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Basal protein expression is associated with worse outcome and trastuzamab resistance in Her2+ invasive breast cancer

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Abstract

Purpose—We investigated the effect of basal protein expression on trastuzamab response in patients with Her2+ breast cancer who received trastuzamab and in Her2+ breast cancer cell lines.

Methods—Expression of CK5/6, CK14, and EGFR was evaluated after immunohistochemical staining in paraffin-embedded tissue of 97 patients with Stage 1-3 Her2+ breast cancer treated with chemotherapy/trastuzamab. Groups with and without basal protein expression were compared with respect to clinicopathologic parameters and survival. We treated 4 cell lines (2 basal-Her2(HCC1569, HCC1954) and 2 non-basal-Her2(BT474, SKBR3)) each with vehicle, trastuzamab (T), Paclitaxel (P), and T+P. Cell viability was assessed and Her2 pathway suppression was compared between groups using immunoblotting. Mammosphere formation was used to assess BCSC properties.

Results—EFGR expression was significant associated with cancer-specific survival (CSS) (p=0.05). CK5/6 expression strongly correlated with overall (OS), disease-free survival (DFS), and CSS (p=0.03, p=0.04, and p=0.03, respectively). Statistical significance was maintained for EGFR and CK5/6 after adjusting for covariates. CK14 was not associated with survival. All cell lines expressed similar levels of Her2. Both T and P alone inhibited proliferation of non-basal cell lines; T+P had an additive cytotoxic effect. Basal cells were resistant to T, P inhibited proliferation, but T+P had no additive cytotoxic effect on cell growth in basal cells. Immunoblotting showed a significant decrease in p-Akt levels after treatment with T or T+P in non-basal cells but not in basal cells. Akt blockade suppressed growth of basal and non-basal Her2+ cells. Furthermore, basal Her2 cell lines had increased mammosphere formation suggesting increased stem cell properties compared to non-basal Her2 cell lines.

Conclusions—CK5/6 and EGFR expression are predictive of worse prognosis in Her2+ breast cancer patients treated with trastuzamab. Basal-Her2 breast cancer cell lines are resistant to

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Conflicts of Interest

The authors have no conflicts of interest to disclose.

trastuzamab which is mediated through the Akt pathway; AKT inhibition abrogates this resistance. Basal Her2 cell lines also have increased stem cell properties which may play a role in the resistance pathway

Keywords

basal breast cancer; Her2 overexpression; trastuzamab resistance

Introduction

Human epidermal growth factor receptor 2-overexpressing (Her2+) breast cancer represents 20-25% of breast cancer and is associated with high relapse rates and poor prognosis.[1-3] Trastuzamab is a monoclonal antibody that targets the Her2 extracellular domain of the Her2 gene and inhibits downstream signaling of intracellular transduction cascades that control cell proliferation, survival, and differentiation. [4-8] While the exact mechanism of anti-tumor activity of trastuzamab in Her2+ breast cancer is unknown,[9-22] its introduction significantly impacted the treatment of Her2+ breast cancer with reduction in relapse rates of up to 50%.[23-26] However, some patients with Her2+ tumors have de novo resistance to trastuzamab, and 60-85% of patients with Her2+ metastatic breast cancer that initially respond to trastuzamab acquire resistance within a year.[23, 24, 27, 28]

Multiple targeted therapies have been developed to treat trastuzamab-resistant Her2+ breast cancer. All of these therapies target various downstream components of the pathway associated with Her2 signaling. However, acquired resistance may continue to be a challenge. This raises the question whether mechanisms outside of Her2 signaling should be investigated to target Her2+ breast cancer. In addition, there is evidence suggesting that there is heterogeneity of Her2 overexpression within Her2+ tumors [29-31] and there may be specific biologic features that predict which tumors exhibit more aggressive behavior.

We hypothesized that the basal phenotype, defined by expression of basal proteins, is a distinct biologic property associated with increased risk of recurrence and resistance to trastuzamab in Her2+ breast cancer. This subset of Her2+ breast cancer (basal-Her2) has been shown to carry a worse prognosis, [32-35] but little is known about how those with the basal-Her2 subtype respond to trastuzamab. The purpose of our study was to investigate the effect of basal protein expression on prognosis and trastuzamab response in both patients with Her2+ breast cancer and Her2+ breast cancer cell lines.

Methods

Patient and tumor specimen selection

Patients were identified from the Cedars-Sinai Medical Center (CSMC) Cancer Registry from January, 2005 through December, 2011 with Stage I-III Her2+ breast cancer who had surgery followed by chemotherapy and trastuzamab and were followed at CSMC. Patients who presented with Stage 4 disease, whose tumor tissue was not available for marker evaluation, who did not receive follow-up at CSMC, and who did not receive chemotherapy and trastuzamab were excluded. The following clinicopathologic data was obtained from

review of medical records: age at diagnosis, tumor size, grade, histology, nodal status, estrogen and progesterone receptor (ER and PR) status, Her2 status, type of surgery, chemotherapy, radiation therapy, hormonal therapy, date and status of last follow-up.

Tumor specimen analysis

Archived paraffin-embedded tissue blocks from the primary tumor of patients who met the selection criteria were retrieved from the CSMC Department of Pathology. Pathologic review of slides was performed by a breast pathologist (SB) blinded to the results to confirm Her2+ status according to published guidelines [36, 37]. All 97 cases were confirmed to be amplified by fluorescence in situ hybridization (FISH). Positivity for HER2 amplification was defined by ratio of Her2 to CEP17 of greater than 2.2. All slides were reviewed to identify sections for further analysis. Selected tumor blocks were cut and stained according to standard protocol. Standard IHC techniques were used to stain tumors for CK5/6, CK14, and EGFR antibodies as described in our previously published work[32, 55]. Semi-quantitative analysis was performed and degree of immunoreactivity was independently scored by 2 investigators (SB, AC) blinded to clinical data based on percentage of positively stained slides and intensity of positive staining (0, 1+, 2+, or 3+). For EGFR, intensity score was based on the following scoring system: 1+ for faint or partial membranous staining, 2+ for complete weak membranous staining, and 3+ for strong diffuse membranous staining (Figure 1). Scores were estimated visually. Normal tissue was also selected as a control for all staining procedures. A final basal marker expression score was calculated by multiplying percentage of positively stained cells by intensity score. This score was used to determine EGFR expression, CK14 expression and CK5/6 expression for each of the respective markers. Statistical analysis was conducted to determine whether the expression score of each marker was independently associated with disease-free survival (DFS), cancer-specific survival (CSS) and/or overall survival (OS). Groups with and without significant basal marker expression were compared with respect to clinicopathologic parameters and survival.

Statistical Analysis

We used spearman correlations to test the association between the EGFR expression score and age at diagnosis and between tumor characteristics and the CK5/6 expression score. We used Wilcoxon rank sum test to test the association between the EGFR expression score and ER and PR status. Chi-square tests were conducted to test correlations between two categorical data points and Fisher exact tests were considered whenever there were one or more spare cells in the contingency table of two categorical variables. Survival analysis was performed using Cox regression models. The markers found to have a significant correlation with survival after univariable analysis were used to perform multivariable analysis adjusting for the following covariates: age at diagnosis, grade, tumor size, number of positive nodes, ER status and PR status. Kaplan–Meier curves for CSS and DFS were drawn for EGFR with the cut-point at 60 and the log-rank test was used for testing the significance between those above 60 versus below 60.

Cell Culture

The non-basal Her2+ human breast cancer cell lines, SKBR3 and BT474 from the American Type Culture Collection (ATCC, Rockville, MD), and the basal-Her2+ cell lines, HCC1569,

HCC1954 (ATCC, Rockville, MD), and JIMT-1 (http://www.dsmz.de/), were used. These cell lines are well established as basal or non-basal based on gene expression microarray data[38, 39]. Numerous studies have shown that nonbasal-Her2 cells such as SKBR3 and BT474 are sensitive to Herceptin treatment.[14, 40-43] Cancer cell lines were cultured in DMEM or RPMI containing 10% fetal bovine serum (FBS) in 5% CO2 at 37°C. Cells were plated at 2,000 per well in 96-well microtiter plates and treated with vehicle control, trastuzamab (Herceptin) 20 ug/mL (T), Paclitaxel 0.01 uM (P), and trastuzamab (Herceptin) 20 ug/mL + Paclitaxel 0.01 uM (T+P). Cell proliferation was assessed using the Celltiter-Glo Luminescent Cell Viability kit from Promega Corporation (Madison, WI) according to the manufacturer's instructions.

Immunoblotting

Basal-Her2 and non-basal-Her2 cell lines were then treated for 2 hours with the same treatment regimen as described in the previous paragraph. Proteins were extracted from human breast cancer cells using RIPA lysis buffer (Sigma-Aldrich) and protein concentration was determined by the BCA Protein Assay Kit (Thermo). Proteins (20 μg) were separated by SDS–PAGE on 4-20% gradient gels and transferred onto PVDF membrane using Trans-Blot Turbo transfer buffer (Bio-Rad) and the Trans-Blot Turbo transfer system (Bio-Rad). Membranes were blocked in Odyssey blocking buffer (LI-COR) and incubated with primary antibodies (anti-human HER2, p-AKT, AKT, p-ERK, ERK and beta-actin) overnight at 4°C, and then incubated with IRDye 800CW secondary antibodies (LI-COR) for 1 hour at room temperature. The membranes were scanned using the Odyssey infrared imaging system (LICOR).

Immunofluorescence (IF)

Cells were placed into chamber slides at 70-80% confluence. Cells were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, blocked with 5% BSA, incubated with the same primary antibodies as used for immunoblotting overnight at 4°C and secondary antibodies for 1 hour at room temperature. The nuclei were stained with DAPI (4',6-diamidino-2-phenylindole). Images were acquired with Olympus microscope.

Mammosphere Culture

Complete MammoCult™ Medium was prepared by adding 50 mL of thawed MammoCult™ Proliferation Supplements to 450 mL of MammoCult™ Basal Medium (Human) supplemented with 4μg/ml Heparin and 0.48μg/ml Hydrocortisone per the manufacturer's instruction (StemCell Technologies). Single cells of all cell lines were plated on 6-well ultra-low attachment plates (Corning) at a density of 2000 viable cells/mL in Complete MammoCult™ Medium and incubated for 7 days. Fresh medium was added on day 3. The number of spheres greater than 50 μm in diameter were counted per well and this was repeated in triplicate. Mean number of mammospheres ± SEM were calculated for each cell line.

Results

Patient and tumor specimen analysis

We identified 97 patients with Stage 1-3 Her2+ invasive breast cancer treated with chemotherapy and trastuzamab who had adequate archived paraffin-embedded tissue available for analysis. Mean age at diagnosis was 53 (range 25-82). Table 1 lists the clinicopathologic characteristics (including the number of missing data points) of the 97 patients. Among the 97 patients for whom data was available, there was no significant difference in the number of patients with tumors that were T1 versus T2 or T3, no difference in the number of patients with PR-positive versus PR-negative tumors, nor was there any difference in the number of patients who received hormonal therapy versus those who did not receive hormonal therapy. More tumors were node-negative compared to node-positive ($p<0.01$), more tumors were high grade compared to low or intermediate grade ($p<0.01$), more tumors that were ER-positive tumors than ER-negative ($p<0.01$), more tumors were of ductal histology compared to tumors of other histology ($p<0.01$); more patients had lumpectomy compared to mastectomy ($p<0.01$), and more patients had radiation versus no radiation ($p<0.01$). Expression of at least one basal marker was demonstrated in 36/97 (37.1%): 15/97 (15.4%) had expression of CK 5/6, 8/97 (8.2%) expressed CK14, and 33/97 (34.0%) expressed EGFR. Sixty-one (62.9%) patients did not express any basal markers and only 6 (6.2%) patients had expression of all 3 markers. One patient (1.0%) had expression of only CK5/6; one (1.0%) had expression of CK14 alone; and 20 (20.6%) had only EGFR expression; 14 (14.4%) expressed at least 2 basal markers.

For each marker, we conducted survival analysis. EFGR expression had a significant association with CSS ($p=0.049$ using Cox regression analysis), and there was a trend towards significance for OS ($p=0.056$). Statistical significance was maintained after adjusting for covariates ($p=0.03$). There was no correlation between EGFR expression and DFS. CK5/6 expression had a strong correlation with CSS, DFS and OS ($p=0.03$, $p=0.04$, and $p=0.03$, respectively) . Statistical significance was maintained for CSS, DFS and OS after adjusting for covariates ($p=0.03$, $p=0.01$, and $p=0.02$, respectively). There was no significant correlation between CK14 expression and survival. Five-year CSS, DFS and OS of patients with CK5/6 expression compared to survival in those without basal marker expression was approximately 70% vs. 90.4% ($p=0.39$), 75% vs. 92.3% ($p=0.33$) and 70% vs. 90.6% ($p=0.43$), respectively. Additionally, statistical analysis identified a final basal marker expression score cutoff of 60 for which EGFR was significantly associated with worse CSS and there was a trend towards a significant correlation for this cutoff with DFS ($p=0.06$) but no significant threshold was identified for CK5/6 expression. Figure 2 demonstrates the Kaplan-Meier curves comparing CSS (A) and DFS (B) in patients with EFGR expression > 60 versus those < 60. Five-year CSS, DFS and OS of patients with EGFR expression > 60 compared to survival in those <60 was approximately 57% vs. approximately 90% ($p=0.02$), 67% vs. 90% ($p=0.04$) and 58.3% vs. 93.7% ($p=0.07$), respectively. Since a significant threshold for CK5/6 expression could not be identified, a similar Kaplan-Meier curve was not generated. Tumor factors associated with EGFR score > 60 versus < 60 included ER ($p=0.001$ by Fisher's exact test) and PR status ($p=0.03$ by Fisher's exact test). Factors associated with EGFR expression included younger age at

diagnosis (p=0.035 by Spearman correlation), ER and PR expression (=<0.001 and p=0.0002, respectively, Wilcoxon rank sum). Tumor factors associated with CK5/6 expression included only tumor size (p=0.04 by Spearman correlation). Grade, histology, and nodal status were not associated with EGFR or CK5/6 expression. The spearman correlation between CK5/6 and EGFR was 0.49 (p-value < 0.01), demonstrating that CK5/6 and EGFR expression are highly correlated with one another. This strong correlation suggests that the statistical modeling must be performed individually for each basal marker. Therefore, analyses assessing various combinations of basal marker expression were not performed.

Effects of trastuzamab on cell viability in different Her2 cell lines

To explore the effects of basal protein expression on cellular responses to trastuzamab, we selected 4 breast cancer cell lines: 2 non-basal Her2 (BT474, SKBR3) and 2 basal Her2 (HCC1569, HCC1954) subtypes. All cell lines expressed similar levels of Her2 (Figure A1a). Her2 expression was further confirmed using IF. Basal and non-basal properties were confirmed by IF of basal cytokeratin CK5 and luminal cytokeratin CK18, respectively. Using cell viability assays, we found that H and P each inhibited cell proliferation and H+P enhanced the cytotoxic effect in non-basal-Her2 cell lines (Figure 3a and b). However, HCC1569 basal-Her2 cells were resistant to H when used alone or in combination with P (Figure 3c). Basal-Her2 HCC1954 (Figure 3d) and JIMT-1 (Figure A2) showed some effect with P alone and H+P.

Effects of trastuzamab on Akt signaling

To investigate the molecular events underlying distinct responses to trastuzamab in different Her2 breast cancer cells, we studied the effects of H, P, or H+P on the Akt and Erk signaling pathways. Western blot analysis demonstrated a significant decrease in phospho-Akt levels after treatment with H or H+P in the non-basal-Her2 cell lines but not in the basal-Her2 cell lines (Figure 4a and A3a). In contrast, there were no detectable differences in the phospho-Erk levels in all tested cell lines under the same treatment (Figure 4b and A3b). Therefore, persistent and constitutively active Akt may be involved in resistance to trastuzamab and may be critical for $Her2^{+}$ breast cancer cell growth. This was confirmed by a specific Akt inhibitor AI-IV. Akt blockade suppressed the growth of both basal and non-basal Her2+ cells (Figure 5). Taken together, these results suggest that trastuzamab resistance in basal-Her2 breast cancers may be mediated by the PI3K/Akt cell signaling pathway.

Breast cancer stem cell properties

Cancer stem cells have been shown to be enriched in basal-like breast cancer. To explore whether basal Her2 breast cancer cells exhibit cancer stem cell properties, we examined the mammosphere growth capacity, which is a commonly used marker for BCSC, in the 4 breast cancer cell lines. Compared with non-basal Her2 cells, the mammosphere formation capacity was significantly higher in basal Her2 cells (Figure 6). These data demonstrate that basal Her2 cells have more cancer stem cell properties.

Discussion

Trastuzamab resistance remains a significant problem in the management of Her2+ breast cancer, and heterogeneity among Her2+ tumors may contribute to challenges in treating this disease. The basal-Her2 subtype appears to be a distinct entity that is associated with worse prognosis. Our clinical findings not only corroborate data reported in the literature associating the basal-Her2 subtype with worse survival, [44-46] but also provide evidence that basal-Her2 tumors are more likely to be resistant to trastuzamab.

The incidence of basal marker expression in Her2+ breast cancer is variable among studies. Our patient population had a higher rate of basal marker expression than reported in other studies when evaluating expression of any of the 3 markers. However, the rate of expression of the individual markers was lower: 15% for CK5/6 expression, 8% for CK14 expression and 34% for EGFR. In our previous work we identified 9% of Her2+ cases with expression of either CK5/6 or CK14.[32] Liu et al found the basal Her2 phenotype in 8.2% of cases. [34] Basal tumors are known to be characterized by expression of several markers, including CK5, CK5/6, CK14, CK17 and/or EGFR.[47-50] However, there is inconsistency in the expression of these markers and it is not clear which markers are most accurate in defining basal-like breast cancer either alone or in combination. We found CK5/6 and EGFR to be better predictors of outcome than CK14. We also found that CK5/6 expression was highly correlated with EGFR expression. CK 14 was only expressed in 8% of our cases, which may account for the lack of correlation with survival. However, Alshareeda et al also identified CK14 to be inferior to CK5/6 as a prognostic factor. They evaluated IHC-staining of CK5, CK5/6, CK14, CK17, in 995 invasive breast tumors with long-term follow-up and found that CK14 was expressed in 50% fewer cases compared to CK5/6 [50] and CK5/6 expression significantly correlated with survival but CK14 expression did not. Fulford et al evaluated CK14 expression in 443 invasive tumors and found that long-term survival was better in those with CK14 expression who did not develop distant metastases.[51]

We found CK5/6 and EGFR expression to be significantly associated with CSS, and there was a significant correlation between CK5/6 expression and OS. The association between EGFR expression and OS approached statistical significance. These findings confirm work previously reported in the literature. Bagaria and colleagues found 5-year OS of patients with Her2+ breast cancer and CK5/6 or CK14 expression to be 65% compared to 94% in those without basal marker expression.[32] Blows et al evaluated over 10,000 breast cancer specimens assessed by IHC for CK5/6 or EGFR expression [33] and found that the nonluminal-Her2+ cases had a poorer prognosis compared to luminal-Her2+ cases. Liu and colleagues performed tissue microarray and IHC to characterize 713 ER-negative breast cancers and demonstrated that the basal-Her2 subtype had the worst OS among all of the other subtypes.[34] Complete information on trastuzamab treatment was not available in these studies; therefore, conclusions regarding response to trastuzamab could not be made.

In this study we included only patients that received adjuvant chemotherapy and trastuzamab, in attempt to control for adjuvant treatment as a confounding variable. The patients that recurred in the face of trastuzamab had greater basal marker expression suggesting that these tumors were resistant to systemic therapy. Molecular profiling has

been used to predict response to various chemotherapy regimens, and investigators have found the basal subtype to be associated with stronger response to chemotherapy compared to the other molecular subtypes.[52, 53] Therefore, the higher recurrence rates in patients with basal-Her2 tumors was less likely due to resistance to chemotherapy, rather, we believe the tumors were resistant to trastuzamab. Harris et al evaluated pathologic response of 48 patients with Her2+ breast cancer to neoadjuvant vinorelbine and trastuzamab and found that large tumors expressing basal markers demonstrated intrinsic resistance to this drug regimen.[54]

In addition to the clinical evidence that is growing to support our hypothesis, we have also presented preclinical evidence that the basal phenotype may play a role in trastuzamab resistance. Our cell line work confirms that trastuzamab enhances the effect of chemotherapy by causing a greater decrease in cell survival, and this was clearly demonstrated in the nonbasal cell lines. However, there was a significant increase in the viability of basal cells when treated with trastuzamab compared to nonbasal cells. This change was most pronounced in HCC1569 cells indicating that basal cells do not respond to trastuzamab. Wang et al. treated a panel of 13 Her2+ cell lines with trastuzamab and lapatinib and identified SKBR3 and BT474 cells to be responsive to both drugs and HCC1569 to have minimal growth inhibition, suggesting de novo resistance to both of these drugs in vitro.[55] There have been similar findings in the literature using the JIMT-1 cell line, which was also confirmed by our laboratory (Figure A2). JIMT-1 cells were originally isolated from the pleural effusion of a patient with trastuzamab-resistant Her2+ metastatic breast cancer.[56] This cell line has been used as a model to investigate potential mechanisms for drug resistance in Her2+ breast cancer.

There has been a rising interest in cancer stem cells as a potential biologic cause of resistance to cytotoxic therapy. Several studies have reported enrichment of breast cancer stem cells (BCSC) in breast tumors that are resistant to therapy.[57-62] In our stem cell work, we demonstrated that HCC1569 and HCC1954 cells were associated with a higher level of mammosphere formation compared to SKBR3 and BT474, indicating higher stem cell activity in the basal cells. Mammosphere-forming cells have been shown to be useful as a tool to predict CSC-like cells.[63, 64] Croker and colleagues demonstrated that high ALDH expression and CD44+/CD24− expression were associated with increased colony growth, adhesion, migration and invasion in cell lines and enhanced tumorigenicity and metastasis in mouse models.[65] In a later study the investigators found that inhibition of ALDH activity reduces chemotherapy and radiation resistance of stem-like ALDHhiCD44^{+} human breast cancer cells.[66] Assessment of ALDH or CD44+/24− activity in the 4 cell lines would be strong confirmatory tests for stem cell activity and is a plan for future experimentation in our laboratory.

Trastuzamab blocks Her1/Her3/PI3K complex formation which inhibits Akt.[67] We explored ERK and Akt, 2 major downstream signaling pathways, as potential pathways involved in trastuzamab resistance. Our immunoblotting results suggest that Akt signaling may play a role in trastuzamab resistance of the basal cells, but ERK does not. Our Akt inhibition findings suggest that trastuzamab sensitivity is restored by Akt inhibition. In a previous study of Akt inhibition in basal breast cancer, our laboratory found Akt to play a

role in transcription of FOXC1, a critical mediator of EGFR function in basal breast cancer, and that EGFR activation induces FOXC1 through ERK and Akt pathways.[68] Chandarlapaty et al [69] evaluated specimens from patients with Her2+ trastuzamab-resistant breast cancer and found high rates of PI3K-AKT activation. O'Brien and colleagues [70] evaluated response to trastuzumab and lapatinib in 18 Her2+ cell lines innately resistant to these drugs and found that increased activation of the PI3K/AKT pathway correlated with resistance to trastuzumab, which could be overcome by lapatinib. Basal cell lines were among three of the 18 cell lines evaluated in this study. Fabi et al [71] investigated the clinical impact of the expression of PTEN, p-Akt and PI3K in Her2+ metastatic breast cancer patients treated with trastuzumab and found that patients co-expressing PTEN and p-Akt had a statistically significant longer PFS as compared to the rest of patients ($p = 0.01$). The literature supports involvement of the Akt pathway in resistance to trastuzumab, but many of the studies do not provide information on the molecular subtype of the tumor or cells in question. Our work demonstrates a link between basal marker expression, stem cell marker expression, Akt signaling, and resistance to trastuzamab.

Conclusions

Trastuzamab resistance is a complex and challenging problem in management of Her2+ breast cancer. As new targeted therapies continue to be developed and molecular subtyping becomes further refined, multiple avenues of overcoming drug resistance will likely evolve. Our data support the basal phenotype, defined by basal and stem cell marker expression, and its link to Akt signaling as one clinically relevant pathway of trastuzamab resistance. Further studies are needed to characterize the role of Akt in this phenomenon with the hope that additional therapy may be tailored to improve outcome in patients with this aggressive subtype of breast cancer.

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Appendix

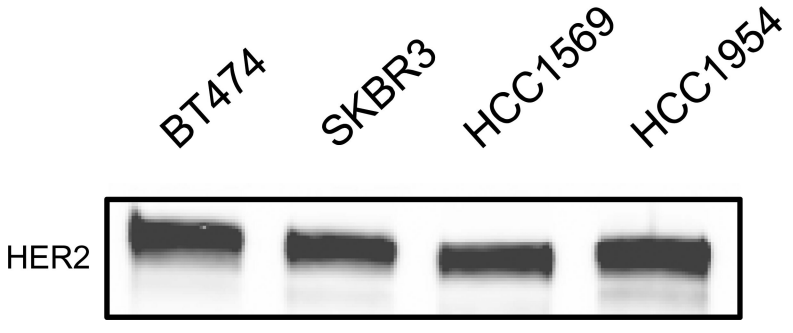

Fig A1.

a Immunoblotting of non-basal Her2 (BT474, SKBR3) and basal Her2 (HCC1569, HCC1954) cell lines with HER2 antibody

Fig A1b Non-basal Her2 and basal Her2 cell lines were co-stained for CK5 (basal cytokeratin) and CK18 (luminal cytokeratin) antibodies. BT474 and SKBR3 were positive for CK18 but not CK5 whereas HCC1569 and HCC1954 were positive for CK5 but not CK18 confirming the molecular phenotypes of all four cell lines

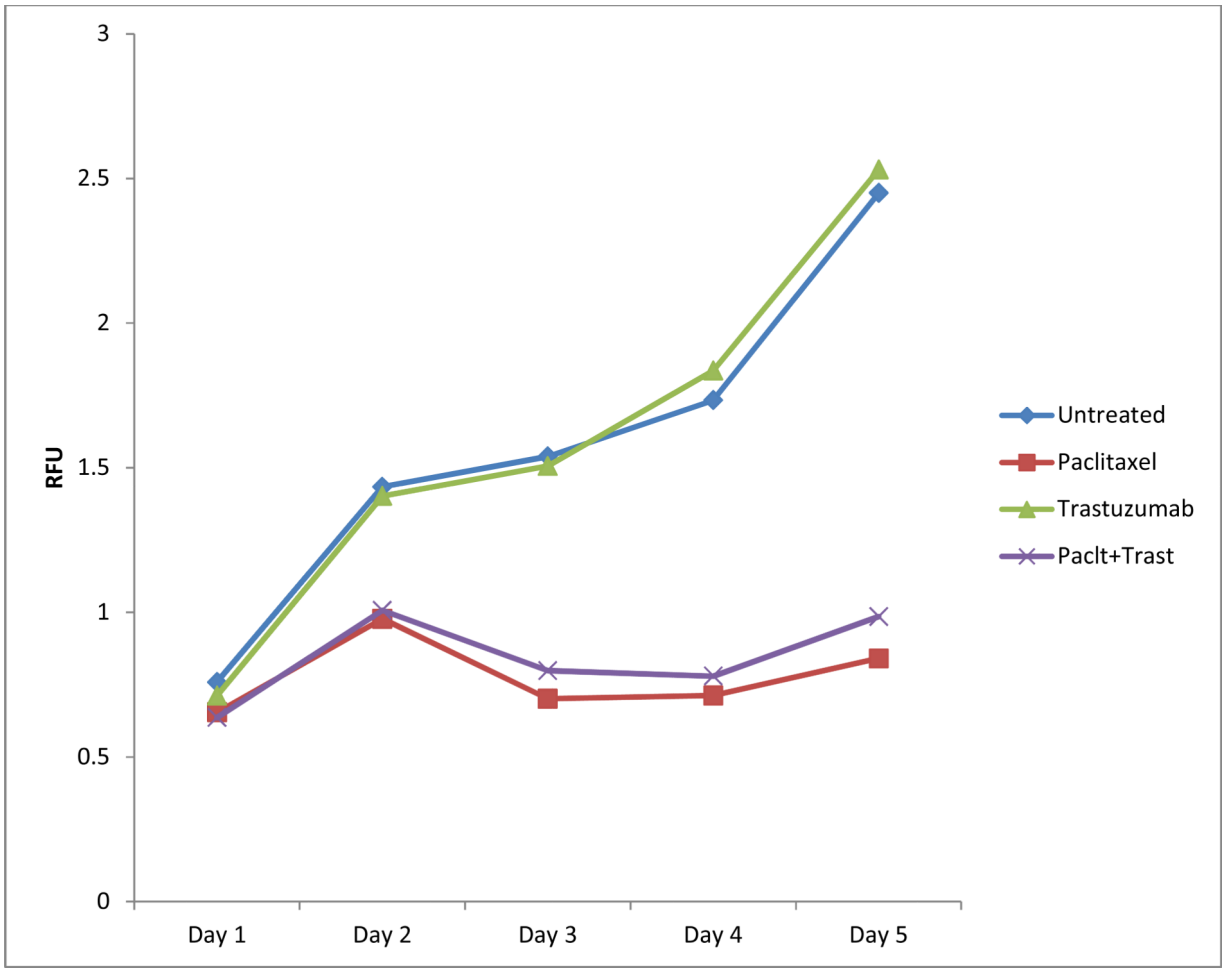

Fig A2.

Cell proliferation was assessed using the CellTiter-Glo luminescent assay after treating basal Her2 cell line JIMT-1 with vehicle control (C), Trastuzamab 20 ug/mL (T), Paclitaxel 0.01 uM (P), and Trastuzamab 20 ug/mL + Paclitaxel 0.01 uM (T+P) over a 5 days. Similar to the other basal Her2 cell lines (HCC1569, HCC1954), JIMT-1 cell proliferation was not inhibited by trastuzamab (grey) relative to the control (blue). Paclitaxel alone (green) did inhibit cell proliferation in JIMT-1 cells but the combination of Trastuzamab and Paclitaxel did not have an additive cytotoxic effect

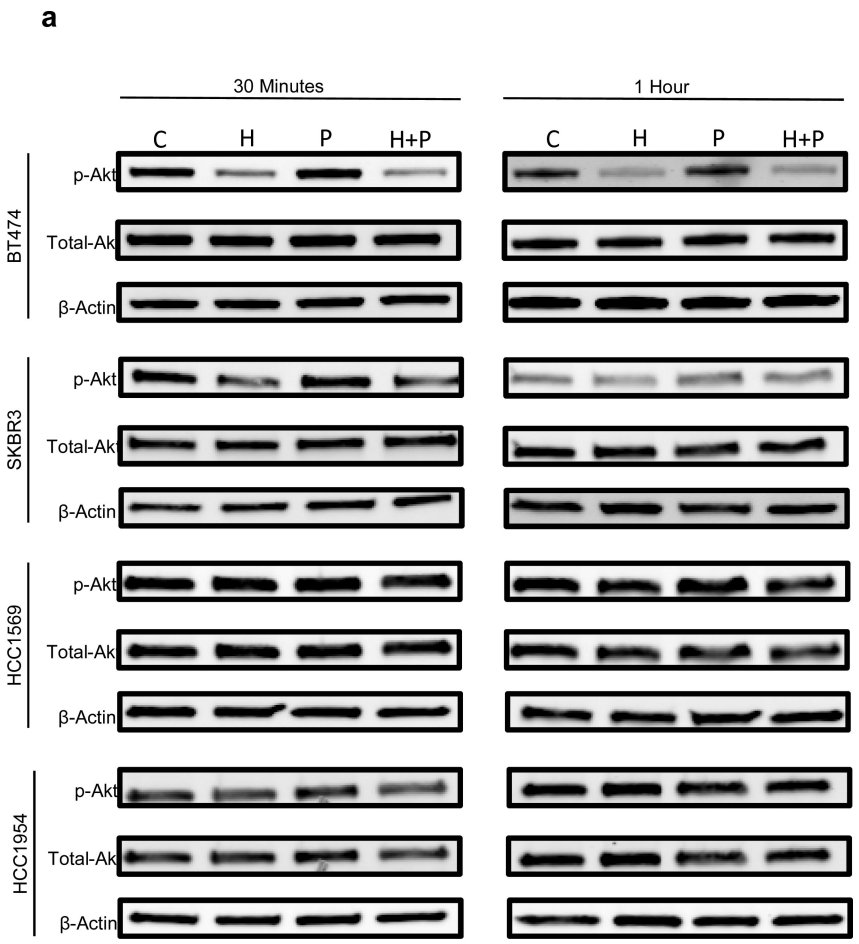

Fig A3a.

Immunoblotting of non-basal Her2 (BT474, SKBR3) and basal Her2 (HCC1569, HCC1954) cell lines with p-Akt, total Akt and β-Actin antibodies after 30 minutes and 1 hour of treatment with vehicle control (C), Trastuzamab 20 ug/mL (T), Paclitaxel 0.01 uM (P), or Trastuzamab 20 ug/mL + Paclitaxel 0.01 uM (T+P)

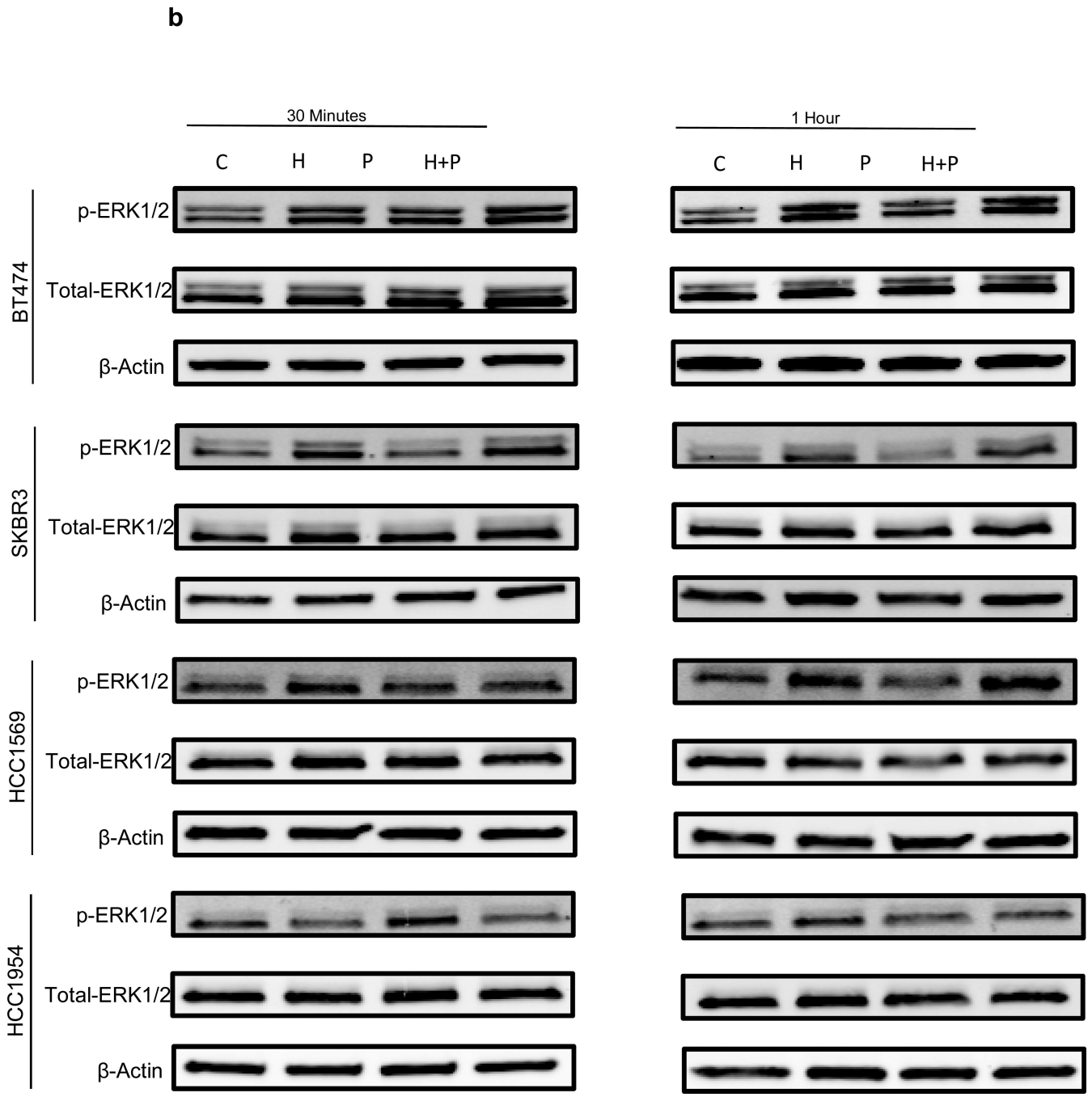

Fig A3b.

Immunoblotting of non-basal Her2 (BT474, SKBR3) and basal Her2 (HCC1569, HCC1954) cell lines with p-ERK 1/2, total ERK 1/2 and β-Actin antibodies after 30 minutes and 1 hour of treatment with vehicle control (C), Trastuzamab 20 ug/mL (T), Paclitaxel 0.01 uM (P), or Trastuzamab 20 ug/mL + Paclitaxel 0.01 uM (T+P)

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Clinical Practice Points

- Tumor expression of basal proteins has been shown to be associated with worse prognosis and can be expressed in Her2+ breast cancer.
- Our research provides clinical and laboratory evidence that basal protein expression in Her2+ tumors is associated with resistance to anti-Her2 therapy, and that Akt signaling may play a significant role in the mechanism of resistance.
- As the mechanisms of drug resistance by basal tumors becomes further elucidated, it may be important to consider alternative therapies in patients with the basal subtype of Her2+ breast cancer.

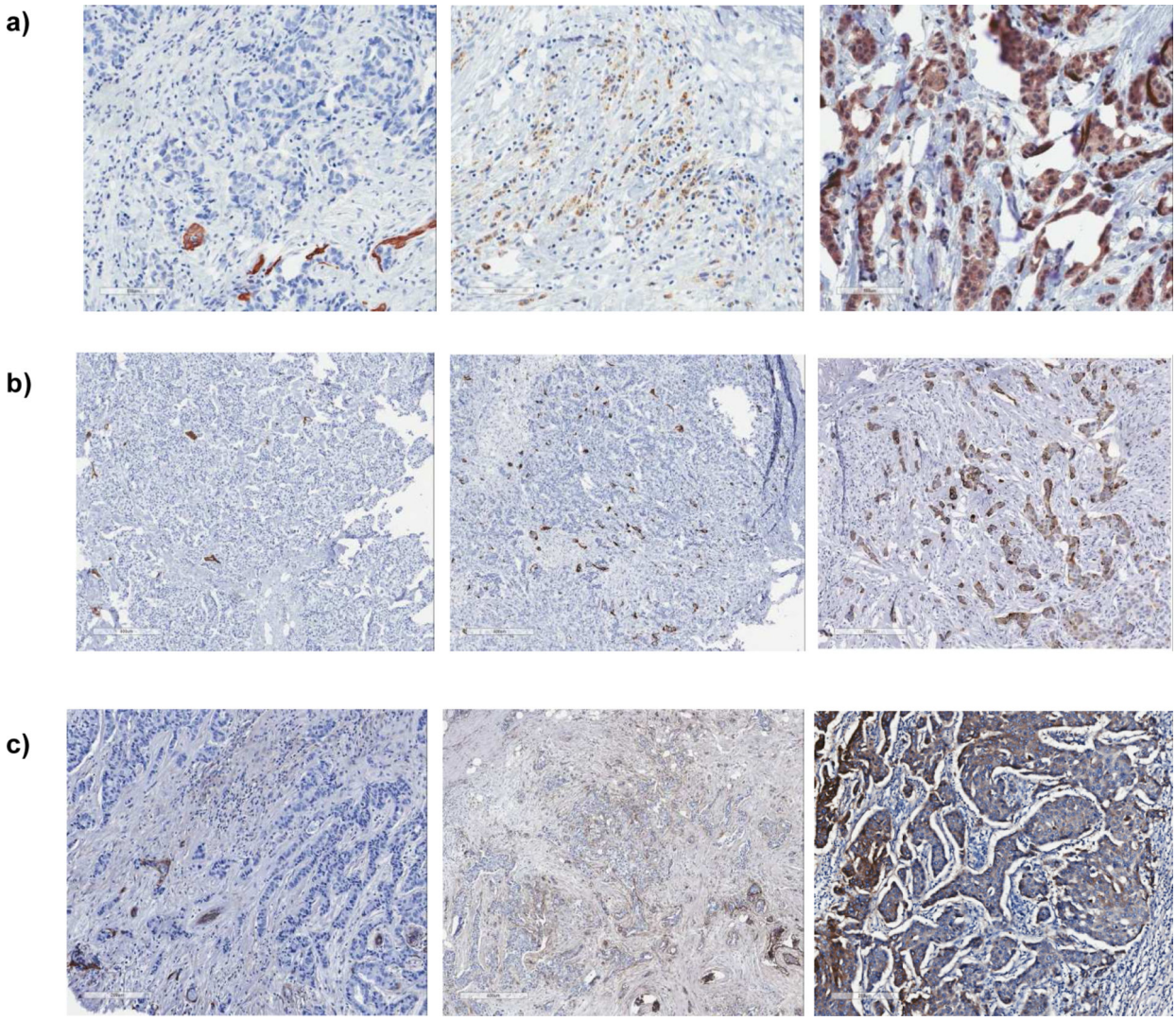
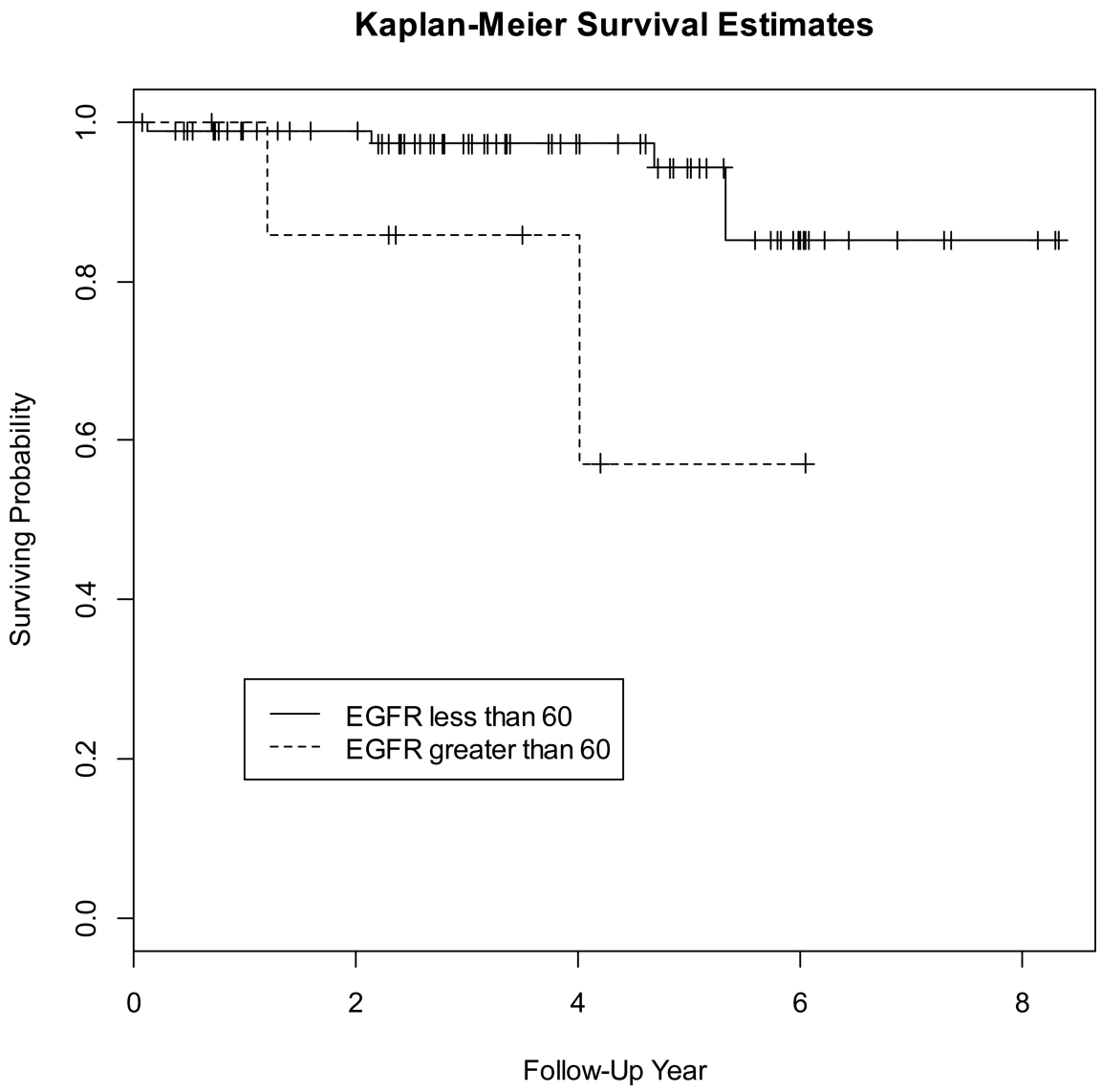


Fig 1.

Representative slides of weak versus moderate versus strong staining for basal markers: a) CK5/6, b) CK14, and c) EGFR

a)



b)

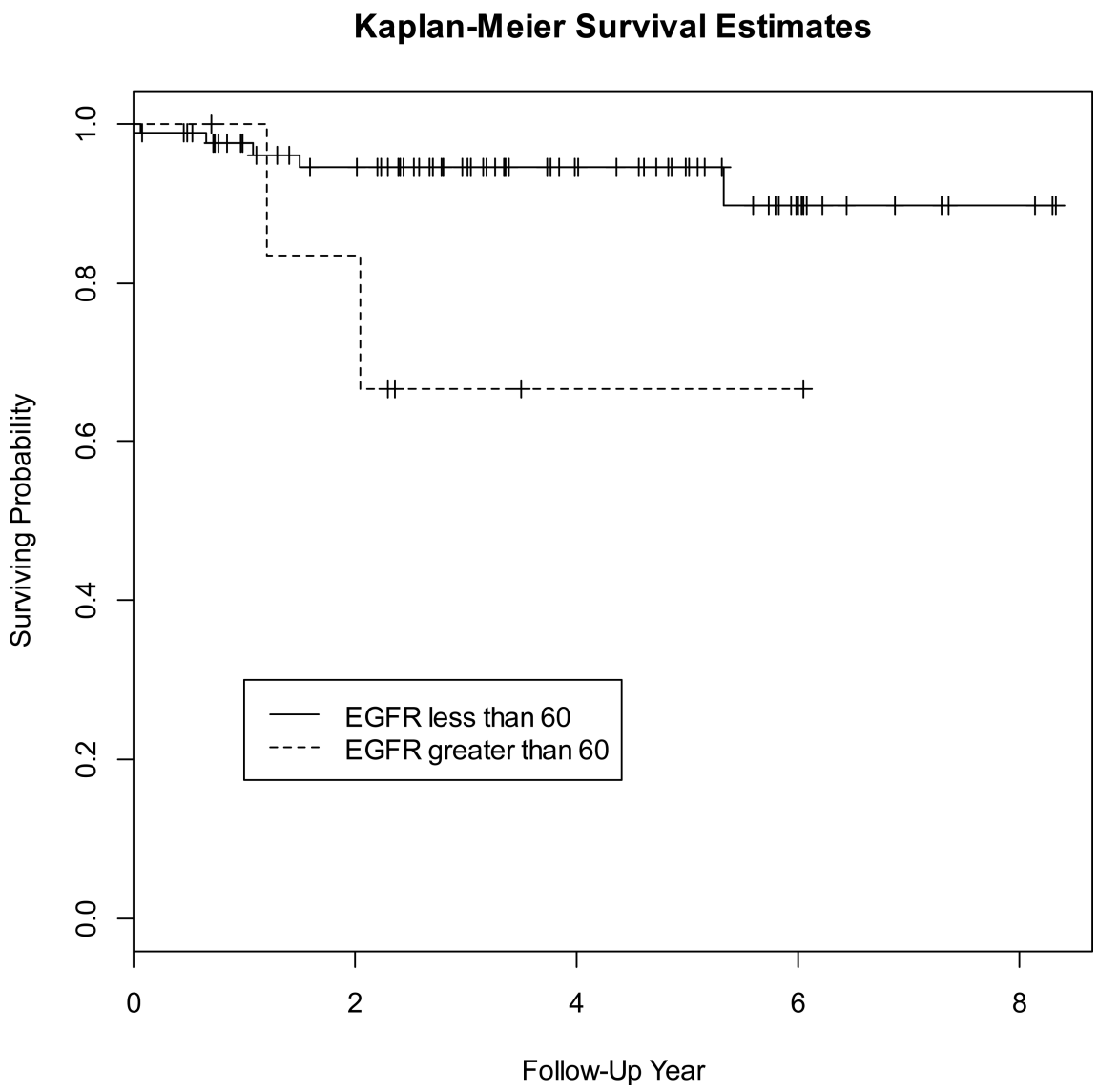


Fig 2.

Comparison of cancer-specific survival (a) and disease-free survival (b) for EGFR expression > 60 (dashed line) versus EGFR expression < 60 (solid line)

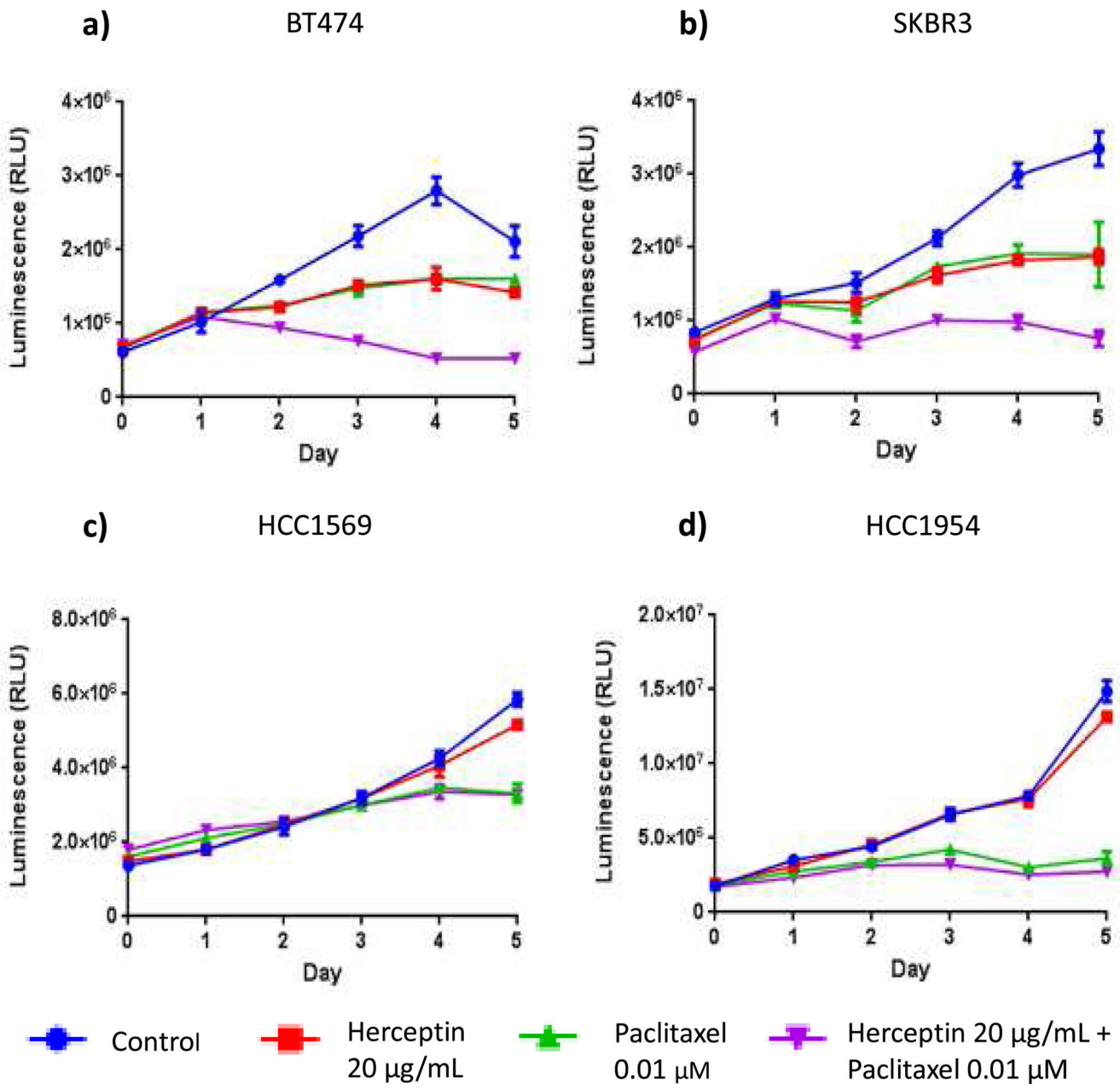


Fig 3.

Non-basal Her2 cell lines BT474 (a) and SKBR3 (b) and basal Her2 cell lines HCC1569 (c) and HCC1954 (d) were treated with vehicle control (C), Trastuzamab 20 ug/mL (T), Paclitaxel 0.01 uM (P), or Trastuzamab 20 ug/mL + Paclitaxel 0.01 uM (T+P) over a 5 day course and cell proliferation was measured using CellTiter-Glo luminescent cell viability assay

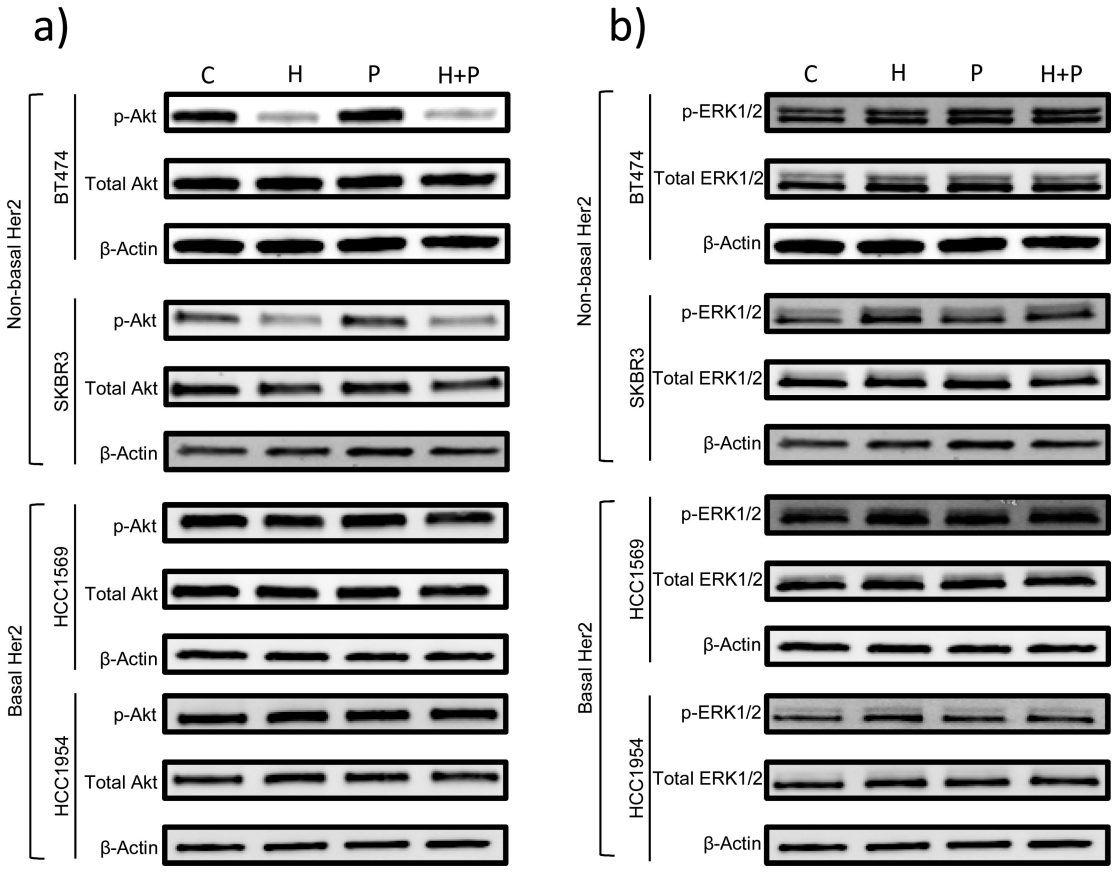


Fig 4.

Immunoblotting of non-basal Her2 (BT474, SKBR3) and basal Her2 (HCC1569, HCC1954) cell lines using a) p-Akt, total Akt and β-Actin antibodies and b) p-ERK 1/2, total ERK 1/2, and β-Actin antibodies after 2 hours of treatment with vehicle control (C), Trastuzamab 20 ug/mL (T), Paclitaxel 0.01 uM (P), or Trastuzamab 20 ug/mL + Paclitaxel 0.01 uM (T+P)

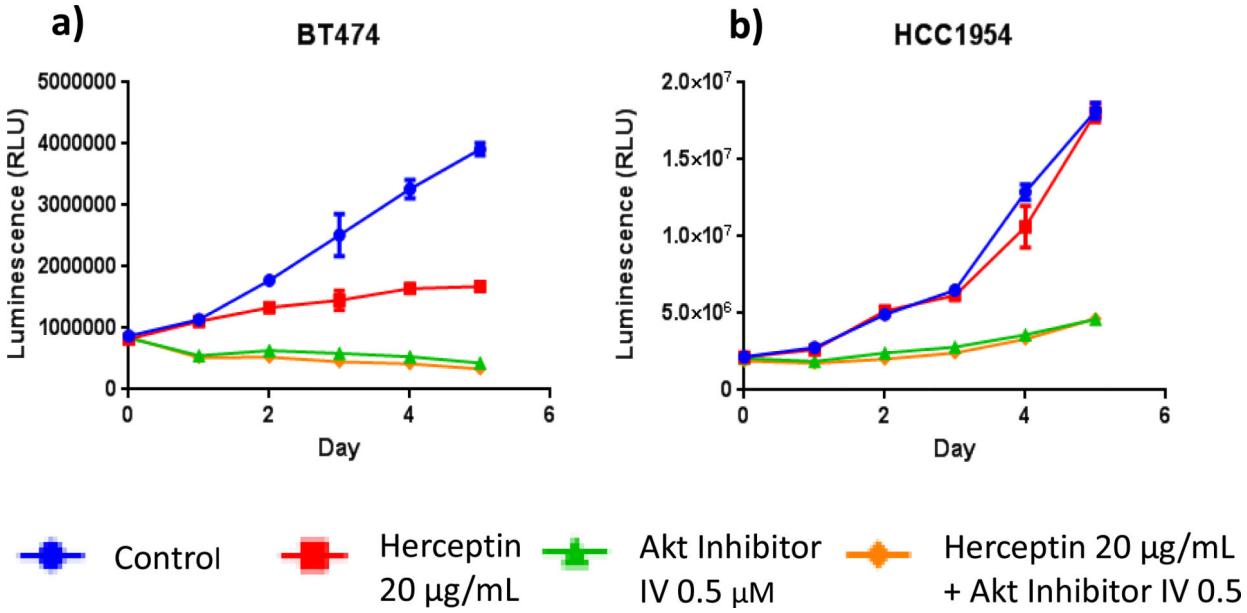


Fig 5.

Akt inhibition results in decreased cell proliferation in basal Her2 cell lines. Non-basal Her2 cell line BT474 (a) and basal Her2 cell line HCC1954 (b) were treated with vehicle control (blue), Trastuzamab 20 ug/mL (red), Akt Inhibitor IV 0.5 uM (green), and Trastuzamab 20 ug/mL + Akt Inhibitor IV 0.5 uM (orange) over a 5 day course and cell proliferation was measured using CellTiter-Glo luminescent cell viability assay

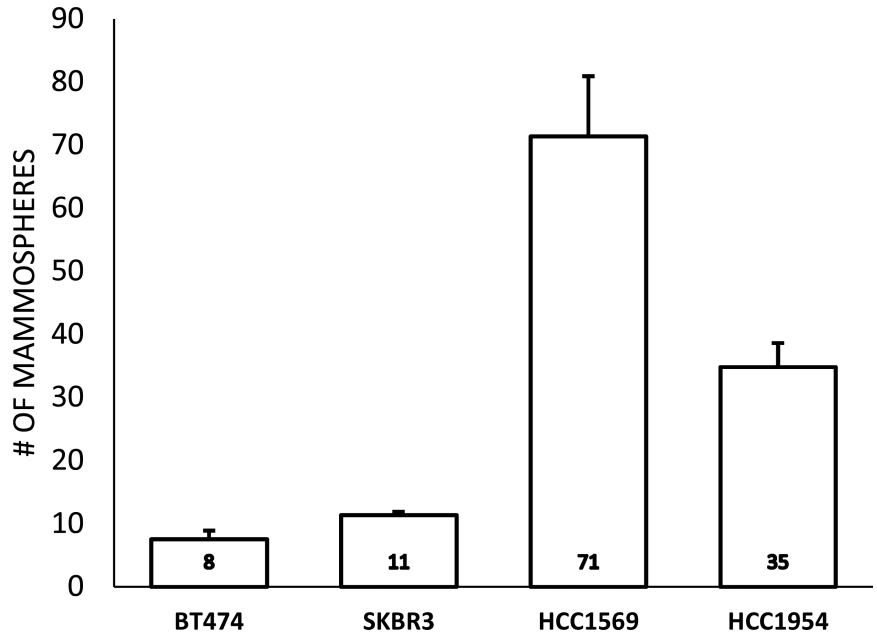


Fig 6.

Breast Cancer Stem Cell (BCSC) Activity: Non-basal Her2 cell lines BT474 (a) and SKBR3 (b) as well as basal Her2 cell lines HCC1569 (c) and HCC1954 (d) were cultured at low density (2000 cells per 9.5 cm^2) in MammoCult™ Media for 7 days forming mammospheres according to previously published protocols. Mammospheres greater than 50 μm in diameter were counted per well and this was repeated in triplicate. Mean number of mammospheres ± SEM were calculated for each cell line

Table 1

Tumor characteristics of patient cohort (n=97) using Chis-squared analysis.

Tumor Characteristic	Number of patients (%)	Number Missing (%)	P-value
Tumor Size		3 (3.1%)	0.06*
T1	38 (39.2%)		
T2	47 (48.5%)		
T3	9 (9.3%)		
Nodal Status		10 (10.3%)	<0.01*
N0	51 (52.5%)		
N1	20 (20.6%)		
N2	11 (11.3%)		
N3	5 (5.2%)		
Tumor Grade		2 (2.1%)	
Low	0		<0.01
Intermediate	18 (18.6%)		
High	77 (79.4%)		
Tumor Histology		0	<0.01*
Ductal	86 (88.7%)		
Lobular	2 (2.1%)		
Mixed Ductal-Lobular	5 (5.2%)		
Other	4 (4.1%)		
ER[1] status		1 (1%)	<0.01
Positive	62 (63.9%)		
Negative	34 (35.1%)		
PR[2] status		3 (3.1%)	1.00
Positive	47 (48.5%)		
Negative	47 (48.5%)		
Primary Surgery Type		4 (4.1%)	<0.01
Lumpectomy	64 (66.0%)		
Mastectomy	29 (29.9%)		
Radiation Therapy		6 (6.2%)	<0.01
Yes	67 (69.1%)		
No	24 (24.7%)		
Hormonal Therapy		1 (1%)	0.22
Yes	43 (44.3%)		
No	53 (54.6%)		

[1] ER=estrogen receptor

[2] PR=progesterone receptor

* groups were combined for Chi-squared analysis (T2 and T3 for Tumor Size; N1, N2, N3 for Nodal Status; Lobular and Mixed Ductal-Lobular for Tumor Histology)