Comparative Proteome Profiling and Mutant Protein Identification in Metastatic Prostate Cancer Cells by Quantitative Mass Spectrometry-based Proteogenomics

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Abstract. Background/Aim: Prostate cancer (PCa) is the most frequent cancer found in males worldwide. The aim of this study was to identify new biomarkers using mutated peptides for the prognosis and prediction of advanced PCa, based on proteogenomics. Materials and Methods: The tryptic peptides were analyzed by tandem mass tag-based quantitative proteomics. Proteogenomics were used to identify mutant peptides as novel biomarkers in advanced PCa. Results: Using a human database, increased levels of INTS7 and decreased levels of SH3BGRL were found to be associated with the aggressiveness of PCa. Using proteogenomics and a cancer mutation database, 70 mutant peptides were identified in PCa cell lines. Using parallel reaction monitoring, the expression of seven mutant peptides was found to be altered in tumors, amongst which CAPN2 D22E was the most significantly up-regulated mutant peptide in PCa tissues. Conclusion: Altered mutant peptides present in PCa tissue could be used as new biomarkers in advanced PCa.

Cancer is known to involve multiple genetic changes that arise through somatic or germline mutations. Most human cancers are caused by the acquisition of somatic mutations throughout a person’s lifetime, but germline mutations, which are responsible for inherited cancer types, are less common, accounting for 3-16% of total cancers (1). Somatic mutations can be caused by environmental factors such as exposure to tobacco and ultraviolet radiation, as well as age, and as such they cannot be inherited to the next generation. Almost 100,000 somatic mutations in various cancer genomes have been reported over the past three decades (2). In a recent genome-wide analysis, between 20 and 100 mutations in protein-coding genes were observed in solid tumors (3). Another study showed that common adult solid tumors such as those in brain, breast, colon, or pancreas, contain an average of 33 to 66 mutations that would be expected to change protein function (4).

Prostate cancer (PCa) is the most common cancer in males, and is responsible for the second highest mortality of all male cancers in the USA (5). When patients have a local or regional cancer, the five-year survival rate is close to 100%. However, the five-year survival rate for later-stage PCa drops to 30%, due to metastasis (6, 7). The pathogenetic molecular risk factors for PCa metastasis have not yet been clarified, but age, race, and inherited genetic factors are known to be risk factors (5). Among the genetic factors, germline mutations in
**BRCA1** and **BRCA2** not only increase the risk of PCa progression, but are also associated with poor prognosis and an aggressive PCa phenotype (8). A mutation in HOXB13 has been associated with a significantly increased risk of PCa (9). Recent genome-wide association studies have identified 77 single nucleotide polymorphisms (SNPs) related to the risk of PCa, which, although common, have low penetrance (10, 11). As with most cancers, over 90% of the mutations in PCa are somatic mutations. Most of the PCa-related somatic mutations occur in TP53, followed by PTEN, KRAS, and FOXA1. In addition, somatic mutations in the androgen receptor (AR), MYC, and PIK3CA have also been identified as being important in PCa (12).

Millions of human SNPs have been identified. Mutations that result in a single amino acid change in a protein are referred to as single amino acid polymorphisms (SAAPs) (13). Although the mutant proteins found in cancer are potentially useful as clinical biomarkers, it is difficult to detect and validate them, and such biomarkers are rarely used clinically (14). Many researchers have therefore tried to identify mutant proteins using proteogenomics, a field which combines genomics with mass spectrometry-based proteomics.

The scarcity of biomarkers that can be used for the prediction and prognosis of PCA is one of the reasons for the high mortality rate seen in PCa patients. To identify new candidate biomarkers for advanced and/or aggressive PCa, we focused on mutant peptides. Since cancer is a genetic disease that can be caused by a range of mutations, we hypothesized that mutant proteins involved in advanced PCa could be used as a new type of candidate biomarkers.

Differentially expressed proteins (DEP) were quantified using tandem mass tag (TMT)-based proteomics approaches in PCa cell lines with four levels of aggressiveness: LNCaP and PC-3 as models of less aggressive PCa, and the sub-lines LNCaP-LN3 and PC-3M as models of more aggressive PCa (15, 16). The metastatic PCa-related mutated peptides were identified using a cancer mutation database consisting of the CanProVar, CMPD and UniProtKB human databases. The expression of selected mutated peptides in more aggressive PCa cell lines was verified in human prostate tissues using parallel reaction monitoring (PRM) after standard peptide synthesis. The aim of this study was to discover and verify candidate biomarkers for the prediction of the prognosis of advanced PCa using a shotgun proteomics approach, and verify that the mutant peptides could be potential new biomarkers in PCa patients.

**Materials and Methods**

**Cell culture.** The PCa cell lines LNCaP, LNCaP-LN3, PC-3 and PC-3M were purchased from the Korea Cell Line Bank (KCLB, Seoul, Korea). Cancer cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, and 1% penicillin G and streptomycin as antibiotics, at 37°C in an atmosphere of 5% CO₂ in a humidified incubator. The normal prostate cell line was grown in Keratinocyte-SFM (Gibco, Waltham, MA, USA) with a Human Keratinocyte Growth Supplement kit (Gibco), including human recombinant EGF and a bovine pituitary extract.

**Preparation of cellular proteins and trypsin digestion.** Cell pellets were washed with 1 mL of PBS and then cells were lysed in lysis buffer containing 100 mM Tris-Hcl pH 7.6, 4% SDS, 1 mM dithiothreitol (DTT) and the Halt Protease Inhibitor Cocktail. The cell lysate was then homogenized with 4% SDS lysis buffer supplemented with the Halt Protease Inhibitor Cocktail. To improve protein extraction, the cell samples were then centrifuged at 16,000 × g for 10 min at 4°C to pellet any debris. Proteins were reduced and alkylated with DTT and iodoacetamide (IAA) for 30 min at room temperature in the dark. The proteins were then precipitated by the addition of 10% trichloroacetic acid for four hours at 4°C to remove contaminating substances such as detergent. After centrifugation at 12,000 × g for 10 min at 4°C, the pellets were washed twice by the addition of 500 μl of –20°C acetone, and then dissolved in 100 mM triethylammonium bicarbonate (TEAB) with sonication. The dissolved proteins were centrifuged at 12,000 × g for 10 min at 4°C to eliminate any insoluble material, and the protein content determined using the BCA protein assay prior to further experiments.

For quantitative proteomics using TMT, proteins were transferred to low-protein binding E-tubes and digested with trypsin at a ratio of 1:50 (w/w) overnight at 37°C, after which the peptides were re-assayed using a quantitative colorimetric peptide assay in order to provide an accurate determination of each protein group. Peptides (100 μg) from each cell line were labeled with 0.8 mg of the sixplex-TMT reagent dissolved in 41 μl of acetonitrile (ACN) and incubated for one hour at RT. To quench the TMT reaction, 5% hydroxyamine in 50 mM TEAB was added to the peptides. Finally, all of the labeled peptides were mixed together in a low protein binding E-tube. The combined peptides were fractionated using two different methods: a High-PH RP fractionation kit, and an OFFGEL fractionator (Agilent, Santa Clara, CA, USA), to enhance the number of identified proteins. Both fractionation methods were performed according to the manufacturer’s procedures.

**Preparation of clinical cancer tissues.** The biological specimens and data used for this study were provided by the National Biobank of Korea-Kyungpook National University Hospital (KNUH), a member of the Korea Biobank Network. KNUH were obtained (with informed consent) under institutional review board (IRB)-approved protocols (No. KNUMC 2016-05-021). Normal tissue and prostate tissue (separated into T2 and T3) were stored at –80°C before use. The prostate tissues were rinsed using 1 ml of PBS, and then homogenized with 4% SDS lysis buffer supplemented with the Halt Protease Inhibitor Cocktail. To improve protein extraction, the homogenized tissues were sonicated for two min on ice. The protein samples were then centrifuged at 16,000 × g for 10 min at 4°C to remove any tissue debris. Proteins extracted from the prostate tissue were measured using a BCA protein assay kit. Proteins (50 μg) were then reduced and alkylated with DTT and IAA as described above.

For Filter-aided sample preparation (FASP), a 30K Centricon was pre-wetted with 60% methanol by centrifugation at 14,000 × g for five min. Samples (50 μg) were transferred into the centricon, and
an 8 M urea solution (8 M Urea, 100 mM Tris-Hcl pH 8.5) was added followed by centrifugation at 14,000 × g for 15 min to remove detergents such as SDS. The centrifron was then washed twice with 200 μl of 8 M urea solution, using centrifugation at 14,000 × g for 15 min. To remove urea, the centrifron was washed twice with 100 μl of 50 mM ammonium bicarbonate (ABC), using centrifugation at 14,000 × g for 10 min. Proteins in the centrifron were digested by the addition of 1 μg of trypsin and incubated at 37°C overnight. Peptides were collected in low-protein binding E-tubes by centrifugation at 14,000 × g for 10 min. An additional 50 μl of 50 mM ABC was added to the centrifron to allow for complete elution of the peptides. Before the LC-MS/MS analysis, the samples were desalted using a C18 Ziptip.

**LC-MS/MS analysis for TMT-based proteomics.** For TMT-based proteomics from the PCa cell lines, high resolution mass-spectrometry was performed using LTQ-velos Orbitrap with the Eksigent nanoLC system. Peptides (1 μg) were loaded into the Eksigent nanoLC and separated using a homemade C12 reverse-phase analytical column. The two-h gradient for the Eksigent nanoLC was 2% solvent B (100% ACN in 0.1% FA) for two min, 2–21% solvent B for 110 min, 21-90% solvent B for three min, and 90% for five min at a flow rate of 300 nl/min. For the LTQ-velos Orbitrap, full MS scans were acquired at m/z 300-1,800 with a resolution of 30,000. The top 10 data-dependent modes were fragmented with NCE at 40% in high-energy collisional dissociation (HCD). MS/MS was obtained using the following settings: MS/MS resolution 7,500, a minimal signal of 5,000, an isolation width of 2.0 m/z, automatic gain control (AGC) target at 1e6, and a 0.1 msec activation time. The lock mass ion from ambient air (m/z 445.120024) was able to improve mass accuracy.

**LC-PRM analysis for peptide quantification.** To evaluate MS accuracy and retention time during PRM, 25 fmoles of a quality control sample was prepared using a peptide mixture (SCIEX, Framingham, MA, USA; P/N 800-00208) consisting of four peptides (VYV, DRVYIHPF, YGGFM, and YGGFL). The synthetic peptide, GYLPNPALQR, as a general internal standard peptide (17) was provided at 98% purity from Lugen Sci. (Bucheon, Gyeonggi, Korea) and was spiked at 25 fmole into the FASP peptides derived from prostate tissue. Q-Exactive with Easy-nLC 1000 was used to quantitatively analyze mutant peptides from the prostate tissues using target quantitation. A sample (2 μg) containing 25 fmoles of internal standard (IS) was injected into the Easy-nLC 1000 with a commercial C18 reversed-phase-column. The samples were separated using a linear gradient of 0-3% solvent B for 2.5 min, 3–25% solvent B for 72 min, and 25-90% solvent B for three min at a constant flow rate of 300 nl/min. A full scan of Q-Exactive was performed using the following settings: source voltage 2.0kV; capillary temperature of 280°C; full MS range m/z 300-1,000; MS resolution 70,000; AGC target 3e6; and maximum IT of 150 msec. The target MS/MS mode employed a resolution of 17,500, a target AGC value of 1e5, a maximum IT of 75 msec, and an isolation window of 2.0 m/z with 27eV of normalized collision energy.

**Data analysis and bioinformatics for proteomics and proteogenomics.** The MS/MS spectra were searched using MaxQuant 1.5.1.0 to identify proteins and peptides (18). The UniProtKB human database was searched for the TMT-based proteomics study. The MS-
to confirm that the mutant peptides were indeed specific mutated peptides. A total of 70 mutant peptides and 68 mutation sites were identified in 66 proteins from the PCa cell line.

All of the mutant peptides, with the exception of CHML S342*, which arose from a nonsense mutation, were missense mutations. Unfortunately, only seven of these mutated peptides were altered in aggressive PCa; two were up-regulated and five were down-regulated. However, since all of the mutations were derived from the PCa cell line, the mutant peptides were considered as candidate biomarkers associated with PCa.

**Results**

Quantitative TMT-based proteomics of prostate cancer cell lines. A TMT-based proteomics approach was designed to discover DEPs and mutant peptides which can be used as cancer biomarkers associated with the aggressiveness of PCa in different PCa cells (Figure 1A). The MS/MS spectra produced were assessed using MaxQuant 1.5 to identify and quantify the peptides and proteins involved. The MaxQuant
analysis was performed using the same procedures used for both proteomics and proteogenomics. Two different databases were used with MaxQuant to identify the DEPs using proteomics approaches, and the mutated peptides using proteogenomics (Figure 1B). The UniProtKB human database was used to identify DEPs, and the cancer mutant database, consisting of the CanProVar, CMPD and UniProtKB human databases, was used to identify PCa-related mutant peptides. The MaxQuant search results were filtered for scores greater than 40 and FDR values of below 1%, to discard false positive protein and peptide hits. All quantitative values were calculated based on reporter ion intensities and were quantified as LNCaP-LN3/LNCaP and PC-3M/PC-3 ratios representing the androgen-dependent (AD) and independent (AI) stages of PCa. We confirmed that the technical correlations of the TMT quantitation were very high, being above 0.86 on average. These quantification data indicated that our proteomics approach is experimentally valid.

**Bioinformatics analysis of prostate cancer cells.** The UniProtKB human database was searched to detect DEPs associated with aggressiveness in AD and AI PCa. A total of 3,939 proteins were identified, and of these 3,409 (86.5%) could be quantified. There were 134 DEPs including 49 DEPs identified from the LNCaP-LN3/LNCaP ratio, and 89 DEPs identified from the PC-3M/PC-3 ratio, that had a more than two-fold (log2 scale ≤ –1 and ≥1) change in their protein expression levels (Figure 1C). The DEPs in AI and AD were identified as having opposing patterns of expression. Four DEPs were present in both AI and AD, and of these, SH3BGRL and SEPT10 were found to be present at decreased levels in aggressive PCa cell lines compared with the non-aggressive parental PCa cell lines. In contrast, the other two proteins, ATP6AP1 and ALDH7A1, were found to be present at increased levels.

**GO, KEGG, and InterPro enrichment analyses were performed using the David software to investigate the biological functions of DEPs identified as related to aggressiveness between AD and AI (Figure 2).** The functions of the altered proteins were significantly different based on AI and AD. Proteins whose levels were increased in LNCaP-LN3 compared with those in LNCaP cells were associated...
with the regulation of entry of bacteria into host cells in GOBP, endoplasmic reticulum and melanosome in GOCC, protein disulfide isomerase activity in GOMF, and disulfide isomerase thioredoxin in Interpro. Proteins up-regulated in PC-3M cells as compared with PC-3 cells were associated with toxin transport and cell proliferation in GOBP, nuclear heterochromatin and extracellular exosome in GOCC, and transporter activity in GOMF. Aggressiveness-related proteins that were reduced in LNCaP-LN3 cells compared with LNCaP cells were associated with extracellular related proteins and the biosynthesis of antibiotics according to the KEGG analysis. However, proteins associated with less aggressive AD PCa, those that were down-regulated in PC-3M cells as compared with PC-3 cells, were more prominently associated with focal adhesion and extracellular exosomes in GOCC, integrin binding proteins in GOMF, and focal adhesion and ECM-receptor interaction in KEGG. In summary, most of the proteins related to aggressiveness in PCa appeared to be involved in different biological functions depending upon whether the cancer is AD or AI.

To identify DEPs that changed statistically significantly in the aggressive AD or AI cell lines, a volcano plot was

Figure 3. Volcano plot of the total quantified proteins in prostate cancer with different degrees of aggressiveness (A). Verification of the mRNA levels of INTS7 and SH3BGRL from the cBioPortal database (B) and correlation of the expression of both genes (C). *p<0.05 and **p<0.01.
produced using a one-way t-test (Figure 3A). All of the proteins quantified in AD and AI were used in the one-way t-test, and the results were filtered using a p-value of less than 0.05 and at least a two-fold change in expression. Altered expression of two proteins that were associated with PCa aggressiveness was found. The protein levels of INTS7 (Q9NVH2; p-value=0.01) were higher in the aggressive PCa cell lines PC-3M and LNCaP-LN3, than in the parental cells. In contrast, SH3BGRL expression (O75368; p-value=0.04) was found to be significantly reduced in the aggressive PCa cell lines PC-3M and LNCaP-LN3 compared with the parental cells.

Using cBioPortal, these two proteins were identified, using the TCGA mRNA database, as being expressed in prostate adenocarcinoma (Figure 3B). The mRNA levels of INTS7 and SH3BGEL were compared based on the PCa GS, and showed the same tendencies as in the proteomics study. In particular, the mRNA levels of INTS7 were slightly increased in both Gleason 7 and ≤8 compared to Gleason 6 (both p-values were 0.030). Conversely, the mRNA levels of SH3BGEL were significantly reduced in both GS 7 and ≤8 patients compared with patients having a GS of 6 (p-values 0.042 and 0.005, respectively). A significant negative correlation was found between SH3BGEL and INTS7 mRNA levels in prostate adenocarcinoma (Spearman’s correlation coefficient, r=-0.22, p=0.0004), using the TCGA database and cBioPortal (Figure 3C).

**Figure 4.** Schematic of the workflow for the identification and verification of mutant peptides (A). Workflow schematic of the parallel reaction monitoring (PRM) for mutant peptides in prostate cancer tissues (B).

Profiling of mutant peptides from prostate cancer cell lines. As described above, a cancer mutation database was created and analyzed using MaxQuant 1.5 to identify mutant proteins and peptides present in PCa. Figure 4A shows a detailed schema of the procedure used to profile cancer mutant proteins. After searching 479,093 MS/MS spectra using
MaxQuant 1.5 and the cancer mutation database, the results were filtered using a score cut-off of over 40, and an FDR of less than 1%, and excluding human protein and peptides present in existing reference databases. As a result, 428 non-redundant peptides were identified from 1,141 mutant peptides in the database. From these, peptides not containing mutated sites were excluded, as well as the mutation between leucine and isoleucine, because mass spectrometry is not able to distinguish between leucine and isoleucine, since they have the same mass. These results were confirmed using manual

Figure 5. Continued
verification of the MS/MS spectra, as described previously (26). PSI-BlastP was used to determine that the mutant peptides did not overlap with the human protein sequence, to confirm that the mutant peptides were indeed specifically mutated peptides. We identified 70 mutant peptides and 68 mutation sites in 66 proteins in the PCa cell lines.

All mutant peptides, with the exception of CHML S342*, which arose from a nonsense mutation, were missense mutations. Only seven of these mutated peptides were altered in aggressive PCa, with two being up-regulated and five down-regulated. However, since all of the mutations were derived from the PCa cell line, the mutant peptides were

Figure 5. Changes in the relative peak area of mutant peptides between normal and prostate cancer tissue (A) and among normal prostate tissue and prostate cancer tissue from stage T2 and T3 patients (B). Underlining indicates the mutant sites. *p<0.05.
considered to be candidate biomarkers associated with PCa. Verification of mutant peptides in prostate tissues using PRM. From the 70 mutant peptides, several mutations were selected based on previous guidelines to verify whether the mutant peptides have the potential to be new biomarkers in PCa patients (27) (Figure 4B). The criteria used for selection were as follows: the selected mutations were within peptides of length 7 to 22 amino acids; there were no internal lysine or arginine residues.; each peptide was completely cleaved and 45 in terms of SSRC score, and had been observed at least twice in previous TMT-based results. Modified peptides were excluded, as well as some mutated peptides containing cysteine and methionine residues that could lead to PTMs or arginine residues,; each peptide was completely cleaved and 45 in terms of SSRC score, and had been observed at least twice in previous TMT-based results. Modified peptides were excluded, as well as some mutated peptides containing cysteine and methionine residues that could lead to PTMs such as oxidation. A total of 26 mutated peptides were considered to be potential biomarkers in PCa patients (27) (Figure 4B). The criteria used for selection were as follows: the selected mutations were within peptides of length 7 to 22 amino acids; there were no internal lysine or arginine residues.; each peptide was completely cleaved by trypsin; and the peptide hydrophobicity was between 10 and 45 in terms of SSRC score, and had been observed at least twice in previous TMT-based results. Modified peptides were excluded, as well as some mutated peptides containing cysteine and methionine residues that could lead to PTMs such as oxidation. A total of 26 mutated peptides were identified to be verified in patients with PCa, using targeted MS. The selected mutant peptides were also re-analyzed for their m/z and charge for use in MS, since the TMT 6-plex modifies both peptide N-terminus and lysine residues.

PRM was used for label-free quantification by a mass spectrometer to verify the presence of the mutant peptide in PCa patient tissues (Figure 4B). These prostate tissues, classified as PVA stages normal, T2, and T3, were prepared using the FASP method for digested peptides. All peptides from tissues were desalted with 5 μg of ziptip and then dissolved in 10 μl of solution A (98% water with 0.1% FA) that included 25 fmole of GYLPNPALQR as a synthetic internal standard peptide. Peptides (1 μg/2 μl) were separated from a commercial C18 column using Q-Exactive high-resolution mass spectrometry. The raw MS files were searched using MaxQuant 1.5 for the spectral library, and analyzed using Skyline to quantify the mutant peptides. In total, thirteen peptides were detected, including the internal standard peptide, in the prostate tissues, which were quantified by the sum of three fragment ions (Table I). When examining the coefficient of variation values of the internal standard peptide, the coefficients of variation for retention time and peak area were within 5% and 20%, respectively. Previous criteria for quantifying target peptides showed that a coefficient of variation between 20 and 25% had high accuracy (28). Therefore, this PRM study showed little technical variability.

The peak area of the mutant peptide was divided by the peak area of the internal standard to calculate the relative quantification of the mutant peptide. Seven of the 12 mutant peptides had different expression levels in PCa tissues as compared to normal tissues (Figure 5A). Three of these mutant peptides: SERPINB5 I319V (GVALSNVVEK); PCMT1 V120I (ELVDSSINNVR); and AIP Q228K (EQPGSPEWQILDK) were decreased. In particular, CAPN2 D22E showed a significant increase in tumors compared with normal tissue ($p=0.040$). No mutant peptides that differ significantly between T2 and T3 were identified, (Figure 5B). However, AIP Q228K (EQPGSPEWQILDK) and NES V130A (AWLSSQAELE) was observed to be increased and decreased at T3 compared to T2, respectively.

### Table I. Characteristics of mutant peptides from prostate tissue obtained using parallel reaction monitoring (PRM).

| #  | Uniprot ID | Gene name | dbSNP ID | Sequence$^\dagger$ | Mutant site | Precursor m/z | Charge | Product m/z | Fragment ion |
|----|------------|-----------|----------|---------------------|-------------|---------------|--------|-------------|-------------|
| 1  | O75083     | WDR1      | rs13441  | FTVGDHSR           | I185V       | 306.8191      | 3      | 571.2582, 399.2098, 670.3267 | y5, y3, y6  |
| 2  | P17655     | CAPN2     | rs25655  | EAEEGLGSHER         | D22E        | 578.2731      | 2      | 755.3794, 585.2739, 201.0869 | y7, y5, b2 |
| 3  | P36952     | SERPINB5  | rs145555 | GVALSNVVEK         | I319V       | 341.8697      | 3      | 683.3834, 284.1717, 157.0971 | y6, y2, b2 |
| 4  | P14618     | PKM       | rs112954819 | ITLDNAYVEK        | M149V       | 583.3086      | 2      | 1052.5258, 147.1128, 951.4781 | y9, y1, y8 |
| 5  | P22061     | PCMT1     | rs4816   | ELVDSSINNVR        | V120I       | 637.3228      | 2      | 932.4431, 1031.5116, 702.3893 | y8, y9, y6 |
| 6  | P48681     | NES       | rs4278369 | AWLSSQAELEER      | V130A       | 680.8464      | 2      | 990.485, 258.1237, 1103.5691 | y9, b2, y10 |
| 7  | O00170     | AIP       | rs641081 | EQPGSPEWQILDK      | Q228K       | 763.8779      | 2      | 1269.6473, 1028.5411, 147.1128 | y11, y8, y1 |
| 8  | O00330     | PDHX      | rs35560997 | LSVNDFIIK         | V326L       | 524.8055      | 2      | 935.5196, 749.4192, 848.4876 | y8, y6, y7 |
| 9  | Q7UNM6     | PSMD13    | rs1045288 | DVPFLQSSQSS        | N13S        | 793.9327      | 3      | 765.4154, 1047.5482, 498.2571 | y6, y9, y3 |
| 10 | Q15149     | PLEC      | rs11136363 | AGVAAQPATQV      | A641V       | 675.0480      | 3      | 831.4682, 1058.5952, 930.5367 | y7, y9, y8 |
| 11 | Q01518     | CAP1      | rs666944  | SALFAQINQG         | S256A       | 643.3461      | 2      | 1197.6222, 955.5207, 1310.7062 | y11, y9, y12|
| 12 | P30084     | ECHS1     | rs1049951 | IFEDPAVGAI         | T75I        | 915.9778      | 2      | 1197.6837, 930.5254, 261.1597 | y13, y10, b2|

$^\dagger$ Bold and underlined indicate the mutant sites.
Discussion

Our data indicate that changes in proteins and biological functions are very different in the different PCA cell lines, AI and AD, and with different degrees of aggression. Based on previous proteomics data obtained using PCA cell lines such as LNCaP and PC-3 and its sub-cell lines, LDHB and PDIA6 were decreased and increased, respectively, in LNCaP-LN3 cells compared with LNCaP cells (29, 30). ANXA10 was increased in PC-3M cells compared with PC-3 cells, while ACTN1 was decreased, a finding which is similar to our results (30). Another previous study comparing AD and AI cells found that the same protein is involved in different processes depending on whether a cell is AI or AD. For example, in AD cells Stat5a/b protects against the degradation of the anti-androgen-ligated AR by the proteasome and therefore activates AR gene expression by amplifying AR signaling. In contrast, Stat5a/b-regulated proliferation, and survival genes are induced by Stat5a/b in AI cells (31). However, previous proteomic studies in PCA cell lines have only described changes in proteins and poor results, but GO of KEGG analysis of DEPs have not been reported (29, 30, 32-34). Based on these findings we propose that different approaches to the prognosis and treatment of PCA should be used, depending on the stage of the cancer.

A volcano plot was used to identify the DEPs that were commonly changed in the AD and AI cells, and identified two significantly altered proteins (Figure 3). The protein levels of INTS7 were increased and those of SH3BGRL were decreased in the sub-cell lines compared with levels in the parent cells, and were comparable with the mRNA levels in cBioPortal. Levels of both proteins were significantly changed in patients with a high GS. INTS7 is an RNA polymerase II complex subunit, important for transcriptional regulation. A recent study has shown that INTS7 is highly mutated and is significantly overexpressed in a diverse number of cancers including PCs (35). According to the human protein atlas, the mRNA levels of INTS7 are significantly higher in testis normal tissues. However, as described above, there are other cancers, mutations in EGFR E868K (rs74807) and APC F1354L (rs7900) have been associated with lung and intestinal cancers, respectively (47-49). The Q228K variant in the AIP gene has been associated with a significantly increased susceptibility to pituitary adenoma in a Turkish patient population (50). Two mutations, SERPINB5 I319V (rs1455555) and RAD18 R302Q (rs373572), have been shown to increase the risk of hepatocellular carcinoma and colorectal cancer, respectively. Furthermore, SERPINB5 I319V mutation may alter the catalytic activity of the SERPINB5 protein (51, 52). However, many of the other mutations are known as non-cancer specific variations.

Since most peptides are not verifiable in a mass spectrometer for a variety of reasons, based on our guidelines 26 mutated peptides were selected for qualitative and quantitative analysis in PCA patient tissues. Amongst these, 12 mutant peptides were qualified and quantified in prostate tissue using PRM and the Skyline software (Table I). It was assumed that the mutant peptides which were not confirmed by PRM could not be detected because only a very small amount of them are present in vivo. Only seven peptides, three of which were up-regulated and four down-regulated, were also altered in PCA tissues compared with normal tissues. However, as described above, there are other mutant peptides which have not been studied, except for SERPINB5 I319V and AIP Q228K, among the seven mutant peptides that showed altered expression patterns. Of the seven mutations that were occurred in PCA, only CAPN2 D22E was significantly increased, especially at the T2 stage. Unfortunately, we could not find any mutant peptide that was significantly associated with aggressive and/or advanced PCA. Nevertheless, since some mutations did show differences in expression levels between T2 and T3, it is necessary to be verified in more samples and different PCA groups to assess if they could be used as biomarkers for advanced PCA.

In summary, 70 mutant peptides were discovered from PCA cell lines using a proteogenomics approach. Twelve of the mutant peptides from prostate tissue were quantified and quantified using PRM. CAPN2 D22E, which is significantly increased in PCs, has the possibility of being a candidate biomarker for the diagnosis of PCs.
Conflicts of Interest

The Authors report no conflicts of interest. The Authors alone are responsible for the content and writing of this article.

Authors’ Contributions

Conceptualization: Kwon, O.K., Kwon, T.G. and Lee, S.; Methodology and Formal analysis: Kwon, O.K., Kim, S. and Ha, Y.; Resources: Lee, J.N., Lee, H., Chum, S.Y. and Kwon, T.G.; Data Curation: Kwon, O.K. and Lee, S.; Writing: Kwon, O.K., Ha, Y. and Lee, S.; Funding Acquisition: Ha, Y., Kwon, T.G., Chum, S.Y. and Lee, S.

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