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Award Number: W81XWH-11-1-0021

TITLE: Molecular Characterization of Human MUC16 (CA125) in Breast Cancer

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REPORT DATE: February 2013

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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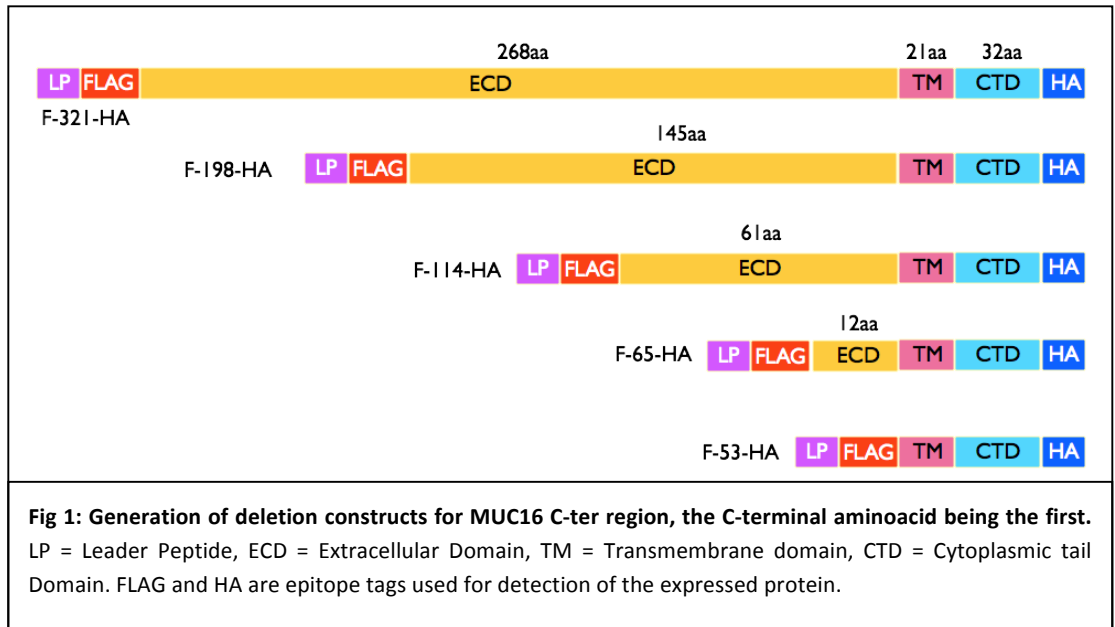
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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE February 2013		2. REPORT TYPE Annual Summary		3. DATES COVERED 3 January 2012 - 2 January 2013	
4. TITLE AND SUBTITLE Molecular Characterization of Human MUC16 (CA125) in Breast Cancer.				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-11-1-0021	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Srustidhar Das E-Mail: sdas@unmc.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Nebraska Medical Center Omaha, NE 68198-7835				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Abstract: This study was designed to understand the role and implications of MUC16 cytoplasmic tail in breast cancer pathogenesis. We would like to update our findings with respect to it since our last report submission. One important question towards this end was whether MUC16 indeed undergoes cleavage, which was addressed using a dual-epitope tagging (N-ter FLAG and C-ter HA) (last report). Having established this, we wanted to identify the potential cleavage site on MUC16, for which we generated a series of deletion constructs suggesting this cleavage might be in the proximal juxta-membrane region. In addition, we have demonstrated that MUC16 undergoes ubiquitylation in the cytoplasmic lysine (K) residue and N-glycosylation in the extracellular asparagine (N) that are responsible its stability. In an effort to identify any intracellular trigger for the cleavage of MUC16 (e.g. phosphorylation on the tyrosine (Y), serine (S) and/or threonine (T)), we have carried out SDM of these residues, but none of them was able to prevent the cleavage of MUC16. While further biochemical characterizations are being carried out, to gauge at the functional significance we have generated stable MCF7 cells and the functional studies are being carried out. The significance of this study is manifold: (i) This will enable us to establish the precise site of cleavage, cellular location of cleavage and the trigger (we do not know at this point whether it is induced or constitutive), which is very much necessary in order to design any kind of therapeutics, (ii) post-translational modifications affecting the trafficking and stability and interaction(s) of it are of immense importance to understand the functional mechanism(s).					
15. SUBJECT TERMS Cytoplasmic tail, Ubiquitylation, Glycosylation, cleavage					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 8	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

Table of Contents

	<u>Page</u>
Introduction.....	4
Body.....	4-6
Key Research Accomplishments.....	7
Reportable Outcomes.....	7
Conclusion.....	7
References.....	8

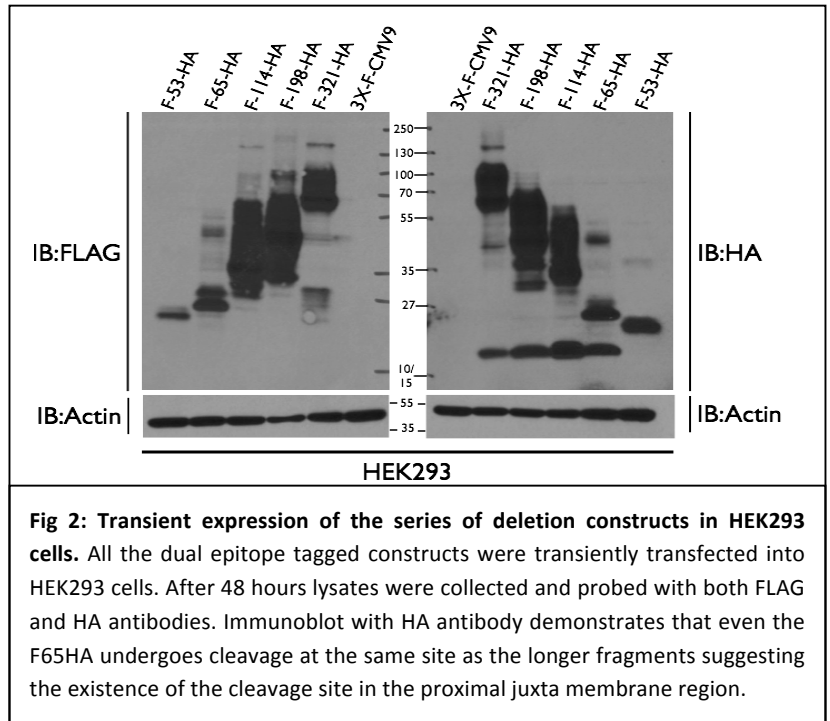
Introduction: Breast cancer (BC) is a major health problem worldwide and is the second leading cause of cancer related deaths in American women with 232,340 and 39,620 estimated new cases and death respectively in 2013 (1). This accounts for 14% of all cancer related deaths among women in USA (1). Therefore, efforts should be undertaken to improve the survival of breast cancer patients with respect to early diagnosis and therapeutic intervention. Mucins are high molecular weight glycoproteins expressed by various epithelial cells and deregulated mucin production is increasingly being recognized as the hallmark feature of the inflammatory and neoplastic disorders of several organs including breast (2, 4). The ability of the membrane-bound mucins to sense the extracellular environment and carryout the outside-in signaling (2) and their differential expression in cancer compared to the normal tissues (particularly in breast and pancreas) makes these interesting candidates for study with respect to diagnosis and therapeutics. The C-terminal regions of these trans membrane mucins have been shown to be most vital with respect to their signaling functions, MUC1 being the most widely characterized (3). In this study we are characterizing the MUC16 C-terminal region. MUC16 is a type-I membrane-bound mucin with a heavily glycosylated N-terminal domain, a transmembrane (TM) region and a 32-residue cytoplasmic tail (CT) domain. Our recent studies demonstrate that normal breast do not express MUC16; however, its expression progressively increases in primary and metastatic breast cancer (4). In addition, MUC16 knock down in breast cancer (BC) cells resulted in significant reduction in the growth and tumorigenic properties of MDAMB231 cells (4). In the present study, to understand the mechanistic involvement of MUC16 in BC, we have focused on its C-terminal region. Although it has been predicted that MUC16 undergoes cleavage/shedding in the extracellular SEA domain(s) by proteases such as MMP7 and neutrophil elastase, the exact nature and site of cleavage and the fate of the membrane bound portion (C-terminal) is not entirely understood. The post-cleavage C-ter region is interesting due to presence of (a) stretch of polybasic



aminoacids, the site of interaction for cytoskeletal proteins like Ezrin/Radixin/Moesin, (b) potential nuclear localization signal (bioinformatics prediction) which might facilitate its nuclear localization with associated transcription factors modulating the transcription of various target genes and (c) several potential serine, threonine and tyrosine phosphorylation sites facilitating its interaction(s) with proteins influencing oncogenic signaling pathways. *Therefore, we **hypothesize** that the oncogenic potential of MUC16 is in part, mediated by the potential involvement of C-terminal domain of MUC16.* Two specific aims were laid out for the study. Aim 1: To investigate the MUC16 cytoplasmic tail domain in tumor growth and metastasis of BC cells. Aim 2: To determine the mechanism(s) of MUC16CT mediated BC progression by identifying potential phosphorylation sites and interacting partners of MUC16CT.

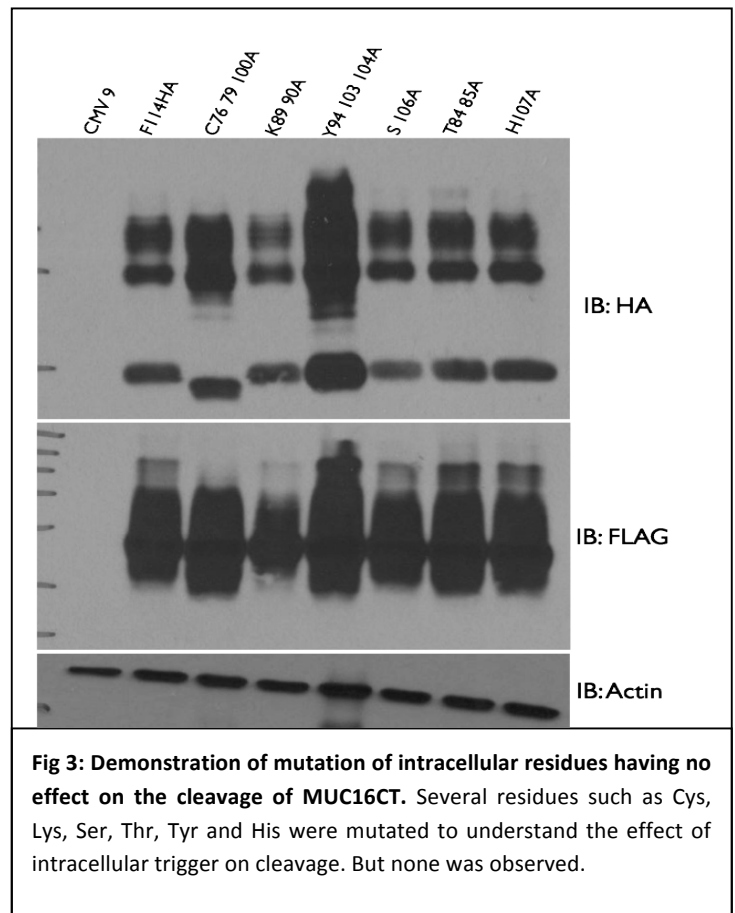
Body: (a) Cleavage of MUC16: Last time, we reported that the cleavage site of MUC16 is downstream to the previously predicted 114 amino acids from the C-ter region. To identify the exact cleavage site, we have

generated several truncated versions of the dual epitope (N-ter FLAG and C-ter HA) tagged constructs (**Fig 1**) to identify the minimal region where cleavage is taking place. These constructs were transiently transfected into the HEK293 cells and the lysates were immunoblotted with both FLAG and HA antibodies. The findings (**Fig. 2**) demonstrate that the cleavage is taking place in the membrane proximal region (12 extracellular aminoacids) as demonstrated by the F65HA construct. Further mutagenesis studies such as Alanine scan are being undertaken to address the issue in a more precise manner. **Significance:** Identifying the cleavage site is critical as this will help us to precisely determine the fragment that is left attached to the cells after cleavage and hence diagnostic and/or therapeutic strategy in terms of developing specific antibody would be much more specific.

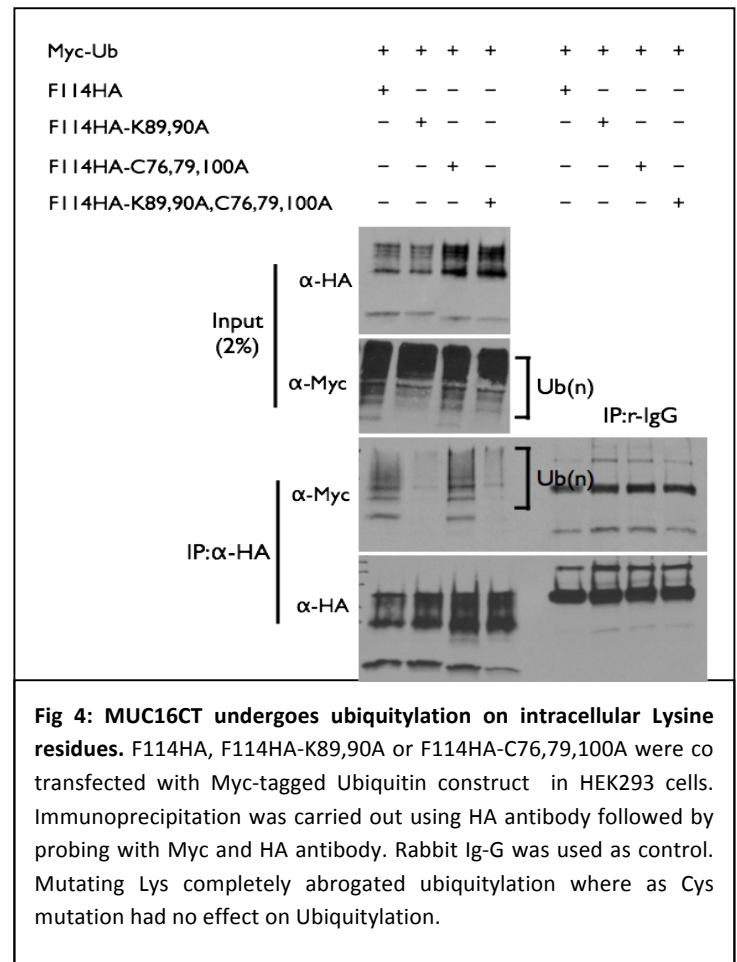


(b) What stimulates cleavage or is it constitutive? Our initial hypothesis was that probably an intracellular event (such as phosphorylation event on either tyrosine, serine or threonine residues) would be acting as a trigger for the induction of cleavage. In order to find this out, we mutated all the tyrosine (Y), serine (S) and threonine (T) residues, but none of them prevented the cleavage (**Fig 3**). This probably suggests this is a constitutive event. Further experiments are necessary to confirm the finding.

(c) Post-translational modifications of MUC16 C-terminal region: As mentioned in our previous report that we observed higher molecular weight protein than the predicted molecular weight of the primary sequence and demonstrated that it in fact undergoes ubiquitylation. As an extension of the study he we have conclusively demonstrated that it is the lysines (K) in the cytoplasmic tail region, which is ubiquitylated. To address this, we generated the lysine and cysteine mutations using the F114HA construct using site-directed mutagenesis. Further, the F114HA, F114HA-K89,90A or F114HA-



C76,79,100A constructs were co-transfected with Myc-tagged Ubiquitin construct (a kind gift from Prof. Youping Sun, Columbia University) in HEK293 cells. Following this, immunoprecipitation was performed using the HA-antibody and the pulled down fractions were probed with both HA and Myc antibody. Rabbit immunoglobulin G was used as control. The findings (Fig 4) demonstrate that, when lysines were mutated, Ubiquitylation was largely abrogated while cysteine mutations did not. To understand what could be the implications of this ubiquitylation, MCF7 cells stably transfected with F114HA construct was treated with MG132 (a proteasomal inhibitor) leading to accumulation of MUC16CT (Fig 5). This demonstrates that ubiquitylation of MUC16CT targets it for proteasomal degradation. Since, mutation of lysines did not completely abrogate the higher mol wt. bands, we hypothesized that it might be undergoing N-glycosylation (three predicted N-Glycosylation site, no O-glycosylation was predicted). To demonstrate this we treated the MCF7-F114HA cells with Tunicamycin (N-glycosylation inhibitor) and this led to removal of the higher mol wt. bands (Fig 5) confirming our prediction. Further mutational analyses of these sites are being carried out to find out the exact site of N-glycosylation. **Significance:** As demonstrated, the stability of MUC16 C-terminal region is dependent on the kind of post-translational modification it undergoes (Ubiquitylation and N-Glycosylation) and therefore understanding this is very crucial to understanding its function.



(d) Functional significance and Identification of interacting partners: We would like to mention that, while we have made significant progress in the biochemical characterization of MUC16 CT, further studies aiming at understanding the functional implications are currently being carried out with respect to sub cellular localization, in vitro and in vivo studies as well as identification of interacting partners. While we are facing some technical challenges in purifying the protein in bacteria due to its transmembrane nature we hope to overcome these challenges in near future. In addition, we are in the process of generating a rabbit polyclonal antibody against the MUC16 cytoplasmic tail, which will enable us to understand the biology of MUC16CT more precisely.

Key Research Accomplishments:

- (a) Cleavage of MUC16 is probably taking place either in the proximal juxta-membrane region.
- (b) Intracellular phosphorylation of neither tyrosine, or serine nor threonine critical for cleavage.
- (c) MUC16-CT undergoes ubiquitylation on the intracellular lysine residues leading to its proteasomal degradation.
- (d) MUC16-CT undergoes N-glycosylation, which is also responsible for its stability.
- (e) Participated in the Journal club and Breast cancer research focus group meetings conducted at University of Nebraska Medical Center.

Reportable Outcomes:

Srustidhar Das, Maria P Torres-Gonzalez, Prabin Dhangada Majhi, Imayavaramban Lakshmanan, Moorthy P. Ponnusamy, Satyanarayana Rachagani, Eric Cruz, Dhanya Haridas, Surinder K. Batra. Carboxyl terminal region of MUC16 is critical for tumor cell invasion and drug resistance. **American Association for Cancer Research**, Wasington DC, USA, April 6-10, 2013 (POSTER PRESENTATION).

Conclusions and Future Directions: Previous studies from our lab (Lakshmanan et al., 2012) have demonstrated that MUC16 plays a critical role in the breast cancer pathogenesis we focused our attention on the C-terminal region of MUC16 to be a major player in this process. In order to understand this, the first and most important question we wanted to address was whether it undergoes cleavage or not. By using dual epitope tagging we have convincingly demonstrated that it indeed undergoes cleavage. Once we identify the cleavage site, this will help us to precisely determine the fragment that is left attached to the cells after cleavage and hence diagnostic and/or therapeutic strategy in terms of developing specific antibody would be much more specific. As our understanding of human diseases grow deeper, we realize the fact that post-translational modification(s) of protein are extremely important for its function. Here we demonstrate that MUC16CT undergoes ubiquitylation and N-glycosylation, which determines the stability of it, may turn out to be the most critical factor for its functions. Further experimental validations are being carried out to understand the role of MUC16-CT in neoplastic diseases with particular emphasis on breast cancer pathogenesis.

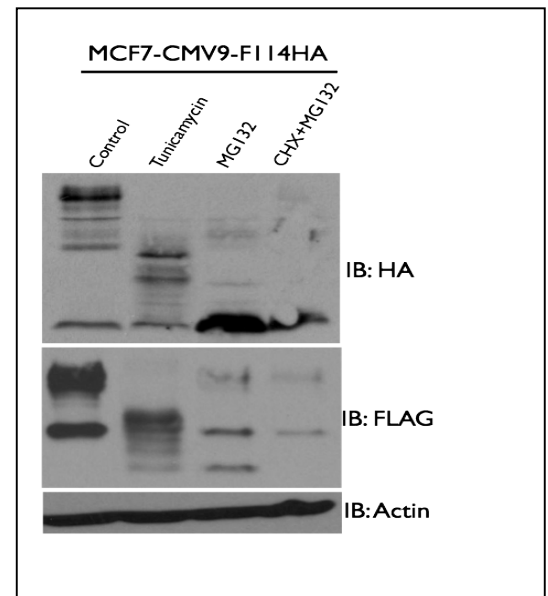


Fig 5: MUC16-CT undergoes N-Glycosylation and proteasomal degradation. MCF7 cells stably transfected with F114HA, were treated with Tunicamycin (5ug/ml), MG132 (10uM) and cyclohexamide (CHX, 100uM) for 12 hours. The resulting lysates were immunoblotted with FLAG and HA antibodies. Actin was used as a loading control. Tunicamycin treatment abrogates the higher mol wt bands suggesting that it undergoes N-glycosylation and it is critical its stability. Treatment with MG132 led to accumulation of MUC16CT suggesting that ubiquitylation of MUC16CT leads to proteasomal degradation.

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