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TITLE: AXL-Targeting Antibody-Drug Conjugate as Novel Therapy for Triple-Negative Breast Cancer

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CONTRACTING ORGANIZATION: Mayo Clinic

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| 13. SUPPLEMENTARY NOTES | | | | | | |
| 14. ABSTRACT Our recent studies discovered that AXL, a receptor protein tyrosine kinase (RTK), is selectively overexpressed and activated in highly aggressive TNBC cells. Based on this discovery in this study, we propose to establish AXL as a novel therapeutic target in the treatment of patients with TNBC. To achieve this, we will develop a novel therapeutic agent, a humanized anti-AXL ADC to specifically and effectively target TNBC with AXL overexpression. Specific Aims: Aim 1. To develop a humanized antibody-drug conjugate (ADC) that can effectively target the AXL membrane receptor tyrosine kinase. Aim 2. To test the efficacy of the AXL-targeted ADC in preclinical animal models Aim 3. To develop a mass spectrometry-based method to effectively monitor AXL expression and activation in xenograft tissues and clinical samples. | | | | | | |
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1. Introduction

Triple negative breast cancers (TNBC) represent 10-15% of all breast cancers. Patients with TNBC are treated with systemic chemotherapy with mostly short-term benefits and associated adverse effects. There is currently no targeted therapy for TNBC. Aberrant activation of oncogenic tyrosine kinase receptors in cancer initiates a phosphorylation cascade in cells to deliver signals that ultimately translate into suppression of apoptosis, invasion and metastasis. Therefore, selective inhibition of tyrosine kinases has emerged as a novel paradigm for targeted cancer therapeutics. Our recent studies have discovered that AXL, a receptor protein tyrosine kinase (RTK), is selectively overexpressed and activated in highly aggressive TNBC cells (Wu et al. 2015). Based on this discovery in this study, we propose to create a novel humanized anti-AXL monoclonal antibody drug conjugate (ADC) as a novel therapy to treat TNBCs with AXL overexpression.

We will first synthesize the ADC by conjugating hMAb173 with mertansine, a highly potent microtubule inhibitor. The ADC will specifically target AXL overexpressing cells and effectively kill the targeted cells. We will also develop near-infrared (NIR) labeled ADC analogs to non-invasively and sensitively monitor the ADC delivery and distribution *in vivo*. We will evaluate the therapeutic potential of this novel ADC in preclinical primograft models that are directly derived from human triple negative breast tumors. We will also develop a highly sensitive and accurate mass spectrometry based method to examine the AXL expression and activation for patients with TNBC.

2. Keywords

triple negative breast cancer
receptor tyrosine kinase
AXL
Phosphorylation
antibody drug conjugates (ADC)
proteomics
mass spectrometry
Patient derived xenograft (PDX)

3. Accomplishments

The study is partnering between Dr. Xinyan Wu at Mayo Clinic and Dr. Parkash Gill at the University of Southern California. This report is based on the progress at the site of Mayo Clinic.

What were the major goals of the project?

The major goals of the project in year 1 were as follows:

- To develop a humanized antibody-drug conjugate (ADC) that can effectively target the AXL membrane receptor tyrosine kinase.
- To develop a mass spectrometry-based method to effectively monitor AXL expression and activation in xenograft tissues and clinical samples.

What was accomplished under these goals?

1) Major activities

- a During the Year 1, we cloned and established MCF10A cells with stable expression of AXL.
- b We tested the AXL expression in archival PDX tumor samples.
- c We did preliminary study using 3-D in vitro culture system to evaluate the therapeutic efficacy hMAb173.
- d We also performed phosphoproteomics to study the AXL downstream signaling network.

2) Specific objectives

- a. To establish cell line models to evaluate the efficacy of ADC developed in Year 2.
- b. To identify PDX models using archival tumor tissues to identify PDX models with high expression of AXL
- c. To identify high performance AXL peptides for PRM assays

3) Significant results

Specific Aim 1: To develop a humanized antibody-drug conjugate (ADC) that can effectively target the AXL membrane receptor tyrosine kinase

Subtask 4: To generate MCF10A cells with stable expression of AXL at different levels for the in vitro assessment of the cytotoxicity and internalization induced by AXL-ADC.

Partially completed

We have cloned the AXL CDS into different retroviral vectors that have different promoters. This allows us to generate stable cell lines with different expression levels of AXL. We cloned AXL into pBABE-puro in which the AXL expression is driven by viral 5-LTR promoter that gives low to median expression level (Figure 1A). We have also cloned AXL into pLEX-puro in which the AXL expression is driven by a CMV promoter that gives relative high expression (Figure 1B). We packaged viral supernatant and infected MCF10A cells. The cells are currently under puromycin selection. We will assess the expression of AXL once the stable expression cell lines are expanded.

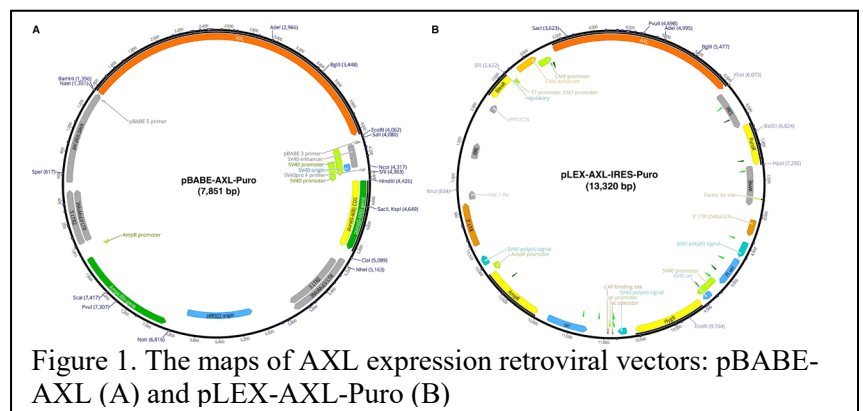


Figure 1. The maps of AXL expression retroviral vectors: pBABE-AXL (A) and pLEX-AXL-Puro (B)

Specific Aim 2: To test the efficacy of the AXL-targeted ADC in preclinical animal models

Subtask 1: To create an animal protocol for PDX study and get approval from Mayo Clinic IACUC and ACURO

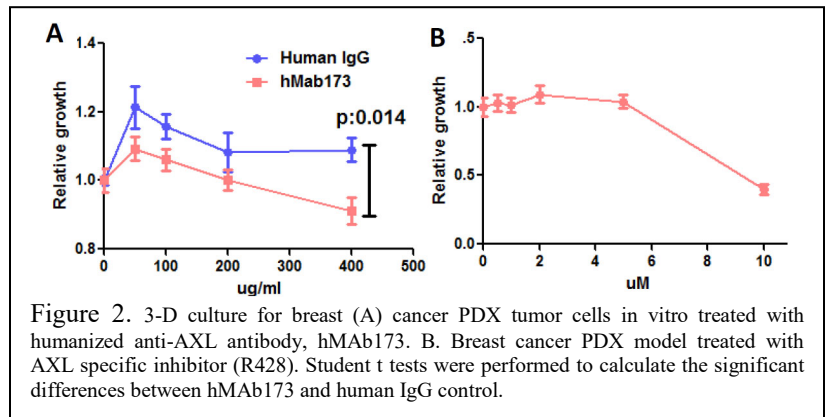
Subtask 2: To create an IRB protocol for using TMA and tumor tissues and get approval from Mayo Clinic and HRPO

Subtask 3: To set up TNBC patient-derived xenografts (PDX) in immune-deficient mouse models for the study of evaluating therapeutic efficacy of ADCs in Major task 4. Cell lines used: MDA-MB-231 [ATCC], PDX [Mayo Clinic] NSG Mice: 20-40 total [JAX] NSG Mice: 30-40 total [JAX] [USC]

Not started.

Due to the outbreak of COVID-19 virus, we were not able to carry out our animal studies. We will start the animal work in the year-two.

However, working with Dr. Liewei Wang, the Co-Investigator of this study, we examined AXL expression in previously collected archival breast cancer PDX tumors and identified several PDXs with median to high expression of AXL. We also performed *in vitro* treatment for previously established PDX tumor cells in a 3-D culture platform. We found that using our humanized monoclonal anti-AXL antibody (hMAb173) to target AXL could suppress AXL+ PDX tumor cell growth in 3-D culture (TNBC PDX-BJ43 P-value: 0.014) (Figure 2A). In addition, we also *in vitro* treated the TNBC PDX model (BJ43) with an AXL specific inhibitor (R428) that could also significantly suppress PDX tumor cell growth in 3-D culture (Figure 1B). (Please note that all the tumor samples were harvested under Dr. Wang's program before the initiation of this study. So far, there was no animal work involved.).



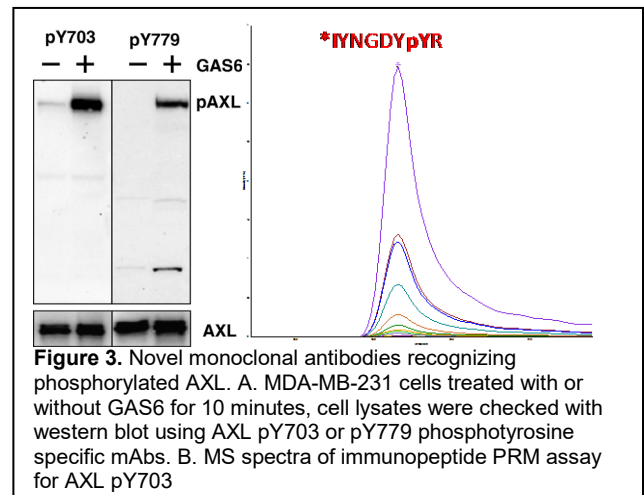
Specific Aim 3: To establish a mass spectrometry-based method to effectively monitor AXL expression and activation in xenograft tissues and clinical samples

Major Task 5: To develop highly sensitive and specific parallel reaction monitoring (PRM) methods to monitor the AXL protein expression and phosphorylation

Subtask 1: To perform AXL IHC, IP and/or phosphopeptides enrichment followed by mass spectrometry analysis Cell line: MDA-MB-231 [ATCC]

Completed

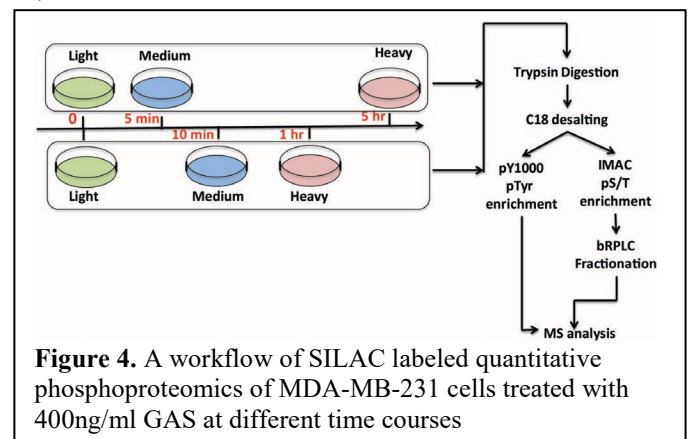
In order to specifically enrich and monitor AXL phosphorylation, we worked with NCI DSHB (Developmental Studies Hybridoma Bank) program and raised two rabbit monoclonal antibodies that can specifically detect AXL phosphorylation at Y703 and Y779 both of which are critical for the activation of the kinase activity of AXL (Figure 3A). Furthermore, the pY703 antibody has been validated to be able to perform immunoprecipitation for phospho-AXL and we have developed sensitive mass spectrometry-based parallel reaction monitoring (PRM) method to detect AXL pY703 phosphopeptide at femtomole level (Figure 3B).



Subtask 2: To analyze the mass spectrometry data and select high performance peptides for PRM analysis

Ongoing

In order to determine the high performance phosphopeptides of AXL to monitor its activity, we performed SILAC-based phosphoproteomics analysis on MDA-MB-231 cells treated with GAS6, an AXL ligand to activate AXL at different time courses (Figure



4). Our preliminary analysis found dynamic phosphorylation of AXL (Figure 5), and activation of its downstream signaling molecules including upregulation of phosphorylation of GAB1, SHC, NCK, CBL and ABL. Surprisingly, we found that tyrosine phosphorylation of several RTKs such as EGFR, ERBB3, ERBB4, MET, EPHA2 and EPHB2 are reduced upon the activation of AXL.

4) Other achievements.

None

What opportunities for training and professional development has the project provided?

Nothing to report

How were the results disseminated to communities of interest?

Nothing to report

What do you plan to do during the next reporting period to accomplish the goals?

Next year, we will have initial batch of the synthetic AXL ADC. We will start to test the toxicity, specificity and efficacy of ADC in cell line models, PDX *in vitro* 3-D culture and *in vivo* treatment. We will optimize the PRM assays to detect AXL in PDX tumors and clinical tumor samples. We will also carry out more mass spectrometry analysis, cell biology and biochemistry studies to understand the signaling network regulated by AXL.

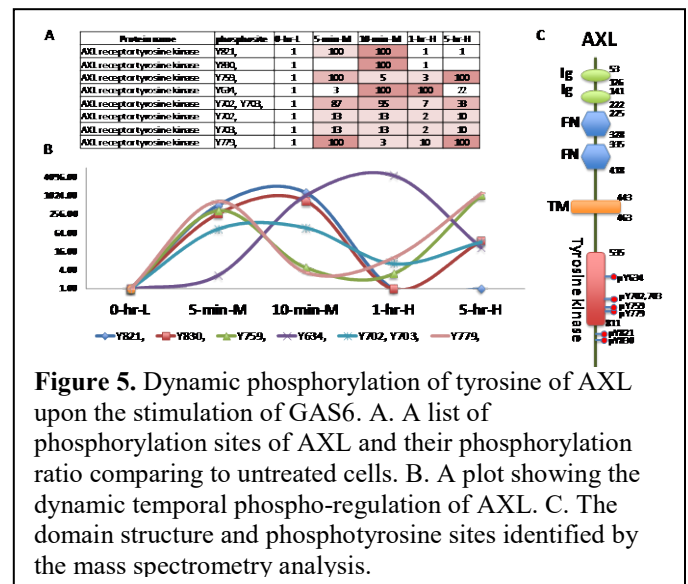


Figure 5. Dynamic phosphorylation of tyrosine of AXL upon the stimulation of GAS6. A. A list of phosphorylation sites of AXL and their phosphorylation ratio comparing to untreated cells. B. A plot showing the dynamic temporal phospho-regulation of AXL. C. The domain structure and phosphotyrosine sites identified by the mass spectrometry analysis.

4. Impact

What was the impact on the development of the principal discipline(s) of the project?

Nothing to report

What was the impact on other disciplines?

Nothing to report

What was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology?

Nothing to report

5. Changes/Problems

The outbreak of COVID-19 significantly delayed our study. We will try to make up during the next project year.

6. Products

We generated two AXL phosphosite (pY703 and pY779) specific monoclonal antibodies.

7. Participants & Other Collaborating Organizations

What individuals have worked on the project?

| | |
|--|--|
| Name: | <i>Xinyan Wu</i> |
| Project Role: | <i>PI</i> |
| Researcher Identifier (e.g. ORCID ID): | 0000-0002-7745-1614 |
| Nearest person month worked: | 3.6 months |
| Contribution to Project: | <i>Dr. Wu designed, oversee and perform experiment</i> |
| Funding Support: | <i>None</i> |

| | |
|--|---|
| Name: | Matthew Philip Goetz |
| Project Role: | <i>Co_investigator</i> |
| Researcher Identifier (e.g. ORCID ID): | 0000-0002-4383-270X |
| Nearest person month worked: | 0.36 months |
| Contribution to Project: | <i>Dr. Goetz provided critical suggestions to the project</i> |
| Funding Support: | <i>None</i> |

| | |
|--|---|
| Name: | Seul Kee Byeon |
| Project Role: | <i>Postdoctoral fellow</i> |
| Researcher Identifier (e.g. ORCID ID): | 0000-0002-7745-1614 |
| Nearest person month worked: | 6 months |
| Contribution to Project: | <i>Dr. Byeon performed mass spectrometry based analysis for the study</i> |
| Funding Support: | <i>None</i> |

| | |
|--|--|
| Name: | Nicole Pearson |
| Project Role: | <i>Technician</i> |
| Researcher Identifier (e.g. ORCID ID): | |
| Nearest person month worked: | 3.6 months |
| Contribution to Project: | <i>Nicole performed cell biology and molecular biology experiments for the study</i> |
| Funding Support: | <i>None</i> |

| | |
|---------------|----------------------------|
| Name: | Li Wang |
| Project Role: | <i>Postdoctoral fellow</i> |

| | |
|--|---|
| Researcher Identifier (e.g. ORCID ID): | |
| Nearest person month worked: | 3.6 months |
| Contribution to Project: | <i>Dr. Wang performed cell biology and molecular biology experiments for the study. She also involved in the mass spectrometry data analysis.</i> |
| Funding Support: | <i>None</i> |

8. Special Reporting Requirements

Nothing to report

9. Appendices

Nothing to report