Applications of HPLC-MALDI-TOF MS/MS Phosphoproteomic Analysis in Oncological Clinical Diagnostics

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Abstract: Recent developments in mass spectrometry have brought clinical proteomics to the forefront of cancer diagnosis and treatment, offering reliable, robust and efficient analysis methods for biomarker discovery and monitoring. Proteins control cellular processes through intricate signaling cascades and protein function is often determined by post-translational modifications. However, alteration of post-translational modifications that control normal cellular processes, such as differentiation, proliferation and apoptosis, has been significantly related to tumorigenesis. Aberrations in protein phosphorylation, for example, offer promising opportunity for disease diagnosis and prognosis and could also serve as a therapeutic indicator for cancer treatment. Recent studies have identified biomarkers involving several cancer types using high-performance liquid chromatography separation and enrichment techniques coupled with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry for protein identification and quantification. However the clinical implementation of proteomic technologies is not without shortcomings and is still dependent on laboratory research to expand identification of relevant biomarkers.

Keywords: Clinical proteomics, mass spectrometry, phosphoproteome, protein biomarker.

1. INTRODUCTION

The logarithmic progress in genomic, epigenomic, and transcriptomic analytics has expanded our knowledge of biomolecular functional regulation but, at the same time, has shattered the reductionist "one-gene, one- protein" paradigm. Transcriptional variation and mRNA splicing are conduits for processing one gene into multiple RNA and protein sequences. As a result, proteomics has emerged as a key methodology in the diagnosis and treatment of various diseases, including cancer, and has evolved to encompass reliable, robust and high-throughput technologies based largely on mass spectrometry (MS). MS offers the ability to determine protein structure and expression levels with unparalleled sensitivity, elucidating underlying cellular mechanisms and circumstances in which aberrations in those mechanisms give way to tumorigenesis. This allows comparative proteomic analysis between malignant and non-malignant samples to identify protein biomarkers. Perhaps the most influential controls of protein activity are post-translational modifications (PTMs), which dictate protein function and interaction through the controls of fluctuating modifications. Abnormalities in the presence of such modifications, including location and quantity, have been shown to be significantly related to the development of cancer and can serve as powerful, discriminating biomarkers for the presence and progression of disease [1-6]. MS technologies have been used to identify and monitor biomarkers representative of different disease states [7-12] and while the analysis of PTMs, such as phosphorylation, as biomarkers for clinical oncology is a promising possibility for disease treatment there are limitations that must be addressed before proteomic technologies can be applied in a clinical environment on a large-scale. Presently, the ability to employ proteomics in the clinical realm is not hindered by a lack of capable technology but rather the lack of knowledge and understanding of the proteome itself and phosphorylated protein biomarkers. As a naturally occurring PTM, phosphorylation maintains normal cellular processes, which highlights the importance of determining basal levels and locations of modifications in order to determine when abnormalities arise. Modifications also vary between different cancer types so unique PTMs must be identified for each cancer that are able to distinguish between different cancers and also differentiate between cancer and other non-cancerous disease states in order to be used as a diagnostic tool.

Clinical proteomics has gained interest in oncology because of its potential for early disease diagnosis, prognostic evaluation, disease progression monitoring and the ability to choose and monitor therapeutic treatment response at the molecular level based on the detection of relevant disease biomarkers [13-15]. However the need to identify multiple robust biomarkers for each disease state is imperative, as some biomarkers can be inadequate when used alone [16]. The ultimate goal of clinical proteomics is to increase patient survival rate through individualized treatment therapies [17, 18]. Current therapeutic standards administered to a broad population can have a low response rate coupled with high toxicity, making it even more evident that individually tailored treatment methods are necessary to achieve the best standard of care for each patient [19, 20]. The potential of using proteomic technologies for individualized patient

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therapeutic development is profound and could significantly change the application of scientific and medical practices in future drug development and patient care.

Clinical samples can include tissue, both malignant and non-malignant, as well as various body fluids, such as plasma, serum and urine. Therefore, analytical techniques must be able to accommodate all of these sample types as well as the vast amount of proteins present in a given sample. In addition to sample complexity, pre-analytical sample treatment can also compromise the reliability of mass spectrometry-based proteomic analysis. It has been observed that differences in sample collection, storage and preparation methods can lead to inconsistencies in resultant mass spectrometry data concerning protein and biomarker identification [21-23]. Samples should be treated consistently for each analysis and between different locations in order to obtain the most reliable mass spectrometry data to determine differences in disease state. Validated guidelines for sample handling and analysis methods have not been determined or applied in clinical proteomics and represent an obstacle that must be addressed before clinical sample biomarker analysis can be used for wide-spread diagnostic purposes. Here, the advantage of clinical proteomics is that procedures can be standardized at every point of the analysis process, providing the most accurate data concerning cancer biomarkers.

Identification of protein biomarkers by evaluation of up- and down-regulated proteins can be performed by several common proteomic methods involvong MS technologies (Table **1**). However, the use of these methods for the identification of cancer-related protein biomarkers is too large in scope to be addressed in this review. Here, we focus on the use of 2-dimensional high-performance liquid chromatography (2D-HPLC) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and tandem mass spectrometry (MALDI-TOF MS/MS) for the enrichment, identification and quantification of protein biomarkers and phosphorylated protein biomarkers related to clinical oncology, as well as current limitations involved in clinical proteomics.

2. PROTEIN FUNCTION AND MODIFICATION

Cancer is a diverse and devastating disease, exhibiting differences in pathology and treatment response between patients that require a comprehensive profile to screen for and diagnose disease, establish disease prognosis, monitor disease progression and determine the best treatment method on an individual patient basis. In the past, genomic patterns were relied upon to elucidate protein function from mRNA gene expression. This was thought to be a reliable method, in theory, because expressed proteins are derived from an RNA blueprint. However, several studies have shown little or no correlation exists between mRNA transcript levels and corresponding protein expression [24, 25]. This is due in part to the various enzymatic and non-enzymatic post-translational modifications (see Table **2**, *modified from* [26]) a protein can undergo that serve to alter its function, such as phosphorylation, glycosylation and nitration, as well as differential splicing, which results in the translation of multiple proteins from a single gene. Thus, the most accurate way to evaluate protein expression is to examine the proteome itself. This can be used to directly monitor changes in protein form, function and abundance.

Phosphorylation is one of the most prevalent and widely researched post-translational modifications, affecting as much as 30% of proteins in the human proteome [27]. Protein phosphorylation is a subject of focus in the proteomic community because it is involved in many inherent and fundamental cellular processes, including signal transduction, differentiation, proliferation, degradation and apoptosis [28-31]. Aberrations in phosphorylation can alter cellular processes and have been linked to the cause or effect of various states of oncogenesis, including human breast, lung, endometrial, hepatocellular and colorectal cancer [32-39]. Discovering and pursuing abnormalities in protein phosphorylation as a target for therapeutic treatment has therefore, been a growing focus in proteomics over the past decade [4, 40].

Post-translational modifications alter protein function by changing the protein structure, which leads to changes in protein-protein interaction and signal transduction. Phosphorylation is recognized as a reversible modification whose effects are exerted by kinases and phosphatases by adding or removing, respectively, a phosphate group on serine, threonine and tyrosine amino acid residues by adenosine triphosphate (ATP) (Fig. **1**). The presence or lack of a phosphate group in certain locations within the amino acid sequence marks the protein for its specific function. It is widely recognized that phosphorylation does not act as an independent event but is likely part of a signaling cascade in which functions are carried out based on multiple phosphorylation events often depending on location within the cell and stages of cellular response [29, 41, 42].

Protein phosphorylation is a dynamic modification that requires a delicate balance of enzyme activity to orchestrate the signaling cascades vital to cellular function. Phosphorylation events involving various proteins can alter cell signaling cascades and are known to be related to oncogenesis and metastasis by effecting cell differentiation, proliferation, migration, growth and survival [28, 42-47]. Enumerating the key sites in these cascades where phosphorylation can go awry offers possibilities for identifying diagnostic, prognostic, and predictive biomarkers as well as therapeutic targets potentially allowing for correction of the altered mechanism present in the disease state or, by inhibition of function and disruption, death of the cancer [27]. This can only be done however, once the protein of interest, the post-translation modification and the modification site are determined. This requires a proteomic analysis method capable of analyzing clinical samples that is automated, high-throughput, reliable and robust to determine normal and altered phosphorylation events and possible biomarkers derived from those events.

3. PROTEOMICS OF SERUM

Proteomic investigations have discovered relevant tumor biomarkers for breast [48-51], ovarian [14-16, 52], hepatocellular [53] and head-and-neck squamous cell carcinoma [54] by determining differences in the protein expression profiles of normal and malignant tissues. Although the analysis of relevant tissue types for protein biomarker discovery is valuable, it is not always feasible due to limitations of surgical accessibility.

Table 1. Summary, Advantages and Disadvantages of Commonly Used Protein Profiling Techniques for Sample Preparation, Separation and MS Analysis

	Method	Material	Separation	Separation Properties	Advantages	Disadvantages
Separation Methods	2D-PAGE	Gel-based	Proteins	Separates based on protein charge and mass	Single gel can accommodate thousands of proteins; can label sample for PTMs	Low throughput (1 sample/gel); not automated; poor repoducibility; poor resolution for low abundance proteins or extreme protein masses; cannot interface with MS; requires large protein input
	DIGE	Gel-based	Proteins	Up to 3 samples are labeled with fluorescent dyes (Cy2, Cy3 and Cy5), combined and run on a 2D-PAGE gel. Fluoresence intensity from the label is measured and compared between the samples.	Can run 3 samples/gel (advantage over 2D-PAGE); high separation capability	(See 2D-PAGE disadvantages)
	Antibodies	Antibody	Proteins	Antibodies can be raised against a certain protein that can be separated from a protein mixture by immunoprecipitation	Can isolate phosphoproteins	Low throughput (1antibody/protein); antibodies for phosphoproteins only bind stongly to phospho-tyrosine when isolating PTMs; binding is not always specific for the protein of interest (non-specific binding effects)
	HPLC	Solid-phase column	Peptides	Liquid-based separation of peptides depending on column stationary phase for simplification of a complex protein digest by fractionation	High throughput; automated; small sample input; better dynamic range of detection for low abundance and extreme mass proteins; capable of separating complex mixtures; can enrich phosphopeptides; capable of 2D separation; can interface directly with on-line MS; reduces ion suppression events during MS analysis	Initial start-up costs of equipment
	Method	**Label**	**Label Target**	**Labeling Method**	**Advantages**	**Disadvantages**
Labeling Strategies	^{32}P	^{32}P radioactive label	Phosphoproteins	Radioactive 32P is incorporated into the protein by ATP to detect the presence and amount of phosphoprotein by Edman degradation	Compatible with gel-based and HPLC fractionation methods; can visualize low abundance and low stoichiometric phohsphorylation events; high sensitivity	Low throughput; radioactivity is necessary for labeling technique

Table 1. Contd.…

	Method	Label	Label Target	Labeling Method	Advantages	Disadvantages
	ICAT	Isotope coded affinity tag (ICAT) - light and heavy	Proteins (cysteine residues)	Differential chemical labeling of 2 protein samples with light or heavy ICAT tags, samples are then combined, trypsin digested and isolated by affinity chromatography for phosphopeptides. LC-MS analysis provides ratios of signal intensities from the ICAT tags that can be used to quantify relative protein levels	Is a gel-free, quantitative proteomic method; can identify low abundance proteins (phosphoproteins); can detect changes in pair-wise comparisons between 2 protein samples	Low throughput; could miss PTM identification because labeling method is not dependant on PTM properties
	iTRAQ™	iTRAQ™ Reagent isobaric tag	Peptides (lysine side chains and N-terminus of each peptide)	Complex protein mixtures are trypsin digested, labeled with an iTRAQ™ Reagent isobaric tag, combined and fractionated by HPLC prior to MS/MS analysis. Peptide abundance is derived from ratios given by the reporter group in the iTRAQ™ tag during MS/MS analysis to provide quantitative data	High throughput, multiplex capability (either 4- or 8-plex available); is not limited to phosphoprotein analysis but can enrich for phosphopeptides during HPLC separation; can determine relative and absolute protein and phosphoprotein quantification; has high labeling efficiency to include all peptides in a complex digest; no increase in sample complexity; can detect changes in pair-wise comparisons of protein samples	Requires MS/MS for quantitative sample analysis; prone to masking effects during MS/MS if the sample is not properly enriched and fractionated first
	MS Ionization	**Ionization technology**		**Application to MS**	**Advantages**	**Disadvantages**
Ionization Techniques	ESI	Electrospray ionization		Spray	On-line interface of LC to MS; is time efficient; ionizes some peptides better than MALDI	Requires more sample imput; once run the sample cannot be reanalyzed without performing the entire analysis again
	MALDI	Matrix-assisted laser desorption ionization		Solid-phase platform	High-throughput; off-line analysis decouples LC and MS so the sample can be stored and reanalyzed for peptides of interest when needed; enhanced observation of low abundance peptides; exhibits limited sample consumption; can analyze complex samples; can more accurately analyze peptides with multiple phosphorylation sites; ionizes some peptides better than ESI	Off-line analysis requires more time during analysis and during MS analysis preparation due to the use of matrix during MALDI plate spotting

Table 1. Contd….

	MS Ionization	Ionization technology	Application to MS	Advantages	Disadvantages
	SELDI	Surface-enhanced laser desorption ionization	Solid-phase platform	High-throughput;capable of analyzing complex samples; limited sample preparation before chip chromatography; limited sample input; can be selective for PTMs based on chip properties	Cannot analyze both non-phosphorylated and phosphorylated peptides in one analysis (is limited to peptides on the chip); lower resolution than MALDI

Table 2. Post-Translational Modifications.

Types	Modified Residues
Phosphorylation	Serine, threonine, tyrosine, histidine
N-linked-glycosylation	Aspargine, lysine
O-linked glycosylation	Lysine, praline, serine, threonine, tyrosine
C-linked glycosylation	Tryptophan
Acetylation	N-terminal of some residues and side chain of lysine or cysteine
Amidation	Generally at the C-terminal of a mature active peptide after oxidative cleavage of last glycine
Hydroxylation	Generally of asparagine, aspartate, proline or lysine
Methylation	Generally of N-terminal phenylalanine, side chain of lysine, arginine, histidine, asparagine or glutamate, and C-terminal cysteine
Pyrrolidone carboxylic acid	N-terminal glutamine which has formed an internal cyclic lactam
Gamma-carboxyglutamic acid	Glutamate
Farnesylation	Cysteine
Myristoylation	Glycine
N-Palmitoylation	Cysteine
S-Palmitoylation	Cysteine
Geranyl-geranylation	Cysteine
S-diacylglycerol cysteine	Cysteine
GPI anchoring	C-terminal asparagine, aspartate, serine
Deamidation	Amidated asparagine and glutamine (needs to be followed by a G)
Sulfation	Serine, threonine, tyrosine
Sumoylation	Lysine
Ubiquitylation	Lysine
ADP-ribosylation	Arginine
Formylation	Of the N-terminal methionine
Citrullination	Arginine
Nitration	Tyrosine
Bromination	Tryptophan
FAD	O-8alpha-FAD tyrosine, Pros-8alpha-FAD histidine, S-8alpha-FAD cysteine, and Tele-8alpha-FAD histidine
S-nitrosylation	Cysteine

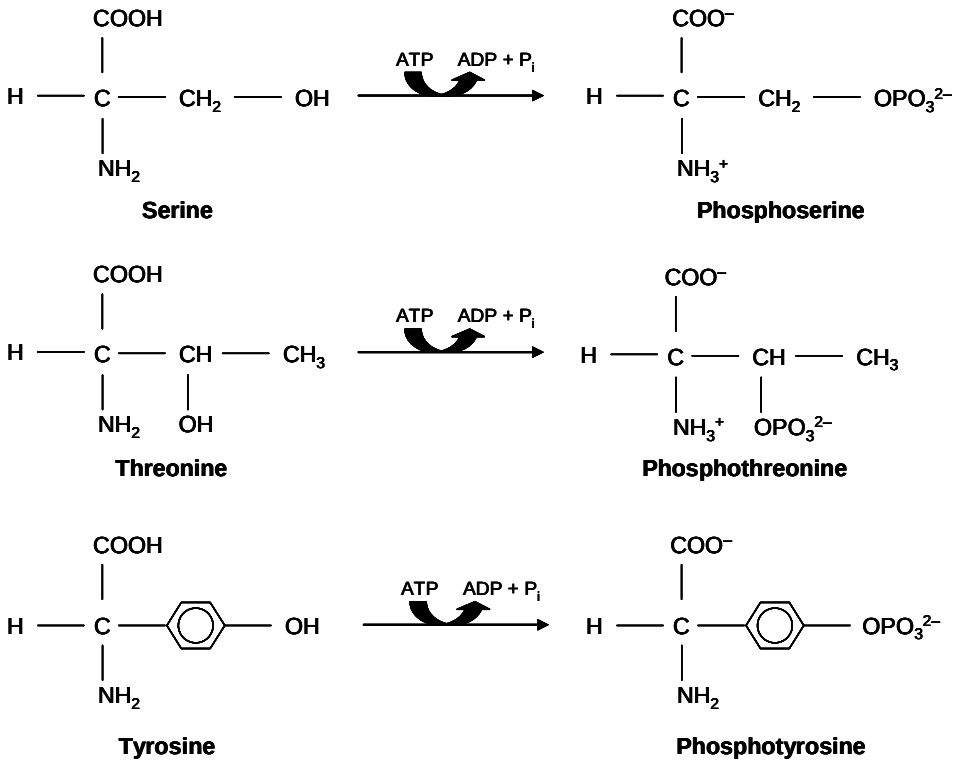

Fig. (1). Phosphorylation of serine, threonine and tyrosine.

As a consequence, serum has been extensively tested as a source for proteomic assessment. Tumors deposit proteins into serum, thereby creating a unique serum profile of relevant disease proteins [18, 55]. Serum proteomics allows the opportunity to detect the presence of specific biomarkers that would not only allow diagnosis of the cancer type but also evaluate prognosis, disease progression, appropriate treatment therapies and treatment response.

Serum proteomics has been used to evaluate tumor specific protein profiles and discriminate peptide biomarkers in a variety of cancers, including: thyroid cancer [55], pancreatic cancer [56] and breast cancer [49]. Signature serum peptides were also able to differentiate between breast, prostate and bladder tumors as well as distinguish these from normal samples [57]. Serum screening for breast cancer biomarkers identified three unique biomarkers that were able to discriminate between early stage disease and normal samples [49]. Serum peptide patterns were also used to evaluate treatment outcome over time in non-small cell lung cancer patients [12].

Comparison of malignant and normal tissue protein profiles is informative in a research environment and is sometimes necessary to identify proteins that may be up- or down-regulated or post-translationally modified, indicating tumorigenesis or various disease states in relevant disease tissues. However, once found it is necessary to detect and quantify these biomarkers in a clinical setting. Serum proteomics offers a method for both protein-specific and global protein analysis with minimally invasive collection techniques that can be implemented in a clinical setting and is compatible with high-throughput and reliable MS technologies.

4. HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY SEPARATION METHODS

Use of high-performance liquid chromatography (HPLC) as a means of sample separation in proteomic analysis has increased over the past decade, simplifying complex protein samples by fractionation and enriching modified peptides prior to MS analysis. HPLC is a sensitive technology that is not prone to some of the complications and limitations experienced with other commonly used separation techniques, such as gel electrophoresis, which can limit its application in a clinical environment. This technology holds promise as a part of the reliable protein analysis of oncologic clinical samples and offers the advantage of being high-throughput, cost and time efficient and compatible with MS protein analysis methods.

A vast amount of proteins are present in human body tissues and fluids, comprising a level of sample complexity that requires separation of proteins prior to MS in order to reliably identify and quantify proteins or modified proteins. Separation methods depending on 2-dimensional gel electrophoresis (2D-GE) have been widely employed in proteomic studies for separation of proteins based on charge and molecular weight [58] and have vast applicability in the proteomics community. However, these separation methods have several limitations that hinder separation of complex protein samples. Gel-based separation methods are laborious, time consuming and often require expensive reagents. Other disadvantages include protein masking, the inability to resolve low abundance membrane proteins and a poor dynamic range of protein detection [24]. When analyzing protein modifications, such as phosphorylation, other limitations

arise since gel-separation techniques cannot detect low copy proteins, a characteristic of most modified proteins. 2D gel-based separation methods have been combined with liquid chromatography separation and enrichment of phosphorylated samples prior to MS analysis to achieve better separation sensitivity than by performing gel-separation alone [59, 60]. These limitations prevent the practical application of gel-based separation methods for complex protein samples in a clinical setting and call for the use of a reliable, reproducible and efficient separation method that can accommodate complex and modified protein samples.

HPLC relies on a pump system, separation columns and UV detectors to separate a peptide mixture based on interactions with the column stationary phase and application of a mobile phase. Protein samples are prepared by proteolytic digestion, thus allowing the analysis of complex protein samples based on their constitutive peptides. Use of smaller columns and a lower flow rate, often termed nano-HPLC, requires less sample input and increases the separation sensitivity exponentially, making this an ideal enrichment method for low stoichiometric modifications [61-63]. HPLC can also be directly interfaced with MS through both on-line electrospray ionization (ESI) and off-line matrix-assisted laser desorption/ionization (MALDI) techniques, both of which are commonly used in proteomic analyses [64]. The high level of sensitivity at the separation phase of nano-flow HPLC provides a high quality and fully represented peptide sample, thus comprising a high-throughput and reliable method for further identification and quantification of peptides and phosphopeptides by MS.

4.1. HPLC Stationary Phase Columns

Peptide separation and enrichment by HPLC can also be customized based on the stationary phase columns used during protein-digest separation (Table **3**). The most common separation method in HPLC is by reverse-phase (RP) chromatography, which depends on the interaction of peptides with a non-polar stationary phase column and a polar mobile phase [62]. In RP separation, polar compounds elute from the column first and non-polar compounds are retained by the column. However, RP separation is usually combined with another separation column to create a separation technique based on two identifying characteristics, or 2-dimensional HPLC (2D-HPLC) for improved resolution (Fig. **2**).

4.1.1. HPLC Separation of Phosphorylated Peptides

HPLC of phosphorylated peptide samples can include a column for strong-cation exchange (SCX) chromatography, immobilized metal affinity chromatography (IMAC) or a titanium dioxide column (TiO_2). SCX chromatography separates peptides based on the presence of the negatively charged phosphate molecule [65]. It has been shown that 2-dimensional SCX/RP-HPLC identifies significantly more unique peptides in a complex protein mixture compared to 1-dimensional RP-HPLC [61, 66]. This 2-dimensional, orthogonal approach ensures the ultimate enrichment and fractionation of phosphopeptides in a sample, which is necessary to avoid complications during MS when analyzing a complex peptide digest.

Use of TiO_2 columns prior to RP chromatography in a 2D-HPLC analysis has been shown to selectively and reliably adsorb and release phosphopeptides under acidic and alkaline conditions, respectively, with up to 90% recovery [67, 68]. Pinkse *et al*. used this method to bind phosphopeptides to the TiO_2 column, enabling MS analysis of non-phosphorylated peptides that eluted off the column first and then eluting the phosphorylated peptides for subsequent analysis [69]. The addition of 2,5-dihydroxybenzoic acid during peptide loading was also shown to considerably enhance selective enrichment of the TiO_2 column for phosphopeptides [70]. Phosphopeptide TiO_2 column enrichment was combined with nano-LC ESI-QTOF MS/MS to detect 21 specific phosphorylation sites and could accommodate peptides containing up to four phosphorylated residues [68]. However, TiO_2 enrichment of phosphopeptides in conjunction with nano-LC MALDI-TOF MS was shown to identify more phosphorylated peptides than the ESI technique and also gave evidence that ESI is biased toward detection of monophosphorylated peptides [70].

Table 3. HPLC Column Summary for Phosphopeptide Enrichment

HPLC Column	Material	Separation	Separation Properties
RP	Non-polar C18 or C8 bonded silica	Peptides	Use of a non-polar stationary phase with alkyl chains bonded to the surface to retain non-polar and elute polar moities.
SCX	SCX resin	Peptides and phosphopeptides	Separates charged molecules for peptide purification based on the exchange of cations. When combined with RP increases the amount and quality of analytical data.
IMAC	Immobilized metal ions - commonly Fe^{3+} and Ga^{2+}	Phosphopeptides	Selectively binds and enriches phosphopeptides due to affinity for negatively charged peptide regions. Can exhibit non-specific binding of non-phosphorylated peptides.
TiO_2	TiO_2 nanoparticles	Phosphopeptides	Selectively enriches phosphorylated species based on high affinity of TiO_2 for phosphopeptides. Phosphopeptides are eluted from the column using an alkaline buffer. Selectivity can often be non-specific but is enhanced when the sample is loaded in DHB or another acidic solution onto the TiO_2 column.

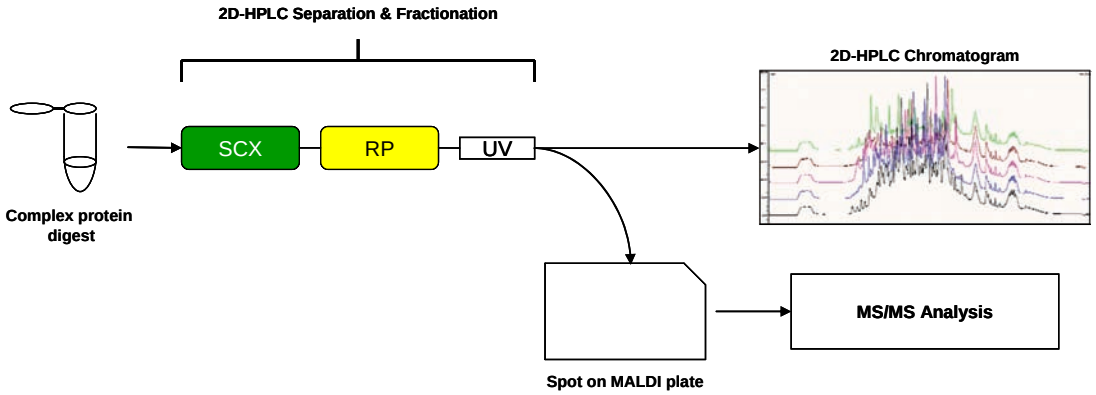

Fig. (2). 2D-HPLC separation of complex protein digests. Digested protein samples are first separated by charge on the SCX column then by hydrophobicity on the RP column. Peptide elution from the column is measure by UV to generate a chromatogram and eluted peptides are spotted on the MALDI solid-phase plate.

IMAC chromatography takes advantage of the affinity between metal ions and phosphorylated peptides to selectively enrich them by interaction with the metal ions in the column [71]. Extra care must be taken however, when using metal affinity chromatography to avoid simultaneous retention of non-phosphorylated species on the column [62, 70]. When compared with TiO_2 columns, IMAC was found to be equivalent in phosphopeptide isolation when using the proper metal ion and experimental adaptations for the sample being analyzed [71].

Ultimately, it is necessary to simplify a complex protein digest to achieve the most accurate and reliable MS data concerning the presence, location and quantity of phosphorylated proteins in a given sample. Although several separation methods have been highly relevant and informative in a laboratory setting for research development it is necessary to propel technology that can be implemented in a clinical setting for the efficient and accurate enrichment and fractionation of patient samples prior to disease biomarker detection and monitoring. The use of HPLC in such an environment has the potential of offering a high-throughput sample fractionation method that is adaptable to enriching modified peptides for PTM monitoring. This allows global proteome analysis for an individual patient and individualized screening for post-translational changes which could be responsible for tumorigenesis or tumor progression.

5. MALDI-TOF MASS SPECTROMETRY

Several recent reviews highlight the use of mass spectrometry for the detection and quantification of clinical biomarkers, emphasizing the potential for this technology in a clinical setting [9, 11, 72]. MS has also been widely employed in the detection and mapping of phosphopeptides[73] (reviewed in [74-77]), but its application in phosphoprotein-related biomarker identification has several complications that have thus far prevented widespread application in clinical settings.

Analytical challenges encountered when analyzing phosphopeptides by MS are a continuing concern. Complications arise due to the characteristics of the phosphate group and the abundance of the phosphorylated protein. Phosphorylation is a low abundance and low stoichiometric modification. During MS analysis of phosphopeptides suppression effects can occur when more abundant non-phosphorylated peptides mask the detection of the less abundant phosphorylated species. Suppression effects also occur upon ionization during MS analysis due to the slightly acidic nature of the peptide caused by the additional phosphate group. Furthermore, phosphorylation is a labile modification and can be reversed upon ionization by the UV-laser used in MS. These characteristics pose challenges during MS analysis and have lead to methodologies that aim to avoid such complications and are able to obtain the most accurate mass data. This depends mainly on proper separation and enrichment techniques of phosphopeptides by 2D-HPLC to avoid suppression effects and MS technologies that are sensitive enough to detect low abundance peptides.

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS has been successfully utilized for protein analysis since the late 1980's and since then has been adapted into a high-throughput, simple and reliable technology with high sensitivity and nearly unlimited mass range that makes it a highly suitable method for the discovery and detection of clinical biomarkers [78, 79]. MALDI-TOF MS has also been used in research as a means of discovering relevant disease biomarkers and when coupled with fluidic separation and enrichment for phosphopeptides by 2D-HPLC provides a promising analysis method for clinical biomarkers.

5.1. Ionization Techniques

Although MALDI and ESI MS ionization techniques are both used in proteomic studies it is important to note that they are inherently different and so, tend to yield varying results for protein analysis [70]. On-line ESI uses a capillary to inject the sample directly into the mass spectrometer as a fine spray, whereas off-line MALDI samples are prepared by mixing the digested protein sample with an ultraviolet absorbing matrix. The matrix/peptide mixture is then applied, or spotted, onto a MALDI plate. When mixed, it is important that the matrix be in excess to the sample in order to "protect" the sample by absorbing energy from the laser, which could fragment the peptides, and separating the peptides to prevent aggregation [80]. Often, the remainder of the method

is fully automated, another feature of MALDI-TOF MS that would be highly beneficial in a clinical setting. Over the past decade the use of MALDI and ESI together has increased to obtain the most data possible from both ionization techniques [75].

5.2. Protein Quantification

MS identification of proteins and phosphoproteins has provided essential data for proteomic studies [81-83]. However clinical studies demand the ability to detect the most finite changes in PTM occurrence, location and quantity to determine the effect on tumorigenesis or presence of a disease related biomarker, requiring a highly sensitive, high resolution analysis method. To meet this requirement TOF MS is often run sequentially, termed tandem MS (MS/MS), to determine the location of the phosphorylated residue by peptide sequencing in the second MS analysis [75, 84]. A survey scan identifies precursor ions from the MS spectra based on ion intensity [77]. The most intense, or most prevalent, ions are then analyzed by MS again to determine the peptide sequence. Tandem MS can also be used for protein quantification when differentiating between two sample types and is often required for quantification when using common peptide labeling techniques.

Protein quantification depends on mass differences between two samples contributed by sample labeling where the peak areas of MS/MS mass spectra are measured for quantitative data. However common labeling methods are problematic because they increase sample complexity, cannot accommodate more than two samples at a time and cannot identify samples containing PTMs, such as phosphorylation [85]. The advent of isobaric tags for peptide labeling has provided a more sensitive, multiplex quantification strategy that can identify more proteins without increased sample complexity, with increased confidence and greater peptide coverage than previously used labeling methods. Isobaric tags are also capable of quantifying sample peptides with modifications as well as unmodified peptides and are useful in global protein scanning. Quantification of peptides and phosphopeptides by MS can determine variation in basal levels of proteins and identify a tumorigenic environment.

The iTRAQ™ method (Applied Biosystems, Carlsbad, CA) utilizes isobaric tags and has a vast advantage over other labeling techniques by accommodating up to 8 samples in an 8-plex multiplex reaction (Fig. **3**). This is vital when patient sample analysis must be completed in a short amount of time without sacrificing reliability. The iTRAQ™ Reagent isobaric tags consist of a reporter group, each with a unique ion mass, and a balance group that makes the total mass of each Reagent tag identical (Fig. **4**). A protein sample is digested and labeled with one of the tags, which are linked to lysine side chains and the N-terminus of each tryptic peptide. Each sample in the multiplex is labeled with a different tag but no mass difference is introduced between the peptides due to the isobaric nature of the tags. Thus, isobaric peptides cannot be differentiated by liquid chromatography and are enriched and fractionated in the same manner as unlabeled samples. It has been shown that there is little to no effect on peptide retention time during HPLC of iTRAQ™ labeled peptides, so the labeling technique does not interfere with HPLC separation methods [85]. iTRAQ™ has been successfully combined with HPLC fractionation for the analysis of

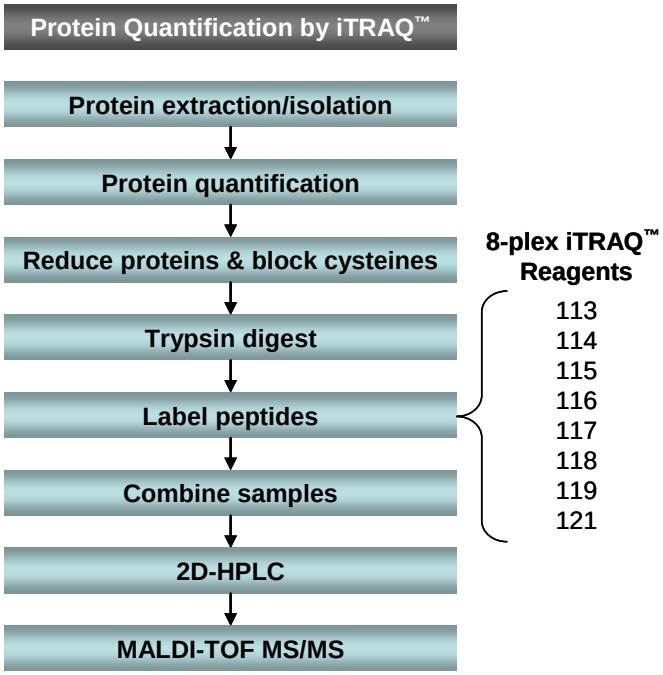

Fig. (3). Sample preparation by iTRAQ™ Reagent labeling. Protein is extracted from sample tissue, fluid or cells and quantified. Proteins are reduced and cysteines blocked using iTRAQ™ provided reagents. Proteins are digested with trypsin to yield a peptide mixture. Peptides are then labeled with an iTRAQ™ Reagent, 113-119 or 121 (8-plex reaction). Labeled peptide digests are combined into a single sample and fractionated by HPLC prior to MALDI-TOF MS/MS analysis.

complex protein mixtures [86] and for phosphorylated peptides [87].

During MS/MS, fragmentation by collision induced dissociation (CID) causes the balance group to be lost from the mass tag, which allows the reporter group mass signal to be detected. Since the different tags have different mass signals it is possible to differentiate the samples from each other based on their *m/z* values. The area of the reporter group peaks reflects the proportion of labeled peptides, allowing sample quantification by comparing the peak areas of the different reporter groups by computational analysis. Phosphoproteins can be quantified in the same way by iTRAQ labeling after phosphopeptide enrichment by HPLC. It is possible to determine differences in protein regulation and affected molecular pathways to establish disease biomarkers as well as possible treatment targets for tumorigenic environments.

The 4-plex iTRAQ™ method was first used to determine relative protein levels and measure target protein quantity in yeast strains to establish up- and down-regulation protein patterns [85]. This study identified over 1,000 proteins and found that iTRAQ™ is able to identify more peptides per protein than the ICAT labeling method, thus confidently identifying more proteins by a two-peptide match. iTRAQ™ quantification has since been applied to identify differentially expressed proteins in endometrial carcinoma, identifying over 1,000 proteins and 3 new biomarkers applicable to early disease diagnosis [88]. Recently, new, differentially expressed proteins were observed in head-and-neck squamous cell carcinoma, elucidating 3 biomarkers for the disease with a sensitivity of 92% [89].

The mass spectrometer is configured to detect the 10 or 15 largest ions in the first run in MALDI-TOF mode. The plate is then rerun in the MALDI-TOF-TOF mode, which analyzes these 10 to 15 fragments in TOF-TOF mode using chemical ionization. These spectra are then analyzed for fragmentation patterns and searched by several international databases using computational algorithms such as MASCOT and Paragon™ (Applied Biosystems). These algorithms can be adjusted for the iTRAQ™ mass offset and are adjusted to reflect tryptic digestion sites or other methods of digestion. Sequence values are computed for the TOF/TOF spectrum and compared to the database with several "goodness of fit" parameters to assign the structure to the peptide. The structural assignments of multiple peptides are then compared to the data base to assign the identification of the protein.

iTRAQ™ isobaric labeling has been utilized to identify protein changes associated with disease progression in both prostate and breast cancer. Prostate cancer cell lines were analyzed by iTRAQ™ labeling and 2D-HPLC-QTOF-MS/MS to identify and monitor protein expression changes related to prostate cancer progression [90]. This study identified 280 unique proteins and 14 potential biomarkers that were significantly differentially expressed between poorly and highly metastatic cell lines. Of these potential biomarkers, gp96 was identified as a possible novel indicator of prostate cancer progression. In another example a breast cancer cell line that mimics various stages of disease progression, including normal, premalignant, low grade and high grade disease state, was analyzed by iTRAQ™ labeling and 2D-HPLC-QTOF-MS/MS to investigate novel changes in EGFR expression associated with breast cancer progression [91]. It was found that tyrosine-phosphorylated EGFR decreases with disease progression and could be used as a biomarker to differentiate between normal and malignant tissue by detecting early stage disease. Both of these studies show that the iTRAQ™ method holds potential for biomarker quantification in many different cancer models.

The 8-plex iTRAQ™ method was able to differentiate differences in the proteome caused by 6 separate leukemogenic protein tyrosine kinases [92]. iTRAQ™ labeling was also combined with IMAC enrichment for phosphopeptides and LC-TOF MS to quantify tyrosine phosphorylation on proteins over time [87]. This method was able to identify 78 tyrosine phosphorylation sites on 58 key proteins. When combined with 2D-HPLC MALDI-TOF MS/MS enrichment and analysis techniques iTRAQ™ quantification of PTMs such as phosphorylation may be a valuable asset for future analysis to identify biomarkers related to tumorigenesis, disease progression and prognostic and therapy evaluation.

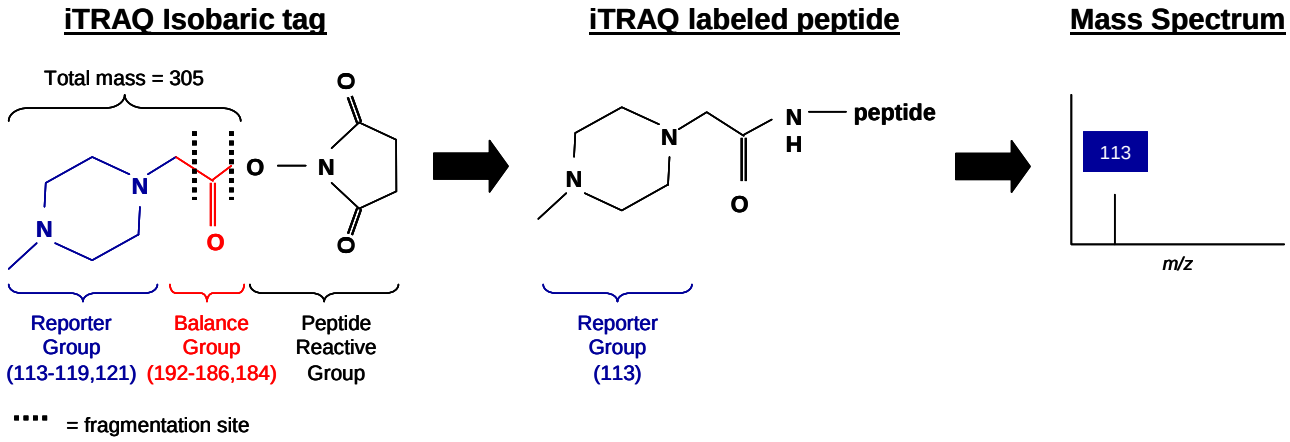

Fig. (4). The iTRAQ™ Reagent isobaric tag is comprised of a reporter group of known mass, a balance group and a peptide reactive group (left). When mixed with the peptide sample, the tag binds to lysine side chains and the N-terminus of each peptide (center). The reporter group and balance group remain attached to the peptide during HPLC separation and fractionation. During MALDI-TOF MS/MS the reporter group is released and detected to represent the abundance of the peptide to which it was attached (right).

6. MALDI-TOF MASS SPECTROMETRY IN CANCER BIOMARKER DISCOVERY

MALDI-TOF MS has played a key role in the development of clinical proteomics by using cancer related biomarkers identified in cell lines, animal models and clinical samples to determine the presence of disease and relevant treatment therapies. Valerio *et al*. utilized MALDI-TOF MS to analyze serum samples in an attempt to identify biomarkers to differentiate between malignant and non-malignant disease states of pancreatic cancer and chronic pancreatitis [56]. Several disease-related peptides were observed in both disease states, distinguishing between normal and diseased patient serum samples. However no biomarkers were found to characterize the two disease states from each other. It remains to be seen whether MS/MS peptide sequencing would have enabled identification of disease-specific biomarkers that could have determined the presence of carcinoma.

Analysis of mouse colorectal tissue blots from colon tumors and normal adjacent tissue by MALDI-TOF MS/MS identified three unique, highly abundant peptides in the tumor profile [93]. These peptides are related to calgranulins A and B and calgizzarin and hold potential as biomarkers to detect the presence of colorectal tumors. Interestingly, peptides were identified in direct tissue blots that had not been previously fractionated by gel-based or HPLC separation methods. However a tissue blot may not represent the entire spectrum of proteins present in a biological tissue sample, thus presenting a less complicated peptide collection for mass spectrometry analysis that is not truly representative of the tissue being analyzed.

Serum proteomic studies have also elucidated possible biomarkers to determine disease presence and differentiation for prostate, bladder, breast and thyroid cancers. Villanueva *et al*. found patterns of proteolytic degradation in serum proteome profiles that can serve as cancer-specific and cancer-type specific biomarkers [57]. MALDI-TOF MS/MS identified 61 signature peptides and evaluated key peptides found within the signature peptides. These key peptides were able to distinguish prostate, bladder and breast cancers as well as between normal control serum samples and cancerous serum samples, while providing an outcome prediction for advanced stage progression of all three cancer types. Additionally, a 12-peptide thyroid cancer signature was determined by comparing serum profiles from thyroid cancer patients and normal control patients [55]. This signature is 95% sensitive and 95% selective for the classification of malignant samples. The high sensitivity of the MALDI-TOF MS/MS technology allowed the identification of two novel, unique peptides specific to thyroid cancer, dehydroalanine-fibrinopeptide A and 54-amino acid-long fibrinogen-α fragment, possible biomarkers to confirm the presence of thyroid cancer.

A mixed profiling and imaging strategy with targeted protein identification was used to compare stage III and IV ovarian carcinomas and benign ovarian tumors to evaluate the presence or absence of biomarkers in the malignant and non-malignant tumor tissues [13]. MALDI-TOF MS identified 8 peptides specific to ovarian cancer that can differentiate between normal and malignant cells. One key biomarker, PA28, had a prevalence of 80% in malignant tumors and holds potential as a biomarker for cancerous ovarian tumors. This study directly analyzed tissue sections by MALDI-TOF MS, proving that MALDI-TOF technology is able to analyze a variety of sample types for various proteomic applications.

Treatment response and outcome was evaluated in non-small cell lung cancer (NSCLC) patients before and during treatment with chemotherapy and bortezomib [12]. The aim of this study was to identify biomarkers that could predict survival based on protein changes during treatment. Serum proteins were analyzed by MALDI-TOF MS/MS and 13 signature peptides were found that could characterize short and long survival rates with 86% accuracy, 100% sensitivity and 73% specificity. A 5 peptide signature able to identify patients likely to exhibit a partial response to treatment, compared to non-responders, was also identified and found to have 89% accuracy.

6.1. Phosphorylated Biomarkers

Vincristine is an antitumor drug that induces apoptosis and is used in chemotherapy treatment. However, some cancers are not responsive to this mode of therapy, warranting further investigation of molecular process during treatment. Treatment of MDF7 breast cancer cell line samples with vincristine was shown to cause up-regulation of the heat-shock protein HSP27, leading to metastasis, poor prognosis and resistance to therapeutic treatment methods [43]. MALDI-TOF MS analysis of the up-regulated form of HSP27 resulting from vincristine treatment revealed a phosphorylation PTM at Ser 82, indicating that HSP27 protein activity is regulated by phosphorylation and could be used as a biomarker to predict treatment response to chemotherapy drugs [43].

Estrogen receptor α (ERα) is involved in the development and growth of breast cancer. Thus, ERα serves as a biomarker for disease prognosis and treatment response to common endocrine breast cancer therapies, such as tamoxifen [94]. The presence of ERα provides a target for drug therapy, however only ~50% of ERα-positive breast cancer patients exhibit response to endocrine treatments, indicating resistance to drug therapies [95, 96]. It is thought that this resistance is due to abnormal phosphorylation events in ERα and the ability to discover and target these PTMs offers promising possibilities for the future of breast cancer treatment [97]. Serine-phosphorylation in the N-terminal domain of the protein causes ERα activation, so possible causes of endocrine resistance due to other phosphorylation events in ERα were investigated by MALDI-TOF MS/MS and ESI-QTOF MS analysis of cell line samples [1]. Previously known phosphorylation events at Ser-118 and Ser-167 [97] were confirmed by this study, however a new phosphorylation PTM was discovered. Ser-154 was found to be partially phosphorylated under breast cancer growth conditions and could act as a biomarker to indicate resistance to endocrine therapies. It is interesting to observe that in this study, Ser-118 was detected by MALDI and not ESI MS, while Ser-167 was detected by ESI and not MALDI MS. This is further proof that phosphopeptides react differently to different ionization techniques, highlighting the use of both methods to detect as many phosphopeptides as possible in a sample of interest.

7. CONCLUSION

Clinical proteomics provides the core database for bioinformatic elucidation of protein function and protein-protein interactions to understand when abnormalities in protein expression either cause or indicate a tumorigenic environment by interfering with normal cellular processes. Since many protein functions and interactions are dictated by post-translational modifications it is vital to investigate when, where and how often these modifications arise during disease occurrence and how they vary from the normal molecular state. Using these modifications as potential biomarkers for disease or targets for treatment is an increasing focus in cancer proteomics that holds vast potential for application in clinical proteomics.

The use of cancer related biomarkers in clinical proteomics offers new possibilities for personalized medicine. Once developed and validated potential uses could include: 1) screening for the likelihood of "molecular" disease development before onset, 2) diagnosis of early stage asymptomatic disease, 3) distinguishing benign from malignant tumors based on molecular expression patterns, 4) determination of "molecular" disease progression, 5) selection of a relevant treatment method based on the "molecular" likelihood of tumor sensitivity/resistance, and 6) monitoring of treatment "molecular" effectiveness over time and, coupled with genomic analysis, especially massive parallel sequencing, 7) identification of key 'bottleneck' therapeutic targets. However, before clinical proteomics can be implemented on a broad scale several issues must be addressed. Sample handling standard operating procedures must be established and applied from the time of sample collection through sample analysis to ensure sample quality, accuracy and reproducibility of results between assessments and different clinical locations [98]. The analysis technology best suited for specific biomarker detection must be determined for efficient and accurate analysis of clinical patient samples. Research of relevant biomarker identification is an on-going endeavor. As the future moves forward understanding between relevant clinical biomarker discrepancies and high throughput molecular technology to efficiently detect key biomarkers is a critical relationship.

ABBREVIATIONS

2D-GE	=	2-Dimensional gel electrophoresis
2D-HPLC	=	2-Dimensional high-performance liquid chromatography
ATP	=	Adenosine tri-phosphate
CID	=	Collision induced dissociation
EGF	=	Epidermal growth factor
ERα	=	Estrogen receptor α
ESI	=	Electrospray ionization
HSP27	=	Heat shock protein 27
ICAT	=	Isotope coded affinity tags
IMAC	=	Immobilized metal affinity chromatography
iTRAQ™	=	Isobaric tags for relative and absolute quantitation
MALDI	=	Matrix-assisted laser-desorption ionization
MALDI-TOF MS/MS	=	Matrix-assisted laser-desorption ionization time-of-flight tandem mass spectrometry
MS	=	Mass spectrometry
NSCLC	=	Non-small cell lung cancer
m/z	=	Mass to charge ratio
PA28	=	Proteasome activator 28
PTM	=	Post-translational modification
Q-TOF MS/MS	=	Quadrupole time-of-flight tandem mass spectrometry
RP	=	Reverse phase
SCX	=	Strong cation exchange
TiO_2	=	Titanium dioxide

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