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

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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.

15. SUBJECT TERMS
None listed.

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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 the 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 the 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?

- 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 In Year 3, we performed IMAC enrichment-based global phosphoproteomic analysis for the MDA-MB-231 cells treated with AXL ligand GAS6. This is an extension of our tyrosine phosphoproteome study (PMID: 34439388) that was completed in Year 2.

b We established an inducible AXL overexpression cell line in MCF10A cells. We also performed functional studies to examine the oncogenic role of AXL.

c We performed IHC staining for a set of TNBC TMA.

d We identified cleaved intracellular isoforms of AXL that encompass the intact tyrosine kinase domain, suggesting the cleaved AXL intracellular domain (ICD) can function as a non-receptor tyrosine kinase in cytoplasm and/or nucleus.

2) Specific objective

a. To study the AXL downstream signaling pathways in TNBC

b. To examine the oncogenic role of AXL in breast cancer

3) Significant results

During Year 2, we established multiple AXL inducible knockout cell lines, including MCF10A and MDA-MB-231 cells. We also performed phosphotyrosine proteomics analysis to dissect the signaling networks regulated by AXL activation. Due to the significant delay in delivering AXL hMAb173 antibody-drug conjugate (AXL-ADC) at Dr. Gill Parkash's site at the University of South California, we could not carry out the proposed studies to evaluate the efficacy of AXL-ADC *in vitro* or *in vivo*. We focused our studies on investigating AXL oncogenic signaling pathways.

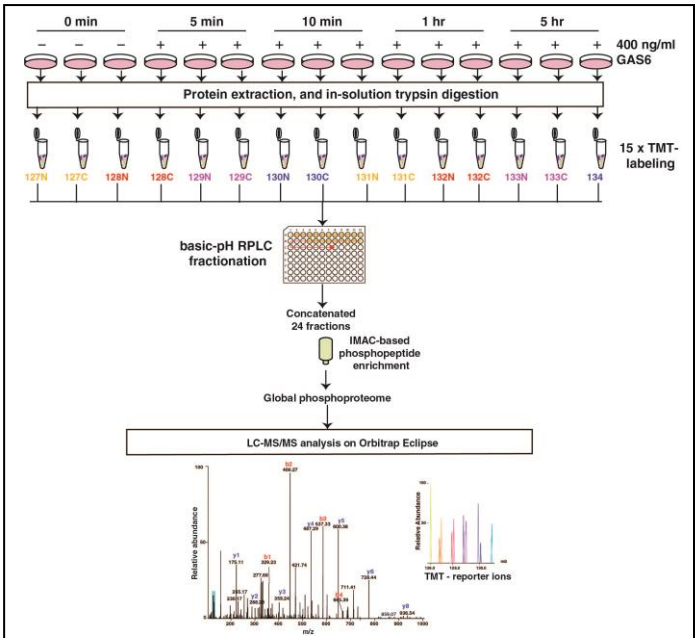

Figure 1. A schematic depicting the strategy used for temporal quantitative phosphotyrosine proteomic profiling of GAS6-treated MDA-MB-231 cells. All experiments were performed in triplicate. Cells were serum starved for 18 h and treated with 400 ng/mL GAS6 for 5 min, 10 min, 1 h or 5 h. After the GAS6 treatment, cells were harvested and lysed in 8 M urea buffer, followed by trypsin digestion and desalting. Peptides were labeled with the TMTpro kit and fractionated with basic reversed-phase liquid chromatography (bRPLC). The fractionated peptides were subjected to IMAC-based phosphopeptide enrichment and analyzed by Orbitrap Eclipse.

- Explore the downstream signaling pathways regulated by the activation of AXL signaling

In Year-3, we performed immobilized metal affinity chromatography (IMAC) phosphopeptide enrichment-based phosphoproteome analysis for TNBC cells treated with AXL ligand, GAS6 (Figure 1). Compared to our previous tyrosine phosphoproteomics analysis that identified 1,145 phosphotyrosine (pTyr) sites from 721 proteins, this study identified 29,175 phosphosites from 6,604 proteins. It provided a more comprehensive survey of the global phosphoproteome regulated by AXL activation. Among them, 5,268 phosphosites have significant differences between GAS6-treated and untreated samples (Ratio >1.5 or <0.67, $p<0.05$). ~ 89% phosphosite changes took place during 5-10min after treatment with GAS6. We used fuzzy-C mean clustering to classify the

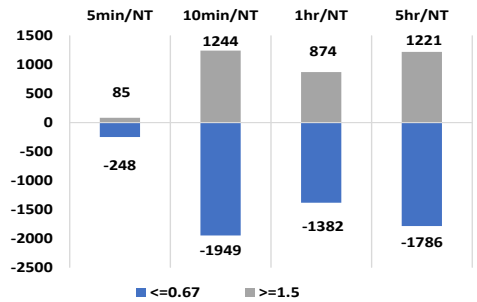

Figure 2. Dynamic regulation of phosphorylation induced by the activation of GAS6/AXL signaling cascades. Plot showing the number of upregulated and downregulated phosphorylation sites at the indicated time points.

dynamic changes induced by GAS6-AXL signaling cascades and discovered three clusters of phosphosites with distinguished phosphorylation patterns (Figure 3). Cluster 1 represents the early hyperphosphorylated sites activated by GAS6 treatment (Figure 3A). Clusters 2 and 3 represent phosphorylation sites negatively regulated by the activation of AXL (Figure 3B, C). We then performed gene set enrichment analysis using the KEGG pathway database (Figure D-F). Multiple cancer signaling pathways, including ErbB, mTOR, and HIF1 signaling pathways, were significantly upregulated by the activation of AXL. Interestingly, we found several DNA repair-related pathways, such as DNA replication, base excision repair, p53 and Fanconi anemia pathways, were also regulated by AXL activation. In addition, we also noticed that multiple key proteins in the HIPPO signaling pathway were hypo-phosphorylated during the activation of AXL (Figure 4 A, B). We are performing western blot analysis to validate the mass spectrometry-based discoveries. We will perform functional assays to explore the potential roles of AXL in DNA repair and Hippo signaling pathways in NCE.

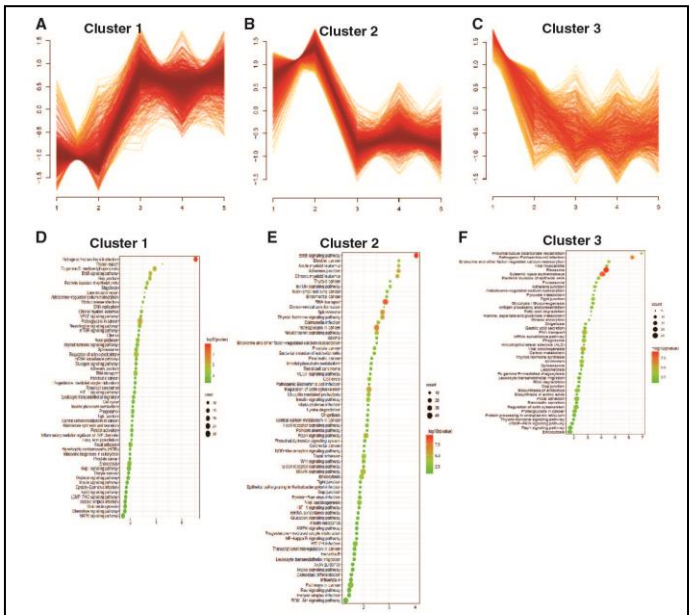

Figure 3. Dynamic regulation of phosphoproteome by AXL activation. A-C, Fuzzy-C clustering analysis revealed three regulated patterns. D-F, Gene set enrichment analysis for regulated phosphoproteins in cluster1, 2 and 3, respectively.

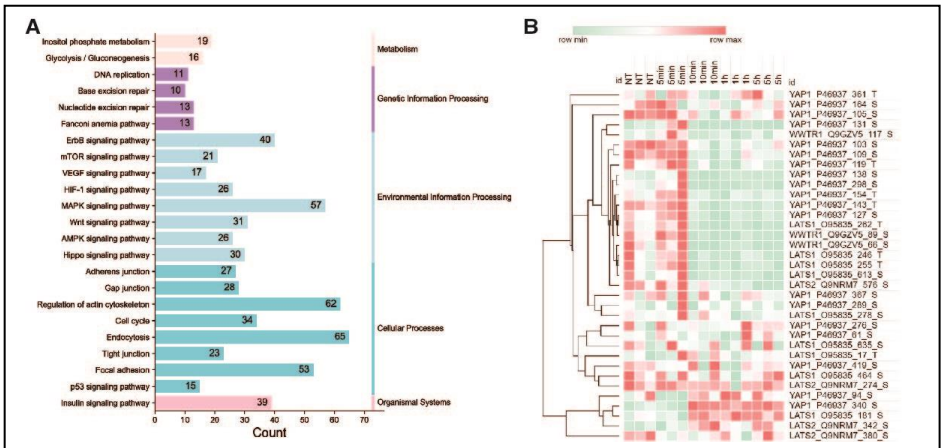

Figure 4. A. Bar plot showing enriched significantly regulated pathways. B. heatmap of phosphorylation sites of key proteins in the Hippo signaling pathway.

- **To explore the oncogenetic role of AXL in breast cancer**

To explore the oncogenetic role of AXL, we established a doxycycline-based AXL inducible overexpression cell line in MCF10A cells (Figure 5 A, B). MCF10A is a spontaneously immortalized normal mammary gland epithelial cell line. The growth of MCF10A cells is dependent on the stimulation of EGF. Our preliminary study found that MCF10A cells can proliferate in the absence of EGF when AXL expression is induced by doxycycline (Figure 5C). In soft agar colony formation assays, we also observed similar phenomena that induction of AXL expression could completely

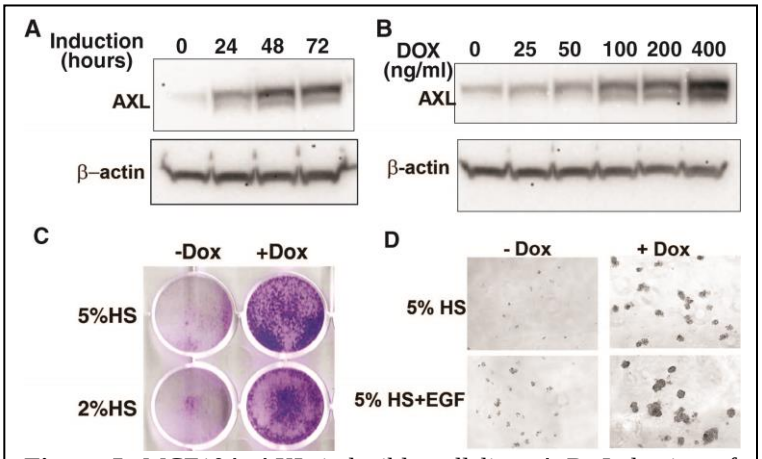

Figure 5. MCF10A AXL inducible cell line. A-B, Induction of AXL expression with different dose of doxycycline at different time points. C-D, Proliferation assay and soft agar colony formation assay of MCF10A-AXL cells with or without induction of AXL.

compensate for the loss of the function of EGF-EGFR signaling. Remarkably, we found that activation of AXL signaling enables much more robust colony formation of MCF10A than EGF stimulation alone (Figure 5D). These data strongly suggested the oncogenic transformation capacity of activation of AXL in mammary gland epithelial cells.

- To examine AXL expression in TNBC tumors and correlate with patient disease prognosis

During the last reporting cycle, we identified a very specific anti-AXL antibody for IHC and optimized the staining protocol. As shown in Figure 6, we can observe robust and specific staining signals in wild-type MDA-MB-231 cells (left panel) and no staining in AXL knockout MDA-MB-231 cells (middle panel). Importantly, we can also observe specific peripheral cell membrane staining in a commercial cervix FFPE section (right panel).

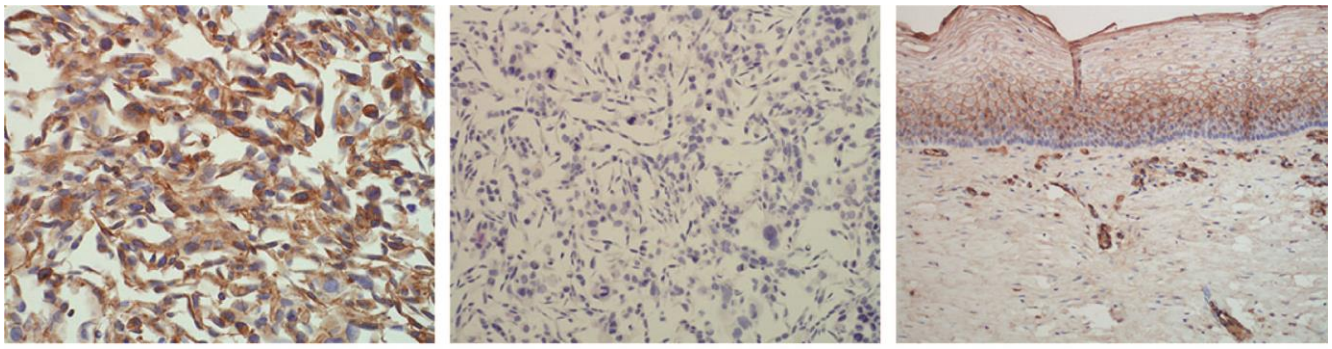

Figure 6. IHC staining with anti-AXL (AF154, R&D) antibody for wild type (left), AXL-KO (right) MDA-MB-231 cells and cervix FFPE section.

In this reporting cycle, we performed IHC staining for a set of TNBC TMAs collected by the Mayo Breast Cancer SPORE program. These TMAs contain TNBC tumors from 291 patients with more than 10 years of follow-up. The IHC staining results were reviewed by Dr. Jodi Carter. There are 48 tumors with negative AXL staining, 107 tumors have medium-level staining, and 136 tumors have high-level staining. After we correlated the staining intensity with the patient survival data, we found that the patients with AXL negative expression tended to have better recurrence-free survival and overall survival compared to patients with a medium to a high level of AXL expression (Figure 7). However, due to the relatively low number (48) of patients with negative AXL expression, we did not observe statistical significance between the groups with different levels of AXL expression. We will try to include more TMAs to improve the statistical power.

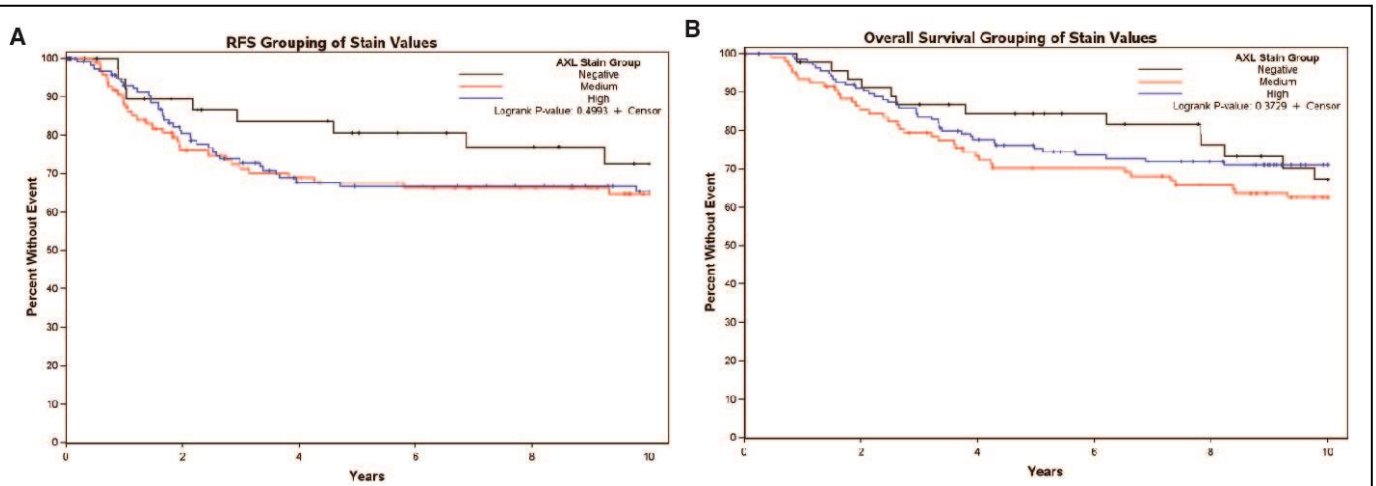

Figure 7. AXL IHC staining for TNBC TMAs. A. Kaplan Meier (KM) plot showing the recurrence free survival for patients with different expression of level AXL. B. KM plot showing the overcall survival for patients with different expression levels of AXL

- ***Identification of cleaved AXL intracellular domain (AXL-ICD)***

While optimizing the IHC staining of AXL, we tested multiple antibodies. Two antibodies provided specific staining patterns in our MDA-MB-231 AXL knockout cells. One (AF154) of them recognizes the extracellular domain of AXL, and the other (C89E7) recognizes the intracellular domain of AXL. In western blot assays, AF154 targeting the AXL extracellular domain detected a single band at ~110 kd. However, C89E7 targeting the intracellular domain can always detect multiple bands, including the 110 kd band and the other two bands at 45 kd and 55 kd. We further confirmed this observation using our established MCF10A cells with inducible expression of AXL. As shown in Figure 8A, we can clearly detect three major bands (110 kd, 55kd and 45kd) in MDA-MB-231 cells and MCF10A cells with the induction of AXL expression. More importantly, with the increase in the dose of doxycycline, the expression levels of all three bands were also augmented. We also performed the immunoprecipitation of AXL with C89E7 antibody followed by protease digestions with trypsin, Lys-C and Glu-C, respectively. The digested peptides were analyzed by mass spectrometry. We identified multiple cleavage sites, mainly localized close to the downstream moiety of the transmembrane domain of AXL (Figure 8B). We have performed a pilot study in which we cloned the longest form of AXL-ICD and fused it with EGFP protein. Compared to the AXL full-length protein, the AXL-ICD demonstrated strong nuclear localization patterns (Figure 8C). These data indicated that AXL-ICD isoforms might play roles in regulating the tyrosine phosphorylation signaling networks in cell nucleus.

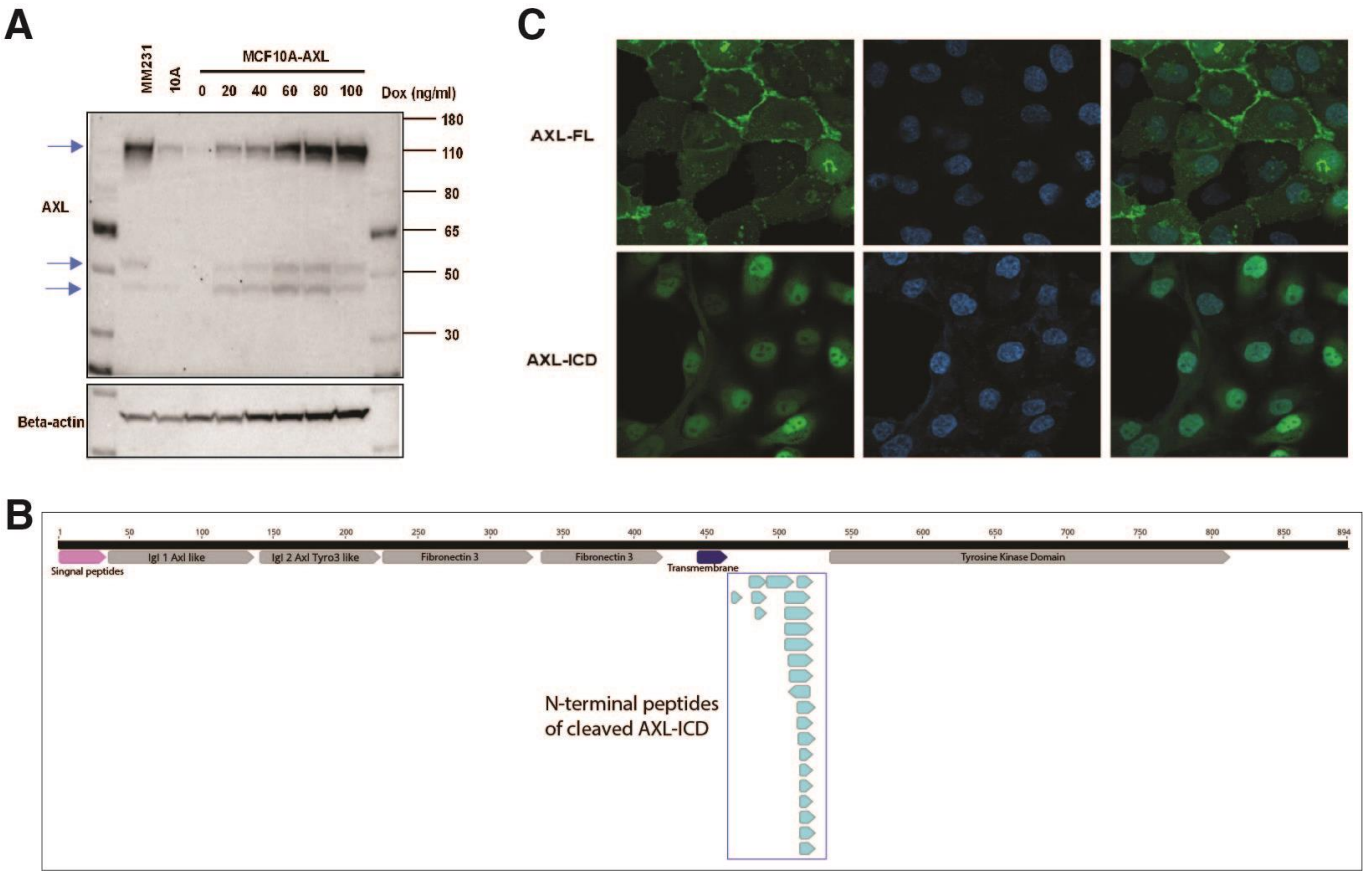

Figure 8. Novel AXL cleaved intracellular domain isoforms (AXL-ICD). A. Western blot showing AXL expression in MDA-MB-231, MCF10A and MCF10A AXL inducible cells that was induced with indicated doses of doxycycline. B. The domain structure of AXL and the identified N-terminal peptides of cleaved AXL-ICD. C. Fluorescent microscope showing the subcellular localization of AXL full-length (AXL-FL, top pannels) and AXL-ICD (bottom pannels).

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, if we can receive the initial batch of the synthetic AXL ADC, we will start to test ADC's toxicity, specificity and efficacy in cell line models, PDX models with *in vitro* 3-D culture and *in vivo* treatment.
- We will try to include more TNBC TMAs for the IHC study to improve the statistical power.
- We will explore the role of AXL in regulating the Hippo signaling pathway and DNA repair pathway.
- We will perform functional studies and phosphoproteomic studies to investigate the role of AXL-ICD in breast cancer progression.

4. Impact

What was the impact on the development of the principal discipline(s) of the project?
- We have established the inducible AXL overexpression cell lines that will help us investigate the role of AXL in promoting tumorigenesis of TNBC.
- The IMAC enrichment-based quantitative phosphoproteomics study provides a detailed landscape of dynamic regulation of global phosphorylation network driven by AXL activation, which demonstrates the complexity of AXL signaling and reveals the role of AXL in modulating signaling networks to promote breast cancer progression. These discoveries should serve as a potentially useful resource for studying AXL functions as well as for the development of effective therapeutic options to target AXL.
- We performed IHC staining studies using a set of precious TMAs with TNBC tumors from more than 290 patients. We observed trends that patients with a high expression level of AXL tend to have poorer recurrence-free survival and overall survival. However, we will need more patients with low/negative expression of AXL to increase the statistical power.
- We identified novel AXL-cleaved intracellular isoforms, indicating novel functions of AXL in the cytoplasm and nucleus.

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. The significant delay on Dr. Gill's site in developing anti-AXL ADC dramatically slowed the progress of the proposed study. We will try to make it up during the next project year.

6. Products
We generated two AXL phosphosite (pY703 and pY779) specific monoclonal antibodies. We generated AXL knockout cell lines. We also established AXL inducible overexpression cell lines.

7. Participants & Other Collaborating Organizations

What individuals have worked on the project?

Name:	*Xinyan Wu*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	0000-0002-7745-1614
Nearest person month worked:	6 months
Contribution to Project:	*Dr. Wu designed, oversaw and performed experiments*
Funding Support:	*None*

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

Name:	Jodi Carter
Project Role:	*Co_investigator*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	0.36 months
Contribution to Project:	*Dr. Carter helped establish IHC staining assays for AXL and interpretted the results.*
Funding Support:	*None*

Name:	Li Wang
Project Role:	*Postdoctoral fellow*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	6 months
Contribution to Project:	*Dr. Wang performed cell biology and molecular biology experiments for the study. She was also involved in mass spectrometry data analysis.*
Funding Support:	*None*

8. Special Reporting Requirements

Nothing to report

9. Appendices

Nothing to report