6-2016

Proteomic Profiling of Serum Derived Exosomes from Prostate Cancer Patients

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LOMA LINDA UNIVERSITY
School of Medicine
In conjunction with the
Faculty of Graduate Studies

Proteomic Profiling of Serum Derived Exosomes from Prostate Cancer Patients

by

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A Dissertation submitted in partial satisfaction of
The requirements for the degree
Doctor of Philosophy in Anatomy

June 2016

Each person whose signature appears below certifies that this dissertation in his/her opinion is adequate, in scope and quality, as a dissertation for the degree Doctor of Philosophy.

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DEDICATION

This also is true, and that is, not all are privileged to often witness the culmination of their efforts or fortunate to see the fruits of their labors. The same could be said of my dear mother (Mary Conteh) who at the tender age of 44, was 'cut down by the knees', and prematurely hurried to the grave by a mysterious malady. Her clinical presentation could be summed-up as; 'Headache in the morning, Coma by the afternoon and dead by the evening'. Should the grave give up its secret and she was alive just for a day; how elated she will be today. Herself a high school 'drop-out' but oh how much she valued education. Of the worlds basics she lacked (including proper and adequate food), but to school she made sure I went regular, regardless of the personal sacrifice. Long before a thought ever appeared on my mind, she desired for me a life that she did not have; one full of reason, service, duty to God and man and yes plenty of food. I did not fulfill her dream of becoming a Nursing Assistant (which she always wanted to be), but I am confident of her approval of my current direction.

It will only be fitting to dedicate this to my dear mother.

ACKNOWLEDGMENTS

"And one will say to him, what are these wounds between your arms? Then he will answer, *Those with which I was wounded in the house of my friends*." Zehariah 13:6 (NKJV). This is a rather peculiar and difficult statement to come from a Semite, given their long history of 'extreme hospitality' even at the expense of one's life. I have in fact been treated quite to the contrary 'at my friends house.' I have received healing at 'my friend's house'.

I will forever be grateful to my friend and mentor Dr. Nathan Wall who ignoring the adage 'can't teach an old dog new tricks' was brave enough to put a pipette in my hand and trusted me to go pour gels. He then sat me down to painstakingly explain the meaning of it all. Who though battered by the harsh realities of today's scientific research world with its 'cut throat' competition for scarce resources yet never stopped being human. Still knows how to give a brotherly bear hug and very slow to chastise. Whose tolerance and indeed appreciation of the inherent value of peoples or all ethnicities and creed is an example of the true heart of God. This entire endeavor would not have been possible without him.

I also pause to acknowledge the role of Dr. Salma Khan who over the years has become a big sister and a true friend to me. She was often the teacher at the bench side introducing me to newer lab techniques, scrutinizing my results and for helping me trouble shoot when things were not going well. As if that was not enough, she often made sure me and my lab mates were well fed.

I am very grateful to Drs. De Leon and Casiano whose steady leadership at the Center for Health Disparities have created the environment and support that makes this work possible.

To my committee members and friends Drs. Ken Wright opening the doors for me to engage in in-depth study of Anatomy and Mark Reeves for intervening to prevent a complete demise of my dreams of becoming a practitioner of the art of Surgery. They both intervened at critical junctures in my life when failure and retreat seemed all but inevitable. They've exercised tremendous patience in days when I did not perform at my best and to you I will always be thankful.

To my friend and committee member Dr. Langridge do I owe a tremendous debt of gratitude for his great friendship and exemplary life. For his tremendous ability to relate to the young and the odd. His voracious appetite for knowledge and for scientific discovery even after decades of pursuing the same, is a true inspiration to me.

Grateful to my committee member and friend Dr Saied Mirshahidi without whose contribution of material and intellectual support, this work would not have been possible and to Dr. Jonathan Neidigh for devoting his time and effort to helping us acquire and make sense of the Mass Spectrometry data.

I will eternally bear a heart full of gratitude to my lab mates Drs. Jessica Jutzy, Malyn Asuncion, Jonathan Aspe and Heather Fergusson-Bennett, Amber and Ronnie who shared everything with me including ideas, computers, materials and indeed joys and sorrows.

Finally I reserve a loving gratitude to my wife June for being my greatest cheerleader. For believing in me and for her unconditional love.

CONTENTS

TABLES

FIGURES

Figures Page

ABBREVIATIONS

Prostate Cancer	PCa
Inhibitor of Apoptosis	IAP
Tumor-Derived Exosome	TEX
National Cancer Institute	NCI
Micro RNA	mRNA
Short Inhibitory RNA	siRNA
Hypoxia Inducing Factor	HIF
Hepatitis B X Interacting Protein	HBXIP
Apoptosis Activating Factor 1	Apaf1
Benign Prostatic Hyperplasia	BPH
Peripheral Blood Leukocytes	PBL
Cytotoxic T Lymphocyte	CTL
Granulocyte-Macrophage Colony-Stimulating Factor	GM-CSF
T-Regulatory Cell	Treg
Interferon	IFN
Interleukin	IL
Serial Analysis of Gene Expression	SAGE
Prostate Specific Antigen	PSA
Lipoxygenase	LOX
Cyclooxygenase	COX
Multiple Myeloma	MM
Acute Lymphoblastic Leukemia	ALL

Chronic Myelogenous Leukemia	CML
Acute Myelogenousc Leukemia	AML
Chronic Lymphocytic Leukemia	CLL
Single Nucleotide Polymorphisms	SNPs
Enzyme Linked Immunoabsorbant Assay	ELISA
Lysosomal-Associated Membrane Protein 1	LAMP1
Alpha Fetoprotein	AFP
Hepatitis C Viral	HCV
Hepatocellular Carcinoma	HCC
Phosphate Buffered Saline	PBS
Phenylmethanesulfonylfluoride	PMSF
Multiple Analysis of Variance	MANOVA
African American	AA
Caucasian American	CC
Hereditary PCa Gene	HPC-1
Dithiothreitol	DTT
Mass Spectrometry	MS
Endoplasmic Reticulum	ER
Kilodaltons	Kda
Iroquois Homeobox Protein 5	IRX5
Mitochondrial Tumor Suppressor 1 Isoform 4	MTS1
Trinucleotide Repeat Containing 6B Isoform 3	TNR6B
Vitamin D Receptor	VDR

ABSTRACT OF THE DISSERTATION

Proteomic Profiling of Serum Derived Exosomes from Prostate Cancer Patients

by

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Doctor of Philosophy, Graduate Program in Anatomy
Loma Linda University, March 2016
Dr. Nathan R. Wall, Chairperson

Touted among the major achievements in the diagnosis and management of Prostate cancer (PCa) in the past few decades has been, the dramatic decline of men with advanced/metastatic PCa at diagnosis coupled with a significant improvement (>90%) in the five and ten year survival rates of the disease. Non-palpable PCa (potentially clinically treatable disease) now accounts for 70-80% of all newly diagnosed cases of PCa. Preceding these changes by about a decade was the introduction of Prostatic Specific Antigen (PSA) into clinical practice; first as biomarker for monitoring response to therapy and subsequently as a complementary screening tool. It is not surprising then that a cause-effect relationship has been suggested. Like a double-edged sword, the use of PSA as a screening tool has also been blamed for the rise of unnecessary prostate related invasive procedures including biopsies and surgeries. Some of the documented criticisms of PSA include its lack of specificity (elevated in inflammation) for PCa and the difficulty of establishing a cut-off value that is highly sensitive and specific for the disease. It is estimated that perhaps up to half of the PCa diagnosis are patients whose tumors would have been clinically undetectable had PSA not been included in the screening process. The dissatisfaction with PSA has created opportunities to search for novel biomarkers for screening and monitoring of treatment in PCa. Some of the more

novel biomarkers that have been examined as potential replacements for PSA are the RNA product prostate cancer antigen-3 (PCA 3), the enzyme alpha methylacyl-CoA, and the gene fusion product TMPRSS2-ERG. More recently Zhang, Casiano and colleagues described an increased predominance of autoantibodies to Cyclin-B1 in the sera of PCa patients compared to controls with Benign Prostatic Hyperplasia (BPH). All these efforts are in different stages of maturity but yet to have ground breaking clinical impact.

We hereby examined the role of exosomes in PCa and a qualitative profile of its proteomic composition. The higher levels of circulating exosomes in sera of PCa patients is directly prostate-derived and could be stress-induced as the non-cancer prostate stroma and the immune system interact with neoplastic cells. This could be reflected in some of the similarities in the proteomic profile of serum-derived exosomes and exosomes derived from direct PCa in vitro cell line cultures. Moreover, PCa has a higher incidence and a greater disease severity in non-Hispanic African Americans and this difference in disease biology can be reflected in the difference in the proteomic profile of exosomes across ethnicities/races. Serum exosomes originate from a diverse population of normal, neoplastic or inflammatory cells. Tumors do shed membrane vesicles directly into serum or extracellular space. These vesicles are referred to as tumor derived exosomes (TEX). Our overall objective was to use a seroproteomics approach focused on profiling serum exosomes in PCa patients. This profile would then be compared with that of non-cancer patients in an effort to identify proteins unique to cancer patients. A comparative study across racial groups will help us begin to identify possible protein markers/players involved in determining disease aggressiveness. The project hopes to one day fill the gap of the lack of biomarker identification in PCa patients and perhaps give us potential therapeutic targets to help lower morbidity in the black and indeed all cohorts of patients. It builds on preliminary data indicating that PCa patient sera have exosomes containing stress survival and cancer related proteins.

CHAPTER 1

LOCALIZATION AND UP-REGULATION OF SURVIVIN IN CANCER HEALTH DISPARITIES: A CLINICAL PERSPECTIVE.

Published:

Khan S, Ferguson Bennit H, Valenzuela MMA, **Turay D**, Diaz Osterman CJ, Moyron RB, Esebanmen GE, Ashok A, Wall NR. Localization and up regulation of Survivin in cancer health disparities: a clinical perspective. Biologics: Targets and Therapy, 9: 57-67, 2015.

Abstract

Survivin is one of the most important members of the inhibitors of apoptosis (IAP) protein family as it is expressed in most human cancers but is absent in normal, differentiated tissues. Lending to its importance, Survivin has proven associations with apoptosis and cell cycle control and has more recently been shown to modulate the tumor microenvironment and immune evasion as a result of its extracellular localization. Up regulation of Survivin has been found in many cancers including breast, prostate, pancreatic, and hematological malignancies and may prove to be associated with the advanced presentation, poorer prognosis and lower survival rates observed in ethnically diverse populations.

Key Words: Survivin, cancer, exosomes, health disparity

Introduction

Cancer is a major public health problem in the United States and the world. Recent epidemiological statistics indicate that cancer will develop in one in three women and one in two men in the US over their lifetime.(Siegel *et al*., 2013) The three most common cancers among males are prostate, colorectal and melanoma of the skin, and among females, breast, uterine corpus and colorectal.(Sharp *et al*., 2014) Although deaths attributed to cancer have declined among both Caucasians and African Americans, African Americans continue to suffer a greater burden for each of the most common types of cancer.(Siegel *et al*., 2013) This discrepancy recorded among cancer patients from different ethnicities is termed cancer health disparity. The National Cancer Institute (NCI) defines cancer health disparity as an adverse difference in cancer incidence (new cases), cancer prevalence (all existing cases), cancer death (mortality), cancer survivorship, and burden of cancer or related health conditions that exist among specific population groups.(Siegel *et al*., 2013) When investigating the factors that contribute to cancer health disparities, the most obvious are access to health care and socioeconomic status.(Beydoun & Beydoun, 2008, Guessous *et al*., 2010, Jadav *et al*., 2015) However, evidence exists that dietary fat can influence carcinogenesis.(Tsai & Giovannucci, 2012) In 1982, the US National Academy of Sciences committee on Diet, Nutrition and Cancer, using both epidemiological and experimental data, concluded that a causal relationship between fat intake and the occurrence of cancer exists.(Council, 1982) However, the strongest evidence that environmental factors give rise to an etiology of cancer comes from the studies of cancer incidence in different ethnic populations and their migrations and lifestyle habits. Specifically, the adoption of a Westernized diet appears causal in the significant increase in annual deaths, in native Japanese, from colon,(Takachi *et al*., 2011) breast(Kono, 2010) and pancreatic cancers(Kasuga *et al*., 2013) upon their moving from Japan to the United States. In addition, experimental animal studies agree that both specific and non-specific evidence exists for the occurrence of cancer being strongly associated with consumption of a diet high in fat.(Guthrie & Carroll, 1999) Contradictory studies also exist in which lifestyle factors in cancer incidence have been described. Whereas years smoking and number of cigarettes smoked had a correlation with increased incidence of pancreatic cancer, there was no correlation with pancreatic cancer and body mass index, physical activity, alcohol, coffee and green tea consumption.(Nakamura *et al*., 2011)

Early detection is important in cancer discovery, treatment and survival. In order to better understand cancer incidence and mortality in diverse populations it has become imperative that we identify and then characterize markers of cancer development and progression to include both pathways and molecular mechanisms associated with these disparities. Given the strong link between cancer incidence, oxidative stress and diets high in fat,(De Pergola & Silvestris, 2013) we must map these associations and identify the survival entities and pathways as potential targets. A long-term goal in health disparities research is to understand how an increase in oxidative stress will ultimately promote cancer cell resistance to therapy-induced death and how to overcome this resistance.

Survivin is an important member of the inhibitors of apoptosis (IAP) protein family because its tumor specific expression is unique out of all of the human gene products.(Reed, 2001) Survivin expression is evident during embryonic and fetal development but not in terminally differentiated tissue.(Li *et al*., 1998) It is expressed in virtually all of the different types of human cancers (**Table I**), making Survivin an alluring protein in the study of carcinogenesis.(Andersen *et al*., 2007) Survivin is referred to as a bifunctional protein, having essential roles in inhibiting apoptosis and controlling proper cell division.(Altieri, 2003) In our most recent work we have begun to refer to Survivin as a multifunctional protein as it does much more, to include controlling diverse cellular functions, including surveillance checkpoints, suppression of cell death, the regulation of mitosis, and adaptation to unfavorable environments.(Altieri, 2003, 2006)

Localization of Survivin

The multifaceted functionality of Survivin is still being intensely scrutinized, and it appears that protein compartmentalization may be important. Survivin has been shown to localize in mitochondria, where it modulates tumor cell apoptosis similar to the Bcl-2 family.(Dohi, Beltrami*, et al*., 2004b) Its localization to nucleus and cytosol confers its role in mitosis regulation and apoptosis inhibition, respectively.(Fortugno *et al*., 2002) Furthermore, we have identified the existence of Survivin extracellularly, contained in small membrane bound vesicles known as exosomes (**Figure 1**), and have shown that exosome-bound Survivin protein can be secreted by cancer cells to be taken up by surrounding cells, producing a field effect that confers a general stress-survival phenotype.(Khan *et al*., 2009, Khan S *et al*., 2011, Webber *et al*., 2015).

Table: Influence of Survivin on Clinical Prognosis.

Cancer type	**On prognosis/staging of the disease**	**Survivin expression**	**Reference**
1. Breast cancer	Good to poor	Moderate to high	Kalla Singh 2010, Adamkov 2012, Rexhepaj 2010, Xu 2012, Dedić Plavetić 2013, Span 2006, Li 2004, Boidot 2009, Khan 2014
2. Prostate cancer	Fair to poor	Moderate to high	Zaffaroni 2005, Shariat 2004, Koike 2008, Zhang 2009
3. Pancreatic cancer	Poor	High	Xie 2013
4. Leukemia	Good to poor	Low to high	Fulda 2009, Kamihira 2001, Park 2011 , Kelly 2011, Troeger 2007, Esh 2011, Tyner 2012, Ahmed 2012, Morrison 2012, Grzybowska-Izydorczyk 2010, Carter 2012, Small 2010
5. Other cancers	Good to poor	Moderate to high	Waligorska-Stachura 2009

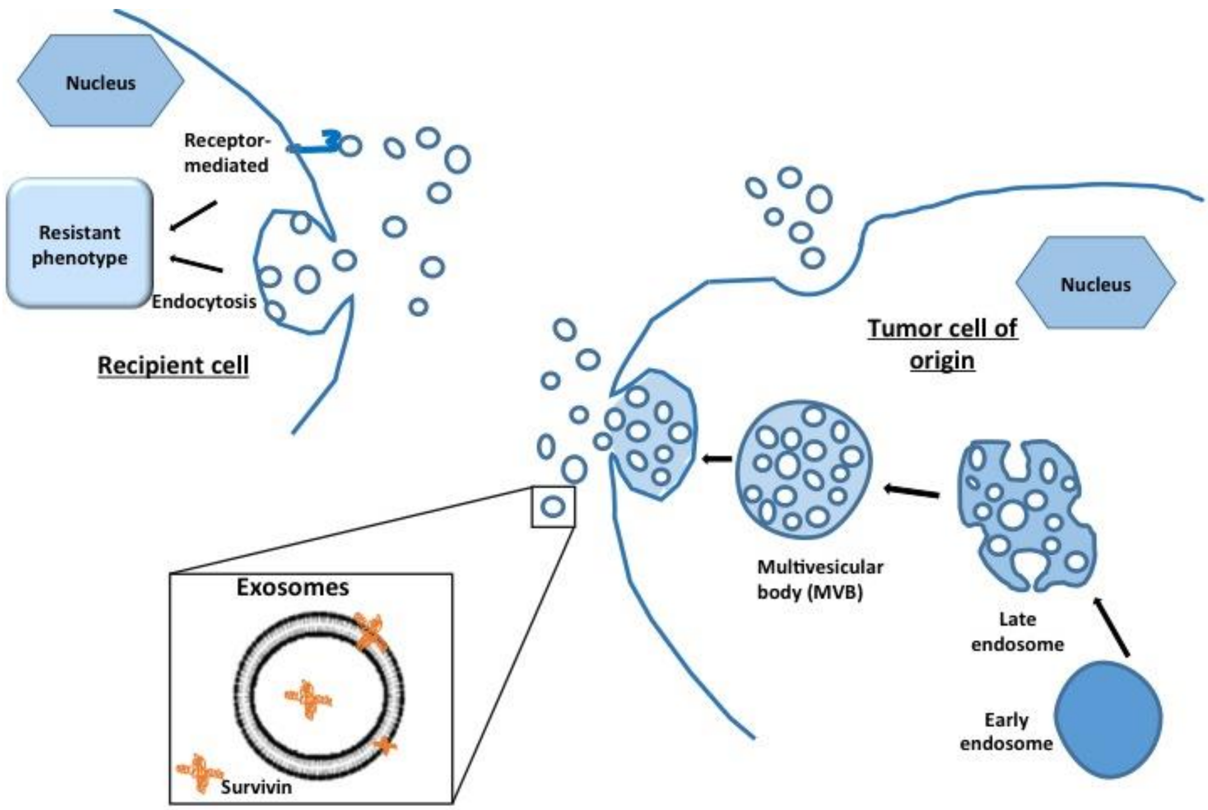

Figure 1. Exosomes play important roles in intercellular communication. A tumor cell communicates with the tumor microenvironment and may be able to affect cancer cell aggressiveness as a result of the protein, RNA and/or miRNA payload found within. One potentially important protein to the proliferative, invasive or therapy resistance nature of the tumor is Survivin which has been found released from cancer cells in exosomes.

Consistent with Survivin's association with unfavorable clinicopathological parameters, extracellular trafficking of Survivin throughout the tumor microenvironment could be responsible for augmenting the aggressive status of a tumor while prohibiting or minimizing therapeutic results.(Li F *et al*., 1999, Li & Ling, 2006, Khan *et al*., 2009) This review focuses on the multifaceted roles of Survivin in cancer biology, its cellular localization and its cancer health disparity-specific up regulation, specifically in breast, prostate, pancreas and hematological cancers.

Nuclear Survivin as a Cell Cycle Regulator

Nuclear Survivin is known to be a cell cycle associated protein. Investigations of cell division regulation during the depletion of Survivin by siRNA demonstrated an increase in mitotic arrest and chromosomal misalignment. Furthermore, this study confirmed that Survivin is involved in microtubule assembly and centromere stabilization during mitosis.(Yang *et al*., 2004) Survivin's role in mitosis regulation is associated with its involvement in the chromosomal packaging complex and its contribution to the formation of the mitotic spindle.(Altieri, 2008, Church & Talbot, 2012) IAP family proteins cIAP2 and Survivin have been shown to dramatically increase upon exposure to hypoxia.(Dong *et al*., 2001, Dohi, Okada*, et al*., 2004) Furthermore, survivin's promoter has been shown to contain 3 putative HIF-1 binding or response elements.(Zagorska & Dulak, 2004) Nuclear Survivin was found to be distinctly involved in the prognosis of different cancers as will be discussed in our specific cancers section.

Cytoplasmic/Mitochondrial Survivin as an Apoptosis Inhibitor

Survivin's ability to interfere with cellular death pathways appears to reside in the cell's cytoplasm. Survivin localizes to the mitochondria(Dohi, Beltrami, *et al*., 2004a) and therefore may provide, like Bcl-2, a role in mitochondrial stability. Cellular stress was shown to modulate the expression and localization of Survivin with hypoxia-induced Survivin found exclusively in the mitochondria. Furthermore, upon apoptotic stimulation, mitochondrial Survivin is rapidly released to the cytosol where its cytoprotective effects prevent the activation of the initiator caspase 9.(Dohi, Beltrami, *et al*., 2004a)

Early studies showed that Survivin and XIAP protected cells from undergoing caspase-dependent apoptosis. Subsequently, *in vitro* binding experiments showed that Survivin, like XIAP and other IAPs, bound to the terminal effector cell death proteases, caspase 3 and 7, but not to initiator caspase 8.(Tamm *et al*., 1998) Controversy in the field arose when a study by the Altieri group showed that Survivin did not inhibit caspase 3 activity and where recombinant Survivin failed to decrease recombinant caspase 3 activity *in vitro*.(Banks *et al*., 2000) Current evidence suggests that Survivin acts on caspases in an indirect manner by binding to the hepatitis B X interacting protein (HBXIP) and forming a complex with pro-caspase 9, inhibiting the apoptosome formation.(Marusawa *et al*., 2003) This Survivin-HBXIP complex, not individual Survivin or HBXIP proteins, binds to pro-caspase 9 and works to prevent recruitment of apoptosis activating factor 1 (Apaf1) thus suppressing intrinsic apoptosis. In addition, Survivin binds to and regulates the stability of XIAP, which is a direct caspase 3 and 9 inhibitor.(Church & Talbot, 2012) More specifically, the formation of a Survivin-XIAP complex promotes increased XIAP stability, protecting XIAP from proteasomal degradation, resulting in a facilitated inhibition of caspase-dependent cell death.(Dohi, Okada, *et al*., 2004)

Extracellular Survivin as a Modulator of Tumor Microenvironment

Survivin has recently been shown to exist in the extracellular space,(Khan *et al*., 2009) via 40-100 nm membrane vesicles called exosomes.(Khan S *et al*., 2011) Various cell types, such as B- and T- lymphocytes, dendritic cells, neurons, intestinal epithelial cells, as well as tumor cells release exosomes.(Denzer K *et al*., 2000, Keller S *et al*., 2006, Simpson RJ *et al*., 2009, Greening *et al*., 2015) In particular, it has been shown that both human and mouse tumor cells release tumor cell-derived exosomes (TEX) constitutively.(Wolfers J *et al*., 2001) Additionally, specific protein content found both on and within TEX give an indication of their functional and biological roles, and their cell of origin, making TEX excellent biomarkers.(Zitvogel L *et al*., 1998, Andre *et al*., 2002, Wieckowski E & TL, 2006, Aleckovic & Kang, 2015) Early detection, aggressive determination, and therapeutic efficacy may one day be possible through the use of these exosomes and their contents.

Our lab has shown that the extracellular pool of Survivin has the ability to cause neighboring cancer cells to increase resistance to therapy, rapidly proliferate and acquire an increased potential to become invasive *in vitro*,(Khan *et al*., 2009) providing a protective role to the neighboring tumor cells.(Khan S *et al*., 2011) The ability of extracellular Survivin to cause these effects in the surrounding cancer cells correlates with the fact that Survivin overexpression is observed in virtually every human cancer type.(Altieri DC, 2003a) TEX may also be used as a tool to detect malignant conditions.(Aleckovic & Kang, 2015) Serum taken from cancer patients has an increased level of TEX,(Ginestra A *et al*., 1998, Ginestra A *et al*., 1999) which has a positive correlation with the progression of the tumor.(Khan S *et al*., 2011) In addition to serum, TEX were shown to be isolated from malignant tumor fluids, urine,(Nilsson *et al*., 2009, Rolfo *et al*., 2014) ascites fluids(Adams *et al*., 2005, Shender *et al*., 2014) and pleural effusions.(Andre *et al*., 2002, Park *et al*., 2013) We have recently shown that exosomal Survivin may be a useful tool for early detection, diagnosis, and even monitoring prostate cancer progression.(Khan *et al*., 2012) Newly diagnosed and advanced prostate cancer patients with high or low-grade cancer had significantly higher levels of exosomal Survivin compared to control subjects or patients with pre-inflammatory benign prostatic hyperplasia (BPH).(Khan *et al*., 2012)

Survivin in Cancer Immunity Evasion

Survivin has been ascribed multiple roles not only in malignancy, but also in immunity and differentiation.(Zangemeister-Wittke & Simon, 2004) Survivin has been shown to be essential for T-cell maturation, homeostasis and proliferation at various stages of development.(Xing *et al*., 2004) It has also been shown to modulate peripheral blood leukocytes (PBL) when in the extracellular space by binding to leukocytes, thereby inducing molecular processes implicated in the pathogenesis of inflammation.(Mera *et al*., 2008) On the basis of the literature and our data, Survivin may be said to exhibit duplicity in cancer immunity as it can act as a tumor associated antigen, or modulate immune environment to permit tumor growth.

Recently, an artificial antigen-presenting cell, developed to study anti-Survivin $CD4^+$ T-cell responses in cancer patients, was shown to elicit both Th1 and Th2 responses against Survivin. The level of avidity was appropriate to recognize tumor cells.(Tanaka *et al*., 2011) Previously, constructed DNA-peptide complexes (mimovirus) of Survivin epitopes, have been shown to stimulate strong cytotoxic T lymphocyte (CTL)-mediated long-term memory of murine immune response and exact a high anti-tumor effect in BALB/c mice.(Yang *et al*., 2008) Furthermore, a DNA construct encoding a secreted version of Survivin, along with a plasmid coding for murine granulocyte-macrophage colony-stimulating factor (GM-CSF) as a molecular adjuvant, were observed to elicit humoral responses against Survivin in sera collected from mice. IgG2a antibody was the prevalent antibody sub-class, thereby implicating the induction of a Th1-CD4+ cellular response.(Lladser *et al*., 2006)

We have recently shown that when T-cell cultures were incubated with Survivin, surface binding and intracellular uptake of Survivin by these T-cells occurred. Upon further investigation, a Survivin-associated decreased proliferation was observed in these T-cells. In addition, analysis of CTLs revealed a reduction in their functional cytotoxicity. However, Treg (T-regulatory cell) function remained unaltered. Importantly, the numbers of Th1 and Tc1 cells were significantly reduced, together with the cytokines associated with them (IFN-γ and IL-2), while an increase in $IL\text{-}4^+$, $IL\text{-}5^+$ and $IL\text{-}13^+$ T-cells was observed. These results suggest a skewing from a type 1 response, which mediates immunity with cytokines that enhance cellular cytolytic activity and can elicit an effective anti-tumor response to a type 2 T-cell response which does not lead to tumor rejection and is frequently observed in cancer patients.(Wan, 2010, Amarnath *et* *al*., 2011, Pietra *et al*., 2012, Jutzy *et al*., 2013) The verification and molecular mechanism underlying this Th cell plasticity is yet to be fully elucidated.

Cancer Specific Up Regulation of Survivin: Breast Cancer

Breast cancer is the second most common cancer type (following lung cancer) and the most common cancer among women worldwide. It is estimated that in the United States alone there are nearly 3 million women with diagnosed breast cancer and approximately 227,000 more will be added to that number this year.(Siegel *et al*., 2013) African American women are more likely than all other women to die from breast cancer as their tumors often are discovered at a later, more advanced stage, leaving them fewer treatment options.(Dehal *et al*., 2013, Johnson *et al*., 2013) There are several pathways involved in breast cancer pathogenesis with pathways of tumor cell death playing an important role in its development and maintenance. Among the proteins involved in cell death/survival pathways, Survivin is one of the most studied. Using serial analysis of gene expression (SAGE), Survivin was found to be the fourth highest expressed transcript in a number of common cancers including breast cancers.(Velculescu *et al*., 1999) In a study examining the interaction of the insulin-like growth factor II and Survivin, Kalla Singh S et al, found that high IGF-II expression-regulation of Survivin correlated and was significantly higher in African Americans than in Caucasians.(Kalla Singh *et al*., 2010) In this study it was shown that IFG-II regulates Survivin, leading to inhibition of mitochondrial membrane depolarization, cell survival and chemoresistance. Furthermore, the effect of IGF-II and IGF-II siRNA on the expression of Bcl-2, Bcl-XL and Survivin in African American and Caucasian breast cancer cells was measured. IGF-

II expression was shown to be causative in the upregulation of these antiapoptotic proteins, while IGF-II siRNA was prohibitive.(Kalla Singh *et al*., 2010) This intriguing observation will require further investigation.

The different sub-cellular pools of Survivin in breast cancer appear to have distinct functions. Adamkov et al, suggested that nuclear staining of Survivin antigen could be used as a marker of the degree of neoplasia,(Adamkov *et al*., 2012) while Rexhepaj et al, suggested that increased levels of nuclear Survivin are associated with a proliferative phenotype.(Rexhepaj *et al*., 2010) One thing that is clear is that Survivin plays a key role in the initiation and progression of breast cancer. High mRNA expression was found to be an independent prognostic marker in breast cancer patients(Xu *et al*., 2012) and Survivin up regulation significantly correlated to lymph node involvement, tumor stage and histological type.(Dedić Plavetić *et al*., 2013) By contrast, others have shown that high levels of its expression are associated with a beneficial response to chemotherapy.(Span *et al*., 2006) This could be due to alternative splicing of Survivin. Multiple studies demonstrate that alternative splicing patterns are altered during cancer progression.(Li, 2004) Several different mechanisms contribute to changes in the regulation of alternative splicing including stress, stimulation of receptors by growth factors, cytokines, or hormones, etc. Survivin, to date, has six different described variants with different apoptotic properties and intracellular localization (**Figure 2**).(Necochea-Campion *et al*., 2013) Protein and mRNA levels of the pro- and anti-apoptotic isoforms of Survivin correlate with cancer prognosis.(Boidot *et al*., 2009)

Early diagnosis of breast cancer is challenging due to a lack of serum biomarkers and inadequate as it is performed through invasive means such as needle biopsy, scanning

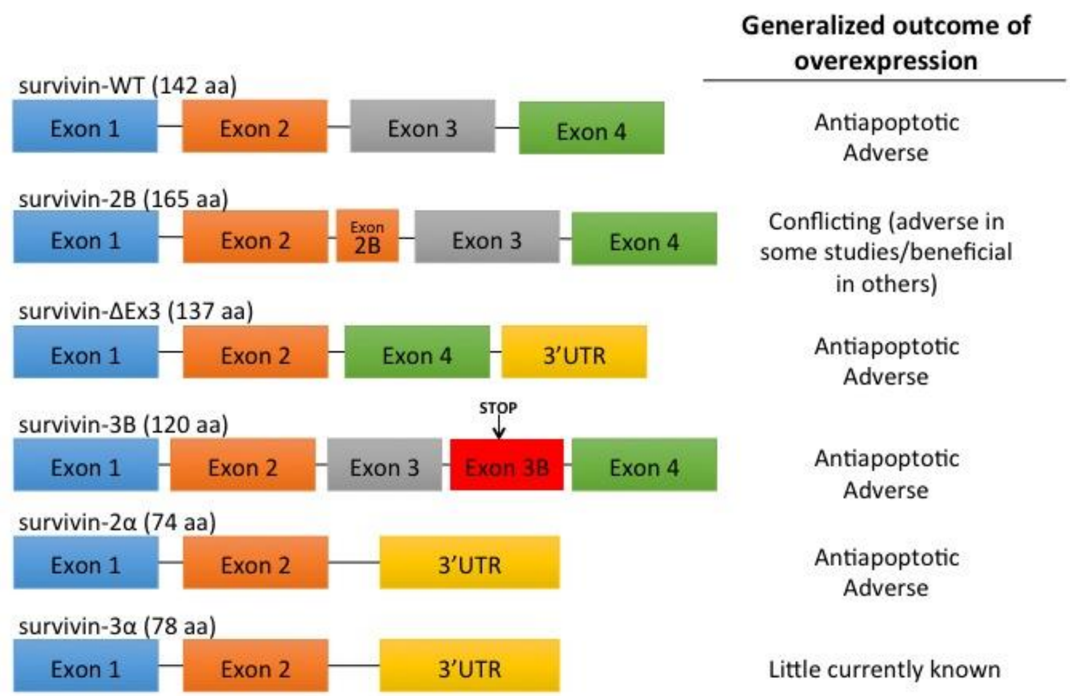

Figure 2. Splicing of the human survivin pre-mRNA produces six different splice variants. With the exception of survivin-3□ and survivin-2B, survivin-WT, survivin-ΔEx3, survivin-3B, and survivin-2□ are all associated with an unfavorable antiapoptotic phenotypes. The survivin-2B has been shown to have either a favorable or unfavorable association depending upon the cancer type it is expressed in, and survivin-3□ has yet to be determined.

and invasive pathological examination. Despite the availability of numerous diagnostic and prognostic methods, there remains a need for an easy, sensitive and non-invasive way to track tumor activity. We propose that through analysis of tumor exosomes and by specifically assaying these exosomes for tumor-specific antigens such as Survivin, XIAP, cIAP1/2, chaperone proteins such as HSP70 and 90, just such a biomarker discovery may one day be realized. We have found an extracellular Survivin pool in serum exosomes in prostate(Khan *et al*., 2012) and breast cancers.(Khan *et al*., 2014) In these breast cancer patients' sera, we found Survivin levels and exosome numbers to be significantly increased over controls with a disparate expression of the Survivin splice variants similar to that observed in tissues. It is important though that we recognize the possible confounding factors such as co-morbidities, psychological complications, genetics, and environmental exposures that could affect these results.

Cancer Specific Up Regulation of Survivin: Prostate Cancer

Prostate cancer (PCa) is the most frequently diagnosed non-skin cancer in men and the second leading cause of male cancer deaths in the U.S., accounting for 238,590 new cases and 29,720 deaths in 2013.(Brawley, 2012b, Siegel *et al*., 2013) These statistics have undergone minimal changes despite advances in screening and early diagnosis and therefore still require a significant investment if prostate cancer is to be defeated. As has recently been described by our colleagues, African American men have a growing disparity in their prostate cancer incidence and mortality compared to other ethnic groups,(Basu *et al*., 2011) and they present with the disease at a much younger age than do Caucasian men which is a trait common in more aggressive cancers.(Hoffman *et al*., 2001, Karami *et al*., 2007)

Survivin is expressed in prostate cancer and has been shown to be up regulated in order to protect the prostate cancer microenvironment against apoptosis and oxidative stress-induced damage.(Zaffaroni *et al*., 2005) Survivin therefore directly and/or indirectly influences cell survival and death. Shariat et *al*., using immunohistochemistry, compared Survivin protein expression in normal and malignant prostate tissue and lymph node tissue from prostate cancer patients. There appeared to be a gradual but consistent rise of Survivin expression from normal prostate specimens (36%), to prostate cancer (71%), with the highest expression found in metastatic lymph nodes (81%).(Shariat *et al*., 2004) Survivin expression therefore seemed to correlate with the degree of transition from normal prostate epithelia to a more aggressive form of prostate cancer (metastatic prostate cancer).

Our group recently looked at relative levels of Survivin in the sera of prostate cancer patients and compared it to that of patients with BPH and from subjects with no diagnosis of cancer or BPH.(Khan *et al*., 2012) Survivin levels proved to exhibit a stronger correlation in our hands than prostate specific antigen (PSA) when it came to distinguishing the two clinical conditions. We therefore propose that exosomal Survivin evaluation should be given serious consideration as a plausible biomarker for early detection of prostate cancer and perhaps could be used to monitor treatment efficacy and disease recurrence. Higher levels of not only Survivin, but its splice variants 2B and 2α, both *in vitro* and in tissue, seem to correlate with prostate cancer cell proliferation and a more aggressive phenotype.(Koike *et al*., 2008) The intracellular compartment localization of Survivin has been suggested to be of prognostic value. When tissues of patients with locally advanced prostate cancer were stained and examined for Survivin, patients with higher levels of intranuclear Survivin exhibited improved survival, whereas those with higher levels of cytoplasmic Survivin exhibited a poorer prognosis.(Zhang *et al*., 2009)

In summary, Survivin, in prostate cancer has a dual role as an inhibitor of apoptosis and cell cycle mediator. Its level of expression appears to correlate with the progression from normal to indolent and to a more aggressive form of prostate cancer. Our demonstration of exosomal Survivin in the plasma of patients with newly diagnosed low-grade prostate cancer(Khan *et al*., 2012) provides a rationale for studies to investigate the utility of exosomal Survivin as an early, easily measured biomarker for prostate cancer diagnosis as well as a marker to monitor treatment efficacy and tumor recurrence.

Cancer Specific Up Regulation of Survivin: Pancreatic Cancer

Cancer of the pancreas is the fourth most common cause of cancer death in men and women in the United States. In 2013, an estimated 45,220 new cases and 38,460 deaths from pancreatic cancer occurred.(Siegel *et al*., 2013) It is a highly malignant disease and lacks clear early warning signs or symptoms thus remaining silent in its victims until it is well advanced. The vast majority of patients are not diagnosed until stage III or IV and once diagnosed exhibit a median survival of 4-8 months with a 5-year survival rate being $<5\%$.(Lowenfels & Maisonneuve, 2006) Risk factors include gender, age, diabetes, chronic pancreatitis, family history, smoking, alcohol abuse and possibly diets high in fat.(Lowenfels & Maisonneuve, 2006) Early diagnosis continues to be the greatest obstacle and there is an urgent need for screening biomarkers.

Pancreatic cancer incidence in the United States is higher in African Americans and Hispanics than in Caucasians.(Gordis, 1993, Woutersen *et al*., 1999) In a number of recent studies, the risk factors in men (cigarette smoking and diabetes mellitus) and women (moderate/heavy alcohol consumption and an elevated body mass index (obesity)), explain almost the entire African American/non-Hispanic White disparity in incidence. In the absence of these risk factors, pancreatic adenocarcinoma incidence rates among African Americans do not exceed those of Caucasians from either men or women.(Chang *et al*., 2005, Hayanga, 2005, Singal *et al*., 2012) In 2003, a group at the Barbara Ann Karmanos Cancer Institute analyzed a group of pancreatic cancer patients (166 African American, 244 Caucasian) for clinicopathologic characteristics of the disease as well as immunohistochemical expression of commonly found pancreatic cancer biomarkers: Fas, FasL, p21/waf-1, p27, p53 and Her2. They also investigated the presence and types of K-ras mutations at codon 12.(Pernick *et al*., 2003) African Americans were found to have significantly higher rates of K-ras mutations than did Caucasians, and their treatment with chemotherapy or radiation therapy was also much less effective than that recorded in Caucasians. African Americans more frequently than Caucasians were found with positive surgical margins and many clinicopathologic variables such as median survival, 5-year survival, and stage at presentation were different. African Americans were less immunoreactive to Fas expression and had a much stronger Her2 expression than did Caucasians.(Pernick *et al*., 2003)

As in prostate and breast cancer as previously discussed, epidemiological evidence exists for a strong association between pancreatic cancer and a high consumption of dietary fat. Dietary fat is made up of fatty acids and lipids that are metabolized into arachidonic acid. The key enzymes for arachidonic acid metabolism are lipoxygenases (LOXs) and cyclooxygenases (COXs) which outside of dietary fat research have been shown associated with the development and progression of pancreatic cancer.(Ding *et al*., 2001) LOX and COX inhibitors prohibit the continued progression of pancreatic cancer and induce intrinsic mitochondria-associated apoptotic cell death.(Tong *et al*., 2002)

There have been numerous studies performed on the prognostic implications of Survivin in pancreatic cancer. A high expression of Survivin was found to be related to shorter survival in patients with resected pancreatic adenocarcinoma.(Xie *et al*., 2013) In contrast, high nuclear levels of Survivin predicted better prognosis than cytoplasmic Survivin.(Tonini *et al*., 2005) Furthermore, Sagol et al and Sun et al showed no significant association between Survivin and long-term survival.(Sagol *et al*., 2005, Sun *et al*., 2007) Targeting Survivin early on in the process could play an invaluable role in preventing the progression to malignancy. In addition, a screening biomarker that could potentially detect early stages of the disease is of utmost importance.

Cancer Specific Up Regulation of Survivin: Hematological Malignancies

Hematological malignancies such as leukemia, lymphoma, myeloma and myelodysplastic syndromes affect the bone marrow, the blood cells, the lymph nodes and other parts of the lymphatic system. These pathologies are interrelated, likely the result of acquired changes to the DNA of a single stem cell. Approximately 140,000 people will be diagnosed with leukemia, lymphoma or myeloma, accounting for approximately 9 % of all new cancers diagnosed each year in the United States.(Society, 2012) Of particular interest is multiple myeloma (MM), which accounts for approximately 10 % of all hematologic malignancies diagnosed in the United States annually.(Pulte *et al*., 2012) Among the hematological malignancies, MM is known to affect individuals from ethnically diverse populations in a disparate manner.

In accordance with reports for many types of solid tumors,(Church & Talbot, 2012, Waligorska-Stachura *et al*., 2012) cancer specific up regulation of Survivin also occurs in hematological malignancies,(Fulda, 2009, 2012) though to date there have been no published reports taking ethnicity into account. In hematological cancers, expression of Survivin is associated with poor clinical outcomes and resistance to chemotherapy.(Adida *et al*., 2000, Kamihira *et al*., 2001, Kelly, 2011, Park *et al*., 2011) Survivin expression levels are linked to risk of early relapse in pediatric B-cell acute lymphoblastic leukemia (ALL)(Troeger *et al*., 2007, A. Esh, 2011, Tyner *et al*., 2012) and to tumor aggressiveness(Ahmed *et al*., 2012) and chemoresistance in adult ALL.(Morrison *et al*., 2012)

High levels of Survivin expression have also been linked to cell proliferation and antiapoptotic characteristics in chronic myelogenous leukemia (CML),(Wang *et al*., 2005) and chronic lymphocytic leukemia (CLL).(Grzybowska-Izydorczyk *et al*., 2010) In acute myeloid leukemia (AML), levels of Survivin expression were found to be significantly predictive of shorter overall and event-free survival.(Carter *et al*., 2012) In addition, the highest Survivin expression levels are detected in the CD34(+)CD38(-) AML stem/progenitor cell populations,(Carter *et al*., 2012) further validating Survivin's potential as a prognostic biomarker and therapeutic target. Overexpression of Survivin in

CD34+ hematopoietic cells has been found to induce hematological malignancies *in vivo*, suggesting that it has a role in the development of these diseases.(Small *et al*., 2010)

Localization of Survivin to the nucleus versus cytoplasm is very important because the functional dynamics of Survivin are dependent on the site of Survivin expression.(Kumar *et al*., 2012) Using chemotherapeutic drugs in hematologic cancer, Bernardo et al., reported that cytoplasmic Survivin was more relevant to the apoptotic index than that associated with nuclear Survivin.(Bernardo *et al*., 2012) Investigating Survivin's cellular locations, alternative splice variant profiles within the context of cancer health disparities and novel therapeutic modalities will continue to be important areas of study.

Liquid Biopsy

The tumor microenvironment is being increasingly recognized as providing many key factors necessary for many of the stages of disease progression including local resistance, immune escape, and distant metastasis. Understanding this tumor microenvironment, including the cells involved and the communications ongoing between them will continue to prove instrumental in our understanding of cancer and eventually our ability to control it if not terminate it. In order to fully "learn the language", there is a need for new biomarker discovery. Specifically, biomarkers that are easily isolated and identified from blood, urine, saliva, cerebral spinal fluid, ascites, etc., as well as from tissue biopsies, will need to be identified. The term "liquid biopsy" has been used recently to describe the source of these biomarkers and could be defined as broadly as circulating tumor cells, circulating tumor DNA, exosomes and secretomes.(Rolfo *et al*., 2014) Differential expression of exosomal Survivin may serve as a diagnostic and or prognostic marker, in early cancer patients and may soon lead to the development of potential therapeutics for the treatment of these diseases.

Conclusions

Most efforts on the identification of candidate cancer biomarkers, and on analyzing differences in the cancer biology that exists between African Americans and Caucasian patients have focused on gene expression differentials in tumor tissues, epigenetic issues such as methylation patterns and on single nucleotide polymorphisms (SNPs). While these efforts have been necessary in providing important clues for understanding biochemical mechanisms associated with cancer health disparities, it is also imperative to develop non-invasive approaches that analyze indirectly and early in the disease process, the molecular profiles of tumors. One recent study has investigated the -31G>C promoter polymorphism across approximately 7,500 cancer cases and 9,000 controls.(Qin *et al*., 2014) This polymorphism was significantly associated with an increased cancer risk in colorectal, gastric, and urothelial cancers. In contrast, this SNP was remarkably decreased in patients with hepatocellular carcinoma. With regard to ethnic diversity, this SNP was shown to increase cancer risk in Asian populations(Qin *et al*., 2014) as well as a higher Wilms' tumor risk in Serbian children.(Radojevic-Skodric *et al*., 2012) Findings such as these encourage us to not only look at the overall abundance of gene or protein products in racial disparities and cancer but to look deeper into the minutia that may have been historically overlooked and may provide important insights not before recognized as factors in cancer development and resistance.

Recent studies have shown that small membrane-bound vesicles called exosomes constitute the latest mode of intercellular information transfer or communication. This exchange of molecular information is facilitated by their unique composition, which is enriched with enzymes, structural proteins, adhesion molecules, lipid rafts, microRNA and RNA. Importantly, cancer cells have been shown to secrete more exosomes than do their normal counterparts indicating that exosomes can be used as diagnostic markers and their active secretion has functional implications. In addition, recent studies revealed that genes involved in inflammation and autoimmune responses are differentially up regulated in cancer patients compared to controls. This could imply that differences in antitumor immune responses may exist between racial groups in tumors.

It is very important to specifically target Survivin in a defined location for therapeutic purposes. Survivin is a unique inhibitor of apoptosis with triple functionality: in cell cycle regulation when it is present in the nucleus, inhibition of apoptosis when it is in the mitochondria, and resistance to chemotherapy when it exists in the tumor microenvironment packaged in exosomes. Survivin's up regulation in specific cancers, in addition to its presence in serum exosomes, make it an important molecule both as a diagnostic as well as prognostic marker. Unfortunately, controversy exists as to whether Survivin expression is favorable or unfavorable in the outcome of cancer. Survivin expression is an unfavorable prognostic indicator in esophageal, hepatocellular, and ovarian cancers, cholangiocarcinoma, and endometrial cancers but has associated favorable outcomes in gastric, bladder, breast, ependymoma osteosarcoma and pancreatic ductal adenocarcinomas.(Li *et al*., 2005, Carter *et al*., 2012) To validate its role, a large number of case-control studies need to be adapted. Subsequent studies exploiting the exosomal packaging of Survivin may also one day be used in cancer therapeutics.

In conclusion, this review addresses an urgent need in the fight against cancer health disparities: the need to identify and evaluate novel serum biomarkers such as Survivin and its alternative splice variants for non-invasive early detection of cancer in interventions that can be tailored to Americans of different ethnicities, ultimately, paving the way for future studies focused on analyzing these biomarkers in larger cohorts of ethnically diverse cancer patients.

Conflict of Interest

Salma Khan, Heather Ferguson Bennit, Malyn M. Asuncion Valenzuela, David Turay, Carlos J. Diaz Osterman, Ron B. Moyron, Grace E. Esebanmen, Arjun Ashok, and Nathan R. Wall declare that they have no conflict of interest.

Informed Consent and Animal Studies

No animal or human studies were carried out by the authors for this article.

Acknowledgements

Funding for our laboratory comes from grants for health disparity research: NIH-NCMHD Project EXPORT Program 5P20MD001631/Project 3 (NRW) and NIH-NIMHD P20-MD006988 subproject 2. Funding also comes from a National Merit Test Bed (NMTB) award sponsored by the Department of the Army under Cooperative Agreement Number DAMD17-97-2-7016 (NRW). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

We would like to thank the entire NRW lab for careful review of the manuscript.

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CHAPTER 2

PLASMA-DERIVED EXOSOMAL SURVIVIN, A PLAUSIBLE BIOMARKER FOR EARLY DETECTION OF PROSTATE CANCER

Published:

Khan S, Jutzy JMS, Valenzuela MMA, **Turay D**, Aspe JR, Ashok A, Mirshahidi S, Mercola D, Lilly MB, Wall NR. Plasma-Derived Exosomal Survivin, a Plausible Biomarker for Early Detection of Prostate Cancer. PLoS One, 7(10): 1-10, 2012.

Abstract

Background: Survivin is expressed in prostate cancer (PCa), and its downregulation sensitizes PCa cells to chemotherapeutic agents *in vitro* and *in vivo*. Small membrane-bound vesicles called exosomes, secreted from the endosomal membrane compartment, contain RNA and protein that they readily transport via exosome internalization into recipient cells. Recent progress has shown that tumor-derived exosomes play multiple roles in tumor growth and metastasis and may produce these functions via immune escape, tumor invasion and angiogenesis. Furthermore, exosome analysis may provide novel biomarkers to diagnose or monitor PCa treatment.

Methods: Exosomes were purified from the plasma and serum from 39 PCa patients, 20 BPH patients, 8 prostate cancer recurent and 16 healthy controls using ultracentrifugation and their quantities and qualities were quantified and visualized from both the plasma and the purified exosomes using ELISA and Western blotting, respectively.

Results: Survivin was significantly increased in the tumor-derived samples, compared to those from BPH and controls with virtually no difference in the quantity of Survivin detected in exosomes collected from newly diagnosed patients exhibiting low (six) or high (nine) Gleason scores. Exosome Survivin levels were also higher in patients that had relapsed on chemotherapy compared to controls.

Conclusions: These studies demonstrate that Survivin exists in plasma exosomes from both normal, BPH and PCa subjects. The relative amounts of exosomal Survivin in PCa plasma was significantly higher than in those with pre-inflammatory BPH and control plasma. This differential expression of exosomal Survivin was seen with both newly diagnosed and advanced PCa subjects with high or low-grade cancers. Analysis of

plasma exosomal Survivin levels may offer a convenient tool for diagnosing or monitoring PCa and may, as it is elevated in low as well as high Gleason scored samples, be used for early detection.

Key Words: biomarker, resistance, early detection, prostate, Survivin

Introduction

Worldwide, prostate cancer (PCa) is the second most frequently diagnosed cancer and the sixth leading cause of cancer death in males (Jemal *et al*., 2010, Jemal *et al*., 2011). Increasing age, ethnicity and family history are the only established risk factors and there are no known preventable risk factors established to date (Jemal *et al*., 2011). Prostate cancers (PCa) are generally slow-growing malignancies that are characterized by an imbalance in the rates of cell division and cell death (Lu S *et al*., 1999). Surgery and radiation therapy are effective for localized disease but there is no effective treatment strategy for recurrent or metastatic PCa that has failed surgery, radiation or hormonal therapy (Klein EA & Kupelian PA, 2003). An important challenge to develop treatments that are more effective depends upon our understanding of the molecular mechanism(s) of PCa progression, which will lead us to identify many potential therapeutic target genes and processes that are involved in apoptosis, cell proliferation, metastasis, and growth factor signaling. Total prostate-specific antigen (PSA) has revolutionized PCa screening and has resulted in an overall decrease in PCa metastasis and death (Shariat *et al*., 2011). Unfortunately, the application of PSA screening has also led to over-detection and overtreatment as PSA is neither cancer specific nor a surrogate for the biologic behavior of PCa (Han *et al*., 2003, Shariat *et al*., 2011). Elevations in PSA levels can reflect a cancer presence but can also be present as a result of infection, chronic inflammation or benign prostatic hyperplasia (BPH) (Freedland *et al*., 2005, Bjartell *et al*., 2011). BPH has been shown to exist in greater than 70% of men over the age of 70 but is not considered to be a precursor of prostate cancer though they frequently coexist (Chang *et al*., 2012). It is therefore necessary to continue to screen for biomarkers that are cancer-specific and that are detectable early in the course of the disease.

The processes of both cell survival and cell death have involved highly regulated signaling pathways that are currently the subject of intense investigation. It is known that regulation of apoptosis has a central role in the development of prostate cancer and its progression to an androgen-independent state, which is due, in part to up regulation of antiapoptotic genes after androgen deprivation (Li Y *et al*., 2004, Zhang M *et al*., 2005, Guo Z *et al*., 2006). Several lines of evidence suggest that one of the main events associated with progression after therapeutic failure is increased resistance to apoptosis (Denmeade SR, 1996, Howell SB, 2000), mainly due to the up regulation of antiapoptotic genes, including Bcl-2, Bcl-X_L, Mcl-1 (Krajewska M *et al*., 1996), and Survivin (Altieri DC, 2003b). Survivin, an inhibitor-of-apoptosis (IAP) protein family member, is associated with PCa development, progression, and drug resistance (Krajewska M *et al*., 2003, Kishi H *et al*., 2004, Shariat SF *et al*., 2004, Koike H *et al*., 2008). Recent evidence indicates that the overexpression of Survivin in PCa tumors is associated with poor prognosis and increased tumor recurrence (Nakahara T *et al*., 2007). In contrast, it has also been shown that knockdown of survivin expression by siRNAs enhances the chemosensitivity of prostate cancer cells, reducing tumorigenicity (Shen *et al*., 2009).

Traditionally, Survivin has been viewed as a cytoplasmic or nuclear protein. Recently, Survivin has been also shown to exist extracellularly, contained in small membrane bound vesicles known as exosomes (Khan S *et al*., 2009, Khan S *et al*., 2011). Exosomes are present in serum and urine and contain a wide range of proteins and RNAs and represent their tissue of origin making them a possible source or pool of novel PCa biomarkers (Duijvesz *et al*., 2011). Consistent with Survivin's association with unfavorable clinicopathological parameters, extracellular trafficking of Survivin throughout the tumor microenvironment could be responsible for augmenting the aggressive status of a tumor while prohibiting or minimizing therapeutic results. We have recently shown that exosome-bound Survivin protein can be secreted by cancer cells and be taken up by surrounding cells, producing a field effect that confers a general stress-survival phenotype .

Our present study was designed to investigate the existence of exosomal Survivin in the plasma of PCa patients with a variety of PCa presentations and to compare its exosomal expression levels to those found in control volunteers with no diagnosis of cancer and to patients diagnosed with benign prostatic hyperplasia or BPH. For the past 25 years the Gleason grading system has been used to help evaluate the prognosis of men with prostate cancer. Together with other parameters, prostate cancer has been staged as a means to predict prognosis and guide therapy (Fine & Epstein, 2008). Extracellular Survivin was found highly expressed in the plasma exosomes of PCa patients exhibiting Gleason scores of 6 (low) and 9 (high), and in patients who had relapsed on chemotherapy. However, there were no significant differences in Survivin levels between subjects with low or high Gleason scores. In addition, though exosomes containing Survivin were found in the serum from patients with a diagnosis of BPH, the overall level was significantly lower than that found in the plasma from PCa patients. We believe that in addition to diagnostic markers, prognostic, predictive and therapeutic markers are needed to act as surrogate endpoints in forecasting disease severity, choosing treatments, and monitoring responses to therapies (Mikolajczyk *et al*., 2004, Fradet, 2009, Fiorentino *et al*., 2010, Ploussard & de la Taille, 2010). Our demonstration of exosomal Survivin in the plasma of patient with newly diagnosed low-grade PCa provides a rationale for studies to investigate the utility of exosomal Survivin as an early, easily measured biomarker for PCa diagnosis. Exosomal Survivin may also be studied as a biomarker to monitor treatment of subjects with advanced PCa.

Results

Plasma Levels of Survivin in Healthy Controls and PCa Patients

Survivin was detectable in the plasma from all healthy control subjects and PCa patients. The measurement results of plasma Survivin in normal healthy controls and PCa patients are shown in **Figure 1A**. The mean plasma Survivin levels were significantly different between the healthy control subjects (61.5 pg/ml in controls [n=10]) and the different cancer patient groups (Gleason 6 = 401.7 pg/ml [n=10], Gleason 9 = 375.2 pg/ml [n=10], and subjects resistant to the the chemotherapy agent Taxotere = 410 pg/ml [n=8]; $P < 0.05$ for each comparison vs. control). When the Survivin levels were compared among the three groups of PCa patients in two-way comparisons, none were significantly different from the others.

Survivin Can Be Collected From Serum Taken From Healthy Controls, BPH and PCa Patients

Like what has been measured above in plasma, Survivin was also detectable in the serum from patients having no prior diagnosis of cancer as well as in patients having the diagnosis of benign prostatic hyperplasia (BPH) and those diagnosed with prostate cancer (PCa) (**Figure 1B**). The mean serum Survivin levels were significantly ($P<0.001$) higher

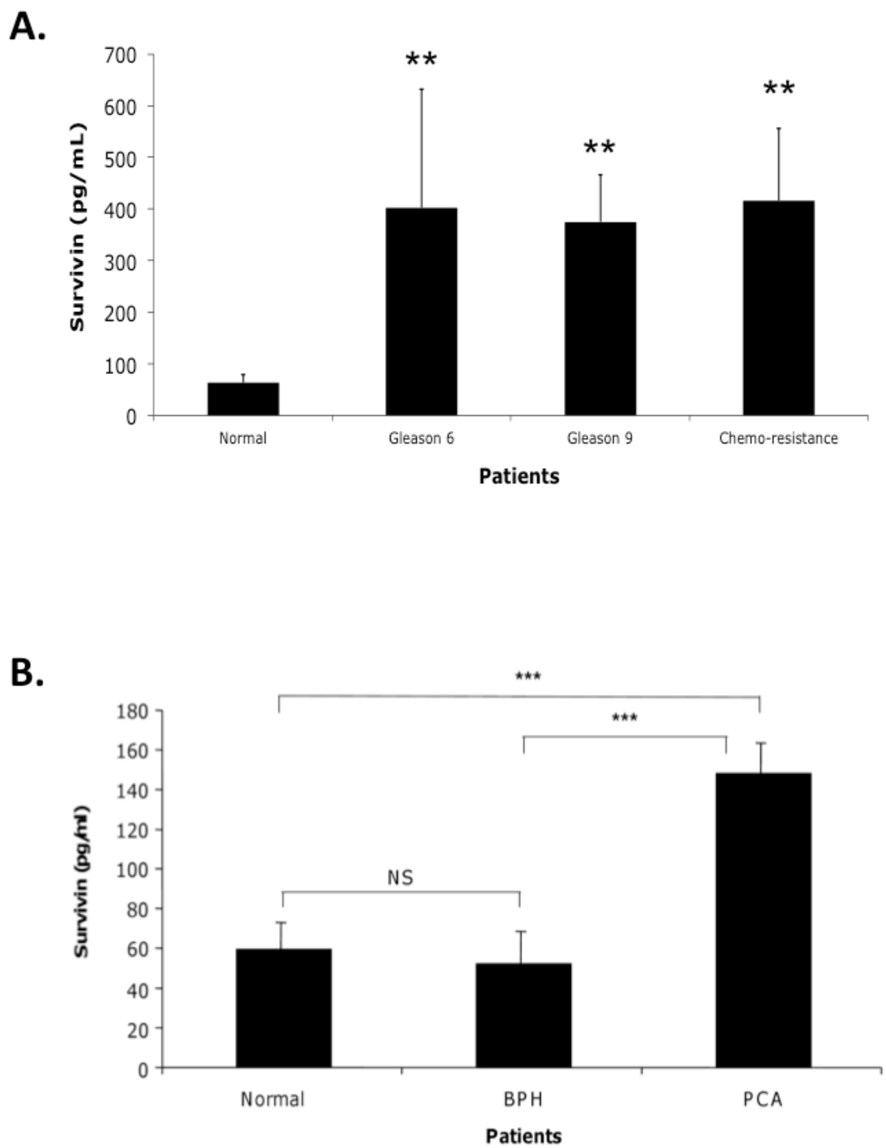


Figure 1. Quantification of Survivin levels in PCa plasma (**A**) and serum (**B**) samples by ELISA. **A.** Survivin levels were measured in plasma derived from Gleason 6 (n=10), Gleason 9 (n=10), and Taxotere-resistant subjects (n=8). **B.** Survivin levels were measured in serum derived from BPH (n=20), and PCa (n=19). Comparisons were accomplished using MANOVA with normal healthy controls (n=10 and 6 respectively). (**, p<0.05, ***, p<0.001; statistically significant).

in the PCa subjects (150 pg/ml [n=19]) than in the serum processed from normal controls (59.7 pg/ml [n=6]) and from BPH patients (55 pg/ml [n=21]).

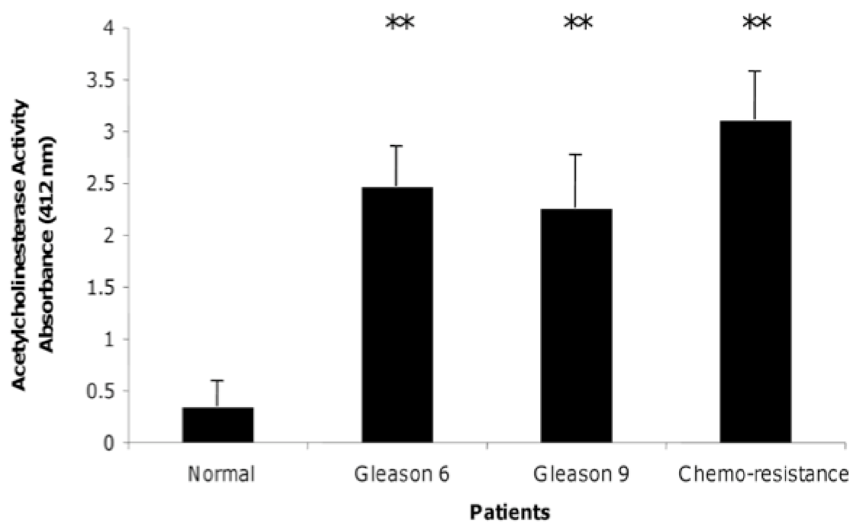
Plasma and Serum Survivin in PCa Patients Exists in an Exosomal Pool

We have previously demonstrated that cultured PCa cells release Survivin into the extracellular milieu within exosomes (Khan S *et al*., 2009). These small, membrane-vesicles are also known to occur in the plasma as well as serum of cancer patients (Mitchell *et al*., 2009, Koumangoye *et al*., 2011). Exosomes were therefore collected by differential centrifugation, and quantitated using the acetylcholinesterase enzymatic assays as we and others have previously described (Johnstone, 2006, Khan S *et al*., 2011). The mean plasma exosome levels were significantly different between the healthy control subjects and the different cancer patients groups (P< 0.001) whereas significant differences in exosome quantity were not found among the PCa patient sample groups (**Figure 2A**). This was also the case when comparing the exosome quantities found in BPH and PCa samples to that of the healthy control subjects (**Figure 2B**). Interestingly, there appears to be a measureable difference in Survivin and in exosomes depending upon the source as both Survivin and exosome quantities were higher when purified from plasma then when purified from serum.

Plasma-Derived Exosomes Contain Survivin

Exosomes were characterized by immunoblotting for the amount of Survivin protein and Lysosomal-associated membrane protein 1 (LAMP1). LAMP1 is a known exosome protein which is commonly used to ensure proper Western blot loading (Quah & O'Neill, 2005, Bhatnagar *et al*., 2007, Khan S *et al*., 2011). Exosomes isolated from

A.



B.

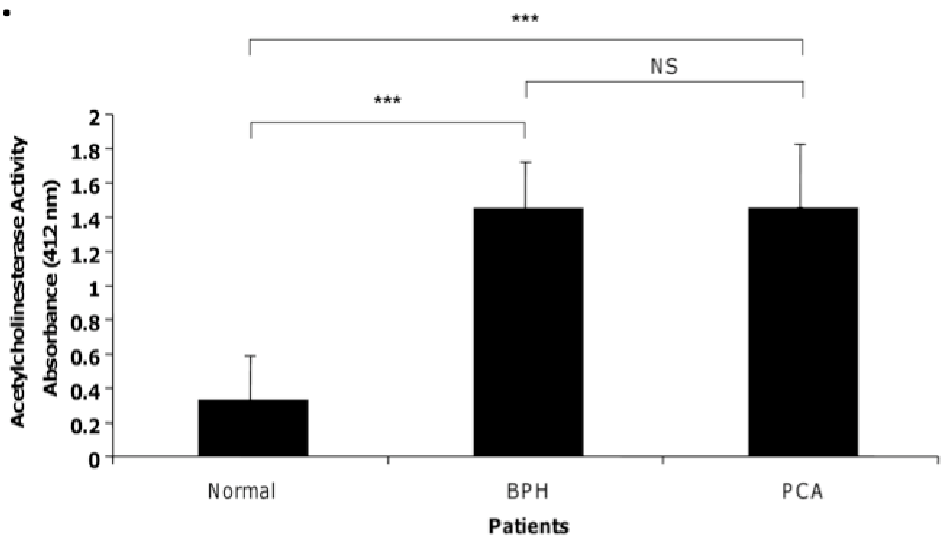


Figure 2. Exosomal contents in PCa patients plasma (**A**) and serum (**B**) samples by using the acetylcholinesterase activity assay. **A.** Exosome levels were measured in plasma derived from Gleason 6 (n=10), Gleason 9 (n=10), and Taxotere-resistant subjects (n=8). **B.** Exosome levels were measured in serum derived from BPH (n=20), and PCa (n=19). Comparisons were accomplished using MANOVA with normal healthy controls (n=10 and 6 respectively). (**, p<0.05, ***, p<0.001; statistically significant).

PCa patient plasma exhibited enhanced Survivin loads compared to exosomes isolated from controls (**Figure 3**). Western blot analysis showed that little exosomal Survivin was detectable in plasma samples collected from six controls having no previous diagnosis of cancer in comparison to the exosome-specific protein Lamp1 (**Figure 3A**). In contrast, exosomes isolated from all twenty pre-treatment PCa subjects contained high amounts of Survivin protein compared to LAMP1 protein levels (**Figure 3B**). Interestingly, there was no significant difference in exosomal Survivin content between patients with Gleason 6 PCa and those with Gleason 9 PCa (**Figure 3C**) when normalized against Lamp1 ($p<0.05$).

Differential Expression of Survivin Exists in BPH and PCa-Derived Exosomes

As above, exosomes collected from BPH and PCa patient serums were characterized by immunoblotting for Survivin and LAMP1 protein (**Figure 4A**). Exosomes isolated from PCa and BPH patient serums exhibited enhanced Survivin loads compared to exosomes isolated from controls (**Figure 4A, Figure 4B and Figure S1**). Interestingly, though Survivin was detected in most and elevated in certain BPH patients, its overall level was significantly less than that found in PCa and there was no significant difference measured between BPH and control patient serums (**Figure 4B**).

PCa Patients with Disease Progression Express High Levels of Exosomal Survivin

Lastly, we evaluated exosomes collected from PCa patients who experienced disease progression while on treatment with chemotherapy. Like in our Gleason patient exosomes, Western blotting showed Survivin protein levels (**Figure 5A**) were markedly higher when compared to cancer-free control subjects, which was confirmed to be

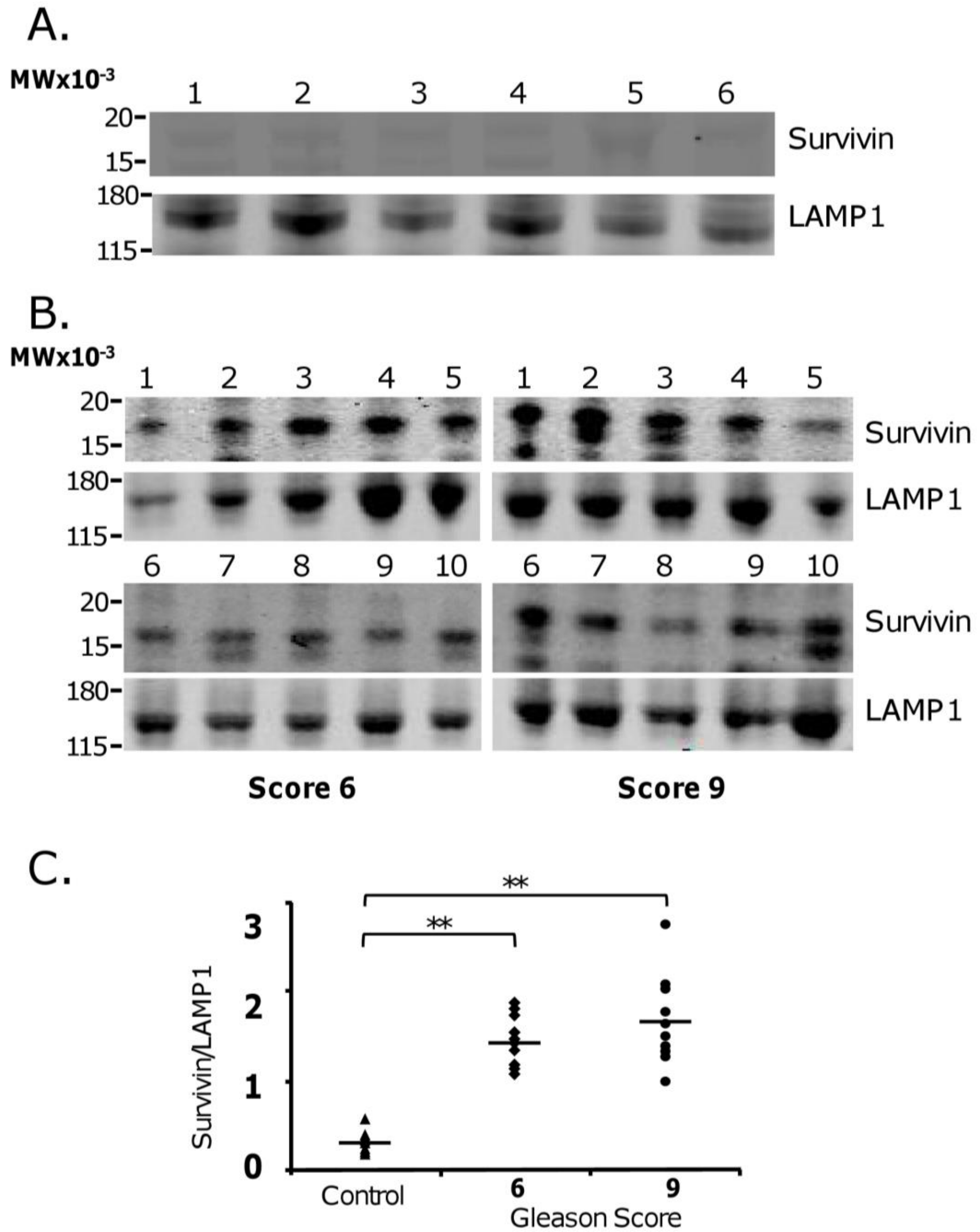


Figure 3. Western Blot Analysis of exosomal Survivin in untreated PCa plasmas. **A**. Antibodies for Survivin and Lamp1 were used for Western blotting of control patient-purified exosomal protein. **B**. Both Survivin and Lamp1 antibodies were detected in the Western Blotting of exosomes-derived from Gleason 6 and Gleason 9. **C**. Proportion analysis of Survivin density to Lamp1 density were shown in both Gleason 6, and Gleason 9 with normal healthy controls (**, $p<0.05$, statistically significant) with no significance recorded between Gleason 6 and Gleason 9 plasma-derived exosomes.

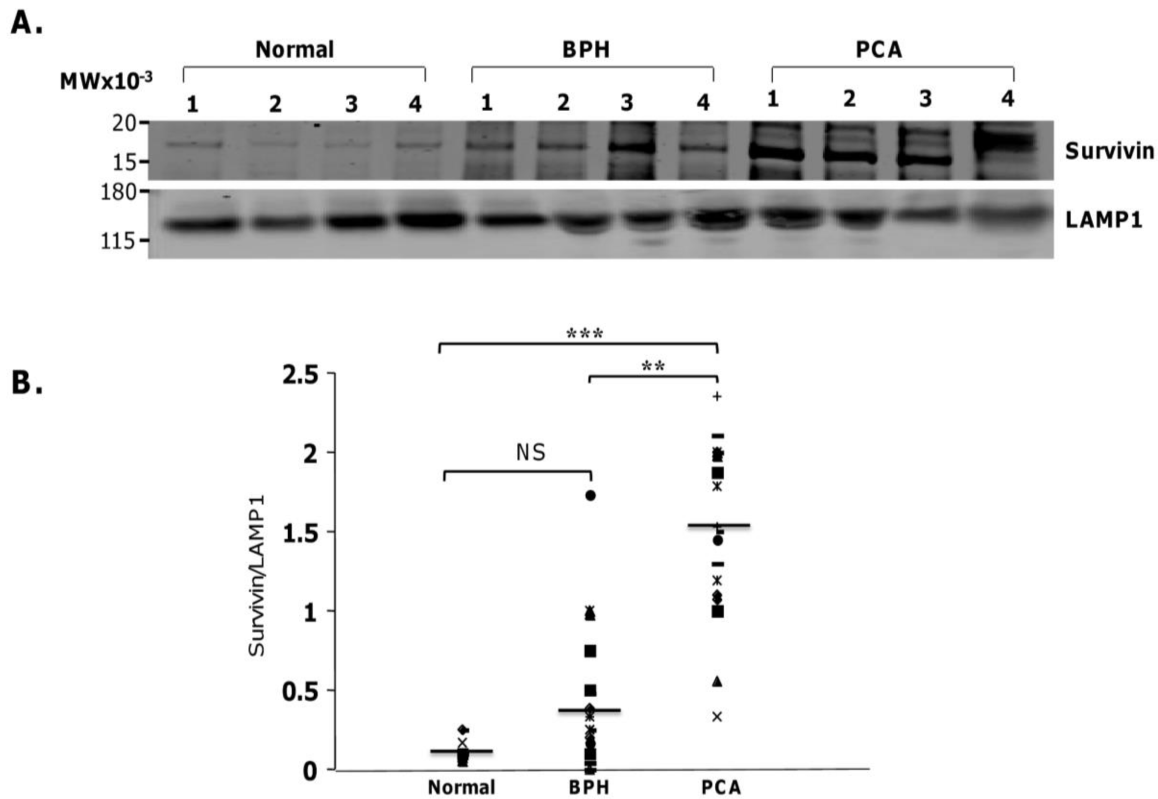


Figure 4. Western Blot Analysis of exosomal Survivin in normal control, BPH and untreated PCa serum samples. **A**. Antibodies for Survivin and Lamp1 were used for Western blotting of patient-purified exosomal protein. **B**. Proportion analysis of Survivin density to Lamp1 density were shown in both BPH and PCa with normal healthy controls (**, p<0.05, ***, p<0.001; statistically significant) with no significance recorded between normal controls and BPH.

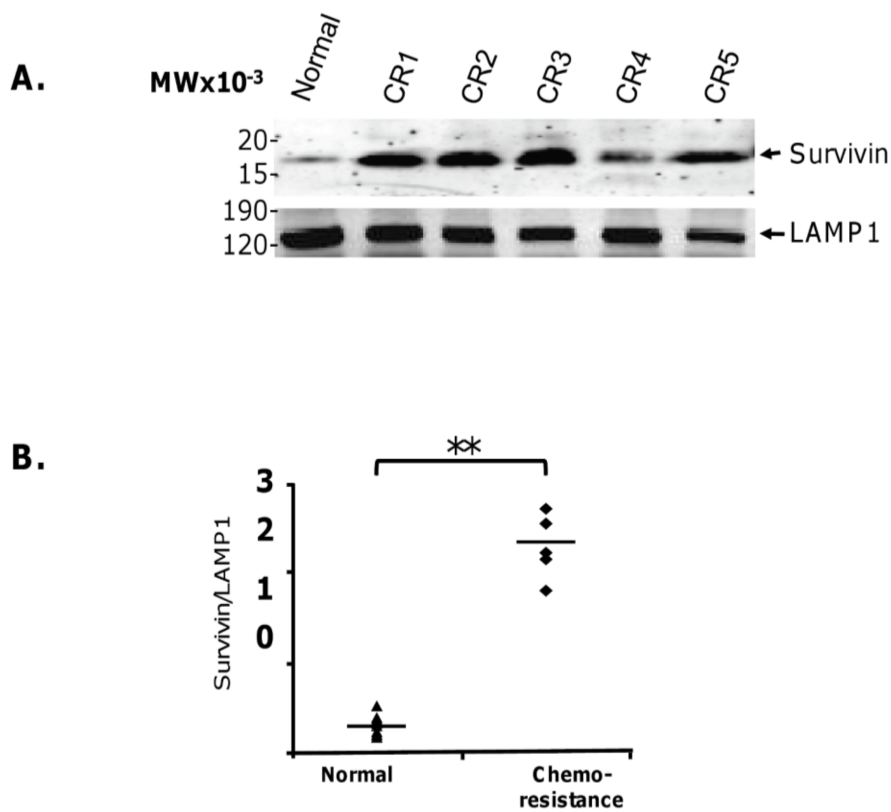


Figure 5. Western Blot Analysis of exosomal Survivin and Lamp1 in Taxotere-resistant PCa patients. **A.** Survivin and Lamp1 antibodies were shown positive. **B.** Densitometric analysis of Survivin/Lamp1 expression in a healthy control (normal) and chemoresistance (CR) cases (**, $p<0.05$, statistically significant).

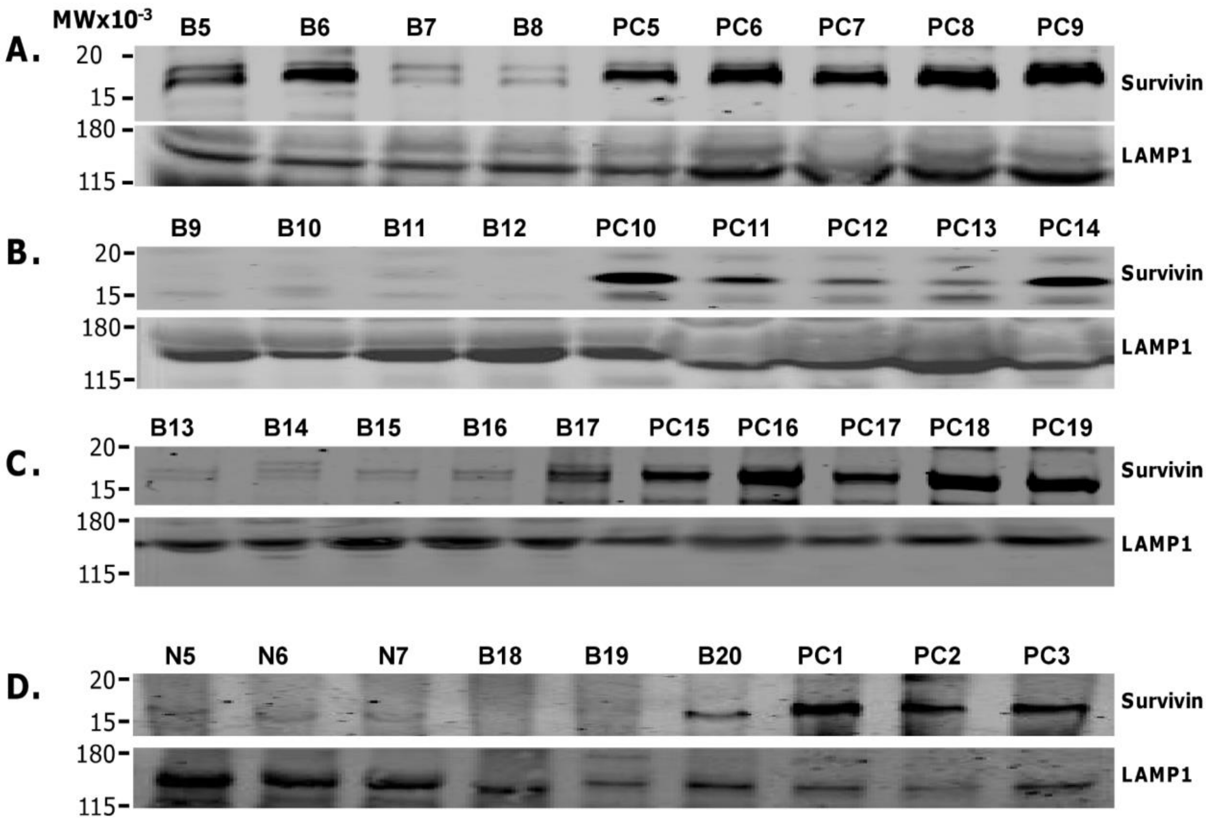


Figure S1. Western Blot Analysis of exosomal Survivin in normal control, BPH and untreated PCa serum samples. Antibodies for Survivin and Lamp1 were used for Western blotting of patient-purified exosomal protein.

significant after densitometric analysis (**Figure 5B**).

Clinical and Pathological Characteristics

Pretreatment data regarding initial plasma Survivin and PSA levels and Gleason scores were acquired for the patients studied. These pathological characteristics are detailed in **Tables 1-4**. In all, eight cases of prostate cancer recurrence after conventional treatment, ten cases of Gleason 6, ten cases of Gleason 9, (**Table 1**); and ten control cases (**Table 2**) were collected with plasmas analyzed. PSA from the plasma (clinical report) and from the ultracentrifuge-purified exosomes showed that though the protein concentrations were not identical, there was similarity in the trend of plasma to plasma-derived exosome PSA values. Importantly, Survivin amounts though significantly less in the exosomes were more consistent across the patients evaluated, and given the sensitivity of the ELISA, picograms for Survivin compared to nanograms for the PSA, is more accurate.

Further studies using sera instead of plasma were performed on six controls, twenty BPH cases (**Table 3**) and 19 additional PCa cases (**Table 4**). Like before, ELISA results from Survivin provided a more sensitive and stable quantitation than did the ELISA for PSA. Quantitation from the sera of BPH patients showed an average of 52.9 pg/ml Survivin and 1.3 ng/ml PSA. In comparison, when evaluated from PCa patients, Survivin averaged 149 pg/ml and PSA averaged 0.3 ng/ml (**Tables 3 and 4**). BPH numbers for both Survivin and PSA are nearly 3 fold lower than in the PCa patient sera. Unfortunately, purchased BPH samples did not come with the clinical PSA ELISA quantities that we have from the PCa cases (**Table 3**).

Correlations of Survivin with PSA

Of the patient's plasma/serum samples evaluated for Survivin, all samples (Gleason 6, Gleason 9, recurrence, or PCa samples) exhibited greater than 100 pg/mL Survivin (**Table 5**). In contrast, only 60% of Gleason 6, 80% of Gleason 9 plasmas and 84% of PCa sera had a PSA of greater than 4 ng/mL. Recurrent patient plasmas all had greater than 4 ng/mL. In control plasmas and BPH sera, no sample had a Survivin concentration greater than 100 pg/mL and no PSA concentration greater than 4 ng/mL (**Table 5**).

Discussion

Prostate carcinoma resists apoptosis with altered expression of both pro- and on Survivin, a multifunctional member of the inhibitor of apoptosis (IAP) gene family that counteracts cell death and controls mitotic progression. Selective overexpression of Survivin has been associated with higher tumor grade, advanced disease stage, rapid tumor progression, short patient survival, and resistance to therapy in patients with various malignancies (Krajewska M *et al*., 2003, Kishi H *et al*., 2004, Koike H *et al*., 2008).

Survivin exists in a number of subcellular locations such as the mitochondria, cytoplasm, and nucleus. Recently it has also been found in the extracellular space (Khan S *et al*., 2009, Khan S *et al*., 2011). We have shown that extracellular Survivin exists in multimolecular complexes which include heat shock proteins (Khan S *et al*., 2011).

Table 1. **Survivin ELISA and PSA levels of plasma from Chemo-resistance, Gleason 6, and Gleason 9 patients are shown.**

Patients ID#	Figure ID#	Survivin ELISA(pg/mL)	PSA* (ng/mL)	Exosomal PSA (ng/mL)
Chemo-Resistance				
006	CR1	503+/-81.9	110	62.28+/-1.2
009	CR2	382+/-29.1	51.5	38.13+/-1.5
010	CR3	435+/-32.4	15.8	4.37+/-7
012	CR4	305+/-19.9	55.4	304.85+/-76.9
013	CR5	413+/-141.5	970	443.25+/-
002a	N/A	258+/-5.2	235	79.84+/-11.7
002b	N/A	246.6+/-183	8.9	1.32+/-0.4
007	N/A	327+/-10.8	62.9	31.91+/-6.6
Gleason 6				
028	1	332+/-15.3	3	2.43+/-0
044	2	328+/-54	2.4	2.90+/-0.2
061	3	330+/-34	5.8	0+/-1.1
066	4	332+/-4.5	8.1	6.10+/-0.5
077	5	313+/-26.8	4.4	5.63+/-0.7
085	6	1366.5+/-82.7	4	1.30+/-0.3
089	7	311+/-7.5	2.9	4.08+/-0.9
093	8	306+/-25.4	4.1	0.39+/-0
101	9	317+/-30.6	3.1	0.93+/-0
117	10	311+/-22.9	7	0+/-0.7
Gleason 9				
270	1	386+/-27.6	11	0.21+/-0.1
367	2	336+/-15.8	6.7	0.42+/-3.4
381	3	393+/-48.1	4.2	0+/-0.9
396	4	348+/-13.7	3.1	2.67+/-0
401	5	287+/-9.1	4.7	4.33+/-18
410	6	603+/-19.6	9.3	2.14+/-0
440	7	301+/-19.9	9.3	2.08+/-0.3
456	8	306+/-18.2	5.54	0.61+/-0.3
474	9	417+/-121.7	3.65.1	0.36+/-0.2
517	10	375+/-7.9	17.8	0+/-1.4

N/A: Not available ***Clinical PSA values**

Table 2. Survivin ELISA and PSA levels of plasma from controls with no diagnosis of cancer.

Patients ID#	Figure ID#	Survivin (pg/mL)	Exosome PSA (ng/mL)
OPN 118	N1	67.5+/-0	0+/-0
OPN 119	N2	65+/-14.9	0+/-0.2
OPN 120	N3	75+/-7	0+/-0.3
OPN 121	N4	60+/-7	0+/-0.2
OPN 122	N5	45.75+/-1.4	0+/-1.0
OPN 123	N6	60+/-7.1	0+/-1.5
OPN 124	N7	67.5+/-0	0+/-.5
OPN 125	N8	71.6+/-10	0+/-1.5
OPN 126	N9	35+/-14	0+/-.5
OPN 127	N10	67.5+/-0	2.17+/-0.4

Table 3. **Survivin ELISA and PSA levels of Sera from BPH patients are shown.**

Patients ID#	Figure ID#	Survivin ELISA(pg/mL)	Exosomal PSA (ng/mL)
VCT08	1	44.5+/-4.94	0.955+/-
G918	2	46.5+/-0.7	0
4PHH	3	45+/-5.6	1.955
OSWG3	4	46+/-7	2.17
XHZN	5	64.5+/-6.36	0
87LUH	6	25+/-35.3	2.67
CC51Y	7	24.5+/-34.64	2.305
XHWCG	8	45.5+/-0.7	1.88
IOAYY	9	49.5+/-0.7	0
ZUQ1B	10	76.5+/-21.2	2.275
L8K8F	11	54.5+/-2.12	2.745
WBZVY	12	52.5+/-7.7	2.5
X3GDZ	13	49+/-1.14	1.955
9UXFW	14	89+/-2.8	0
JCO8M	15	55.5+/-2.12	2.35
XWKEL	16	53+/-11.3	0
H2GY6	17	45.5+/-4.9	2.035
PPAQH	18	54.5+/-12	0
AU4AD	19	66.5+/-3.5	0
DXCOB	20	70.5+/-0.7	0

Table 4. **Survivin ELISA and PSA levels of Sera from Prostate Cancer patients.**

Patients ID#	**Figure ID#**	**Survivin ELISA(pg/mL)**	**PSA (ng/mL)**	**Exosomal PSA (ng/mL)**
642	1	140.1+/-0.34	6.2	0
6227	2	147.13+/-4.4	3.29	0
6636	3	154.21+/-7.24	4.5	0
3918	4	142.93+/-1.88	5.2	0
3960	5	140+/-0.03	13.5	0
2060	6	139+/-1.25	10.6	0
304	7	133.13+/-7	5.3	0
624	8	123.4+/-7.39	8.8	0
286	9	157.16+/-2.44	2.73	0
2828	10	155.75+/-5.7	3.9	0
14739	11	154.58+/-2.71	6.82	0
13077	12	155.7+/-2.1	7.1	1.73
6129	13	183+/-19.79	5.4	0
9508	14	179+/-26.87	9.4	1.835
14824	15	135.5+/-7	7	0
13406	16	146+/-2.82	8.8	2.335
9594	17	145+/-2.12	6.56	0
5117	18	149+/-7	42	0
69	19	146+/-5.6	58	0

*Clinical PSA Values

Table 5. **Correlation of Survivin and PSA levels from Plasma.**

Patients	**Survivin (pg/mL)**	**PSA (ng/mL)**
Normal	<100 (n=10) (**<u>100%</u>**) >100 (n=0) (0%)	<4 (n=10) (**<u>100%</u>**) >4 (n=0) (0%)
Gleason 6	<100 (n=0) (0%) >100 (n=10) (**<u>100%</u>**)	<4 (n=4) (40%) >4 (n=6) (**<u>60%</u>**)
Gleason 9	<100 (n=0) (0%) >100 (n=10) (**<u>100%</u>**)	<4 (n=2) (20%) >4 (n=8) (**<u>80%</u>**)
Recurrences	<100 (n=0) (0%) >100 (n=8) (**<u>100%</u>**)	<4 (n=0) (0%) >4 (n=10) (**<u>100%</u>**)
BPH	<100 (n=20) (**<u>100%</u>**) >100 (n=0) (0%)	<4 (n=20) (**<u>100%</u>**) >4 (n=0) (0%)
PCA	<100 (n=0) (0%) >100 (n=19) (**<u>100%</u>**)	<4 (n=3) (16%) >4 (n=16) (**<u>84%</u>**)

Furthermore, we have also shown that these complexes reside in or on exosomes, and that cancer treatment can enhance release of such exosomes from cancer cells (Khan S *et al*., 2011). Extracellular Survivin is able to mediate a pro-survival field effect through its secretion by cancer cells and uptake by surrounding normal and transformed cells (Khan S *et al*., 2009). Linking extracellular Survivin's ability to enhance cellular proliferation, survival and tumor cell invasion with a membrane-protective trafficking modality provides additional support for the hypothesis that Survivin plays a pivotal role in the pathobiology of cancer cell growth and protection from therapeutic interventions.

Several previous studies have examined the plasma levels of Survivin in cancer patients (Sugahara *et al*., 2004, El-Attar *et al*., 2010). In adult T-cell leukemia and the supernatants from in vitro cultures of solid-tumor cells, the levels of Survivin protein was low compared to the cellular Survivin protein and mRNA levels (Sugahara *et al*., 2004). This finding resulted in the conclusion that Survivin protein levels in plasma do not reflect cellular Survivin levels. Furthermore, Survivin plasma levels were evaluated compared to alpha fetoprotein (AFP) in patients with chronic hepatitis C viral infection (HCV) with and without hepatocellular carcinoma (HCC) (El-Attar *et al*., 2010). Though not concluded to be as reliable in these studies as AFP, Survivin levels were determined to be measurable and significant in patients suffering with HCV and measurable but not significant in those with HCC. Though not entirely clear, these results could be explained by the finding that HCV-infected HCC cells became more resistant to cell death and thus Survivin release compared to control or HCV-infected cells alone (El-Attar *et al*., 2010).

The prostate specific antigen (PSA) assay has been controversially utilized in prostate cancer screening though initially it was envisioned as a tool for evaluating treatment response (Croswell *et al*., 2011). Its use as a screening tool was driven by both the US prostate cancer burden and a need to detect its presence at a time that would allow for curative treatment to begin. Unfortunately, its use is believed to have led to both over diagnosis and overtreatment and hence the urgent need for novel biomarkers to be found to supplement PSA for management and treatment (Mazzola *et al*., 2011). Exosomal Survivin measurements may provide another plasma-based assay for the presence of PCa. In the present study, we have identified for the first time that exosomes containing Survivin can be purified from plasma collected from patients with a diagnosis of PCa. Although there was little difference in exosome quantity, exosomal Survivin levels were higher in exosomes purified from PCa patients than from the sera of normal controls. In addition, in this study and that performed by others (Sugahara *et al*., 2004, El-Attar *et al*., 2010), plasma-quantitated Survivin in samples taken from patients with no confirmed cancer diagnosis, also had reduced Survivin levels compared to PCa patient sera. The source of this exosomal Survivin pool, though unclear, may originate in immune cells (Iero *et al*., 2008) such as lymphocytes (Clayton *et al*., 2005), monocytes (Valenti *et al*., 2007) and dendritic cells (Quah & O'Neill, 2005), all Survivin containing cells (Fukuda & Pelus, 2006) which have been shown to release exosomes. Survivin expression is associated with established features of biologically aggressive prostate carcinoma, such as higher final Gleason score and metastasis to regional lymph nodes (Shariat SF *et al*., 2004). In our study we found that patients who had failed treatment with Taxotere had elevated levels of exosomal Survivin. In addition, and of special interest, we found that patients presenting with either mid (Gleason 6) or high (Gleason 9) Gleason scores exhibited higher exosomal Survivin levels with no significant difference in Survivin content between them. Our findings, though more robust in the plasma we studied appear in agreement with what has been found with urine, tumor exosomes and PSA (Mitchell *et al*., 2009). In these studies, urine exosome PSA is described as present in 20 of 24 PCa specimens while in our hands, tumor exosome Survivin is found in 47 of 47 specimens. Whether or not cancer treatment will affect tumor exosome Survivin levels has yet to be evaluated.

In this current study, we provide compelling evidence that circulating Survivin may be a useful diagnostic and prognostic marker for human PCa. Our results indicate that Survivin, analyzed directly from serum/plasma or from serum/plasma-derived exosomes, was lower in patients with BPH and healthy controls than in men with PCa. Survivin levels did not change, being consistently high, with regard to Gleason score and patients having recurrence, suggesting that Survivin levels could be used for early detection and could perhaps one day more accurately differentiate BPH from PCa. Comparing patients with and without tumors, but both having high PSA values will also be an important next step, as in our hands all the BPH samples we have acquired have relatively non-cancer PSA values (Williams & Naz, 2010).

The role of exosomal Survivin is still unknown. It is possible that a therapy-stressed, cellular release of exosomes containing Survivin and other antiapoptotic proteins, RNAs or miRNAs is performed as a final attempt to protect themselves from the stress that exists within the tumor microenvironment. Larger studies with more events and longer follow-up will be required to develop a more definitive statement regarding the association of Survivin expression in exosomes or in the tumor microenvironment with prostate carcinoma progression and as importantly, metastasis and survival. These findings will provide a rationale for further evaluation of exosomal Survivin and its role in resistance to androgen-based therapy in prostate carcinoma and raise the possibility of targeted combination therapy for advanced prostate cancer.

Our finding that Survivin, a unique human inhibitor of apoptosis (IAP), has intercellular transport and signaling capabilities is significant. Consistent with Survivin's association with unfavorable clinicopathological parameters, trafficking Survivin throughout the tumor microenvironment can drive the aggressive status of the tumor, prohibiting or minimizing therapeutic results. In our current work we show that though the overall number of exosomes being shed into patient plasma does not significantly change during the development of cancer, the level of Survivin in those exosomes increases significantly. Importantly, progression from mid- to late-stage does not drive an appreciable Survivin increase indicating that Survivin may prove useful as a biomarker for earlier detection of prostate cancer. Indeed, Survivin-based testing, performed on tumor-exosomes, will allow molecular-based diagnosis that in time may also aid in therapy decisions and disease response surveillance leading to better management of prostate cancer.

Materials and Methods

Patient Plasma and Serum

Plasma samples were collected from ten healthy male volunteers, and twenty-eight PCa patients. Plasma from twenty PCa subjects was obtained from the specimen bank of the SPECS consortium which is an observational clinical trial which utilizes prostate tissue and clinical values obtained by informed consent to derive gene signatures predictive of outcome at the time of diagnosis. SPECS is directed by Dr. Dan Mercola from the University of California, Irvine. Samples were pre-prostatectomy plasma randomly selected from ten low-grade PCa cases (Gleason 6) and ten high-grade PCa cases (Gleason 9). In addition, plasma from eight advanced-disease PCa patients participating in a second-line chemotherapy trial was also collected. These patients had failed chemotherapy with Taxotere. Nineteen serum samples were collected from PCa patients and six controls through the San Manuel Band of Mission Indians Biospecimen Laboratory at Loma Linda Universities Cancer Center. Twenty BPH samples were purchased from Bioserve Biotechnologies, Beltsville, MD. Bioserve Biotechnologies as provider of these samples is covered as defined in the HIPPA Act of 1996 as is providing them to us with a limited data set of protected health information.

Blood was collected in vacuum tubes containing sodium heparin. The tubes were centrifuged at 2000g x 7 minutes, and the plasma was then removed and aliquoted for storage at -80°C. All samples were obtained in the course of IRB-approved studies, following the documentation of informed consent in accordance with university policy at both Loma Linda University and the University of California at Irvine.

Human Survivin Immunoassay

Whole plasma samples were subjected to a commercially available human Survivin Immunoassay (R&D systems, Minneapolis, MN) using the manufacturer's instructions in order to quantitate Survivin concentrations.

Exosome Isolation

Plasma microvesicles were isolated as previously described, with minor modifications (Caby MP *et al*., 2005). Thawed, cryopreserved plasma (2 ml) was centrifuged for 30 min at 500 x g, 45 min at 12,000 x g and 18 h at 110,000 x g. Pellets were resuspended in a large volume of PBS, filtered through a 0.22-µm filter (Millipore, Billerica, MA) and centrifuged at 110,000 x g for 1 h. Microvesicle pellets were washed once in a large volume of PBS, centrifuged at 110,000 x g for 1 h and re-suspended in 50–200 ml of PBS. The amount of 110,000 x g pellet proteins recovered was measured using the BCA protein assay kit (Pierce, Rockford, IL). Exosomes were used as fresh preparations for immunoblotting or were conserved at -80 □C for later use. For serum samples, the commercially available ExoQuick (SBI, Mountain View, CA) was employed as described by the vendor. Briefly, 100 µL of serum was incubated with 100 µL of ExoQuick solution followed by a 2 hr incubation at 4 °C followed by centrifugation at 1500 x g for 30 minutes. After centrifugation the exosomes appear as a beige or white pellet at the bottom of the vessel which is then reconstituted with 500 µL of dH_2O.

Exosome Quantification

To quantify the amount of exosomes released, we assessed the activity of acetylcholinesterase, an enzyme that is associated with these vesicles. Acetylcholinesterase activity was assessed as described by Savina et al. (Savina A *et al*., 2003). Briefly, 40 µl of the exosome fraction was suspended in 110 µl of PBS. 37.5 ml of this PBS-diluted exosome fraction was then added to individual wells on a 96-well flat-bottomed microplate. 1.25 mM acetylthiocholine and 0.1 mM 5,50-dithiobis(2-nitrobenzoic acid) were then added to exosome fractions in a final volume of 300 µl, and the change in absorbance at 412 nm was monitored every 5 min for 30 min.

Human PSA Immunoassay

Isolated exosomes from patients` plasma were subjected to human Prostate Specific Antigen (PSA) using a PSA Immunoassay (American Research Products, Inc., Waltham, MA, USA) kit following the manufacturer's instructions in order to quantitate PSA concentrations in exosomes.

Western Blot Analysis

For Western blot analysis, cells or exosomal preparations were lysed using lysis buffer (50 mM Tris (pH 7.5), 1% NP40, 0.25% DOC, 150 mM $NaCl_2$, 1 mM PMSF, 10 µg/ml Aprotinin/ leupeptin/pepstatin, 20 mM NaF, 0.2 mM EGTA, 1 mM EDTA (pH 8.0), H2O). For protein concentrations the BCA assay (Pierce, Rockford, IL) was used. Proteins from exosomes (20–40 µg) were separated using 12% Bis–Tris polyacrylamide gels, transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA) and probed using the following antibodies: mouse monoclonal anti-LAMP1 (Abcam, Cambridge, MA), and rabbit polyclonal anti-Survivin (Novus, Littleton, CO). Secondary antibodies (IR-Dye conjugated) were goat anti-rabbit and goat anti-mouse immunoglobulin (LICOR, Lincoln, Nebraska). Immunoreactive bands were detected using the Odyssey Imaging System (LICOR, Lincoln, Nebraska) and quantified using ImageQuant software.

Statistical Analysis

Multiple comparisons among different groups were calculated by using Multiple Analysis of Variance (MANOVA). Student t-test (two-tailed) was used to evaluate the significance of changes between control groups and experimental groups. Probability values $P<0.05$ were considered statistically significant.

Acknowledgements

The author's would like to thank the Center for Health Disparities & Molecular Medicine for resource support of this project and the Graduate Students that it involved. Also, we would like to thank the Department of Medicine at the University of California, Irvine for sample acquisition as well as Loma Linda Universities Cancer Center & San Manuel Band of Mission Indians Biospecimen Laboratory.

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CHAPTER 3

PROTEOMIC PROFILING OF SERUM-DERIVED EXOSOMES FROM ETHNICALLY DIVERSE PROSTATE CANCER PATIENTS

Published:

Turay D, Khan S, Osterman CD, Curtis MP, Khaira B, Neidigh JW, Mirshahidi S, Casiano CA, Wall NR. Proteomic profiling of serum-derived exosomes from ethnically diverse prostate cancer patients. Accepted for Publication, Cancer Investigation, 2015.

Abstract

Prostate cancer (PCa) remains the most frequently diagnosed male malignancy in Western countries and the second most common cause of male cancer death in the United States. The relatively elevated PCa incidence and mortality among African American men makes this cancer type a challenging health disparity disease. To increase the chance for successful treatment, earlier detection and prediction of tumor aggressiveness will be important and need to be resolved. This study demonstrates that small membrane-bound vesicles shed from the tumor called exosomes contain ethnically and tumor-specific biomarkers, and could be exploited for their diagnostic and therapeutic potential.

Introduction

Prostate cancer (PCa) is the most frequently diagnosed non-skin cancer in men and the second leading cause of male cancer deaths in the United States, accounting for 233,000 new cases and 29,480 deaths in 2014 (Siegel *et al*., 2014). The incidence of the disease has an uneven geographic distribution with the highest rates recorded in Australia, North and Western Europe and the United States (Jemal *et al*., 2011). The incidence and mortality of the disease is disproportionately high among African American (AA) men compared to other ethnicities in the United States (Aizer *et al*., 2014). An interesting note is that the disease incidence appears to be lower among Black men in Africa, although this has been attributed to the lack of adequate and sufficient statistics from this region of the world (Tindall *et al*., 2014).

While the basis for PCa health disparities is not well understood, evidence points to the interplay between socioeconomic, environmental, and biologic/genetic factors (Aizer *et al*., 2014). A number of risk factors for PCa have been examined in the literature including chronic inflammation of the prostate (infectious and non-infectious), hormonal factors (elevated testosterone, leptin etc.), smoking, dietary and genetic factors; among others. Smith et al., described the link between the susceptibility gene HPC-1 (Hereditary PCa) and an increased risk of developing PCa, especially in younger patients (Smith *et al*., 2011). Overall, PCa is believed to be a disease of multifactorial origin.

Prostate Specific Antigen (PSA) combined with a digital rectal exam has remained the standard of screening for PCa for the past two to three decades. PSA was first described as a marker for human semen in forensics but it was subsequently demonstrated in the serum of men with prostate disease (Verma *et al*., 2014). The test was however intended for surveillance of diagnosed PCa patients. There was a sharp rise in newly diagnosed cases in the early 1990's attributed to the introduction of PSA as an FDA-approved screening test for PCa. Coincident with this increase in the number of new cases diagnosed was a pathologic migration toward a more favorable (early disease) stage at diagnosis (Bryant & Lilja, 2014, Darwish-Yassine *et al*., 2014). Non-palpable PCa (and therefore perhaps clinically treatable disease) now accounts for 70-80% of newly diagnosed cases (Kardasevic & Delic-Redzepagic, 2014, Powell *et al*., 2014). This may at least in part explain why the combined 5 and 10 year survival for all stages of PCa is currently at 99% and 91% respectively. The true clinical value/advantage of using serum PSA as a screening test has been recently called into question. While the use of this test has increased the proportion of patients with lower tumor stage at the time of diagnosis, its value is still debated because of its limitations. These limitations include the cut-off PSA value of 4.0 ng/ml which fails to detect a significant number of prostate tumors; PSA screening has not been demonstrated to improve health outcomes, particularly in older men; PSA is not specific for PCa since its levels can be elevated in patients with benign prostatic hyperplasia (BPH) and prostatitis, leading to a false-positive finding of up to 60-80% of prostate biopsies; and obesity lowers PSA levels, which in theory could lead to delayed detection of PCa and worse clinical outcome (Bradley *et al*., 2013). Moreover, the lack of specificity of PSA as a screening tool has also led to many possibly unnecessary diagnostic (biopsies) and therapeutic procedures. The conclusions from the European Trial of PCa screening was that 1410 screenings/biopsies had to be performed and 48 additional cases treated in order to prevent one death from PCa (Loeb *et al*., 2011). It should be noted, however, that most of the information available on the efficacy of the PSA test is based on studies of men of European descent and that despite its limitations, PSA screening is still highly recommended for AA men ages 45 and above (Faraday *et al*., 2009). The limitations of the PSA test demand increasing the efforts to identify novel biomarkers that will supplement this test and enhance early PCa detection, management and therapeutic response. For this purpose we have initiated the evaluation of small membrane-bound vesicles called exosomes derived from PCa patients from different ethnicities for the possibility that they may contain yet undiscovered biomarkers. Potentially, these biomarkers could be tailored for early PCa detection and management in AA men, with the ultimate goal of reducing or eliminating PCa health disparities.

Recent studies have shown that tumor-derived exosomes are key modulators of cell to cell and cell to extracellular communication within the tumor microenvironment (Johnstone, 2006). Exosomes play an important part in this communication due to their ability to transport cancer-promoting material such as protein, RNA and miRNA (Johnstone, 2006, Khan S *et al*., 2011, Khan *et al*., 2015). In order to successfully treat cancer, it will be crucial to develop therapeutic strategies that not only target tumor cells but also the mediators within the tumor microenvironment that modulated many aspects of the etiology of this disease (Aspe *et al*., 2014, Asuncion Valenzuela *et al*., 2015, Valenzuela *et al*., 2015). However, enhanced treatment capabilities are the direct result of earlier detection which will depend significantly on the early discovery of biomarkers shed by these tumors (Properzi *et al*., 2013).

Exosomes are present in serum, plasma and urine and contain a wide range of proteins and RNAs, representing their tissue of origin thus making them a possible source or pool of novel PCa biomarkers (Duijvesz *et al*., 2011). Our present study was designed to profile the exosomal proteins from the plasma of ethnically diverse PCa patients and control individuals with no diagnosis of PCa, and to compare these exosomal profiles between the different ethnic groups. We believe that in addition to diagnostic markers, prognostic, predictive and therapeutic markers are needed to act as surrogate endpoints in forecasting disease severity, choosing appropriate treatment modalities, and monitoring responses to therapies (Mikolajczyk *et al*., 2004, Fradet, 2009, Fiorentino *et al*., 2010, Ploussard & de la Taille, 2010). Our previous demonstration of exosomal Survivin, an inhibitor of apoptosis and indicator of cancer severity, in the plasma of patients with newly diagnosed PCa (Khan *et al*., 2012) provided a rationale for studies to investigate the utility of exosomal content as a source of early, easily measured PCa biomarkers in ethnically diverse populations. Here we present our initial results in this effort.

Materials and Methods

Patient Plasma

Plasma samples were collected from 9 healthy male volunteers, and 12 PCa patients (**Table I**). These 12 patients self defined themselves as AA (n=4), Caucasian (n=4) and Hispanic (n=4). Blood was collected in vacuum tubes containing sodium heparin. The tubes were centrifuged at 2000 x g for 7 minutes, and the plasma was then removed and aliquoted for storage at -80°C. All samples were obtained in the course of IRB-approved studies, following the documentation of informed consent in accordance with Loma Linda University policies.

Table 1. Demographic Data of PCa Patients

	No Diagnosis (n=9)	Caucasian (n=4)	African American (n=4)	Hispanic (n=4)
MEAN AGE	38.0 (26-45)	62.5 (48-73)	58.1 (48-75)	66.0 (64-68)
PSA VALUE	N/A	5.52 (2.7-8.8)	14.04 (4.5-58)	18.16 (6.5-42)
GLEASON SCORE	N/A	6.75	7.14	7.75
MEDIAN PATHOLOGIC STAGING	N/A	pT2NoMx	pT2NoMx	pT3NoMx

Exosome Isolation

For plasma microvesicle samples, the commercially available ExoQuick (SBI, Mountain View, CA) was employed as described by the vendor. Briefly, 100 µl of plasma was incubated

with 100 µL of ExoQuick solution followed by a 2 hr incubation at 4 °C followed by centrifugation at 1500 x g for 30 minutes. After centrifugation the exosomes appear as a beige or white pellet at the bottom of the vessel which is then reconstituted with 500 µl of dH_2O (Caby MP *et al*., 2005).

Exosome Quantification

To quantify the amount of exosomes released, we assessed the activity of acetylcholinesterase, an enzyme that is associated with these vesicles (Savina A *et al*., 2003). Acetylcholinesterase activity was assessed as described by Savina et al. (Savina A *et al*., 2003). Briefly, 40 µl of the exosome fraction was suspended in 110 µl of PBS. 37.5 ml of this PBS-diluted exosome fraction was then added to individual wells on a 96-well flat-bottomed microplate. 1.25 mM acetylthiocholine and 0.1 mM 5,50-dithiobis(2-nitrobenzoic acid) were then added to exosome fractions in a final volume of 300 µl, and the change in absorbance at 412 nm was monitored every 5 min for 30 min.

Protein Separation

For protein analysis, exosomal preparations were lysed using lysis buffer (50 mM Tris (pH 7.5), 1% NP40, 0.25% DOC, 150 mM $NaCl_2$, 1 mM PMSF, 10 µg/ml Aprotinin/ leupeptin/pepstatin, 20 mM NaF, 0.2 mM EGTA, 1 mM EDTA (pH 8.0), H2O). For protein concentrations the BCA assay (Pierce, Rockford, IL) was used. Proteins from exosomes (20–40 μg) were separated using 12% Bis–Tris polyacrylamide gels.

In-Gel Trypsin Digestion and MS

Protein bands were excised manually and washed with 50% (v/v) methanol and 5% (v/v) acetic acid. The gel pieces were then dehydrated in acetonitrile and dried in a SpeedVac concentrator (Savant, Farmingdale, NY). Proteins were reduced using 10 mM dithiothreitol (DTT) in 100 mM ammonium bicarbonate for 30 min at room temperature. The DTT solution was removed and the proteins were alkylated for 30 min at room temperature using 100 mM iodoacetamide after which the gel pieces were dehydrated as before. Gel pieces were rehydrated in 100 mM ammonium bicarbonate and then dehydrated and dried as previously described. Proteins were tryptically digested using MS grade trypsin (Promega, Madison, WI), added at a final concentration of 20 ng/μl to fully cover the gel pieces. Digestion was performed at 37 °C overnight. Peptides were recovered with 30 μl, 50% (v/v) acetonitrile and 5% (v/v) formic acid twice. All supernatants were pooled and dried in a SpeedVac concentrator for 1 hr.

Tryptic peptides were analyzed on a ThermoFinnigan LCQ Deca XP system that includes a surveyor HPLC and a PicoView 500 (New Objective, Woburn, MA) for performing nanoflow electrospray ionization. The flow of the surveyor HPLC pump was split to achieve a 200–300 nanoliter/min flow exiting a PicoFrit column (New Objective) packed with BioBasic C18 beads (10 cm, 5 l m, 300 A°). Samples were loaded onto a Michrom Bioresources (Auburn, CA) cap-trap at 5 l l/min and washed with mobile phase A (aqueous 2% acetonitrile with 0.1% formic acid). Peptides were then eluted onto the column and into the mass spectrometer using a gradient of 0–75% mobile phase B (aqueous 90% acetonitrile with 0.1% formic acid). The mass spectra acquisition was operated in the data dependent mode with one MSscan (300–1,500 m/z) and three MS/MS scans of the most intense ions in the MS scan. We used the Sequest algorithm implemented on the TurboSequest software package to identify proteins based on the MS/MS spectra. The resulting Sequest hits were filtered based on the charge state and Xcorr value to require Xcorr C 1.5, 2.0, and 2.5 for single, double, and triple charged ions, respectively.

The MS/MS fragmentation spectra were searched against a current human protein database (March 2009) containing 37,391 reference sequences. The search algorithms Sequest (Eng *et al*., 1994), Mascot (Perkins *et al*., 1999), and X! TANDEM (Craig & Beavis, 2004), were used to identify peptides and proteins. The significance of identified peptides and proteins were determined using the Peptide-Prophet (Keller *et al*., 2002) and Protein-Prophet (Nesvizhskii *et al*., 2003), respectively, algorithms as implemented in Scaffold 2 (Proteome Software, Portland, OR). We included only peptides with a Scaffold score of C 95% (5% false discovery rate) in the results.

Statistical Analysis

Multiple comparisons among different groups were calculated using Multiple Analysis of Variance (MANOVA). A Student t-test (two-tailed) was used to evaluate the significance of changes between control groups and experimental groups. Probability values $P<0.05$ were considered statistically significant.

Results

PCa Patients Exhibit Increased Exosome Numbers

We have previously demonstrated that cultured PCa cells release exosomes into the extracellular milieu (Khan S *et al*., 2009). These small, membrane-vesicles are also known to occur in the plasma as well as serum of cancer patients (Mitchell *et al*., 2009, Koumangoye *et al*., 2011, Khan *et al*., 2012). Exosomes were therefore collected using ExoQuick, and quantitated using the acetylcholinesterase enzymatic assay as we and others have previously described (Johnstone, 2006, Khan S *et al*., 2011, Khan *et al*., 2012). The mean plasma exosome levels varied significantly between the healthy control subjects group and the different PCa patient groups ($P< 0.001$). However, there was no significant difference in exosome quantity between the different PCa patient groups (**Figure 1**).

Plasma-Derived Exosomes Contain Potential Biomarkers

Exosomes purified using ExoQuick from PCa patient plasma were characterized by mass spectrometry. After the extraction from a preparative gel (**Figure 2**), proteins were identified by LC-MS. The ten most abundant proteins identified were: Apolipoproteins, Pregnancy Zone Protein, Macroglobulins, Keratin, Albumin Precursors, Haptoglobin, Ceruloplasmins, Transferrin, Complement Proteins and Fibronectin (**Table 2**). Exosomal proteins were next categorized for localization using Scaffold 2 software and found to have the majority of their proteins determined to be intracellular (57%) and extracellular (32%) with only 7% identified as cell membrane and 4% as indeterminate (**Figure 3A**). Intracellular proteins present in the exosomes were next annotated for distribution and

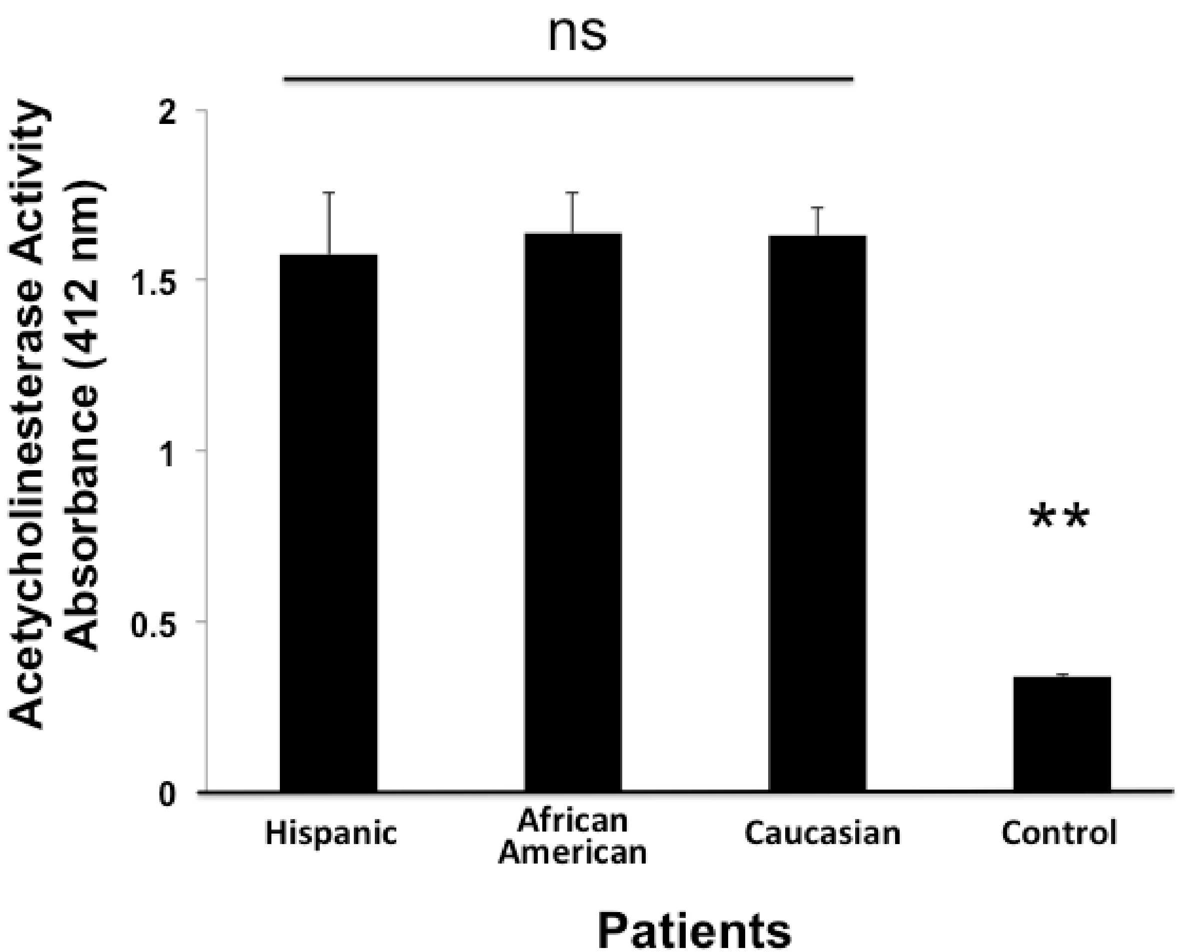


Figure 1. Exosomal contents in PCa patients plasma samples by using the acetylcholinesterase activity assay. Exosome levels were measured in plasma derived from subjects representing Hispanic, African American and Caucasian PCa patients and control collected from the three ethnic groups. Exosome levels were measured in serum derived from 9 control, 4 Caucasian, 4 African American and 4 Hispanic subjects. Comparisons were accomplished using MANOVA. (ns = not significant; **, $p<0.05$, statistically significant).

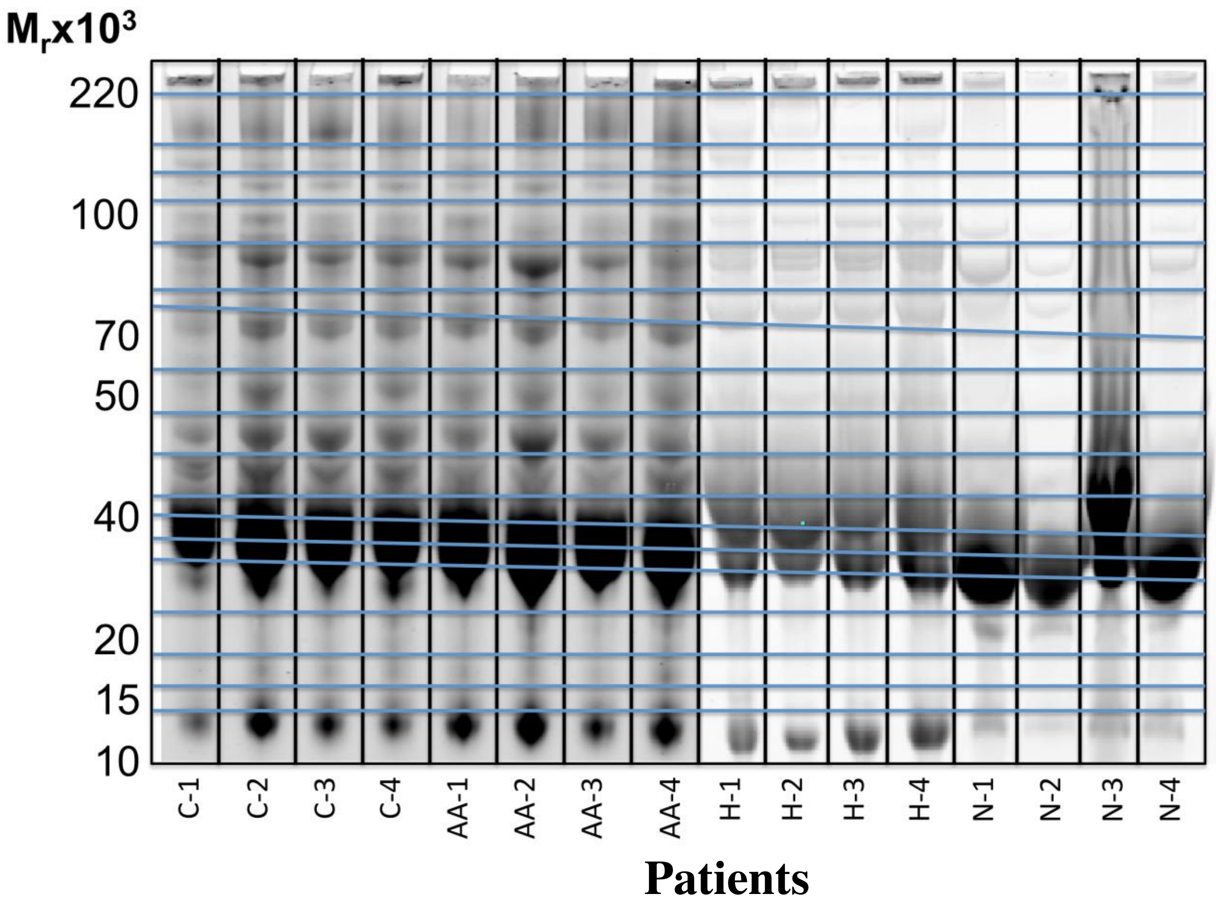


Figure 2. Exosomal proteins taken from PCa patients plasma were separated on a 4-12% gradient SDS gel and stained with Coomassie brilliant blue. Gels are representative of 2 individual gels ran, showing 4 individuals of each ethnicity (C-Caucasian, AA-African American, H-Hispanic, N-no PCa diagnosis). The major bands were analyzed by trypsin digestion and LC-MS/MS mass spectrometry. Molecular-weight markers in kilodaltons (Kda) are shown on the *left*.

Table 2. Ten Most Abundant Proteins Discovered

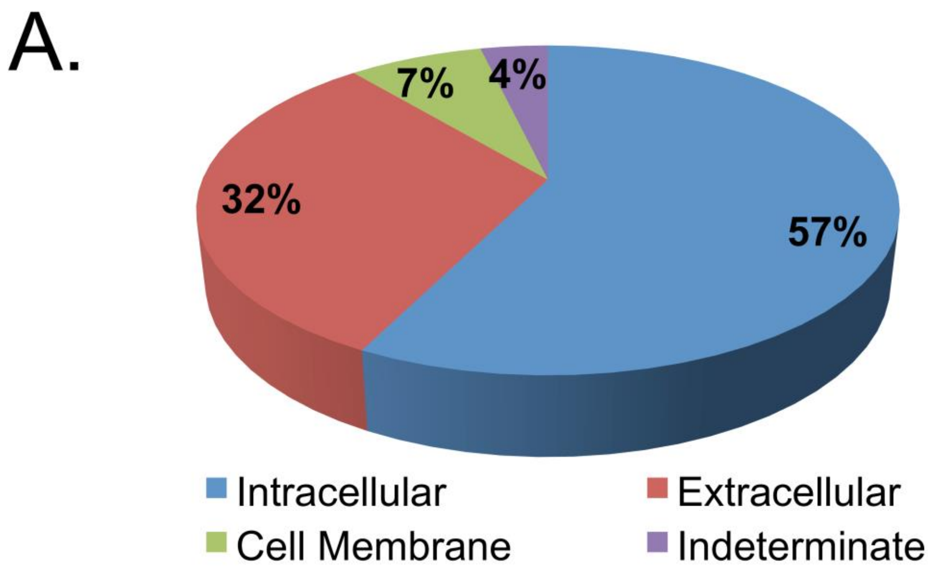
Macroglobulins	Keratin
Albumin Precursors	Haptoglobin
Ceruloplasmins	Transferrin
Complement Proteins	Fibronectin
Apolipoproteins	Pregnancy Zone Protein

found to be mostly cytosolic (45%) and nuclear (24%), with the remaining 31% spread as cytoskeletal (9%), Golgi (2%), ER (4%), mitochondrial (3%) and ubiquitous (13%) (**Figure 3B**).

Proteomic Comparison Across Different Ethnicities

Exosome protein compositions were compared between those purified from individuals with no diagnosis of PCa (non-Ca) and those with PCa (**Figure 4A**). The exosomes from the non-Ca individuals provided the identities of 145 proteins (74+71) while the exosomes collected from the PCa patients provided the identities of 184 proteins (71+113). Though there were 71 proteins found in common, we believe that the analysis of the 74 non-Ca- specific and 113 PCa-specific proteins that could lead to the discovery of a novel biomarkers, either a molecule which has become reduced as tumors develop, identified from the unique non-Ca samples, or proteins with enhanced expression as the tumor develops, identified from the unique PCa samples. When comparing proteins identified from exosomes of our non-Ca volunteers to ethnicity-specific- associated PCa exosome proteins, the attribute most striking is the reduced number of proteins held in common and the low number of proteins unique to each ethnicity. The sum of the proteins from AA (82), Hispanic (149) and Caucasian (63) do not add to the number of unique proteins from PCa (113) as there are a number of common proteins found among these three ethnic groups.

Ethnicity-specific exosomal proteins were next investigated in order to define if any of the 184 PCa-specific proteins, shared amongst the different ethnic groups were held in common with all ethnicities, pairs of ethnicities, or definitive for a single ethnicity. Any protein held in common would be a potential biomarker for PCa, while proteins that were



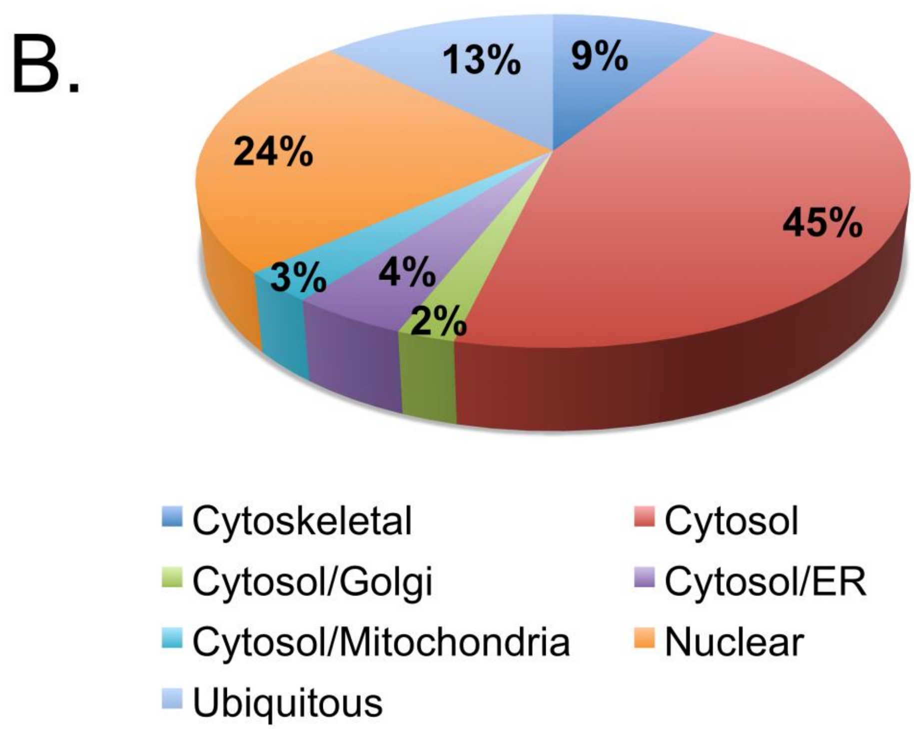


Figure 3. PCa exosome protein localization. **A.** Exosomal proteins were categorized for primary localization and found to have the majority of their proteins determined to be intracellular and extracellular with only a small percentage of them in the cell membrane or indeterminate. **B.** Intracellular proteins were next categorized as being cytosolic, nuclear, cytoskeletal, golgi, ER, mitochondrial and ubiquitous in their distribution.

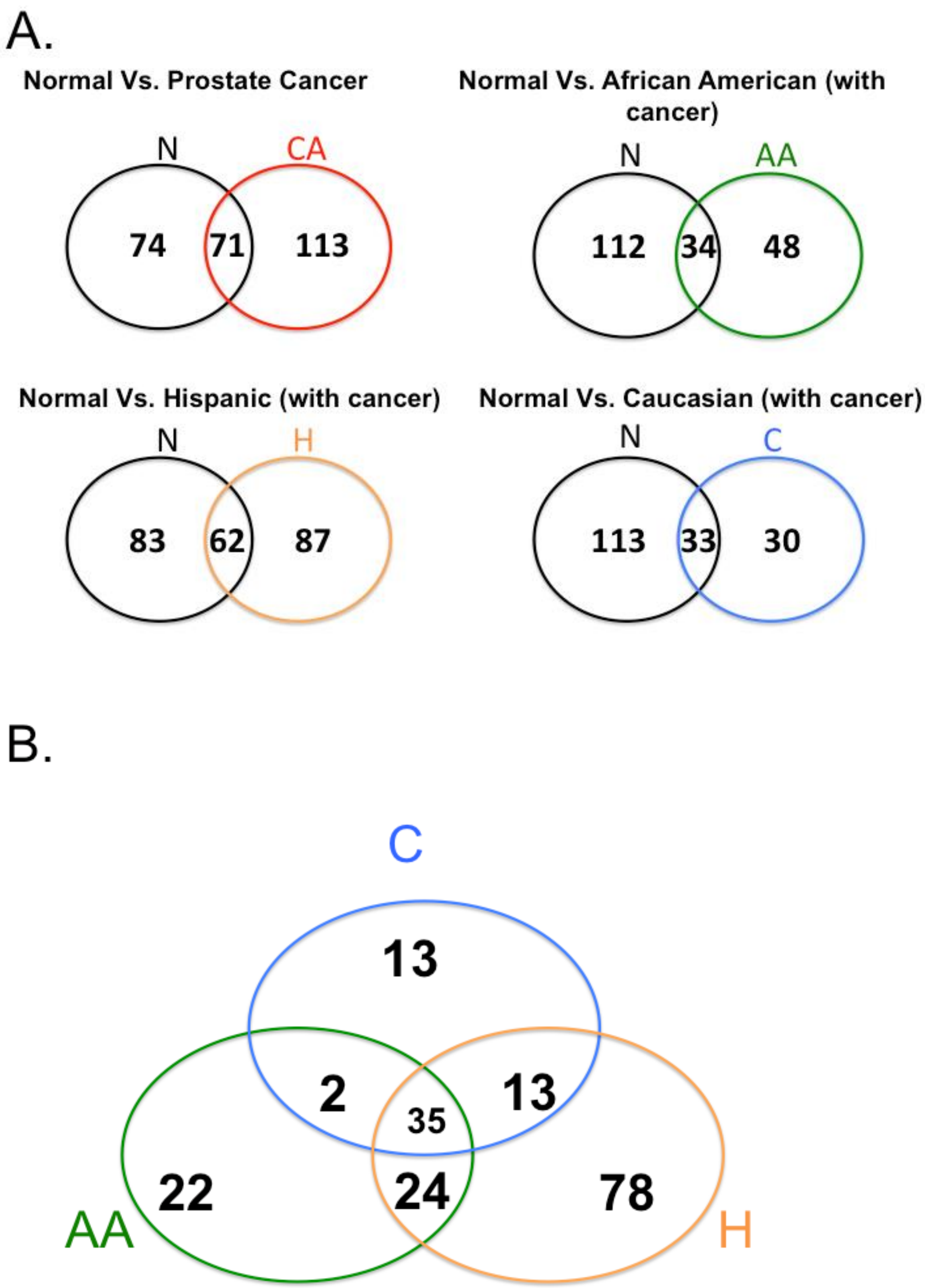


Figure 4. Exosomal protein enrichment was monitored in each sample by mass spectrometry. **A.** Cancer changes were assessed by first comparing PCa (CA) to the no PCa (N) diagnosis control and then controls to each ethnicity specific exosomal proteome. **B.** Intra-ethnicity was assessed by simply comparing within the ethnic-specific exosomal proteomes. Venn diagrams show the comparisons in terms of exosome proteomes.

only found in a specific ethnicity would be ideal as a putative biomarker for PCa in that ethnicity. **Figure 4B** shows that there are 35 proteins held in common between the three ethnic groups studied (**Table 3**) and there are 2, 24 and 13 shared proteins in AA/Caucasian, AA/Hispanic and Caucasian/Hispanic respectively, that should be further evaluated (**Table 4**). We also identified proteins that are unique, in our analysis, to each individual ethnicity represented (**Table 5 and Supplemental Table I**).

Among all of these putative biomarkers are proteins of specific interest because they have been previously described as cancer-associated proteins **(Table 6)**. These includes: DNA helicase homolog PIF1, Four and a Half LIM Domain 3, Glutathione S transferase omega 2, Maternal embryonic leucine zipper kinase, Iroquois homeobox protein 5, Leucine rich zipper containing 4, minichromosome maintenance complex component 5, Mitochondrial tumor suppressor 1 isoform 4, nasopharyngeal epithelium specific protein, Ubiquitin-like with PHD and ring finger domains, and Trinucleotide repeat containing 6B isoform 3.

Discussion

The PSA test has been controversially utilized in PCa screening though initially it was envisioned as a tool for evaluating treatment response. Its use as a screening tool was driven by both the United States PCa burden and a need to detect its presence at an early stage that would allow for curative treatment. Unfortunately, its use is believed to have led to both over diagnosis and over treatment, and there remains an urgent need for novel biomarkers to be found to supplement PSA for early PCa detection, management, and treatment (Mazzola *et al*., 2011). Recent efforts in PCa biomarker discovery and

Table 3. Proteins held ethnically in common.

African American, Hispanic & Caucasian (35)	
5'-nucleotidase, cytosolic III-like	Inter-alpha (globulin) inhibitor H1
Albumin preproprotein	Inter-alpha globulin inhibitor H2 polypeptide
Alpha-2-macroglobulin precursor	Keratin 2
Apolipoprotein A-I preproprotein	Lipoprotein Lp(a) precursor
Apolipoprotein B precursor	Phosphatidylinositol-3,4,5-trisphosphate-dependent Rac exchange factor
Ataxin 7-like 1 isoform 3	Pregnancy-zone protein
Ceruloplasmin precursor	Propionyl-Coenzyme A carboxylase, alpha polypeptide isoform b
Complement component 3 precursor	RUN and FYVE domain containing 3 isoform 3
Complement component 4 binding protein, alpha chain precursor	Serum amyloid P component precursor
Complement factor H isoform a precursor	Similar to C4A protein isoform 1
Complement factor H-related 1	Similar to complement component 3
Fibrinogen, alpha polypeptide isoform alpha-E preproprotein	Similar to complement component C3, partial
Fibrinogen, beta chain preproprotein	Similar to immunoglobulin lambda-like polypeptide 1
Fibrinogen, gamma chain isoform gamma-A precursor	Transferrin
Fibronectin 1 isoform 6 preproprotein	UDP glycosyltransferase 1 family, polypeptide A8 precursor
Haptoglobin isoform 1 preproprotein	URB2 ribosome biogenesis 2 homolog
Haptoglobin-related protein	Vinculin isoform meta-VCL
Hypothetical protein XP_002343504	

Table 4. Proteins shared with two or more ethnicity.

Black/Caucasian	Black/Hispanic	Caucasian/Hispanic
Cytochrome P450, family 7, subfamily B, polypeptide 1	AHNAK nucleoprotein 2	Ankyrin repeat domain 47
Ribosomal protein L24	Amphiregulin B	Cell division cycle 20
	Ankyrin repeat domain 30A	Cysteine-rich motor neuron 1
	Complement component 1, q subcomponent, B chain precursor	Hypothetical protein LOC9907
	Complement factor B preproprotein	Hypothetical protein XP_002343483
	Dystonin isoform 1e precursor	Hypothetical protein XP_002347522
	Family with sequence similarity 120B	Hypothetical protein XP_002348153
	GDP dissociation inhibitor 1	LGP1 homolog isoform 2
	GPR158-like 1	Plasminogen
	GRIP1 associated protein 1 isoform 1	RAD50 homolog isoform 1
	Histidine-rich glycoprotein precursor	Similar to hCG2043206
	Homeobox B13	Zinc finger protein 594
	Hypothetical protein XP_002342686	Zinc finger, ZZ type with EF hand domain 1
	Keratin 1	
	Keratin 10	
	Keratin 6B	
	Keratin 76	
	Kinesin family member 14	
	MIT, microtubule interacting and transport, domain containing 1	
	Mitochondrial ribosomal protein S22	
	Regulator of G-protein signaling 4 isoform 1	
	Spectrin, beta, non-erythrocytic 2	
	Ubiquitin-like with PHD and ring finger domains 1 isoform 2	

Table 5. Proteins unique to each ethnicity.

BLACKS (22)	CAUCASIANS (13)	HISPANIC (78)
Alpha 1B-glycoprotein precursor	Albumin preproprotein isoform 2	A-kinase anchor protein 9 isoform 3 [Homo sapiens]
Complement component 3	DAZ interacting protein 3, zinc finger	Actin, gamma 1 propeptide
Complement component C3, partial	Erythrocyte membrane protein band 4.9 isoform 1	Actin, gamma 2 propeptide
Dynein heavy chain domain 2 isoform 1	Hypothetical protein LOC93081	Aiolos isoform 1
G protein-coupled receptor 171 [Homo sapiens]X&	Hypothetical protein XP_002347495	Alpha 3 type VI collagen isoform 4 precursor
Heat shock 70kDa protein 8 isoform 1	Integrin-linked kinase	Alpha-1-microglobulin/bikunin preproprotein]
Hemopexin precursor	Leucine rich repeat containing 4	Alpha-2-glycoprotein 1, zinc
Hypothetical protein LOC338094	Leucine rich repeat containing 4B	Alpha-2-HS-glycoprotein
Hypothetical protein LOC54980	NAD kinase	Amphiphysin isoform 1
Hypothetical protein XP_002342292	Nuclear receptor subfamily 1, group H, member 3 isoform a	Apolipoprotein A-II preproprotein
Hypothetical protein XP_002342604	Phosphodiesterase 6A	Apolipoprotein C-I precursor
Immunoglobulin lambda-like polypeptide 1	RNA polymerase I associated factor 53	Apolipoprotein D precursor
KDEL (Lys-Asp-Glu-Leu) containing 1	Transferrin isoform 4 precursor	Arginine vasopressin receptor 1A
Keratin 71		Arginyl-tRNA synthetase
Kinesin family member 4		CD5 molecule-like
Maternal embryonic leucine zipper kinase		Coagulation factor II preproprotein
Minichromosome maintenance complex component 5		Coiled-coil domain containing 104
Mitogen-activated protein kinase 3 isoform 1		Complement component 1, q subcomponent, C chain precursor
Pericentrin		Complement component 5 preproprotein]
RAR-related orphan receptor A isoform a		Complement component 6 precursor
Smad ubiquitination regulatory factor 1 isoform 2		Complement component 8, gamma polypeptide
Solute carrier family 6 member 8 isoform 2		Further Proteins are listed in Supplemental Table 1.

Table 6. Known cancer associated proteins.

PROTEIN	CANCER TYPE	FUNCTION
DNA Helicase Homolog PIF1	Multiple	Inhibits Telomerase and suppresses Apoptosis
Four and a Half LIM Domain 3	Multiple	Interacts with SMAD Proteins, Tumor suppressor Zinc finger type protein
Glutathione S Transferase Omega 2	Ovarian	Involved in the metabolism of Carcinogens and Xenobiotics
Maternal Embryonic Leucine Zipper Kinase	Colorectal	Neural Stem cell marker, Overexpression in tumors confers Chemoresistance
Iroquois Homeobox Protein 5	Prostate	Transcription Factor involved in Body Patterning. Assists proliferation of cancer cells
Leucine Rich Zipper Containing 4	Brain	Enhances migration of Glioblastoma cells
Minichromosome Maintainance complex Component 5	Skin	Initiation of DNA replication.
Mitochondrial Tumor Suppressor 1 Isoform 4	Prostate	Prostate
Nasopharyngeal epithelium Specific Protein	Head and Neck Cancer	Inhibits Invasion and proliferation of Nasopharyngeal Cancer
Ubiquitin-like with PHD and ring finger domains	Lung cancer	Silences Tumor Suppressor genes in Lung Cancer
Trinucleotide repeat containing 6B Isoform 3	Prostate	Degrades mRNA. A SNP at 22q13 confers risk for Prostate Cancer

Supplemental Table I. Proteins unique to Hispanics.

HISPANIC (57)		
Complement factor H-related 3	Keratin 27	SH2 domain containing 4B isoform 1
Complement factor I preproprotein	Keratin 32	Similar to elongation factor Tu GTP binding domain cont
Cortactin binding protein 2	Keratin 4	Similar to immunoglobulin lambda-like polypeptide
Cytochrome P450, family 2, subfamily E, polypeptide 1	Keratin 9	solute carrier family 35, member F5
Dynein heavy chain domain 3	Mitochondrial tumor suppressor 1 isoform 4	Synaptotagmin-like 2 isoform a
Epsilon globin	Myelin expression factor 2	Transient receptor potential cation channel, subfamily M, member 3
Eukaryotic translation initiation factor 2B, subunit 4 delta isoform	Nasopharyngeal epithelium specific protein 1	Transthyretin precursor
Eukaryotic translation initiation factor 3, subunit 10 theta, 150/170kD	Outer dense fiber of sperm tails 2-like isoform a	Trinucleotide repeat containing 6B isoform 3
FERM domain containing 5 isoform 2	Paraoxonase 1 precursor	Tubulin, gamma complex associated protein 2
Fibronectin 1 isoform 7 preproprotein	PDZ domain-containing guanine nucleotide exchange factor I	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase-like 1
Ficolin 3 isoform 1 precursor	Pleckstrin and Sec7 domain containing	UNC13 (C. elegans)-like
G protein-coupled receptor 148	Protein kinase N2	Upstream of NRAS isoform 2
Gelsolin isoform b	Protein serine kinase H1	Vesicle-fusing ATPase
Glutathione peroxidase 3 precursor	receptor activity modifying protein 3 precursor	Vitamin D-binding protein precursor
Hypothetical protein LOC51237	Retinol-binding protein 4, plasma precursor	Vitronectin precursor
Hypothetical protein LOC643677	Sec6 protein	Zinc finger and BTB domain containing 22
Immunoglobulin J chain	Semenogelin II precursor	Zinc finger protein 100
Inter-alpha (globulin) inhibitor H4	Serine proteinase inhibitor, clade A, member 1	
Keratin 12	Serpin peptidase inhibitor, clade G, member 1 precursor	
Keratin 16	Serum amyloid A4, constitutive	

evaluation remain scarce and those that have been published describe proteins that though perhaps tumor specific, continue to show little specificity for PCa or require invasive or surgical procedures (Welsh *et al*., 2003, Lin *et al*., 2013).

The goal of this work was to further evaluate clinical samples of PCa taken from patients representing AA, Caucasian and Hispanic ethnicities for potential biomarkers of tumor definition and aggressiveness. Collecting blood samples or a relatively non-invasive "liquid biopsy" could provide clinicians with a superior diagnostic and therapeutic effectiveness-defining tool. Analyzing the secretome, or the secreted biomolecules, from the developing tumor could more rapidly pinpoint the type of cancer and best practice for treatment. Here we analyzed microvesicles called exosomes which are ubiquitously released into the serum/plasma during tumorigenesis and have been shown to be specifically enhanced in the serum of PCa patients compared to their representative controls (Khan *et al*., 2012). Furthermore, recent studies have demonstrated the importance of exosomal proteins in cancer cell function with a particular interest paid to their role in tumorigenesis (Khan S *et al*., 2009, Park *et al*., 2012, Demory Beckler *et al*., 2013, Ji *et al*., 2013). Our own recent work has defined the role of the inhibitor of apoptosis protein Survivin, released in tumor exosomes, in cellular proliferation, invasiveness and resistance to apoptosis (Khan S *et al*., 2009, Khan S *et al*., 2011). By comparing the secretome of PCa patients representing various ethnic groups, we may also gain key insights into tumor-associated molecular determinants of PCa health disparities.

The molecular content of exosomes represents the molecular profile of the cell type that released it (Properzi *et al*., 2013). Though a number of studies now describe the association of exosomal markers with several cancer types, only a few exosome based diagnostic assays are currently available for clinical use (Properzi *et al*., 2013). The present study was designed to dissect the PCa exosome from patients of varying ethnicities and determine by mass spectrometry if there are ethnic differences in exosome protein profiles. Though we found a large number of novel proteins that appeared to be ethnicity-specific, additional studies with larger population cohorts are needed before one could unequivocally state that these proteins will help define molecular determinants of PCa health disparities. There may be other factors influencing the shedding of these exosomes and their content such as age, diet, and environmental exposures that may result from occupation or residence. The etiology of disease, access to healthcare, or the compliance with interventional recommendations may be as important, or more important, than the secretome that results from the above etiology. However, associating molecular content with disease content may prove effective in early detection, resulting in the saving of thousands of lives.

In this study, we were able to acquire a random sample of patients from AA, Caucasian and Hispanic ethnicities and through molecular and cellular processes extract a secretome from the microvesicles called exosomes. Using mass spectrometry we have identified approximately 50 newly identified exosomal proteins that are added to the exosome and tumor exosome databases. We are now beginning the arduous process of evaluating one, or combinations of these, as putative PCa biomarkers. The list of identified exosomal proteins includes three proteins with previous PCa association: Iroquois Homeobox Protein 5 (IRX5), Mitochondrial Tumor Suppressor 1 Isoform 4 (MTS1), and Trinucleotide repeat containing 6B Isoform 3 (TNR6B) (Table 6).

Interestingly, the expression of IRX4 protein, a similar protein to IRX5, has been shown in normal prostate tissue and its knockdown is associated with enhanced growth (Nguyen *et al*., 2012). However, its interaction with vitamin D receptor (VDR) is speculated to be necessary for its anticancer function since in the absence of the VDR, PCa susceptibility increases (Nguyen *et al*., 2012). MTS1, which is also known as a microtubule-associated tumor suppressor, inhibits PCa cell proliferation, delays the progression of mitosis by prolonging metaphase and reducing tumor growth (Ye *et al*., 2007). It too requires a number of interacting proteins for its function and is not solely associated with PCa as it has also been shown to play a role in breast, brain, ovarian, pancreas, colon, and head & neck cancers (Ye *et al*., 2007). Of the three putative PCa-associated exosomal proteins, TNR6B appears to hold the most promise. This protein has known interactions with novel loci, single nucleotide polymorphisms (SNPs), on TET2 and SYK with interactions modifying the risk for PCa (Tao *et al*., 2012). However, as in the case of MTS1 and IRX5, additional studies are needed before these putative biomarkers may be used to define risk, presence or even therapeutic response in PCa.

An important limitation of this study lies in the small number of patients that were evaluated from each ethnicity. In spite of this, we were able to find differences in exosomal protein contents between PCa patients and non-Ca controls, and between PCa patients from the different ethnic groups. These results provide initial evidence that exosomal profiling may not only yield new PCa biomarkers but also putative molecular determinants of PCa health disparities. Additional studies with larger cohorts of ethnically diverse patients and matched controls are guaranteed to identify unequivocally exosome derived PCa biomarkers and health disparity determinants. Another limitation was that potentially important proteins were either too small to identify or masked by other higher content proteins. In addition, it may also be problematic that proteins may be present in both normal individuals and prostate cancer patients, and it is their expression level that defines them as pathologic. This will always be the case with these types of proteomic analyses. Also, patients were selected prior to undergoing any form of therapy which would also be informative as the therapeutic stress causes different proteins and pathways to be affected, potentially resulting in therapy-associated changes in exosomal protein content. It should be noted, however, that to date, it has not been established if treatment causes a radical change in exosome numbers or exosome macromolecule content. Clearly, the clinical relevance of these initial observations remain to be established, and it is too early to predict whether the potential exists to adopt the three newly identified exosomal proteins in platforms for screening patients for the presence of PCa. Studies with larger cohorts are currently underway in our laboratories to evaluate whether these new findings will lead to the identification of molecular determinants of PCa health disparities and ultimately to their reduction or elimination.

Acknowledgements

The author's would like to thank the Center for Health Disparities & Molecular Medicine for supporting this project and the graduate students that it involved. We would also like to thank the Loma Linda Universities Cancer Center & San Manuel Band of Mission Indians Biospecimen Laboratory for providing valued samples for this study. We would also like to thank members of the Wall Laboratory as well as Shahrzad Khosrowpour from Chapman University for careful review of our manuscript.

Declaration of Interest

The research reported in this publication was supported by NIH awards P20MD006988 and 2R25 GM060507 (CAC & NRW). Funding was also made possible through a National Merit Test Bed (NMTB) award sponsored by the Department of the Army under Cooperative Agreement Number DAMD17-97-2-7016 (NRW). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Conflict of Interest

All authors declare no conflict of interest.

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CHAPTER 4

CONCLUSION

Prostate cancer (PCa) is the most frequently diagnosed non-skin cancer in men and the second leading cause of male cancer deaths in the U.S., accounting for 240,890 new cases and 33,730 deaths in 2011(Brawley, 2012a). The incidence of the disease has an uneven geographic distribution with the highest rates recorded in Australia, North and Western Europe and The United States(GLOBOCAN). The incidence and mortality of the disease is almost doubled among blacks compared to other ethnicities in the United States(Parkin *et al*., 2005). An interesting note is that the disease incidence is lowest among African Blacks although some have attributed that to the lack of availability of proper statistics from this region of the world(Ferlay *et al*., 2010).

A plethora of risk factors for Prostate cancer have been questioned in the literature including chronic inflammation of the prostate (infectious and non-infectious), hormonal factors (elevated testosterone, leptin etc), smoking, dietary and genetic factors; just to name a few. Smith et al described the link between the susceptibility gene HPC-1 (Hereditary Prostate Cancer) and an increased risk of developing prostate cancer, especially in younger patients(Smith *et al*., 1996). Overall, prostate cancer is believed to be a disease of multifactorial origin.

Prostate Specific Antigen (PSA) combined with a digital rectal exam has remained the mainstay of screening for prostate cancer for the past two to three decades. PSA was first described as a marker for human semen in forensics but it was subsequently demonstrated in the serum of men with prostate disease(Stamey *et al*., 1987). The test was however intended for surveillance of diagnosed prostate cancer patients. There was a sharp rise in newly diagnosed cases in the early 1990's attributed to the introduction of PSA as a screening test for prostate cancer. Coincident with this increase in the number of new cases diagnosed was a pathologic migration toward a more favorable (early disease) stage at diagnosis(Derweesh *et al*., 2004). Non-palpable prostate cancer (and therefore perhaps clinically treatable disease) now accounts for 70-80% of newly diagnosed cases according to the same authors. This may at least in part explain why the combined 5 and 10 year survival for all stages of prostate cancer is currently at 99% and 91% respectively. The true clinical value/advantage of using serum PSA as a screening test has been recently called into question. While the use of this test has increased the proportion of patients with lower tumor stage at the time of diagnosis, its value is still debated because of its limitations: 1) the cut-off PSA value of 4.0 ng/ml fails to detect a significant number of prostate tumors; 2) PSA screening has not been demonstrated to improve health outcomes, particularly in older men; 3) PSA is not specific for PCa since its levels can be elevated in patients with benign prostatic hyperplasia (BPH) and prostatitis, leading to a false-positive finding of up to 60-80% of prostate biopsies; and 4) obesity lowers PSA levels(Werny *et al*., 2007), which in theory could lead to delayed detection of PCa and worse clinical outcome. Moreover, the lack of specificity of PSA as a screening tool has also led to too many and sometimes unnecessary diagnostic (biopsies) and therapeutic procedures. The conclusions from the European Trial of Prostate-Cancer screening was that 1410 screenings/biopsies had to be performed and 48 additional cases treated in order to prevent one death from prostate cancer(Schroder *et al*., 2009). It is the realization of this need that has led us to devote effort to the identification of novel biomarkers that, will enhance early PaCa detection, management and therapeutic response. We recognized that we are not alone in this quest but our motivation comes even more so from the fact that efforts in this direction are at the best promising but inconclusive.

The "holy grail" of PCa diagnosis and management is to determine an optimal combination of clinical indicators or biomarkers that could detect tumors early with high specificity/sensitivity and with limited invasiveness, and that could accurately predict which diagnosed men will develop aggressive tumors requiring treatment, and which treated men are likely to undergo recurrence and develop advanced, chemoresistant disease. In spite of the availability of a plethora of gene products considered as promising PCa biomarkers, it is recognized that their combined use with the available clinical information is still insufficient for early diagnosis and for guiding individualized therapeutic interventions and predicting outcomes. Their main limitation is that they lack specificity and some may require invasive procedures such as biopsies. However, there is growing interest in using proteomics approaches to identify tumor-derived serum microvesicles called exosomes and their content, as serological biomarkers. This interest stems from the notion that these blood components are considered "sensors" of molecular events associated with tumorigenesis. The proteomic and ribonucleic make-up of tumor derived exosomes, offers us a snapshot into the 'internal milieu' of the tumor microenvironment(Yang & Robbins, 2011).

The very first step toward this journey of novel Biomarker discovery for prostate cancer commences with a descriptive analysis of the protein make-up of exosomes derived from the sera of patients with prostate cancer. By comparing this to the proteomic profile of exosomes from non-cancer subjects, one hopes to begin to define potential biomarker targets. Moreover, comparing proteomic profiles across different ethnicities may just unmask molecular mechanisms involved in conferring a more aggressive disease phenotype among blacks of African descent.

This quest for a novel biomarker has to begin with a complete and exhaustive seroproteomic profiling of the entire proteome of serum derived exosomes of PCa patients. A secondary but equally important objective is to identify molecular marker(s) that might begin to explain the higher incidence and relatively higher disease severity and mortality in the black population.

The proposed projects associated with this dissertation focused on a critical need in the fight against PCa: the identification of novel and promising blood biomarkers that could enhance the early detection and management of PCa and the identification of molecular markers that may begin to explain the ethnic disparity of the disease.

Most efforts on the identification of candidate PCa biomarkers, have emphasized the analysis of differential gene expression in tumor tissues, methylation patterns, or single nucleotide polymorphisms (SNPs) (Saif *et al*., 2005, Gonda *et al*., 2011, Gonda & Saif, 2011, Smith *et al*., 2012). While these efforts are necessary and provide important clues for understanding biological mechanisms associated with PCa, it is also imperative to develop innovative, non-invasive approaches that analyze indirectly and early in the disease process, the molecular profile of prostate tumors. Recent studies have shown that small membrane-bound vesicles called exosomes constitute the latest mode of intercellular information transfer or communication (Kharaziha *et al*., 2012, Vlassov *et al*., 2012). This exchange of molecular information is facilitated by their unique composition, which is enriched with enzymes, structural proteins, adhesion molecules, lipid rafts, microRNAs (miRNAs) and RNAs. An international database (exocarta.org) of exosome biomolecules has recorded well over 4,000 proteins and over 2,000 RNA's. Importantly, cancer cells have been shown to secrete more exosomes than do normal tissues indicating that exosomes can be used as diagnostic markers and their active secretion has functional implications. Not only do cancer cells release more exosomes than normal cells but their biomolecular make-up reflects their cell/tissue of origin(Ge *et al*., 2012).

The studies that have been undertaken in this process have described for the first time the entire proteomic make-up of exosomes from PCa patients using exo-profiling and seroproteomics approaches, currently considered the most promising strategy for the identification of serum biomarkers in human cancers (Tjalsma *et al*., 2008, Tan *et al*., 2009, Kobold, Luetkens*, et al*., 2010, Kobold, Lutkens*, et al*., 2010). We are pleased with the works success but in no way believe we have done any more than just begun to understand a better method for early detection of cancer in general and prostate cancer in specifics.

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Dr. David Turay's BIOGRAPHICAL SKETCH

Position Title:
Assistant Professor of Surgery, Chief – Division of Acute Care Surgery, Dept of Surgery

Education and Training:

Institution	Year	Field of Study	Degree
Universidad de Montemorelos	1999	Medicine	MD
Loma Linda University	2005-2006	Internship, Gen. Surgery	
Loma Linda University	2006-2010	Resident, Gen. Surgery	
Massachusetts General Hospital Harvard Medical School	2013-2014	Fellowship, Critical Care Surg	
Loma Linda University	2010-2016	Anatomy	PhD

A. Personal Statement
My desire to be grounded in the Basic Sciences and my realization of the increasing role of Molecular Medicine in the treatment of multiple conditions, led me back to the lab. My mentor and friend Dr. Nathan Wall was kind enough to open the doors of his Laboratory to me and in so doing, opened my eyes to a whole new reality of Cancer Biology and biomarker molecular biology. I initially wondered how this will fit into my current clinical interest in caring for the injured and those with critical illness. It was at Dr. Wall's Lab that I came to increasingly see some similarities between cancer; a final pathway to chronic disordered inflammation and the inflammatory process that inevitably follows injury. In this research project, we set out to discover novel extracellular (exosomal) biomarkers and their role in resistance of cancers to stress-induced cell death in the tumor microenvironment. I intend to eventually apply the knowledge gained in this endeavor to examine serum exosomes of head injury patients for biomarkers of therapeutic and prognostic significance. I am uniquely prepared and delighted to work with Dr. Wall and this group on the translational phase of his important research.

B. Positions/Appointments

07/2015 – Present	Division Chief, Acute Care Surgery Medical Director of Trauma Services	Loma Linda Univ. Medical Center
07/2014 – Present	Associate Director, Surgical Intensive Care Unit	Loma Linda Univ. Medical Center
07/2010 –06/2013	Attending Surgeon & Assistant Professor of Surgery	Loma Linda Univ. Medical Center

C. Prior Positions

07/1998 –06/1999	Community Health Officer (Required for MD degree)	El Camaron Municipality

07/1989 –08/1991	Math and Science Teacher	Oaxaca, Mexico Masanga Primary School Masanga, Sierra Leone

D. Honors and Awards

2013	Clinical Teacher of the year *Loma Linda University*
2010	Teaching Resident of the year *Loma Linda University*
2010	Alpha Omega Alpha *Loma Linda University*
2009	Vascular Surgery Senior Resident of the year *Loma Linda University*
1994	Best Student in the Basic Sciences *Universidad de Montemorelos*
1987	Student of the year in Chemistry and Mathematics Award *Christ the King College, Sierra Leone*

E. Memberships in professional Organizations

2013	Society of Critical Care Medicine
2013	Massachusetts Medical Society
2012	Society of American Gastrointestinal and Endoscopic Surgeons
2010	Alpha Omega Alpha Honor Medical Society
2006	American College of Surgeons (Current Fellow)
2005	American Association of Anatomists

F. Boards & Certifications

09/2014	Surgical Critical Care (Subspecialty of the American Board of Surgery)
02/2011	American Board of General Surgery
07/2014	Advance Trauma Life Support Instructor
06/2011	Fluoroscopy and X-Ray Supervisor and Operator
05/2010	Fundamentals of Laparoscopic Surgery
02/2009	Advanced Cardiac Life Support – Experienced Provider
09/2006	Advance Trauma Life Support
03/2000	Educational Commission for Foreign Medical Graduates

G. Current Professional State Licenses

06/2007	Physician & Surgeon’s License, Medical Board of California
07/2013	Physicians License, Commonwealth of Massachusetts Board of Medicine

H. Selected recent and relevant peer-reviewed publications

Turay D, Khan S, Osterman CD, Curtis MP, Khaira B, Neidigh JW, Mirshahidi S, Casiano CA, Wall NR. Proteomic profiling of serum-derived exosomes from

ethnically diverse prostate cancer patients. Accepted for Publication, Cancer Investigation, 2015.

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