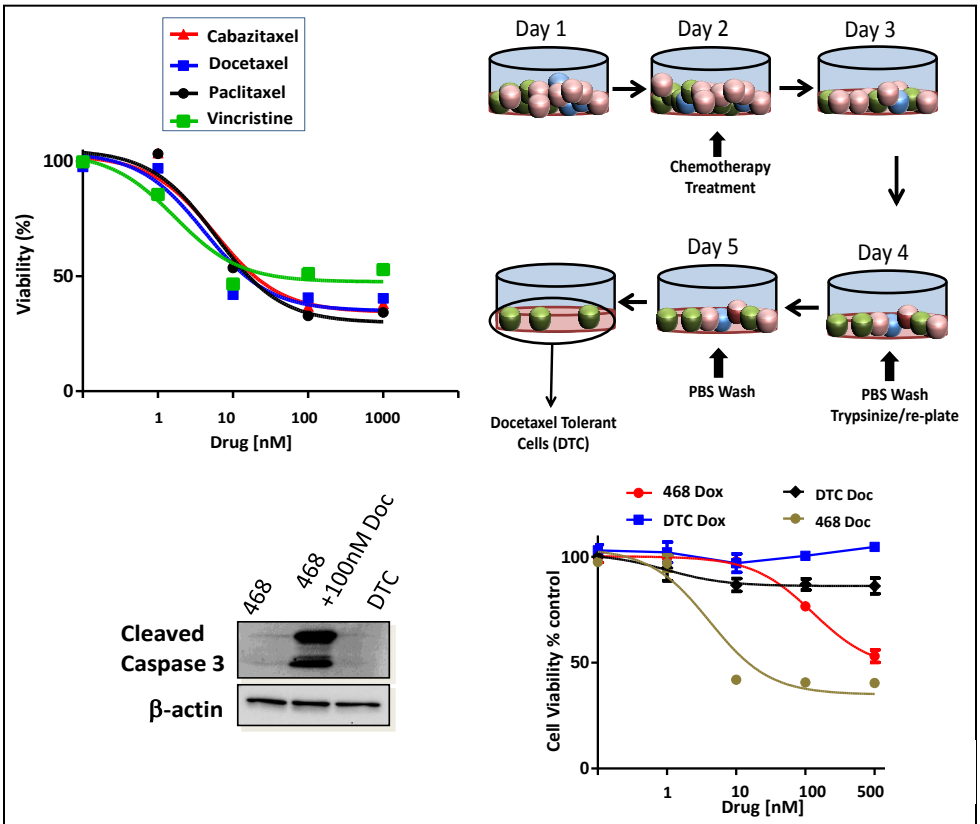

Fig. 1 A) Cell viability of MDA-MB-468 in the presence of indicated chemotherapy for 48 hours. B) Schematic for generating a subpopulation of chemotherapy tolerant breast cancer cells. Cells which survive 100nM Docetaxel were termed docetaxel tolerant cells (DTC). C) Western blot of active caspase 3 from MDA-MB-468 cells in the presence or absence of 100nM docetaxel and DTC, b-Actin loading control. D) MTS cell viability analysis of MDA-MB-468 parent or DTC cells incubated for 48 hours in indicated chemotherapy.

3.2 CD44 induces ERM mediated AKT survival signaling in a subpopulation of chemotherapy tolerant breast cancer cells

Activation of survival kinases is postulated to be one of the regulators of oncogenic survival signaling and in chemotherapy resistance. However the mechanistic role played by the kinases and its role in survival is poorly understood. We observed an increase in expression levels of phosphorylated Akt in DTC, suggesting survival is largely driven by this kinase (Fig. 2A). Consistently, DTC maintain their sensitivity to P13K/Akt inhibitor LY294002 (Fig. 2B), despite

their broad chemotherapy tolerance. However, Akt signaling has proven to be a difficult target for clinical intervention.

A number of upstream and adaptor proteins function to activate Akt. We observed an increase in the expression levels of cell cortex proteins involved in survival such as Ezrin and also the complex activation of ERM in DTC compared to the parent cells. Moreover, heterogeneity within tumors suggests that single-kinase targeting is an effective method of breast cancer therapy. Therefore, we began to investigate scaffold-kinase interactions, upstream of Akt and ERM, which may precipitate subsistence of DTC.

CD44 and EGFR have been independently associated with activation of ERM and Akt, respectively. Gene silencing of CD44 with siRNA transfection and subsequent immunoprecipitation and western blotting demonstrated that the Akt/ERM/EGFR complex is forms in response to antitubulin chemotherapies when CD44 is expressed (Fig. 2C).

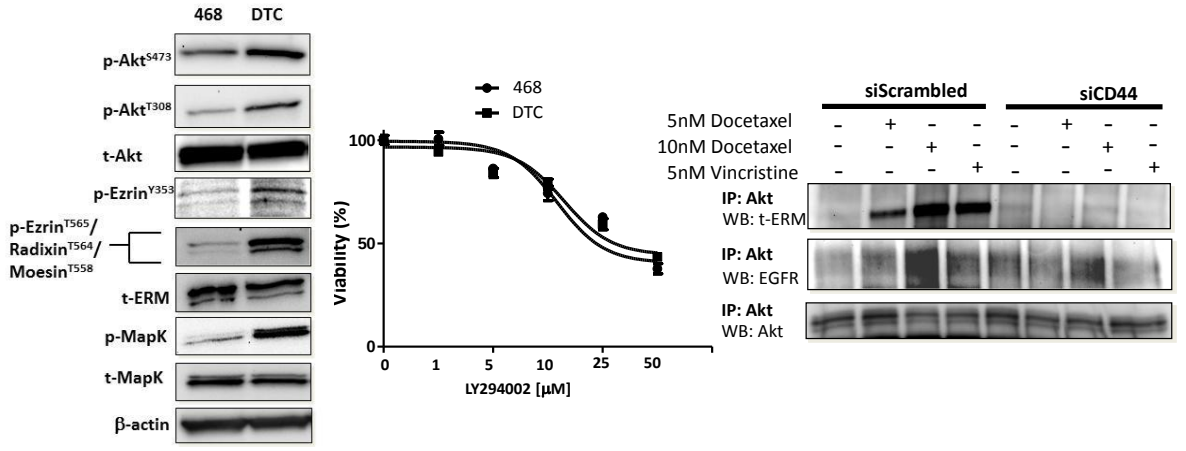

Fig. 2 A) Western blot of 468 and chemotherapy tolerant cells (DTC) shows increased EGFR-associated survival and oncogenic protein activation B) Cell viability of 468 and DTC in the presence of the PI3K/Akt inhibitor LY294002 C) Immunoprecipitation of Akt from 468 cells +/- chemotherapy demonstrates a requirement for CD44 expression for full complexation of the ERM-Akt survival pathway.

3.3 Enhanced EGFR-CD44v6 interaction is induced in in-vivo and in-vitro models of chemotherapy tolerance.

Recent clinical evidence indicates that the variant isoform 6 of CD44 (CD44v6) correlates with aggressive forms of breast cancer. Immunoprecipitation of EGFR from unstimulated 468 and

chemotherapy tolerant (DTC) cells demonstrates a heavy physical interaction with CD44v6 (Fig. 3A). These findings were confirmed when we analyzed residual tumor tissue harvested from *in-vivo*, xenograft mice treated with docetaxel (Fig. 3B). Based on these findings we hypothesized that neutralizing antibodies targeting CD44v6 will abrogate survival signaling induced by chemotherapy. Our preliminary studies have investigated this hypothesis by examining the effect of a neutralizing antibody targeting CD44v6 to silence Akt survival signaling(Fig. 3C).

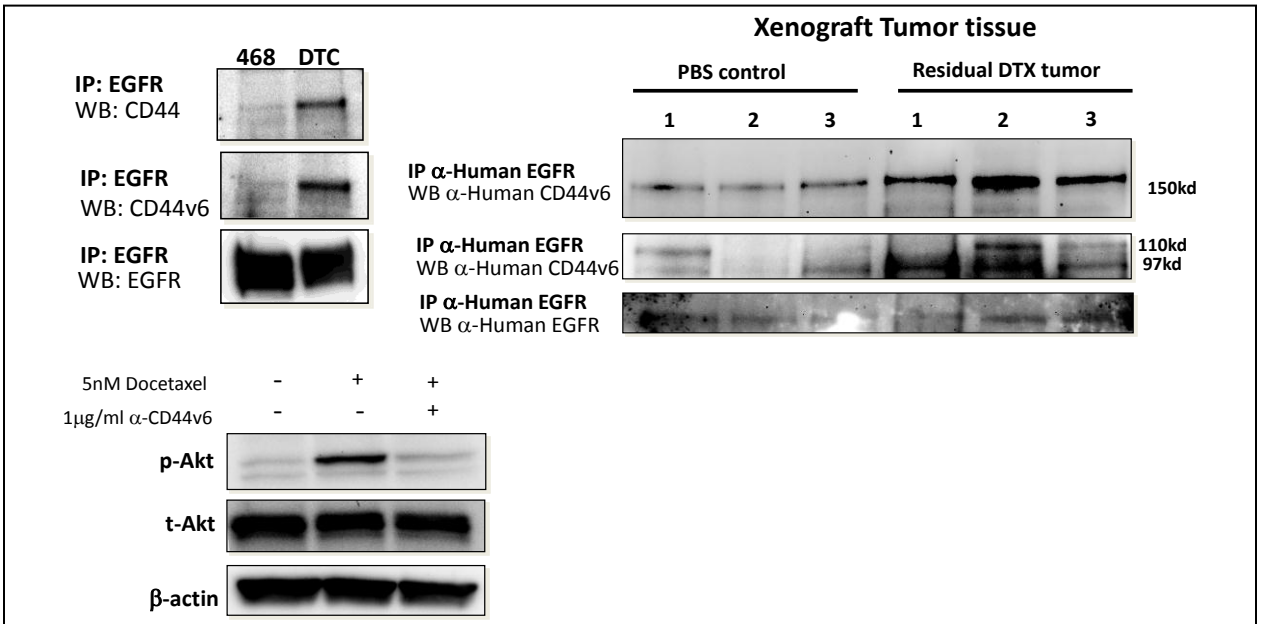

Fig. 3 A) Western blot of 468 and chemotherapy tolerant cells (DTC) shows increased EGFR-associated survival and oncogenic protein activation B) Cell viability of 468 and DTC in the presence of the PI3K/Akt inhibitor LY294002 C) Immunoprecipitation of Akt from 468 cells +/- chemotherapy demonstrates a requirement for CD44 expression for full complexation of the ERM-Akt survival pathway.

3.4 Chemical recombination of EGFR and CD44v6 monoclonal antibodies sensitizes a subpopulation of chemotherapy tolerant cancer cells

Based on these observations, we speculated that independent inhibition of EGFR or CD44v6 will augment survival of breast cancer cells following exposure to antitubulin chemotherapy. According to cytotoxicity assays performed in-vitro, our preliminary evidence suggests that neutralizing antibodies will synergize with low dose chemotherapy to significantly attenuate breast cancer cell viability following sustained exposure with chemotherapy (Fig. 4B).

The emerging paradigm of breast cancer treatment emphasizes target specificity, reduction of side-effects, toxicities and cost. Bi-specific antibodies (BsAb) are a unique, evolving field of cancer therapy. With a reduction in single epitope affinity, BsAb retain high affinity upon contact with dual epitopes making their affinity several orders of magnitude greater than monoclonal antibodies alone. Following a scheme outlined in figure 4A, F(ab)2 are obtained from ficin digestion and chemically recombined using mercaptoethylene reduction, protection and selective regeneration of thiols. The resulting BsAb maintains an epitope specific for CD44v6 (blue) and EGFR (Green) (Fig. 4A). Our preliminary studies have elucidated that chemical recombination of monoclonal antibodies elicits a novel, bispecific antibody successfully recognizing both CD44v6 and EGFR (Fig. 4C).

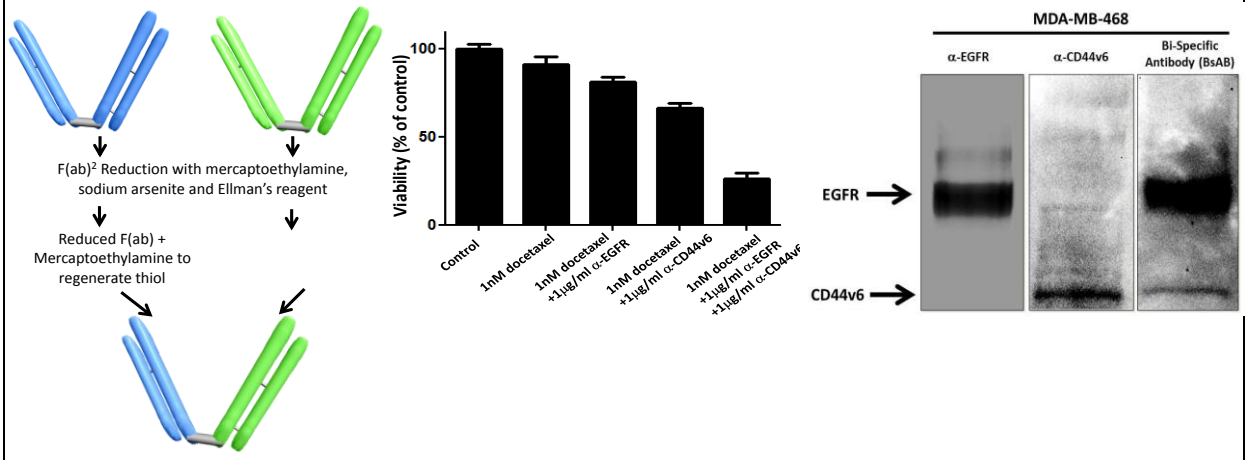

Fig. 4 A) Schematic of Bi-Specific Antibody preparation by chemical recombination B) Cell viability analysis of 468 cells in the presence of docetaxel in combination with anti-EGFR or anti-CD44v6 or a combination of both antibodies. Note synergism when antibodies are combined C) Western blot of unstimulated lysate from MDA-MB-468 probed with monoclonal EGFR, CD44v6 or Bi-specific antibody targeting CD44v6 and EGFR

4. Future Direction

We have shown the role played by CD44 in conferring a subpopulation of cancer cells tolerant to chemotherapy. Cancer stem cell markers such as CD44 serve as biological tools and these can be harnessed to target them. CD133 was also shown to be unregulated in a subpopulation of cancer cells shown to be resistant to treatment. These can implicate novel therapeutic and diagnostic implication in relapse of breast cancer.

5. References

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