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Identification, prioritization and evaluation of glycoproteins for aggressive prostate cancer using quantitative glycoproteomics and antibody-based assays on tissue specimens

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Abstract

Prostate cancer is highly heterogeneous in nature; while the majority of cases are clinically insignificant, some cases are lethal. Currently, there are no reliable screening methods for aggressive prostate cancer. Since most established serum and urine biomarkers are glycoproteins secreted or leaked from the diseased tissue, the current study seeks to identify glycoprotein markers specific to aggressive prostate cancer using tissue specimens. With LC-MS/MS glycoproteomic analysis, we identified 350 glycopeptides with 17 being altered in aggressive prostate cancer. ELISA assays were developed/purchased to evaluate 4 candidates, i.e. cartilage oligomeric matrix protein (COMP), periostin, membrane primary amine oxidase (VAP-1) and cathepsin L, in independent tissue sets. In agreement with the proteomic analysis, we found that COMP and periostin expressions were significantly increased in aggressive prostate tumors while VAP-1 expression was significantly decreased in aggressive tumor. In addition, the expression of these proteins in prostate metastases also follows the same pattern observed in the proteomic analysis. This study provides a workflow for biomarker discovery, prioritization and evaluation of aggressive prostate cancer markers using tissue specimens. Our data suggest increase in COMP and periostin and decrease in VAP-1 expression in the prostate may be associated with aggressive prostate cancer.

Keywords

aggressive; prostate cancer; biomarker; glycoproteomics; OCT

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Introduction

Prostate cancer is the most common cancer and the second leading cause of cancer death in men in the United States. While the incidence of prostate cancer remains high, most of the cases diagnosed today represent slow growing, clinically insignificant forms of the disease with some cases being the aggressive, metastasizing and lethal form. Currently, Gleason grading system is the predominant grading system used to evaluate prostate carcinoma. Based on glandular architecture, the carcinoma is graded from 1 to 5 and the Gleason score is the addition of the Gleason grade of the most prevalent and the second most prevalent pattern [1]. Serum prostate specific antigen (PSA) blood testing is a standard screening assay for prostate cancer in use in some populations. Unfortunately, PSA testing does not distinguish between aggressive and indolent prostate cancer. Therefore, to initiate early treatment for clinically significant (aggressive) prostate cancer patients and to alleviate unnecessary pain of patients with indolent (non-aggressive) prostate cancer, the current study seeks to identify glycoprotein markers specific to aggressive prostate cancer.

Serum and urine biomarkers which have been proven of great value in disease diagnosis are generally extracellular glycoproteins expressed in disease tissues that are secreted or shed into bodily fluids [2, 3]. Therefore, to identify potential serum/urine biomarker, we carried out mass spectrometry analysis on glycoproteins in prostate specimens, using a solid phase extraction of N-linked glycopeptide (SPEG) method. The SPEG method, with effective isolation of peptides with N-linked glycosylation sites, results in great reduction in sample complexity and therefore increased detection sensitivity [4]. In addition, this method, by removing optimal cutting temperature (OCT) medium, enables proteomic analysis of frozen tissues embedded in OCT [5]. The ability to use OCT-embedded frozen tissue is a great advantage, as not only are the proteins well preserved, but also serial sectioning, histologic characterization and selection can be performed for internal validation of sample quality.

In this study, we present a workflow for biomarker discovery, prioritization and evaluation using glycoproteomic analysis on OCT-embedded frozen tissue specimens. This study is an extension of our previous work on quantitative glycoproteomic analysis of OCT-embedded tissue [5]. While our previous work provide an initial discovery of potential prostate biomarkers with only 102 glycopeptide identified, in the current study, with label-free LC-MS/MS analysis, we identified a total of 350 unique N-linked glycopeptides with 17 associated with aggressive prostate cancer. In addition, for better candidate identification, both aggressive and non-aggressive tumors as well as the normal tissues adjacent to the tumors and normal prostate tissues from healthy subjects were utilized for biomarker discovery. The candidates were further prioritized based on their association with aggressive prostate cancer and literature reports. Finally, we performed verification work on additional sample sets to better account for biological variations in the population.

Materials and Methods

Materials

Hydrazide resin and sodium periodate were from Bio-Rad (Hercules, CA); sequencing-grade trypsin and TMB reagent were from Promega (Madison, WI); PNGase F was from New

England Biolabs (Ipswich, MA); C18 columns were from Waters (Milford, MA); Recombinant protein, capture and detection antibody of human cartilage oligomeric matrix protein (COMP), periostin, cathepsin L, clusterin and galectin-3 binding protein, streptavidin-HRP conjugates, ELISA plates and human VAP-1 Quantikine ELISA Kit were from R&D systems (Minneapolis, MN); All other chemicals were from Sigma-Aldrich (St. Louis, MO).

Clinical Specimens

Samples and clinical information were obtained with informed consent and performed with the approval of the Institutional Review Board of the Johns Hopkins University. The non-aggressive and aggressive primary prostate tumors were collected from radical prostatectomy or transurethral resection of prostate at Johns Hopkins Hospital and Johns Hopkins Bayview Medical Center under the NCI-funded Johns Hopkins prostate cancer SPORE project. The non-aggressive prostate cancer group includes 31 specimens. Twenty-seven specimens are from tumors with Gleason score 6; four specimens are from tumors with Gleason score 7 with no evidence of recurrence up to 15 years of follow-up. The aggressive prostate cancer group includes 24 specimens. Fifteen specimens are from tumors with Gleason score 8 or 9; five specimens are from tumors with Gleason score 7 where the patients died of cancer metastasis within 6 years of surgery (Suppl. Table 1). Normal prostate tissues were from healthy transplant donors; prostate metastases were from men who died of prostate cancer and underwent autopsy at the Johns Hopkins Medical Institutions. All specimens were snap-frozen, embedded in OCT and stored at -80 °C till use.

Glycopeptide Isolation from Prostate Specimens

OCT-embedded prostate frozen tissues were sectioned and stained with hematoxylin and eosin to facilitate cryostat micro-dissection for enrichment of the tumor tissue or the adjacent normal tissue. Six μm -thick tissue sections for each specimen were collected in sterile screw-cap bullet tubes. Proteins were extracted using cell lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% Na Deoxycholate, 1% Triton X-100). BCA assay was performed to determine the protein concentration. For each tissue specimen, 100 μg of proteins were used to extract formerly N-linked glycopeptides using SPEG as described in our previous publication [5].

LC-MS/MS Analysis

Formerly N-linked glycopeptides were analyzed using a linear ion trap mass spectrometer (LTQ, Thermo Fisher, Waltham, MA) after separation with a 15 cm $\times$ 75 μm C18 column (5 μm particles with 100 Å pore size). A nanoAquity UPLC at 300nL/min with a 100-min linear ACN gradient (from 5 to 32% B over 90min; A=0.1% formic acid in water, B=0.1% formic acid in ACN) was used. Top 8 data-dependent MS/MS spectra with exclusion for 20s and a repeat count of 2 were set. CID in the ion trap was used with a collision energy setting of 35%. The voltage was set at 2.0kV. Each sample was subjected to LC-MS/MS analysis once.

Identification of Protein and Glycosylation Sites and Glycopeptides Quantification

Raw MS data from LTQ were converted into mzXML files by a MassWolf file converter 20 [6]. MS/MS spectra were searched with X!Tandem (released on 01/01/2010) against a human International Protein Index (IPI, version 2.28) database containing 40110 entries. For the database search, the peptide mass tolerance was set at 3.0Da, MS/MS tolerance was 0.5Da, and flexible parameters were set as follows: cysteine modification (add cysteine with 57Da), methionine oxidization (add Met with 16Da), and a (PNGase F-catalyzed) conversion of Asn to Asp (add Asn with 1Da). One missed tryptic end and a maximum of two missed cleavage sites were permitted. The assigned peptides were evaluated by Peptide Prophet [7] and only peptides with N-glycosites (NXS/T, where X can be any amino acid except P) and minimum probability score of 0.8 (with error rate less than 0.027) were reported in this study [8, 9]. Spectral counting was used to analyze the LC-MS/MS data obtained from LTQ to determine the protein relative abundance in prostate specimens as described before [10]. Due to random sampling of mass spectrometer in collecting MS/MS spectra used for spectral count, we only quantified proteins with at least 5 spectral counts in total from tissue specimens compared. The statistical analysis of spectral counting data was performed using our previously described procedure [11].

Pubmed Based Literature Search

Through the Pubmed database, 5 searches with different search terms were performed for each candidate protein. Term searched included: 1) recommended, alternative and short name of each candidate; 2) search term 1 and cancer; 3) search term 1 and prostate cancer; 4) search term 1, aggressive or aggressiveness, and cancer; 5) search term 1, aggressive or aggressiveness, and prostate cancer. The numbers of publications generated by these searches were recorded for each protein candidate and the respective publications were screened.

ELISA Development and analysis

Sandwich ELISA assays were developed for cartilage oligomeric matrix protein (COMP), periostin and cathepsin L. Briefly, one hundred microliter of 1 µg/mL COMP, periostin or cathepsin L capture antibody diluted in PBS (PH 7.4) were coated in 96-well plates at room temperature overnight, followed by blocking with 1% BSA. The wells were then incubated with 100 µL sample diluted in PBS+1% BSA for 1 h followed by incubation with 100 µL COMP (0.2 µg/mL), periostin (2 µg/mL) or cathepsin L (0.5 µg/mL) biotinylated detection antibody for 1 h, and with streptavidin-HRP conjugates (1:200) for 30 min. The assays were developed with 100 µL TMB substrate for 5-20 min and the reactions were stopped by adding 50 µL H₂SO₄. The measurements were acquired by reading the plate at 450 nm with a spectrophotometer. COMP, periostin, VAP-1 and cathepsin L recombinant proteins were used as calibrators. For each assay, an 8 point calibration curve was generated with serial dilution of the recombinant protein. Sandwich ELISA for membrane primary amine oxidase (VAP-1) was carried out per manufacture's instruction.

For ELISA analysis of tissue specimens, proteins from OCT-embedded prostate frozen tissues were extracted using cell lysis buffer as described before. The order of tissue specimens to be tested was randomized for ELISA analysis. BCA assay was performed to

determine the protein concentration. Total protein in the samples were diluted to 40 µg/mL for COMP, 2.5 µg/mL for periostin and 20 µg/mL for cathepsin L using PBS+1% BSA. Total protein concentration was used to normalize ELISA data. For each detection, 100 µL sample was used. For each sample, duplicate measurements were performed.

Statistical Analysis

Calibration curves for protein quantification were established using a 4-parameter nonlinear regression in Gen5 1.04.5 data analysis software. Student's t-test was used for determination of statistical significance of spectral count results. ANOVA followed by Fisher's least significant difference test was used for determination of statistical significance of ELISA results.

Results

Quantitative Proteomic Analysis of OCT-embedded Prostate Tumor Specimens

To identify the glycoproteins in prostate tumor, we analyzed the formerly N-linked glycopeptides from non-aggressive and aggressive prostate tumors by LC-MS/MS using LTQ. With a minimum probability of 0.8, we identified 350 unique N-linked glycopeptides containing consensus N-linked glycosylation motif (NXS/T, where X is any amino acid except P) representing 242 unique glycoproteins (Suppl. Table 2). We then used the number of spectra assigned to all glycosites of the same glycoprotein to determine the relative abundance of each glycoprotein between aggressive and non-aggressive tumor, between aggressive tumor and normal prostate tissue, between aggressive tumor and the normal tissue adjacent to non-aggressive tumor, and between aggressive tumor and the normal tissue adjacent to aggressive tumor. Since the glycopeptide isolation employed in this study is based on hydrazide chemistry, where lots of aldehyde groups from the oxidation of glycans from each glycosite was available for bonding with hydrazide, the structure of glycans usually does not affect the capture of glycopeptides. Only in extreme cases, where glycan structures are totally missing from a glycosite (glycol-occupancy), will glycopeptide abundance be affected. The spectral count results, therefore, mostly represent glycoprotein changes and may also reflect the glyco-occupancy changes of glycopeptides.

As the goal of this study is to identify proteins specifically altered in aggressive tumor, we were interested in proteins that are commonly up-regulated or down-regulated in aggressive tumor as compared to both non-aggressive tumor and normal prostate tissue. Table 1 shows the total spectral count of glycoproteins from all 4 specimens of each group and the ratio of glycoproteins between aggressive tumors and non-aggressive tumors as well as the 3 groups of normal tissues, where there is at least a 50% increase or decrease in aggressive tumors compared to both non-aggressive tumors and the normal prostate tissues. The ratios of the proteins identified only in aggressive tumors but not in non-aggressive tumors or normal tissues were arbitrary assigned to 100. P values were calculated between aggressive tumors and non-aggressive tumors. With these criteria, we identified 11 glycoproteins that were up-regulated and 6 glycoproteins that were down-regulated in aggressive prostate cancer, 5 of which were statistically significantly different between aggressive and non-aggressive tumors.

Candidate Prioritization

To determine which candidate glycoproteins are likely to serve as novel biomarkers for aggressive prostate cancer and therefore of more importance, literature analysis was carried out to further prioritize the candidates associated with aggressive prostate cancer.

As described in “materials and methods” section, 5 searches were carried out to determine the number of publications on the candidate protein, on the candidate protein related to cancer, on the candidate protein related to prostate cancer, on the candidate protein related to aggressive cancer and on the candidate protein related to aggressive prostate cancer respectively. While the number of publications on a certain candidate predicts the ease of verification, e.g. availability of antibodies, the number of publications of a candidate on prostate cancer and cancer aggressiveness was used to evaluate the potential of the candidate protein as an aggressive prostate cancer marker. Meanwhile, whether the candidate has been reported before as an aggressive prostate cancer marker is also critical as the novelty of candidates is an important factor in prioritization.

Out of the 17 candidate markers, we found all have publications on their function and their role in cancer; 12 have been associated with prostate cancer; 11 have been associated with cancer aggressiveness; 8 have been associated with prostate cancer aggressiveness (Table 2). Subsequently, we screened literatures reporting association of these proteins with prostate cancer, cancer aggressiveness and aggressive prostate cancer. As indicated in table 2, all 11 proteins were reported to be associated with both prostate cancer and cancer aggressiveness with the associations at either mRNA or protein level. On the other hand, although 8 candidates were found to have publication on prostate cancer aggressiveness, most articles were from mechanistic studies based on in vitro experiments on prostate cancer cell lines, suggesting the novelty of these proteins to be verified at the tissue level with clinical specimens. The only two proteins that have been studied before at a tissue level as potential markers of aggressive prostate cancer were periostin and cathepsin L, both identified with our previous study on aggressive prostate cancer with limited tissue specimens [5]. We therefore decided to also include these proteins in our verification with ELISA.

Verification with ELISA Assays

To evaluate the results from the proteomic data, we tried developing ELISA assays for the 11 proteins that have been associated before with prostate cancer or cancer aggressiveness but not/barely with aggressive prostate cancer. We successfully developed ELISA assays for 3 candidates, i.e. COMP, periostin and cathepsin L and purchased ELISA kit for VAP-1. The analytical performances of the developed ELISA assays were determined using human recombinant proteins. The limit of detection (LOD) calculated as background OD $\pm$ 3SD was 7, 4 and 27 pg/mL for COMP, periostin and cathepsin L respectively; the dose responsive range was from 0 to 5 ng/mL for COMP and periostin and from 0 to 2 ng/mL for cathepsin L (Fig. 1). At the lowest standard point, the coefficient of variation (CV) was 3.5%, 0.80% and 6.3% for COMP, periostin and cathepsin L respectively. The intra-assay CVs were 5.9%, 16% and 9.9% for COMP, periostin and cathepsin L respectively. Human VAP-1 ELISA kit has a detection limit of 24 pg/mL and a dose responsive range from 0 to 50 ng/mL.

Subsequently, the protein levels of COMP, periostin, cathepsin L and VAP-1 were measured in prostate specimens including normal prostate tissues from 12 subjects, non-aggressive prostate tumors from 27 subjects, aggressive prostate tumors from 20 subjects and 10 prostate metastases from 7 subjects. In agreement with the proteomic analysis, we found a significant increase in the expression of COMP and periostin in aggressive prostate tumor compared to both non-aggressive tumor and normal prostate tissue; average COMP expression was elevated in aggressive tumor by ~ 7.3 fold compared to normal prostate tissue and by ~ 2 fold compared to non-aggressive tumor; average periostin expression was elevated in aggressive tumor by ~ 254 fold compared to normal prostate tissue and by ~ 2.6 fold compared to non-aggressive tumor. Compared to normal prostate tissue, COMP and periostin expression were also higher in non-aggressive tumor although the differences were not statistically significant. In addition, similar to that shown in proteomic analysis, we found that periostin expression was also significantly elevated in prostate metastases (by ~ 186 fold compared to normal tissue). Also in agreement with the proteomic analysis, we found a significant decrease of VAP-1 in aggressive prostate tumor compared to both non-aggressive prostate tumor and normal prostate tissue; average VAP-1 expression in aggressive tumor was decreased by ~ 2.5 fold compared to normal prostate tissue and by ~ 1.4 fold compared to non-aggressive tumor. Compared to normal prostate tissue, VAP-1 expression was also significantly decreased in non-aggressive tumor (by ~ 1.7 fold). The expression of VAP-1 measured was the lowest in prostate metastases which was significantly decreased from aggressive tumor by ~ 6.5 fold. Cathepsin L expression, on the other hand, was not distinctly different between aggressive and non-aggressive prostate tumor. These data show that COMP, periostin and VAP-1 are differentially regulated in aggressive prostate tumor and may be used distinguish patients with aggressive cancer.

Discussion

In this study, glycoproteomic analysis using SPEG method was employed for the identification of protein changes associated with aggressive prostate cancer. The method has been shown before to be compatible with OCT-embedded frozen materials [5] which not only preserves protein integrity, but also allow sectioning for tumor characterization and enrichment. To better identify candidate protein markers associated with aggressiveness, both aggressive and non-aggressive tumors as well as the normal tissues adjacent to the tumors and normal prostate tissues from healthy transplant donors were utilized for biomarker discovery. For more focus verification of candidate markers, candidates were prioritized based on the novelty and potential of the candidate markers determined through literature report based searches. Finally, for verification of the proteomic analysis, the candidate proteins were further evaluated by ELISA assays in a different sample set. To our knowledge, this study pioneers a workflow on biomarker discovery, prioritization and evaluation of aggressive prostate cancer candidate marker with quantitative glycoproteomic analysis on OCT-embedded tissues.

COMP, a 524 kDa pentameric glycoprotein is an extracellular matrix protein that is abundantly expressed in cartilage, tendon and vascular smooth muscle [12-14]. Studies show that COMP promoted growth factor-stimulated cell proliferation [15, 16] and prevent apoptosis by elevating anti-apoptotic proteins [17]. In the prostate, COMP was shown to be

expressed by prostate fibroblasts and its mRNA expression was highly elevated in grade 3 reactive stroma [18] and during transdifferentiation of fibroblasts [19] suggesting a possible role of COMP in prostate cancer tumorigenesis. COMP was reported to be associated with hepatocellular carcinoma and breast cancer and was only recently reported to be associated with prostate adenocarcinoma [20]. Here, we found that COMP expression is elevated in prostate tumor, especially in aggressive prostate tumors. Together with the previous report, it'd be interesting to evaluate the performance of COMP as a potential marker in a larger cohort for aggressive prostate cancer.

VAP-1 is a homodimeric 170 kDa glycoprotein that is present in essentially all human tissues [21]. As one of the amine oxidase family member, VAP-1 catalyzes the oxidative conversion of amines to aldehydes. Studies show amine oxidases, in general, are biological regulators for cell growth and differentiation [22]. As there are higher biogenic amines in tumor cells compared to normal cells, amine oxidases by generating reactive oxygen species, may be cytotoxic for cancer cells through apoptosis. In rats, decreased VAP-1 activity was observed in chemical-induced mammary cancer [23]. Decreased amine oxidase activity was also observed in human bowel cancer [24]. It was also reported that VAP-1 may participate in tumor surveillance; VAP-1 was shown to mediate the adhesion of tumor-infiltrating lymphocytes to various carcinomas to kill cancer cells [25, 26]. Our data showed that VAP-1 was significantly reduced in non-aggressive and further reduced in aggressive as well as prostate metastases, suggesting VAP-1 reduction may be associated with the tumor aggressiveness.

Periostin is a 90 kDa extracellular matrix glycoprotein that is highly expressed in bones and connective tissues [27, 28]. It is an adhesion molecule and has been shown to modulate biochemical properties of fibrotic tissues through interacting with various extracellular matrix proteins [29, 30]. Recently, periostin overexpression was associated with a number of cancers, including prostate cancer [5, 31, 32]. With a small sample set, our group previously identified overexpression of periostin with iTRAQ labeling in aggressive prostate tumors and confirmed the overexpression through Western blot and IHC [5]. Here we showed that periostin expression was elevated in prostate cancer and confirmed again that periostin expression was further elevated in aggressive prostate cancer. These results warrant further studies into periostin on its potential clinical utility of as a biomarker for aggressive prostate cancer.

Cathepsin L was identified as a candidate in this study as well as in our previous study [5], where proteomic analysis showed an upregulation in aggressive tumor. With ELISA analysis, however, we find a significant decrease of cathepsin L in aggressive tumor. This discrepancy may be explained by the results from our previous study with immunohistochemistry staining where cathepsin L was found predominantly expressed in epithelium cells and the expression was comparable in both normal epithelium and cancer epithelium [5]. Epithelium percentage, as a result, is positively correlated with cathepsin L reading when cathepsin L level is compared between NAG tumors and AG tumors. While our verification set had similar epithelium percentage between NAG and AG tumors, 2 of the 4 NAG samples in the discovery set contained low amounts of tumor and therefore low

epithelium percentages, which may in part explain the discrepancy between ELISA and glycoproteomics analysis.

While the focus of this study is to identify glycoproteins differentially expressed between aggressive and non-aggressive prostate tumor, both normal tissue from healthy donors and from tumor adjacent areas were included for candidate discovery. The inclusion of these normal tissues is very important and facilitates better candidate identification. For example, clusterin was identified in our previous study as a potential prostate candidate marker, where no normal prostate tissue was utilized for candidate discovery. With the current spectral counting analysis, clusterin expression was also found highly increased by 4.5 fold in aggressive tumor compared to non-aggressive tumor. However, further analysis by comparing clusterin expression in aggressive tumor to its adjacent normal tissue revealed that the change is not tumor specific. Similarly, galectin-3 binding protein was dropped from the candidate list during spectral counting analysis. Indeed, both of these proteins were not significantly associated with aggressive prostate tumor as determined by ELISA (Suppl. Fig. 1).

To evaluate the candidates from the proteomic data, ELISA was employed in this study for its sensitivity and adaptability [33]. However, successful development of ELISA is largely based on the availability of sensitive and specific antibodies. Development of ELISA is time-consuming, and hard to achieve. During the course of the study, we found the most time-consuming phase to be the evaluation of candidates (Fig. 3). While it only took us 1 month to carry out the initial discovery phase using glycopeptide capture and mass spectrometry analysis, we spent 6 months on preparation for evaluation of the candidates on a larger sample set, including the development of ELISA assays. In addition, we spent about 3 months on analyzing the proteomic data and prioritizing the candidates, since developing ELISA on a large number of candidates is not practical. More importantly, development of suitable ELISA assays depends largely on the availability of antibodies with good affinity and specificity to the candidate proteins. Therefore, to expedite candidate marker evaluation, a high throughput antibody independent method may be highly desirable in the future. In this respect, specific reaction monitoring (SRM), a method that is compatible with assessment of large number of candidates without the need of antibodies, may be more advantageous for efficient evaluation of candidates [34, 35].

In conclusion, in this manuscript, we present a workflow for biomarker discovery, prioritization and evaluation of aggressive prostate cancer markers with OCT-embedded tissues. We found that COMP, periostin and VAP-1 were regulated in the same pattern as what we observed in glycoproteomic analysis and may be associated with aggressive prostate cancer. Further studies may be done to evaluate whether these protein are useful markers in distinguishing aggressive prostate cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviation

COMP	cartilage oligomeric matrix protein
VAP-1	membrane primary amine oxidase
PSA	prostate specific antigen
SPEG	solid phase extraction of N-linked glycopeptides
OCT	optimal cutting temperature

Figure 1.

Standard Curve of ELISA assays for protein quantification of cartilage oligomeric matrix protein (COMP), periostin, membrane primary amine oxidase (VAP-1) and cathepsin L. A), dose-response curves for COMP. B), dose-response curve for periostin. C), dose-response curve for VAP-1. D), dose-response curve for cathepsin L.

Figure 2.

ELISA quantitation of cartilage oligomeric matrix protein (COMP), periostin, membrane primary amine oxidase (VAP-1) and cathepsin L expression in normal prostate tissue (N), non-aggressive (NAG), aggressive (AG) and metastatic prostate tumors. Protein expression of COMP (A), periostin (B), VAP-1 (C) and cathepsin L (D) was measured in 12 N, 27 NAG, 20 AG and 10 MET. *, $p < 0.05$, ***, $p < 0.001$ compared to normal prostate tissue. #, $p < 0.05$, ##, $P < 0.01$ compared to NAG tumor. &&&, $P < 0.001$ compared to AG tumor.

Figure 3.
Timeline for identification, prioritization and evaluation of candidate proteins with altered expression in aggressive prostate carcinoma.

Glycoproteins specifically associated with aggressive prostate cancer, as determined by spectral counting

Table 1

Spectral count analysis was carried out on LC-MS/MS data of glycopeptides isolated from 4 normal prostate tissues, 4 metastatic tumor, 4 aggressive and 4 non-aggressive prostate tumors as well as the normal prostate tissues adjacent to prostate tumors. The protein name, Swissprot accession number, the total spectral counts of each candidate from each tissue group and the P value and fold change between aggressive and non-aggressive tumor as well as the fold change between aggressive and normal tissue, between aggressive tumor and normal tissue adjacent to aggressive tumor and between aggressive tumor and normal tissue adjacent to non-aggressive tumor are listed. #: identified glycosites; AG-T: aggressive prostate tumor; NAG-T: non-aggressive prostate tumor; AG-N: normal tissue adjacent to aggressive prostate tumor; NAG-N: normal tissue adjacent to non-aggressive prostate tumor; N: normal prostate tissue from transplant donor; MET: prostate metastases.

Name	Swiss Prot	Total Spectral Count						Fold Change				P Value
		AG-T	NAG-T	AG-N	NAG-N	N	MET	AG-T/NAG-T	AG-T/N	AG-T/AG-N	AG-T/NAG-N	
Asporin	Q9BXN1	15	7	4	2	5	5	2.14	3	3.75	7.5	0.00016
Cartilage oligomeric matrix protein	P49747	12	0	0	1	0	0	100	100	100	12	0.00228
Versican	P13611	21	0	1	4	0	6	100	100	21	5.25	0.03367
Acid ceramidase	Q13510	4	22	12	12	8	6	0.18	0.5	0.33	0.33	0.04983
EMILIN 3	Q9H8L6	6	1	2	2	1	3	6	6	3	3	0.04983
Alpha-2-glycoprotein 1, zinc	P25311	3	9	5	16	9	2	0.33	0.33	0.6	0.19	0.05326
Adipocyte plasma membrane-associated protein	Q9HDC9	8	4	1	2	1	3	2	8	8	4	0.06085
Metalloproteinase inhibitor 1	P01033	3	6	9	9	6	0	0.5	0.5	0.33	0.33	0.06861
Lymphocyte antigen	Q30161	10	3	0	3	0	5	3.33	100	100	3.33	0.07611
Periostin	Q8IZF9	17	10	8	6	3	5	1.7	5.67	2.125	2.83	0.08382
Cathepsin L	P07711	7	0	2	2	0	2	100	100	3.5	3.5	0.12107
Alpha-2-HS-glycoprotein	P02765	3	10	6	11	5	0	0.3	0.6	0.5	0.27	0.12531
Collagen alpha 1(VI) chain	P12109	13	8	7	8	6	2	1.63	2.17	1.86	1.63	0.27098
150 kDa oxygen-regulated protein	Q9Y4L1	20	6	6	10	10	15	3.33	2	3.33	2	0.27953
Membrane copper amine oxidase	Q16853	15	26	20	29	20	0	0.58	0.75	0.75	0.52	0.30198
immunoglobulin J chain	P01591	6	2	4	4	4	3	3	1.5	1.5	1.5	0.47801
Integrin beta-1	P05556	3	7	4	5	6	1	0.43	0.5	0.75	0.6	0.59797

Literature search based analysis of glycoproteins specifically associated with aggressive prostate cancer

Table 2

Pubmed based literature searches were carried out for 17 candidate proteins. The candidate protein names, Swissprot accession numbers, the number of publications on the candidates, the number of publications of the candidates on cancer, the number of publications of the candidates on prostate cancer, the number of publications of the candidates on cancer aggressiveness and the number of publications of the candidates on aggressive prostate cancer are listed.

Name	Swiss Prot	Name # of Hits	& "Cancer"	& "Prostate Cancer"	& (Aggressive OR Aggressiveness) & "Cancer"	& (Aggressive OR Aggressiveness) & "Prostate Cancer"
Cartilage oligomeric matrix protein	P49747	3553	100	5	7	0
Lymphocyte antigen	Q30161	7316	553	33	6	0
Membrane copper amine oxidase	Q16853	978	29	1	1	0
Cathepsin L	P07711	2377	253	8	20	1
Splice isoform V0 of P13611 Versican core protein	P13611	1056	100	8	11	1
Acid ceramidase	Q13510	178	43	12	2	1
Alpha-2-glycoprotein 1, zinc	P25311	74	18	5	1	1
Periostin	Q8ZF9	443	109	7	8	2
Splice isoform Beta-1A of P05556 Integrin beta-1	P05556	5420	879	34	36	3
Metalloproteinase inhibitor 1	P01033	5557	885	41	49	4
150 kDa oxygen-regulated protein	Q9Y4L1	847	185	20	7	4
Alpha-2-HS-glycoprotein	P02765	850	50	0	0	0
Collagen alpha 1(VI) chain	P12109	160	17	0	0	0
Asporin precursor	Q9BXN1	78	2	1	0	0
Adipocyte plasma membrane-associated protein	Q9HDC9	41	2	0	0	0
Immunoglobulin J chain	P01591	34	1	0	0	0
EMILIN 3	Q9H8L6	4	2	0	0	0
Summary		17/17	17/17	12/17	11/17	8/17