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Antibody-independent targeted quantification of TMPRSS2-ERG fusion protein products in prostate cancer

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ABSTRACT

Fusions between the transmembrane protease serine 2 (TMPRSS2) and ETS related gene (ERG) represent one of the most specific biomarkers that define a distinct molecular subtype of prostate cancer. Studies of TMPRSS2-ERG gene fusions have seldom been performed at the protein level, primarily due to the lack of high-quality antibodies suitable for quantitative studies. Herein, we applied a recently developed PRISM (high-pressure high-resolution separations with intelligent selection and multiplexing)-SRM (selected reaction monitoring) strategy for quantifying ERG protein in prostate cancer cell lines and tumors. The highly sensitive PRISM-SRM assays provided confident detection of 6 unique ERG peptides in both TMPRSS2-ERG positive cell lines and tissues, but not in cell lines or tissues lacking the TMPRSS2-ERG rearrangement, clearly indicating that ERG protein expression is significantly increased in the presence of the TMPRSS2-ERG gene fusion. Significantly, our results provide evidence that two distinct ERG protein isoforms are simultaneously expressed in TMPRSS2-ERG positive samples as evidenced by the concomitant detection of two mutually exclusive peptides in two patient tumors and in the VCaP prostate cancer cell line. Three peptides, shared across almost all fusion protein products, were determined to be the most abundant peptides, providing "signature" peptides for detection of ERG over-expression resulting from TMPRSS2-ERG gene fusion. The PRISM-SRM assays provide valuable tools for studying TMPRSS2-ERG gene fusion protein products in prostate cancer.

Abbreviations: TMPRSS2, transmembrane protease serine 2; ERG, ETS related gene; FISH, fluorescence in situ hybridization; SRM, selected reaction monitoring; PRISM, high-pressure high-resolution separations with intelligent selection and multiplexing; LOD, limit of detection; LOQ, limit of quantification; XIC, extracted ion chromatogram; CV, coefficient of variation.

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1. Introduction

The identification of gene fusion events between the androgen-responsive transmembrane protease serine 2 (TMPRSS2) 5′ region and the proliferation-associated ETS related gene (ERG) transcription factor in over 50% of prostate cancers (Kumar-Sinha et al., 2008; Mosquera et al., 2009; Tomlins et al., 2008, 2005) has provided novel insights into the possible mechanisms of prostate cancer progression, by providing a direct link between androgen sensitivity and proliferation-associated changes in gene expression (St John et al., 2012). However, despite the mechanistic significance of this observation, most studies of the TMPRSS2-ERG gene fusion to date have relied upon observations at the gene level, using either fluorescence in situ hybridization (FISH) to identify the chromosomal translocation, or fusion-specific PCR to identify fusion transcripts (Demichelis et al., 2007; Maher et al., 2009; Perner et al., 2006; Tomlins et al., 2005). Since the hypothetical role of TMPRSS2-ERG gene fusions in prostate carcinogenesis is dependent on the transcription activating functions of the ERG gene product, it is important to both verify the protein-level expression of the TMPRSS2-ERG gene fusion and quantify the levels of protein expression in tumors of varying stage, grade, and outcome. The TMPRSS2-ERG fusion gene encodes a truncated ERG protein and the study of TMPRSS2-ERG gene fusion at the protein level will contribute to the understanding of the roles of this protein in critical signaling pathways in prostate cancer such as the androgen signaling pathway and the phosphoinositide 3-kinase (PI3K) pathway (Zong et al., 2009). To date, most of the published data on ERG protein expression has relied on immunohistochemical detection of this protein in tissue samples (Falzarano et al., 2011; Furusato et al., 2010; Park et al., 2010; van Leenders et al., 2011). Moreover, these ERG antibodies cannot distinguish between various versions of the ERG protein, such as the different isoforms generated by distinct fusion sites and alternative splicing.

The availability of a sensitive, robust, and antibody-independent method for identifying and quantifying individual peptides within ERG that are differentially present in the various gene fusion-specific isoforms would provide researchers with a useful tool for prognostic and mechanistic studies of the potential role of truncated ERG proteins in prostate cancer development and progression. Unlike immunoassays, mass spectrometry (MS)-based assays can be completely independent of antibodies. Selected reaction monitoring (SRM)-MS represents a major advance in both sensitivity and specificity for quantitative analysis of target proteins and has frequently been used as an alternative to antibody-based assays (Addona et al., 2009; Anderson and Hunter, 2006; Gerber et al., 2003; Huttenhain et al., 2012; Keshishian et al., 2009; Picotti et al., 2009, 2013; Simicevic et al., 2013). However, the sensitivity of conventional SRM is still insufficient to allow for accurate measurement of some extremely low-abundance proteins, such as ERG protein. To improve the sensitivity of SRM-based assays, Anderson and colleagues (Anderson et al., 2004; Kuhn et al., 2012; Whiteaker et al., 2010) have developed a SISCAPA method where target peptides were enriched using anti-peptide antibodies prior to SRM analysis and the assay sensitivity was greatly increased. A major limitation of the SISCAPA method was that it required generation of high-quality antibodies against target peptides, introducing many of the same challenges restricting typical antibody production, e.g., moderate success rates and long lead times. More recently, our group has developed an antibody-independent PRISM (high-pressure high-resolution separations with intelligent selection and multiplexing)-SRM strategy where target peptides were enriched using high pH reversed-phase (RP) LC prior to the second dimension LC-SRM analysis (Shi et al., 2012). This method improved the sensitivity of target protein detection and quantification by at least 100-fold compared with conventional SRM.

In this study we have developed a series of targeted PRISM-SRM assays capable of specifically recognizing 16 distinct peptides from various domains of TMPRSS2-ERG gene fusion protein products, and demonstrated expression of seven of these peptides in TMPRSS2-ERG positive prostate cancer cell lines and patient-derived tumor samples. These results provide quantitative and isoform-specific information about ERG protein expression in prostate cancer cells and tumors; application of these assays for measurements of the TMPRSS2-ERG gene fusion protein products in large patient cohorts has the potential to significantly enhance our understanding of the role of ERG protein in the biology of prostate cancer.

2. Materials and methods

2.1. Prostate cancer cell line and tissue samples

Six prostate cancer cell lines were analyzed in this study, including two TMPRSS2-ERG gene fusion positive cell lines, VCaP and NCI-H660, as well as four TMPRSS2-ERG gene fusion negative cell lines LNCaP, DU145, PC3 and 22RV1. All cell lines were purchased from the American Type Culture Collection (Manassas, VA). Ten metastatic castration-resistant prostate cancer (CRPC) tissue samples were also analyzed in this study, including five TMPRSS2-ERG gene fusion positive tissues PT1 – PT5 and five TMPRSS2-ERG gene fusion negative tissues NT1 – NT5 provided by the University of Michigan, Ann Arbor (J. Wei and A. Chinnaiyan). For detailed information about these samples, refer to the previous publication by Grasso et al. (2012). Two additional TMPRSS2-ERG gene fusion positive localized prostate cancer tissues PT6 and PT7 were provided by Weill Cornell Medical College (M. Rubin). The information of the 12 tissue samples is listed in Table S1. All experimental procedures were approved by the Institutional Review Boards of the University of Michigan (Ann Arbor, MI), Cornell University (New York, NY), and Pacific Northwest National Laboratory (Richland, WA) in accordance with federal regulations.

2.2. Protein extraction and digestion

Proteins were extracted from each cell line using a urea solution (8 M urea in 50 mM NH_4HCO_3). Cells were sonicated for 1 min twice and the protein concentration was determined using the bicinchoninic acid (BCA) assay (Pierce). Proteins in each cell line sample were reduced with 10 mM dithiothreitol

at 37 °C for 1 h and alkylated using 40 mM iodoacetamide at room temperature for 1 h in the dark. Samples were diluted 10-fold with 50 mM NH_4HCO_3 and 1 M $CaCl_2$ was added to each sample to reach a concentration of 1 mM. Protein digestion was performed at 37 °C for 3 h using trypsin (Affymetrix, Santa Clara, CA) at a 1:50 ratio (w/w). Each sample was desalted using a C18 SPE column (Discovery DSC-18, SUPELCO, Bellefonte, PA) and concentrated to a volume of ~50 μL. The peptide concentration was measured using the BCA assay. Samples were stored at −80 °C.

A different procedure was used to process the tumor tissue samples obtained by coring of OCT frozen tissue blocks. Briefly, a lysis buffer containing 8 M urea, 75 mM NaCl, protease inhibitor cocktail (Sigma–Aldrich, St. Louis, MO), complete protease inhibitor tablet (Roche, Mannheim, Germany), 50 mM NH_4HCO_3, pH 7.8, was used to lyse tissues. Samples were sonicated for 3 min and the protein concentration was determined using the BCA assay. Proteins in each tissue sample were reduced with 5 mM dithiothreitol at 37 °C for 1 h and alkylated using 10 mM iodoacetamide at room temperature for 1 h in the dark. $CaCl_2$ was added to each sample to obtain a final concentration of 1 mM. Protein digestion was performed at 37 °C overnight at a 1:50 ratio (w/w). Tryptic digestion was quenched using trifluoroacetic acid (final pH: 2–2.5). Each sample was desalted using a SCX SPE column (Discovery DSC-SCX, SUPELCO, Bellefonte, PA). The peptide concentration was measured using the BCA assay. Samples were stored at −80 °C for future analysis.

2.3. SRM assay development

Sixteen proteotypic peptides (Table S2) covering different sequence regions of various TMPRSS2-ERG gene fusion protein products were selected and stable isotope-labeled heavy peptides with C-terminal [$^{13}C_6{}^{15}N_2$] lysine or [$^{13}C_6{}^{15}N_4$] arginine were synthesized (Thermo Fisher Scientific) for SRM assay development. The peptides were selected following the standard criteria as described by Aebersold and coworkers (Lange et al., 2008). The purity of these synthetic peptides was >97% as determined by amino acid analysis (AAA). SRM parameters were optimized by direct infusion experiments on a TSQ Quantum Ultra triple quadrupole mass spectrometer (Thermo Fisher Scientific), where the 6–8 most intense fragment ions for each peptide were selected as precursor-to-fragment transitions and the collision energy (CE) of each transition was optimized automatically in SRM mode. The peptides were dissolved in a buffer containing 50% acetonitrile and 0.1% formic acid and the infusion rate was 300 nL/min.

The transitions and corresponding optimal CE values from the infusion experiments were further validated for optimal detection of the target peptides in actual LC-SRM analysis. In this step 50 fmol/μL of heavy peptide standards were spiked with 0.5 μg/μL of VCaP-derived tryptic peptides and 2 μL of the sample were analyzed using a nanoACQUITY UPLC® system (Waters Corporation, Milford, MA) and a TSQ Vantage triple quadrupole mass spectrometer (Thermo Fisher Scientific). A transition with a low intensity or a high level of interference was not selected. Three transitions per peptide were retained for the final SRM assays.

2.4. PRISM fractionation

Five fmol/μL of high-purity heavy peptides (purity > 97%) were spiked with 1 μg/μL of peptides from each cell line or tissue sample and the peptides were separated following the PRISM workflow using high pH reversed-phase capillary LC on a nanoACQUITY UPLC® system as described previously (Shi et al., 2012). Briefly, separations were performed using a capillary column packed in house (3 μm Jupiter C18 bonded particles, 200 μm i.d. × 50 cm long) at a flow rate of 3.3 μL/min on binary pump systems, using 10 mM ammonium formate (pH 10) as mobile phase A and 10 mM ammonium formate in 90% acetonitrile (pH 10) as mobile phase B. Forty-five microliters of each sample (1 μg/μL) were loaded onto the column and separated using a binary gradient of 5–15% B in 15 min, 15–25% B in 25 min, 25–45% B in 25 min, and 45–90% B in 38 min. Following the LC separation, the eluent from the capillary column was split into two flowing streams (1:10 split) via a Tee union. The smaller fraction of eluent was sent at a flow rate of 300 nL/min to a TSQ Quantum Ultra triple quadrupole mass spectrometer for on-line SRM monitoring of heavy peptide standards. TSQ Quantum Ultra was operated with ion spray voltages of 2400 ± 100 V, a capillary offset voltage of 35 V, a skimmer offset voltage of −5 V, and a capillary inlet temperature of 220 °C. Tube lens voltages were obtained from automatic tuning and calibration without further optimization. Both Q1 and Q3 were set at unit resolution of 0.7 FWHM and Q2 gas pressure was 1.5 mTorr. A scan width of 0.002 *m/z* and a dwell time of 10 ms were used. A large fraction of the capillary column eluent flowing at a rate of 3 μL/min was automatically collected every 1 min into a 96-well plate using a Triversa NanoMate® system (Advion BioSciences, Ithaca, NY) over the course of ~100 min LC separation. Prior to peptide fraction collection, 17 μL of water was added to each well in the plate to avoid peptide loss and also to dilute the peptide fraction for LC-SRM analysis. The fraction containing a target peptide was intelligently selected based on the retention time of the peptide obtained by on-line monitoring. The detailed method for intelligent selection was described in our previous study (Shi et al., 2012).

2.5. LC-SRM analysis

Following high pH capillary RPLC separation and intelligent selection, the fractions containing the target peptides were subjected to LC-SRM analysis. All peptide fractions were analyzed using a nanoACQUITY UPLC® system coupled on-line to a TSQ Vantage triple quadrupole mass spectrometer. The UPLC® system was equipped with an ACQUITY UPLC BEH 1.7 μm C18 column (75 μm i.d. × 25 cm), which was connected to a chemically etched 20 μm i.d. fused-silica emitter via a Valco stainless steel union. Four microliters of each peptide fraction were loaded onto the column at a flow rate of 1 μL/min for 5 min. Peptides were separated at a flow rate of 500 nL/min using a 10 min linear gradient from 5 to 65% acetonitrile in water. The TSQ Vantage was operated in the same manner as the TSQ Quantum Ultra.

2.6. Calibration curve experiments

Tryptic peptides from four TMPRSS2-ERG negative tissue samples were pooled and used as the matrix for the

calibration curve experiments. Heavy standard peptides were spiked into the matrix at a constant concentration of 5 fmol/μL, while light peptides (purity >97%) were spiked at 0, 0.5, 1, 2, 5, 10, 20, 50, 100 and 500 amol/μL levels. The concentration of peptide mixture in each individual data point was adjusted to 1 μg/μL. All spike-in samples were analyzed using the same PRISM-SRM method used for analysis of prostate cancer cell line and tumor tissue samples as mentioned above. Light to heavy peak area ratios were plotted against the corresponding light peptide concentration values to build a calibration curve for each peptide. The limit of detection (LOD) and limit of quantification (LOQ) were defined as the lowest concentration point of target peptides at which the S/N of surrogate peptides was at least 3 and 10, respectively. Signal to noise ratio (S/N) was calculated by the peak apex intensity over the highest background noise in a retention time region of ±10 s for the target peptides.

2.7. Data analysis

The raw data acquired on the TSQ Vantage triple quadrupole MS were initially imported into Skyline software (MacLean et al., 2010) for visualization of chromatograms of target peptides and to determine which peptides can be detected. The detected peptides were further quantified using Xcalibur 2.0.7 (Thermo Fisher Scientific). The most abundant transition for each peptide was used for quantification unless interference was observed. Peak detection and integration were based on two criteria: 1) the same retention time and 2) approximately the same relative peak intensity ratios across multiple transitions between light peptide and heavy peptide standards. All data were manually inspected to ensure correct peak detection and accurate integration. Light to heavy peak area ratios were used to quantify target peptides. The expression level of each peptide in cell line or tissue samples (amol/μg of total protein) was calculated by the following equation: (amol/μL concentration of endogenous target peptide calculated based on calibration curve) × (μL loading volume on-column)/(μg loading amount on-column). Extracted ion chromatograms (XICs) were created using Skyline.

3. Results

3.1. Study design

The goal of this study was to accurately detect and quantify TMPRSS-ERG fusion products in prostate cancer at the protein level. To achieve this goal, we have applied an antibody-independent PRISM-SRM method. Briefly, a list of peptides that uniquely represent ERG protein were selected and synthesized for development of SRM assays, and then ERG-derived target peptides in prostate cancer samples were enriched and intelligently selected using PRISM and were further quantified by LC-SRM. The workflow for this study is summarized in Supplementary Figure S1.

3.2. Peptide selection and SRM assay development

The selection of peptides is a critical step in the development of sensitive SRM assays. A commonly used criterion is to select peptides that have previously been detected through large-scale shotgun analyses (Picotti and Aebersold, 2012). However, the transcriptional regulator ERG is such a low abundance protein that it has not been detected in either our internal shotgun data sets or publicly available databases such as PeptideAtlas (Desiere et al., 2005). To ensure that ERG protein can be detected with high sensitivity, we selected 16 unique peptides covering different sequence regions of various ERG protein isoforms. Four out of the 16 peptides are shared by almost all ERG isoforms while the other 12 peptides are less common (Table 1).

SRM parameters were manually optimized by direct infusion to ensure high sensitivity of SRM assays. The 6 most intense transitions of each peptide resulting from the infusion analyses were further inspected by LC-SRM where VCaP lysate was used as matrix. The transitions showing low intensity signals or high levels of interference were eliminated. Finally, the top 3 transitions were selected for monitoring each target peptide. The transitions for the 16 peptides and the corresponding optimal collision energy for each transition are listed in Table S2.

3.3. Detection of multiple isoforms of ERG protein in TMPRSS2-ERG gene fusion positive prostate cancer cell lines and tumor tissues

Using the PRISM-SRM strategy, TMPRSS2-ERG fusions were detected at the protein level in TMPRSS2-ERG gene fusion positive prostate cancer cell lines and tumor tissues with confirmed fusions at the genome level.

A total of 9 TMPRSS2-ERG gene fusion positive controls were included in this study, where 2 were cell lines (VCaP and NCI-H660) and 7 were prostate tumor tissues (PT1 to PT7). SRM monitoring of the 16 selected peptides showed that multiple peptides were simultaneously detected in 8 out of the 9 positive controls. The only exception was the tissue sample PT3 where only 1 unique peptide was detected.

Substantial expression of ERG protein was observed in the VCaP cell line, one of the most extensively used *in vitro* models for TMPRSS2-ERG gene fusion. Figure 1A depicts XICs of transitions monitored for the 6 peptides detected in the VCaP cell line, where the bottom panel indicates the responses of the endogenous ERG peptides. Interestingly, peptide VIVPADPTLWSTDHVR showed ~50- to 100-fold higher response than the other peptides in the VCaP cell line. In the NCI-H660 cell line, only 2 ERG-derived peptides were detected and the responses of these peptides were ~10-fold lower than those in the VCaP cell line (Supplementary Figure S2).

ERG protein was also detected in TMPRSS2-ERG gene fusion positive prostate tumor tissues. As an example, Figure 1B demonstrates that 5 unique ERG peptides were detected in sample PT1 where peptide VIVPADPTLWSTDHVR again displayed the highest response. ERG protein was also detected in all the other 6 TMPRSS2-ERG positive tissue samples (Supplementary Figure S3), indicating that the expression of ERG protein may be a common feature for prostate cancer patients with the TMPRSS2-ERG gene fusion.

Overall, a total of 7 unique ERG peptides were detected in TMPRSS2-ERG positive samples; 5 of these were consistently

Table 1 – ERG-derived peptides selected for PRISM-SRM assays as well as their expression in various ERG protein isoforms. "X" means the ERG protein isoform listed in a row contained the peptide listed in a certain column. The peptides detected in this study were highlighted in either green or red. The peptides not detected were shown in black. The two peptides highlighted in red are mutually exclusive in each ERG isoform.

Peptide	MIQTVPDPAAHIK	MASTIK	TEMTASSSSDYGQTSK	MTASSSSDYGQTSK	MECNPSQVNGSR	MVGSPDTVGMNYGSYMEEK	MNYGSYMEEK	HMPPPNMTTNER	VIVPADPTLWSTDHVR	VIVPADLPYEPPR	ITTRPDLPYEPPR	ITTRPAAQPSPSTVPK	NTDLPYEPPR	ITTRPGTK	ITTRPVSYR	ALSHVIQR
Exon	E4	E4	E6	E6	E6	E7	E7	E7	E7/E8	E7/E11	E10/E11	E10/E12	E9/E11	E10/E11	E10/E11	E10/E11
T1_E3_type_II_met1/1-462	X		X		X	X		X	X				X			
T2_E2_type_V_met1/1-462	X		X		X	X		X	X				X			
T1_E2_type_I_met1/1-462	X		X		X	X		X	X				X			
NM_004449/1-462	X		X		X	X		X	X				X			
NM_001243428/1-486	X		X		X	X		X	X		X					
NM_001136154/1-486	X		X		X	X		X	X		X					
T3_E4_type_VIII_met1/1-423				X	X	X		X	X				X			
T2_E4_type_VI_met2/1-423				X	X	X		X	X				X			
T1_E3_type_II_met2/1-423				X	X	X		X	X				X			
T2_E2_type_V_met2/1-423				X	X	X		X	X				X			
T1_E2_type_I_met2/1-423				X	X	X		X	X				X			
T1_E4_type_III_met1/1-423				X	X	X		X	X				X			
T1_E4_type_III_met2/1-409					X	X		X	X				X			
T3_E4_type_VIII_met2/1-409					X	X		X	X				X			
T2_E5_type_VII_met2/1-354							X	X	X				X			
T1_E5_type_IV_met2/1-354							X	X	X				X			
T2_E5_type_VII_met1/1-363						X		X	X				X			
T1_E5_type_IV_met1/1-363						X		X	X				X			
NM_001243429/1-363						X		X	X				X			
NM_001136155/1-387						X		X	X		X					
ENSP00000381877/1-455		X	X		X	X		X	X				X			
NM_182918/1-479		X	X		X	X		X	X		X					
T2_E4_type_VI_met1/1-454			X		X	X		X	X				X			
ENSP00000381879/1-456		X	X		X	X		X	X			X				
ENSP00000381881/1-463	X		X		X	X		X	X			X				
NM_001243432/1-317	X		X		X	X		X	X		X					
ENSP00000415659/1-172		X	X		X	X		X		X						
ERG8/1-325	X		X		X	X		X	X					X		X
TEPC/1-220				X	X	X		X	X						X	

present in both cell lines and prostate cancer tissues. We mapped these peptides to various isoforms of ERG protein where the exon information was included (Supplementary Figure S4). While most of the detected peptides are encoded by different exons, peptides MVGSPDTVGMNYGSYMEEK and HMPPPNMTTNER are translated from the same exon (exon 7). Of significance was that peptides ITTRPDLPYEPPR and NTDLPYEPPR are mutually exclusive; that no single isoform contains both of these peptides (highlighted in red in Table 1; Figure S4). The detection of both ITTRPDLPYEPPR and NTDLPYEPPR in the VCaP cell line as well as in the PT1 and PT2 tissues indicated that at least two distinct isoforms of ERG protein were simultaneously expressed in these TMPRSS2-ERG positive samples.

3.4. *Quantification of ERG-derived peptides in prostate cancer cell lines and tumor tissues*

The abundance of a target peptide can be determined by the peak area ratio of light (endogenous) peptide to a heavy peptide standard spiked into each sample at known concentration. In this study the most abundant transition for each peptide was used for quantification. The peak area ratios of

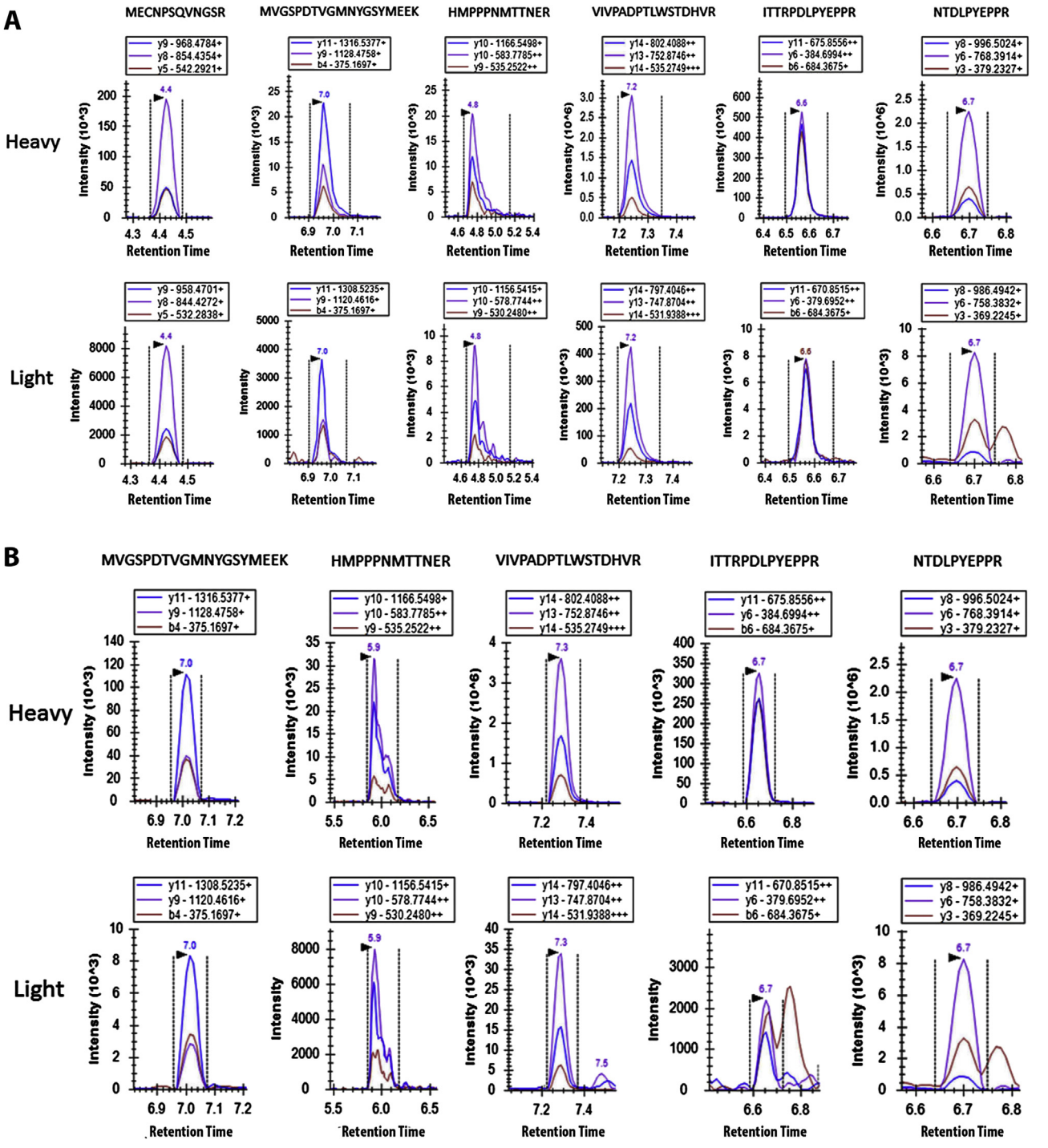

Figure 1 – Multiple peptides derived from ERG protein are simultaneously detected in TMPRSS2-ERG gene fusion positive prostate cancer cell line VCaP (A) and tumor tissue PT1 (B). The transitions monitored for each peptide were plotted as overlaid extracted ion chromatograms (XICs). XICs of a target peptide (Light) and the corresponding heavy internal standard (Heavy) were shown as a group where the peptide sequence was indicated on top of each group. All peptides were analyzed in triplicate and one representative XIC for each peptide was shown here.

all the 7 peptides detected in the 9 TMPRSS2-ERG positive samples are listed in Table S3. These values ranged from 0.00027 to 0.26. We also calculated the coefficient of variation (CV) values based on the peak area ratio values for each peptide in triplicate measurements of each cell line and patient sample. The observed CV values across almost all samples were less than 15% (Table S3), suggesting that high reliability was achievable with our SRM assays.

To accurately quantify the ERG-derived peptides, we built a calibration curve and determined the LOD and LOQ values for

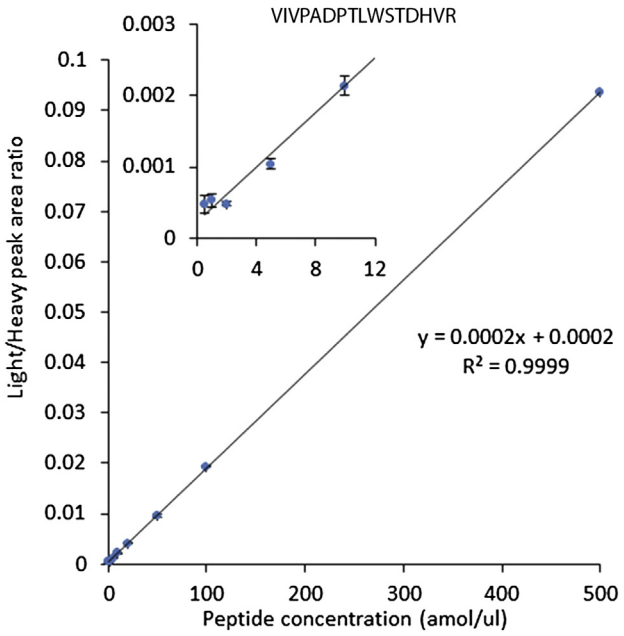

Figure 2 – Calibration curve of peptide VIVPADPTLWSTDHVR. The light to heavy peak area ratio of the transition *m/z* 602.66 → 747.87 was plotted against the corresponding light peptide concentration (amol/μL). The heavy peptide standard was consistently spiked at 5 fmol/μL. Nine data points were used to build the curve, where each data point was measured in triplicate. Error bars represent standard deviation of three measurements for each data point. The insert plot shows a more detailed view of lower end of the curve.

each peptide. This was achieved by a spike-in experiment where a tryptic peptide mixture of 4 TMPRSS2-ERG negative tissue samples was used as matrix (see "Experimental Procedures" for details of this experiment). Figure 2 shows the calibration curve of peptide VIVPADPTLWSTDHVR based on the transition 602.66 → 747.87. Excellent linearity was observed over a concentration range of 2–500 amol/μL. The peptide can still be detected at a concentration as low as 0.5 amol/μL, but the response becomes nonlinear when the concentration is lower than 2 amol/μL. Based on these observations, we concluded that the peptide VIVPADPTLWSTDHVR had an LOD of 0.5 amol/μg of total protein and an LOQ of 2 amol/μg of total protein, which are equivalent to 4.5 amol and 18 amol of peptide on column for LOD and LOQ, respectively. The calibration curves of all the other 6 detected ERG peptides are shown in Supplementary Figure S5. The LOD for these peptides ranged from 4.5 to 45 amol or 0.5 to 5 amol/μg of total protein, and the LOQ ranged from 18 to 450 amol or 2 to 50 amol/μg of total protein (Table 2).

We quantified those ERG-derived peptides based on the peak area ratio values and the calibration curves, where the expression level of each peptide was represented by amol/μg of total protein. The calculation was described in "Experimental Procedures". The expression levels of the 6 peptides detected in the TMPRSS2-ERG positive cell lines (VCaP and NCI-H660) ranged from ~20 to 1200 amol/μg of total protein (Table 3). Similar ERG expression patterns were observed in prostate tumor tissue samples, where ERG-derived peptides were also expressed at widely variable levels – ranging from ~10 to 1200 amol/μg of total protein in the 7 TMPRSS2-ERG positive tissues (Table 4). The abundance of all 7 detected ERG peptides in TMPRSS2-ERG positive cell lines and tissues was depicted in a clustered bar chart (Supplementary Figure S6). Note that the most abundant peptides were MVGSPDTVGMNYGSYMEEK, HMPPPNMTTNER, and VIVPADPTLWSTDHVR that are shared by almost all ERG isoforms (Table 1). These peptides are suitable as "signature" peptides for highly sensitive, highly specific detection of ERG protein expression.

3.5. *ERG protein expression is highly correlated with ERG gene rearrangement*

We also analyzed 9 TMPRSS2-ERG fusion negative controls, including 4 classic prostate cancer cell lines (LNCaP, DU145, PC3 and 22RV1) and 5 prostate tumor tissues. PRISM-SRM analysis showed that none of the ERG unique peptides were detected in the TMPRSS2-ERG negative samples with one exception, sample NT2, which had low level expression of a single peptide (Table 4). Figure 3 compares the expression patterns of the ERG peptide VIVPADPTLWSTDHVR in TMPRSS2-ERG gene fusion positive versus negative samples. While this peptide was clearly detected in all TMPRSS2-ERG positive cell lines (Figure 3A) and in 6 out of 7 TMPRSS2-ERG positive tumor tissues (Figure 3B), it was not detected in the 4 negative cell lines (Figure 3A) or the 5 negative tumor tissues (XICs not shown). There was a consistently high correlation between ERG protein levels, detected by PRISM-SRM, and the presence of TMPRSS2-ERG gene rearrangements.

4. Discussion

The TMPRSS2-ERG gene fusion represents one of the most specific biomarkers for prostate cancer, having nearly 100% specificity (Mosquera et al., 2009; Tomlins et al., 2009). Numerous studies have demonstrated that TMPRSS2-ERG transcripts are over-expressed in prostate cancer due to rearrangement of TMPRSS2 and ERG genes (Demichelis et al., 2007; Perner et al., 2006; Tomlins et al., 2005). TMPRSS2-ERG transcripts are translated into a truncated ERG protein product, which may serve as a diagnostic marker for prostate cancer and may also play a critical role in cell signaling (for example, androgen signaling) involved in the development and progression of prostate cancer (Zong et al., 2009). However, most of the studies on TMPRSS2-ERG gene fusion have thus far been performed at the mRNA level rather than at the protein level due to the lack of high-quality antibodies against ERG protein. MS-based SRM assays provide a powerful alternative to antibody-based assays for quantification of target proteins. However, the sensitivity of conventional SRM is much less than immunoassays and does not allow for detection of the low-abundance ERG protein. Recently, our group developed the 2D-LC-based PRISM-SRM strategy which allows for >100-fold improvement in the sensitivity of target protein detection and quantification (Shi et al., 2012). Using this method, we successfully quantified ERG protein at levels as low as ~10 amol/μg of total protein in prostate tumor tissue samples

Table 2 – LOD and LOQ of each ERG peptide detected in this study.

Peptide	Transition[a]	LOD		LOQ	
		amol	amol/μg total protein	amol	amol/μg total protein
TEMTASSSSDYGQTSK	840.36 → 1146.49	18	2	180	20
MECNPSQVNGSR	689.80 → 844.43	45	5	180	20
MVGSPDTVGMNYGSYMEEK	1047.94 → 1308.52	45	5	450	50
HMPPPNMTTNER	712.82 → 578.77	18	2	180	20
VIVPADPTLWSTDHVR	602.66 → 747.87	4.5	0.5	18	2
ITTRPDLPYEPPR	518.95 → 379.70	45	5	90	10
NTDLPYEPPR	601.30 → 758.38	45	5	90	10

a The transition that was used to determine LOD and LOQ values.

Table 3 – Quantification of ERG peptides in ERG$^+$ and ERG$^-$ cell lines. The expression levels of these peptides were represented by amol/μg of total protein. Each value represents the mean and standard deviation (SD) of three replicate measurements. VCaP and NCI-H660 are TMPRSS2-ERG fusion positive cell lines; LNCaP, DU145, PC3 and 22RV1 are TMPRSS2-ERG fusion negative cell lines.

Sequence	amol/μg of total protein					
	VCaP	NCI-H660	LNCaP	DU145	PC3	22RV1
MECNPSQVNGSR	189 ± 20					
MVGSPDTVGMNYGSYMEEK	618 ± 26					
HMPPPNMTTNER	1240 ± 17	84 ± 11				
VIVPADPTLWSTDHVR	726 ± 16	98 ± 3				
ITTRPDLPYEPPR	73 ± 2					
NTDLPYEPPR	17 ± 1					

(Table 4), and demonstrated superior sensitivity of PRISM-SRM over in-house ERG antibodies in detecting recombinant ERG proteins (unpublished results).

In addition to sensitivity, specificity is a key factor influencing the accuracy of an assay. The specificity of immunoassays can be compromised due to cross-reactivity of antibodies with nonspecific molecules. One of the major advantages of an SRM assay over an immunoassay is its high specificity, as the peptide sequence is confirmed at the product ion level. In this study all 7 detected peptides are unique to ERG protein as confirmed by a BLAST search. Moreover, both the parent ion and several product ions were monitored for each unique peptide in SRM analyses, which further improved the analytical specificity. Based on specific detection of peptides uniquely representing our target protein ERG, we achieved ultra-high specificity for ERG analyses.

In principle SRM-based assays can provide information on protein isoforms by monitoring of isoform-specific peptides. As shown in Table 1 and Figure S4, given the substantial sequence homology among potential TMPRSS2-ERG gene fusion protein products and the limited repertoire of specific enzyme-cleavage (e.g., tryptic) peptides, it is difficult to distinguish among potential isoforms (in fact some TMPRSS2-ERG fusions produce the same truncated ERG proteins; see Table 1 and Figure S4 for details). In addition the inability to detect given isoform-specific peptide(s) does not exclude the

Table 4 – Quantification of ERG peptides in ERG$^+$ and ERG$^-$ tissues. The expression levels of these peptides were represented by amol/μg of total protein. Each value represents the mean and SD of three replicate measurements. NQ means the peptide was detected, but its amount was lower than the LOQ and could not be accurately quantified. PT1-PT7 were TMPRSS2-ERG fusion positive prostate tumor tissues; NT1-NT5 were TMPRSS2-ERG fusion negative prostate tumor tissues.

Sequence	amol/μg of total protein											
	PT1	PT2	PT3	PT4	PT5	PT6	PT7	NT1	NT2	NT3	NT4	NT5
TEMTASSSSDYGQTSK							287 ± 11					
MVGSPDTVGMNYGSYMEEK	339 ± 6	50 ± 17		80 ± 16	382 ± 9	117 ± 16	546 ± 38					
HMPPPNMTTNER	1210 ± 35	169 ± 34	17 ± 4	78 ± 12	760 ± 41	543 ± 42	762 ± 28		198 ± 46			
VIVPADPTLWSTDHVR	48 ± 1	NQ		NQ	30 ± 2	74 ± 6	35 ± 1					
ITTRPDLPYEPPR	31 ± 3	10 ± 1										
NTDLPYEPPR	26 ± 2	10 ± 1		NQ								

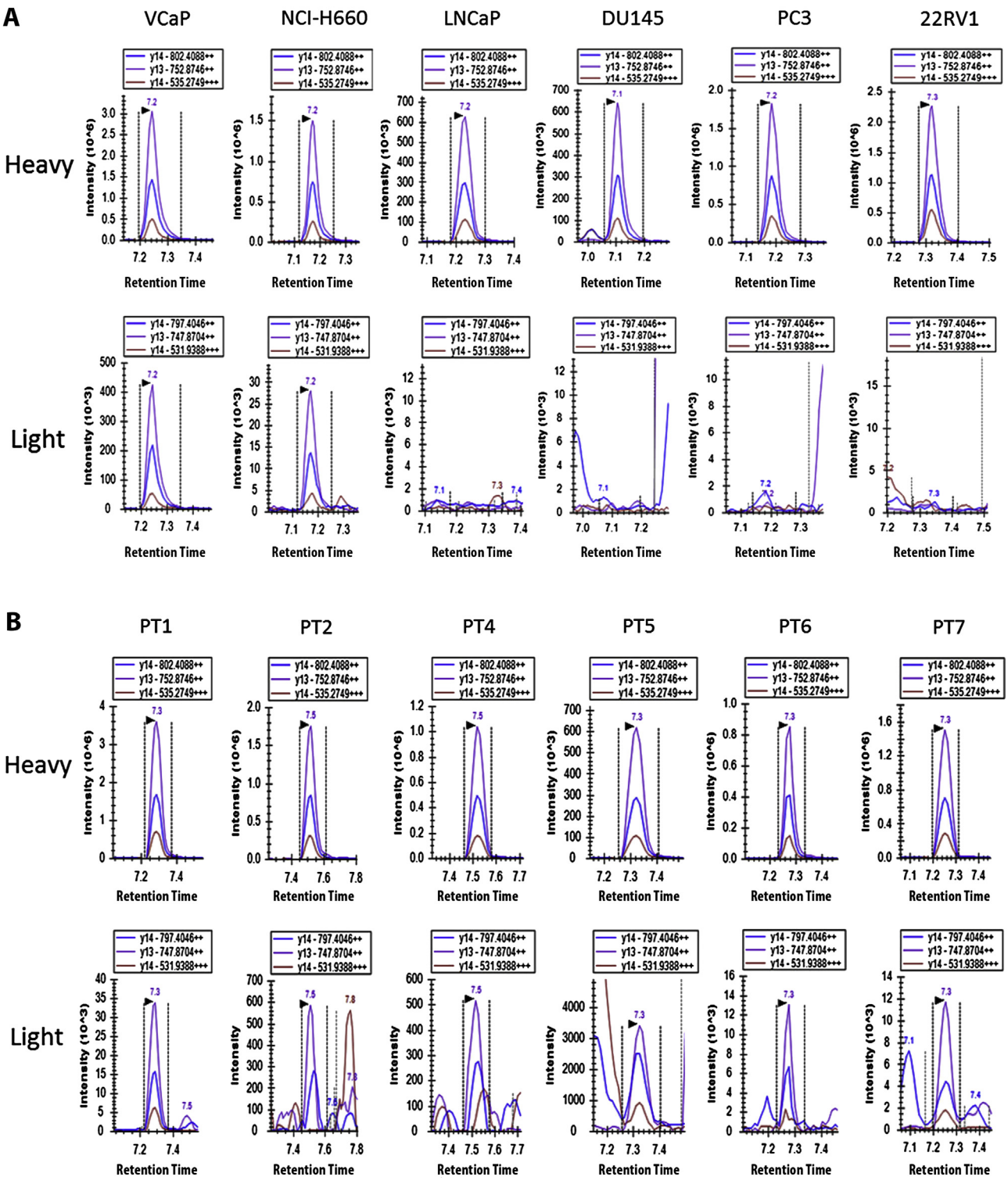

Figure 3 – Expression of an ERG derived peptide VIVPADPTLWSTDHVR in prostate cancer cell lines (A) and tumor tissues (B). (A) The peptide was detected in TMPRSS2-ERG gene fusion positive prostate cancer cell lines (VCaP and NCI-H660), but was not detected in TMPRSS2-ERG gene fusion negative prostate cancer cell lines (LNCaP, DU145, PC3 and 22RV1). (B) The peptide was detected in 6 out of the 7 TMPRSS2-ERG gene fusion positive prostate tumor tissues (PT1, PT2, PT4–PT7), but was not detected in the 5 TMPRSS2-ERG gene fusion negative prostate tumor tissues (XICs not shown). All samples were analyzed in triplicate and one representative XIC for each sample was shown here.

presence of a particular isoform either, because there are other "shared" peptides that are detected. In this study we confirmed the concurrent presence of at least two clearly distinguishable groups of isoforms of ERG protein in the same TMPRSS2-ERG positive samples, based on our observation that two mutually exclusive ERG peptides (ITTRPDLPYEPPR and NTDLPYEPPR; Table 1 and Figure S4) were simultaneously detected in several samples (Tables 3 and 4). This is the first documentation at the

protein level of a potential multi-focal origin for prostate cancer, driven by distinct TMPRSS2-ERG fusion events or by the translation of multiple alternative transcripts. The quantitative SRM results on the limited set of peptides are not able to identify exactly which fusion protein products are expressed in the samples, however, the detection of certain peptides, as well as their abundance information, can serve as unique "profiles" for the expression status of various TMPRSS2-ERG fusion protein products in a given sample (Supplementary Figure S7).

The broad range of abundances of the detected ERG peptides (Tables 3 and 4) indicated that multiple isoforms of ERG protein were potentially expressed at widely variable levels in TMPRSS2-ERG positive prostate cancer cell lines and tumor tissues. In addition there are other factors that could cause variable detection levels of different peptides in a sample, such as the differences in tryptic digestion efficiency, peptide-specific interference, and endogenous proteolytic cleavage. Nevertheless, the comparison of abundances of specific peptides across different clinical samples is expected to provide valuable information for diagnosis or monitoring of TMPRSS2-ERG prostate cancer. For the sake of determining ERG over-expression as a result of TMPRSS2-ERG fusion, we recommend the SRM analysis of peptides MVGSPDTVGMNYGSYMEEK, HMPPPNMTTNER, and VIVPADPTLWSTDHVR which are shared across almost all possible fusion protein products and have shown to be the most abundant peptides in either cell line or tissue samples.

Our results also demonstrated that ERG protein expression was highly correlated with TMPRSS2-ERG rearrangements (Table 3, Table 4, and Figure 3). Indeed, Park et al. previously explored ERG protein expression by immunohistochemistry and observed high concordance between ERG gene rearrangement and ERG protein expression (Park et al., 2010). Compared with immunohistochemistry used in the study by Park et al., our PRISM-SRM method does not rely on antibodies and hence, PRISM-SRM was both cost- and time-efficient, and allowed for obtaining (1) accurate quantification and (2) isoform information of ERG protein expressed in the samples. This information should be valuable for an in-depth understanding of the role of ERG protein isoforms in the biology of prostate cancer. Considering the high sensitivity, high specificity, and antibody-independent features of the PRISM-SRM approach, the method is expected to enable simultaneous quantification of many other protein biomarkers in various types of biological samples.

5. Conclusion

We have successfully quantified the TMPRSS2-ERG gene fusion protein products in prostate cancer cell lines and prostate tumor tissues using an antibody-independent PRISM-SRM approach. By monitoring for multiple unique ERG peptides, we proved that at least two ERG protein isoforms were simultaneously expressed in some TMPRSS2-ERG gene fusion positive samples. We also identified three "signature" peptides MVGSPDTVGMNYGSYMEEK, HMPPPNMTTNER, and VIVPADPTLWSTDHVR for detecting general ERG over-expression resulting from TMPRSS2-ERG fusion. Our results showed that ERG protein expression was highly correlated with TMPRSS2-ERG gene rearrangement. TMPRSS2-ERG gene fusion transcripts play an important role in stratifying prostate cancer risk (Tomlins et al., 2011). Our results indicated that ERG protein expression may be useful for molecularly subtyping prostate cancer and prostate needle biopsy evaluation (Park et al., 2010). PRISM-SRM measurements provided not only quantitative, but also isoform-specific information (information that other technologies are currently "blind" to) about ERG protein expression in prostate cancer samples. It is anticipated that broad application of the PRISM-SRM assays in prostate cancer studies will further improve our understanding of the role of TMPRSS2-ERG gene fusion in the progression of prostate cancer.

6. Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molonc.2014.02.004.

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