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TITLE: Targeting Signal Transducers and Activators of Transcription-3 (STAT3) as a Novel Strategy in Sensitizing Breast Cancer to EGFR-Targeted Therapy

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13. SUPPLEMENTARY NOTES

14. ABSTRACT
Our research effort in the past award year has resulted in several interesting findings that support the study hypothesis: deregulated EGFR and STAT3 pathways synergistically contribute to the malignant biology of breast cancer and that combined uses of anti-EGFR and anti-STAT3 treatments result in significantly increased breast cancer cell death compared to single agent treatments. First, we have created isogenic breast cancer cell lines to stably express modestly activated and highly activated STAT3, STATCA. Second, using these established isogenic breast cancer cell lines, we found that increased STAT3 activation rendered breast cancer cells resistant to EGFR-targeted therapy. Third, we showed that STAT3 expression knock-down sensitized EGFR-expressing breast cancer cells to anti-EGFR therapy. Finally, our in vitro results showed that combined used of a STAT3 small molecular weight inhibitor AG490 and a clinically used EGFR inhibitor Iressa synergistically targeted EGFR-expressing breast cancer cells to anti-EGFR therapy.

15. SUBJECT TERMS
EGFR, STAT3, drug resistance, signal cross-talks

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INTRODUCTION

The proposal is built on the following observations from our studies and others. **(1)** Single use of EGFR-targeted therapy, gefitinib/Iressa (an EGFR tyrosine kinase inhibitor) and cetuximab (an EGFR blocking antibody), demonstrated a moderate therapeutic effect in breast cancer patients. **(2)** Both EGFR and STAT3 are oncoproteins and frequently over-expressed and/or constitutively activated in breast cancer (Lo et al. 2005b). We observed co-overexpression of both EGFR and p-STAT3 in 60% of EGFR-positive breast carcinomas (Lo et al. 2005a). **(3)** STAT3 is constitutively activated in 50% of the breast cancer and can be activated by EGFR and other growth factor- and cytokine pathways (Garcia et al. 1997; Yu and Jove 2004). **(4)** Iressa-treated breast cancer cells and clinical specimens displayed insufficient suppression of STAT3 activity despite with complete EGFR inhibition (Albanell et al. 2001; Lo et al. 2005a). **(5)** Although EGFR and STAT3 can mediate different downstream targets, our recent study indicated that they cross-talk at multiple levels (Lo et al. 2005a). Based on these rationales, we **hypothesize** that deregulated EGFR and STAT3 pathways synergistically contribute to the malignant biology of breast cancer and that combined uses of anti-EGFR and anti-STAT3 treatments result in significantly increased breast cancer cell death compared to single agent treatments. The hypothesis is being tested by the following three Specific Aims.

AIM 1: To determine whether increased STAT3 activity confers resistance to anti-EGFR therapies in EGFR-expressing breast cancer cells.

AIM 2: To investigate whether suppression of STAT3 expression/activity sensitizes EGFR/p-STAT3-expressing breast cancer cells to anti-EGFR therapies.

AIM 3: To determine the therapeutic effects of combined use of anti-EGFR and anti-STAT3 treatments in a mammary tumor-bearing animal model.

The outcome from this proposal is likely to help us achieve the long-term goal of the study, which is to better understand the malignant biology of breast cancer including those with de-regulated EGFR pathway and to provide rationales for more effective therapies for women with breast cancer.

BODY

Creation of isogenic breast cancer cells that stably express modestly and highly activated STAT3 (Task 1-b). To determine whether increased STAT3 expression/activity confers resistance to anti-EGFR therapy in EGFR-expressing breast cancer cells, we first generated isogenic SK-BR-3 breast cancer cell lines to express the control vector or constitutively activated STAT3, STAT3CA. SK-BR-3 cells express modest levels of activated STAT3 and are relatively sensitive to Iressa, and are thus ideal for the proposed study. It is worthwhile to mention that STAT3CA is a modified variant of STAT3 that contains substitution of two Cys residues within the SH2 domain and dimerizes spontaneously, binds to DNA, activates transcription, and transforms normal fibroblasts (Bromberg et al. 1999). More recently, STAT3CA has been linked to tumorigenesis of prostate (Qin et al. 2008) and skin (Pedranzini et al. 2004; Chan et al. 2004). STAT3CA also transforms mouse bone marrow cells into highly aggressive T cell leukemia in mice (Ecker et al. 2009).

As shown by the immunoblotting in **Figure 1**, we have successfully established two isogenic SK-BR-3 breast cancer stable transfectants to express the control vector and STAT3CA vector. In these studies, we transfected SK-BR-3 cells with corresponding vectors and subjected them to G418 treatments for one month to allow for stable selection. Stable clones were then examined for STAT3CA expression using immunoblotting. Together, these results indicate that these isogenic cells are ideal for examining the effects of increased STAT3 activity on the resistance of breast cancer cells to EGFR-targeted therapy (Task 1-c).

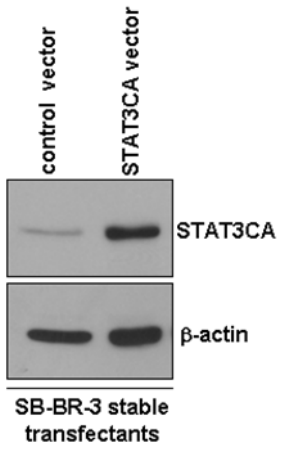

Figure 1. Creation of isogenic SK-BR-3 cell lines stably expressing the control vector and STAT3CA. Immunoblotting indicates increased expression of STAT3 in STAT3CA-transfected SK-BR-3 cells but not in control vector-transfected counterparts.

Increased STAT3 activation rendered breast cancer cells resistant to EGFR-targeted therapy

(Task 1-c). Using the isogenic SK-BR-3 cell lines, we examined their response to various concentrations of Iressa following 48 hr treatments. Cell survival was determined using Celltiter Blue Cell Viability assay kit (Promega), a fluorescent method that is based on the ability of living cells to convert a redox dye (resazurin) into a fluorescent end product, resorufin, as we previously described (Lo et al., 2008a,b). As shown by the results in **Figure 2**, SK-BR-3-STAT3CA cells were more resistant to Iressa-mediated growth suppression compared to SK-BR-3-vector cells. These results indicate that increased STAT3 activity rendered breast cancer cells more resistant to anti-EGFR agent, Iressa.

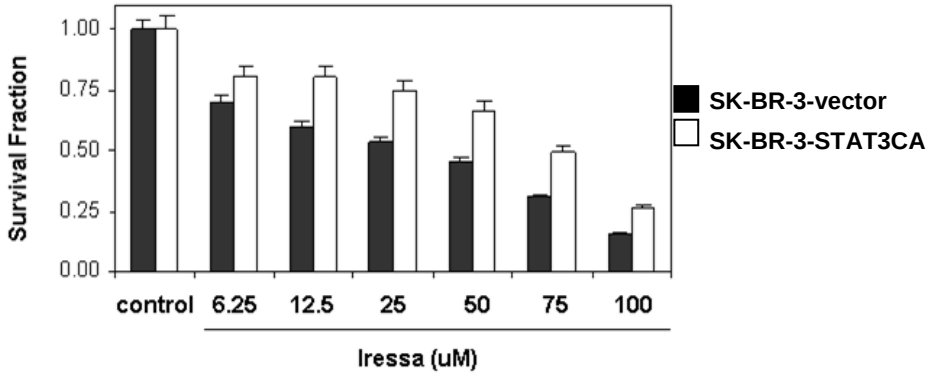

Figure 2. Increased STAT3 activation rendered SK-BR-3 breast cancer cells resistant to EGFR-targeted therapy. SK-BR-3-vecor and SK-BR-3 STAT3CA cells were treated with vehicle control (1% DMSO) and 6.25-100 uM Iressa for 48 hrs. Survival rates were then determined using Celltiter Blue Cell Viability assay.

STAT3 expression knock-down sensitizes EGFR-expressing breast cancer cells to anti-EGFR therapy (Task 2-a). In the 2008-progress report, we reported that suppressing STAT3 activity using dominant-negative STAT3 mutant (STAT3-DN) led to increased sensitivity of breast cancer cells to EGFR-targeted therapy. Here, we further suppressed STAT3 expression using STAT3-specific siRNA to determine the effects of this on sensitivity of breast cancer cells to Iressa. In these studies, we used MDA-MB-468 breast cancer cells that are known to express high levels of both EGFR and p-STAT3 (Y705) and resistant to EGFR-targeted agents, as we previously reported (Lo et al. 2005a). These cells were untransfected or transfected with STAT3-specific siRNA or non-specific siRNA (Dharmacon). Forty-eight hrs following transfections, the cells were subjected to cell viability assay, as we described earlier. As shown by the results in **Figure 3A**, STAT3 expression down-regulation sensitized MDA-MB-468 cells to Iressa treatment. Non-specific siRNA did not affect cell survival indicating assay specificity. As expected, STAT3 siRNA specifically and effectively reduced STAT3 protein expressing, as indicated by immunoblotting **(Figure 3B)**. Collectively, the results from the STAT3 siRNA experiments indicate that targeting STAT3 sensitizes EGFR-expressing breast cancer cells to EGFR-targeted therapy *in vitro*.

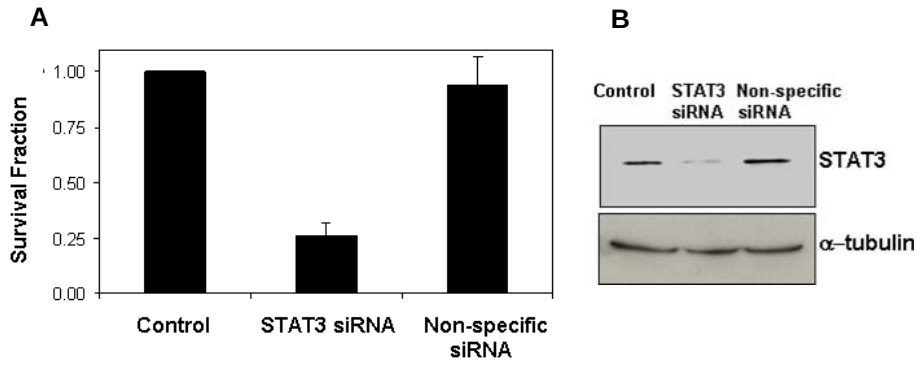

Figure 3. STAT3 siRNA sensitized MDA-MB-468 cells to anti-EGFR treatments.
A: STAT3 expression knockdown by siRNA led to sensitization of MDA-MB-468 cells to Iressa treatment.
B: STAT3-specific siRNA but non-specific siRNA reduced STAT3 expression in MDA-MB-468 cells, indicating specificity, as shown by immunoblotting.

A STAT3 small molecular weight inhibitor AG490 sensitizes EGFR-expressing breast cancer cells to anti-EGFR therapy (Task 2-b). To further investigate Specific Aim 2, we determined the extent to which STAT3 inhibition sensitizes breast cancer cells to EGFR-targeted therapy using AG490, a small molecular weight inhibitor that targets STAT3-activating kinase, JAK2. Interestingly, we found breast cancer cells to respond to AG490+Iressa combination better than AG490 alone and Iressa alone **(Figure 4A)**. Using the Median-effect analysis (Chou and Talalay 1984) that computes combination index (CI), we determined whether AG490 and Iressa targeted breast cancer cells synergistically, as we previously described (Lo et al. 2008). As indicated by the low CI values, we found combination of STAT3 and EGFR inhibitors to be synergistic in targeting breast cancer cells **(Figure 4B)**. Collectively, the results from STAT3-DN (2008-report), STAT3 siRNA experiments (Figure 3) and STAT3 inhibitor study (Figure 4) indicate that targeting STAT3 sensitizes EGFR-expressing breast cancer cells to EGFR-targeted therapy *in vitro*. These *in vitro* results also strongly justify the *in vivo* studies proposed in Specific Aim 3 to examine the efficacy of the combination regimen using an orthotopic breast cancer xenograft model.

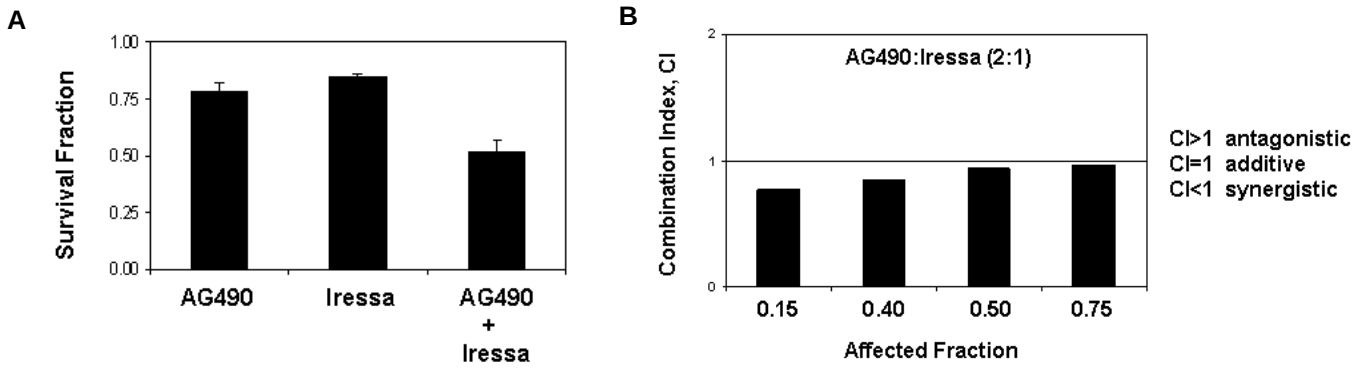

Figure 4. A STAT3 small molecular weight inhibitor AG490 sensitizes EGFR-expressing breast cancer cells to anti-EGFR therapy.
A: Combined uses of AG490 and Iressa resulted in a greater growth suppression than single agents in EGFR-expressing breast cancer cells. H3578T cells were treated with AG490 (50 uM), Iressa (25 uM) or in combination for 48 hrs and survival fractions determined using Celltitier Blue Cell Viability assay.
B: AG490 and Iressa synergistically target breast cancer cells. H3578T cells were subjected Median-effect analysis to derive CI that indicates synergistic effect (CI<1), additive effect (CI=1) and antagonistic effect (CI>1). As indicated the low CI values. These agents synergistically inhibit the growth of breast cancer cells.

KEY RESEARCH ACCOMPLISHMENTS

- Task 1-b: Establishment of isogenic breast cancer cells that stably express modestly and highly activated STAT3.
- Task 1-c: Increased STAT3 activation rendered breast cancer cells resistant to EGFR-targeted therapy.
- Task 2-a: STAT3 expression knock-down sensitizes EGFR-expressing breast cancer cells to anti-EGFR therapy.
- Task 2-b: A STAT3 small molecular weight inhibitor AG490 sensitizes EGFR-expressing breast cancer cells to anti-EGFR therapy.

REPORTABLE OUTCOMES

Funding applied based on work supported by this award
Research Scholar Grant Lo (PI)
American Cancer Society
De-regulated EGFR and STAT3 pathways in breast cancer EMT and intravasation

CONCLUSION

Our research effort in the past award year has resulted in several interesting findings that support the study hypothesis: deregulated EGFR and STAT3 pathways synergistically contribute to the malignant biology of breast cancer and that combined uses of anti-EGFR and anti-STAT3 treatments result in significantly increased breast cancer cell death compared to single agent treatments. First, using established isogenic breast cancer cells that stably express modestly and highly activated STAT3, we found Increased STAT3 activation rendered breast cancer cells resistant to EGFR-targeted therapy. Second, we found that STAT3 expression knock-down sensitizes EGFR-expressing breast cancer cells to anti-EGFR therapy. Finally, our results showed that combined used of a STAT3 small molecular weight inhibitor AG490 and a clinically used EGFR inhibitor synergistically target EGFR-expressing breast cancer cells to anti-EGFR therapy. Together, these promising results prompt us to further explore the role of STAT3 activation in the resistance of human breast cancer cells to anti-EGFR therapy in animals in the next award year.

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Lo, H. W., X. Cao, H. Zhu, and F. Ali-Osman. 2008. Constitutively activated STAT3 frequently coexpresses with epidermal growth factor receptor in high-grade gliomas and targeting STAT3 sensitizes them to Iressa and alkylators. *Clin Cancer Res* 14 (19):6042-6054.

Lo, H. W., S. C. Hsu, and M. C. Hung. 2005b. EGFR signaling pathway in breast cancers: from traditional signal transduction to direct nuclear translocalization. *Breast Cancer Res Treat*:1-8.

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APPENDIX

Updated Biosketch

BIOGRAPHICAL SKETCH

NAME	POSITION TITLE
LO, HUI-WEN, Ph.D.	**Assistant Professor**
eRA COMMONS USER NAME HUIWENLO	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Chung-Shan Medical & Dental College	B.S.	1986	Nutrition
The University of Texas at Austin	M.A.	1990	Nutritional Sciences
The University of Texas-Health Science Center at Houston	M.S.	1994	Biomedical Sciences
The University of Texas-Health Science Center at Houston	Ph.D.	2002	Biochemistry and Molecular Biology
MD Anderson Cancer Center	POSTDOC	2004	Cancer Research

A: POSITIONS AND HONORS

Positions and Employment

2006-present Assistant Professor (tenure-track), Department of Surgery, Duke University School of Medicine
2005-2006 Instructor, University of Texas M.D. Anderson Cancer Center
2002-2004 Postdoctoral Fellow, University of Texas M.D. Anderson Cancer Center
1998-2000 Research Investigator, University of Texas M.D. Anderson Cancer Center
1993-1998 Senior Research Assistant, University of Texas M.D. Anderson Cancer Center

Awards and Honors (selected)

2008 Outstanding Alumni Award, Chung-Shan Medical University, Taichung, Taiwan
2008 Career Development Award, Duke Brain Cancer SPORE, NCI
2007-present Idea Award, Department of Defense
2006-present The Howard Temin Award, NCI
2005-2006 American Cancer Society Postdoctoral Fellowship
2004 AFLAC Scholar-in-Training Award, American Association for Cancer Research
2004 Trainee Excellence Award, M.D. Anderson Cancer Center
2002 WICR Brigid G. Leventhal Scholar Award, American Association for Cancer Research-Women in Cancer Research
2001 John P. McGovern Award, Graduate School of Biomedical Sciences The University of Texas-Health Science Center

Professional Memberships

2007-present Member, Duke Comprehensive Cancer Center
2006-present Member, Society of Neuro-oncology
1997-present Member, American Association for Cancer Research
1997-present Member, Women in Cancer Research-American Association for Cancer Research

B. SELECTED PEER-REVIEWED PUBLICATIONS (FROM 24 PUBLICATIONS)

1. **Lo, H.-W.** and Ali-Osman, F. Genomic cloning of hGSTP1*C, an allelic human pi class glutathione S-transferase gene variant and functional characterization of its retinoic acid response elements. *Journal of Biological Chemistry 272:32743-32749, 1997.*

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11. Hanada*, N., **Lo*, H.-W.,** Day, C.-P., Pan, Y., Nakajima, Y. and Hung, M-C. Co-regulation of B-Myb Expression by E2F1 and EGF Receptor. *Molecular Carcinogenesis 45:10-17, 2006. (*These authors contributed equally to this work.)*
12. **Lo, H.-W.** and Hung, M.-C. Nuclear EGFR Signaling Network in Cancers: linking EGFR pathway to cell cycle progression, nitric oxide pathway and patient survival. *British Journal of Cancer 94:184-188, 2006.*
13. **Lo, H.-W.**, Ali-Seyed M., Wu, Y., Bartholomeusz, G., Hsu, Sheng-Chieh, and Hung, M.-C. Nuclear-cytoplasmic Transport of EGFR Involves Receptor Endocytosis, Importin β1 and CRM1. *Journal of Cellular Biochemistry 98:1570-1583, 2006.*
14. **Lo, H.-W.,** Hsu, S.-C., and Hung, M.-C. EGFR Signaling Pathway in Breast Cancers: from traditional signal transduction to direct nuclear translocalization. *Breast Cancer Research and Treatment 95:211-218, 2006.*
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18. **Lo, H.-W.**, Stephenson, L., Cao, X., Milas, M., Pollock, R. and Ali-Osman, F. Identification and functional characterization of the human GSTP1 gene as a novel transcriptional target of the p53 tumor suppressor gene. *Molecular Cancer Research 6:843-850, 2008.*

19. **Lo*, H.-W.**, Cao, X., Zhu, H. and Ali-Osman, F. Constitutively activated STAT3 frequently co-expresses with EGFR in high-grade gliomas and targeting STAT3 sensitizes them to Iressa and alkylators. *Clinical Cancer Research 14:6042-6054, 2008. (*Corresponding Author; provided as Appendix 1)*
20. **Lo*, H.-W.**, Zhu, H., Cao, X., Aldrich, A.J. and Ali-Osman, F. A novel splice variant of GLI1 that promotes glioblastoma cell migration and invasion. *Cancer Research 2009 (In Press; *Corresponding Author)*

C. RESEARCH SUPPORT

ACTIVE:

The Howard Temin Award (5K01-CA118423-03) (Lo, PI) 9/25/2006-7/31/2011
National Cancer Institute
Nuclear EGFR Signaling Network in Human Cancers

The goal of this application is to understand the biological role of the nuclear EGFR signaling pathway in human cancers. Aim 1: Characterize the transcriptional co-regulation of the iNOS gene by nuclear interaction of EGFR and STAT3 and determine its role in tumor survival. Aim 2: Characterize interaction of nuclear EGFR with c-jun and determine its effect on TWIST gene activation and TWIST-mediated breast cancer progression. Aim 3: determine the role of nuclear EGFR and underlying mechanisms in chemo-resistance.

Idea Award (W81XWH-07-1-0390) (Lo, PI) 6/1/2007-6/30/2010
Breast Cancer Research Program
Department of Defense
Targeting signal transducer and activator of transcription 3 as a novel strategy in sensitizing breast cancer to anti-EGFR therapy

The goal of this project is to determine the role of STAT3 activation in the resistance of breast cancer to EGFR-targeted therapy. AIM 1: To determine whether increased STAT3 expression/activity confers resistance to anti-EGFR therapy in EGFR-expressing breast cancer cells. AIM 2: To investigate whether suppression of STAT3 expression/activity sensitizes EGFR-expressing breast cancer cells to anti-EGFR therapy. AIM 3: To determine the therapeutic effects of combined use of anti-EGFR and anti-STAT3 treatments in a mammary tumor-bearing animal model.