Cancer Letters 313 (2011) 54–63

Contents lists available at SciVerse ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Inactivation of Rac1 reduces Trastuzumab resistance in PTEN deficient and insulin-like growth factor I receptor overexpressing human breast cancer SKBR3 cells

Yong Zhao [a,1], Zhishan Wang [a,1], Yiguo Jiang [b], Chengfeng Yang [a,c,*]

[a] Department of Physiology, Michigan State University, 2201 Biomedical Physical Sciences, East Lansing, MI 48824, United States
[b] Institute for Chemical Carcinogenesis, State Key Laboratory of Respiratory Diseases, Guangzhou Medical University, Guangzhou 510182, PR China
[c] Center for Integrative Toxicology, Michigan State University, East Lansing, MI 48824, United States

ARTICLE INFO

Article history:
Received 17 June 2011
Received in revised form 21 August 2011
Accepted 22 August 2011

Keywords:
Trastuzumab resistance
Rac1
PTEN
IGF-IR
Akt
HER2

ABSTRACT

Drug resistance remains to be a big challenge in applying anti-HER2 monoclonal antibody Trastuzumab for treating breast cancer with HER2 overexpression. Amplification of insulin-like growth factor I receptor (IGF-IR) and deletion of tumor suppressor phosphatase and tensin homolog (PTEN) are implicated in Trastuzumab resistance, however, the underlying mechanisms have not been clearly defined. Activation of Rac1, a member of Rho GTPase family, is capable of causing cytoskeleton reorganization, regulating gene expression and promoting cell proliferation. To investigate the mechanism of Trastuzumab resistance, PTEN knockdown and IGF-IR overexpressing stable cell lines were generated in HER2 overexpression human breast cancer SKBR3 cells. Rac1 was highly activated in PTEN deficient and IGF-IR overexpressing Trastuzumab-resistant cells in a HER2-independent manner. Inactivation of Rac1 by using a Rac1 inhibitor NSC23766 or siRNA knocking down the expression of Tiam1, a guanine nucleotide exchange factor for Rac, significantly reduced Trastuzumab resistance in SKBR3 cells. Inhibition of Rac1 had no effect on the levels of phosphor-HER2 and phosphor-Akt, but significantly decreased the levels of cyclin D1 in Trastuzumab-resistant cells. Inhibition of Akt with an Akt inhibitor also significantly reduced Trastuzumab resistance. However, simultaneous inhibition of both Rac1 and Akt resulted in a significantly more decrease of Trastuzumab resistance than inactivation of Rac1 or Akt alone. These results suggest that Rac1 activation is critically involved in Trastuzumab resistance caused by PTEN deletion or IGF-IR overexpression. Simultaneous inhibition of Rac1 and Akt may represent a promising strategy in reducing Trastuzumab resistance in HER2 overexpression breast cancer.

1. Introduction

Human epidermal growth factor receptor-2 (HER2 or ERBB2) is overexpressed in approximately 15–25% of human breast cancers and HER2 overexpression is associated with poor prognosis [1,2]. Trastuzumab (Herceptin) is the first rationally designed anti-HER2 monoclonal antibody approved for the treatment of breast cancers with HER2 overexpression. While wide applications of Trastuzumab achieved clinical therapeutic efficacy in some patients with HER2 overexpression, a large portion of selected patients did not respond well to Trastuzumab treatment. It was reported that the overall response rate to Trastuzumab treatment as a single agent was 23–26%

* Corresponding author at: Department of Physiology, Michigan State University, 2201 Biomedical Physical Sciences, East Lansing, MI 48824, United States. Tel.: +1 517 884 5153; fax: +1 517 355 5125.
E-mail address: yangcf@msu.edu (C. Yang).
[1] These authors contribute equally to this work.

0304-3835/$ - see front matter © 2011 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.canlet.2011.08.023

[3,4]. When used in combination with adjuvant chemotherapies, about 40% response rate was observed and Trastuzumab significantly improved disease-free survival among women with HER2 overexpression breast cancers [5,6]. However, despite the very promising therapeutic effects at the beginning, resistance to Trastuzumab developed within about 1 year in the majority of patients who initially respond [7–9].

The mechanism of Trastuzumab resistance in HER2 overexpression breast cancer has not been completely understood. Two kinds of Trastuzumab resistance have been observed: primary resistance occurring in those patients who never respond to Trastuzumab treatment as a single agent; and acquired resistance occurring in the patients who initially show good responses but develop resistance after repeated Trastuzumab treatment. It has been suggested that overexpression of human epidermal growth factor receptor-1 (HER1 or EGFR), Her3, c-Met, insulin-like growth factor I receptor (IGF-IR), or loss of tumor suppressor phosphatase and tensin homolog (PTEN) may contribute to primary and acquired Trastuzumab resistance, though the involved mechanisms have not been clearly defined [7–9]. One common feature of those proposed events that are implicated in Trastuzumab treatment resistance is that they all can lead to Rac1 activation [10–13].

Rac1, a member of the Rho family small GTPases that belong to Ras GTPase super family, is capable of regulating cytoskeleton reorganization, promoting cell survival, proliferation, transformation, migration and invasion [14,15]. Three Rac isoforms have been identified: Rac1, Rac2 and Rac3. While Rac2 is exclusively expressed in hematopoietic cells and Rac3 is mainly expressed in brain, Rac1 is ubiquitously expressed. Like other small GTPases, Rac acts as a molecular switch cycling between an active GTP-bound state and an inactive GDP-bound state. It is the active form of Rac (Rac-GTP) that interacts with the downstream effectors and regulates a variety of important cellular functions. Rac is activated by guanine nucleotide exchange factors (GEFs), which can usually be activated by the overexpression of oncogenic tyrosine kinase receptors or deletion of tumor suppressor PTEN [16,17].

Studies have shown that Rac GTPases are overexpressed in human breast cancer [18,19]. Ours and other studies have demonstrated crucial roles for Rac1 activation in promoting breast cancer cell survival, proliferation and migration [10,20–23]. Although IGF-IR overexpression or PTEN deletion are implicated in primary and acquired resistance to Trastuzumab treatment, it is not known whether IGF-IR overexpression or PTEN deletion increase Rac1 activation in HER2 overexpression human breast cancer cells and whether Rac1 activation contributes to Trastuzumab resistance caused by IGF-IR overexpression or PTEN deletion. In this study we found that Rac1 was highly activated in IGF-IR overexpressing or PTEN deficient HER2 overexpression human breast cancer SKBR3 cells in a HER2-independent manner, and inactivation of Rac1 significantly reduced Trastuzumab resistance. Our findings suggest that Rac1 activation is critically involved in multiple mechanisms-caused Trastuzumab resistance and inhibiting Rac1 could be efficient in reducing Trastuzumab resistance in HER2 overexpression breast cancer.

2. Materials and methods

2.1. Cell line and reagents

HER2 overexpression human breast cancer SKBR3 cell was purchased from ATCC (Manassas, VA) and cultured in DMED/F12 medium (Invitrogen, Carlsbad, CA) supplemented with 10% FBS at 37 °C in a humidified 5% CO_2 atmosphere. Trastuzumab was generously provided by Genentech, Inc. (South San Francisco, CA). The Rac1 inhibitor NSC23766 was obtained from Tocris Cookson, Inc. (Ellisville, MO). The Akt inhibitor VIII trifluoroacetate salt hydrate and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were purchased from Sigma (St. Louis, MO). Control shRNA vector (pMKO.1-puro-shRNA) and PTEN knockdown shRNA vector (pMKO.1-puro-PTEN shRNA) were obtained from Addgene. Human full length IGF-IR cDNA clone was purchased from Open Biosystems (Huntsville, AL). Lentiviral expression vector pLenti7.3/V5-DEST™ was obtained from Invitrogen.

2.2. Generation of PTEN knockdown and IGF-IR overexpressing stable cells

To general shRNA Control and PTEN stable knockdown cells, SKBR3 cells were transfected with Control shRNA vector (pMKO.1-puro-shRNA) or PTEN knockdown shRNA vector (pMKO.1-puro-PTEN shRNA), respectively, followed by puromycin selection. Single colonies were picked up and propagated. The PTEN protein levels in Control and PTEN knockdown colonies were determined by Western blot. To generate IGF-IR overexpressing stable cells, human full length IGF-IR cDNA was cloned into pLenti7.3/V5-DEST™ vector using Gateway® cloning technology (Invitrogen) following manufacturer's instructions. Control (pLenti7.3) and IGF-IR expressing (pLenti7.3-IGF-IR) lentiviral particles were packaged using 293T cells as previously described [24]. SKBR3 cells were transduced with GFP control (pLenti7.3) or IGF-IR-expressing (pLenti7.3-IGF-IR) lentiviral particles and subcultured 48 h after lentiviral particle transduction. Fluorescence Activated Cell Sorting was performed to sort GFP positive cells. IGF-IR overexpression was confirmed by Western blot.

2.3. Cell viability analysis

Cell viability was determined by MTT assays. Briefly, cells were plated in 96-well plates. After overnight culture, different concentrations of Trastuzumab (0.05–1.0 μg/ml) and/or Rac1, Akt inhibitors were added to wells and incubated for 7 days. At the end of treatment, the culture medium was replaced with 50 μl of MTT-serum-free fresh medium (MTT final concentration: 0.5 mg/ml) followed by a 4 h incubation; 200 μl of dimethylsulfoxide (DMSO) was then added to dissolve the formazan crystals. Absorbance was measured with a microplate reader Spectra Max plus (Molecular Devices, Sunnyvale, CA) at a wavelength of 570 nm.

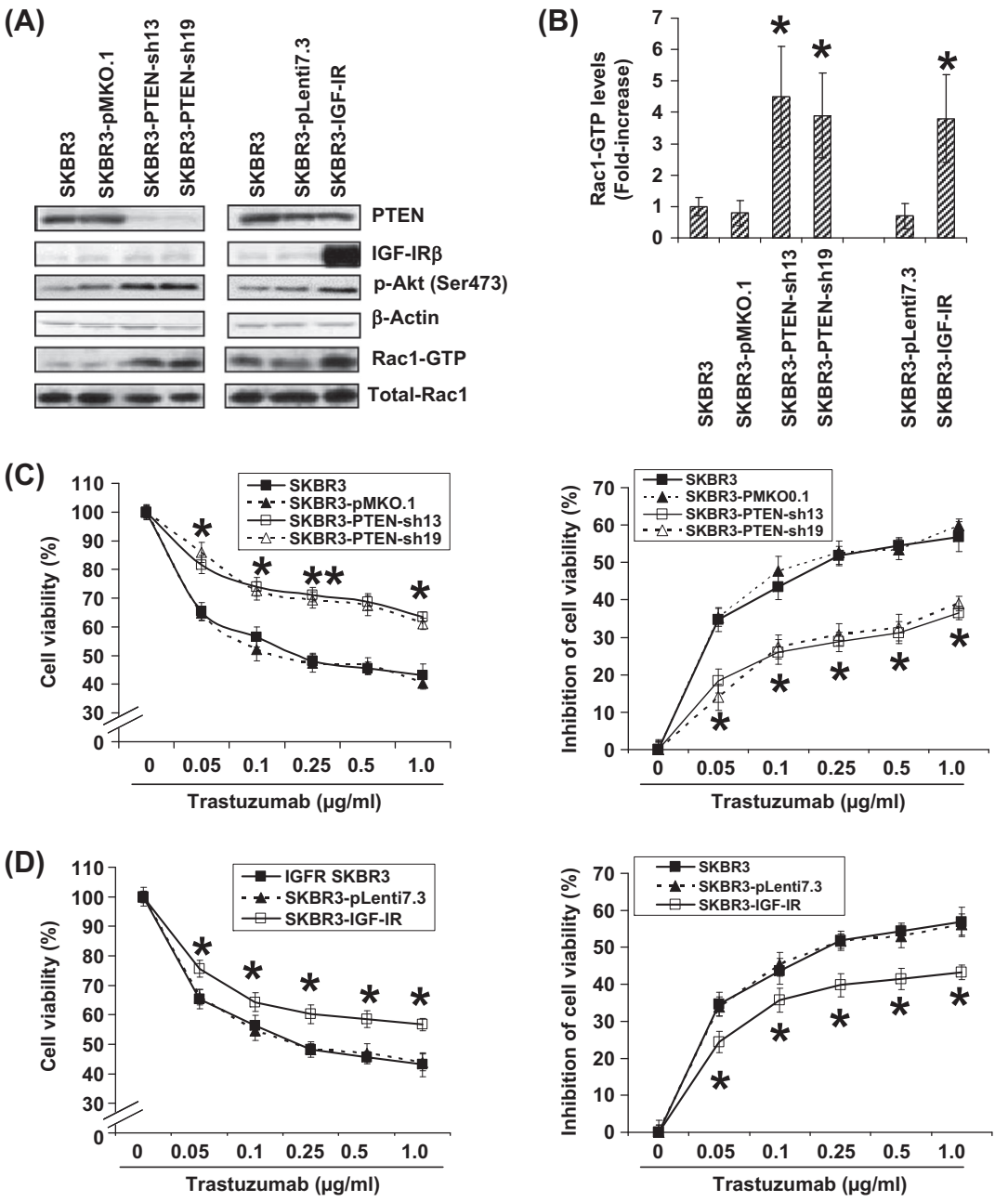

Fig. 1. Rac1 is highly activated in PTEN stable knockdown and IGF-IR overexpressing Trastuzumab-resistant SKBR3 cells. (A) Phosphor-Akt and Rac1-GTP levels are increased in PTEN stable knockdown and IGF-IR overexpressing SKBR3 cells. PTEN knockdown and IGF-IR overexpressing stable cells were generated as described in Section 2. Cells were cultured in DMEM/F12 supplemented with 10% FBS and collected at about 70–80% confluence for Western blot analysis of PTEN, IGF-IR and phosphor-Akt levels. Rac1-GTP levels were determined using GST-PBD pull-down assay as described in Section 2. (B) Densitometric analysis of Rac1-GTP levels normalized to the corresponding total Rac1 levels. Data are presented as means ± standard deviations ($n = 3$). $^{*}p < 0.05$, compared with parental SKBR3 or vector control SKBR3 cells. (C and D) PTEN stable knockdown SKBR3 cells (C) and IGF-IR stable overexpressing SKBR cells (D) are resistant to Trastuzumab. Cells were cultured in 96-well plates and treated with vehicle control (0 μg/ml) or various concentrations of Trastuzumab (0.05–1.0 μg/ml) for 7 days. Cell viability (%) was determined using MTT assay as described in Section 2 and expressed relative to vehicle control treatment. Inhibition of cell viability (%) was calculated using the formulae: 100% – vehicle control- or Trastuzumab-treated cell viability (%). Data are presented as means ± standard deviations ($n = 8$). $^{*}p < 0.05$, compared with the same concentration of Trastuzumab-treated parental or vector control SKBR3 cells. Similar results were obtained in two additional experiments.

2.4. Rac-GTP pull-down assays

Cells were plated to 10 cm tissue culture dishes and allowed to grow to 70–80% confluence. Cells were then treated with vehicle control (DMSO), Rac1 inhibitor NSC23766, or Akt inhibitor VIII for 24 h in culture medium with 0% FBS. Rac-GTP levels were determined with a pull-down assay using the GST-p21-binding domain (PBD) of

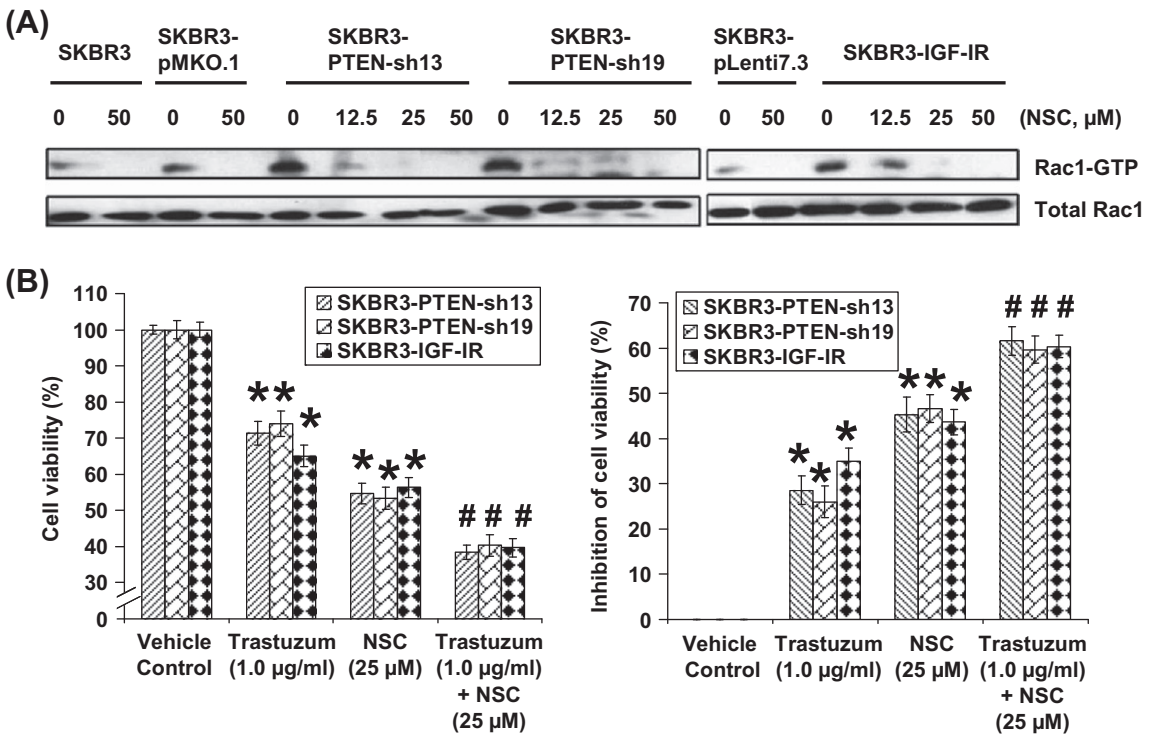

Fig. 2. Inhibition of Rac1 reduces Trastuzumab resistance in PTEN deficient and IGF-IR overexpressing SKBR3 cells. (A) Inhibition of Rac1 activation by a Rac1 inhibitor NSC23766. Cells were cultured in DMEM/F12 supplemented with 10% FBS and allowed to grow to about 70–80% confluence. Cells were then treated with vehicle control or different concentrations of NSC23766 (NSC) in the absence of FBS for 24 h. Rac1-GTP levels were determined using GST-PBD pull-down assay as described in Section 2. (B) Inhibition of Rac1 reduces Trastuzumab resistance. PTEN stable knockdown or IGF-IR overexpressing cells were cultured in 96-well plates and treated with vehicle control, Trastuzumab (1.0 μg/ml), NSC (25 μM), or Trastuzumab (1.0 μg/ml) plus NSC (25 μM) for 7 days. Cell viability (%) was determined using MTT assay as described in Section 2 and expressed relative to vehicle control treatment. Inhibition of cell viability (%) was calculated using the formulae: 100% – vehicle control- or Trastuzumab-treated cell viability (%). Data are presented as means ± standard deviations ($n = 8$). $^{*}p < 0.05$, compared with vehicle control-treated cells; $^{\#}p < 0.05$, compared with vehicle control-, Trastuzumab alone- and NSC alone-treated cells. Similar results were obtained in two additional experiments.

p21-activated kinase (PAK), as described previously [10,20,21], and using an anti-Rac1 antibody for Western blot detection.

2.5. Western blot analysis

Cells were lysed using tris-sodium dodecyl sulfate (SDS) as described by Yang et al. [10] and subjected to SDS–polyacrylamide gel electrophoresis (PAGE) (10–30 μg of protein/lane). The following primary antibodies were used: anti-Rac1 (Millipore, Billerica, MA); anti-cyclin D1 (BD Pharmingen, Bedford, MA); anti-HER2, anti-phospho-HER2 (Tyr877), anti-phospho-HER2 (Tyr1221/1222), anti-Akt, anti-phosphor AKT (Ser473), anti-PTEN, anti-IGF-IRβ (Cell Signaling Technology, Beverly, MA); anti-Tiam1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); and anti-β-actin (Sigma, St. Louis, MO).

2.6. Tiam1 and HER2 knockdown using small interfering RNAs (siRNAs)

Negative Control and ON-TARGETplus SMARTpool siRNA for Her2 were obtained from Thermo Scientific Dharmacon (Lafayette, CO). Tiam1 siRNA and Control siRNA were purchased from Santa Cruz Biotechnology, Inc. SiRNA duplexes (100 nM) were transfected into cells using Lipofectamine 2000 (Invitrogen) in serum-free medium following the manufacturer's instructions. Twenty-four hour after transfection, cells were collected and plated to 96-well plates. After overnight culture, cells were then treated with Trastuzumab for cell viability analysis. For analyzing Rac-GTP levels, Rac-GTP pull down assays were performed 72 h after siRNA transfection. Successful knockdown of Tiam1 or Her2 was confirmed by Western blot.

2.7. Statistic analysis

The statistical analyses for the significance of differences in numerical data (means ± standard deviations) were performed using two-tailed *t*-tests for comparison of two data sets or one-way analysis of variance (ANOVA) for multiple data sets. A p value of <0.05 was considered statistically significant.

3. Results

3.1. Rac1 is highly activated in PTEN deficient and IGF-IR overexpressing Trastuzumab-resistant SKBR3 cells

Transfection with control shRNA vector (pMKO.1-puro-shRNA) showed no effect on PTEN protein levels in SKBR3 cells, three randomly selected clones were pooled together and used as shRNA vector control

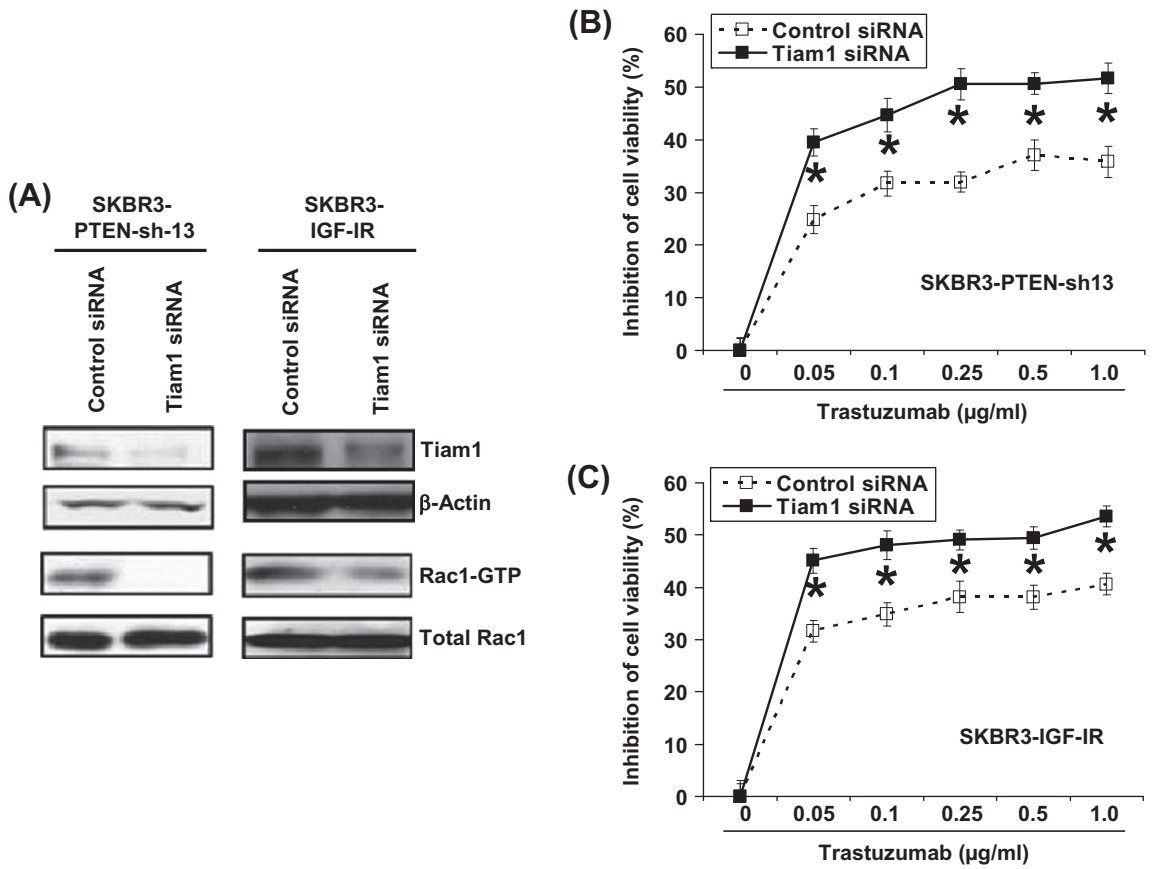

Fig. 3. SiRNA knocking down the expression of Tiam1 impairs Rac1 activation and reduces Trastuzumab resistance in PTEN deficient and IGF-IR overexpressing SKBR3 cells. (A) SiRNA knocking down the expression of Rac-GEF Tiam1 impairs Rac1 activation in Trastuzumab-resistant SKBR3 cells. Cells were transfected with control or Tiam1 siRNA as described in Section 2. Forty-eight hour after siRNA transfection, cells were cultured in the absence of FBS for 24 h. Rac1-GTP levels were then determined using GST-PBD pull-down assay as described in Section 2. Tiam1 protein levels were detected by Western blot analysis. (B and C) SiRNA knocking down the expression of Rac-GEF Tiam1 reduces Trastuzumab resistance. PTEN stable knockdown (B) or IGF-IR overexpressing (C) cells were transfected with control or Tiam1 siRNA in 6-cm tissue culture dishes. Twenty-four hour after siRNA transfection, cells were detached by trypsinization and cultured into 96-well plates and treated with vehicle control or different concentrations of Trastuzumab (0.05–1.0 μg/ml) for 7 days. Cell viability (%) was determined using MTT assay as described in Section 2 and expressed relative to vehicle control treatment. Inhibition of cell viability (%) was calculated using the formulae: 100% – vehicle control- or Trastuzumab-treated cell viability (%). Data are presented as means ± standard deviations ($n = 8$). $^{*}p < 0.05$, compared with the same concentration of Trastuzumab-treated control siRNA-transfected cells. Similar results were obtained in two additional experiments.

cells (SKBR3-pMKO.1) (Fig. 1A). After Western blot screening of 30 single clones obtained from PTEN shRNA knockdown vector transfection, several single clones with more than 70% knockdown of PTEN protein levels were obtained. PTEN knockdown was confirmed by Western blot analysis and two single PTEN shRNA knockdown clones (SKBR3-PTEN-sh13 and SKBR3-PTEN-sh19) were used in this study (Fig. 1A). Control (SKBR3-pLenti7.3) and IGF-IR overexpressing (SKBR3-IGF-IR) cells were pooled-population generated by fluorescence activated cell sorting after lentiviral transduction of SKBR3 cells with plenti7.3 or pLenti7.3-IGF-IR particles. IGF-IR overexpression in SKBR3 cells was confirmed by Western blot analysis (Fig. 1A).

Since PTEN is a negative regulator of phosphoinositide 3-kinase (PI3K)/Akt pathway and high IGF-IR expression enhances PI3K/Akt activation, increased levels of phosphor-Akt were observed in PTEN knockdown and IGF-IR overexpressing SKBR3 cells (Fig. 1A). Comparing with parental or vector control SKBR3 cells, PTEN stable knockdown and IGF-IR overexpressing cells had significantly higher active Rac1 (Rac1-GTP) levels (Fig. 1A). Densitometric analysis of Rac1-GTP levels that were normalized to the corresponding total Rac1 levels revealed 2.5- to 4.5-fold increases of Rac1 activation in PTEN stable knockdown and IGF-IR overexpressing cells (Fig. 1B).

Trastuzumab treatment caused decreases of SKBR3 cell viability in a dose-dependent way. However, PTEN stable knockdown and IGF-IR overexpressing cells displayed significantly higher viability in each corresponding Trastuzumab treatment (Fig. 1C and D). Inhibition of cell viability by 1 μg/ml of Trastuzumab treatment was reduced from ~60% in parental and shRNA vector control cells to ~40% in PTEN stable knockdown cells; and from ~59% in pLenti7.3 vector control cells to ~45% in IGF-IR overexpressing cells (Fig. 1C and D). Together, these results indicate that Rac1 was highly activated in PTEN stable knockdown and IGF-IR overexpressing SKBR3 cells, which became resistant to Trastuzumab treatment.

3.2. Inactivation of Rac1 reduces Trastuzumab resistance in PTEN deficient and IGF-IR overexpressing SKBR3 cells

We next wanted to determine whether inhibiting Rac1 reduces Trastuzumab resistance. We first used a small molecule Rac1 inhibitor NSC23766 to inhibit Rac1 activation. NSC23766 is a selective inhibitor of Rac-specific guanine nucleotide exchange factors (Rac-GEFs) TrioN and Tiam1 (IC50: ~50 μM), preventing Rac activation without affecting

the activity of closely related Cdc42 and RhoA Rho GTPases [25]. NSC23766 (NSC) dose-dependently and efficiently inhibited Rac1 activation in SKBR3 cells (Fig. 2A). A complete inhibition of Rac1 activation in both PTEN stable knockdown and IGF-IR overexpressing cells was achieved with 25 μM or a higher concentration of NSC treatment for 24 h.

To examine the role of Rac1 activation in PTEN deletion- or IGF-IR overexpression-caused Trastuzumab resistance, cells were treated with Trastuzumab (1 μg/ml), NSC (25 μM), or both (Trastuzumab, 1 μg/ml + NSC, 25 μM). While treatment with Trastuzumab or NSC alone significantly reduced the viability of PTEN stable knockdown and IGF-IR overexpressing SKBR3 cells, combined treatment with both Trastuzumab (1 μg/ml) and NSC (25 μM) further significantly decreased cell viability more than any of either single treatments (Fig. 2B). Inhibition of cell viability was increased from ~30% by 1 μg/ml of Trastuzumab treatment alone to ~63% by 1 μg/ml of Trastuzumab plus NSC treatment in PTEN stable knockdown cells; and from ~36% by 1 μg/ml of Trastuzumab treatment alone to ~62% by 1 μg/ml of Trastuzumab plus NSC treatment in IGF-IR overexpressing cells (Fig. 2B).

To exclude the potential non-specific effect of Rac1 inhibitor NSC23766, we used another approach to inhibit Rac1 activation by knocking down a Rac-GEF Tiam1, a target of NSC23766. SiRNA knocking down the expression of Tiam1 impaired Rac1 activation in PTEN stable knockdown and IGF-IR overexpressing SKBR3 cells (Fig. 3A). As a result, Trastuzumab treatment was capable of significantly more efficiently reducing the viability of Tiam1-knocked down SKBR3-PTEN-sh13 and SKBR3-IGF-IR cells than control siRNA-transfected cells (Fig. 3B and C). These results indicate that Rac1 was activated mainly by Rac-GEF Tiam1 in SKBR3 cells; Rac1 activation contributed to PTEN deletion- or IGF-IR overexpressing-caused Trastuzumab resistance; and inhibition of Rac1 activation significantly reduced Trastuzumab resistance.

3.3. Rac1 activation in PTEN deficient and IGF-IR overexpressing SKBR3 cells is independent of HER2 overexpression

It has been proposed that alternative cell signaling from other abnormally-expressed and/or activated molecules contributes to Trastuzumab resistance in HER2 overexpression breast cancer. To further determine the mechanism of Trastuzumab resistance caused by PTEN deletion or IGF-IR overexpressing in SKBR3 cells, we next examined whether Rac1 activation depended on HER2 overexpression in PTEN stable knockdown and IGF-IR overexpressing SKBR3 cells. As shown in Fig. 4, although siRNA knocking down HER2 expression significantly decreased Rac1 activation in parental SKBR3 cells (Fig. 4A), HER2 knockdown showed no effect on Rac1 activation in PTEN stable knockdown and IGF-IR overexpressing SKBR3 cells (Fig. 4B). These results indicate that while Rac1 activation in parental SKBR3 cells depended on HER2 overexpression, Rac1 activation in PTEN deficient or IGF-IR overexpressing Trastuzumab-resistant SKBR cells did not rely on HER2 overexpression anymore.

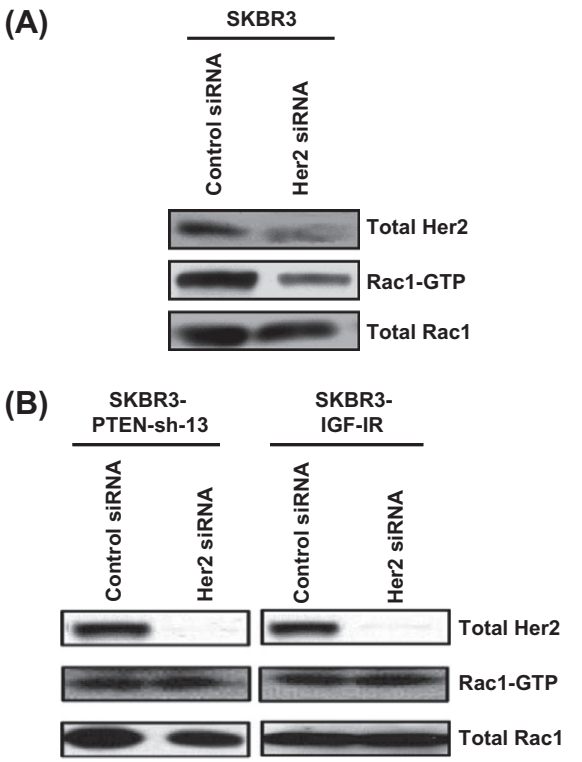

Fig. 4. Rac1 activation in PTEN deficient and IGF-IR overexpressing SKBR3 cells is independent of HER2 overexpression. (A) Knocking down HER2 expression reduces Rac1 activation in parental SKBR3 cells. Cells were transfected with control or HER2 siRNA as described in Section 2. Forty-eight hour after siRNA transfection, cells were cultured in the absence of FBS for 24 h. Rac1-GTP levels were then determined using GST-PBD pull-down assay as described in Section 2. HER2 protein levels were detected by Western blot. (B) Rac1 activation in PTEN stable knockdown and IGF-IR overexpressing Trastuzumab-resistant cells is independent of HER2 overexpression. Cells were transfected with control or HER2 siRNA as described in Section 2. Forty-eight hour after siRNA transfection, cells were cultured in the absence of FBS for 24 h. Rac1-GTP levels were then determined using GST-PBD pull-down assay as described in Section 2. HER2 protein levels were detected by Western blot analysis. Similar results were obtained in two additional experiments.

3.4. Inhibition of Rac1 has no effect on the levels of phosphor-HER2 and phosphor-Akt but decreases the level of cyclin D1

We next examined how inactivation of Rac1 may reduce Trastuzumab resistance. We first determined whether inhibition of Rac1 had an effect on the levels of phosphor-HER2 and total HER2. Western blot analysis revealed that there were no significant differences of phosphor-HER2 (Tyr877 and Tyr1221/1222) levels between vector control cells and PTEN stable knockdown cells or IGF-IR overexpressing cells (Fig. 5). A 24 h treatment with the Rac1 inhibitor at a concentration (NSC, 25 μM) that reduced Rac1 activation and Trastuzumab resistance (Fig. 2) showed no effect on phosphor-HER2 levels (Fig. 5). Similarly, nor did inhibition of Rac1 affect total HER2 levels (Fig. 5). Since increased levels of phosphor-Akt were detected in PTEN stable knockdown cells and IGF-IR overexpressing cells (Fig. 1A), we also determined whether inhibition of Rac1 had an effect on the levels of phosphor-Akt. As shown in Fig. 5, neither the levels of phosphor-Akt nor the levels of total Akt were changed by the 24 h treatment with the Rac1 inhibitor. These results indicate that inhibition of Rac1 had no effect on the activation of HER2 and Akt.

Ours and other previous studies showed that Rac1 activation increased cyclin D1 expression and promoted breast cancer cell proliferation [20,21,26]. We then investigated whether inhibition of Rac1 had an effect on cyclin D1 levels. Treatment with the Rac1 inhibitor (NSC, 25 μM) for 24 h not only diminished cyclin D1 expression in vector control SKBR3 cells, but also significantly decreased cyclin D1 protein levels in PTEN stable knockdown cells and IGF-IR overexpressing cells (Fig. 5). Given the crucial role of cyclin D1 in breast cancer cell survival and proliferation, these results suggest that down-regulation of cyclin D1 expression may contribute to reduced Trastuzumab resistance resulting from inhibition of Rac1.

3.5. Simultaneous inhibition of both Rac1 and Akt significantly further reduces Trastuzumab resistance in PTEN deficient and IGF-IR overexpressing SKBR3 cells

Increased Akt activation is implicated in Trastuzumab resistance in HER2 overexpression breast cancer and inhibition of Akt may reduce Trastuzumab resistance [27]. Since phosphor-Akt levels were increased in PTEN stable knock down and IGF-IR overexpressing Trastuzumab-resistant SKBR3 cells (Figs. 1A and 5), and treatment with Rac1 inhibitor that reduced Trastuzumab resistance did not alter phosphor-Akt levels (Fig. 5), we then wanted to determine whether simultaneous inhibiting both Rac1 and Akt will further reduce Trastuzumab resistance. Akt inhibition was achieved by treating cells with an Akt kinase inhibitor VIII trifluoroacetate salt hydrate (AKTi). As shown in Fig. 6A, AKTi treatment dose-dependently and efficiently reduced phosphor-Akt levels in both

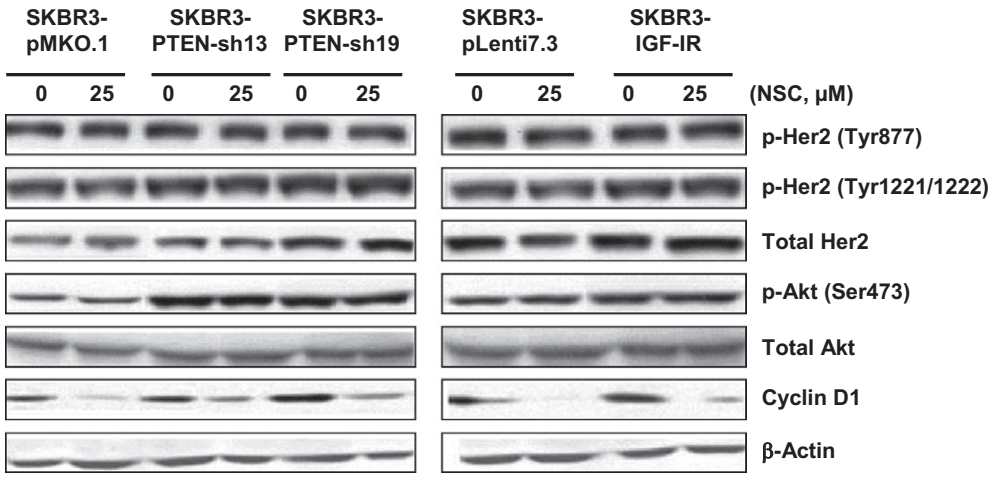

Fig. 5. Inhibition of Rac1 has no effect on the levels of phosphor-HER2 and phosphor-Akt but decreases the level of cyclin D1. Cells were cultured in DMEM/F12 supplemented with 10% FBS and allowed to grow to about 50–60% confluence. Cells were then treated with vehicle control or NSC (25 μM) in the presence of 10% FBS for 24 h and collected for Western blot analysis of phosphor-HER2, total HER2, phosphor-Akt and total Akt levels. To examine the effect of Rac1 inhibition on cyclin D1 expression, cells were first serum-starved for 48 h and then treated with vehicle control or NSC (25 μM) in the presence of 10% FBS for 24 h and collected for Western blot analysis of cyclin D1 levels. Similar results were obtained in two additional experiments.

vector control cells and PTEN stable knockdown and IGF-IR overexpressing cells. However, AKTi treatment showed no effect on Rac1 activation in PTEN stable knockdown and IGF-IR overexpressing cells (Fig. 6A).

To simultaneously inhibit both Rac1 and Akt, cells were treated with both NSC (25 μM) and AKTi (5 μM). As shown in Fig. 6B, comparing with Trastuzumab alone treatment, Trastuzumab plus Rac1 inhibitor NSC treatment or Trastuzumab plus Akt inhibitor AKTi treatment had significantly stronger inhibitory effect on the viability of Trastuzumab-resistant SKBR3 cells. The inhibition of cell viability was increased from ~28–34% by Trastuzumab alone treatment to ~56–61% by Trastuzumab plus NSC or Trastuzumab plus AKTi. However, Trastuzumab plus both NSC and AKTi treatment showed the strongest inhibition on cell viability. The inhibition of cell viability was increased from ~56–61% by Trastuzumab plus NSC or Trastuzumab plus AKTi treatment to ~80–83% by Trastuzumab plus NSC and AKTi (Fig. 6B). These results indicate that simultaneous inhibition of both Rac1 and Akt further reduced Trastuzumab resistance.

4. Discussion

Trastuzumab is the first approved monoclonal antibody against HER2 and its wide application in HER2 overexpression breast cancer treatment represents a milestone in rationally designed targeted therapy. However, despite very promising therapeutic effects at the beginning, resistance occurred in the majority of patients who initially responded to Trastuzumab. Moreover, there was also a large portion of patients who never responded to Trastuzumab. The mechanism underlying the occurrences of Trastuzumab resistance has not been clearly defined. Therefore, it is imperative to elucidate the mechanism of resistance and identify additional molecular targets for improving Trastuzumab therapeutic efficacy. We found in this study that Rac1 was highly activated in PTEN stable knockdown and IGF-IR overexpressing Trastuzumab-resistant SKBR3 cells in a HER2-independent manner, and inactivation of Rac1 resulted in a significant decrease of resistance to Trastuzumab. Moreover, we also found that simultaneous inhibition of both Rac1 and Akt led to a further significant reduction of Trastuzumab resistance in PTEN deficient and IGF-IR overexpressing SKBR3 cells. This study alone with a previous report showing that Rac1 activation contributes to Trastuzumab resistance in SKBR3 cells selected from long term repeated Trastuzumab treatment [28] indicate that Rac1 activation is critically involved in multiple mechanisms-caused Trastuzumab resistance, and inhibition of Rac1 may significantly improve the therapeutic efficacy in a large number of HER2 overexpression breast cancer patients with primary and/or acquired Trastuzumab resistance.

Overexpression of EGFR, HER3, c-Met, IGF-IR or depletion of PTEN are implicated in primary and/or acquired Trastuzumab resistance in HER2 overexpression breast cancer. However, it may not be useful or practical to target aforementioned each individual molecules in order to decrease Trastuzumab resistance because they share some common signaling pathways. Instead, the strategies targeting a common downstream key signaling molecule may reduce Trastuzumab resistance more efficiently. For example, it is believed that HER2 overexpression causes PI3K/Akt activation, which promotes breast cancer cell survival, proliferation, migration and invasion. Alternative signaling from either overexpression of other oncogenic tyrosine kinase receptors (EGFR, HER3, c-Met, IGF-IR, etc.) or deletion of tumor suppressor PTEN in HER2 overexpression breast cancer cells also causes PI3K/Akt activation, which is thought to play a critical role in Trastuzumab resistance. Therefore, it has been proposed to inhibit PI3K or Akt to reduce Trastuzumab resistance in HER2 overexpression breast cancer [27]. Interestingly, a recent study reported that the non-receptor tyrosine kinase Src was also commonly activated by overexpression of HER2 or other oncogenic tyrosine kinase receptors or deletion of PTEN [29]. Inhibition of Src significantly reduced Trastuzumab resistance in cell culture and mouse xenograft tumor model studies using HER2 overexpression human breast cancer cells [29].

The small Rho GTPase Rac1 is another important downstream signaling molecule that is usually activated

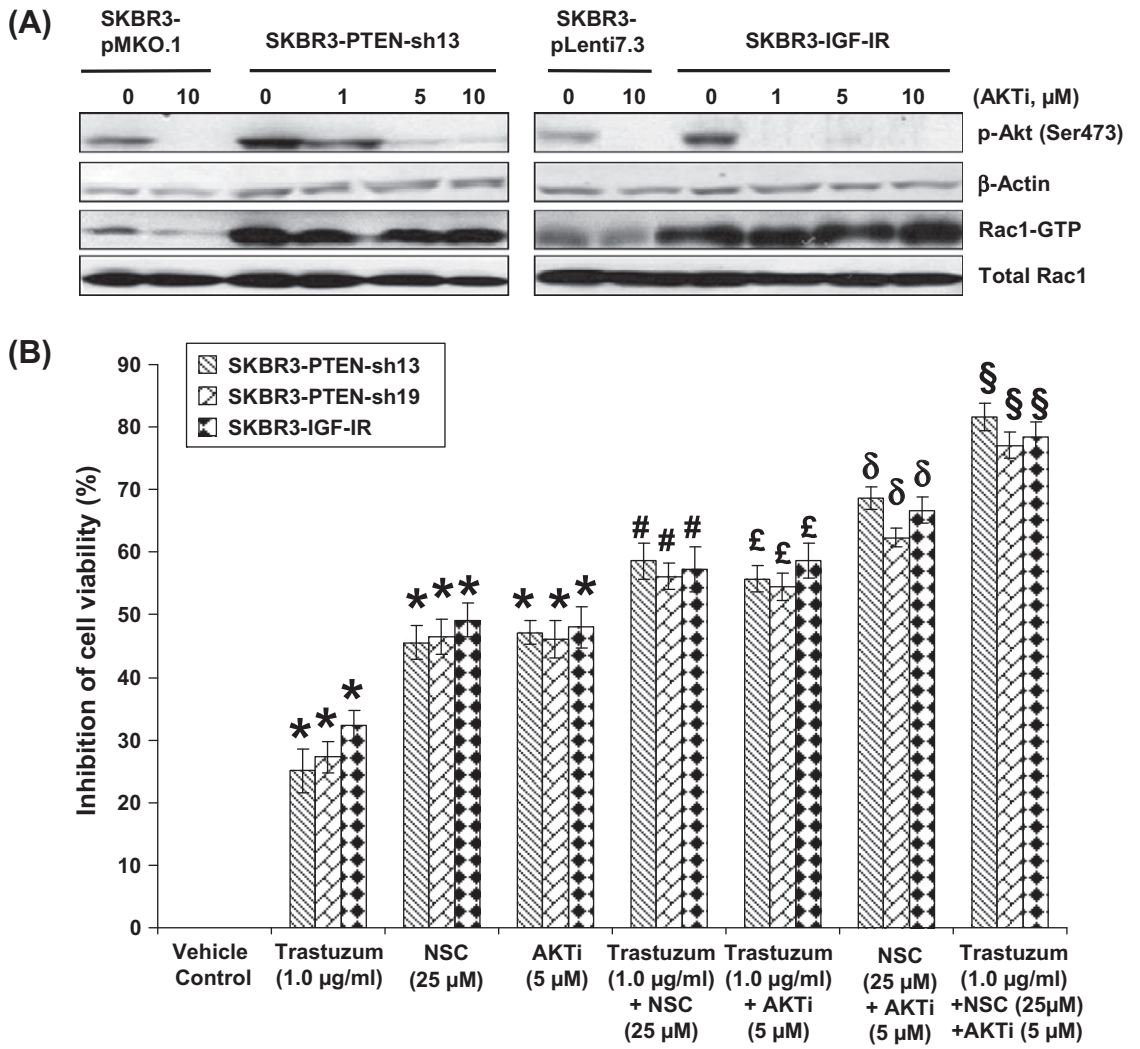

Fig. 6. Simultaneous inhibition of both Rac1 and Akt significantly further reduces Trastuzumab resistance in PTEN deficient and IGF-IR overexpressing SKBR3 cells. (A) Inhibition of Akt has no effect on Rac1 activation. Cells were cultured in DMEM/F12 supplemented with 10% FBS and allowed to grow to about 70–80% confluence. Cells were then treated with vehicle control or different concentrations of an Akt inhibitor (AKTi) in the absence of FBS for 24 h. Phosphor-Akt levels were detected by Western blot. Rac1-GTP levels were determined using GST-PBD pull-down assay as described in Section 2. (B) Simultaneous inhibition of both Rac1 and Akt significantly further reduces Trastuzumab resistance. Cells were cultured in 96-well plates and treated with vehicle control, Trastuzumab (1.0 μg/ml), NSC (25 μM), AKTi (5 μM), NSC (25 μM) plus AKTi (5 μM), Trastuzumab (1.0 μg/ml) plus NSC (25 μM), Trastuzumab (1.0 μg/ml) plus AKTi (5 μM), or Trastuzumab (1.0 μg/ml) plus NSC (25 μM) and plus AKTi (5 μM) for 7 days. Cell viability (%) was determined using MTT assay as described in Section 2 and expressed relative to vehicle control treatment. Inhibition of cell viability (%) was calculated using the formulae: 100% – vehicle control- or Trastuzumab and/or NSC, AKTi-treated cell viability (%). Data are presented as means ± standard deviations ($n = 8$). $^{*}p < 0.05$, compared with vehicle control-treated cells; $^{\#}p < 0.05$, compared with vehicle control-, Trastuzumab alone- and NSC alone-treated cells; **£**$p < 0.05$, compared with vehicle control-, Trastuzumab alone- and AKTi alone-treated cells; δ $p < 0.05$, compared with vehicle control-, NSC alone- and AKTi alone-treated cells; § compared with vehicle control-, Trastuzumab + NSC-, and Trastuzumab + AKTi-treated cells. Similar results were obtained in two additional experiments.

by overexpression of various oncogenic tyrosine kinase receptors or PTEN deletion via PI3K-dependent and –independent mechanisms [30,17]. Animal model studies showed that gene targeting (knockout) of Rac1 and Rac2 abrogated chronic myelogenous leukemia (CML) development in mice [31]. Therefore, Rac GTPases have been proposed as novel molecular targets for cancer therapy [32–35]. The specific small molecular inhibitors for Rac GTPases have been developed [25,36]. Treatment of mice with the Rac1 inhibitor NSC23766 suppressed the tumor growth in human CML cell mouse xenograft tumor model studies [31]. Our findings that Rac1 was highly activated in PTEN deficient and IGF-IR overexpressing Trastuzumab-resistant SKBR3 cells and inactivation of Rac1 significantly reduced Trastuzumab resistance provide additional strong rationale for developing new generation and clinically applicable Rac1 inhibitors. Combination of Trastuzumab treatment with a Rac1 inhibitor may represent another promising strategy for improving Trastuzumab efficacy in HER2 overexpression breast cancer.

Another interest finding from this study is that neither inactivation of Rac1 affected phosphor-Akt levels nor did inhibition of Akt affect Rac1 activation. However, inhibiting either Rac1 or Akt reduced Trastuzumab resistance. These findings suggest that Rac1 and Akt may work separately to promote Trastuzumab resistance resulting from PTEN deletion or IGF-IR overexpression. Indeed, only limited success of application of Akt inhibitors in reducing Trastuzumab resistance were observed in recent clinical trials [37]. It is thus likely to achieve a better Trastuzumab efficacy by combination with simultaneous inhibition of both Rac1 and Akt. In this study we found that treatment with Trastuzumab plus a Rac1 inhibitor NSC and an Akt inhibitor AKTi achieved a significantly stronger inhibition of cell viability than treatment with Trastuzumab plus NSC or AKTi alone. These findings provide further rationale for developing more efficient and less toxic Rac1 and Akt inhibitors for future combined application to fight better against Trastuzumab resistance.

In summary, we found that the small GTPase Rac1 was highly activated in PTEN deficient and IGF-IR overexpressing Trastuzumab-resistant SKBR3 cells in a HER2-independent manner. Inactivation of Rac1 significantly reduced Trastuzumab resistance. Simultaneous inhibition of both Rac1 and Akt resulted in a significantly more decrease of Trastuzumab resistance. These findings provide new evidence indicating that Rac1 activation is critically involved in multiple-mechanisms-caused Trastuzumab resistance. Combinational treatment of Trastuzumab with clinically applicable Rac1 and Akt inhibitors may achieve a better outcome for a large portion of breast cancer patients with HER2 overexpression.

Acknowledgements

This study was funded by ELSA U. PARDEE FOUNDATION (Midland, MI). The Trastuzumab was provided by Genentech, Inc. (South San Francisco, CA).

References

[1] D.J. Slamon, G.M. Clark, S.G. Wong, W.J. Levin, A. Ullrich, W.L. McGuire, Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene, Science 235 (1987) 177–182.
[2] M.M. Moasser, The oncogene HER2: its signaling and transforming functions and its role in human cancer pathogenesis, Oncogene 26 (2007) 6469–6487.
[3] C. Vogel, M.A. Cobleigh, D. Tripathy, J.C. Gutheil, L.N. Harris, L. Fehrenbacher, D.J. Slamon, M. Murphy, W.F. Novotny, M. Burchmore, S. Shak, S.J. Stewart, First-line, single-agent Herceptin(R) (trastuzumab) in metastatic breast cancer. A preliminary report, Eur. J. Cancer 37 (Suppl. 1) (2001) 25–29.
[4] C.L. Vogel, M.A. Cobleigh, D. Tripathy, J.C. Gutheil, L.N. Harris, L. Fehrenbacher, D.J. Slamon, M. Murphy, W.F. Novotny, M. Burchmore, S. Shak, S.J. Stewart, M. Press, Efficacy and safety of trastuzumab as a single agent in first-line treatment of HER2-overexpressing metastatic breast cancer, J. Clin. Oncol. 20 (2002) 719–726.
[5] A.D. Seidman, Is trastuzumab active following conventional adjuvant chemotherapy in HER2-positive early breast cancer?, Nat Clin. Pract. Oncol. 3 (2006) 178–179.
[6] A.D. Seidman, D. Berry, C. Cirrincione, L. Harris, H. Muss, P.K. Marcom, G. Gipson, H. Burstein, D. Lake, C.L. Shapiro, P. Ungaro, L. Norton, E. Winer, C. Hudis, Randomized phase III trial of weekly compared with every-3-weeks paclitaxel for metastatic breast cancer, with trastuzumab for all HER-2 overexpressors and random assignment to trastuzumab or not in HER-2 nonoverexpressors: final results of Cancer and Leukemia Group B protocol 9840, J. Clin. Oncol. 26 (2008) 1642–1649.
[7] C.A. Hudis, Trastuzumab – mechanism of action and use in clinical practice, New Engl. J. Med. 357 (2007) 39–51.
[8] R. Nahta, F.J. Esteva, Trastuzumab: triumphs and tribulations, Oncogene 26 (2007) 3637–3643.
[9] J.T. Garrett, C.L. Arteaga, Resistance to HER2-directed antibodies and tyrosine kinase inhibitors: mechanisms and clinical implications, Cancer Biol. Ther. 11 (2011) 793–800.
[10] C. Yang, Y. Liu, M.A. Lemmon, M.G. Kazanietz, Essential role for Rac in heregulin beta1 mitogenic signaling: a mechanism that involves epidermal growth factor receptor and is independent of ErbB4, Mol. Cell. Biol. 26 (2006) 831–842.
[11] J.S. Kim, X. Peng, P.K. De, R.L. Geahlen, D.L. Durden, PTEN controls immunoreceptor (immunoreceptor tyrosine-based activation motif) signaling and the activation of Rac, Blood 99 (2002) 694–697.
[12] D. Ferraro, S. Corso, E. Fasano, E. Panieri, R. Santangelo, S. Borrello, S. Giordano, G. Pani, T. Galeotti, Pro-metastatic signaling by c-Met through RAC-1 and reactive oxygen species (ROS), Oncogene 25 (2006) 3689–3698.
[13] L.R. Fiedler, E. Schonherr, R. Waddington, S. Niland, D.G. Seidler, D. Aeschlimann, J.A. Eble, Decorin regulates endothelial cell motility on collagen I through activation of insulin-like growth factor I receptor and modulation of alpha2beta1 integrin activity, J. Biol. Chem. 283 (2008) 17406–17415.
[14] K. Burridge, K. Wennerberg, Rho and Rac take center stage, Cell 116 (2004) 167–179.
[15] F.M. Vega, A.J. Ridley, Rho GTPases in cancer cell biology, FEBS Lett. 582 (2008) 2093–2101.
[16] K.L. Rossman, C.J. Der, J. Sondek, GEF means go: turning on RHO GTPases with guanine nucleotide-exchange factors, Nat. Rev. Mol. Cell Biol. 6 (2005) 167–180.
[17] M.R. Schiller, Coupling receptor tyrosine kinases to Rho GTPases–GEFs what's the link, Cell. Signal. 18 (2006) 1834–1843.
[18] G. Fritz, I. Just, B. Kaina, Rho GTPases are over-expressed in human tumors, Int. J. Cancer 81 (1999) 682–687.
[19] A. Schnelzer, D. Prechtel, U. Knaus, K. Dehne, M. Gerhard, H. Graeff, N. Harbeck, M. Schmitt, E. Lengyel, Rac1 in human breast cancer: overexpression, mutation analysis, and characterization of a new isoform, Rac1b, Oncogene 19 (2000) 3013–3020.
[20] C. Yang, E.A. Klein, R.K. Assoian, M.G. Kazanietz, Heregulin beta1 promotes breast cancer cell proliferation through Rac/ERK-dependent induction of cyclin D1 and p21Cip1, Biochem. J. 410 (2008) 167–175.
[21] C. Yang, Y. Liu, F.C. Leskow, V.M. Weaver, M.G. Kazanietz, Rac-GAP-dependent inhibition of breast cancer cell proliferation by {beta}2-chimerin, J. Biol. Chem. 280 (2005) 24363–24370.
[22] P.J. Baugher, L. Krishnamoorthy, J.E. Price, S.F. Dharmawardhane, Rac1 and Rac3 isoform activation is involved in the invasive and metastatic phenotype of human breast cancer cells, Breast Cancer Res. 7 (2005) R965–R974.
[23] M.S. Sosa, C. Lopez-Haber, C. Yang, H. Wang, M.A. Lemmon, J.M. Busillo, J. Luo, J.L. Benovic, A. Klein-Szanto, H. Yagi, J.S. Gutkind, R.E. Parsons, M.G. Kazanietz, Identification of the Rac-GEF P-Rex1 as an essential mediator of ErbB signaling in breast cancer, Mol. Cell 40 (2010) 877–892.
[24] Z. Wang, Y. Zhao, E. Smith, G.J. Goodall, P.A. Drew, T. Brabletz, C. Yang, Reversal and prevention of arsenic-induced human bronchial epithelial cell malignant transformation by micoRNA-200b, Toxicol. Sci. 12 (2011) 110–122.
[25] Y. Gao, J.B. Dickerson, F. Guo, J. Zheng, Y. Zheng, Rational design and characterization of a Rac GTPase-specific small molecule inhibitor, Proc. Natl. Acad. Sci. USA 101 (2004) 7618–7623.
[26] D. Cai, A. Iyer, K.N. Felekkis, R.I. Near, Z. Luo, J. Chernoff, C. Albanese, R.G. Pestell, A. Lerner, AND-34/BCAR3, a GDP exchange factor whose overexpression confers antiestrogen resistance, activates Rac, PAK1, and the cyclin D1 promoter, Cancer Res. 63 (2003) 6802–6808.
[27] M.S. van der Heijden, R. Bernards, Inhibition of the PI3K pathway: hope we can believe in?, Clin Cancer Res. 16 (2010) 3094–3099.
[28] M. Dokmanovic, D.S. Hirsch, Y. Shen, W.J. Wu, Rac1 contributes to trastuzumab resistance of breast cancer cells: Rac1 as a potential therapeutic target for the treatment of trastuzumab-resistant breast cancer, Mol. Cancer Ther. 8 (2009) 1557–1569.
[29] S. Zhang, W.C. Huang, P. Li, H. Guo, S.B. Poh, S.W. Brady, Y. Xiong, L.M. Tseng, S.H. Li, Z. Ding, A.A. Sahin, F.J. Esteva, G.N. Hortobagyi, D. Yu, Combating trastuzumab resistance by targeting SRC, a common node downstream of multiple resistance pathways, Nat. Med. 17 (2011) 461–469.

[30] Y. Zheng, Dbl family guanine nucleotide exchange factors, Trends Biochem. Sci. 26 (2001) 724–732.
[31] E.K. Thomas, J.A. Cancelas, H.D. Chae, A.D. Cox, P.J. Keller, D. Perrotti, P. Neviani, B.J. Druker, K.D. Setchell, Y. Zheng, C.E. Harris, D.A. Williams, Rac guanosine triphosphatases represent integrating molecular therapeutic targets for BCR-ABL-induced myeloproliferative disease, Cancer Cell 12 (2007) 467–478.
[32] T.K. Lee, K. Man, J.W. Ho, X.H. Wang, R.T. Poon, C.K. Sun, K.T. Ng, I.O. Ng, R. Xu, S.T. Fan, Significance of the Rac signaling pathway in HCC cell motility: implications for a new therapeutic target, Carcinogenesis 26 (2005) 681–687.
[33] K. Walker, M.F. Olson, Targeting Ras and Rho GTPases as opportunities for cancer therapeutics, Curr. Opin. Genet. Dev. 15 (2005) 62–68.
[34] G. Fritz, B. Kaina, Rho GTPases: promising cellular targets for novel anticancer drugs, Curr. Cancer Drug Targets 6 (2006) 1–14.
[35] N. Nassar, J. Cancelas, J. Zheng, D.A. Williams, Y. Zheng, Structure-function based design of small molecule inhibitors targeting Rho family GTPases, Curr. Top. Med. Chem. 6 (2006) 1109–1116.
[36] C. Onesto, A. Shutes, V. Picard, F. Schweighoffer, C.J. Der, Characterization of EHT 1864, a novel small molecule inhibitor of Rac family small GTPases, Methods Enzymol. 439 (2008) (1864) 111–129.
[37] J. LoPiccolo, G.M. Blumenthal, W.B. Bernstein, P.A. Dennis, Targeting the PI3K/Akt/mTOR pathway: effective combinations and clinical considerations, Drug Resist. Update 11 (2008) 32–50.