

The Role of Elevated Hyaluronan-Mediated Motility Receptor (RHAMM/HMMR) in
Ovarian Cancer

by

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Dedication

This dissertation is dedicated to my loving family and friends. For the completion of this journey would have never come to fruition without you. To my parents, who always gave me their never-ending support. To my sister, Julie, for always being my biggest fan and supporter. To my best friend, Jeff Nippard, for always being there for me no matter what. You are the reason this endeavor saw its way to completion. I would also like to give a special thank you to my mentor, Dr. Patricia Kruk, who saw potential and intellect in me when I never did. Her kindness and patience are the reasons I never gave up. I would have never completed this wild ride without you, pushing me and challenging my scientific mind. And lastly, I would like to thank my amazing colleague/best friend/sister, Mai Mohamed. You are the one who always picked me up when I was down and went through all of my highs and lows as if they were your own.

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List of Abbreviations

AA	Amino acids
AP-1	Activator protein 1
AURKA	Aurora Kinase A
BC	Breast cancer
BRCA1	Breast cancer susceptibility gene 1
CA125	Circulating cancer antigen 125
CCC	Clear cell carcinoma
CCM	Concentrated conditioned medium
CD44	Cluster differentiation 44
CRC	Colorectal carcinoma
D	Domain
EC	Endometrioid carcinoma
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
ELISA	Enzyme linked immunosorbant assay
ELK-1	ETS domain-containing protein
EOC	Epithelial ovarian cancer
ER	Endoplasmic reticulum

ERK	Extracellular signal regulated kinase
FAK	Focal adhesion kinases
FBS	fetal bovine serum
FH	Family history
FIGO	International Federation of Gynecology and Obstetrics
FT	Fallopian tube
FTE	Fallopian tube epithelium
GOF	Gain of function
H&E	Hematoxylin and eosin
H&N	Head and neck
HA	hyaluronan
HE4	Human epididymis protein 4
hESC	human embryonic stem cell
HGSC	High grade serous carcinoma
HGSOC	High grade serous ovarian carcinoma
HIOSE	SV-40 large T antigen transfected human ovarian surface epithelium
HRP	Horse radish peroxidase
IgG	Immunoglobulin G
IHC	immunohistochemical
IHC	Immunohistochemistry
LAA	Leukemia associated antigen
LGSC	Low grade serous carcinoma
LMP	Low malignant potential

LPA	Lypophosphatidic acid
MAP	Mitogen activated kinase
MEK	Mitogen activated protein kinase 1
MI	Milliliter
MM	Multiple myeloma
MUC	Mucinous
NFH	Non family history
NW	Normal weight
OB	Obese
OC	Ovarian cancer
ON	Overnight
OPN	Osteopontin
OSE	Ovarian surface epithelium
OW	Overweight
PBS	Phosphate Buffered Saline
PD	Poorly differentiated
PDGF	Platelet derived growth factor
pg	Picogram
PVDF	polyvinylidene fluoride
qPCR	Quantitative polymerase chain reaction
RHAMM	Receptor for hyaluronan-mediated motility
ROMA	Risk for ovarian malignancy alogorithm
RSK	Ribosomal protein S6 kinase

RT	Room temperature
RT-PCR	Real-time polymerase chain reaction
SE	Standard error
SC	Serous carcinoma
scrRNA	scramble RNA
scrRNA	Scrambled RNA
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEREX	Serological analysis of recombinant tumor cDNA expression libraries
siRNA	Silencing RNA
SMC	Smooth muscle cells
STIC	Serous tubal intraepithelial carcinoma
TAA	Tumor associated antigen
TPX	Microtubule Nucleation Factor
TTR	transthyretin
WB	Western immunoblot
WD	Well differentiated
<i>wtp53</i>	Wild type p53

Abstract

Ovarian cancer (OC) has the highest mortality among gynecological cancers. The high mortality is associated with the lack of an accurate screening tool to detect disease in early stage. As a result the majority of OCs are diagnosed in late stage. Further, the molecular events responsible for malignant transformation in the ovary remain poorly understood. Consequently, delineating key molecular players driving OC could help elucidate potential diagnostic, prognostic and therapeutic targets.

Receptor for hyaluronan-mediated motility (RHAMM) belongs to a group of hyaladherins, which share a common ability to bind to hyaluronan (HA). Intracellularly, RHAMM is involved in microtubule spindle assembly contributing to cell cycle progression. On the cell surface, loosely tethered RHAMM forms a complex with cluster differentiation 44 and HA to activate cell signaling pathways that promote cellular migration, invasion and proliferation. Since RHAMM is overexpressed in a number of cancer types and it is often associated with an aggressive cancer phenotype, I sought to determine if RHAMM similarly contributes to OC.

I found that RHAMM is overexpressed in clinical specimens of OC by immunohistochemistry and although both primary and metastatic OCs stain equally for RHAMM, RHAMM staining was most intense among clinically aggressive OC histologic subtypes. Further, using an in vitro model system, I was able to show that OC cells express and secrete RHAMM. Abrogation of RHAMM using silencing RNA technology inhibited OC cell migration

and invasion suggesting that RHAMM may contribute, at least in part, to the metastatic propensity of OC.

Since RHAMM lacks an export signal peptide sequence and has not been reported to employ alternate mechanisms for extracellular secretion, I utilized computational analyses to predict post-translational glycosylation events as a novel mode for RHAMM secretion. N-glycosylation inhibitors abrogated RHAMM secretion by OC cells in vitro validating my prediction and identify a novel and potentially unconventional mode for RHAMM secretion.

Lastly, since RHAMM is secreted by OC cells, I sought to determine whether RHAMM could be detected in bodily fluids. In a pilot study, I found that urinary levels of RHAMM are elevated in OC patients as measured by enzyme-linked immunosorbant assays. Decreased urinary RHAMM levels noted following cytoreductive surgery support OC as the source of elevated urinary RHAMM levels. Finally, while obesity was associated with high urinary RHAMM levels in OC patients, combined measurements of urinary RHAMM and serum CA125 improved prediction of OC.

Taken together, the studies described herein suggest that RHAMM contributes to OC and that further studies are warranted to further elucidate the clinical role of RHAMM in OC.

Chapter 1: Introduction

Ovarian Cancer Overview

Commonly described as the “silent killer”, ovarian cancer (OC) is the fifth leading cause of cancer related deaths in women and it has the highest mortality of all gynecological malignancies¹. In the United States, over 22,000 women will be diagnosed annually with over 14,000 deaths from OC. Roughly one in seventy women will develop OC in their lifetime with only a 45% five year survival². When disease has undergone metastatic spread, the survival rate drastically decreases from >90% in early stage disease to less than 30% in late stage¹. Late diagnosis is largely a result of ambiguous symptoms like bloating, stomach pain, back pain and irregular vaginal bleeding which can be attributed to female irregularities. This prevents women from seeking medical attention until it is too late and the disease has spread.

Risk factors for OC

The majority of OCs are sporadic in origin, but about 10% of all epithelial ovarian carcinomas (EOC) are associated with a hereditary predisposition and are characterized by an increased incidence and earlier onset of disease³. Therefore, family history (FH) is the biggest risk for OC with the majority of hereditary OCs associated with breast cancer susceptibility 1, (BRCA1), gene mutations (equating 1500 deaths annually)^{4,5,6,7}.

Epidemiological studies suggest that, besides race⁸ and FH of breast cancer (BC) or OC⁹, events associated with ovarian surface epithelial (OSE) traumatization may result in aberrant OSE growth leading to ovarian epithelial carcinogenesis^{10,11,12,13}. Specifically, it has been suggested that incessant ovulation^{10,11,12,13,14} causes rapid cycles of OSE division leading to errors in DNA replication and repair resulting in the inactivation and overexpression of tumor suppressor genes and oncogenes, respectively¹⁵. Thus, increased age, reproductive history (nulliparity), early menarche, late menopause and fertility drug use increase the risk for OC. In contrast, suppression of ovulation by pregnancy, lactation or oral contraceptive use decrease the risk for OC. Lifestyle factors including dietary fat intake and smoking may also increase the risk for OC while dietary intake of vitamins A,C, D and E may protect against OC^{16,17,18,19}.

OC Subtypes

Despite the fact that the simple squamous-cuboidal OSE^{20,21} comprises only a minute fraction of the total ovarian mass and is traditionally thought to be the source of OCs, the reasons for the propensity of OC development are still poorly understood. Nonetheless, EOCs account for the majority of OCs. Due to the common embryologic origin of OSE with the epithelia lining fallopian tube (FT), endometrium and cervix, histologic subtypes of epithelial ovarian tumors include serous (SC), endometrioid (EC) and mucinous (MUC) tumors²².

Malignant epithelial tumors comprise the most common types of OC, which includes SC, EC, MUC and clear cell carcinoma (CCC). EC and CCC are believed to arise from the endometriosis to the ovary²³ and are associated with pelvic endometriosis²⁴. EC typically

presents in stage I with a unilateral mass still confined to the ovary and makes up about 10% of all OC cases²⁴. CCC also presents as a unilateral mass in stage I or II, however if diagnosed late, CCC often has a poor prognosis due to high prevalence of drug resistance²³. MUC is the rarest subtype, only comprising 3% of OC and often presents as a large unilateral mass where bilateral nodes are often metastatic²⁴. Historically, MUC was commonly over-diagnosed due to the difficulty in distinguishing it from the gastrointestinal tract, however advances in gross examination and immunohistochemical staining markers have improved diagnostic accuracy²³.

Serous OC is most common type of EOC and makes up 70% of all cases of OC¹. There are two disease entities split into a dualistic model characterizing SC into type 1 or type 2 OC by their resemblance to non-neoplastic epithelia and their somatic mutation profile. Type 1 OCs are commonly low grade serous carcinomas (LGSC), relatively less aggressive, often arise from precursor lesions and have specific molecular markers including KRAS, BRAF, PTEN and CTNNB1²⁴. Conversely, type 2 OCs typically present as high grade serous carcinomas (HGSC) and have an aggressive phenotype. HGSC is the most common OC subtype and encompasses 60-80% of all ovarian epithelial malignancies². They are characterized by their chromosomal instability and mutant p53 profile^{25,26}.

The Recent Paradigm Shift in OC Etiology

While OC is traditionally thought to arise from the OSE, more recent evidence suggests an alternate extra-ovarian origin for HGSC. HGSC has a poorly defined pathogenesis, but is suggested to arise in the secretory epithelium of the distal FT which

then easily transfers to the ovarian surface^{27,28,29,30}. Pre-malignant lesions found in the FTs prophylactically removed by salpingectomy as well as serous tubal intraepithelial carcinomas (STIC)²⁸ support the FT as the potential source of HGSC^{27,31}. Further, HGSC resembles tissue from the FT epithelium (FTE)³². However, despite many attempts, precursor lesions that lead to HGSC have not yet been identified and progression to HGSC from a borderline low-grade tumor is rare.

Current Screening Methods and Biomarkers

As noted above, OC is a heterogeneous disease with multiple subtypes; all proven difficult to detect at an early stage. To date, pelvic examination, ultrasound or circulating cancer antigen 125 (CA125) serum levels are evaluated for OC detection. CA125 is elevated in about 80% of OC patients, but fails to detect approximately 50% of stage 1 OC patients^{33,34}. Serum CA125 is also frequently up-regulated in normal conditions, benign reproductive disease and other cancer types³⁵. Measuring CA125 levels has been reported to cause patient anxiety and, in many cases, unnecessary surgery³⁴.

To increase specificity of OC detection, multi-marker panels, such as combinations of CA125 and human epididymis protein 4 (HE4), are being studied, but require further validation³⁶. HE4 has been shown to be elevated in the serum of OC and endometrial cancer patients³⁷. Although early reports of using sera HE4 as a biomarker for OC appeared very promising, drawbacks have been noted. Like CA125, HE4 has been shown to be elevated in other diseases such as lung cancer³⁸, kidney fibrosis³⁹ and chronic kidney disease⁴⁰. In addition, recent comparative analysis studies of HE4 and risk for ovarian malignancy algorithm (ROMA), which scores the likelihood of malignancy

being seen at surgery based off the results of HE4 assay, ARCHITECT CA 125 II and menopause status, did not increase the detection of OC compared to CA125 alone.³⁷ However, other applications of HE4 as a cancer biomarker are being investigated, including its use to detect mucinous OC or disease progression of endometrial cancer.⁴¹

Other biomarkers are being evaluated in combination with CA125 to improve OC detection, especially as that pertains to detecting early stage OC. For example, mesothelin is elevated in ~70% of early stage serous OC, thereby improving OC detection rates by CA125 alone⁴². Osteopontin (OPN) is under review as a combinatorial marker with CA125 because OPN levels are histologically elevated in metastatic OC lesions⁴³, serum OPN levels decrease following cytoreductive surgery⁴⁴ and an unique COOH-terminal truncated OPN form has been reported to be elevated in OC patient urine⁴⁵. Prior studies have also suggested that measuring serum CA125 and lysophosphatidic acid (LPA) together could be clinically important since LPA is found in ascites fluid of OC patients and is also elevated in OC patient plasma⁴⁶. Previous work in our lab reported that urinary levels of the anti-apoptotic protein, Bcl-2, are elevated in early and late stage OC with a tendency for increased urinary Bcl-2 levels with increasing tumor grade and stage. We also showed that the combination of urinary Bcl-2 and serum CA125 increased detection of both early and late stage OC patients.⁴⁷ Lastly, the multi-biomarker panel consisting of transthyretin (TTR), ApoA1, transferrin, beta 2 microglobulin and CA125 comprise the OVA1 test is already used clinically. This test best exemplifies the use of multiple markers for the most effective results assessing the risk for OC in women who present with an adnexal mass when radiological evaluation is unsure for malignancy⁴⁸.

Despite many advances in medical research, neither the survival rate nor the

treatment for OC has changed significantly for over 30 years. Optimal cytoreductive surgery followed by platinum based chemotherapy remains the mainstay of therapy in the management of advanced EOCs²⁰. However, while the response rate to primary chemotherapy can be as high as 76%, response rate is dramatically reduced after relapse of disease⁴⁹. Platinum resistance, defined as disease recurrence less than six months from completion of therapy is an important prognostic predictor. Patients with platinum-resistant tumors have a response rate of less than 10% when retreated with platinum compounds⁵⁰. Alternative options also have poor response rates of 18-30%^{51,52,53,54,55,56,57,54,58,59}. Together, this underscores the need to improve our understanding of this disease and its etiology. Delineating key players driving OC could help elucidate potential molecular diagnostic, prognostic and therapeutic targets.

RHAMM: Overview

Receptor for hyaluronan-mediated motility (RHAMM) belongs to a group of hyaladherins, which share a common ability to bind to hyaluronan (HA). RHAMM is located on chromosome 5, q arm, band 34, has a molecular mass of 85kDa and is 724 amino acids (AA) in length. Based on subcellular localization, RHAMM performs multiple functions. Intracellularly, RHAMM is involved in microtubule spindle assembly, thereby contributing to cell cycle progression⁶⁰. On the extracellular surface, RHAMM forms a trimeric complex with cluster differentiation 44 (CD44) and HA to activate cell signaling pathways that promote migration, invasion and cell proliferation⁶¹. While RHAMM is overexpressed in hematological malignancies and solid tumors arising from prostate⁶², bladder⁶³ and breast⁶⁴, it is not known whether RHAMM contributes to OC.

Although minimally expressed in normal tissue, elevated RHAMM in BC and colorectal cancer (CRC) is associated with poor clinical outcome and a more aggressive cancer phenotype^{64,65}.

RHAMM Domains

RHAMM consists of five known functional domains. Each domain consists of an alpha helical coiled-coil separated by non-coiled sections. The first domain, encompassing the N-terminus, is a non-essential domain that is often missing in the truncated forms commonly seen in cancer. The function of domain 1 (D1) is not entirely clear, however, it is hypothesized to negatively regulate the remaining functional domains of RHAMM (D2-D5). D2 is reportedly a well conserved leucine-zipper motif and has been shown to enable extracellular matrix (ECM) fibronectin to bind to cell surface RHAMM, in order to promote podosome formation and cell motility⁶⁶. The function of D3 appears to be intracellular such that D3 encodes a region of RHAMM that associates with intracellular mitogen activated protein kinase 1 (MEK1) to promote an intracellular RHAMM/MEK1/extracellular signal regulated kinase 1(ERK1) complex⁶¹. D4 has both extracellular and intracellular functions. D4 has been shown to mediate cell motility and focal adhesion turnover by cell surface RHAMM while the D4 domain of intracellular RHAMM encodes a region required for RHAMM to bind to ERK1, which then activates the MAP kinase signaling cascade. Finally, D5 of extracellular RHAMM is the HA binding domain composed of 9 to 11 amino acids ordered in a basic BX₇B motif⁶⁷. This domain consists of basic amino acid sequences that bind solely to HA and is key for cell motility driven by extracellular RHAMM. The D5 domain of intracellular

RHAMM also binds to ERK1 and blocking this region prevents intracellular formation of the RHAMM/ERK1 complex⁶⁸.

RHAMM Splice Variants

RHAMM is known to have at least 5 splice variant isoforms. Full length RHAMM (RHAMMv5) is the canonical form of RHAMM and consists of all 5 functional domains. Alternative splice variant RHAMMv5(-48bp) and RHAMMv5(-147bp)⁶⁹ truncations are seen variably overexpressed in different malignancies. Abnormal quantities of the splice variant RHAMMv5(-48bp) compared to full length RHAMM are associated with poor prognosis in multiple myeloma (MM), indicating deregulation of these proteins in cancer can interrupt cell cycle progression, increase cell motility and promote cell proliferation⁷⁰.

Additionally, specific RHAMM isoforms contribute wound healing. For example, immediately following injury in smooth muscle cells (SMCs), western immunoblots (WB) detected a 65kDA RHAMM isoform band and 1 hour after injury, a 70kDA RHAMM isoform demonstrating a progressive shift in RHAMM isoforms band expression associated with advancing stages of wound healing⁷¹.

A minor transcript of RHAMM containing exon 4 (RHAMMv4) is transforming when it is overexpressed and it has been shown to mediate Ras/ERK signaling⁷². This variant encodes a RHAMM isoform found on the cell surface and in the cytoplasm as determined by epitope-tagging flow cytometry⁷³ which has been shown to regulate Ras-ERK signaling. This RHAMMv4 variant, then, plays different depending on its cellular

location⁷³ although are both intracellular and extracellular RHAMMv4 must be present for ERK activation by platelet derived growth factor (PDGF)⁷³.

Intracellular RHAMM

Functions of intracellular RHAMM are centered on mitotic spindle assembly, Ras signaling and actin binding. RHAMMs association with centrosome formation⁶⁶ and mitotic spindle assembly⁷⁴ strongly suggests intracellular RHAMM is important for cell cycle progression. During post-mitotic interphase, RHAMMs' degradation through ubiquitination allows mitotic spindle assembly through the BRCA1/BARD1 complex to mediate microtubule nucleation factor (TPX2) localization. RHAMMs' ubiquitination releases TPX2 from the spindle pole signaling aurora kinase A (AURKA) activation and, thus, driving cell cycle progression⁷⁵. Additionally, prolonged presence of RHAMM can attenuate cell cycle progression by suppressing the protein complex cdc2/cyclinB1, leading to cell cycle arrest⁷⁶. Intracellular RHAMM has also been shown to directly bind to ERK within the cell and can activate signaling downstream of RAS. Within the cytoplasm, activated ERK can indirectly activate ribosomal protein S6 kinase (RSKs) and ERK 1,2 translocation to the nucleus is critical for gene expression and DNA replication⁷⁷. This subsequently leads to the activation of transcription factors activator protein 1 (AP-1), ETS domain-containing protein (ELK-1) and Myc that promote downstream transcription of proteins involved with proliferation, migration, invasion and adhesion. Lastly, RHAMM can associate with cell actin filaments as well as microtubules to modulate the cytoskeletal network⁷⁴. In this way, RHAMM/tubulin interactions

influence MEK/ERK1,2 mediated instability of the mitotic spindle since the stability/instability of this scaffold is critical for cellular proliferation and migration⁷⁸

Extracellular RHAMM

Cell surface RHAMM has been reported to be key in activating signaling cascades. Extracellular RHAMM binds to exogenous HA and acts as a co-receptor for HA with CD44⁷⁹ and PDGF⁸⁰ which transduce the ERK 1,2 MAPKinase signaling cascade. Subsequently, this activates Erk kinases⁷³, Src⁸¹, protein kinase C, and focal adhesion kinases⁷² (FAK) leading to increased migration, invasion, proliferation and adhesion.

Upon binding with HA, RHAMM activates the phosphorylation of ERK 1,2. This promotes ERK 1,2 translocation into the nucleus and contributes to the transcriptional machinery of mitogenic genes⁸². Downstream effectors of the ERK 1,2 pathway as noted above then lead to tumor progression and invasion⁸³ and cell motility after injury^{71,81}.

RHAMM in Wound Healing

Originally found secreted from embryonic chick heart fibroblasts, RHAMM has been well studied as a key player in normal wound repair. When studying the HA content surrounding excisional fetal skin wounds compared to incisional fetal skin wounds, Lovvoran et al. 1998 reported markedly more CD44 and RHAMM immunohistochemical (IHC) staining along the excisional wounds from day 1 to day 7 suggesting a role for RHAMM in fibroplasia⁸⁴. Similarly, fibroblasts from mice with a genetic deletion of

RHAMM failed to heal scratch wounds > 3mm or invade HA supplemented collagen gels in culture⁸⁵. These researchers also showed that RHAMM is required for CD44 cell surface localization, ERK1,2/CD44 trimeric complex and ERK 1,2 localization to the cell nucleus confirming that RHAMM is essential for fibroblast mitogenic signaling in wound repair. It is not surprising, then, that exogenous HA is now used extensively in the dermatological field to decrease wound-healing time and scar formation through RHAMM signaling pathway.

Interestingly, arterial wound repair and contraction by SMCs, characterized by ECM remodeling and collagen deposition, is similarly mediated by HA/RHAMM as in cutaneous wound repair. SMCs devoid of RHAMM demonstrated reduced adhesion to collagen both in vivo and in vitro indicating that clinical inhibition of RHAMM may reduce constrictive arterial remodeling and stenosis⁸⁶.

RHAMMN in Breast Cancer

As demonstrated in BC, both intracellular and extracellular RHAMM contributes to malignant disease. Early studies by Zhang et al. 1998 using real-time polymerase chain reaction (RT-PCR) and IHC demonstrated RHAMM overexpression correlated with poor prognosis in BC⁶¹. These researchers also reported that RHAMM overexpression in human specimens correlated with overexpression of ERK and Ras, thereby confirming the association of RHAMM with Ras and ERK previously seen in murine fibroblasts. Similarly, using RT-PCR, florescent staining and subcellular fractionation, Assman et al. 1998 reported high levels of cytoplasmic RHAMM in BC cell lines⁸⁷ which

complemented IHC studies indicating overexpression of intracellular RHAMM in invasive lobular carcinomas related to reduced overall patient survival time⁸⁸.

As noted earlier, it has also been shown that, in invasive BC cell lines, RHAMM forms a trimeric complex with CD44 on the cell surface to initiate the ERK 1,2 MAP kinase pathway⁸². Sustained ERK 1,2/MAP kinase signaling driven by the RHAMM/CD44 complex appears necessary to maintain and drive BC cell motility. Likewise, particularly aggressive subsets of BC cells localized to local and lung micrometastases demonstrate high levels of RHAMM located in extracellular CD44/RHAMM complexes⁸⁹.

Lastly, a link between RHAMM and BRCA1 has been identified by genetic analyses which shows that a specific genetic variation in RHAMM appears to modify BC risk in BRCA1 mutation carriers⁹⁰. Blanco et al. 2015 showed that interaction between RHAMM, BRCA1, TPX and AURKA was essential for microtubule organization that contributes to the regulation of apicobasal polarity in BC cells. Modulation/loss of any of these components appeared to increase risk for BC. Genotyping performed on 15,252 BRCA1 and 8,211 BRCA2 mutation carriers further confirmed the potential for RHAMM as a BC susceptibility gene⁸⁹.

RHAMM in Hematological Malignancies

Serological analysis of recombinant tumor cDNA expression libraries (SEREX) identified RHAMM as a leukemia associated antigen (LAA) and tumor associated antigen (TAA). LAAs elicit high-titer host immunoglobulin G (IgG) reactivity from B-cells stimulated by antigen-specific helper T cells since it is believed that LAAs may

have epitopes recognized by CD4⁺ and CD8⁺ T lymphocytes⁹¹. Consequently, LAAs present as potential antigenic structural targets for cellular immunotherapies and antibody therapies. In this way, RHAMM is currently under investigation as a target for vaccine immunotherapy to enhance the effect of graft-vs.-host benefits after stem cell transplantation in AML patients.

Alternate Protein Secretion

The classical secretory pathway for protein export is well conserved, well defined and requires an endoplasmic reticulum (ER) signal transport sequence typically located at the protein N-terminus⁹². Newly translated proteins destined for extracellular secretion or integration into the cell membrane travel through the rough ER and the Golgi complex where they undergo similar modifications and are packaged for cell surface transport⁹². Secreted proteins that lack a signal peptide sequence have been shown to employ alternate export mechanisms including transporter channels, protein-release complexes, flippase activity, exocytosis and membrane blebbing⁹³. Interestingly, like RHAMM, many proteins lacking an export peptide sequence have cytoplasmic functions that widely differ from their extracellular function⁹⁴. Additionally, cytoplasmic proteins that employ alternate export mechanisms are often overexpressed and secreted as a result of cellular transformation^{72,95,96}. While it has been well established that RHAMM lacks a signal export peptide sequence and there are no reports that it is secreted by alternate export mechanisms, its mechanism for secretion is still unknown.

Rationale

RHAMM is overexpressed in a number of cancer types. It is often associated with an aggressive cancer phenotype due, in part, to its ability to promote cellular migration and invasion, thereby contributing to poor clinical outcome. Herein, I sought to determine if RHAMM similarly contributes to OC.

Central Hypothesis

Overexpression and secretion of RHAMM contributes to OC disease progression. Three specific aims are proposed to test this hypothesis.

Specific Aims

Aim 1. Determine the contribution of RHAMM to OC progression.

To begin to assess the role of RHAMM in OC, I will first demonstrate overexpression of RHAMM in clinical samples of OC and then, using an in vitro cell culture model system, I will determine whether RHAMM promotes OC cell migration/invasion.

Aim 2. Delineate the mechanism of RHAMM secretion in OC.

Although RHAMM has discrete intra- and extracellular functions that could promote tumor progression, it lacks a signal peptide transport sequence. Therefore, the mechanism by which RHAMM is secreted is unknown. Using computational analyses supported by in vitro validation, I will suggest an alternate, signal peptide transport sequence-independent mechanism by which RHAMM may be secreted by OC cells.

Aim 3. Evaluate RHAMM as a novel urinary biomarker for OC.

Since RHAMM is only loosely tethered to the external cell surface, elevated levels of RHAMM may be found in bodily fluids of OC patients. Therefore, I will measure and evaluate the potential of urinary RHAMM levels among healthy controls, women with benign gynecologic disease and OC to serve as a novel OC biomarker.

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Chapter 2: RHAMM is overexpressed in OC

Background

As discussed in chapter 1, intracellular RHAMM is involved in microtubule spindle assembly and it contributes to cell cycle progression¹. On the extracellular surface, RHAMM forms a trimeric complex with cluster differentiation 44 (CD44) and HA to activate extracellular signal regulated kinase 1/2 (ERK1/2) cell signaling pathways to promote migration, invasion and cell proliferation². Although minimally expressed in normal tissue, RHAMM is overexpressed in hematological malignancies and solid tumors arising from prostate³, bladder⁴ and breast⁵. In addition, elevated RHAMM in breast cancer (BC) and colorectal cancer (CRC) has been associated with poor clinical outcome and a more aggressive cancer phenotype^{5,6, 7,8}. In order to determine whether Rhamm might likewise contribute to ovarian cancer progression, I began my studies by determining whether RHAMM is overexpressed in clinical specimens.

Methods

Clinical Specimens

With University of South Florida Institutional Review Board approval and patient consent, tissues were collected from a cohort of women who had undergone primary surgery with complete surgical staging for epithelial ovarian cancer (EOC) or borderline tumors at the Moffitt

Cancer Center and the University of South Florida (Table 2.1). This gynecologic oncology database was also used to select women who had undergone oophrectomy due to cystadenoma or had their ovaries removed for unrelated pathology. All tissue specimens were di-identified prior to release for research. All tissues were fixed with 10% formalin, paraffin-embedded, sectioned and stained with hematoxylin and eosin (H & E). The slides were reviewed by Drs. Champeaux and Nicosia to confirm histologic diagnosis according to the International Federation of Gynecology and Obstetrics (FIGO) classification system.

Immunohistochemistry

For immunohistochemical (IHC) studies, formalin-fixed paraffin sections were cut at 3 microns and dried overnight at room temperature (RT) then deparaffinized and rehydrated. Sections were incubated in BLOXALL™ Blocking Solution (Vector Laboratories, Burlingame, Ca) for 20 minutes to block endogenous peroxidase activity. Antigen retrieval was achieved by placing slides in 1× solution Antigen Unmasking Solution (Vector Laboratories, Burlingame, Ca) brought to a boil and maintained at 95 °C for 30 minutes on a hot plate. Specimens were then immunostained using Rabbit Anti-Human CD168 (ThermoFisher Scientific, Waltham, MA) primary antibody (1:100) for 1 hour and Vectastain® Elite ABC Kit (Vector Laboratories)+ Dako's EnVision™ + HRP Rabbit (DAB+) kit according to the manufacturer's instructions, then counterstained with modified Mayer's haematoxylin, dehydrated through graded alcohol, cleared with xylene, and mounted with resinous mounting medium. To control variability, all samples were stained at the same time and with the same lot of reagents. Breast carcinoma (BC) was used as an internal positive control while negative controls were obtained by substitution of primary antibody with normal rabbit serum.

Evaluation of RHAMM staining

A minimum of 100 cells were counted at a final magnification of 400X per tissue section. Immunostaining of RHAMM was scored based on average percent of positive epithelial cells (0, negative or no staining; 1+, <30%; 2+, 30-50% and; 3+, >50%) and on staining intensity (negative, weak, moderate and strong). Cellular localization of RHAMM was also assessed as cytoplasmic, membranous, nuclear or stromal.

Images

Images were acquired on a digital *Olympus DP-20* camera under a *Leica dmire2* microscope. *Olympus* micro imaging software *CellSens* platform was used to acquire and process images. Images were taken with a final magnification of 100x and 400x.

Results

RHAMM is overexpressed in OC.

Immunological staining was performed on 44 tissue sections from 36 women. The sample population provided sections of normal ovarian surface epithelium (OSE) (n=5), serous OC (n=22), omental metastasis (n=7), lymph node metastasis (n=1), endometrioid OC (n=1), clear cell carcinoma of the ovary (CCC) (n=1), mucinous OC (n=1) and normal fallopian tube (FT) (n=6). Though these samples comprise a small pilot study, they are representative of a typical clinical practice with regards to histological distribution (Table 2.1). BC was used as a positive control (n=2).

Overall, I found that 91% (20/22) of serous OC stained positively for RHAMM with levels of staining intensity ranging from weak (<30% from each field, N=4), moderate (30-50%, N=7) to

strong (>50%, N=9) while 0% (0/5) of normal OSE stained for RHAMM. (Figure 2.1, Table 2.2) Staining patterns, as depicted by intense, punctate/diffuse cytoplasmic staining, seen in BC positive control were consistent with previous reports of RHAMM in BC where intense staining is predominately in the cytoplasm and nucleus, but negative in the stroma^{9,10}.

RHAMM expression was predominately localized in the cytoplasm in 91% (20/22) of the serous OC specimens while the surrounding stromal tissue remained negative (Figure 2.1, Table 2.3). Further, membranous staining was seen in 27% (6/22) of serous OC (Figure 2.1D, E, F &H) and 18% (4/22) of serous OC specimens demonstrated nuclear staining (Figure 2.1H). Well differentiated (WD) serous OC displayed membranous RHAMM staining localized to the apical cell surface (Figure 2.1D, E, F & H). Interestingly, I noted RHAMM staining in OC cysts (Figure 2.1D&F).

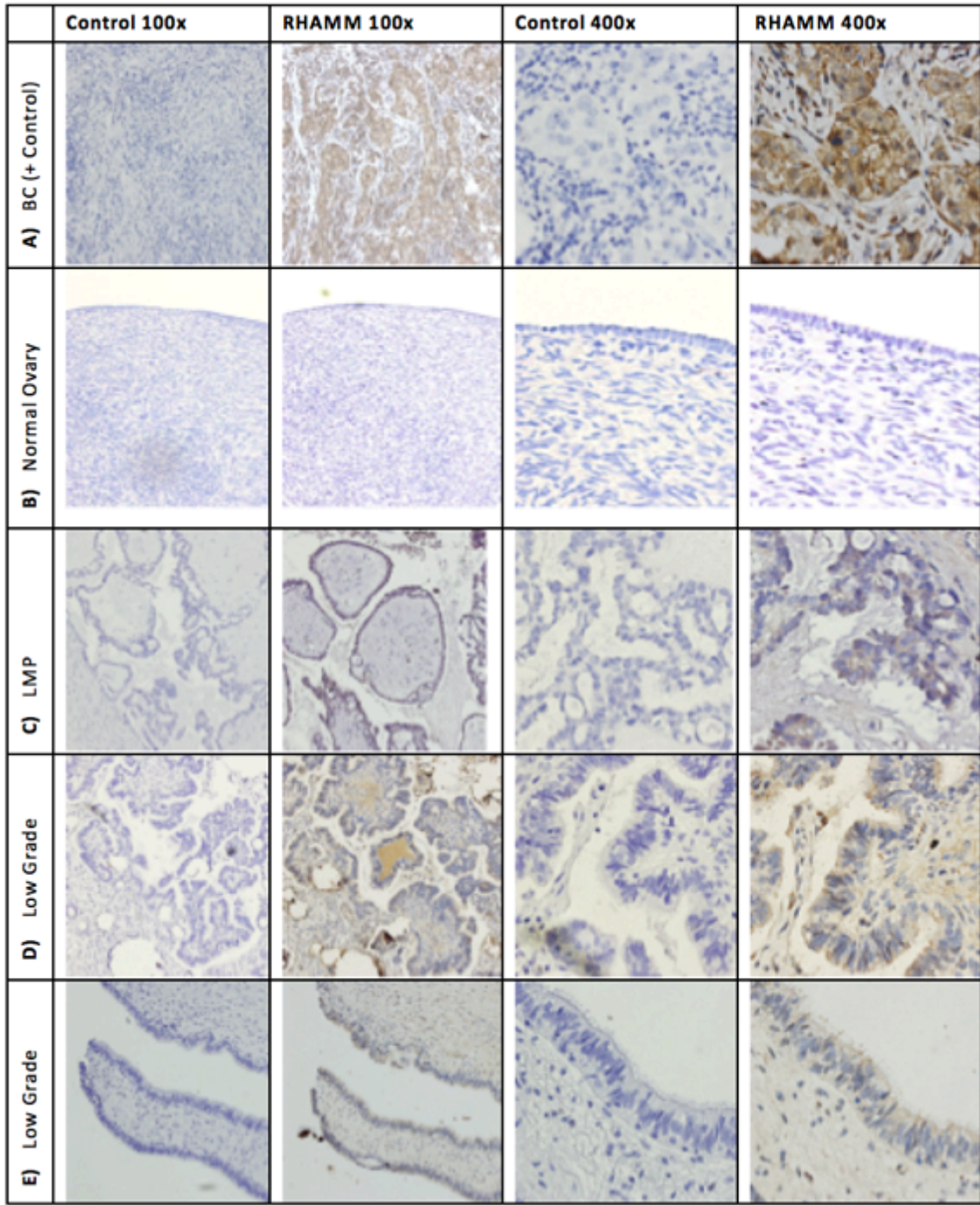


Figure 2.1: RHAMM staining is elevated in OC tissue specimens (Continued on Next Page)

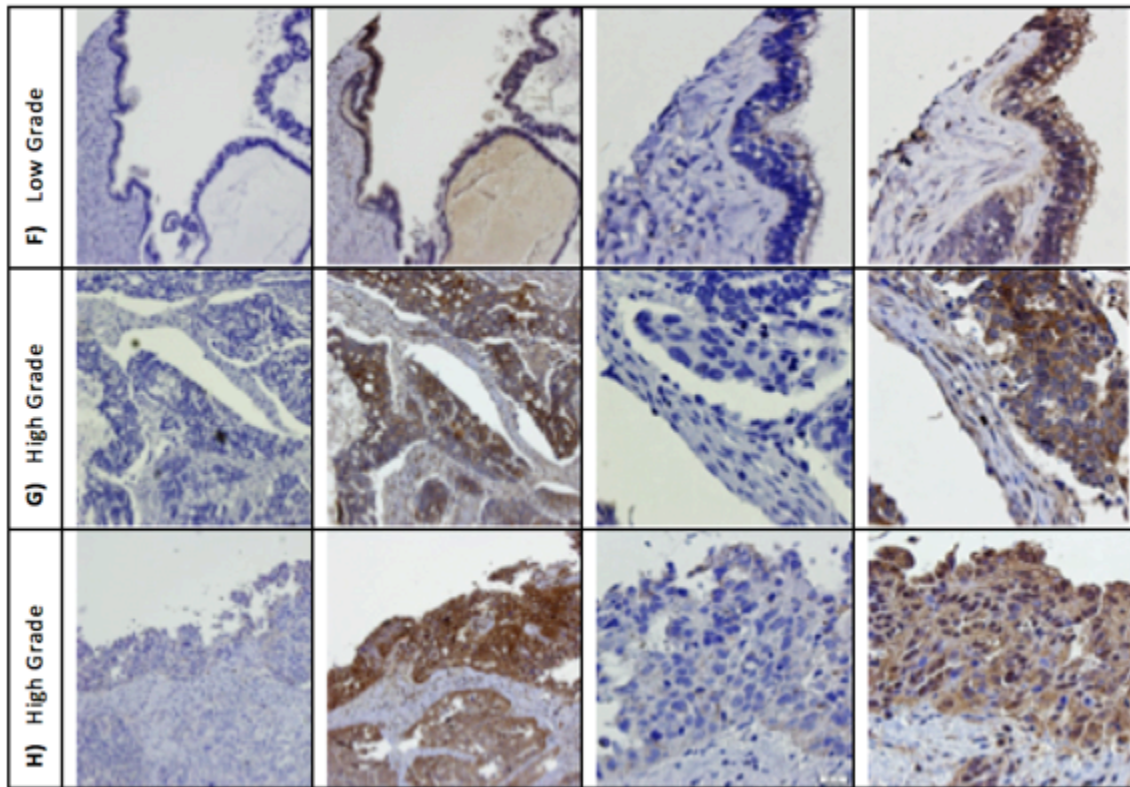


Figure 2.1: RHAMM staining is elevated in OC tissue specimens

Representative photographs of IHC staining for RHAMM [anti-CD168 polyclonal antibody (PA5-32309) with a 1:100 dilution] in B) normal, C) low malignant potential (LMP), D&E) WD and F&G) PD serous OC. PD breast carcinoma was used as a positive control (A). Control sections were incubated with non-immune serum. Original magnification 100x and 400x.

RHAMM staining intensity increases with grade in serous OC.

Poorly differentiated (PD) OC sections displayed intense punctate staining while WD OC displayed mostly weak and homogeneously diffuse staining (Figure 2.1, Table 2.2). Although percentages of positive staining were similar in WD (8/10 or 80%) and PD (14/15 or 93%) serous OC, increased staining intensity correlated with increasing grade (Figure 2.1, Table 2.1).

RHAMM is overexpressed in metastatic disease.

When I compared RHAMM staining in primary tumors and their respective metastases for eight serous OC patients, I found positive RHAMM staining in 88% (7/8) of the primary tumors and 88% (7/8) of their omental or 100% (1/1) of lymph node metastases. Matching RHAMM staining percentages, intensities and cellular localization were seen in primary tumors and their respective metastases among all 8 patient samples (Figure 2.2, Table 2.4).

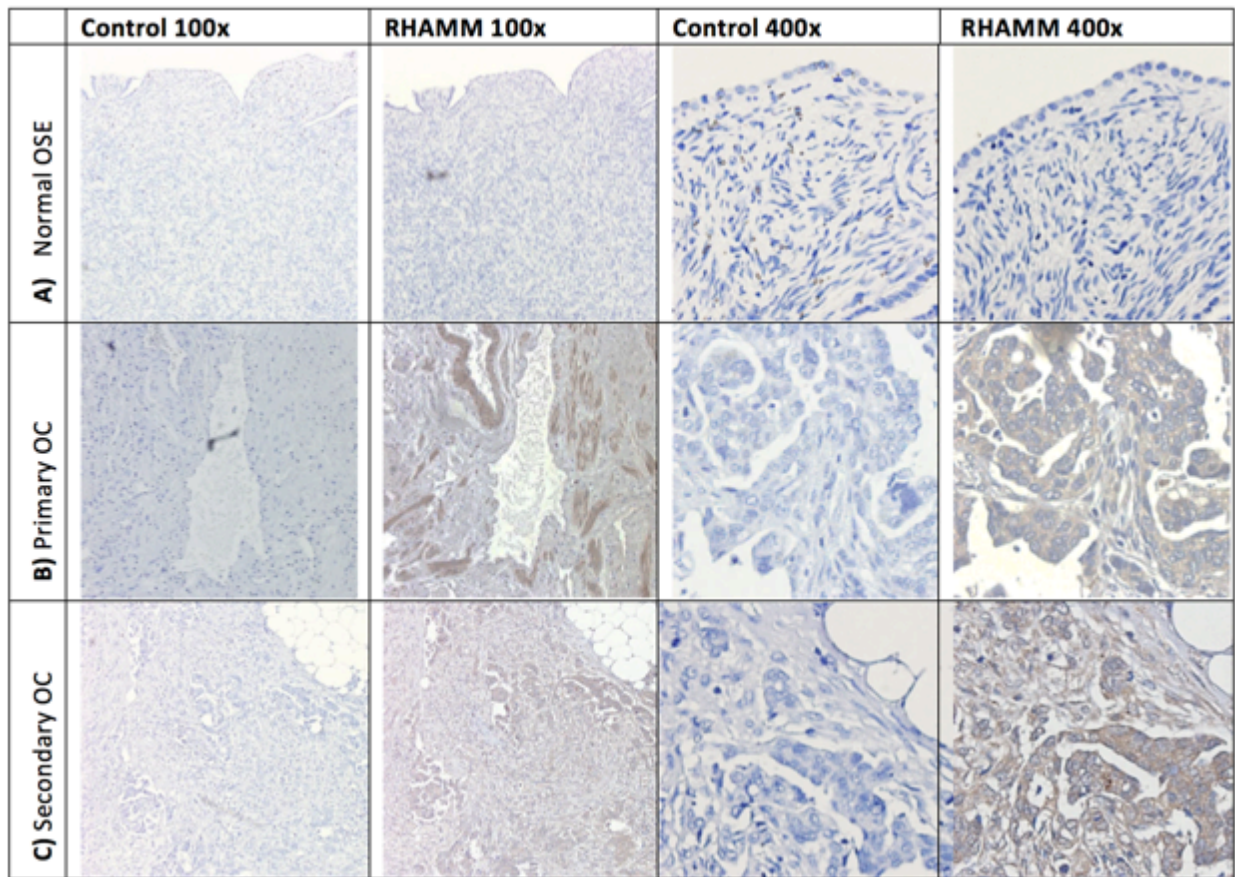
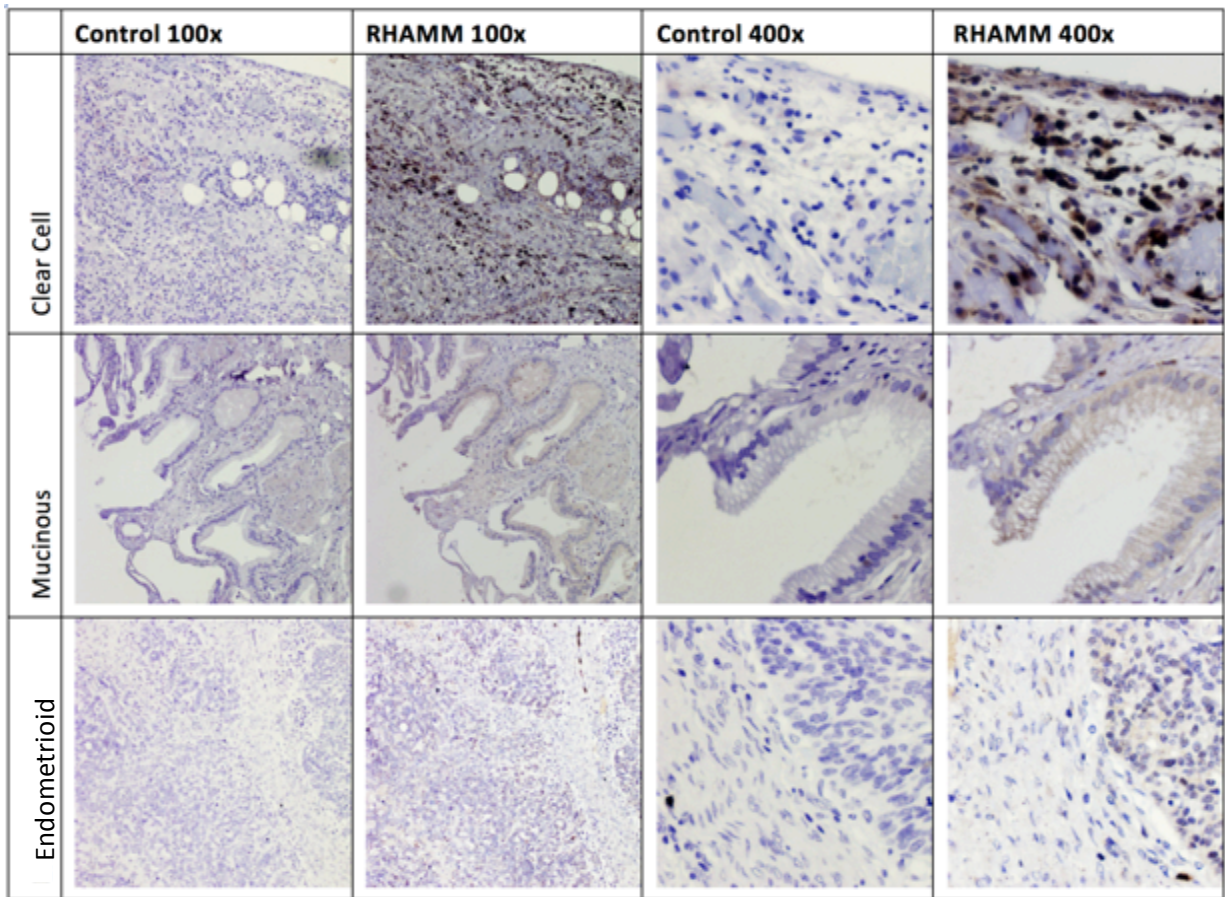


Figure 2.2: Primary and metastatic OC stain equally for RHAMM

Representative photographs of IHC staining for RHAMM in multiple sections from within the same patient of A) normal, B) primary serous adenocarcinoma and C) omental metastatic tissue. Control sections were incubated with non-immune serum. Original magnification 100x and 400x.

RHAMM is variably expressed in different histological subtypes.

Additionally, I examined other histological subtypes of OC and found that endometrioid OC was devoid of RHAMM staining and only weakly positive RHAMM staining was seen in mucinous OC, but RHAMM stained intensely in CCC (Figure 2.3). The CCC specimen demonstrated intense, punctate nuclear RHAMM staining in contrast to predominately cytoplasmic and membranous staining seen in serous OC. Weakly, positive RHAMM staining was seen in the mucinous OC and staining appeared localized to the apical membranous cell surface (Figure 2.3).



**Figure 2.3: RHAMM is differentially expressed among OC histological subtypes.
(Continued on Next Page)**

Figure 2.3: RHAMM is differentially expressed among OC histological subtypes.

Representative photographs of IHC staining for RHAMM from tissues of CCC, mucinous OC and endometrioid OC. Control sections were incubated with non-immune serum. Original magnification 100x and 400x.

Normal fallopian tube epithelium (FTE) stains intensely for RHAMM.

Compared to normal OSE, which failed to stain for RHAMM, I found 100% (6/6) positive RHAMM staining in all normal FTE specimens examined (Figure 2.4). There was moderate to strong staining limited to the surface of the fimbrial epithelium, cytoplasm and nucleus while stromal elements did not stain for RHAMM. Regions with the strongest staining were predominately at the apical surface of the fimbrial epithelial cells (Figure 2.4).

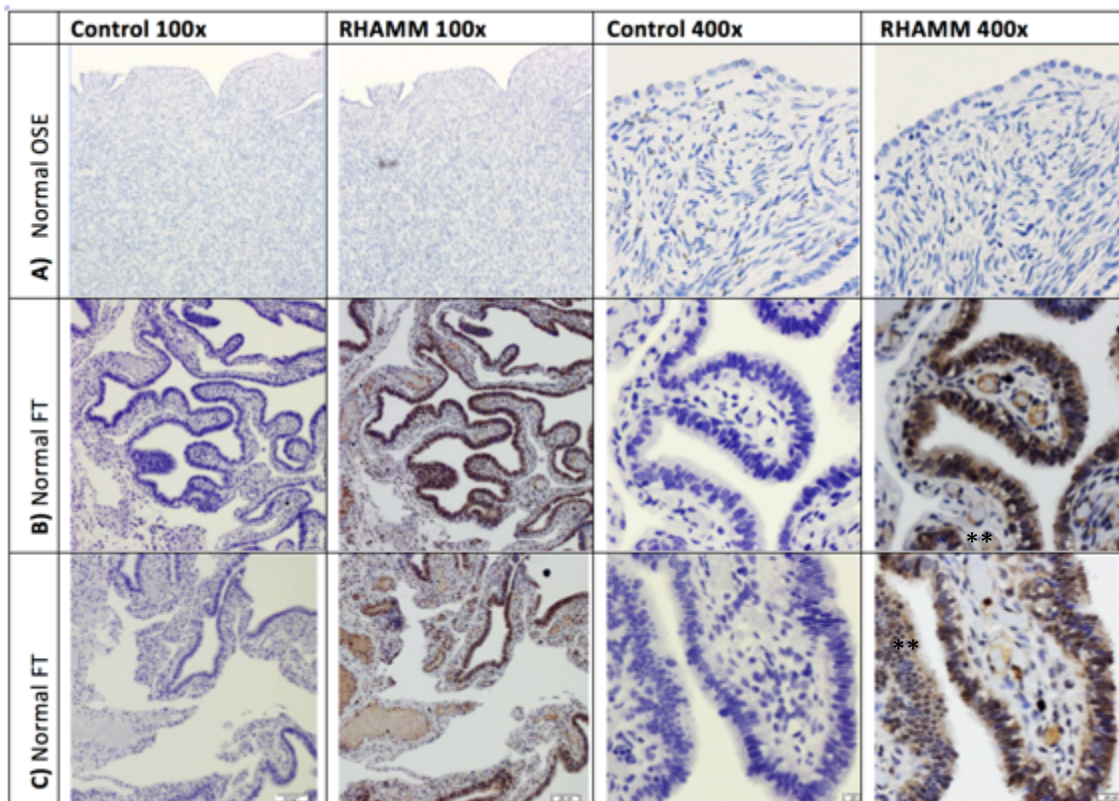


Figure 2.4. Normal FT stains intensely for RHAMM

Representative photographs of IHC staining for RHAMM in normal FT. Control sections were incubated with non-immune serum. Original magnification 100x and 400x.

Conclusion

Overall survival of OC has not improved for several years due to poor understanding of its pathogenesis, late diagnosis, emergence of drug resistance and lack of reliable biomarkers. Consequently, in order to better elucidate the etiology of this disease, the aim of this pilot study was to determine if, like other cancer types, RHAMM is overexpressed in OC and whether RHAMM could, likewise, promote OC progression.

I show for the first time that RHAMM expression is elevated in serous OC compared to normal OSE. I observed 91% of serous OC patients demonstrated positive RHAMM staining which was localized primarily to the cytoplasm, cell membrane, cystic fluid and, occasionally, the nucleus. RHAMM staining appeared to be specific to epithelial tumor cells since the stroma failed to stain. I also showed that RHAMM staining intensity increased with increasing cancer grade. Levels of RHAMM appear to be dependent upon the extent of differentiation where WD specimens demonstrated intense, punctate staining while PD specimens typically demonstrated intense, but rather diffuse RHAMM staining. High levels of RHAMM in aggressive colorectal cancer tumor budding cells are associated with higher grade, poor survival, increased lymphatic invasion and nodal metastasis¹¹. Additionally, invasive BC cell lines express higher levels of RHAMM¹² and IHC staining in 189 mammary carcinomas revealed that elevated RHAMM in lobular carcinomas is correlated with more invasive behavior and reduced overall patient survival time¹⁰. Therefore, high levels of RHAMM seen in high grade OC could contribute to an invasive phenotype. High levels of HA have been reported in the ovarian tumor microenvironment¹³, such that HA may mediate RHAMM overexpression and activation in OC leading to cell transformation¹⁴, initiation of the ERK1/2 MAPkinase pathway¹⁵ and cell proliferation^{16,17}.

Upon binding to HA, extracellular RHAMM activates the ERK1/2 MAP kinase pathway leading to the transcription and translation of proteins associated with migration and invasion. Downstream effectors of this pathway include transcription factors AP-1 and SP1, known to play roles in DNA damage repair, cell cycle control, migration and invasion¹⁸. Cytoplasmic RHAMM binds to actin and microtubules in the cytoskeleton and plays a role in cell cycle progression through the G2M phase¹⁹. Lack of RHAMM in normal tissue suggests elevated RHAMM in OC may contribute to an invasive phenotype in OC.

Though only few samples of endometrioid, mucinous and CCC were available, my samples reflect the incidence of OC histosubtypes. Of OC malignancies, CCC, mucinous and endometrioid only comprise about 5%, 10% and 15% of cases, respectively²⁰. Endometrioid OC failed to stain for RHAMM and mucinous OC stained weakly in agreement with the lesser aggressive clinical behaviors of these OC histosubtypes. In contrast, CCC showed a distinct pattern of intense nuclear RHAMM staining. Recent evidence has shown intense cytoplasmic IHC staining of Naspin A as a specific marker for CCC²¹. Given that CCC can require a specific course of treatment and careful monitoring due to its poor prognosis and resistance to chemotherapeutics²², that addition of nuclear RHAMM staining could expand the known molecular profile of CCC. However, it is possible that these single incidences do not representatively reflect the population of OC cases, therefore more samples would be needed to determine RHAMM staining in endometrioid, mucinous and CCC histosubtypes.

Since recent studies suggest that high grade serous ovarian cancer (HGSOC) arises from the FTE²³, I also subjected normal FTE to IHC staining for RHAMM. Distinct molecular markers of HGSOC include dysregulation of wild type p53 (*wtp53*) which is seen in about 96% of HGSOC cases. Dysregulation of *wtp53* can occur as gain of function (GOF) mutations, termed TP53

mutations. These mutations often lead to the acquisition of oncogenic functions, abrogating the cell cycle constraints controlled by wtp53²⁴. Since wtp53 is a negative transcriptional regulator of RHAMM protein⁷, RHAMM mRNA and protein levels are normally down regulated in the presence of wtp53. Conversely, I speculate that dysregulation of wtp53 in OC could drive overexpression of RHAMM. Interestingly, RHAMM was strongly evident in all of the normal FT specimens examined, showing the most intense staining at the apical end of the fimbriae. Reports of morphological variation among normal FTE cells²⁵ and presence of wt p53 mutations in non-suspicious FTE²⁶, suggest that underlying p53 mutations present in apparently normal FTE could modulate RHAMM expression prior to visualization of overt changes associated with malignant transformation. Dysregulation of RHAMM in the FT in concert with wtp53 mutations could contribute to pre-malignant lesions and, thus, drive oncogenic progression to HGSOc.

Table 2.1 – Summary of the patient cohort.

	N=36 Patients	Low Grade Well Differentiated	High Grade Poorly Differentiated
Normal	5		
Ovarian Cancer	25	10	15
Histology			
Serous	22	7	15
Endometrioid	1	1	
Mucinous	1	1	
Clear Cell	1	1	
Stage			
I	3	3	0
II	3	0	3
III	11	5	6
IV	8	2	6
Other			
Normal Fallopian Tube	6	N/A	N/A
Breast Cancer	2	1	1

Table 2.2- Quantification of RHAMM IHC.

	N	Positive	% Staining			Staining Intensity		
			<30%	30-50%	>50%	Weak	Moderate	Strong
Normal	5	0						
Cancer	25	22/25	5/25	8/25	9/25	6/25	6/25	10/25
Histology								
Serous	22	20/22	4/22	7/22	9/22	5/22	6/22	9/22
Endometrioid	1	0	0	0	0	0	0	0
Mucinous	1	1/1	1/1	0	0	1/1	0	0
Clear Cell	1	1/1	0	1/1	0	0	0	1/1
Grade								
Low	10	8/10	3/10	4/10	1/10	4/10	2/10	2/10
High	15	14/15	2/15	4/15	8/15	2/15	4/15	8/15
Stage								
I	3	3/3	1/3	2/3	0/3	1/3	1/3	1/3
II	3	3/3	1/3	1/3	1/3	0/3	2/3	1/3
III	11	9/11	2/11	3/11	4/11	4/11	1/11	4/10
IV	8	7/8	1/8	2/8	4/8	1/8	2/8	4/8
Fallopian Tube	6	6/6	0	1/6	5/6	1/6	2/6	3/6

Table 2.3- Subcellular localization of RHAMM.

Localization	N	Positive	Cytoplasm	Membrane	Nuclear	Stroma
Normal	5	0				
Cancer	25	22/25	21/25	7/25	5/25	1/25
Histology						
Serous	22	20/22	20/22	6/22	4/22	1/22
Endometrioid	1	0	0	0	0	0
Mucinous	1	1/1	1/1	1/1	0	0
Clear Cell	1	1/1	0	0	1/1	0
Grade						
Low	10	8/10	7/10	2/10	2/10	1/10
High	15	14/15	14/15	5/15	3/15	0
Stage						
I	3	3/3	2/3	0	1/3	0
II	3	3/3	3/3	1/3	1/3	0
III	11	9/11	9/11	4/11	2/11	0
IV	8	7/8	7/8	2/8	1/8	1/8
Fallopian Tube	6	6/6	5/6	5/6	2/6	2/6

Table 2.4- RHAMM immunostaining patterns shown in primary and metastatic tumors.

Patient	Description	Grade	Staining	% Staining	Intensity
P1	Normal Ovary	-	-	0	Negative
P1	Primary OC	low	+	1	Weak
P1	Omental metastasis	low	+	1	Weak
P2	Primary OC	low	+	2	Weak
P2	Omental metastasis	low	+	2	Weak
P3	Normal Ovary	-	-	0	Negative
P3	Primary OC	high	+	3	Strong
P3	Omental metastasis	high	+	3	Moderate
P4	Primary OC	high	+	1	Weak
P4	Omental metastasis	high	+	1	Weak
P5	Primary OC	high	-	0	Negative
P5	Omental metastasis	high	-	0	Negative
P6	Primary OC	high	+	3	Strong
P6	Omental metastasis	high	+	3	Strong
P7	Primary OC	high	+	2	Moderate
P7	Omental metastasis	high	+	2	Moderate
P8	Primary OC	high	+	2	Moderate
P8	Lymph node metastasis	high	+	1	Weak

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Chapter 3: RHAMM promotes OC cell migration and invasion

Background

Cellular migration and invasion are essential for metastatic spread contributing to disease progression and poor clinical outcome. Originally cloned in 1992, multiple studies have demonstrated a role for RHAMM in cell motility^{1,2,3} and malignant transformation^{4,5}. Having shown that RHAMM is overexpressed in OC (Chapter 2) similarly to a number of solid^{6,7,8,9,10} and hematological^{11,12} cancers, herein I sought to establish an in vitro OC model in order to determine whether RHAMM overexpression in OC cells likewise modulates OC cell migration and invasion.

Methods

Cell Culture

The SV 40-Large T-Ag-transfected human OSE (HIOSE-118 and HIOSE-121), normal human dermal fibroblasts (HDF), OC (OVCAR5, OV90, SKOV3 and ES-2), BC (MCF-7) and cervical cancer (OV2008 and C13) cell lines (Table 3.1) were cultured in Medium 199/MCDB 105 (Sigma, St. Louis, MO) with 5% fetal bovine serum and gentamicin. All cells were incubated at 37° C with 5% CO₂.

Western Blot

Cells were washed in PBS, trypsinized, pelleted, and washed 1-2 times in cold PBS. Cells were lysed in CHAPS buffer and 30 µg of protein was separated via 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to polyvinylidene fluoride (PVDF) membranes and blocked in 5% milk in Tween 20-Tris buffered saline. Blots were incubated in their respective primary antibodies overnight, followed by incubation with a horseradish peroxidase-(HRP-) conjugated secondary antibody (Fisher, Pittsburgh, PA), and developed via enhanced chemiluminescence substrate (ECL) (Pierce/Fisher, Pittsburgh, PA). Antibodies used were monoclonal rabbit anti-CD168 RHAMM antibody (abcam®, Cambridge, MA)

Human HMMR/CD168/ RHAMM Sandwich enzyme-linked immunosorbent assay (ELISA)

RHAMM ELISA (LifeSpan BioSciences, Inc., Seattle, WA) was performed according to the manufacturer's recommended instructions for cell culture lysates. For analyses of conditioned medium, volumes of conditioned medium equivalent to 1×10^6 cells were concentrated by centrifugation at $16,000 \times g$ using 30,000 kDa microfilters (Millipore, Bedford, MA). All samples were thawed to room temperature, centrifuged to remove particulate matter and volumes of 100ul normalized by cell number were examined by ELISA. Plates were read using a microplate reader (BioTek ELx800) with a 450nm wavelength filter.

Real-time polymerase chain reaction (qPCR)

Cells were treated with TRIzol reagent from Invitrogen (Carlsbad, CA) and ribonucleic acid (RNA) was isolated according to the manufacturer's instructions. To generate single-stranded

complementary DNA (cDNA), the Applied Biosystems GeneAmp RNA *polymerase chain reaction* (PCR) Core Kit (Foster City, CA) was used with 3 ug/mL total RNA using Biometra UNO-thermo-block and Perkin-Elmer-GeneAmp PCR system 9600. qPCR was carried out with SYBR Green Universal Master Mix (Applied Biosystems), cDNA, and primers. Primers used were sense 5'- ACTCCGCTGTCAGCTTGCTAAA-3' and antisense 5'- AATGGGGTCTTCAGGGCAAA- 3' (Sigma- aldrich Oligoarchitect®, St. Louis, MO) and GAPDH (All-in-One™ qPCR Primer for NM_002046.3, GeneCopeia, Rockville, MD) as control. Amplification was performed with 40 cycles of denaturation (95°C, 10 sec), annealing (60°C, 20 sec), and extension (72°C, 15 sec) using a Bio-RAD Chromo4 Real Time PCR Detector using Opticon Monitor 3 program. Levels of RHAMM were normalized to the GAPDH message values and the fold difference was determined by dividing the threshold cycle (Ct) value by the reference sample.

Cell Transfection

One million cells were transfected using the Nucleofector device (Amaxa, Gaithersburg, MD) with 30nM of siRHAMM Silencer® select validated siRNA that targets all 4 sequenced RHAMM variants (ThermoFisher Scientific, Waltham, MA) or scramble (scr) RNA (ThermoFisher Scientific, Waltham, MA). Briefly, 1×10^6 cells were mixed with 2 µg of the appropriate plasmid in 100 µL of Nucleofector solution (kit #VCA-1003). The cell suspensions were transferred to electroporation cuvettes and transfected using program X-005 on the Nucleofector device for cell lines (HIOSE-121, OV2008 and OCAR5) and program A-023 for (OV90) OC cell line.

To estimate overall transfection efficiency, Green Fluorescent Protein (GFP)-transfected cells were visualized and photographed 24 h post-transfection using a digital camera-equipped fluorescence microscope. Parallel cultures of cells transfected with siRHAMM or scrRNA were incubated overnight at 37° C with 5% CO₂ and collected the following day for analysis. RHAMM silencing was confirmed by WB.

Quantification of Cell Proliferation

In order to account for differences in cell proliferation with or without RHAMM inhibition, cell number was counted using a hemocytometer in order to estimate viable cells per mL. The average cell count was taken from each of the 5 (4x4) square sets and was multiplied by 10,000. Cell numbers were counted before, immediately after, 24h after and 48h after transfection. Each data point was the mean of at least 3 experiments done in triplicate.

Cell Migration Assay

OV90 and HIOSE-121 cells were serum starved overnight (ON) and then transfected with siRHAMM or scr RNA. Untransfected cells served as additional controls. One million cells were washed with PBS, trypsinized and aliquoted onto silicone well inserts (Ibidi USA Inc., Fitchburg, WI). The cells were incubated ON to create a monolayer of adherent cells. The silicone insert was removed after 24h to create the scratch wound. Images were taken at 0h (silicone insert removal), 24h and 48h.

Cell Invasion Assay

As per the manufacturer's protocol, OV90, OVCAR5 and HIOSE-121 cells were serum starved ON, untransfected cells and cells transfected with siRHAMM or scrRNA were maintained for 24h. The following day, the cells were washed with PBS, trypsinized and recounted. Transwell inserts from the CytoSelect™ 96-Well Collagen Cell Invasion Assay (Cell Biolabs, Inc., San Diego, CA) were warmed and rehydrated with serum-free media for 1h before adding cells. Cell suspensions were added to transwell inserts and placed in a feeder tray containing 10% FBS media, covered and incubated for 24h. Cells that had invaded through the collagen coated transwell inserts onto the underlying membrane were dissociated and lysed. Lysed cells are detected with CyQuant® GR Dye and quantified using a fluorescence plate reader at 480 nm/520 nm.

Statistics

For real time PCR, error bars illustrate RQmin and RQmax, which were calculated as: RQ_{ave} divided by $(\text{standard deviation}^{\wedge} \text{student's } t \text{ value at the } 95\% \text{ confidence interval, for } 5 \text{ degrees freedom})$ and RQ_{ave} times $(\text{standard deviation}^{\wedge} \text{student's } t \text{ value at the } 95\% \text{ confidence interval, for } 5 \text{ degrees of freedom})$, respectively. This range represents the 95% confidence level. For ELISA, proliferation and invasion studies, results were analyzed using the student T-test. $p \leq 0.05^*$ and $p \leq 0.0001^{**}$ was considered statistically significant.

Results

RHAMM is Elevated in and Secreted by OC Cells

Since OC patient specimens stained intensely for RHAMM (Chapter 2), I sought to establish an in vitro model in which to study the function of RHAMM for OC progression. I measured levels of RHAMM mRNA, cellular protein and secreted RHAMM protein into concentrated conditioned media (CCM) by qPCR, WB and ELISA, respectively, in MCF-7, OV2008, C13, OVCAR5, OV90, SKOV3, ES-2, HIOSE-118, HIOSE-121 and HDF cells. qPCR revealed varying levels of RHAMM mRNA among the cell lines (Figure 3.1A). However, protein levels of both cellular and secreted RHAMM were consistently elevated, up to 5x, in OC cell lines compared to IOSE cells as measured by WB (Figure 3.1B-D). Protein bands of CCM were quantified by densitometric analysis and shown as a trim signal measured by ImageJ computer software program. CCM was compared to a positive control OV2008 cell lysate (Figure 3.1C). Using ELISA, RHAMM levels in CCM, normalized for cell number, were measured and found to be higher in showing higher OC cells than normal OSE (Figure 3.1D).

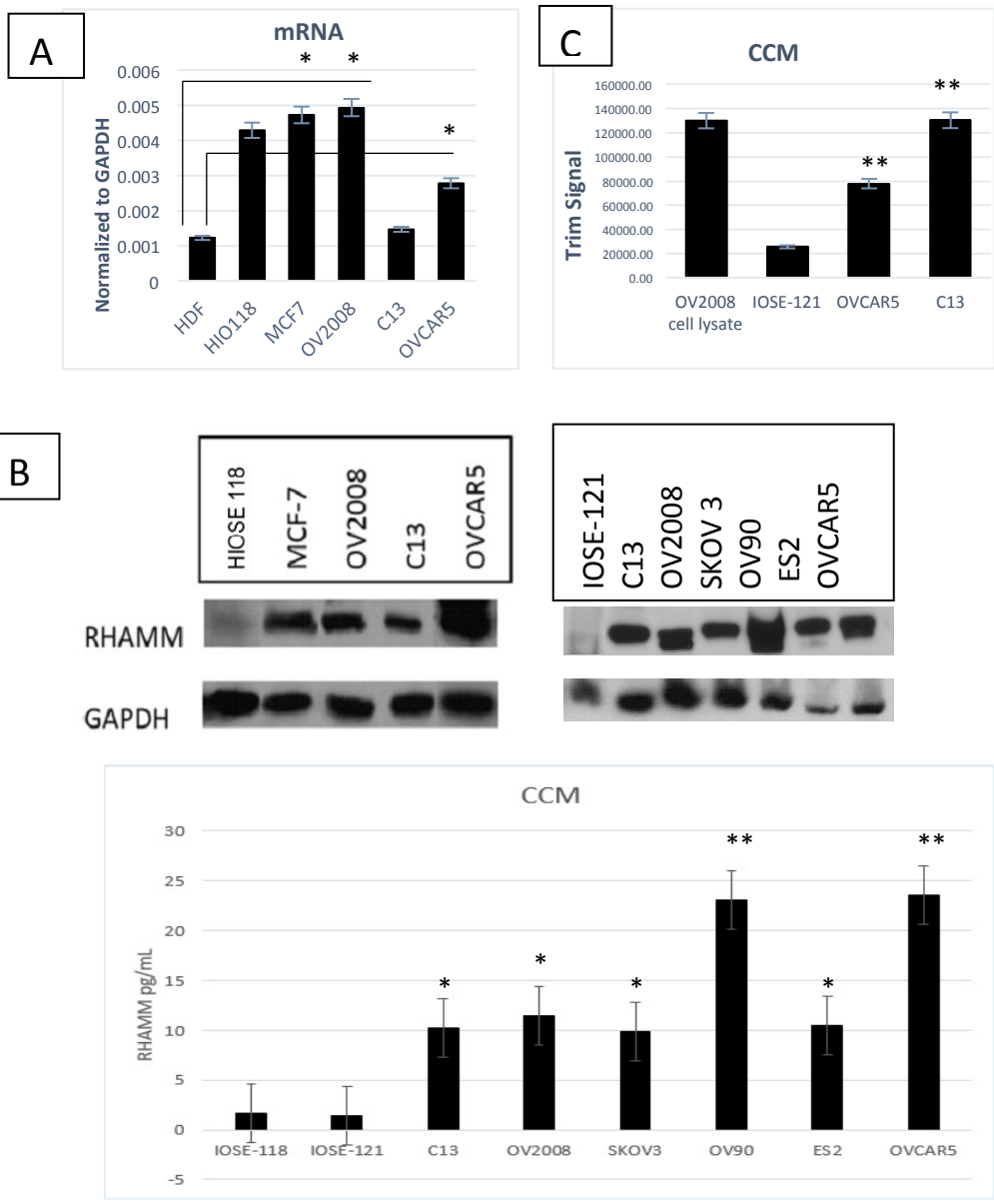


Figure 3.1 RHAMM is overexpressed in cultured OC cells.

Message and protein levels of RHAMM were measured by qPCR (A), WB (B,C) and ELISA (D) in HIOSE-118, HIOSE-121, HDF, MCF-7, OV2008, C13, OVCAR5, SKOV3, OV90 and ES-2 cells. A) qPCR was performed on isolated mRNA using RHAMM sense and anti-sense primers to measure RHAMM expression. WB was performed on protein cell lysates (B) and CCM (C) WB were quantified by densitometric analysis (Image J software for trim signal value) of target protein bands to GAPDH levels where OV2008 cell lysate was used as a positive control. ELISA was performed on CCM normalized for cell number (D). Results are expressed as the average RHAMM level \pm S.E where $p \leq 0.05^*$ and $p \leq 0.0001^{**}$ was considered statistically significant.

RHAMM does not promote OC cell proliferation

Since RHAMM contributes to cell cycle progression and, hence, cellular proliferation through its nuclear role, I sought to determine whether inhibition of RHAMM negatively impacted OC cell growth. HIOSE-121, OVCAR5 and OV90 cells were serum starved ON, then transfected with siRHAMM or scrRNA with untransfected cells serving as additional controls. I found that cell number was not significantly affected by loss of RHAMM expression after 24h or 48h after transfection (Figure 3.2). Successful transfection was validated by WB (Figure 3.2 D-F).

RHAMM Promotes OC Cell Migration

In order to determine RHAMMs' contribution for OC cell migration, HIOSE-121 and OV90 cells were serum starved ON, then transfected with siRHAMM RNA and scr RNA while untransfected cells served as additional controls. Following transfections, cells were subjected to scratch assays and monitored for 'wound repair' for 48 hours. I found that the migration of normal OSE cells (HIOSE-121) was unaffected by abrogation of RHAMM. Regardless of treatment (siRHAMM RNA, scrRNA or untreated controls), all OSE cells equally filled in the scratch gap within 48h (Figure 3.3A). In contrast, reduced OV90 cell migration was noted in cells transfected with siRHAMM RNA compared to untreated controls or cells transfected with scrRNA as early as 24h (Figure 3.3B). By 48h untreated and scrRNA treated OV90 cells had completely filled the scratch gap while OV90 cells transfected with siRHAMM RNA had only filled approximately 32% of the scratch gap (Figure 3.3B).

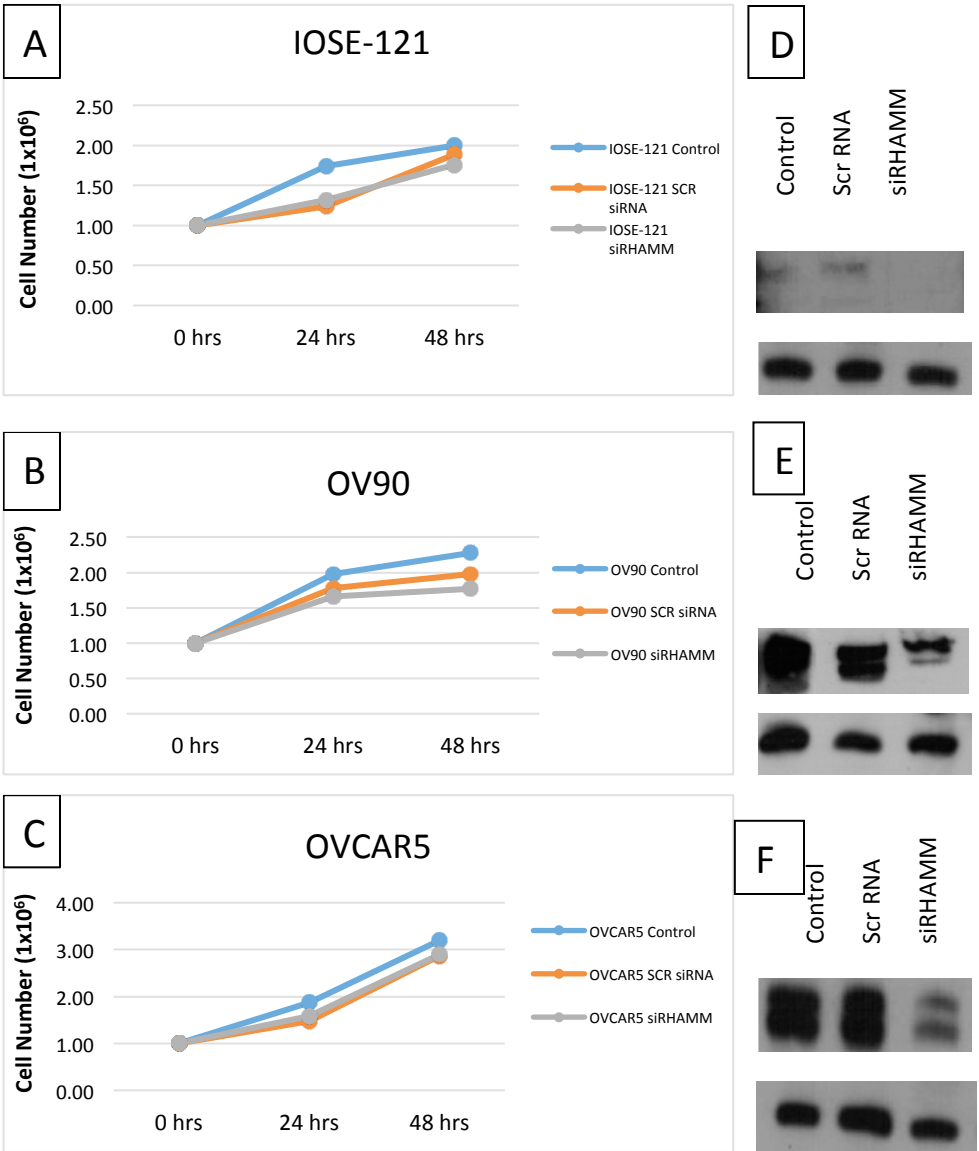


Figure 3.2 RHAMM does not promote OC cell proliferation.

The number of viable (A) HIOSE-121, (B) OV90 and (C) OVCAR5 cells transfected with siRHAMM RNA, scr RNA as well as untransfected cells was determined by cell counts performed at the time of transfection (0h) and 24h and 48h after transfection. The results are expressed as the average cell number \pm S.E. Each data point was the mean of at least three experiments done in triplicate. $p \leq 0.05^*$ was considered statistically significant. Successful silencing of RHAMM was confirmed by corresponding WB (D-F).

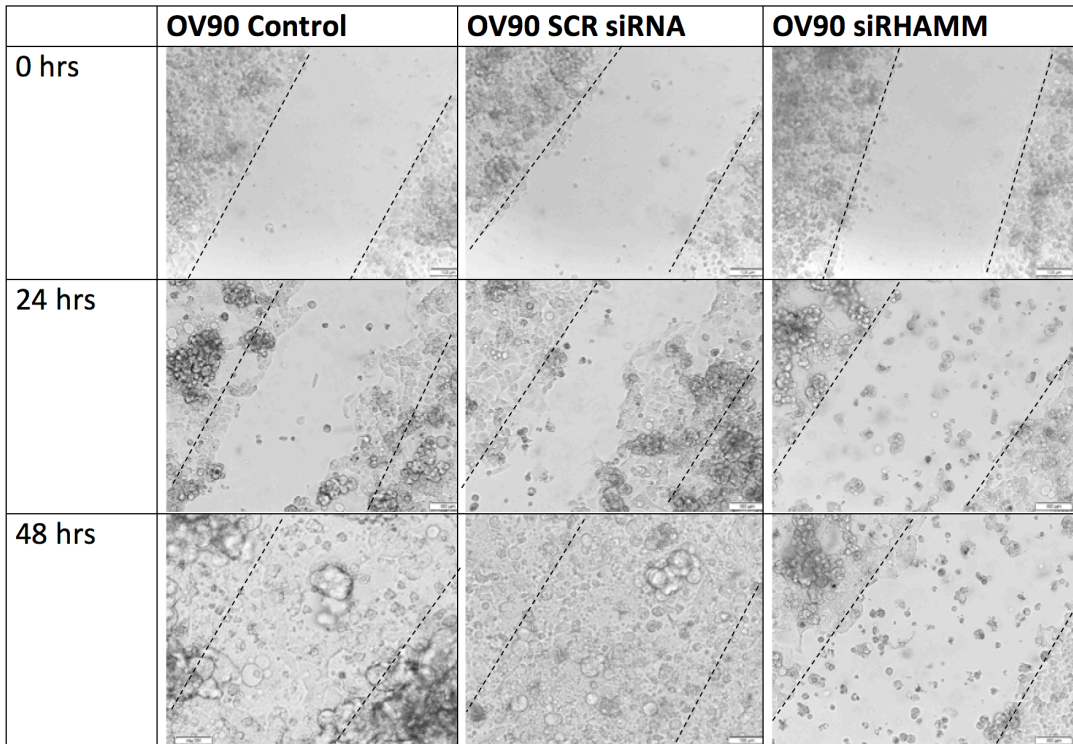
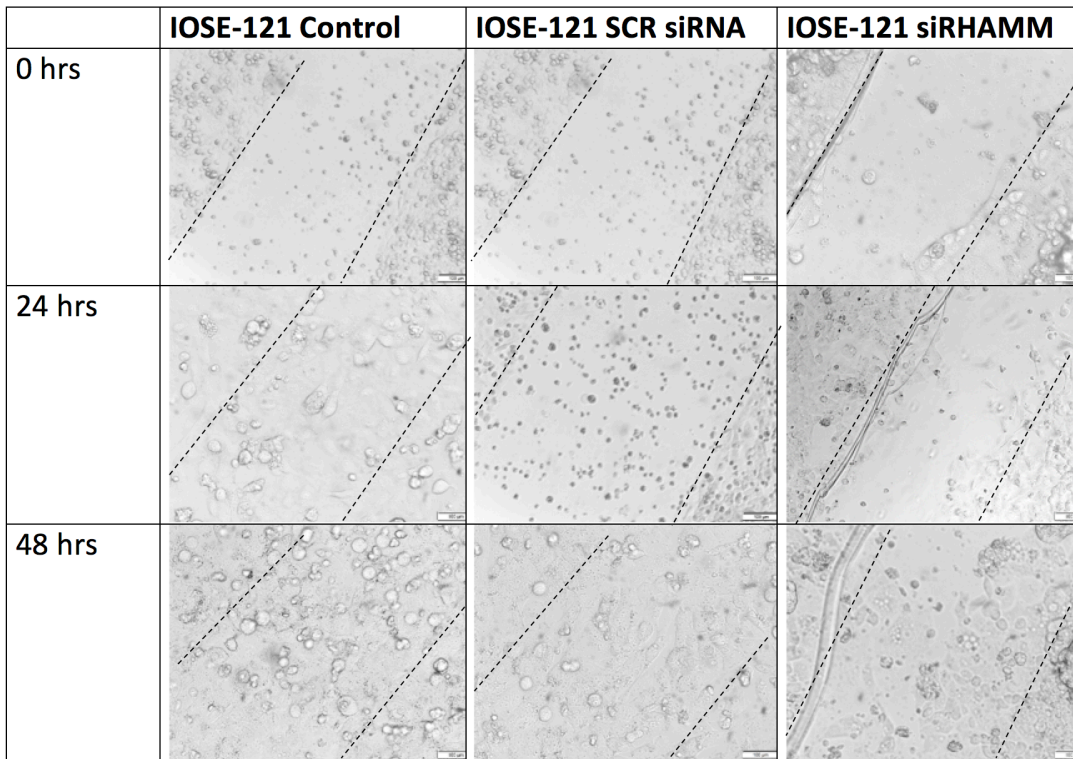


Figure 3.3 RHAMM promotes OC cell migration. (Continued on Next Page)

Figure 3.3 RHAMM promotes OC cell migration.

Representative images from scratch assays of normal HIOSE-121 (A) and OC OV90 (B) cells transfected with siRHAMM RNA or scrRNA and untransfected cells. Images were taken immediately after the silicone insert was removed (0 hrs), 24 hrs and 48 hrs. 100x magnification.

RHAMM Enhances OC Cell Invasion

In addition to mediating cell migration, I wanted know if RHAMM also promoted OC cell invasion. HIOSE-121, OV90 and OVCAR5 cells were serum starved O/N, transfected with either scr RNA, siRHAMM RNA or untransfected (as control) and then subjected to invasion assay using collagen coated Boyden chambers. My results show that regardless of treatment (siRHAMM RNA, scrRNA or untreated controls), all OSE cells equally migrated through the Boyden chambers (Figure 3.4). However, there was significantly less invasion by siRHAMM RNA treated OV90 and OVCAR5 cells compared to their scrRNA and untreated control counterparts (Figure 3.4). Invasion by siRHAMM RNA transfected OV90 cells was reduced by 39.7% and 47.6% compared to untreated and scrRNA treated OV90 cells, respectively. Likewise, invasion by siRHAMM RNA transfected OVCAR5 cells was reduced by 65.9% and 58.9% compared to untreated and scrRNA treated OVCAR5 cells, respectively. Successful inhibition of RHAMM expression was confirmed by WB.

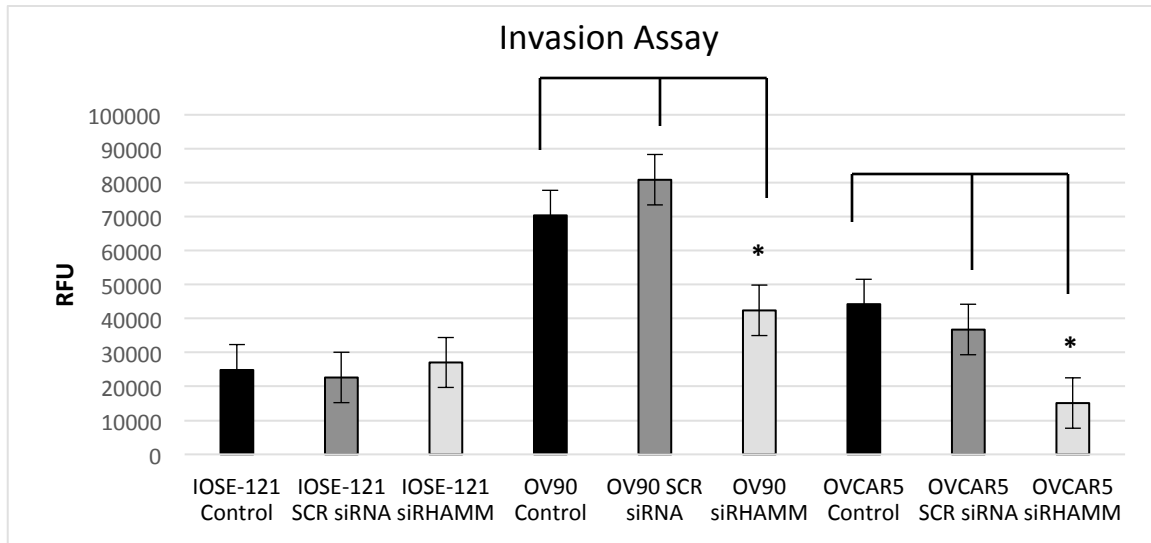


Figure 3.4 RHAMM contributes to OC cell invasion.

HIOSE 121, OV90 and OVCAR5 cells transfected with either siRHAMM RNA, scrRNA or left untreated were seeded onto a Cytoselect collagen-coated transwell membranes in triplicates for 24h. Cells that invaded through the collagen gel onto the underlying membrane were detached and lysed. Lysed cells are detected with CyQuant® GR Dye and quantified using a fluorescence plate reader at 480 nm/520 nm. Results are represented as a mean of RFU \pm S.E. $p \leq 0.05^*$

Conclusion

It is well established that RHAMM plays a profound role in cell migration associated with normal wound healing^{13,14}. RHAMM also contributes to a more aggressive phenotype requiring migratory and invasive cell capabilities seen in malignancies such as breast¹⁵, prostate⁹, bladder⁸ and colon¹⁰ cancer. Having shown that RHAMM is overexpressed in OC (Chapter 2) similarly to a number of solid and hematological cancers, herein, I sought to establish an in vitro OC model in order to determine whether RHAMM overexpression in OC cells likewise modulates OC cell migration and invasion.

Using normal HIOSE and a variety of cancer cell lines, I have been able to show that cellular levels of RHAMM message and protein are elevated in OC cells lines. Likewise,

RHAMM levels were elevated in the CCM of OC cultures indicating that in vitro OC cell lines represent an appropriate model system with which to begin to define the mechanism(s) by which RHAMM may contribute to OC progression.

Surprisingly, when RHAMM expression was inhibited by siRNA silencing technology, I was unable to detect any significant change in OC cell proliferation. Given that the multiple functions associated with RHAMM are defined not only by its subcellular localization, but also by differences in RHAMM isoform expression, it is possible that the predominant RHAMM isoform expressed in these cell lines was not functionally associated with cell cycle progression.

Since cultured HIOSE cells and their in vivo counterparts failed to express significant amounts of RHAMM protein, it was not unexpected that silencing RHAMM in HIOSE cells did not result in any changes in cellular proliferation. It should be noted, however, that I was able to detect RHAMM mRNA in HIOSE cells, but differences in RNA stability and processing between HIOSE and OC cells could limit the degree to which HIOSE RHAMM RNA is translated into protein compared with OC RHAMM RNA species. In contrast, I found a significant decrease in the migratory and invasive capabilities of OC cells as a result of RHAMM inhibition suggesting that RHAMM may drive OC progression using similar cellular migratory and invasive mechanisms as reported in other cancer types.

Lastly, it is important to note that in addition to wound healing, inherent RHAMM expression/function manifests during embryogenesis where it is essential for proper developmental processes^{16,17,18} especially as that pertains to contributing to and maintaining human embryonic stem cell (hESC) pluripotency. High levels of extracellular RHAMM are evident at very early stages of embryonic development while high levels of both cell surface and intracellular RHAMM are found high in later developmental stages¹⁶. Studies have shown that

RHAMM is responsible for the migration to and differentiation of hESCs¹⁶ within the cranial neural crest¹⁷. Therefore, it is not surprising that re-capitulation of embryonic events resulting in the re-activation of RHAMM could contribute to cellular transformation and cancer progression in OC.

Table 3.1 : Cell Line Descriptions

Cell Line	Cell Type
HIOSE-118/ HIOSE-121	Normal OSE cells with FH / without FH
HDF	Normal human dermal fibroblasts
MCF-7	Breast adenocarcinoma
OV2008/C13	Human papillomavirus-related cervical squamous cell carcinoma
SKOV3	Ovarian serous cystadenocarcinoma
ES-2	Ovarian clear cell adenocarcinoma
OV90	Ovarian adenocarcinoma
OVCAR5	High grade ovarian serous adenocarcinoma

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Chapter 4: Unconventional Secretion of RHAMM

Background

Reports of RHAMMs' tumorigenic functions are related to its contribution to increased cellular migration^{1,2}, invasion^{3,4} and cell proliferation⁵. However, its functions are dictated by its location⁶. Intracellular functions of RHAMM are related to mitotic spindle organization influencing cellular proliferation while extracellular RHAMM binds to HA in the tumor microenvironment activating downstream MAP kinase signaling⁷. RHAMM, discovered in medium of sub-confluent chick heart fibroblasts¹, has no signal peptide transport sequence and, therefore, is not secreted by cells in a traditional manner. Likewise, there have not been any reports of RHAMM secretion by alternate methods such as transporter channels, protein chaperone complexes, flippase activity, exocytosis or membrane blebbing. Therefore, the mechanism by which RHAMM is secreted remains unknown. Having shown that OC cells overexpress RHAMM both in vivo (Chapter 2) and in vitro (Chapter 3) and that secretion of RHAMM by OC cells contributes to cellular migration and invasion (Chapter 3), I will employ computational analyses to predict a novel mode for RHAMM secretion. In vitro studies will also be performed to validate my computational predictions. As a result, I anticipate these studies to identify a mechanism by which OC cells secrete RHAMM and which will provide insight needed to attenuate RHAMMs' cell surface HA receptor function.

Methods

Computational Characterization of disorder: PONDR

All sequences used for computational analyses for RHAMM, CD44, SLC9A and CHP1 were derived from their UniProtID number (Table 4.1). Using computational programs, regions of RHAMMs intrinsic disorder were detected using PONDR VSL2⁸, PONDR VL3⁸, PONDR VLXT⁹ and PONDR-FIT¹⁰. These four algorithms have different methods for predicting regions of intrinsic disorder and PONDR-FIT uses a combination of six different predictors to quantitate a score. PONDR-FIT is a meta-predictor that uses Foldindex, TopIDP, IUPred, PONDR VLXT, PONDR VSL2, and PONDR VL3. PONDR[®] VLXT is capable of identifying potential molecular interactions motifs in disordered proteins and disordered protein regions.

Secondary Structure Prediction: RaptorX

Prediction of the secondary structure for RHAMM was determined by RaptorX. This webserver program uses components of both template-based and template-free strategies for determining secondary structure¹¹. With a given sequence this program can find related templates by comparing BLAST and HHpredz sequences to predict a secondary structure model based upon their algorithms¹¹.

Protein binding regions: ANCHOR analysis

ANCHOR algorithm software was used to predict prospective binding sites within RHAMMs regions of disorder. This algorithm is based on the hypothesis that long regions of disorder contain localized potential binding sites that are unable to form enough favorable intra-chain interactions to fold on their own, but can possibly stabilize energy by binding with a globular

protein¹². ANCHOR works cooperatively with IUPred algorithm which is based on a total pairwise interresidue energy estimation with the assumption that sequences will not be able to fold due to instability in regions of disorder¹³.

Protein Binding Partners: STRING 9.1

Potential protein binding partners of RHAMM were predicted using the String 9.1 Platform.¹⁴ This software is a search tool for retrieving experimental and predicted interaction information between proteins based on a confidence score.

Phosphorylation, O-link and N-link Glycosylation: PTM Analysis using NetPhos, NetOGlyc and NetNGlyc

Sites of post-translational modification (PTM) within RHAMM were analyzed using algorithm software that predicts sites of phosphorylation and glycosylation from a peptide sequence. NetPhos presents an artificial neural network method that predicts the phosphorylation sites in independent protein sequences with a sensitivity in the range from 69% to 96%¹⁵. DIPHOS uses a web-based tool for the prediction of protein phosphorylation sites¹⁶. Together these programs predict the number of potential serine, threonine, and tyrosine phosphorylation sites. NetOGlyc 4.0¹⁷ determines the likely O-linked glycosylation sites on hydroxyl groups of a serine, threonine, tyrosine, hydroxyline or hydroxyproline side chains and NetNGlyc 1.0¹⁸ determines the likely N-linked glycosylation sites on amide groups of an asparagine or arginine side chain by a predetermined threshold. NetOGlyc predicts the number of likely residues containing hydroxyl groups that have the potential for glycosylation and NetNGlyc networks could identify 86% of the glycosylated residues with an overall accuracy of 76%¹⁸.

Cell Culture

The SV 40-Large T-Ag-transfected human OSE cell lines HIOSE-118 and HIOSE-121, BC (MDA-MB-231, MCF-7), cervical cancer (HELA, OV2008, C13), prostate cancer (PC3), pancreatic cancer (Panc1), colon cancer (WiDr) and OC (OVCAR5 and OV90) cell lines were cultured in Medium 199/MCDB 105 (Sigma, St. Louis, MO) with 5% fetal bovine serum and gentamicin. All cells were incubated at 37° C with 5% CO₂.

Cytosolic Extraction by Digitonin Semipermeabilization

Protein separation protocol using Digitonin Semipermeabilization was performed as described by Liu and Fagott, 2011¹⁹. Cell were grown to confluency and then washed with ice-cold PBS. After removal of PBS, 3 ml of Digitonin Solution was added to the dish which was then placed on an orbital shaker for 10 min at 100 rpm at 4°C. The digitonin-solubilized material was collected as the cytosolic fraction.

Western Blot

Untreated and treated cells were washed in PBS, trypsinized, pelleted, and washed 1-2 times in cold PBS. Cells were lysed in CHAPS buffer and 30 µg of protein was separated via 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to polyvinylidene fluoride (PVDF) membranes and blocked in 5% milk in Tween 20-Tris buffered saline. Blots were incubated in their respective primary antibodies overnight, followed by incubation with a horseradish peroxidase- (HRP-) conjugated secondary antibody (Fisher, Pittsburgh, PA), and developed via enhanced chemiluminescence substrate (ECL) (Pierce/Fisher, Pittsburgh, PA). Antibodies used were monoclonal rabbit anti-CD168 RHAMM

antibody at 1:1000 (abcam®, Cambridge, MA), mouse anti-CD44 antibody at 1:1000 (abcam®, Cambridge, MA), and CHP1 at 1:1000 (Thermo Fisher Scientific, Waltham, MA)

Co-Immunoprecipitation (co-IP)

Whole cell lysate and cytosolic protein were mixed with A/G-agarose beads to make a working solution of 50% protein and A/G beads of 100ul for 1 mL of solution. The solution was mixed on a shaker for 10 minutes at 4°C. Mixture was centrifuged and supernatant was extracted. 1ug/mL of protein incubated with the appropriate primary antibody overnight at 4°C. The following day the solution was washed three times with PBS and subjected to western blot.

PNGase F De-Glycosylation

PNGase F assay (New England BioLabs, Ipswich, MA) was conducted as per manufacturer's instructions, 10 ug of cell lysate, denaturing buffer and water were combined, heated at 100 °C for 10 minutes and chilled on ice. Glycobuffer, 10%NP-40 and water was added to make a total reaction volume of 20 ul. One ul of PNGase F was added and mixed gently. The total reaction was incubated for 1 hour at 37 °C and then run on a gel for analysis. Gel was stained with coomassie blue and imaged with a BioRad Chemidoc (BioRad, Hercules CA).

Tunicamycin (TM) Treatment

One million cells were counted and seeded in a 6 well plate. Cells were serum-starved overnight in media supplemented with 0.1% FBS. Media was changed the following day and cells were either treated with methanol (MeOH), 2.5 μM TM or 5uM TM and then incubated for 24 hrs at 37° C with 5% CO. After incubation, conditioned media was collected and cells were lysed and subjected to ELISA and WB.

Human HMMR/CD168/ RHAMM Sandwich enzyme-linked immunosorbent assay (ELISA)

RHAMM ELISA (LifeSpan BioSciences, Inc., Seattle, WA) was performed according to the manufacturer's recommended instructions. Prior to performing the experiments, all samples were thawed to RT and centrifuged to remove particulate matter. Plates were read using a microplate reader (BioTek ELx800, Winooski, VT) with a 450nm wavelength filter.

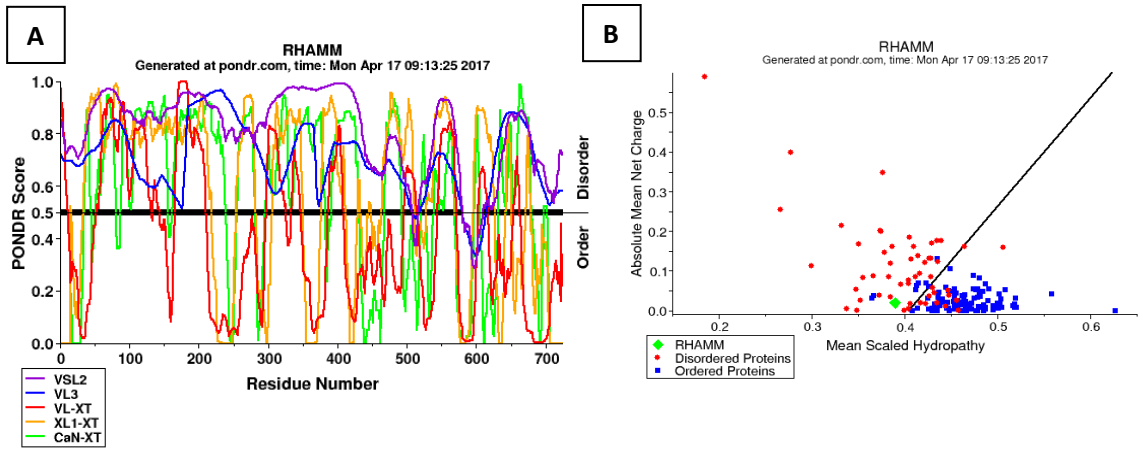
Results

RHAMM is a disordered and hydrophilic protein.

Bioinformatic analysis using disorder prediction software was used to analyze full length RHAMM (Figure 4.1A). Intrinsic disorder analysis using multiple PONDR predictors shows RHAMM is mostly an intrinsically disordered protein (IDP). These predictors consider a residue disordered if the score is greater than 0.5 on the y-axis output, showing that RHAMM has multiple intrinsically disordered regions (IDRs). RHAMM also has a mean hydrophathy output with a low mean net charge (valence). This confers a more open or unfolded conformation in agreement of a protein structure with several regions of intrinsic disorder (Figure 4.1B).

IDPs are highly flexible and undergo constant dynamic conformational change, increasing chances for protein-protein interaction (PPI). Regions likely to undergo PPI in RHAMM were predicted by the ANCHOR algorithm and found to directly correlate with the regions of disorder (Figure 4.1C). These regions are predicted by binding sites undergoing order to disorder transition-binding sites to globular protein binding sites¹³, making them susceptible for interaction with other proteins. RaptorX computational software predicted RHAMMs' secondary structure as more than 80% alpha- helical (Figure 4.1D). However, the software could

not predict RHAMMs tertiary structure because highly disordered proteins that undergo constant conformational change lack a fixed tertiary structure²⁰. RHAMM was also analyzed using HMMpTM computational software, which predicts topology of alpha-helical transmembrane proteins²¹. This software confirmed that the localization of RHAMM is cytoplasmic based on its amino acid (AA) sequence and lack of signal peptide sequence (Figure 4.1E).



Mean net charge: 0.0193 Hydropathy: 0.3895 Boundary Hydropathy=0.4202

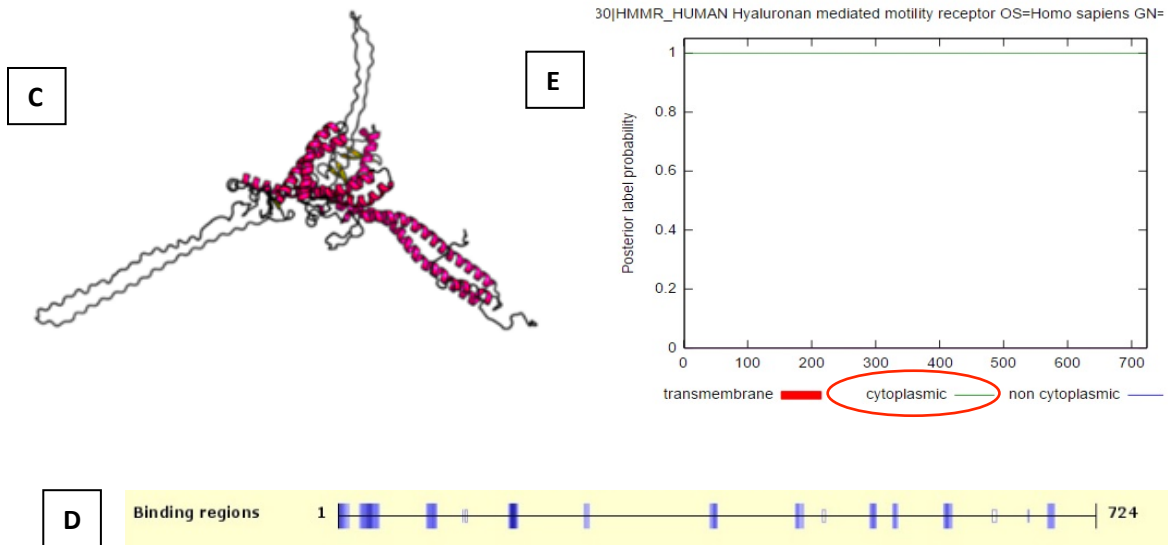
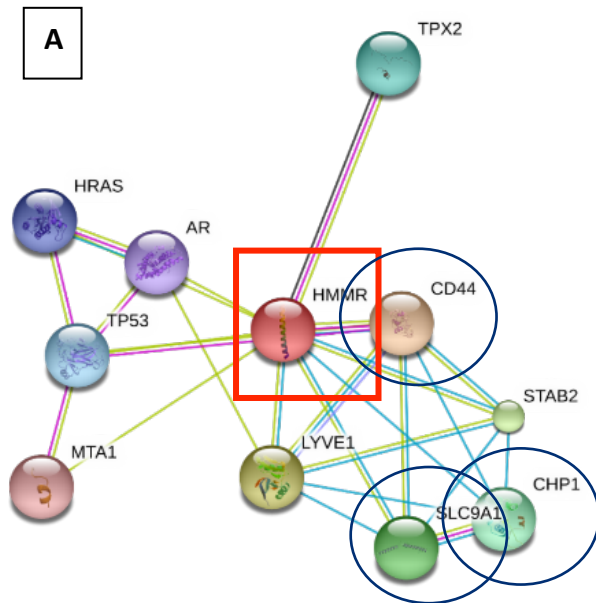


Figure 4.1 RHAMM is a Disordered Protein

A) POND (Predictor of Intrinsically Disordered Regions) detects disordered regions in RHAMM. Regions shown above the 0.5 threshold are disordered and confers protein flexibility and plasticity. B) Prediction of RHAMMs hydropathy mean net charge based off of its AA sequence. C) RaptorX property prediction of RHAMMs secondary structure based off of AA sequence. D) ANCHOR prediction of protein-protein interaction (PPI) binding regions undergoing order to disorder transition-binding sites to globular protein binding site. E) HMMpTM prediction of RHAMMs cellular localization.

In silico identification of possible protein chaperones for RHAMM

Disordered proteins are well documented for their plasticity and promiscuity when interacting with other proteins²². RHAMMs' multiple regions of intrinsic disorder confer a high propensity for PPIs. Therefore I sought to identify proteins which might serve as RHAMM chaperones for extracellular transport of RHAMM. STRING computational software utilizes a database of reported experimental PPI interactions based off of a publication database¹⁴. An interactome of proteins was created and illustrated proteins that directly interact with RHAMM (Figure 4.2A). Proteins that were likely candidates for cell surface exportation were selected for further in silico analysis based on their canonical function. As a result, CD44, SLCA9A and CHP1 were subjected to bioinformatic analyses of predicted intrinsic disorder, hydrophobicity, protein binding regions and cellular localization (Figure 4.2B-E). Protein characteristics utilized for further experimentation were based on canonical cellular function, regions of intrinsic disorder, low mean scaled hydropathy, binding regions for PPI and cellular localization (Figure 4.2A-E). CD44 and CHP1 were the most ideal candidates because they are mostly disordered which increases the chance of PPIs and they have low mean scale hydropathy numbers conferring an open configuration. Predicted localization of CD44 at the cell surface would potentiate the possibility of intracellular interaction prior to the plasma membrane for export. In contrast, CHP1 is a cytoplasmic protein that could bind to RHAMM and mediate RHAMMs' transport for exocytic membrane trafficking. SLC9A was also considered for RHAMM transport, however, it is the least likely candidate due to the fact that it is mostly ordered, has a high mean scale hydropathy and contains PPI binding sites predominantly at the c-terminus, easily hidden by its closed configuration.



CD44- Transmembrane protein receptor for HA and other ligands, such as osteopontin, collagens, and matrix metalloproteinases. Can form a trimeric complex with HA and RHAMM on the cell surface.

Solute carrier protein 9A (SLC9A)- linker between integral membrane and cytoskeletal proteins

Calcium binding protein (CHP1) - regulates vesicular trafficking (exocytic membrane traffic) and mediates the association between microtubules and membrane-bound organelles of ER and Golgi.

Figure 4.2 In silico identification of potential protein binding partners for RHAMM. (Continued on Next Page)

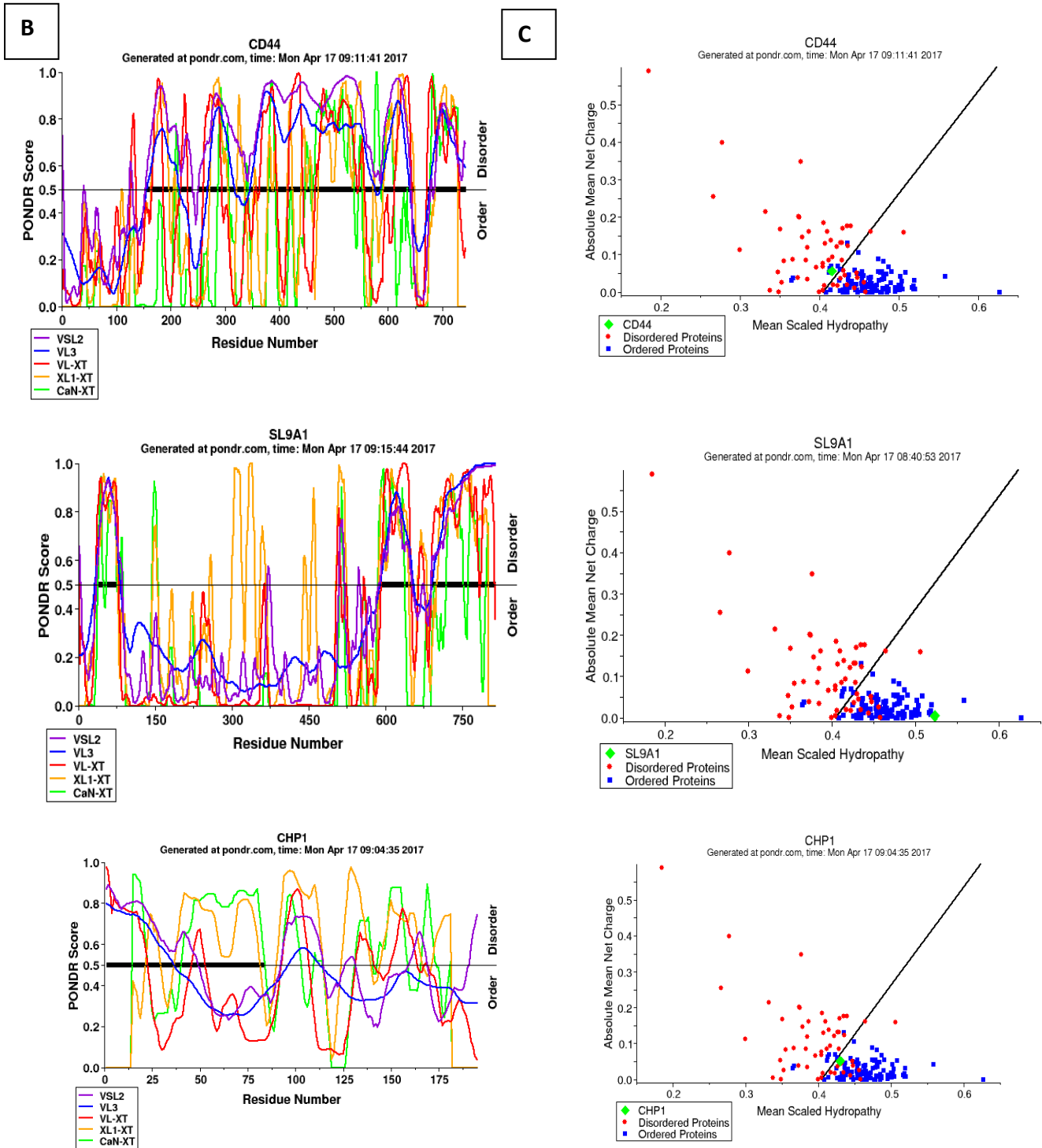


Figure 4.2 In silico identification of potential protein binding partners for RHAMM. (Continued on Next Page)

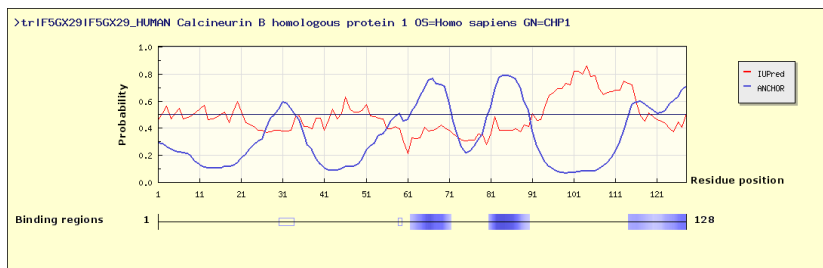
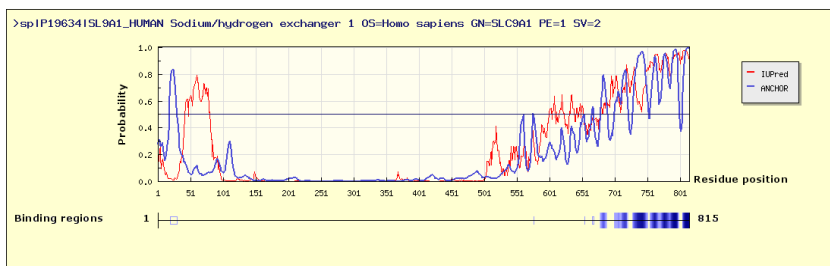
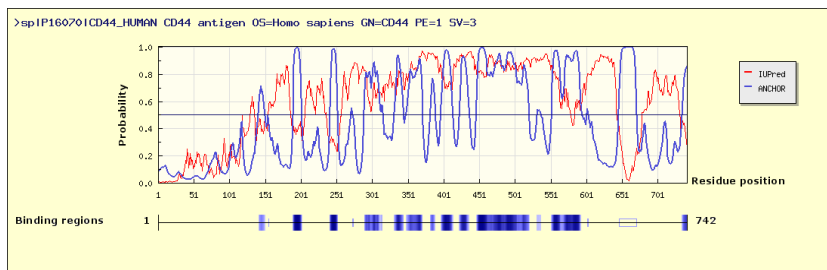
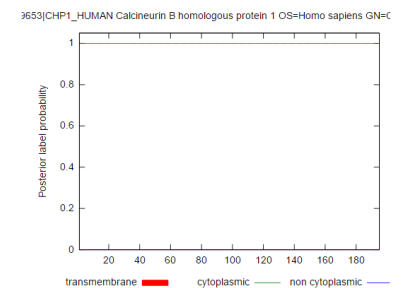
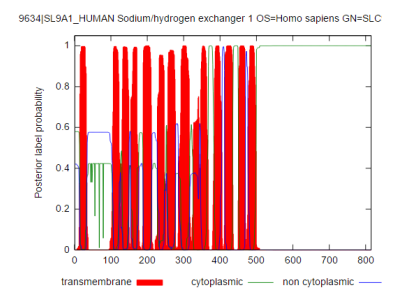
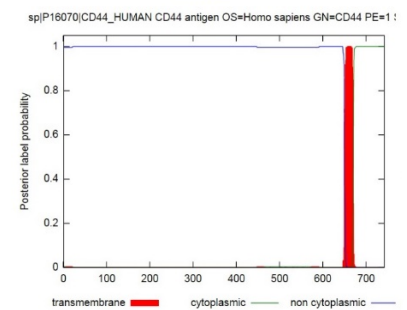
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Figure 4.2 In silico identification of potential protein binding partners for RHAMM.

A) STRING analysis of RHAMMs potential binding partners utilizing a protein database of known interactions. B) PONDR predicted regions of protein disorder in CD44, SLC9A and CHP1 using a threshold of 0.5. C) Prediction of CD44, SLC9A and CHP1 hydrophobicity detected by their mean net charge based off each AA sequence. D) ANCHOR prediction of PPI regions of CD44, SLC9A and CHP1 undergoing order to disorder transition-binding sites to globular protein binding site. E) HMMpTM prediction of CD44, SLC9A and CHP1 cellular localization based off AA sequence.

CHP1 binds to RHAMM in the cytosol

To determine if RHAMM transport and secretion to the cell surface occurs by protein chaperones, cytosolic protein was extracted by cell fractionation in HIOSE, cervical cancer and OC cells. Even though RHAMM and CD44 have a well-documented interaction on the surface of malignant cells and form a trimeric complex for HA interaction^{23,24,25}, I was unable to detect CD44 in the cytosolic protein extract (Figure 4.3A). Likewise, I was unable to detect significant amounts of SLC9A within cellular extracts (Figure 4.3B). Therefore, I eliminated both CD44 and SLC9A as a possible RHAMM chaperones. Without either CD44 or SCL9A in the cytosol, there is little reason to believe that either would form an intracellular complex with RHAMM before exportation to the membrane.

Subsequently, I performed a co-IP on cytosolic protein of OC cells (OVCAR5 and OV90) and found that RHAMM and CHP1 interact within the cytosol (Figure 4.3C). IgG protein was used as a control for non-specific binding. Consequently, a CHP1:RHAMM protein complex may chaperone RHAMM secretion.

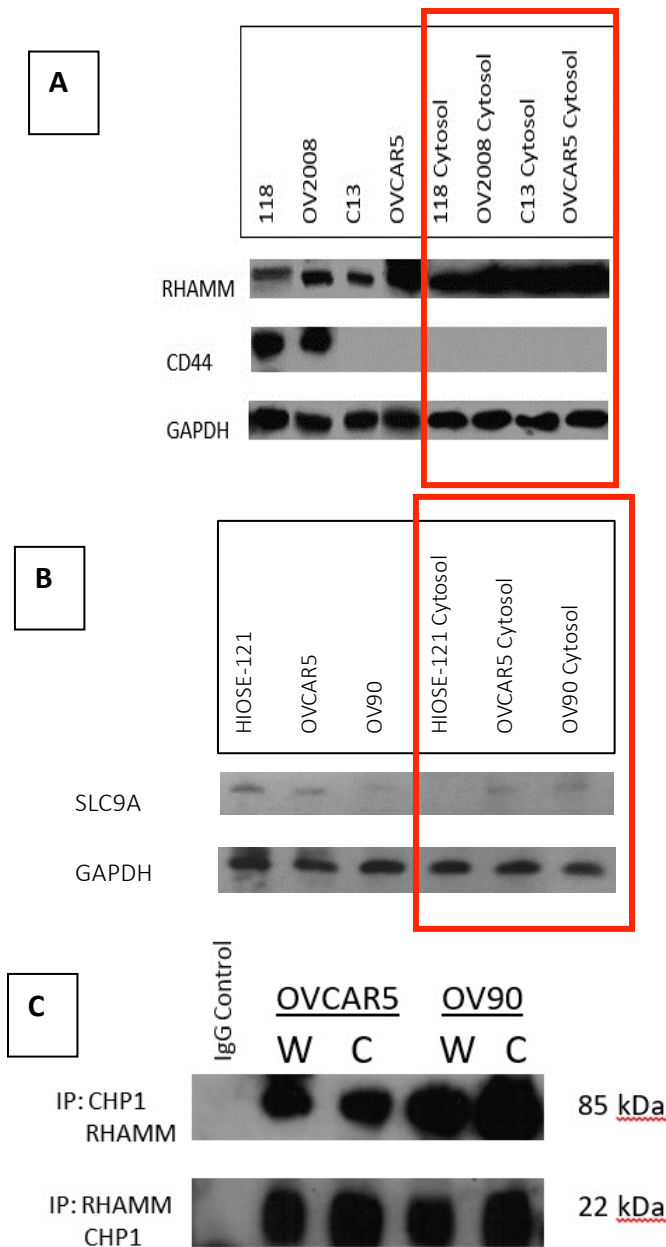


Figure 4.3 CHP1 Binds to RHAMM in the Cytosol

WB of normal (HIOSE-118, HIOSE 121), cervical cancer (OV2008, C13) and OC (OVCAR5, OV90) whole cell lysate and cytosolic protein. GAPDH was used as a loading control (A,B). Primary antibodies anti-RHAMM, anti-CD44 (A) and anti- SLC9A (B) were used. Digitonin semi-permeabilization was performed to separate cytosolic (C) protein from whole cell lysates (W). C) Co-IP was performed on whole cell proteins and cytosolic proteins from OVCAR5 and OV90 cells which were incubated with either anti-RHAMM or anti-CHP1 overnight with protein A/G-PLUS agarose beads, followed by WB. IgG was used as a control for non-specific binding.

RHAMM is not dependent on CHP1 for protein export

CHP1 inhibition was performed using siRNA CHP1 silencer followed by WB. I found that inhibition of CHP1 expression slightly decreased RHAMM levels in OVCAR5 and OV90 cells. (Figure 4.4A) Conversely, cells were also transfected with siRHAMM silencing RNA and WB showed that CHP1 levels did not significantly change with loss of RHAMM. This indicated that their protein levels were not dependent on each other even though they interact within the cell. Importantly, there was no significant difference in secreted RHAMM levels after CHP1 inhibition (Figure 4.4B) suggesting that even though RHAMM can bind to CHP1, CHP1 is not essential for RHAMM secretion.

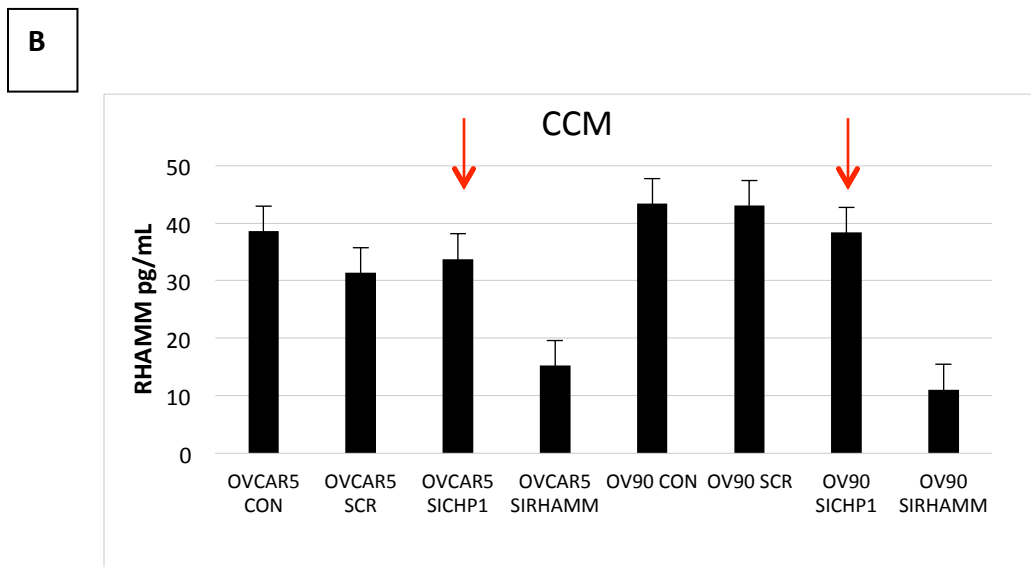
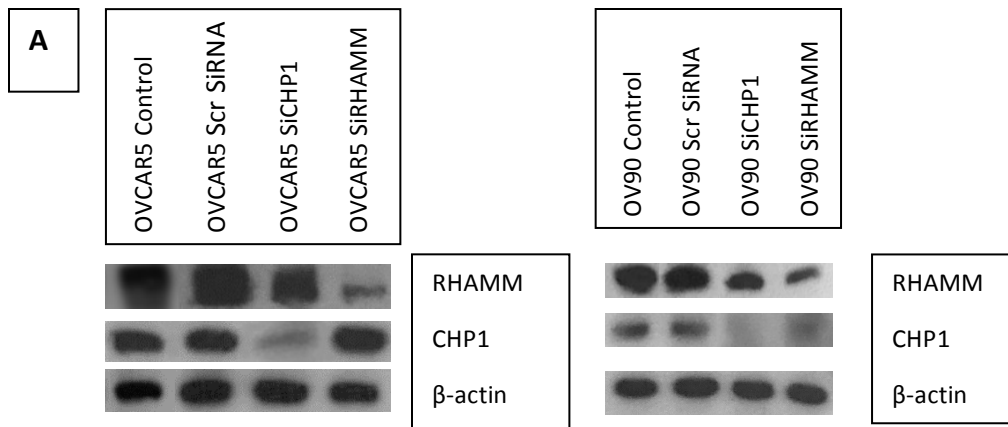


Figure 4.4 RHAMM is not dependent on CHP1 for Protein Export

(A) WB of OVCAR5 and OV90 cells treated with siCHP1, siRHAMM, scrRNA or untreated (as control) incubated with either anti-RHAMM or anti-CHP1. β -actin was used as a loading control. (B) ELISA performed on CCM from 1×10^6 cells treated with siCHP1, siRHAMM, scrRNA or untreated. ELISA results are expressed as a mean pg/mL RHAMM \pm SE and represented as a histogram where $p \leq 0.05$ was considered statistically significant.

RHAMM has a high propensity for PTM

Further computational analysis of RHAMM was needed to delineate RHAMMs' mechanism for secretion. Proper protein folding and function as well as protein interactions through IDPs are typically dictated by PTM²⁶. Potential phosphorylation, O and N-linked glycosylation sites within RHAMM were determined using prediction software NetPhos 2.0¹⁵, NetOGlyc 4.0¹⁸ and NetNGlyc 1.0¹⁸. These programs determine the propensity for phosphorylation, O- and N- linked glycosylation of each residue of RHAMM based on the AA sequence of RHAMM (Figure 4.5A-C). Each program designates a threshold to be passed for significantly high level of confidence for each residue output. As shown, RHAMM has many potential sites for phosphorylation and O- and N- linked glycosylation.

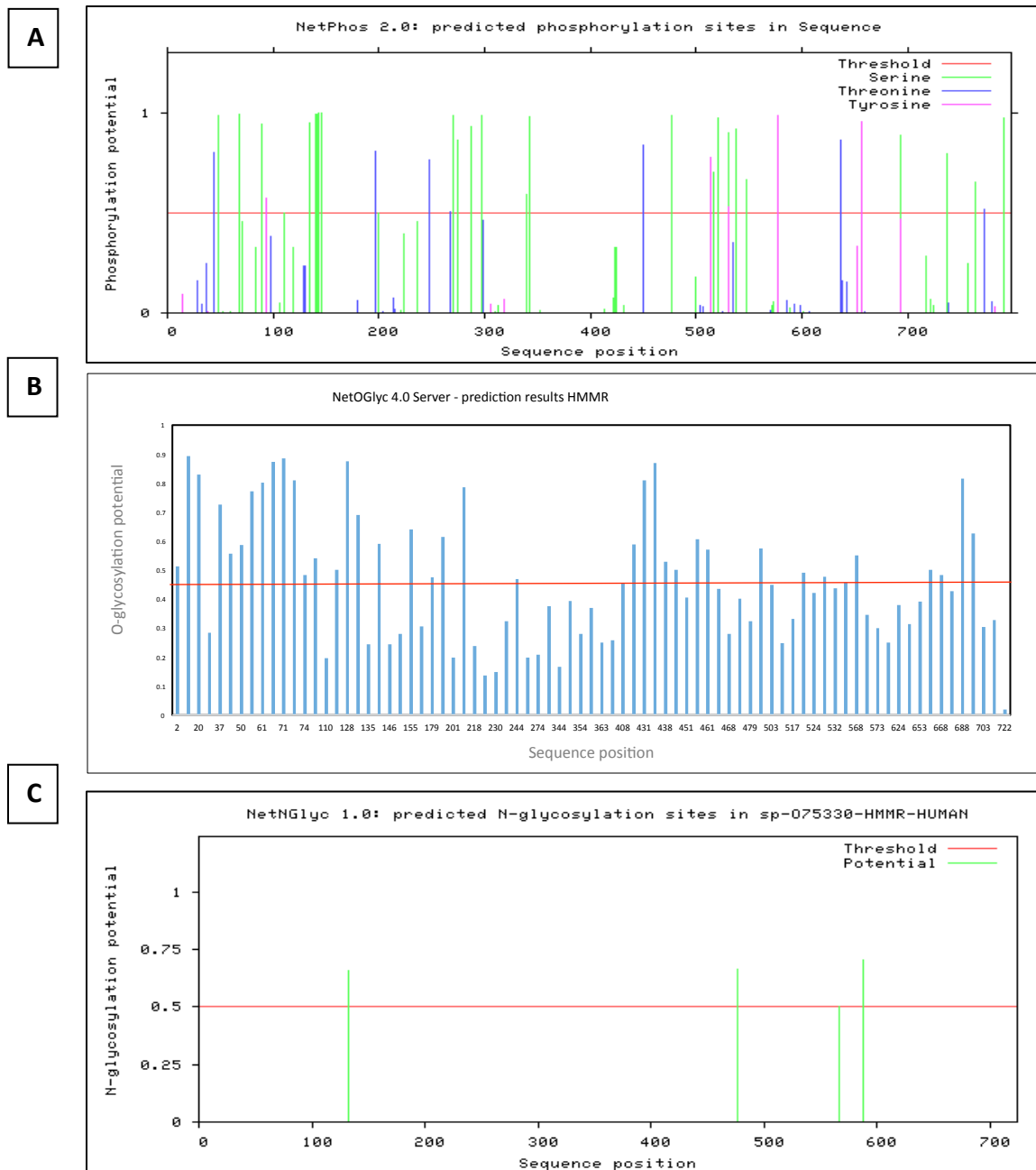


Figure 4.5 RHAMM has a high potential to undergo PTM.

Predictions for RHAMMs potential sites of (A) phosphorylation (B) O-glycosylation and (C) N-glycosylation were obtained using computer software programs NetPhos, NetOGlyc and NetNGlyc and evaluated based on the software's predetermined threshold for confidence, indicated by red line.

Inhibition of glycosylation decreases cellular RHAMM expression and secretion in OC cell lines

To narrow down possible PTM sites, I hypothesized that N-linked glycosylation would be the most likely mechanism driving RHAMM secretion due to its role in classical protein secretion pathway²⁷. To determine whether N-linked glycosylation is essential for RHAMM secretion, OC (OVCAR5), prostate cancer (PC3), cervical cancer (HELA), BC (MDA-MB-231 and MCF7) and pancreatic cancer (Panc1) were treated with 2.5 ug/mL of Tunicamycin (TM), a known general inhibitor of glycosylation for 24h. Cell lysates were examined by WB (Figure 4.6A) and CCM was measured by ELISA, normalized to cell number (1×10^6 cells) (Figure 4.6B). WB only showed a notable reduction in RHAMM levels in OVCAR5 cell lysates suggesting that glycosylation may not be a global mechanism for RHAMM secretion since the levels of cellular RHAMM among the panel of remaining cell lines were not impacted by TM treatment. Likewise, while both MDA-MB-231 and OVCAR5 cells showed reduced secretion of RHAMM following TM treatment, only OVCAR5 cells demonstrated reduction of both cellular and secreted RHAMM following TM treatment.

To verify a role of glycosylation for RHAMM secretion in OC, I examined whether cellular and secreted RHAMM levels are also inhibited following TM treatment in another OC cell line, OV90. OVCAR5 and OV90 cells were treated for 24h with 0 ug/ml, 2.5 ug/mL and 5ug/mL TM. Cell lysates were collected and subjected to both WB and ELISA (Figure 4.6C,D). WB showed a significant decrease in RHAMM protein with both 2.5ug/mL and 5ug/mL treatment while ELISA showed up to 3.7x and 3.29x reduction in cellular RHAMM levels following TM treatment in OVCAR5 and OV90 cells, respectively. Corresponding CCM was also collected, normalized to cell number and measured for RHAMM levels by ELISA (Figure

4.6E). I found that TM reduced secreted amounts of RHAMM by up to 6.23x and 2.65x in OVCAR5 and OV90 cells, respectively suggesting that, by maintaining protein stability and proper protein folding to prevent lysosome or proteasome degradation^{28,29}, glycosylation mediates RHAMM secretion in OC cells.

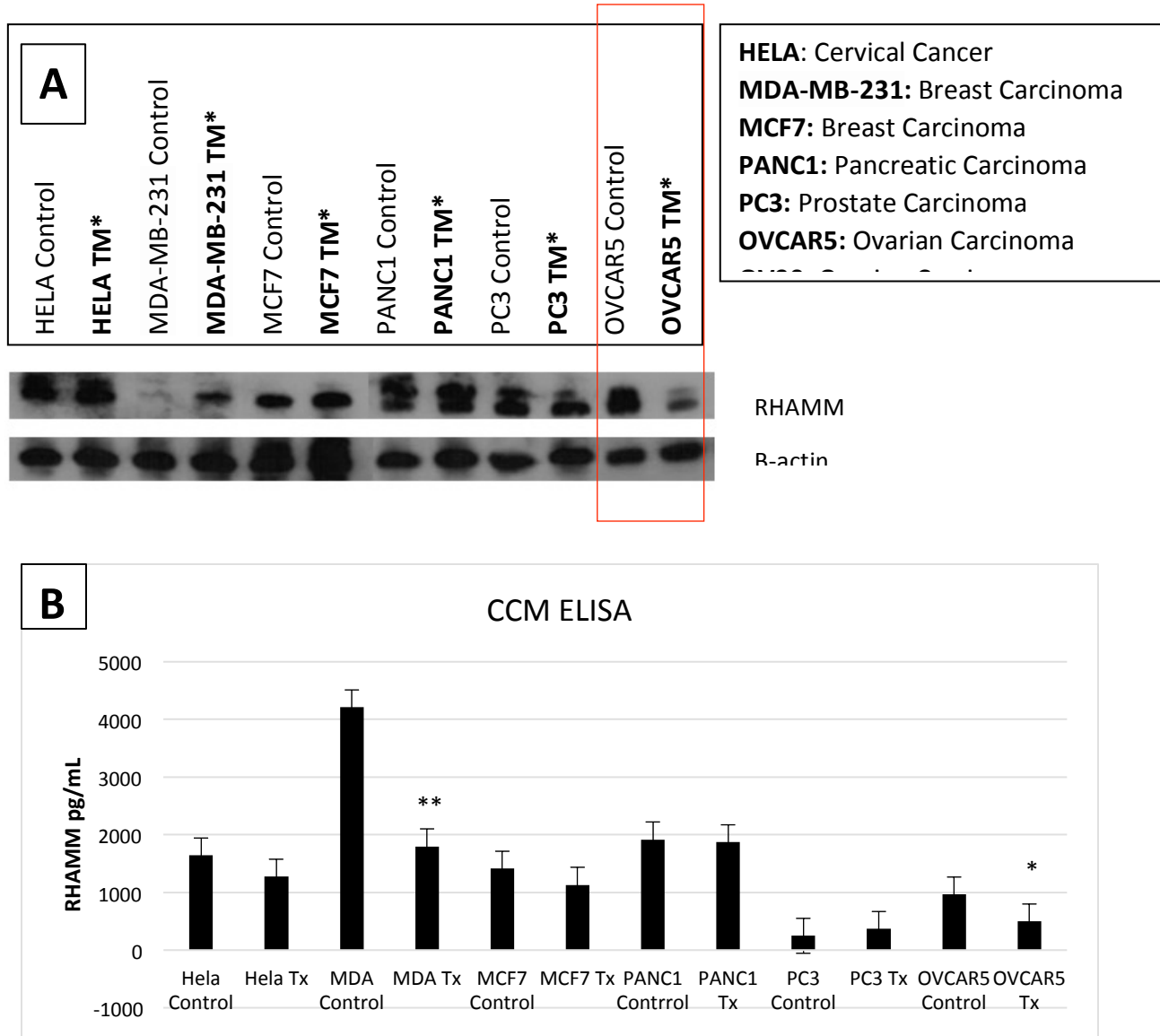


Figure 4.6 TM treatment abrogates RHAMM expression and secretion in OC. (Continued on Next Page)

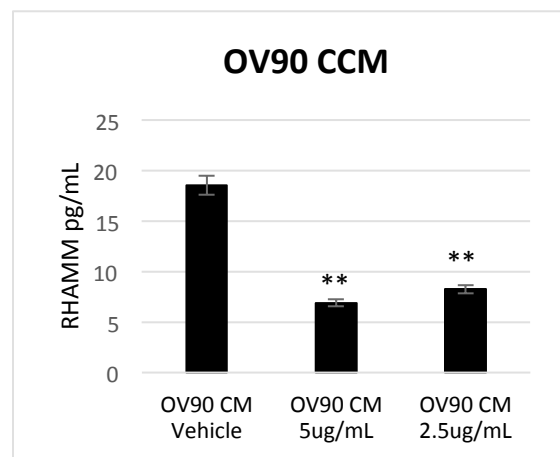
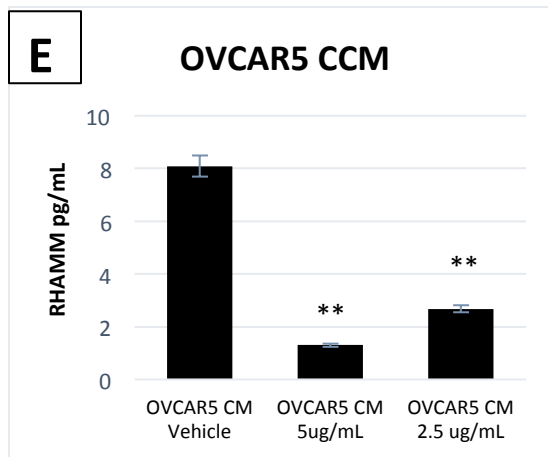
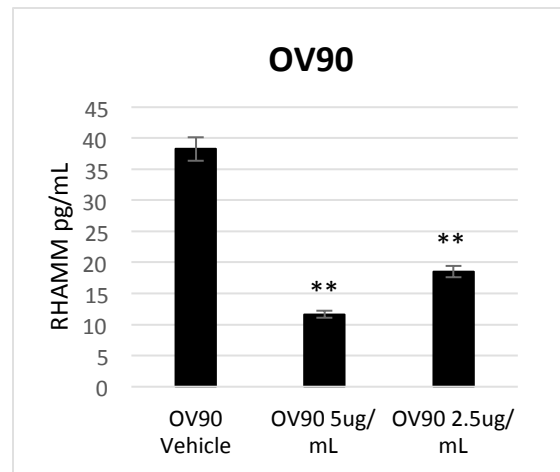
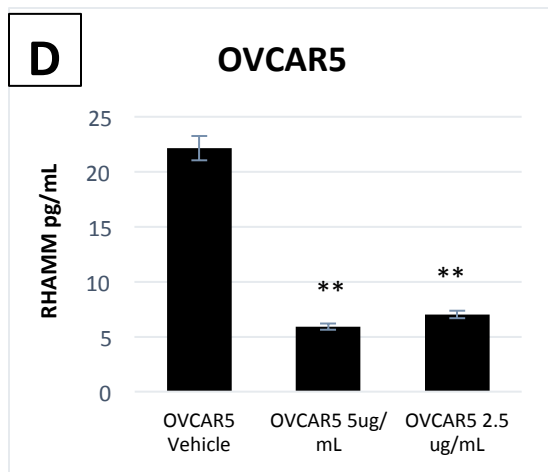
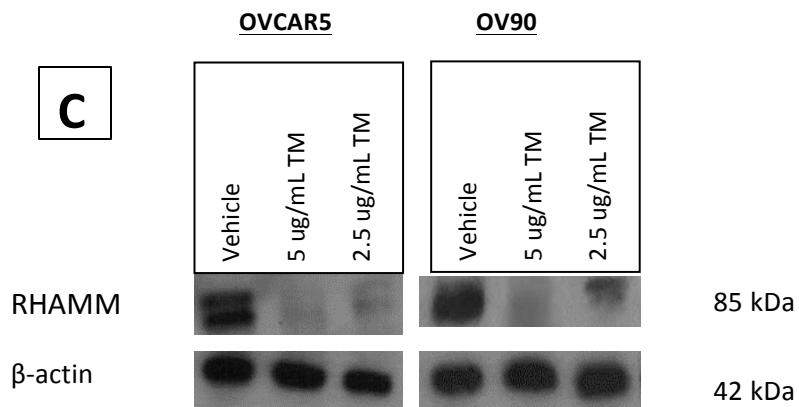


Figure 4.6 TM treatment abrogates RHAMM expression and secretion in OC. (Continued on Next Page)

Figure 4.6 TM treatment abrogates RHAMM expression and secretion in OC.

(A) OC (OVCAR5), prostate cancer (PC3), cervical cancer (HELA), BC (MDA-MB-231 and MCF7) and pancreatic cancer (Panc1) cells were treated with TM for 24h using vehicle control (MeOH) and 2.5 ug/mL and subjected to WB (B). CCM corresponding to (A) was normalized to cell number (1×10^6 cells) and subjected to RHAMM ELISA. (C) OC cells (OVCAR5 and OV90) were treated with TM for 24h using vehicle control (MeOH), 2.5 ug/mL, or 5ug/mL TM. Following TM treatment, OC cell lysates were subjected to WB (C) and ELISA (D) for RHAMM. CCM corresponding to (C) was normalized from 1×10^6 cells and measured by RHAMM ELISA (E). ELISA results are expressed as a mean pg/mL RHAMM \pm SE and represented by histogram where $p \leq 0.05$ * and $p \leq 0.0001$ ** were considered statistically significant.

RHAMM is N-glycosylated

To better characterize RHAMM glycosylation, I sought to determine if RHAMM is specifically N-glycosylated by treating OC cells with Rapid PNGase F. PNGase F enzymatically removes N-linked oligosaccharides from glycoproteins which can be visualized on SDS-PAGE stained with Coomassie as cleavage products of a target protein. Cellular lysates collected from OVCAR5 and OV90 cells following digestion with PNGase F were subjected to SDS-PAGE (Figure 4.7). Compared to untreated controls, I was able to detect cleaved protein bands of RHAMM smaller than their parental protein (<85kDa) indicating that, at least a portion, of cellular RHAMM is N-glycosylated in OC cells.

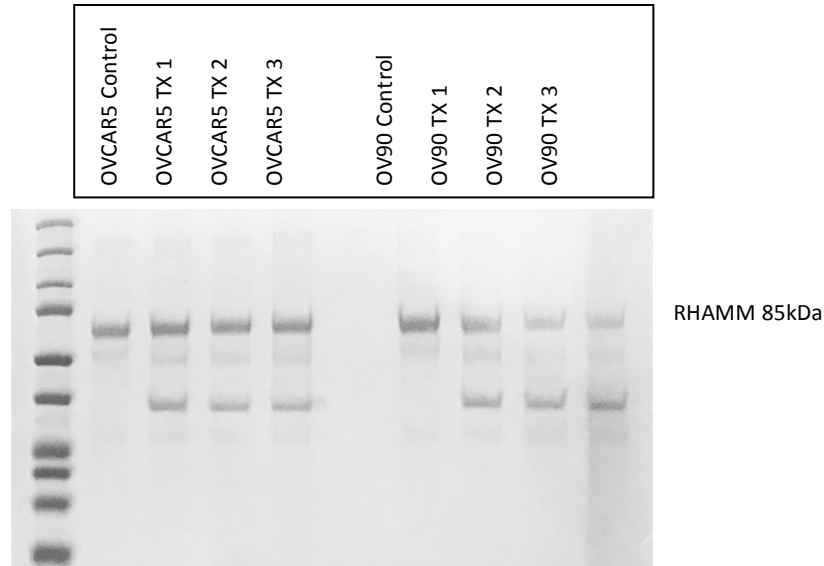


Figure 4.7 RHAMM is N-Linked Glycosylated

Triplicate cultures OC cells (OVCAR5 and OV90) were treated with PNGase F, electrophoresed by SDS-PAGE and coomassie stained. Images were taken with a ChemiDoc.

Conclusion

Cytoplasmic proteins that have dualistic function on the extracellular surface are commonly associated with oncogenic properties such that they are often activated/overexpressed during malignant transformation³⁰. Since I am interested in the role of extracellular RHAMM to promote OC cell migration and invasion and that RHAMM lacks a conventional export peptide sequence or reported use of alternate secretory pathway, it was important to begin to study potential and unconventional export pathways for RHAMM secretion in OC. This prompted me to perform computation analyses of RHAMM in order to better understand and characterize its biochemical structure.

Computational analyses indicated that RHAMM is a very disordered protein, it is comprised mostly alpha-helical domains and it has a high propensity for PPI and PTMs. Potential protein binding partners of RHAMM were identified as CD44, SLC9A and CHP1. Since these proteins have the ability to either transport to the plasma membrane or are known trafficking proteins, there were selected as candidate chaperones for RHAMM secretion. However, I was only able to demonstrate interaction between RHAMM and CHP1 in vitro by WB and immunoprecipitation assays. Unfortunately, abrogation of CHP1 failed to reduce cellular expression of RHAMM or its secretion suggesting that a RHAMM/CHP1 interaction complex is not responsible or necessary for RHAMM secretion by OC cells.

Since PTMs largely contribute to the functional complexity and diversity of the proteome, I subsequently sought to determine whether PTMs could be responsible for RHAMM secretion by OC cells. My computational studies identified several phosphorylation and N-/O-glycosylation sites in RHAMM. Glycosylation is a post-translational protein modification resulting in the addition of oligosaccharides on specific AAs. Since protein glycosylation is often crucial for protein function as it enables proteins to participate in diverse and essential biological functions, it is not surprising that alterations in protein glycosylation can facilitate development of neoplastic disease³¹. In vitro abrogation of global RHAMM glycosylation with TM resulted in significant reduction of both cellular RHAMM and secreted RHAMM indicating that a considerable proportion of intracellular and extracellular RHAMM is glycosylated in OC cells.

N-glycosylated proteins frequently function as cell surface receptors and/or membrane proteins mediating cell-cell interactions³². N-glycosylation (GlcNAc- β -Asn) involves a step-wise process by which preassembled dichol-linked triglucosylate polymannose oligosaccharides are added to asparagine residues in the endoplasmic reticulum (ER)³³. Classical protein transport to

the ER for GlcNAc- β -Asn requires the presence of a specific consensus sequence, which has not been experimentally shown in RHAMM. However, using predictive computational software (NetNGlyc 1.0³⁴), I found that RHAMM was predicted to have multiple sites for potential GlcNAc- β -Asn. Using a PNGase F assay, which cleaves N-glycans and high mannose from glycoproteins, thereby preventing protein N-glycosylation, I was further able to show that a substantial amount of RHAMM is N-glycosylated in OC in keeping with a cell surface function of RHAMM.

In contrast, O-glycosylation (O-GalNAc) is the addition of an oligosaccharide onto the hydroxyl group of a serine or threonine residues. O-GalNAc is carried out by GlcNc-transferase and was identified as the first glycopeptide forming enzyme to act outside of the classically defined secretory pathway³⁵. Since RHAMM contains a myriad of sites for potential O-glycosylation predicted by computational software (NetOGlyc 4.0³⁶), it might be useful in future studies to validate O-GalNAc of RHAMM in OC cells as well as to determine whether this PTM plays a role in RHAMM secretion.

Taken together, my data suggest that glycosylation of RHAMM may promote extracellular RHAMM function and oncogenic potential in OC. The degree to which either O- or N-glycosylation contributes to the etiology and progression of OC, and potentially other cancer types, clearly warrants further study.

Table 4.1: Protein Sequence Identifier used in Computational Analysis

Protein	UniProt Identifier
RHAMM	O75330
CD44	P16070
CHP1	Q99653
SL9A1	P19634

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Chapter 5: Urinary Levels of RHAMM are Elevated in Ovarian Cancer Patients

Background

OC is a heterogeneous disease with multiple subtypes; proven difficult to detect at an early stage. To date, pelvic examination, ultrasound or circulating cancer antigen 125 (CA125) serum levels are evaluated for OC detection. CA125 is elevated in about 80% of OC patients, but fails to detect approximately 50% of stage 1 OC patients¹. Serum CA125 is also frequently upregulated in normal conditions, benign reproductive disease and other cancer types¹. Measuring CA125 levels has been reported to cause patient anxiety and, in many cases, unnecessary surgery¹. To increase specificity, multi-marker panels in combination with CA125, such as human epididymis protein 4, are being studied, but require further validation. This underscores the need to find new OC biomarkers which are capable of detecting disease when treatment options are the most successful.

Since RHAMM expression appeared localized to cytoplasm, cell surface membrane and especially at the apical cell surface in WD OC and normal FT, potentially within ovarian cystic fluids and secreted by OC cells in vitro, I sought to determine whether RHAMM could be secreted by OC cells and, thereby, be detected in bodily fluids. Elevated RHAMM levels in patient urine can potentially be a prognostic marker or diagnostic marker alone or in combination with other markers, warranting RHAMMs clinical importance.

Methods

Patient Cohort

University of South Florida Institutional Review Board approval and patient consent was obtained for prospective (studies #Pro00003119, #Pro00000903) and retrospective (study #106004) collection of urine samples. Anonymized urine samples from healthy male and female controls (N=29), patients with benign gynecological pathology (N=30), OC (N=150), lung cancer (N=20), BC (N=20), brain cancer (N=20), head and neck (H&N) (N=19), prostate cancer (N=22), cervical cancer (N=9), colorectal cancer (CRC) (N=21), melanoma (N=20), endometrial cancer (N=15) as well as sarcoma (N=12) were released for research from the tissue bank at the H. Lee Moffitt Cancer Center and the University of South Florida. All samples were centrifuged at 3,000 x g and the supernatant was aliquoted and frozen at -20 °C before analyses were conducted.

Western Blot (WB) Analysis

Normal and OC urine samples of equivalent volume were centrifuged at 16,000 x g using 30,000 kDa microfilters (Millipore, Bedford, MA) to concentrate the urine specimens. Concentrated urine samples were electrophoresed via 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membrane. Membranes were blocked for 1 hour at room temperature using 5% milk in tris-buffer saline with tween. Membranes were then incubated overnight at 4°C in monoclonal rabbit anti-CD168 RHAMM antibody (abcam®, Cambridge, MA) and then incubated for 1 hour at room temperature in goat anti-rabbit HRP conjugated antibody (Thermo Fisher Scientific, Waltham, MA). Protein bands were visualized using SuperSignal West Femto Substrate (Thermo Fisher Scientific, Waltham,

MA), densitometric analysis was performed using *Image Studio Lite Version 5.0* software program.

Human HMMR/CD168/ RHAMM Sandwich enzyme-linked immunosorbent assay (ELISA)

RHAMM ELISA (LifeSpan BioSciences, Inc., Seattle, WA) was performed according to the manufacturer's recommended instructions for urine sample specimens. Prior to performing the experiments, all samples were thawed to room temperature and centrifuged to remove particulate matter. Plates were read using a microplate reader (BioTek ELx800) with a 450nm wavelength filter.

Statistical Analysis

Samples for RHAMM ELISA were run in duplicate and concentration calculated as per manufacturer's protocol. Data were subjected to descriptive statistics, T-Test, Mann-Whitney U, Wilcoxon W, Kuskal-Wallis, one-way ANOVA, Spearman's rho, and Bonferroni post-hoc analysis. $p \leq 0.05$ was considered statistically significant.

Results

Urinary RHAMM Levels are elevated in OC Patients

By WB, urinary RHAMM protein was negligible in normal control samples (N=10), but elevated in 6/9 (66.67%) OC samples. Lysate from the BC cell line (MCF-7) was used as a positive control and protein bands were quantified by densitometric analysis (Figure 5.1A,B). Further, urinary analysis of RHAMM protein levels from OC patients measured by ELISA (N=150) averaged almost 15X higher than normal controls (N=29) (Figure 5.1C,D & Table 1).

Urinary levels of RHAMM in normal controls averaged 8.16 pg/mL, compared to 116.66 pg/mL in OC patient urine ($p < 0.0001$). Additionally, ELISA measurements of urinary RHAMM from 30 women with benign gynecological diseases including ovarian cysts, uterine fibroids and teratomas averaged 12.85 pg/mL, which is slightly higher than normal controls, but still significantly lower than OC RHAMM levels ($p < 0.0001$) (Figure 5.1C). Lastly, ELISA measurements of urinary RHAMM in patients with sarcoma, melanoma, lung, breast, brain, head and neck, prostate, cervical, CRC, and endometrial cancers were conducted (Figure 5.1D). Although higher than benign disease and normal controls, elevated urinary RHAMM levels in cervical and CRC cancer were not statistically significant. In contrast, minimal levels of urinary RHAMM were found in all other remaining malignancies.

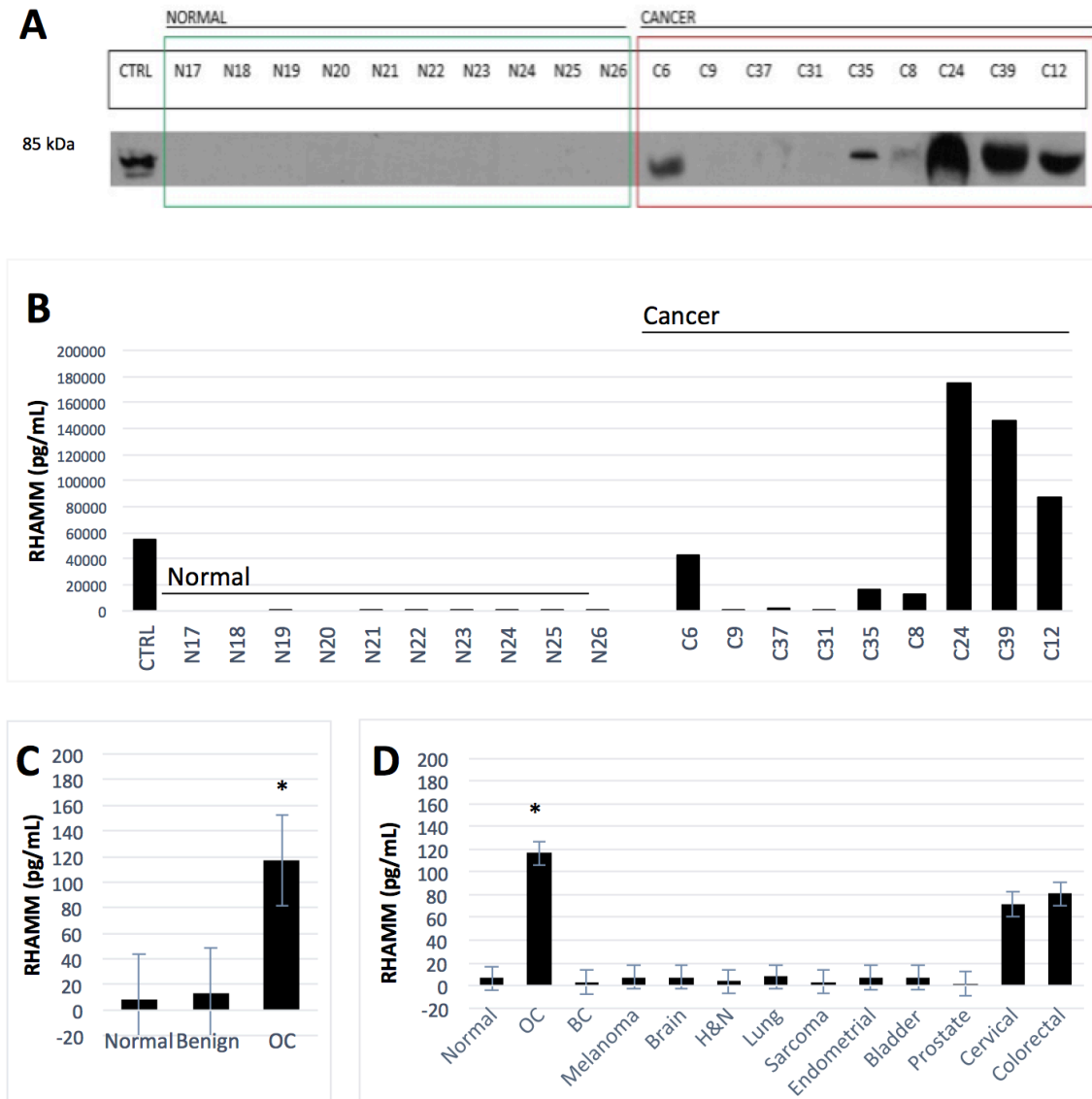


Figure 5.1 Urinary RHAMM levels are elevated in OC patients.

Concentrated urine samples of equivalent volumes from normal controls (N=10) and patients with OC (N=9) were screened for RHAMM by WB (A). Membranes were incubated with anti-RHAMM (1:1000) overnight and visualised with enhanced chemiluminescent. BC cell lysate (MCF-7) was used for positive control. Densitometric values were calculated using *Image Studio Lite Version 5.0* and represented as a histogram (B). Urinary samples were examined by ELISA for urinary RHAMM levels in normal controls (N=29), benign gynecological disease (N=30) and OC (N=150) (C). Urinary RHAMM levels were measured in multiple cancer types including cervical (N=21), CRC (N=9), BC (N=20), brain (N=20), H&N (N=20), lung (N=20), sarcoma (N=19), endometrial (N=20), bladder (N=12), and prostate (N=14) (D). ELISA results (C,D) are expressed as a mean pg/mL RHAMM \pm SE and represented as a histogram where $p \leq 0.05$ was considered statistically significant (*).

Urinary RHAMM Levels Decrease After OC Cytoreductive Surgery

Urinary RHAMM levels were measured by ELISA in 10 OC patients (Figure 5.2A) and two patients with benign low malignant potential (LMP) ovarian tumors (Figure 5.2B) immediately prior to initial cytoreductive surgery, within two weeks of cytoreductive surgery and, where possible, at a three month post-operative follow-up. I found up to 89% reduction in urinary RHAMM levels post-operative compared to pre-cytoreductive surgery in 7/10 OC patients two weeks post-surgery and in 8/10 OC patients three months post-surgery. In contrast, an increase in urinary RHAMM levels two weeks post-cytoreductive surgery was noted in 3/10 OC patients compared to pre- cytoreductive surgery urinary RHAMM levels (Figure 5.2A). Additionally, I measured urinary RHAMM pre and post benign tumor debulking and found minimal changes in urinary RHAMM (Figure 5.2B).

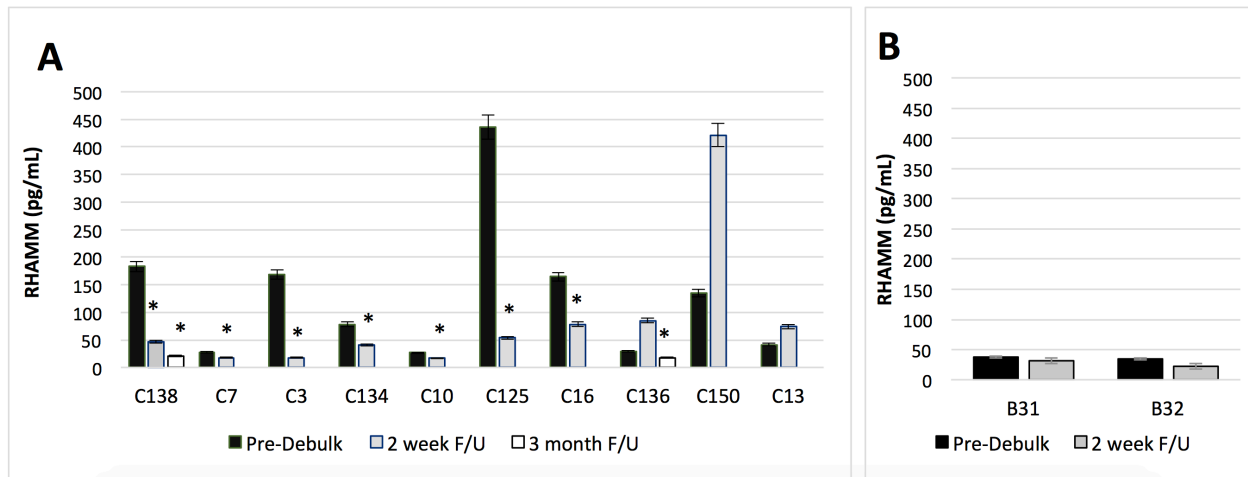


Figure 5.2 Elevated urinary RHAMM levels decrease in OC patients after tumor debulking.

Urinary levels of RHAMM were measured by ELISA from **A)** 10 OC patients and **B)** patients with benign LMP ovarian tumors prior to tumor removal (black bars), within two weeks of debulking surgery (gray bars) and 3 month post debulking surgery where possible (white bars). Results are expressed as a mean pg/mL RHAMM \pm SE and represented as a histogram where $p \leq 0.05$ was considered statistically significant (*).

Urinary RHAMM Levels are Higher in Obese OC Patients

When comparing urinary RHAMM levels and clinical parameters (Table 1 & Figure 5.3), urinary levels of RHAMM did not appear to be related to OC stage (Figure 5.3A), grade (B), patient age (C), family history of BC, OC or CRC (D) or tumor mass (E,F). I found a tendency for a higher average of urinary RHAMM levels in stage 1 and stage 3 OC patients, however, the elevation was not significant (inset, Figure 5.3A). Interestingly, when RHAMM ELISA urinary measurements in OC patients were aligned by body mass index (BMI), urinary RHAMM levels in obese (OB) patients showed a propensity to be higher than overweight (OW) and normal (NW) weight OC patients (Fig. 3G,H). While, not statistically significant, elevated urinary RHAMM levels in obese OC patients averaged 156.2 pg/mL compared to 103.99 pg/mL and 83.55 pg/mL in overweight and normal weight OC patients, respectively.

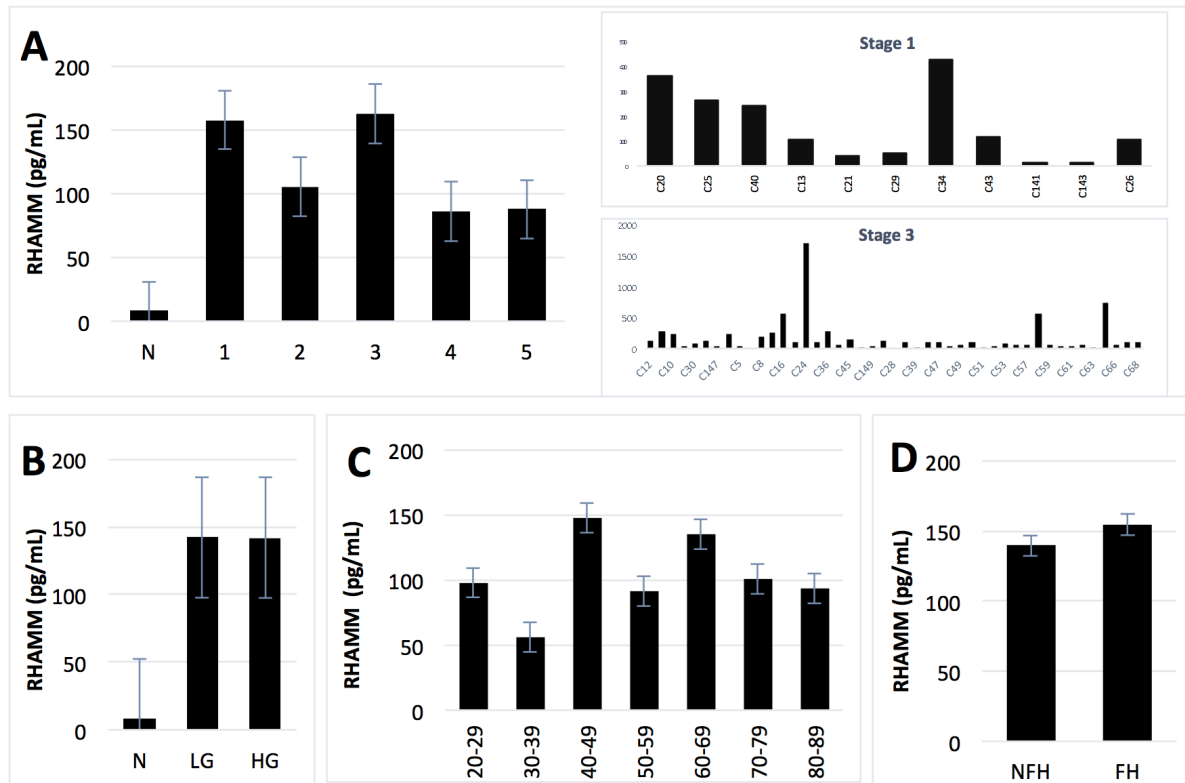


Figure 5.3 Urinary RHAMM levels are higher in obese OC patients. (Continued on Next Page)

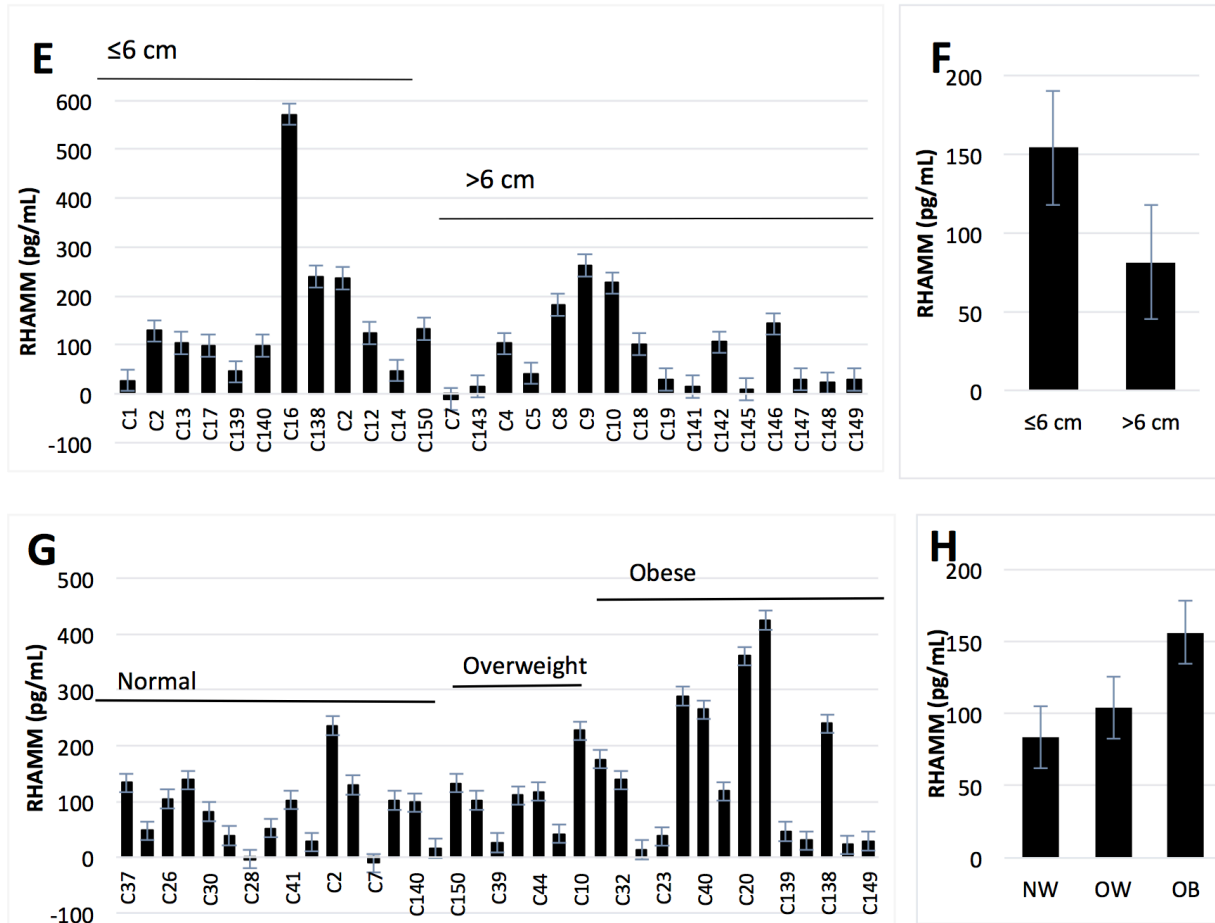


Figure 5.3 Urinary RHAMM levels are higher in obese OC patients.

Urinary RHAMM levels in normal controls (N=29) and OC patients were measured by ELISA and compared with clinical parameters including A) stage [stage 1 (N=11), stage 2 (N=2), stage 3 (N=45), stage 4 (N=6), stage 5 (N=11)], B) grade [low grade (N=23) and high grade (N=27)], C) age (20-29 (N=2), 30-39 (N=5), 40-49 (N=9), 50-59 (N=27), 60-69 (N=31), 70-79 (N=20), 80-89 (N=11)], D) family history of OC, BC or colon cancer [NFH (N=6), FH (N=15)], E,F) tumor size [≤6 cm (N=11), >6 cm (N=15)], and G,H) BMI [OB >30 (N=14), OW 25-29.9 (N=6), NW 18.5-24.9 (N=17)] of OC patients. Results are expressed as a mean pg/mL RHAMM ± SE and represented as a histogram where p≤0.05 was considered statistically significant.

OC Detection Improves when Urinary RHAMM Levels are combined with Serum CA125 Levels

Urinary RHAMM levels were evaluated in 29 OC patient samples with known serum CA125 levels (Figure 5.4). Positive prediction of OC by urinary RHAMM levels was defined by a statistical difference compared to control with a 95% confidence interval whereas positive prediction for OC using serum CA125 levels were defined by clinical standards². Serum CA125 levels in 19/29 or 65.5% OC patient samples predicted positive for OC while failing to predict OC in 10/29 or 34.5% OC samples. In contrast, 26/29 or 89.7% of the samples predicted positive for OC by elevated urinary RHAMM levels while failing to predict 3/29 or 10.3% of the samples. However, the combination of serum CA125 and RHAMM appeared to predict 28/29 or 96.5% of OC samples.

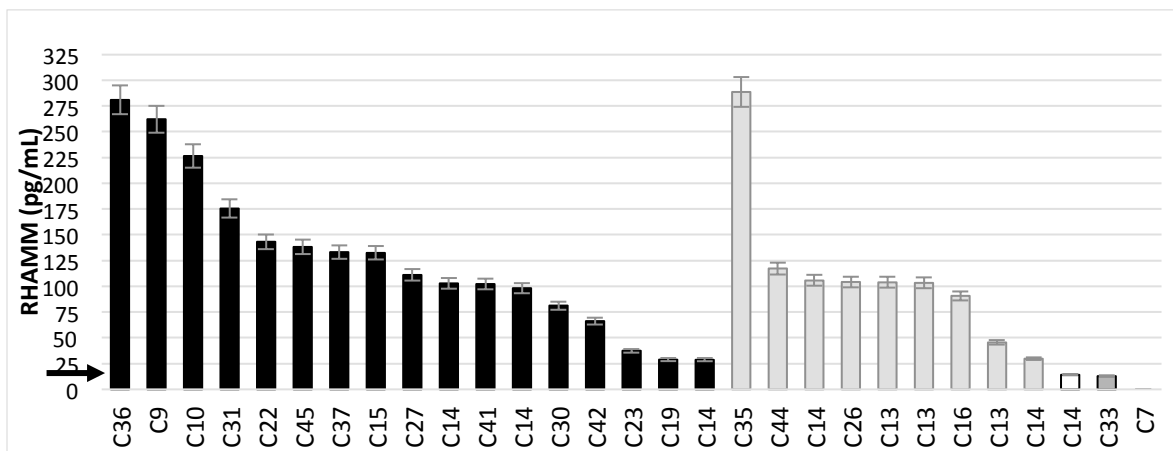


Figure 5.4 OC prediction increases when patient urinary RHAMM levels are combined with serum CA125 levels.

Urinary levels of RHAMM by ELISA from 29 OC patients were shown and compared to clinically positive CA125 serum levels (>65 U/mL, green and yellow bars) or negative CA125 serum levels (<65 U/mL, blue and red bars). Urinary RHAMM was evaluated by a positive prediction (green and blue bars) or negative prediction (yellow and red bars) for OC. Urinary RHAMM positive prediction threshold was considered statistically significant compared to controls with a 95% confidence interval. Results are expressed as a mean pg/mL RHAMM ± SE and represented as a histogram.

Conclusion

RHAMM is a non-integral, extracellular receptor protein, only loosely tethered to the cell membrane. Originally discovered from the conditioned medium of embryonic chick heart fibroblasts, RHAMM is either shed or secreted into the extracellular environment³. Herein, I sought to determine if RHAMM could be detected in bodily fluids. More specifically, we sought to determine whether elevated RHAMM levels could be detected in the urine of OC patients, thereby serving as a potential diagnostic and/or prognostic biomarker of disease. Urinary biomarkers are ideal for disease detection since urine collection is simple, safe, non-invasive and cost effective. In addition, urinary proteins retain high stability and urinary filtration precludes the presence of large proteins found in serum, such as albumin, which can confound test results.

RHAMMs' α -helical and coiled structure confers a hydrophilic and water soluble protein profile⁴ indicating RHAMM might be present in bodily fluids. Given that glomerular filtration typically excludes high molecular weight proteins from the urine, I was surprised to detect full length RHAMM protein at 85 kDa in OC patient urine by WB. However, my results are in keeping with others who also reported high molecular weight proteins in urine of women at high risk for BC including matrix metalloproteinase-2, matrix metalloproteinase-9 (MMP9) and MMP9/neutrophil gelatinase-associated lipocalin complex (MW: 72 kDa, 92kDa and 115 kDa respectively)⁵. High molecular weight proteins present in the urine are commonly associated with renal dysfunction⁶. Donadio et al. (2003) reported renal impairment in both early and late stage OC showing at least a 10% impairment of glomerular filtration rate (GFR) and creatinine clearance in 30% of stage 1, 50% of stage 2, 56% of stage 3 and 64% of stage 4 OC patients⁶ suggesting that renal impairment due to disease could enable urinary transport of full-length RHAMM. Interestingly, despite lacking a secretory signal peptide sequence, RHAMM is

commonly secreted by cells by an alternate and, as yet, poorly understood mechanism⁷ so that RHAMM may bypass glomerular filtration by an alternate transport pathway.

In this pilot study, I consistently found elevated urinary RHAMM levels in OC patients that were significantly higher than normal healthy controls and women with benign gynecological disease (*P<0.0001). While most patients with benign gynecological disease did not demonstrate elevated urinary RHAMM, elevated urinary RHAMM was observed in a patient with uterine fibroids and a patient with endometrioma. During inflammation HA levels increase within the microenvironment which, in turn, promotes increased RHAMM secretion⁸ and involvement of RHAMM in the inflammatory process⁹ so that inflammatory benign gynecologic conditions, as may have been present in these two patients, could result in a transient increase in urinary RHAMM levels. Though not statistically significant, average urinary RHAMM was also elevated in cervical cancer and CRC. Reports of RHAMM mRNA overexpression in women with cervical cancer¹⁰ and intense RHAMM staining in CRC tumor budding cells associated with a more malignant and invasive cancer phenotype¹¹ are in agreement with my findings. Although studies indicate elevated RHAMM in breast¹², prostate¹³ and bladder¹⁴ tissue, my samples did not show urinary elevations in these cancer types. However, since RHAMM contributes to an invasive and metastatic phenotype⁸, it is tempting to speculate that increased levels of RHAMM within the tumor microenvironment, and, subsequently, in the urine, may discriminate between cancers with less than 50% 5 year survival (ovarian, cervical, colorectal) related, in part, to metastatic spread, compared with cancer survival greater than 50% 5 year survival such as breast, prostate, glioma and sarcoma¹⁵.

While urinary RHAMM levels were essentially unchanged in patients with benign disease following cytoreductive surgery, reduced urinary RHAMM levels after cytoreductive

surgery suggests that RHAMM is produced and secreted by OC cells. In contrast, increased urinary⁹ RHAMM after cytoreductive surgery could be associated with post-surgical infections since 35.8% of patients experience symptomatic infections after cytoreductive surgery¹⁶.

When analyzing clinical parameters, average urinary RHAMM levels were independent of age, FH and tumor mass. One might expect higher RHAMM levels in the patients with larger tumors, however, I found no differences in average urinary RHAMM levels based on tumor mass. This may reflect the presence of necrotic tissue often present within the central core of tumors, thereby reducing the effective tumor volume capable of secreting RHAMM. Most notably, I found elevated urinary RHAMM levels independent of tumor stage and grade indicating that RHAMM could be a viable diagnostic marker for detection of early stage disease, which is imperative for successful treatment and patient survival.

Additionally, I found a trend for elevated urinary RHAMM with increasing BMI. Multiple studies have correlated obesity with increasing risk for comorbidities such as diabetes, hypertension and cancer¹⁷. In OC, a meta-analysis of 121 studies provided strong epidemiological correlation between women with a high BMI and increased risk for OC, shorter time to disease relapse and overall survival time¹⁸. Obesity-mediated secretion of pro-inflammatory cytokines (IGF-1, VEGF, TNF- α and IL-6) promotes cellular dysfunction¹⁹ and has been shown to increase the risk of multiple malignancies including high grade serous OC¹⁸. Interestingly, the ability of obesity associated cytokines TNF- α and IL-6 to activate transcription factor NF- κ B, a common downstream effector of RHAMM²⁰, may exacerbate tumorigenic contributions of RHAMM. Lastly, a comparison of urinary RHAMM levels with serum CA125 levels suggested that RHAMM, in combination with CA125, can more accurately predict OC. Going forward, then, it would be interesting to determine whether combinations of urinary levels

of RHAMM with other biomarkers currently under clinical investigation such as mesothelin, Bcl-2, lipoprotein A, osteopontin and the OVA1 test could likewise improve OC detection.

Taken together, I show, for the first time, elevated urinary RHAMM levels in both early and late stage serous OC patients, minimal expression in patients with benign gynecological disease, the potential for obesity to confound test results and improved disease detection when urinary RHAMM levels are combined with serum CA125 measurements. This pilot study suggests, then, that elevated urinary levels of RHAMM are associated with OC and may serve as a novel biomarker contributing to non-invasive, economical and effective diagnostic and prognostic applications.

Table 5.1. Summary of patient clinical parameters

Sample	N	Average Urinary RHAMM (pg/mL)	Range Lower limit	Range Upper limit	p-value
Normal	29	8.16433368	-8.50288	54.43993895	
Benign	30	12.8451222	-1.029525763	85.95183057	p<0.0001
OC	150	116.662456	-27.1929	1702.52388	p<0.0001
Other Cancer					
Breast	20	3.038716	-0.424456354	23.94696898	
Melanoma	20	7.38305	0.55800801	50.54440287	
Brain	20	7.40445	-0.459595635	46.65082733	
Head and Neck	19	3.696184	-1.374710715	16.36502676	
Lung	20	7.797443	-10.16901388	113.6471452	
Sarcoma	12	3.187877	-0.8321832	16.26171595	
Endometrial	14	6.937981	-1.31133649	94.04170598	
Bladder	17	7.020548	-1.37833232	42.53569287	
Prostate	21	1.647099	-2.51055025	19.43540151	
Cervical	9	71.36415	-10.64064941	382.2059057	
Colorectal	21	80.95593	-7.359425625	321.8391349	
STAGE					p=0.249
Stage 1	11	157.635708	14.12994	424.42521	
Stage 2	4	105.348346	37.508515	143.22621	
Stage 3	45	162.35244	-10.77318	1702.52388	
Stage 4	6	85.8344317	12.838485	175.459295	
Stage 5	11	87.5899564	22.47945	239.703255	
GRADE					p=0.280
Low grade	23	142.265278	14.12994	424.42521	
High grade	27	141.993346	-10.77318	1702.52388	
AGE (years)					p= 0.832
20-29	2	98.1338875	20.80848	175.459295	
30-39	5	56.173133	10.16778	103.32459	
40-49	8	147.813483	-7.0938	1702.52388	
50-59	27	91.4446452	-27.1929	571.57434	
60-69	30	134.730681	-6.14796	878.01417	
70-79	20	100.570824	-10.77318	463.93452	
80-89	11	93.1003286	8.74902	226.42794	
Tumor Mass					p=0.102
0-6 cm	11	154.296986	27.177795	571.57434	
>6 cm	15	81.4531359	-10.77318	262.13274	
Family History					p=0.503
NFH	6	139.629013	37.508515	360.736255	
FH	15	154.676083	-3.776025	1702.52388	
BMI					p=0.419
N	17	83.5546827	-10.77318	235.54089	
OW	6	103.986345	25.39085	226.42794	
OB	14	156.19858	12.838485	424.42521	

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Chapter 6: Concluding Remarks

OC is the deadliest gynecologic cancer whose molecular etiology remains poorly understood. It is a complex and heterogeneous disease characterized by numerous potential origins of disease from within different areas of the ovary and surrounding tissues as well as diverse clinical presentations with multiple histologic subtypes characterized by varying molecular profiles. Yet, despite its diversity, there is a general propensity for metastatic spread and emergence of drug resistant disease among OCs.

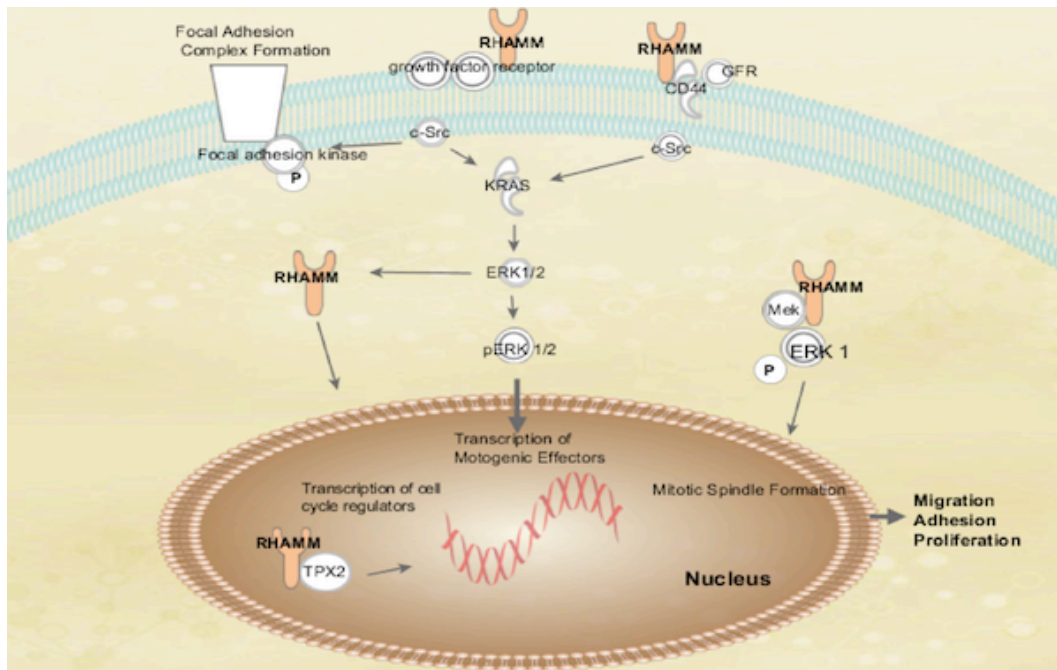


Figure 6.1 Schematic representation of RHAMMs association in oncogenic signaling pathways created with Ingenuity Pathway Analysis software.

RHAMM has dualist functions dependent upon its cellular localization that makes it an attractive entity to drive both metastatic spread and drug resistant disease (Figure 6.1). Intracellularly, RHAMM is involved in microtubule spindle assembly and gene transcription contributing to cell cycle progression. On the cell surface, loosely tethered RHAMM forms a complex with CD44 and HA that promotes cellular migration and invasion. Since the contribution of RHAMM to OC progression has not been delineated, I sought to determine if RHAMM could promote OC invasion/metastasis.

I found that RHAMM is overexpressed in clinical specimens of OC by immunohistochemistry and although both primary and metastatic OCs stain equally for RHAMM, RHAMM staining was most intense among clinically aggressive OC histologic subtypes. Further, using an in vitro model system, I was able to show that OC cells express and secrete RHAMM. Abrogation of RHAMM using silencing RNA technology inhibited OC cell migration and invasion suggesting that RHAMM may contribute, at least in part, to the metastatic propensity of OC.

Since RHAMM lacks an export signal peptide sequence and has not been reported to employ alternate mechanisms for extracellular secretion, I utilized computational analyses to predict post-translational glycosylation events as a novel mode for RHAMM secretion. Treatment with a peptide *N*-glycosidase F enzyme successfully cleaved N-linked oligosaccharides from RHAMM as seen by SDS-PAGE. Glycosylation inhibitors abrogated RHAMM secretion by OC cells in vitro further validating my prediction for an unconventional glycosylation-mediated mode for RHAMM secretion.

Lastly, since RHAMM is secreted by OC cells, I sought to determine whether RHAMM could be detected in bodily fluids. In a pilot study, I found that urinary levels of RHAMM are

elevated in OC patients as measured by ELISA. Decreased urinary RHAMM levels noted following cytoreductive surgery support OC as the source of elevated urinary RHAMM levels. Finally, while obesity was associated with high urinary RHAMM levels in OC patients, combined measurements of urinary RHAMM and serum CA125 improved prediction of OC.

Though my data did not support a role for RHAMM in OC cell growth, future therapeutic interventions targeting extracellular RHAMM could be employed for managing metastatic spread. The use of monoclonal antibodies, such as trastuzumab, in combination with traditional chemotherapeutics has been very successful in BC¹. Trastuzumab is a monoclonal antibody which targets the extracellular domain of HER2², thereby selectively targeting the highly proliferative BC cells that overexpress HER2. Although, not as effective when used alone, it has been shown to have synergistic effects when used in conjunction with cisplatin, carboplatin, radiation and docetaxel¹. Using a monoclonal antibody targeting extracellular RHAMM could similarly reduce metastatic spread when used in combination with taxanes or platinum-based agents in OC.

Recent advances in chemotherapeutic modalities have utilized HA as a drug target or drug delivery carrier³. HA nanoparticle delivery systems exploit the strong binding affinity HAs have to their receptors⁴. Since the HA receptor, RHAMM, is commonly overexpressed in cancer while there is little to no RHAMM expression in normal cells, it appears reasonable to target HA/RHAMM for selective drug delivery. HA has also been coupled to cytotoxic agents, such as paclitaxel, and remains non-toxic until its bioconjugates are internalized³. As a result, using an HA nanoparticle delivery system to target RHAMM could bypass the systemic cytotoxic effects associated with traditional chemotherapy³.

The extracellular form of RHAMM could further be targeted using an inside out approach to prevent its cell surface localization. Glycobiology represents an active and promising area of research for drug discovery since glycosylation patterns of proteins are frequently altered in malignancy⁵. For example, N-glycosylation inhibitors are currently being studied for their effectiveness in overcoming drug resistance in glioma by targeting compensatory mechanisms commonly seen in malignancy⁶. Since I've shown RHAMM is likely exported by N-glycosylation, N-glycosylation inhibitors could be useful therapies to disrupt the extracellular distribution of RHAMM and subsequent activation of the ERK1, 2 MAPkinase pathway⁶, thereby mitigating OC cell metastasis as well as emergence of drug resistant disease.

As previously mentioned, varying levels of different RHAMM isoform confers distinct tumorigenic phenotypes. While my work was focused on the extracellular function of RHAMM in OC, it would be interesting to more deeply explore the role of intracellular RHAMM in OC. RHAMMs' role in the mitotic spindle assembly and interaction with BRCA1, at both the physical and genetic level, has been shown to have profound effects in BC susceptibility⁷. This could be an area to explore in OC patients who are mutant BRCA1 or have family history of BRCA1 mutations. Centrosome amplification is normally regulated by the BRCA-BARD1 complex, which directly interacts with RHAMM, but which results in aberrant mitotic spindle assembly if the BRCA-BARD1/RHAMM interaction is altered⁸. As a result, since the BRCA1-BARD1 complex is essential for proper chromosome stability and spindle formation⁹, mutations in BRCA1 and/or RHAMM could interfere with spindle assembly leading to chromosomal instability⁹.

Taken together, the studies described herein suggest that RHAMM contributes to OC and that further studies are warranted to elucidate the clinical role of RHAMM in OC.

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Appendix A:

IRB Approval Letter



DIVISION OF RESEARCH INTEGRITY AND COMPLIANCE
Institutional Review Boards, FWA No. 00001669
12901 Bruce B. Downs Blvd., MDC035 • Tampa, FL 33612-4799
(813) 974-5638 • FAX (813) 974-5618

March 21, 2012

This letter supersedes the one dated 03/25/11.

Santo Nicosia, M.D.
Pathology and Cell Biology
Dept. Pathology & Cell Biology MDC11
12901 Bruce B Downs Blvd
Tampa, FL 33612

RE: **Expedited Approval** for Initial Review
IRB#: Pro00003119
Title: Ovarian Cancer: A Team Approach Toward Identification of Novel Diagnostic and Therapeutic Targets

Dear Dr. Nicosia:

On 3/31/2011 the Institutional Review Board (IRB) reviewed and **APPROVED** the above referenced protocol. Please note that your approval for this study will expire on 03/31/12

Approved Items:

[Nicosia OCRF research protocol](#)
OCRF Consent.

It was the determination of the IRB that your study qualified for expedited review which includes activities that (1) present no more than minimal risk to human subjects, and (2) involve only procedures listed in one or more of the categories outlined below. The IRB may review research through the expedited review procedure authorized by 45CFR46.110 and 21 CFR 56.110. The research proposed in this study is categorized under the following expedited review category:

(3) Prospective collection of biological specimens for research purposes by noninvasive means.

(5) Research involving materials (data, documents, records, or specimens) that have been collected, or will be collected solely for nonresearch purposes (such as medical treatment or diagnosis).

Please note, the informed consent/assent documents are valid during the period indicated by the official, IRB-Approval stamp located on the form. Valid consent must be documented on a copy of the most recently IRB-approved consent form.

A segment of your study qualifies for a waiver of the requirements for the documentation of informed consent as outlined in the federal regulations at 45CFR46.116 (d) which states that an IRB may approve a consent procedure which does not include, or which alters, some or all of the elements of informed consent, or waive the requirements to obtain informed consent provided the IRB finds and documents that (1) the research involves no more than minimal risk to the subjects; (2) the waiver or alteration will not adversely affect the rights and welfare of the subjects; (3) the research could not practicably be carried out without the waiver or alteration; and (4) whenever appropriate, the subjects will be provided with additional pertinent information after participation.

A segment of your study qualifies for a waiver of the requirement for signed authorization as outlined in the HIPAA Privacy Rule regulations at 45 CFR 164.512(i) which states that an IRB may approve a waiver or alteration of the authorization requirement provided that the following criteria are met (1) the PHI use or disclosure involves no more than a minimal risk to the privacy of individuals; (2) the research could not practicably be conducted without the requested waiver or alteration; and (3) the research could not practicably be conducted without access to and use of the PHI.

Specifically, for the retrospective data collection- PI will be receiving already de-identified data from MCC Tissue bank. For the prospective phase- PI will be obtaining consents and authorizations.

As the principal investigator of this study, it is your responsibility to conduct this study in accordance with IRB policies and procedures and as approved by the IRB. Any changes to the approved research must be submitted to the IRB for review and approval by an amendment.

We appreciate your dedication to the ethical conduct of human subject research at the University of South Florida and your continued commitment to human research protections. If you have any questions regarding this matter, please call 813-974-5638.

Sincerely,



Barry B. Bercu, M.D., Chairperson
USF Institutional Review Board