Published in final edited form as:

Cancer Invest. 2016 January 2; 34(1): 1–11. doi:10.3109/07357907.2015.1081921.

Proteomic Profiling of Serum-Derived Exosomes from Ethnically Diverse Prostate Cancer Patients

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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.

Keywords

Exosome; Prostate cancer; Proteome; Biomarker; Health disparity; Early detection

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DECLARATION OF INTEREST

The authors report no conflicts of interest.

SUPPLEMENTARY MATERIAL ONLINE

Supplemental Table I

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 (1). 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 (2). The incidence and mortality of the disease is disproportionately high among African American (AA) men compared to other ethnicities in the United States (3). 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 (4).

While the basis for PCa health disparities is not well understood, evidence points to the interplay between socioeconomic, environmental, and biologic/genetic factors (3). A number of risk factors for PCa have been examined in the literature including chronic inflammation of the prostate (infectious and noninfectious), 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 (5). 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 (6). 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 (7, 8). Nonpalpable PCa (and therefore perhaps clinically treatable disease) now accounts for 70–80% of newly diagnosed cases (9, 10). 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 (11). 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 (12). 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 (13). 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 (14). Exosomes play an important part in this communication due to their ability to transport cancer-promoting material such as protein, RNA and miRNA (14–16). 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 (17–19). 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 (20).

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 (21). 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 (22–25). Our previous demonstration of exosomal Survivin, an inhibitor of apoptosis and indicator of cancer severity, in the plasma of patients with newly diagnosed PCa (26) 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 1). 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 $2000 \times g$ 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.

Exosome isolation

For plasma microvesicle samples, the commercially available ExoQuick (SBI, Mountain View, CA, USA) was employed as described by the vendor. Briefly, 100 μ L of plasma was incubated with 100 μ L of ExoQuick solution followed by a 2 hr incubation at 4°C followed by centrifugation at 1500 $\times$ 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 μ L of dH₂O (27).

Exosome quantification

To quantify the amount of exosomes released, we assessed the activity of acetylcholinesterase, an enzyme that is associated with these vesicles (28). Acetylcholinesterase activity was assessed as described by Savina et al. (28). Briefly, 40 μ L of the exosome fraction was suspended in 110 μ L of PBS. 37.5 μ L 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 μ L, 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 NaCl₂, 1 mM PMSF, 10 μ g/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, USA) was used. Proteins from exosomes (20–40 μ g) 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, USA). 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, USA), added at a final concentration of 20 ng/ μ L to fully cover the gel pieces. Digestion was performed at 37°C overnight. Peptides were recovered with 30 μ L, 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, USA) 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 μ m, 300 Å). Samples were loaded onto a Michrom

Bioresources (Auburn, CA, USA) cap-trap at 5 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 (29), Mascot (30), and X! TANDEM (31), were used to identify peptides and proteins. The significance of identified peptides and proteins were determined using the Peptide-Prophet (32) and Protein-Prophet (33), respectively, algorithms as implemented in Scaffold 2 (Proteome Software, Portland, OR, USA). 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 (34). These small, membrane-vesicles are also known to occur in the plasma as well as serum of cancer patients (26, 35, 36). Exosomes were therefore collected using ExoQuick, and quantitated using the acetylcholinesterase enzymatic assay as we and others have previously described (14, 16, 26). 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 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 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 (37). Recent efforts in PCa biomarker discovery and 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 (38, 39).

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 (26). Furthermore, recent studies have demonstrated the importance of exosomal proteins in cancer cell function with a particular interest paid to their role in tumorigenesis (34, 40–42). 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 (16, 34). 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 (20). 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 (20). 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 (43). 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 (43). 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 (44). 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 (44). Of the three putative PCa-associated exosomal proteins, TNFR6B 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 (45). 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

The authors would like to thank the Center for Health Disparities & Molecular Medicine for supporting this project and the graduate students that it involved. They 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. They would also like to thank members of the Wall Laboratory as well as Shahrzad Khosrowpour from Chapman University for careful review of our manuscript.

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.

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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*.

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.

Table 1

Demographic data of PCa patients

	No Diagnosis (<i>n</i> = 9)	Caucasian (<i>n</i> = 4)	African American (<i>n</i> = 4)	Hispanic (<i>n</i> = 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

Table 2

Ten most abundant proteins discovered

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

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

African American/Caucasian	African American/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

African American (22)	Caucasian (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 Maintenance 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