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14. ABSTRACT

Breast cancer is the most common cancer in women and the second deadliest. The gene Six1 is aberrantly expressed in half of all breast cancer tumors and in as much as 90% of metastatic tumors and has been shown to lead to increased tumor formation, increased metastasis and shorter survival. In addition, Six1 expression has been shown to confer resistance to TRAIL-induced apoptosis. In this project, we are identifying factors that contribute to this resistance. The TRAIL signaling pathway is part of the body's natural tumor surveillance program and may have a great impact on cells' ability to form tumors and metastasize. In addition, the TRAIL signaling pathway is currently being exploited in clinical trials where resistance is an obstacle in achieving a response. By identifying markers of resistance and underlying mechanisms, resistance may be predicted and circumvented. In this project we have, in addition to assaying traditional apoptosis markers, performed a genome-wide shRNA library screen that could reveal novel genes that mediate TRAIL resistance in breast cancer, with a special focus on genes downstream of six1. Validation of these genes is currently underway.

15. SUBJECT TERMS
Six1, TRAIL resistance, breast cancer, genome-wide shRNA library screen

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Annual Report Lina Dimberg Department of Defense

INTRODUCTION

Breast cancer is the most common cancer in women and the second deadliest. Biological approaches to combat cancer may allow for more targeted therapy and less side effects. The TNF Related Apoptosis Inducing Ligand (TRAIL) pathway is part of the body's natural defense against tumors and is currently being exploited as a cancer drug in clinical trials. Although tumor cells generally are much more sensitive to TRAIL than normal cells, TRAIL resistance is a common obstacle in therapy. Knowledge about underlying mechanisms and ways to predict and circumvent resistance are lacking. We have recently found evidence that expression of Six1 is a novel marker of TRAIL resistance. Six1 is a homeobox gene that is not normally expressed in adults. However, Six1 is overexpressed in 50% of breast cancer tumors and in as many as 90% of metastatic lesions. Six1 expression promotes tumor initiation and progression as well as metastasis, leading to a worsened clinical outcome and decreased survival (Ford et al 1998) (Coletta et al 2008, Micalizzi et al 2009) The aim of this project is to define mechanisms of TRAIL resistance in breast cancer, with a special focus on mechanisms that may be related to Six1 expression. The ultimate goal is to improve the chances of successfully using TRAIL in breast cancer therapy, and to explore the role of TRAIL resistance in the enhanced metastasis associated with Six1 expression.

BODY

Task1: To develop cell line systems where Six1 expression can be regulated by inducible overexpression and/or knockdown and to characterize TRAIL resistance in these cells (Year 1)

A. Six1 vector construction and verification (months 1-3)
B. Inducible knockdown of Six1 in 21PT cells (months 3-5)
C. Inducible overexpression of Six1 in MCF7 and MCF12-A cells (months 5-7)
D. Knockdown of Six1 in 4T1 cells (months 7-9)

For Task1, we considered several different options for cell line systems. It proved harder than we had anticipated to generate cell lines with an inducible overexpression/knockdown of Six1. In addition, some of the cell lines we tested did not show the same consistent phenotype in respect to Six1-induced TRAIL resistance. We decided to change strategy slightly and instead first establish a different model cell line, the Burkitt's Lymphoma cell line BJAB, with which we could perform the screen described under Task 2 (see below) and then verify candidate genes in breast tumor cell lines as well as in primary breast tumor tissue. This cell line worked well for many reasons: it is a suspension cell line so it is easier to grow up large numbers of cells as required for the shRNA screen, the parental cell line expresses virtually no Six1, when transfected with Six1 it maintains a stable Six1 expression over time and it has a clear consistent TRAIL resistance phenotype that can be specifically reversed by knocking down the ectopic Six1 expression (Fig.

1). In addition, we have access to an established cell line, BJAB LexR, which has been made naturally resistant to TRAIL through long-term culture in the presence of increasing concentrations of the drug Lexatumumab, an anti-DR5 antibody. In the LexR cell line, we anticipate to find general resistance mechanisms that are not related to Six1 expression which could then be dissociated from TRAIL resistance mechanisms found in Six1-expressing cells. Once resistance mechanisms are discovered, their generality across different cancers, particularly breast cancer, will be explored.

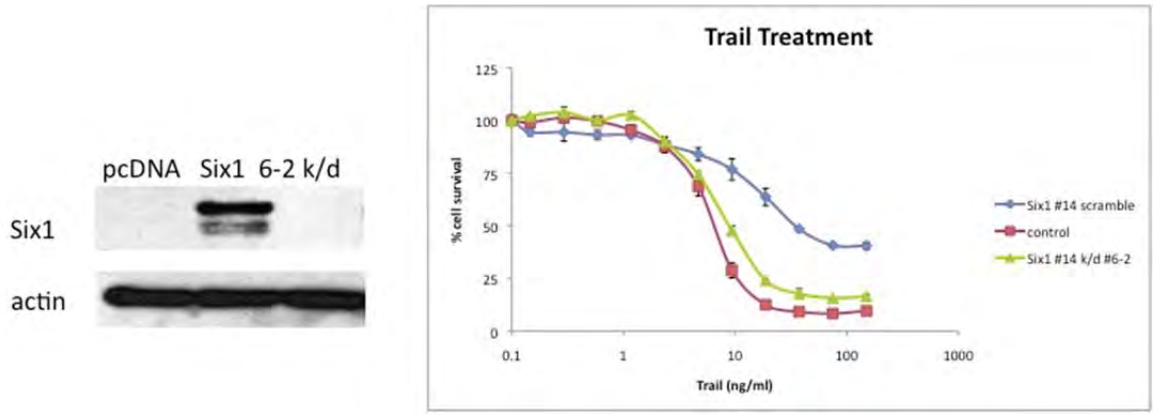
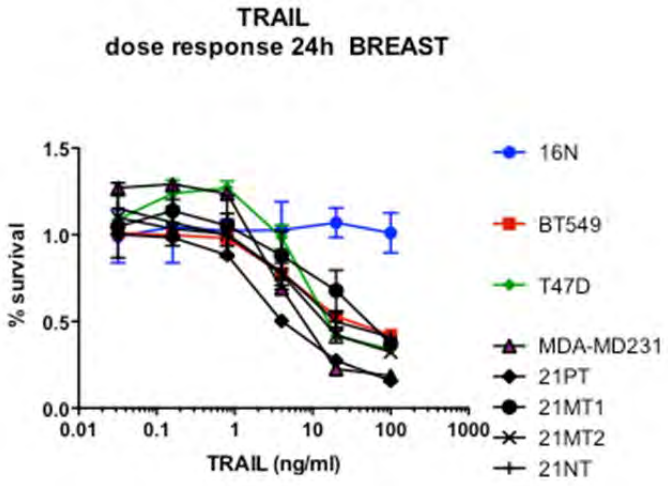


Fig 1: Six1 expression confers TRAIL resistance in BJAB cells. Left panel: Western blot showing Six1 protein expression of BJAB cells stably transfected with empty vector (pcDNA), with pcDNA-Six1 (Six1) , and with pcDNA Six1 followed by shRNA targeting Six1 (6-2 kd). Right panel: Percentage of surviving cells in BJAB-pcDNA cells (control), BJAB Six1-cells control cells with a scrambled shRNA vector (Six1 #14 scramble) and Six-1 cells in which the Six1 expression has been knocked down (Six1 #14 k/d 6-2) .The cells were treated with TRAIL at varying concentration for 24 h and then subjected to MTS assay. % cell survival was calculated from the relative absorbance normalized to untreated cells.

In parallel, we are continuing to explore breast cancer cell line systems for future validation of targets. We have evaluated TRAIL sensitivity in a panel of 8 breast cancer cell lines including the 21 PT series, BT549, T47D and MDA-MB231, with the aim of manipulating Six1 expression and determining the effect on TRAIL resistance (fig2). Importantly, the 16N cell line, which are the normal non-tumor counterpart of the 21PT series of breast cancer cell lines, is almost completely resistant to TRAIL, illustrating the important
concept that TRAIL therapy is likely to have a minimal toxic effect on normal cells in the body during cancer treatment.

Fig2: Varying response to TRAIL in a panel of breast cancer lines after 24 h of treatment as assayed by MTS assay.



It should be noted that all breast cancer cell lines shown in fig. 2 do respond to TRAIL. Nonetheless, in the BT549 cell line which has a high Six1 expression, a transient 70% knockdown of Six1 resulted in an increased sensitivity to TRAIL (fig 3), indicating that this may be a system in which TRAIL sensitivity is negatively influenced by Six1. Thus, targets from our shRNA screen may be verified in this breast cancer cell line setting.

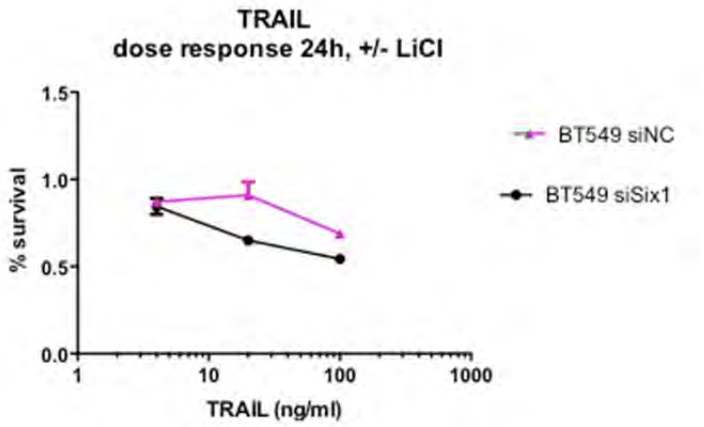


Fig. 3. Response to TRAIL treatment in the BT549 breast cancer cell line transfected with non-argeting siRNA (BT549 siNC) and siRNA targeting Six1 (BT549 siSix1) at 4, 20 and 100ng/ml as compared to untreated controls. The cells were treated with TRAIL at varying concentration for 24 h and then subjected to MTS assay. % cell survival was calculated from the relative absorbance normalized to untreated cells.

We are also in the process of establishing TRAIL-resistant breast cancer cell lines. which, by analogy to the LexR BJAB cell system, will enable us to study mechanisms of TRAIL resistance that can occur during the course of TRAIL treatment that may or may not be associated with Six1 expression.

E. Characterization of response to recombinant TRAIL and agonistic TRAIL antibodies in the generated cell lines (months 9-12)

We compared the expression of a panel of different apoptosis proteins in the BJAB Six1-cell line versus the control cell line by flow cytometry (receptor expression) and by Western blot. We did not find a difference in TRAIL receptor protein expression, IAP proteins, Bcl2 proteins, FLIP or caspase 8, the exception being the pro-apoptotic Bcl-2 protein Bid which was insufficiently cleaved (activated) in the Six1-cells as compared to the control cells. The reduced activation was reversed when Six1 was knocked down. Six1 is a transcription factor and although we have shown it affects Bid cleavage, this effect is unlikely to be direct. Thus, to determine the mechanism by which Six1 causes TRAIL resistance, and to possibly shed light on how Six1 alters Bid cleavage, we continued to the non-biased experimental approach under Task 2.

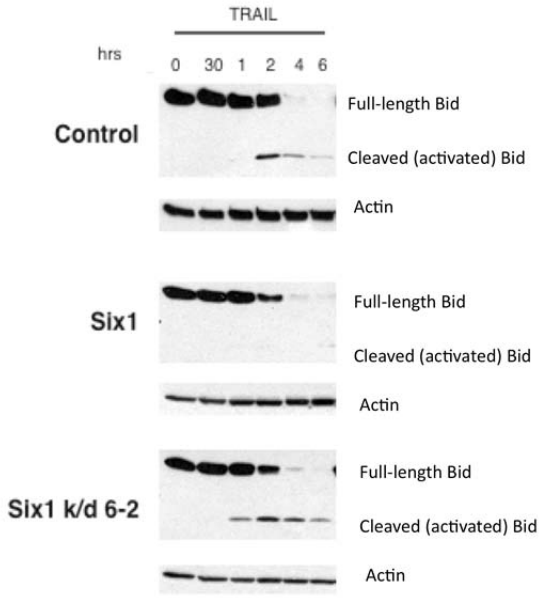


Fig4: Six1expressing BJAB cells exhibit reduced cleavage/activation of pro-apoptotic Bid protein as compared to control cells and cells in which Six1 has been knocked down again.

Task 2: To determine the molecular mechanism by which Six1 alters TRAIL signaling in breast cancer

A. Mining the microarray data of RNA from CAT transfected vs. Six1 transfected MCF12A cells and MCF7 cells previously generated in our laboratory for candidate genes that are upregulated or downregulated by Six1 and that may be involved in TRAIL-induced apoptosis, (month 13)

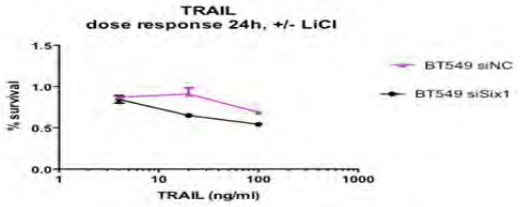
We mined the MCF7 microarray data for Six1-mediated downregulation of pro-apoptotic genes that would be expected to increase the response to TRAIL and upregulation of anti-apoptotic cells that would be expected to decrease the response to TRAIL. We found a downregulation of pro-apoptotic Bcl2-proteins Bak and Bad and an upregulation of XIAP and NF-KB in unstimulated Six1 cells versus

control cells. However, we have been unable to demonstrate in additional experiments that Six1 does in fact mediate TRAIL resistance in MCF7 cells, and thus we are no longer pursuing this avenue. Because MCF7 cells are caspase 3 deficient, their response to TRAIL is complicate by their inability to efficiently undergo apoptosis.

B. Performing and analyzing the shRNA library screen in the cell lines generated under Task1. We will determine which shRNAs reverse the Six1-induced resistance to TRAIL induced apoptosis in Six1-overexpressing cells, i.e.which shRNAs that are under-represented in Six1-overexpressing cells that survive TRAIL treatment vs untreated cells. These shRNAs should target genes that mediate TRAIL resistance induced by Six1. Conversely, shRNAs that induce resistance to TRAIL in cells where Six1 is knocked out would be over-represented in the surviving TRAIL-treated population vs in untreated cells and should be negative regulators of TRAIL resistance (month 14-17)

We used the BJAB cell lines generated under Task1, in addition to the TRAIL-resistant LexR BJAB cell line. Our genome-wide loss of function screen was performed using the GeneNet lentiviral shRNA library. This library contains 200 000 shRNAs with sequencing tags, allowing for high throughput deep sequencing. After selection for a particular phenotype, in this case TRAIL resistance, the shRNAs that promote or prevent this phenotype can be deduced from the frequency in which the shRNA is present in the selected population versus the unselected population. Briefly, we infected BJAB-Six1 cells, BJAB-pcDNA cells (empty vector control) and BJAB-LexR with the library, treated the cells with TRAIL for 24 hours, washed the cells and cultured them for an additional 3 days before harvesting and isolating RNA. A library was constructed using sequence tag specific primers and deep sequencing was performed using Illumina sequencing technology. Bioinformatic analysis yielded a list of putative resitance genes and a list of putative sensitivity genes for each cell line. We were encouraged to see that GALNT14 (Wagner et al 2007), a gene that has recently been identified as a marker of TRAIL sensitivity in numerous cell lines and primary cells, was one of the top hits for mediating TRAIL sensitivity in the empty vector transfected cells and that TAK1, a gene involved in TRAIL resistance (Morioka et al 2009), fell out as a resistance gene. Bioinformatic statistical analysis was performed, and the approximately 100 and 180 candidate resistance gene hits for the BJAB-Six1 and BJAB-LexR lines, respectively, were chosen based on the statistical significance and on information found in the literature. Using this approach, we compiled a pool of shRNA vectors targeting our gene list for both the BJAB-Six1 and BJAB-LexR lines. Importantly, these shRNA vectors are not identical to the shRNAs in the original screen, correcting for off-target effects.

Top candidate sensitivity genes in BJAB-Six1



Top candidate resistance genes in BJAB-Six1

GeneSymbol	weightZ_P	Rank	Evalue
BMPR2	9.59E-05	1	9.59E-05
DDX18	0.00016285	2	0.0003257
SQLE	0.0001679	3	0.0005037
QKI	0.00021672	4	0.00086688
ADAMTS5	0.0002483	5	0.0012415
SLC41A2	0.00036097	6	0.00216582
FOXP1	0.00036991	7	0.00258937
CACYBP	0.0003752	8	0.0030016
ZAK	0.0003768	9	0.0033912
C9orf5	0.00041638	10	0.0041638

Table 1: Candidate sensitivity genes and resistance genes in BJAB-Six1 cells, based on the overrepresentation and underrepresentation, respectively, of the corresponding shRNAs in shRNA library transduced TRAIL treated cells.

We have received the data for these secondary screenings and we are currently validating targets one by one.

Bioinformatic analysis of the hits from the screen revealed frequent targeting of the wnt/beta-catenin pathway. In parallel to the genetic approach, we therefore used several pharmacological inhibitors to explore the effect of this pathway on TRAIL sensitivity. One inhibitor, LiCl, was shown to sensitize BJAB-Six1-cells to TRAIL-induced apoptosis. Synergy experiments using MTS assays and the Calcusyn program revealed that LiCl is indeed highly synergistic in combination with TRAIL in BJAB-Six1 cells (lowest CI value 0.015 at 4ng/ml TRAIL in combination with 10 mM LiCl), and, to a lesser extent BJAB-pcDNA cells (CI value 0.226 in the same experiment and dose). LiCl is known to inhibit GSK3-b, which in turn inhibits beta-catenin. We confirmed that LiCl induces increased nuclear expression of beta-catenin and a transcriptionally active beta-catenin/TCF complex in these cells. We will further explore whether the effect on beta-catenin is required and sufficient for LiCl-mediated enhancement of TRAIL apoptosis in Six1, and whether the synergism that exists between TRAIL and LiCL is indeed enhanced in Six1 overexpressing cells. We will then begin to examine which downstream targets, beta-catenin regulated or not, may be involved. Previously identified GSK3-b-regulated proteins that may be important for TRAIL sensitization are DR4/DR5, TGF-beta, c-myc, NF-kB, FLIP, and, importantly, Bid. Preliminary experiments to examine whether LiCl and TRAIL synergize in breast cancer cells have been perfomed with positive results

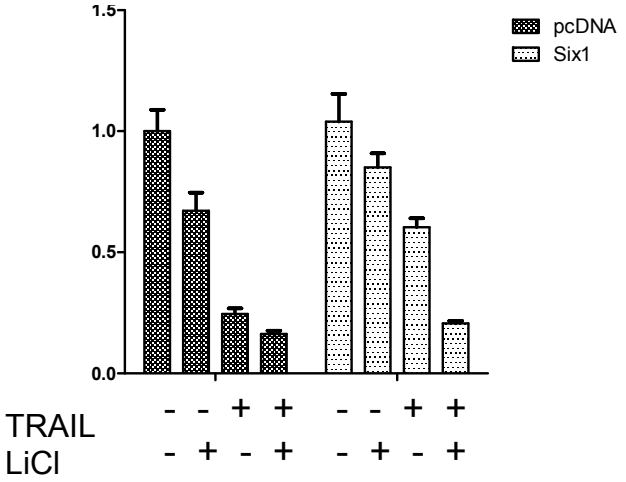


Fig 5: LiCl synergizes with TRAIL in the BJAB-Six1 cell line. BJAB-Six1 and BJAB-pcDNA were treated with TRAIL (4ng/ml) and/or LiCl (10mM) for 24 hours and then subject to MTS assay. The figure shows the MTS absorbance value normalized to untreated pcDNA

C. Corroborating Six1 upregulation of DcR1 + other candidates by Northern, RT-PCR, Western blot, and/or flow cytometry (month 17-21)
D. Designing shRNA + acquiring cDNA for vectors, constructing vectors. These vectors will be used for transient knockdown/overexpression studies (months 21-25)
E. Transient knockdown/ overexpression studies to determine impact of candidate genes on TRAIL sensitivity (months 26-30)

Experiments to complete these tasks are currently underway.

Task 3: To test whether Six1-induced metastasis involves resistance to TRAIL-induced apoptosis

A. Transfect cell lines MCF-7 and 4T1 with candidate gene cDNA or shRNA (month 30-32)
B. Inject MCF7 and 4T1 cell lines into nude mice and immunecompetent mice, respectively. Evaluate metastasis. (month 32-36).

We have not yet begun this task as this task is dependent on the identification of Six1 and/or LexR targets that mediate TRAIL resistance.

KEY RESEARCH ACCOMPLISHMENT

- Establishment of a cell line system in which to study Six1-mediated effects on TRAIL-induced apoptosis
- Evaluating TRAIL sensitivity in a panel of 8 breast cancer cell lines
- Identifying Bid cleavage as a point of differential downstream effect of TRAIL signaling in Six1-expressing cells
- Screening a genome-wide shRNA library as well as secondary shRNA screens, resulting in a list of putative TRAIL resistance genes which are currently being validated

REPORTABLE OUTCOMES

- Poster presentation at the Era of Hope DOD conference in Orlando, Florida August 2011
- Poster presentation at the CSHL Cell Deth Meeting in Cold Spring Harbor, NY, October 2011
- Invited review article for Oncogene, submission Nov 2011:
  Dimberg L Y, Andersson A, Behbakht K, Thorburn A, Camidge R, Ford HL On the TRAIL to successful cancer therapy? Predicting and preventing resistance to TRAIL-based therapies

CONCLUSIONS

We have so far established a cell line system in which to identify Six1-mediated inducers of TRAIL resistance using a genome-wide shRNA library screen. We have performed secondary screens and we are currently validating candidate resistance genes one by one. These genes will then need to be validated in breast cancer cell systems and in primary breast tissue. The inclusion of primary breast cancer tissue in future validation is a suggestion of change to the original proposal. We believe that this will strengthen the clinical breast cancer relevance as compared to using cell line systems only. In addition, in some work performed in ovarian cancer by our collaborator, Kian Behbakht, Six1 correlates more significantly with TRAIL resistance in tumors than in cell lines. Since the screen identified many hits in the Wnt pathway we also evaluated pharmacological inhibitors to this pathway, leading to the identification of LiCl as an agent that synergizes with TRAIL in our system, more so in the Six1 expressing cells than in control cells. The findings from our studies so far provide the framework for identifying TRAIL resistance genes in breast cancer, in particular in association with Six1 expression. We anticipate that this work will lead to a further understanding of how Six1 contributes to the tumor phenotype and that it will improve the possibilities of using TRAIL therapy in breast cancer by providing biomarkers and targets of therapeutic intervention that may be exploited in combinatorial therapy.

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