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Protein isoform-specific validation defines multiple chloride intracellular channel and tropomyosin isoforms as serological biomarkers of ovarian cancer

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Abstract

New serological biomarkers for early detection and clinical management of ovarian cancer are urgently needed, and many candidates have been reported. A major challenge frequently encountered when validating candidates in patients is establishing quantitative assays that distinguish between highly homologous proteins. The current study tested whether multiple members of two recently discovered ovarian cancer biomarker protein families, chloride intracellular channel (CLIC) proteins and tropomyosins (TPM), were detectable in ovarian cancer patient sera. A multiplexed, label-free multiple reaction monitoring (MRM) assay was established to target peptides specific to all detected CLIC and TPM family members, and their serum levels were quantitated for ovarian cancer patients and non-cancer controls. In addition to CLIC1 and TPM1, which were the proteins initially discovered in a xenograft mouse model, CLIC4, TPM2, TPM3, and TPM4 were present in ovarian cancer patient sera at significantly elevated levels compared with controls. Some of the additional biomarkers identified in this homolog-centric verification and validation approach may be superior to the previously identified biomarkers at discriminating between ovarian cancer and non-cancer patients. This demonstrates the importance of considering all potential protein homologs and using quantitative assays for cancer biomarker validation with well-defined isoform specificity.

1. Introduction

Epithelial ovarian cancer (EOC) is the most lethal gynecological cancer in the United States, with an estimated 22,280 new cases detected and 15,500 deaths in 2012.[1] When diagnosed early (Stages I/II), treatment is generally successful, with a five-year survival rate of up to 90%; but unfortunately, most cases are not detected until after the cancer has spread, resulting in a dismal five-year survival rate of 30% or less.[2–3] There are currently no effective screening tests for EOC early detection, and existing clinical tests using protein

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biomarkers, such as cancer antigen 125 (CA-125), human epididymis protein-4 (HE4), or multivariate OVA1, are only approved for monitoring disease recurrence, therapeutic response, or for use in managing women with an ovarian adnexal mass.[4–7] The most commonly used EOC biomarker, CA125, is recognized as a poor biomarker for early detection due to its high false-positive rate and poor sensitivity and specificity.[8–9] Better biomarkers or, more likely, panels of markers are urgently needed to diagnose early-stage EOC with high sensitivity and specificity, and for clinical management of the disease after initial diagnosis.

We and others have leveraged proteomics to discover new EOC biomarkers. Diverse experimental systems, including cancer cell cultures, tissue specimens, ascites fluid, secretomes, and mouse models, have been investigated using numerous proteomics strategies in attempts to identify better EOC biomarkers.[10–21] Using an in-depth 4D analysis of serum from severe combined immunodeficiency (SCID) mice containing a human endometrial ovarian cancer tumor, we recently identified 106 candidate human proteins with at least two peptides.[21] In that study, we performed a pilot validation on candidate biomarkers in the 20–55 kDa region of 1D SDS gels and found that nearly half the proteins discovered in the xenograft mouse model could be detected in human serum using multiple reaction monitoring analysis. Two of the tested candidates, chloride intracellular channel 1 (CLIC1) and cathepsin D 30 kDa fragment (CTSD-30kDa), showed significantly elevated serum levels in cancer patients compared with non-cancer controls.[21]

A major advantage of xenograft mouse models is that proteins shed by human tumors into mouse blood can be unambiguously distinguished by exploiting species differences in peptide sequences identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS). However, the capacity to distinguish species differences diminishes as the sequence homology between the two species for specific proteins increases, especially with lower-abundance proteins where sequence coverage is typically low. Similarly, the capacity to distinguish between homologous human members of protein families during the discovery phase is often limited by low sequence coverage of candidate biomarkers.

The high number of candidates identified using current proteomics methods, coupled with the lack of well-characterized immunoassays for most of the new candidates, necessitates the use of alternative quantitative techniques capable of screening candidates in patient serum or plasma. MRM has recently emerged as the most effective targeted quantitative technique for biomarker verification due to its high selectivity and multiplexing capability. [22–23] Despite the recent wide use of MRM as a quantitative tool in proteomics, concerns have been raised regarding its specificity and selectivity.[24–25] One of the major concerns is the specificity of peptide signals chosen for MRM quantitation, especially in a complex proteome background. In addition, the use of proteolytic fragmentation in discovery experiments creates a fundamental problem of protein inference.[26] In complex proteomes such as serum or plasma, it is often not trivial to reconnect peptides to a precise protein of origin due to the presence of shared tryptic peptides from protein isoforms (alternatively splicing forms from a single gene) and members of a protein family (proteins produced by homologous genes). Although the term “protein isoform” has been variably defined, in this study, protein isoform will be used to indicate related protein sequences from a single gene. These isoforms may be produced by alternative splicing, proteolytic processing, or other posttranslational modifications. Homologs or homologous proteins will be used to refer to members of a protein family produced by different genes.

The protein inference problem is further aggravated by substantial redundancy in the database caused by completely or nearly completely redundant entries, partial sequences, polymorphisms, sequencing errors, etc. In order to prevent inflation of protein counts in

discovery experiments, a common practice is to use the parsimony principle or the Occam's razor constraint to report a minimal list of proteins that can account for all observed peptides.[26] This often results in multiple protein entries being assigned to a set of peptides due to homologous proteins and redundancy in the database, and the actual protein(s) present in the sample can be ambiguous.

Importantly, the failure to detect peptides unique to an isoform does not prove the isoform is not present in the sample. This is particularly critical in biomarker studies, as the correct or best biomarker may be a protein family member or isoform that was not explicitly identified in the discovery phase. It is important to differentiate isoforms and homologous family members because these related proteins are often associated with distinct structural or functional roles [27–29]. This is particularly important in cancer biomarker validation studies, because only the specific members of a protein family that are shed by the tumor into the blood should ideally be quantified. In this regard, any assay, including sandwich ELISA assays, could give misleading results if the isoform and family member specificity is unknown or if multiple related proteins are quantitated as a group. MRM has been used successfully to quantitate specific protein isoforms or mutant proteins especially from cell extracts.[30–32] However, targeted serological quantitation of serum biomarkers using MRM methods is a relatively new approach, and little is known about how the accuracy of protein quantitation is affected by the isoform and family member specificity of the peptides chosen.

We have developed a gel-based, label-free MRM quantitation approach (GeLC-MRM) as a rapid, first-level biomarker verification strategy using human plasma or serum samples.[21, 33–34] This approach consists of major protein depletion, 1-D gel separation, in-gel trypsin digestion, and reversed-phase nanoLC-MRM analysis.[33] We previously demonstrated the reproducibility of the entire GeLC-MRM workflow and showed that it was capable of providing accurate relative quantitation of candidate biomarkers in the low ng/mL range from less than 100 μ L of serum or plasma.[33, 35] It also has the added capability of distinguishing various molecular weight-forms of proteins that often are not distinguished using alternative proteome fractionation strategies and may not be distinguished by some immunoassays. We have successfully applied the GeLC-MRM strategy to validate a number of ectopic pregnancy and ovarian cancer candidate biomarkers, and subsequently confirmed one of the ectopic pregnancy low-abundant candidates using higher throughput immunoassays on a larger patient sample set.[21, 33–35] In the current study, we used in-depth GeLC-MS/MS analysis of patient serum pools and isoform-specific MRM assays to identify and quantitate additional EOC biomarkers related to CLIC1 and tropomyosin 1 (TPM1) in individual patient sera. Additional members in both protein families were detected that significantly distinguished between cancer and control patients, and some of the newly identified biomarkers may be superior to the previously identified homologs.

2. Materials and Methods

2.1 Reagents and Chemicals

Dithiothreitol (DTT) was obtained from GE Healthcare (Piscataway, NJ). Iodoacetamide and 200 proof molecular biology grade ethanol were purchased from Sigma-Aldrich (St. Louis, MO). HPLC-grade acetonitrile was purchased from Thomas Scientific (Swedesboro, NJ). Sequencing-grade modified trypsin was purchased from Promega (Madison, WI).

2.2 Human Serum Collection and Processing

All research in this study involving human specimens was conducted under The Wistar Institute Institutional Review Board-approved protocols #2109171, #EX2110012, and #260221. All human samples were derived from subjects with informed written consent.

Data were analyzed anonymously. Serum samples were processed for MS analysis either individually or as pools, as previously described.[21] Characteristics of each ovarian cancer patient and non-cancer control are shown in Supplemental Table 3.

2.3 Immunoaffinity Depletion and SDS-PAGE

Samples were depleted of 20 abundant serum proteins using a ProteoPrep20 Immunodepletion Column (Sigma-Aldrich) as previously described.[21, 36] Briefly, 30 – 60 μ L of serum was filtered through a 0.22 μ m microcentrifuge filter and injected onto the column. The unbound proteins were collected, pooled, and precipitated with nine volumes of 200 proof ethanol prechilled to -20°C . Ethanol supernatants were carefully removed and protein pellets were dried, frozen and stored at -20°C until further use. Frozen protein pellets were thawed, resuspended in 1% SDS, 50 mM Tris-HCl pH8, reduced and alkylated prior to SDS-PAGE. For all samples, an equivalent of 10 μ L of original serum were loaded into three adjacent lanes, separated for 4 cm, and stained with Colloidal Blue (Invitrogen). All stained gels did not show any major differences in total gel lane intensities. Each gel lane was sliced into uniform 1 mm slices, and adjacent lane slices were combined and digested with modified trypsin (Promega, Madison, WI) as previously described.[36]

2.4 LC-MS/MS of Patient Sample Pools

To identify CLIC and TPM isoforms and family members that could be detected in ovarian cancer patient sera, label-free comparisons of pooled sera were performed. One pool of serum from patients with benign ovarian masses and three pools of advanced ovarian cancer patient serum samples were made. Serum pools were immunodepleted, separated on a 1-D SDS gel for 4 cm, sliced into 40 fractions, and digested with trypsin as described above. Eight μ L of tryptic digests were analyzed using an LTQ Orbitrap XL mass spectrometer (Thermo Scientific, Waltham, MA) connected to a NanoACQUITY UPLC system (Waters, Milford, MA). Peptides were eluted at 200 nL/min using a 229-min discovery gradient consisting of 5–28% B over 168 min, 28–50% B over 51.5 min, 50–80% B over 5 min, 80% B for 4.5 min, before returning to 5% B over 0.5 min. A short blank gradient was run before injecting the next sample. The mass spectrometer was set to scan m/z from 400 to 2000. The full MS scan was collected at 60,000 resolution in the Orbitrap in profile mode followed by data-dependant MS/MS scans on the six-most-abundant ions exceeding a minimum threshold of 1000 collected in the linear trap. Monoisotopic precursor selection was enabled and charge-state screening was enabled to reject $z = 1$ ions. Ions subjected to MS/MS were excluded from repeated analysis for 60 s.

2.5 Data Processing

MS/MS spectra were extracted and searched using the SEQUEST algorithm (v. 28, rev. 13, University of Washington, Seattle, WA) in Bioworks (v. 3.3.1, Thermo Scientific) against the human UniRef100 protein sequence database (v. June 2011) plus common contaminants, and decoy sequences. The decoy database was produced by reversing the sequence of each database entry, and the entire reversed database was appended in front of the forward database. Spectra were searched with a partial tryptic constraint with up to two missed cleavages, 100 ppm precursor mass tolerance, 1 Da fragment ion mass tolerance, static modification of cys (+57.0215 Da), and variable modification of methionine (+15.9949 Da). Consensus protein lists were created using DTASelect (v. 2.0, licensed from Scripps Research Institute, La Jolla, CA) and the following filters were applied: remove proteins that are subsets of others, full tryptic constraint, a minimum of two peptides, mass accuracy 10 ppm, and $C_n = 0.05$. We previously showed that this database search and filtering strategy enhanced high-confidence detection of low-abundance blood proteins compared with commonly used alternative strategies.[37] The peptide false discovery rate (FDR), calculated based on decoy count, was less than 1% based on redundant peptide count

(spectra count). The peptide FDR was 1% based on non-redundant peptide count. Quantitative comparisons of all detected CLIC and TPM family members across serum pools were performed using Rosetta Elucidator software to compare peptide signal intensities in full MS scans. Retention time alignment, feature identification (discrete ion signals), feature extraction, and protein identifications were performed by the Elucidator system as previously described.[21, 33–34]

2.6 Label-free GeLC-MRM Analysis

Serum samples were depleted of 20 abundant serum proteins, separated on a 1-D SDS gel for 4 cm, sliced into 40 fractions, and digested with trypsin as described above. [21] We had previously showed good reproducibility of the overall analytical pipeline, including major protein depletions, gels, trypsin digests, and the MRM analyses with consistent recovery of proteins in technical replicates.[33] MRM experiments were performed on a 5500 QTRAP hybrid triple quadrupole/linear ion trap mass spectrometer (AB SCIEX, Foster City, CA) interfaced with a NanoACQUITY UPLC system. Eight μ L of tryptic digests were injected using the partial loop injection mode onto a UPLC Symmetry trap column (180 μ m i.d. x 2 cm packed with 5 μ m C18 resin; Waters) and then separated by RP-HPLC on a BEH C18 nanocapillary analytical column (75 μ m i.d. x 25 cm, 1.7 μ m particle size; Waters) at 45 °C. Chromatography was performed with Solvent A (Milli-Q water with 0.1% formic acid) and Solvent B (acetonitrile with 0.1% formic acid). Peptides were eluted using a 27-min MRM gradient at 400 nL/min for 5–35% B over 24 min, 35% B for 3 min before returning to 5% B in 0.5 min. To minimize sample carryover, a fast blank gradient was run between each sample. MRM data were acquired at unit resolution in both Q1 and Q3 with a spray voltage of 3300 V, curtain gas of 20 p.s.i., nebulizer gas of 10 p.s.i., interface heater temperature of 150 °C, and a pause time of 3 ms. To monitor system performance, a reference enolase digest sample was interspersed between experimental samples, at approximately every 18 samples. We previously demonstrated the long-term reproducibility of label-free MRM quantitation, where the majority of the enolase peptides were found to have a coefficient of variation (CV) of < 16%.[33] In addition, MRM transitions for 4 trypsin self-digestion peptides (VATVSLPR, LSSPATLNSR, LGEHNIDVLEGNEQFINAAK and IITHPNFNGNTLDNDIMLIK) were also monitored in each sample to ensure retention time reproducibility of the LC system.

In MRM assay development, peptide candidates for targeted proteins were selected from the Orbitrap LC-MS/MS analysis described above. In some cases, peptide candidates were derived from prior human plasma/serum LC-MS/MS proteomic analyses. Initially, 4 or more transitions per peptides, including the most dominant y-ions observed from Orbitrap LC-MS/MS, were targeted in advanced ovarian cancer patient pooled samples known to contain the proteins of interest using the 229-min discovery gradient described above. Correct peptide MRM traces were selected based on overlapping MRM transitions at the retention time that correspond to the peptide retention time observed in the Orbitrap LC-MS/MS analysis. MRM-initiated detection and sequencing (MIDAS) was also used to generate MS/MS data to confirm peptide identities. Subsequently, three to four most intense MRM transitions were selected for each peptide, and samples containing the targeted proteins were re-analyzed using the shorter 27-min MRM gradient to determine the correct peptide retention times. In addition to the proteins described in this study, a number of other proteins were also monitored. A total of 172 transitions were monitored in the final method. Scheduled MRM was used to reduce the number of concurrent transitions and maximize the dwell time for each transition. The detection window was set at 3 min, and the target scan time was set at 1.8 s. With these parameters, the maximum concurrent transitions were 53, and with the expected peak width of ~22 s, a minimum of 10 data points per chromatographic peak was expected. Data analyses were performed using MultiQuant

version 2.1 software (AB SCIEX). Correct peptide MRM transitions have the expected retention times and consistent ratios of overlapping transitions. The most abundant interference-free transition for each peptide was used for quantitation.

Protein levels across samples were determined as previously described.[33] First, each peptide amount was determined by summing the peptide's peak area across all gel slices analyzed. The summed peptide area for each sample was then normalized by dividing it by the average value for that peptide in the advanced cancer samples. Finally, the protein amount in each sample was determined by taking the average of the normalized peptide values (normalized area).

2.7 Statistical Analyses

Serum levels of candidate biomarkers were compared across sample groups using the Mann-Whitney test, and Bonferroni-adjusted *P*-values were reported in scatter plots. Results were considered statistically significant if the Bonferroni-adjusted *P*-value of the test was less than 0.05. Spearman's correlation coefficients were calculated to examine correlations among all tested tropomyosin peptides. For each candidate biomarker, a receiver operator characteristic (ROC) curve was generated and the area under the curve was calculated to reflect biomarker-specific potential sensitivity and specificity for distinguishing non-cancer controls vs. cancer patients.

3. Result and Discussion

3.1 Ambiguities in Identification of EOC Candidate Biomarker Isoforms from Analysis of Xenograft Mouse Serum

We previously identified 106 human proteins with at least two peptides from the serum of a xenograft mouse model of human ovarian endometrioid cancer (TOV-112D tumors) using a gel-based, multidimensional protein profiling strategy.[21] In that study, GeLC-MRM quantitation of candidate biomarkers in the 20–55 kDa region showed that CLIC1 and the mature form of CTSD were significantly elevated in ovarian cancer patients compared with non-cancer individuals. An interesting candidate biomarker that was not included in that initial validation experiment was TPM1 isoform 6. This protein was initially identified as a human protein in the xenograft mouse serum based upon the detection of two human-specific peptides and four peptides common to human and mouse (Supplemental Table 1). But in the course of setting up assays for the current validation study, we observed that the two apparently human-specific peptides based upon use of the UniRef100 v. 2007 database were now shared with new mouse sequences in the UniProtKB 2011 database (Supplemental Figure 1). This meant that if the newer database had been used in the original xenograft mouse discovery experiment, TPM1 would not have been identified as a human protein but would have been categorized as “species indistinguishable,” as all identified peptides would be common to human and mouse. This change in species categorization due to database updates was not a widespread problem, because re-examination of the species specificity of our previously identified putative human peptides revealed that only seven peptides (approximately 1%) were identical to new mouse entries in the 2011 database.

As discussed above, inferring the correct peptide-protein association is often confounded by the presence of shared peptides from homologous proteins, alternative splice variants, or nearly identical database entries.[26] But for MRM quantitation of cancer biomarkers, it is important to accurately determine the peptide-protein relationship to ensure the correct family member and protein isoform is being quantitated. In order to determine all potential peptide-protein associations for the observed TPM peptides, each peptide identified in the xenograft mouse was searched against the human UniProtKB database (February, 2012) using the BLAST algorithm. All database entries containing the peptide sequence were

identified and redundant entries were manually removed. When available, gene names associated with each database entry were extracted (Table 1). These peptides show a great degree of ambiguity in peptide-protein association due to the large number of known TPM isoforms and the very high homology between TPM genes. Tropomyosin is encoded by four genes (*TPM1* to *TPM4*), and each gene can further generate multiple isoforms by the use of alternative promoters and/or alternative RNA splicing. More than 40 distinct TPM sequences have been reported in vertebrates.[38–39] The TPM1 peptides identified from the xenograft model were initially assigned to TPM1 isoform 6 (Q7Z6L8) using the parsimony principle to explain all the identified peptides (Supplemental Table 1). While BLAST indicates TPM1 is present, the exact TPM1 isoform is ambiguous. Furthermore, the presence of TPM2, TPM3, or TPM4 cannot be excluded and needs to be considered.

3.2 Protein Homologs Detectable in Patient Serum Pools that Correlate with EOC

To determine which TPM isoform(s) are detectable in ovarian cancer patient serum, we used an ovarian patient serum protein dataset from in-depth GeLC-MS/MS analysis of the 20–55 kDa region of one benign control and three different late-stage ovarian cancer patient immunoaffinity-depleted serum pools. In addition to TPM isoforms, we searched for additional isoforms and closely related homologs of CLIC1, Peroxiredoxin-6 (PRDX6), and CSTD, as these proteins were previously validated as promising EOC biomarkers from the TOV-112D xenograft model.[21] Results are summarized in Supplemental Table 2. No homologs for PRDX6 or CSTD were identified that had greater than 25% sequence identity, but CLIC4, a CLIC1 homolog, was identified in the ovarian cancer patient sera. Analysis of gel fractions beyond the 20–55 kDa region did not identify additional members of CLIC or TPM protein families. The amounts of all CLIC and TPM proteins identified in the patient sera were quantitated by summing MS intensities for all peptides unique to a specific gene product (Figure 1). There was evidence of protein products for all four TPM genes, and all gene products showed elevated levels in EOC. However, the different TPM gene products did not show consistent abundance level patterns across all cancer pools, indicating that these gene products were not coordinately shed into the blood of cancer patients.

In the case of TPM1, one new TPM1-specific peptide and two shared peptides were discovered in the patient serum in addition to all previously identified TPM1 isoform 6 peptides from the xenograft mouse serum (Figure 2, Table 1, Supplemental Table 2). Based on the newly identified AELSEGQVR peptide, all observed peptides were contained within two TPM1 isoforms, TPM1 variant 6 (Q1ZYL5) or B7Z596. These two sequences share 80% identity and differ from each other at the C-terminus. Distinguishing between these isoforms was not feasible in this study due to the inability to detect any isoform-specific C-terminal peptides. Although no other TPM1 isoforms were conclusively identified in human serum, their presence cannot be ruled out. But the failure to detect any unique peptides to other TPM1 isoforms suggests they are either not present or are present in much lower abundance in human serum.

CLIC1 was confirmed to be both detected and elevated in ovarian cancer patient serum compared to the benign control. Also, CLIC4 was detected by nine specific peptides and showed elevated levels in ovarian cancer patient sera, suggesting that it was an additional EOC candidate biomarker. But, similar to the TPMs, the CLIC gene products did not show consistent abundance level patterns across all cancer pools (Figure 1).

The detection of CLIC4 in ovarian cancer patient sera by nine specific peptides raised the question as to why only human CLIC1 had been previously identified in the xenograft mouse serum.[21] Examination of the xenograft mouse data showed that CLIC4 had been identified by four peptides; however, all peptides were identical to mouse sequences so this protein was identified as species indistinguishable (Supplemental Table 1). This is not

surprising, as the human and mouse CLIC4 sequences are 99% identical (Figure 3A). While distinguishing between mouse and human CLIC4 is very difficult, distinguishing the different CLIC gene products in human serum is more straightforward, as the four CLIC genes with similar molecular weights exhibit only moderate sequence homology (Figure 3B). Specifically, the two isoforms detected in ovarian cancer patient sera, CLIC1 and CLIC4, share 67% identity. Hence, most CLIC peptides observed in the xenograft mouse serum and in patient serum pools were unique to either CLIC1 or CLIC4.

3.3 Development of MRM Assays for Quantitation of CLIC4 and TPM Isoforms

CLIC and TPM isoform levels in individual serum samples that included 15 non-cancer control serum samples and 18 late-stage cancer samples were determined using GeLC-MRM. Peptides were selected based on their isoform specificity and signal intensity in MRM analysis using a 5500 QTRAP mass spectrometer. Peptide candidates for MRM were derived from a combination of the LC-MS/MS analyses reported above and all prior human plasma/serum LC-MS/MS proteomic analyses. In the case of CLIC4, selection of MRM peptides was relatively straightforward because no major homolog issues were encountered with the identified peptides (Figure 3B). Inclusion of peptides identified from other serum proteome analyses allowed selection of peptides with the strongest MRM signal. For example, the CLIC4 peptide, YLTNAYSR, was found to produce a stronger MRM signal than some of the peptides discovered in the above analysis of patient serum pools and was therefore used for MRM quantitation (Supplemental Table 3).

Selecting appropriate peptides for MRM quantitation of TPM isoforms in general, and TPM1 specifically, was more complicated due to the large number of TPM isoforms. While TPM1 variant 6 (or isoform B7Z596) was clearly identified in the human serum samples, other TPM1 isoforms also could be present (Figure 2). Therefore, AELSEGQVR, which was specific to TPM1v6, and three other peptides shared by several TPM1 isoforms were used for MRM quantitation (Table 1, Supplemental Table 3). We also attempted to target and quantitate TPM2, TPM3, and TPM4, as products of these genes were also identified in the GeLC-MS/MS analysis of patient serum pools (Supplemental Table 2). As is often the case when analyzing complex matrices such as serum, strong, consistent, interference-free MRM signals could not be obtained for all desired peptides. The final MRM assay contained one peptide specific to TPM3, two peptides specific to TPM4, one peptide shared by TPM2 and TPM4, and one peptide shared by all four TPM genes (Table 1, Supplemental Table 3).

3.4 Quantitation of TPM and CLIC Isoforms in Individual Patient Serum Samples

The levels of TPM and CLIC isoforms in individual patient samples were quantitated using GeLC-MRM. As in the case for ELISA assays commonly used to quantitate blood proteins, equal volumes of serum from each patient were processed in the same manner for MRM quantitation. We have previously demonstrated the accuracy, sensitivity, reproducibility, and linearity of peptide calibration curves of the label-free GeLC-MRM approach.[33] Specifically, we showed that the overall analytical pipeline, including duplicate immunodepletions and technical replicates were reproducible with most peptides displaying CVs of less than 15%.[33] Reproducibility of major protein depletions and trypsin digestions has also been demonstrated by others.[22, 40] GeLC-MRM quantitation of the CLIC and TPM peptides and normalized protein values (see Materials and Methods) for individual patient samples are summarized in Supplemental Table 3. TPM peptide amounts were graphically compared across all serum samples as a first-level test of potential differences across patients in peptides specific for certain isoforms and those shared by multiple isoforms both within the TPM1 isoform group and across gene products (Supplemental Figure 2). Similarly, the protein levels (normalized and averaged peptides values) across patient samples were compared as shown in Figure 4A. All the TPM peptides

and TPM family members displayed similar quantitative profiles across serum samples (Supplemental Figure 2, Figure 4A). However, a substantial number of the individual samples showed substantial differences in pairwise ratios of TPMs (Figure 4B), suggesting that different TPMs may have differing capacities to distinguish ovarian cancer patients from non-cancer cases. Nevertheless, Spearman's rank correlation coefficient analysis showed that all tropomyosin peptides analyzed here are highly correlated within each patient group with P -values < 0.001 (data not shown), indicating a lack of evidence that specific tropomyosins differ from other isoforms in being able to distinguish between ovarian cancer and non-cancer control. Also, if alternative TPM isoforms are present that were not considered in this study and they share some of the quantified peptides, their contribution is either minor or they track with the isoforms quantitated here. Finally, factor analysis shows that all the tropomyosin isoforms analyzed here are measuring the same factor (data not shown), which is consistent with the similar quantitative profiles shown in Figure 4A. Since the distribution of all tropomyosin peptides is similar, the peptide (K)LVILEGELER that is shared between TPM2 and TPM4 was assigned to TPM4 for the purpose of calculating the TPM4 protein level.

These data show that multiple TPMs that can be detected in human ovarian cancer patient sera in the present cohort appear to fluctuate in abundance levels in concert, although some variations in ratios of family members were observed in individual samples. To determine whether specific TPM family members or combinations of TPMs are superior ovarian cancer biomarkers than TPM1 alone will require testing of additional, larger patient cohorts. Furthermore, specific TPM family members may be more selective for specific clinical applications such as monitoring responses to de-bulking surgery, therapeutic response, or disease reoccurrence. Supporting this possibility, preliminary comparisons of biomarkers in patients with low CA125 levels pre- and post-surgery suggest that TPM3 may be a superior biomarker for this application (data not shown). Tropomyosin is also known to be modified by post-translational modifications such as acetylation and phosphorylation, and the influence of post-translationally modified forms in ovarian cancer diagnosis also could be tested.

GeLC-MRM quantitative results for CLIC1 and CLIC4 protein levels also are shown in Figure 5, and individual peptide levels are shown in Supplemental Figures 3 and 4. Similar to TPM isoforms, the normalized area ratio plots between the two CLIC proteins show considerable variations for some of the samples. As expected, since the same patient samples were used, CLIC1 results were similar to previously reported results for this protein.[21] The moderate differences observed between the two sets of label-free measurements are expected, since the assays were performed at different times and on different instruments. That is, previous analyses were performed on an AB SCIEX 4000 QTRAP and the current results were from a more sensitive AB SCIEX 5500 QTRAP instrument. The CLIC1 measurements were repeated here to provide a direct comparison to CLIC4. For both CLIC proteins, all peptides from the same protein showed similar distributions among the individual patient samples (Supplemental Figures 3 and 4), indicating that the MRM signals used for quantitation were derived from the same protein and quantitation was not appreciably affected by interfering signals.

3.5 CLIC and TPM Isoforms Can Distinguish EOC from Non-cancer Cases

The capacities of the CLIC and TPM isoforms to distinguish EOC cases were assessed using the GeLC-MRM quantitation data (Supplemental Table 3). A two-way comparison between the non-cancer and cancer groups using scatter plots and the Mann-Whitney test showed that all isoforms could significantly distinguish ($P < 0.05$) between cancer and non-cancer (Figure 6). Based on the P -value, CLIC4 appeared to be the best candidate in distinguishing cancer from non-cancer and TPM3 was the weakest, although this relative ranking could easily

change as additional patients are tested. Future analysis of larger cohorts will allow us to more definitively identify which proteins in these two protein families can most reliably distinguish non-cancer from ovarian cancer.

To evaluate the potential diagnostic efficacy for each of these proteins, receiver operating characteristic (ROC) curve analyses were performed on the non-cancer and cancer groups (Figure 7). Consistent with the Mann-Whitney test, CLIC4 showed the largest area under the curve (AUC) and TPM3 showed the lowest area. In future studies of larger patient cohorts, we plan to test these CLIC and TPM homologs in parallel with other biomarkers to identify optimal biomarker combinations that may outperform any single biomarker for detection and clinical monitoring of EOC.

To our knowledge, the plasma levels of CLIC4 and products of different TPM genes have not been reported previously in serum from ovarian cancer patients. CLIC4 is an interesting multifunctional protein that has been shown to be highly expressed in ovarian cancer stroma and may play an important role in cancer development.[41–42] Since CLIC4 has the highest AUC of any of the biomarkers reported here or in our previous xenograft mouse biomarker validation study,[21] it may be the best biomarker within this group, but this must be confirmed in further studies. The tropomyosins are a family of actin filament-binding proteins that have a well-defined central role in regulating muscle contraction and cytoskeletal organization in non-muscle cells. Decreased expression of specific TPMs has been commonly associated with the transformed phenotype and has been reported for cancer cells and tissues, including EOC.[43–45] The serological levels of TPMs have not been well studied, although high plasma levels of TPM4 have been associated with asbestos exposure[46] and increased levels of TPM serum antibodies were observed in colorectal cancer patients.[47]

Although these proteins show substantial promise as EOC biomarkers, the performance of these markers for early detection and/or clinical management of disease after initial diagnosis must be determined in larger cohorts collected from different sites, longitudinal prediagnostic blood specimens, and specimens collected throughout therapeutic treatment.

4. Conclusions

In the current study, we assessed the potential utility of multiple CLIC and TPM family members as serological biomarkers of ovarian cancer. We demonstrated that quantitative MRM assays could be set up to distinguish between all gene products in these families that were detectable in pools of ovarian cancer patient sera. Although in the case of very complex protein families with many highly homologous gene products and isoforms, the presence of additional highly homologous isoforms could not be ruled out. Nonetheless, by using a combination of unique and shared peptides together with correlation and factor analysis, we determined that any unidentified isoforms either do not occur at a significant level or change in parallel with the isoforms explicitly defined by unique peptides. In summary, we found that in addition to the previously identified CLIC1, CLIC4 and all four TPM gene products (TPM1, TPM2, TPM3, and TPM4) are significantly elevated in ovarian cancer patients compared with non-cancer controls. Further testing of larger patient cohorts are needed to determine whether the multiple CLIC and TPM family members consistently correlate with each other. The differing ratios of family member pairs indicate incomplete correlation and suggest that specific family members may be superior for specific biomarker applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Significance

This manuscript addresses the importance of distinguishing between protein homologs and isoforms when identifying and validating cancer biomarkers in plasma or serum. Specifically, it describes the use of targeted in-depth LC-M/MS analysis to determine the members of two protein families (chloride intracellular channel (CLIC) and tropomyosin (TPM) proteins that are detectable in sera of ovarian cancer patients. It then establishes a multiplexed isoform- and homology-specific MRM assay to quantify all observed gene products in these two protein families as well as many of the closely related tropomyosin isoforms. Using this assay, levels of all detected CLICs and TPMs were quantified in ovarian cancer patient and control subject sera. These results demonstrate that in addition to the previously known CLIC1, multiple tropomyosins and CLIC4 are promising new ovarian cancer biomarkers. Based on these initial validation studies, these new ovarian cancer biomarkers appear to be superior to most previously known ovarian cancer biomarkers.

Highlights

- Established serum MRM assays to distinguish detectable CLIC and TPM family members
- CLIC1 and CLIC4 exhibit similar but non-identical trends across patient sera
- All TPM gene products exhibit similar but non-identical trends across patient sera
- Based on current cohort CLIC4 is the best ovarian cancer biomarker

Figure 1. Quantitative comparisons of TPM and CLIC isoforms using label-free discovery mode, GeLC-MS/MS analysis of patient serum pools. LC-MS chromatograms were aligned and quantitatively compared using Elucidator. Intensities of all identified peptides unique to the indicated protein isoform were summed for each of four serum pools (NC – non-cancer; C1, C2, C3 – three different advanced ovarian cancer pools).

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SP|P09493-5|isoform 5 -----MA 2
TR|D9YZV7|isoform 6 -----MA 2
TR|Q59GR8|variant GGRDFFRTAPRRRRRRTERPGRGGPALGSQDSRGSRVRAAAGLSHCSPPARLP SGAMA 60
TR|B7Z722|isoform CRA_i -----MA 2
TR|Q1ZYL5|variant 6 -----MA 2
TR|B7Z596|cDNA FLJ55130 -----MA 2
**

SP|P09493-5|isoform 5 GSSSLEAVRRKINSLQEQAADAEERAGTLQRELDHERKLEETAEADVASLNRIQLVEEE 62
TR|D9YZV7|isoform 6 GSSSLEAVRRKINSLQEQAADAEERAGTLQRELDHERKLEETAEADVASLNRIQLVEEE 62
TR|Q59GR8|variant GSSSLEAVRRKINSLQEQAADAEERAGTLQRELDHERKLEETAEADVASLNRIQLVEEE 120
TR|B7Z722|isoform CRA_i GSSSLEAVRRKINSLQEQAADAEERAGTLQRELDHERKLEETAEADVASLNRIQLVEEE 62
TR|Q1ZYL5|variant 6 GSSSLEAVRRKINSLQEQAADAEERAGTLQRELDHERKLEETAEADVASLNRIQLVEEE 62
TR|B7Z596|cDNA FLJ55130 GSSSLEAVRRKINSLQEQAADAEERAGTLQRELDHERKLEETAEADVASLNRIQLVEEE 62
*****

SP|P09493-5|isoform 5 LDR A QERLATALQKLEEA E K A A D E S E R G M K V I E S R A Q K D E E K M E I Q E I Q L K E A K H I A E D A 122
TR|D9YZV7|isoform 6 LDR A QERLATALQKLEEA E K A A D E S E R G M K V I E S R A Q K D E E K M E I Q E I Q L K E A K H I A E D A 122
TR|Q59GR8|variant LDR A QERLATALQKLEEA E K A A D G S E R G M K V I E S R A Q K D E E K M E I Q E I Q L K E A K H I A E D A 180
TR|B7Z722|isoform CRA_i LDR A QERLATALQKLEEA E K A A D E S E R G M K V I E S R A Q K D E E K M E I Q E I Q L K E A K H I A E D A 122
TR|Q1ZYL5|variant 6 LDR A QERLATALQKLEEA E K A A D E S E R G M K V I E S R A Q K D E E K M E I Q E I Q L K E A K H I A E D A 122
TR|B7Z596|cDNA FLJ55130 LDR A QERLATALQKLEEA E K A A D E S E R G M K V I E S R A Q K D E E K M E I Q E I Q L K E A K H I A E D A 122
*****

SP|P09493-5|isoform 5 DRKYEEVARKLVIIESDLERAEERAE L S E G K C A E L E E E L K T V T N N L K S L E A Q A E K Y S Q K E 182
TR|D9YZV7|isoform 6 DRKYEEVARKLVIIESDLERAEERAE L S E G K C A E L E E E L K T V T N N L K S L E A Q A E K Y S Q K E 182
TR|Q59GR8|variant DRKYEEVARKLVIIESDLERAEERAE L S E G K C A E L E E E L K T V T N N L K S L E A Q A E K Y S Q K E 240
TR|B7Z722|isoform CRA_i DRKYEEVARKLVIIESDLERAEERAE L S E G K C A E L E E E L K T V T N N L K S L E A Q A E K Y S Q K E 182
TR|Q1ZYL5|variant 6 DRKYEEVARKLVIIESDLERAEERAE L S E G Q V R Q L E E Q L R I M D Q T L K A L M A A E D K Y S Q K E 182
TR|B7Z596|cDNA FLJ55130 DRKYEEVARKLVIIESDLERAEERAE L S E G Q V R Q L E E Q L R I M D Q T L K A L M A A E D K Y S Q K E 182
*****

SP|P09493-5|isoform 5 DRYEEEIKVLSDKLEAETRAEFAERSVTKLEKSIDDLQLYQQLEQNRRLTN-ELKLA 241
TR|D9YZV7|isoform 6 DRYEEEIKVLSDKLEAETRAEFAERSVTKLEKSIDDLQLYQQLEQNRRLTN-ELKLA 241
TR|Q59GR8|variant DRYEEEIKVLSDKLEAETRAEFAERSVTKLEKSIDDLQLYQQLEQNRRLTN-ELKLA 299
TR|B7Z722|isoform CRA_i DRYEEEIKVLSDKLEAETRAEFAERSVTKLEKSIDDLQLYQQLEQNRRLTN-ELKLA 241
TR|Q1ZYL5|variant 6 DRYEEEIKVLSDKLEAETRAEFAERSVTKLEKSIDDLQLYQQLEQNRRLTN-ELKLA 241
TR|B7Z596|cDNA FLJ55130 DRYEEEIKVLSDKLEAETRAEFAERSVTKLEKSIDDLQLYQQLEQNRRLTN-ELKLA 242
*****

SP|P09493-5|isoform 5 LNED----- 245
TR|D9YZV7|isoform 6 LNED----- 245
TR|Q59GR8|variant LNED----- 303
TR|B7Z722|isoform CRA_i LLELNNM----- 248
TR|Q1ZYL5|variant 6 LNED----- 245
TR|B7Z596|cDNA FLJ55130 IFQFKGIHIDTLCTCTFFLCVLWGFSWLLNS 275
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Figure 2.

Sequence alignment of selected TPM1 Isoforms. TPM1 isoforms that contain all five xenograft-identified peptides (green highlight) were aligned using the Clustal algorithm. Two additional peptides (grey highlight) were subsequently identified in analyses of ovarian cancer patient sample pools. Inclusion of these peptides suggests the presence of TPM1 Q1ZYL5 or B7Z596. The database type (SP, Swiss-Prot entry; TR, TrEMBL entry), identifier, and brief description are indicated for each sequence. Tryptic sites (K or R) are indicated in red. Boxed sequences – peptides used for MRM quantitation.

Figure 4.

(A) GeLC-MRM quantitation of tropomyosin proteins in non-cancer individuals (WCS and B series) and individuals with late-stage (T) ovarian cancer. The following peptides were assigned to isoforms: TPM1 variant 6 (ETAADVASLNR, SLQEQADAAEER, LVIIESDLER, AELSEGQVR); TPM3 (IQVLQQQADDAEER); TPM4 (AEGDVAALNR, IQALQQQADEAEDR, [K]LVILEGELER) and TPM - Common (all family members) ([R]IQLVEEELDR). Error bars are standard deviations determined from normalized individual peptide levels for each protein. Individual peptide levels across samples are shown in

Figure 5. (A) GeLC-MRM quantitation of CLIC1 and CLIC4 proteins in non-cancer individuals (WCS and B series) and individuals with late-stage (T) ovarian cancer. Peptides used for quantitation are GVTFNVTTVDTK, LAALNPESNTAGLDIFAK and NSNPALNDNLEK for CLIC1, and NSRPEANEALER, YLTNAYS and EVEIAYSQVAK for CLIC4. Individual peptide levels across samples are shown in Supplemental Figures 3 (CLIC1) and 4 (CLIC4). Error bars are standard deviations determined from normalized individual peptide levels for each protein. (B) Normalized area ratio plots of CLIC1/CLIC4 to visualize differences in protein levels across samples.

Figure 6. Scatter plots showing GeLC-MRM quantitation of the CLIC and TPM isoforms in non-cancer controls (n = 15), and ovarian cancer patients (n = 18). *P*-values were calculated using the Mann-Whitney test with Bonferroni adjustment. Horizontal bars in each group indicate the median serum level of the protein. Peptides associated with each tropomyosin isoform are shown in Figure 4.

Figure 7.

ROC curves of the CLIC and TPM isoforms. ROC curves were generated from non-cancer controls and ovarian cancer patients' datasets. The area under the ROC curve is indicated for each candidate. The peptides associated with each tropomyosin isoform are shown in Figure 4.

Table 1

Peptides identified in TOV-112D xenograft mouse serum and human serum pools

Sequence ^a	Number of Unique Database Entries	Gene
<i>TPM1 Peptides Identified in Xenograft Mouse Serum</i>		
ETAEDVASLN ^b	6	TPM1
SLQEQADAAEER ^b	7	TPM1
(K)LVIIESDLER ^b	18	TPM1
MEIQEQLK	38	TPM1, TPM3
QLVEEELDR ^b	43	TPM1, TPM2, TPM3, TPM4
<i>TPM1 Peptides Detected in Patient Serum Pools</i>		
ETAEDVASLN ^b	6	TPM1
SLQEQADAAEER ^b	7	TPM1
(K)LVIIESDLER ^b	18	TPM1
MEIQEQLK	38	TPM1, TPM3
(R)QLVEEELDR ^b	43	TPM1, TPM2, TPM3, TPM4
AELSEGQVR ^b	10	TPM1
YEEEEK ^c	57	TPM1, TPM2, TPM3, TPM4
ATDAEDVASLN ^{c,d}	19	TPM1, TPM2
<i>TPM2 Peptides Detected in Patient Serum Pools</i>		
(K)LVILEGELER ^b	11	TPM2, TPM4
(R)QLVEEELDR ^b	43	TPM1, TPM2, TPM3, TPM4
ATDAEDVASLN ^c	19	TPM1, TPM2
MELQEMQLK	8	TPM2
SLMASSEYESTK	3	TPM2
YEEEEK ^c	57	TPM1, TPM2, TPM3, TPM4
EDKYEEEEK	31	TPM2, TPM3, TPM4
CGDLEELK ^c	6	TPM2, TPM4
<i>TPM3 Peptides Detected in Patient Serum Pools</i>		
(K)QVLQQADDAEER ^b	16	TPM3
(R)QLVEEELDR ^b	43	TPM1, TPM2, TPM3, TPM4
MELQEQLK	38	TPM1, TPM3
(K)LVIIEGDLER	19	TPM3
HIAEEADR	22	TPM3, TPM4
MLDQTLLDLNEM	12	TPM3
YEEEEK ^c	57	TPM1, TPM2, TPM3, TPM4
EDKYEEEEK	31	TPM2, TPM3, TPM4
CLSAAEEK	14	TPM3
AADAEAEVASLN	1	TPM3
<i>TPM4 Peptides Detected in Patient Serum Pools</i>		

Sequence ^a	Number of Unique Database Entries	Gene
(K)IQALQQADEAEDR ^b	3	<i>TPM4</i>
(K)LVILEGELER ^b	11	<i>TPM2, TPM4</i>
EENVGLHQTLNQTLNELNCI	4	<i>TPM4</i>
AEGDVAALNR ^b	4	<i>TPM4</i>
MEIQEMQLK	2	<i>TPM4</i>
YSEKEDKYEEEIK	4	<i>TPM4</i>
CGDLEELK ^c	6	<i>TPM2, TPM4</i>
(R)IQLVEEELDR ^b	43	<i>TPM1, TPM2, TPM3, TPM4</i>
HIAEEADR	22	<i>TPM3, TPM4</i>
EKAEGDVAALNR	3	<i>TPM4</i>
YEEEIK ^c	57	<i>TPM1, TPM2, TPM3, TPM4</i>
EDKYEEEIK	31	<i>TPM2, TPM3, TPM4</i>
TIDDLK	4	<i>TPM4</i>
ASDAEGDVAALNR	1	<i>TPM4</i>

^aDetected peptides shared by multiple gene products are listed under all matching proteins. Underlined sequences were identified as human in the UniProtKB 2007 database, but also matched a mouse homolog in a later database (see text).

^bPeptide targeted for MRM. Successful quantitation.

^cPeptide targeted for MRM. Unsuccessful quantitation.

^dThis peptide is not present in the group of TPM1 isoforms (Figure 2) defined by peptides identified from the xenograft mouse serum.