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14. ABSTRACT

Epidermal growth factor (EGF) receptor tyrosine kinases (erbB family), EGFR (erbB1) and HER2, are highly expressed in breast cancer and are associated with poor prognosis. A number of EGFR and/or HER2-targeted agents are being investigated for breast cancer treatment. Brk (Breast Tumor Kinase) is a nonreceptor tyrosine kinase that has been shown to enhance the mitogenic signaling of EGF, induce phosphorylation of erbB 3 and interact with AKT. In this study, we aim to investigate whether Brk can promote cells to become refractory to EGFR-targeted drugs. PI-3 kinase/AKT pathway mediates EGF-induced cell growth and survival and is involved in cellular resistance to anti-cancer drugs. Because the PI3K/AKT pathway is regulated by multiple activators, downregulation of the EGFR alone may not lead to its inhibition. We will investigate whether Brk promotes growth and survival as well as PI3K/AKT activity in cells treated with EGFR-targeted agents.

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Introduction

Epidermal growth factor (EGF) receptor tyrosine kinases (erbB family), EGFR (erbB1) and HER2, are highly expressed in breast cancer and are associated with poor prognosis. A number of EGFR and/or HER2-targeted agents are being investigated for breast cancer treatment. However, the redundancy of signaling pathways which promote cell growth and prevent apoptosis can cause cells to become insensitive to these drugs. Brk (Breast Tumor Kinase) is a nonreceptor tyrosine kinase that has been shown to enhance the mitogenic signaling of EGF, induce phosphorylation of erbB 3 and interact with AKT. In this study, we aim to investigate whether Brk can promote cells to become refractory to EGFR-targeted drugs. PI-3 kinase/AKT pathway mediates EGF-induced cell growth and survival and is involved in cellular resistance to anti-cancer drugs. Because the PI3K/AKT pathway is regulated by multiple activators, downregulation of the EGFR alone may not lead to its inhibition. We will investigate whether Brk promotes growth and survival as well as PI3K/AKT activity in cells treated with EGFR-targeted agents.

In order to undertake this investigation, four different tasks, as shown in the Statement of Work, will be carried out. The work that has been accomplished is described in Work in Progress. We have had some difficulty in achieving sufficient suppression of endogenous Brk. Since suppression of endogenous Brk is an important step, this problem has prevented us from carrying out the study at the desired pace. However, several strategies have been taken for trouble-shooting including redesigning the RNAi sequence. Simultaneously, the biochemistry and cell biology parts of the study have been carried out to investigate the role of Brk in the regulation of PI3K/AKT pathways as well as the mechanism of this regulation. We have found that Brk enhances EGF-induced AKT activation and alters the localization of AKT in breast cancer cells. Further studies will be carried out to determine the role of Brk in the regulation of EGF signaling, AKT activity and cell apoptosis. Together, these results will aid in determining the role of Brk in anti-EGFR drug resistance.

Statement of Work

Task 1. To determine the role of Brk on the efficacy of EGFR inhibitors to antagonize cell growth and survival (month 1- 7):

1.1). Human breast cancer cell lines will be selected by a RT-PCR screen for cells which overexpress both Brk and EGFR (month 1-2).

1.2) shRNA targeting to 'knock down' Brk gene expression will be designed and transfected into selected cell lines from 1.1. Cells will be tested for the levels of mRNA and protein expression of Brk. Subsequently, two Brk-knockdown clonal cell lines will be established (month 2-6).

1.3) The clonal cell lines, the Brk-knockdown and parental cells, will be treated with different EGFR inhibitors including AG1478, ZD1839 and GW572016. The duration of the treatment will be approximately 2 weeks. The rate of cell proliferation and apoptosis will be assessed during the course of the treatment (month 6-7).

Task 2. To determine whether Brk overexpression induces cells to become refractory to the EGFR inhibitors (month 1-7).

2.1). Human breast cancer cell lines will be selected by a RT-PCR screen for cells which contain high levels of endogenous EGFR and low or undetectable levels of endogenous Brk (month 1-2).

2.2). Brk will be transfected into selected cell lines from 2.1 and tested for the level of Brk protein expression. Two clonal cell lines overexpressing Brk will be established (month 2-6).

2.3). The clonal cell lines overexpressing Brk and parental cells will be treated with EGFR inhibitors AG1478, ZD1839, and GW 572016. The duration of the treatment will be approximately 2 weeks. The rate of cell proliferation and apoptosis will be assessed during the course of the treatment (month 6-7).

Task 3. To test whether Brk upregulates PI3K and AKT activity in the cells exposed to EGFR inhibitors (month 6-7).

The Brk knockdown, overexpressed, and parental cells, established in Task 1 and 2, will be treated with AG1478, ZD1839, or GW 572016. Subsequently, cells will be analyzed for the PI3K and AKT activity at various time-points (day1-14). The analysis will be achieved by a standard *in vitro* PI-3 kinase assay and measurement of the levels of phosphorylated AKT by immunoblotting.

Task 4. To determine the role of Brk on the efficacy of EGFR inhibitors to antagonize cell growth and survival in an *in vivo* setting (month 7-12).

4.1). The Brk-knockdown clonal cells, established in 1.2, and parental cells will be injected subcutaneously into female BALBc $^{nu/nu}$ mice and they will be monitored for tumor establishment. Two cell lines will be utilized. 12 mice will be utilized for each experimental condition. The experimental conditions will be as follow: a) mice injected with Brk-knockdown cells and treated with drug, b) mice injected with Brk-knockdown cells but not treated with drug, c) mice injected with parental cells and treated with drug, d) mice injected with parental cells but not treated with drug. Forty-eight mice will be utilized in the experiment. Two cell lines will be injected into the animals, therefore, total of 96 mice will be utilized in the entire experiment. (month 7-8)

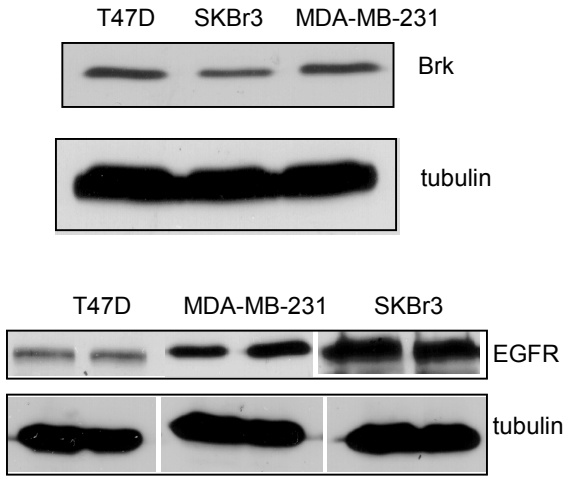
4.2) Following tumor establishment, mice will be administered ZD1839 by oral gavage. After the course of drug treatment (3-4 weeks), mice will be sacrificed. Tumor size will be measured, and tumor growth rate will be assessed and compared between drug-treated and untreated cells (month 8-11).

Key Research Accomplishments

Task 1

1.1 Selection of human breast cancer cell lines.

Human breast cancer cell lines were selected for cells that overexpress both Brk and EGFR. Fifteen breast cancer cell lines were examined by RT-PCR and immunoblot analysis to detect the levels of Brk and EGFR. SKBr3, T47D, and MDA231 cell lines, which express significant levels of Brk and EGFR and can be transfected to at least 70% of total population, were selected to use in the study.



duplicate samples

Fig. 1. Expression of EGFR and Brk in selected breast cancer cell lines

Cell lines SKBr3, T47D, and MDA231 were harvested and cell were lysed in lysis buffer by a standard protocol (ref.1). Cell lysate was subjected to fractionation by standard SDS-PAGE, followed by western blot analysis, utilizing anti-EGFR, anti-Brk, and anti-tubulin antibodies to detect the corresponding protein bands. As shown, all three cell lines express a significant levels of EGFR and Brk. Tubulin serves as a marker for the total whole cell lysate.

1.2. Designing of RNA interference (RNAi) of Brk

We designed 3 different shRNA sequences and constructed them into the pSUPER vector to generate pSUPER shRNA targeting Brk (pSUPER shRNA-Brk) at 3 different target sites. Additionally, a scramble RNA sequence (shRNA-sc) was designed as a control. The efficiency of Brk knockdown was tested in the following experiments. In the first set of experiments, pSUPER shRNA-Brk were cotransfected with flag-tagged Brk expression plasmid into COS-1 and HeLa cells. Subsequently cells were harvested, lysed, and the proteins were fractionated by SDS-PAGE followed by immunoblot analysis with

anti-flag antibody. The protein band corresponding to Brk was quantified by PhosphorImager-scanning. The result shows that the level of flag-tagged Brk expression was significantly decreased when cotransfected with 2 shRNA-Brk sequences, shRNA #1 and #2. The best knockdown effect was 80%, which was achieved by shRNA #1, as shown in Fig. 2. A similar result was obtained in Hela cells.

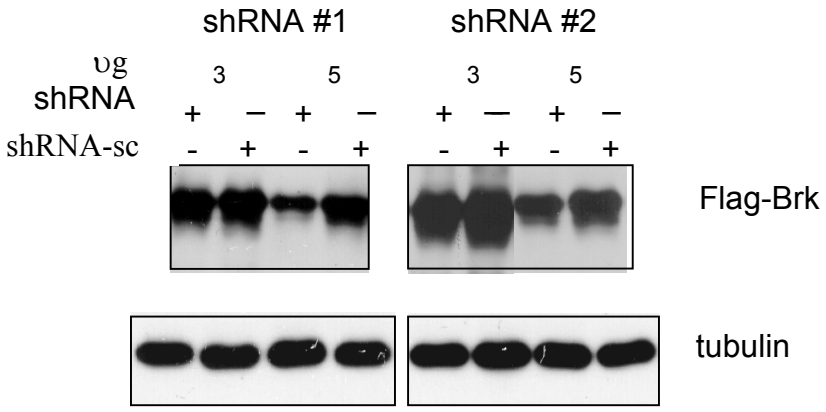


Fig. 2. Flag-tagged Brk was cotransfected with 3 or 5 ug of shRNA-Brk, sequence#1, or #2, or shRNA-sc into COS-1 cells. Sixty hours following transfection, cells were harvested and lysed in lysis buffer. Cell lysate was subjected to SDS-PAGE, followed by western blot analysis with anti-flag and anti-tubulin antibodies.

In the second set of experiments, we tested the RNAi effect of the three shRNA-Brk on endogenous Brk in SKBr3, T47D and MDA231 cells. Each of the three pSUPER shRNA-Brk plasmids or pSUPER shRNA-sc was transfected into the breast cancer cell lines and a homogenous transfected population was achieved by drug selection. Subsequently, cells were harvested and lysed, and SDS-PAGE followed by immunoblot analysis with anti-Brk antibody were performed. The result shows a 20% suppression of Brk expression induced by shRNA #1 (Fig. 3), whereas two other sequences yield no detectable effect. A comparable level of suppression was obtained when the Brk mRNA levels were analyzed by RT-PCR analysis (not shown). Similar results were obtained in all 3 cell lines. These results indicate that the shRNAs cannot efficiently silence the endogenous Brk gene, which may be caused by instability of the binding between the shRNA and its target.

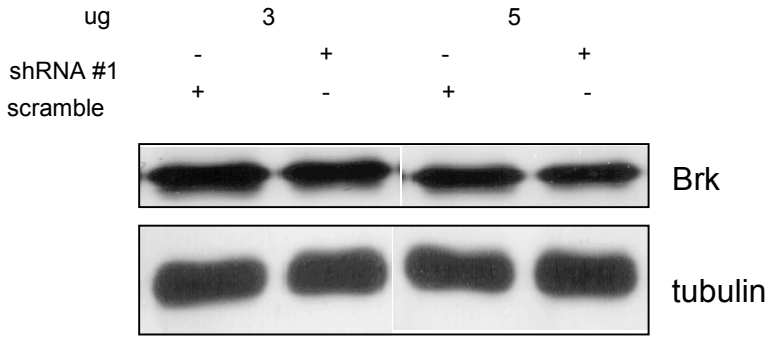


Fig.3. Three or five υg of shRNA-Brk, sequence#1, or shRNA-sc was transfected into T47D cell lines. Two days following transfection, selection drug blasticidin was added to cell culture and cells were incubated for another 4-5 days when all nontransfected cells died. Subsequently, cells were incubated in a fresh media for one more day before being harvested and lysed in lysis buffer. Cell lysate was subjected to SDS-PAGE, followed by western blot analysis with anti-Brk and anti-tubulin antibodies.

To circumvent this problem, we redesigned the RNA by utilizing microRNA (miRNA) instead of shRNA approach. Three different miRNA sequences targeting Brk and one scramble miRNA control were generated and constructed into pSM155. Gene encoding green fluorescent protein (GFP) was inserted into the plasmid to indicate transfected cells. Currently, the effect of miRNA on endogenous Brk expression is being tested. The miRNA, which we have demonstrated to suppress at least 70% of Brk expression, will be used to establish clonal cell lines as proposed in Task 1.3.

1.3 We also generated the following constructs; 1. A vector for tetracycline-regulated miRNA production with GFP tag, and 2. A Brk expression plasmid containing a drug selection marker.

Task 2 To determine whether Brk overexpression induces cells to become refractory to the EGFR inhibitors.

2.1 Selection of breast cancer cell lines

To select breast cancer cell lines, fifteen cell lines were examined by RT-PCR and western blot analysis to detect the levels of EGFR and Brk. Three cell lines, which express high levels of EGFR and low or undetectable levels of Brk were selected. These cell lines include BT 474, MDA 468, and MCF 10A.

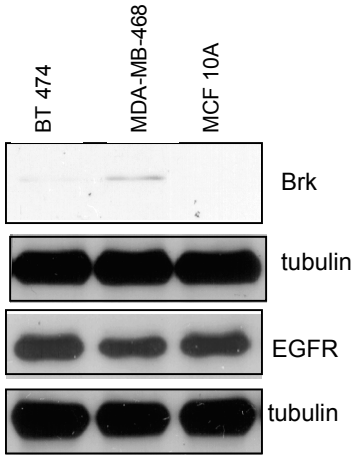


Fig. 4. Expression of EGFR and Brk in selected breast cancer cell lines

Cell lines BT 474, MDA 468, and MCF 10A were harvested and cell were lysed. Cell lysate was subjected to fractionation by SDS-PAGE, followed by western blot analysis, utilizing anti-EGFR and anti-Brk antibodies to detect the corresponding protein bands. As shown, all three cell lines express a significant levels of EGFR and low (BT 474, MDA 468) or undetectable level (MCF 10A) of Brk.

Presently, the clonal cell lines overexpressing Brk and parental cell lines are being established. These cell lines will be used to test for the anti-proliferative effect of the EGFR inhibitors AG1478, ZD1839, and GW572016

Task 3 Determination the role of Brk in the regulation of AKT activity

We determined whether Brk regulated the PI3K/AKT pathway in two breast cancer cell lines T47D and MDA231, which express high levels of Brk. The activation of AKT is judged by its level of phosphorylation. T47D and MDA231 cells were transfected with flag-tagged Brk expression plasmid, serum starved for 18 h, and stimulated with EGF for 10 minutes before being harvested. HA-AKT was immunoprecipitated and subjected to fractionation by SDS-PAGE followed by western blot analysis. Subsequently, the level of phosphorylation of immunoprecipitated AKT was detected by immunoblot analysis using an antibody specific to phosphorylated form of serine residue, or phosphorylated form of AKT. The level of phosphorylation of AKT was quantified by PhosphorImager-scanning. The result showed a detectable increase of AKT phosphorylation in cells transfected with Brk compared to control. In MDA-MB-231 cells, this increase of AKT phosphorylation appears to be EGF independent, since there was a detectable increase in cells expressing Brk compared to control in the absence of EGF stimulation. In T47D cells, however, the increase of AKT phosphorylation is EGF dependent.

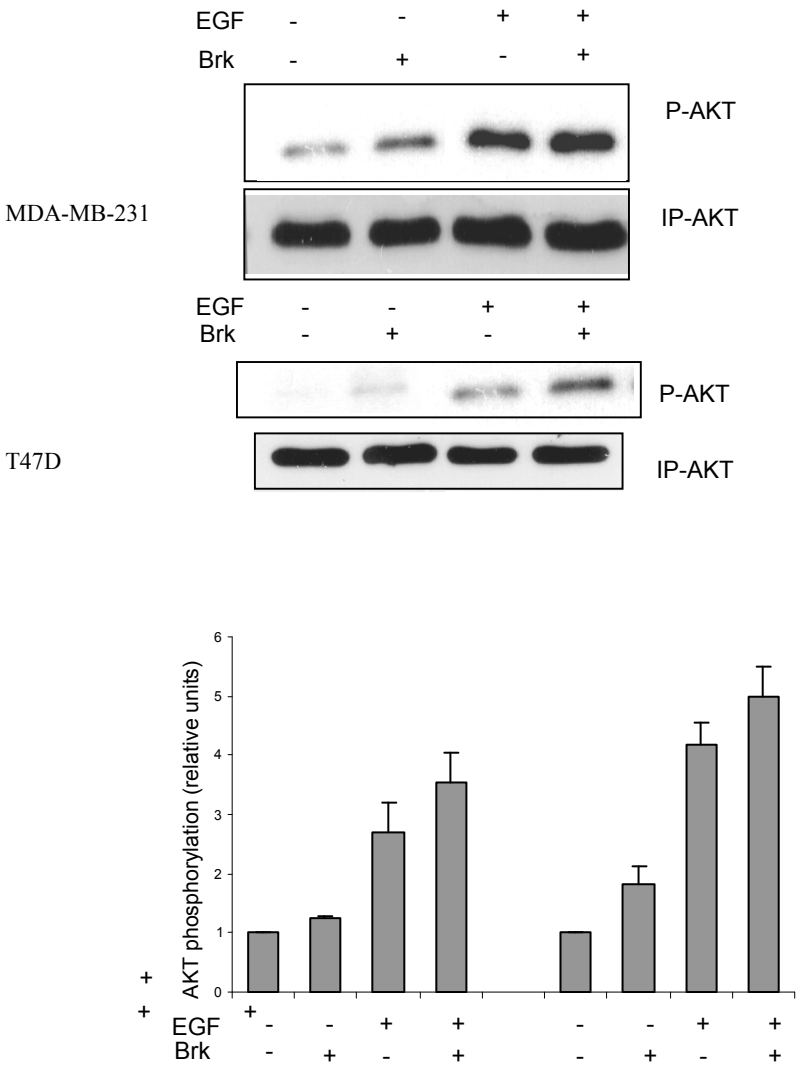


Fig. 5. T47D and MDA-MB-231 cell lines were cotransfected with flag-tagged Brk, or vector control, and HA-tagged AKT. Thirty six hours after transfection, cells were lysed and subjected to immunoprecipitation, as previously described (ref. 2). In brief, cell lysate was incubated with anti-HA antibody and protein A sepharose. The immunocomplex was subjected to SDS-PAGE and western blot analysis, utilizing anti-phosphoserine and anti-HA antibodies. Gels show phosphorylated AKT (p-AKT) and immunoprecipitated HA-tagged AKT (IP-AKT). The protein band corresponding to Brk was quantified by PhosphorImager-scanning. Graph shows the level of AKT phosphorylation in the two cell lines. Data represent average of three independent experiments.

This result shows that Brk promotes the phosphorylation of AKT. Further investigation is being carried out to determine the effect of Brk on AKT downstream

cascades, the mechanism underlying the regulation of AKT activation by Brk, and the link between Brk and EGFR signalings in different cell types. The increase of AKT phosphorylation however, is low, and a plausible explanation is that AKT activation may have already reached its maximal level in this experimental condition. Further test will be carried out to observe the effect of Brk on Akt phosphorylation and activity when cells expose to low levels of EGF, and for a prolonged duration of EGF treatment.

We are currently examining the effect of Brk on AKT activation and cell proliferation when EGF receptor is blocked by EGFR inhibitors. Moreover, we are investigating the pathway by which Brk is involved in the regulation of AKT. These experiments include analyzing the effect of Brk on the localization of AKT and the interaction of AKT with EGFR-family proteins.

Conclusion

In conclusion, we have generated shRNA and miRNA that suppress Brk expression. Furthermore, we have constructed plasmids and selected breast cancer cell lines for further establishment of clonal cell lines tetracycline-regulated, stably producing miRNA targeting Brk. Finally, we have determined the effect of Brk on the EGF-induced activation of AKT in breast cancer cells.

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