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(54) Title: SRM/MRM ASSAY FOR THE FATTY ACID SYNTHASE PROTEIN

(57) Abstract: Specific peptides, and derived ionization characteristics of the peptides, from the Fatty acid synthase (FASN) protein are provided that are particularly advantageous for quantifying the FASN protein directly in biological samples that have been fixed in formalin by the method of Selected Reaction Monitoring (SRM) mass spectrometry or Multiple Reaction Monitoring (MRM) mass spectrometry.

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SRM/MRM Assay for the Fatty Acid Synthase Protein

This application claims the benefit of U.S. Provisional Application No. 61/538,091, filed September 22, 2011, entitled "SRM/MRM Assay for the Fatty Acid Synthase Protein," the content of which are hereby incorporated by referenced in their entirety.

Introduction

Specific peptides derived from subsequences of the Fatty Acid Synthase protein (referred to herein as FASN, and which also is referred to as FAS), are provided. The peptide sequence and fragmentation/transition ions for each peptide are particularly useful in a mass spectrometry-based Selected Reaction Monitoring (SRM) assay, which can also be referred to as a Multiple Reaction Monitoring (MRM) assay. Such assays are referred to herein as SRM/MRM. The use of peptides for quantitative SRM/MRM analysis of the FASN protein is described.

This SRM/MRM assay can be used to measure *relative* or *absolute* quantitative levels of one or more of the specific peptides from the FASN protein. This provides a means of measuring the amount of the FASN protein in a given protein preparation obtained from a biological sample by mass spectrometry.

More specifically, the SRM/MRM assay can measure these peptides directly in complex protein lysate samples prepared from cells procured from patient tissue samples, such as formalin fixed cancer patient tissue. Methods of preparing protein samples from formalin-fixed tissue are described in U.S. Patent No. 7,473,532, the contents of which are hereby incorporated by reference in their entirety. The methods described in U.S. Patent No. 7,473,532 may conveniently be carried out using Liquid Tissue™ reagents and protocol available from OncoPlexDx (formerly Expression Pathology Inc., Rockville, MD).

The most widely and advantageously available form of tissues from cancer patients tissue is formalin fixed, paraffin embedded tissue. Formaldehyde/formalin fixation of surgically removed tissue is by far and away the most common method of preserving cancer tissue samples worldwide and is the accepted convention for standard pathology practice. Aqueous solutions of formaldehyde are referred to as formalin. "100%" formalin consists of a saturated solution of formaldehyde (this is about 40% by volume or 37% by mass) in water, with a small amount of stabilizer, usually methanol to limit oxidation and degree of polymerization. The most common way in which tissue is preserved is to soak whole tissue for extended periods of time (8 hours to 48 hours) in aqueous formaldehyde, commonly termed 10% neutral buffered formalin, followed by embedding the fixed whole tissue in paraffin wax for long term storage at room temperature. Thus, molecular analytical methods to analyze formalin fixed cancer tissue will be the most accepted and heavily utilized methods for analysis of cancer patient tissue.

Results from the SRM/MRM assay can be used to correlate accurate and precise quantitative levels of the FASN protein within the specific tissue samples (e.g., one or more cancer tissue samples) of the patient or subject from whom the tissue (biological sample) was collected and preserved. This not only provides diagnostic information about the cancer, but also permits a physician or other medical professional to determine appropriate therapy for the patient. For example, such an assay can be designed to diagnose the stage or degree of a cancer and determine a therapeutic agent to which a patient is most likely to respond. Such an assay that provides diagnostically and therapeutically important information about levels of protein expression in a diseased tissue or other patient sample is termed a *companion diagnostic* assay.

Summary

The assays described herein measure *relative* or *absolute* levels of specific unmodified peptides from the FASN protein and also can measure absolute or relative levels of specific modified peptides from the FASN protein. Examples of modifications include phosphorylated amino acid residues (*e.g.* phosphotyrosine, phosphoserine and phosphothreonine) and glycosylated amino acid residues (*e.g.* glycosylated asparagine residues) that are present on the peptides.

Relative quantitative levels of the FASN protein are determined by the SRM/MRM methodology, for example, by comparing SRM/MRM signature peak areas (e.g., signature peak area or integrated fragment ion intensity) of an individual FASN peptide in different samples. Alternatively, it is possible to compare multiple SRM/MRM signature peak areas for multiple FASN signature peptides, where each peptide has its own specific SRM/MRM signature peak, to determine the relative FASN protein content in one biological sample with the FASN protein content in one or more additional or different biological samples. In this way, the amount of a particular peptide, or peptides, from the FASN protein, and therefore the amount of the FASN protein, is determined relative to the same FASN peptide, or peptides, across 2 or more biological samples under the same experimental conditions. In addition, relative quantitation can be determined for a given peptide, or peptides, from the FASN protein within a single sample by comparing the signature peak area for that peptide by SRM/MRM methodology to the signature peak area for another and different peptide, or peptides, from a different protein, or proteins, within the same protein preparation from the biological sample. In this way, the amount of a particular peptide from the FASN protein, and therefore the amount of the FASN protein, is determined relative one to another within the same sample. These approaches generate quantitation of an individual peptide, or peptides, from the FASN protein to the amount of another peptide, or peptides, between samples and within samples wherein the amounts as determined by peak area are relative one to another, regardless of the absolute weight to volume or weight to weight amounts of the FASN peptide in the protein preparation from the biological sample. Relative quantitative data about individual signature peak areas between different samples are normalized to the amount of protein analyzed per sample. Relative quantitation can be performed across many peptides from multiple proteins and the FASN protein simultaneously in a single sample and/or across many samples to gain insight into relative protein amounts, one peptide/protein with respect to other peptides/proteins.

Absolute quantitative levels of the FASN protein are determined by, for example, the SRM/MRM methodology whereby the SRM/MRM signature peak area of an individual peptide from the FASN protein in one biological sample is compared to the SRM/MRM signature peak area of an exogenously added “spiked” internal standard. In one embodiment, the internal standard is a synthetic version of the same exact FASN peptide that contains one or more amino acid residues labeled with one or more heavy isotopes. Suitable isotope-labeled internal standards are synthesized so that, when analyzed by mass spectrometry, each standard generates a predictable and consistent SRM/MRM signature peak that is different and distinct from the native FASN peptide signature peak and which can be used as a comparator peak. Thus, when the internal standard is spiked in a known amount into a protein preparation from a biological sample and analyzed by mass spectrometry, the SRM/MRM signature peak area of the native peptide from the sample can be compared to the SRM/MRM signature peak area of the internal standard peptide. This numerical comparison provides either the absolute molarity and/or absolute weight of the native peptide present in the original protein preparation from the biological sample. Absolute quantitative data for fragment peptides are displayed according to the amount of protein analyzed per sample. Absolute quantitation can be performed across many peptides, and thus proteins, simultaneously in a single sample and/or across many samples to gain insight into absolute protein amounts in individual biological samples and in entire cohorts of individual samples.

The SRM/MRM assay method can be used to aid diagnosis of the stage of cancer, for example, directly in patient-derived tissue, such as formalin fixed tissue, and to aid in determining which therapeutic agent would be most advantageous for use in treating that patient. Cancer tissue that is removed from a patient either through surgery, such as for therapeutic removal of partial or entire tumors, or through biopsy procedures conducted to determine the presence or absence of suspected disease, is analyzed to determine whether or not a specific protein, or proteins, and which forms of proteins, are present in that patient tissue. Moreover, the expression level of a protein, or multiple proteins, can be determined and compared to a "normal" or reference level found in healthy tissue. Normal or reference levels of proteins found in healthy tissue may be derived from, for example, the relevant tissues of one or more individuals that do not have cancer. Alternatively, normal or reference levels may be obtained for individuals with cancer by analysis of relevant tissues not affected by the cancer.

Assays of protein levels (e.g., FASN levels) can also be used to diagnose the stage of cancer in a patient or subject diagnosed with cancer by employing the FASN levels. Levels or amounts of proteins or peptides can be defined as the quantity expressed in moles, mass or weight of a protein or peptide determined by the SRM/MRM assay. The level or amount may be normalized to total the level or amount of protein or another component in the lysate analyzed (*e.g.*, expressed in micromoles/microgram of protein or micrograms /microgram of protein). In addition, the level or amount of a protein or peptide may be determined on volume basis, expressed, for example, in micromolar or nanograms/microliter. The level or amount of protein or peptide as determined by the SRM/MRM assay can also be normalized to the number of cells analyzed. Information regarding FASN can thus be used to aid in determining stage or grade of a cancer by correlating the level of the FASN protein (or fragment peptides of the FASN protein) with levels observed in normal tissues.

Once the stage and/or grade, and/or FASN protein expression characteristics of the cancer has been determined, that information can be matched to a list of therapeutic agents (chemical and biological) developed to specifically treat cancer tissue that is characterized by, for example, abnormal expression of the protein or protein(s) (*e.g.*, FASN) that were assayed. Matching information from an FASN protein assay to a list of therapeutic agents that specifically targets, for example, the FASN protein or cells/tissue expressing the protein, defines what has been termed a *personalized medicine* approach to treating disease. The assay methods described herein form the foundation of a *personalized medicine* approach by using analysis of proteins from the patient’s own tissue as a source for diagnostic and treatment decisions.

Detailed Description

In principle, any predicted peptide derived from FASN protein, prepared for example by digesting with a protease of known specificity (*e.g.* trypsin), can be used as a surrogate reporter to determine the abundance of FASN protein in a sample using a mass spectrometry-based SRM/MRM assay. Similarly, any predicted peptide sequence containing an amino acid residue at a site that is known to be potentially modified in FASN protein also can be used to assay the extent of modification of FASN protein in a sample.

FASN fragment peptides may be generated in a variety of ways, including using the Liquid Tissue™ protocol described, for example, in US Patent 7,473,532. Liquid Tissue™ protocol and reagents produce peptide samples suitable for mass spectroscopic analysis from formalin fixed paraffin embedded tissue by proteolytic digestion of the proteins in the tissue/biological sample. Suitable reagents and protocols also are commercially available from OncoPlexDx (formerly Expression Pathology Inc., Rockville, MD).

In the Liquid Tissue™ protocol the tissue/biological sample is heated in a buffer for an extended period of time (e.g., from about 80° C to about 100° C for a period of time from about 10 minutes to about 4 hours) to reverse or release protein cross-linking. The buffer employed is a neutral buffer, (e.g., a Tris-based buffer, or a buffer containing a detergent). Following heat treatment the tissue/biological sample is treated with one or more proteases including, but not limited to, trypsin, chymotrypsin, pepsin, and endoproteinase Lys-C for a time sufficient to disrupt the tissue and cellular structure of said biological sample and to liquefy the sample. Exemplary conditions for the protease treatment are from 30 minutes to 24 hours at a temperature from 37° C to 65° C). Advantageously, endoproteases, and particularly combinations of two or three endoproteases, used either simultaneously or sequentially, are employed to liquefy the sample. For example, suitable combinations of proteases can include, but are not limited to, combinations of trypsin, endoproteinase Lys-C and chemotrypsin, such as trypsin and endoproteinase Lys-C. The result of the heating and proteolysis is a liquid, soluble, dilutable biomolecule lysate. Advantageously, this liquid lysate is free of solid or particulate matter that can be separated from the lysate by centrifugation.

Surprisingly, it was found that many potential peptide sequences from the FASN protein are unsuitable or ineffective for use in mass spectrometry-based SRM/MRM assays for reasons that are not immediately evident. As it was not possible to predict the most suitable peptides for MRM/SRM assay, it was necessary to experimentally identify modified and unmodified peptides in actual Liquid Tissue™ lysates to develop a reliable and accurate SRM/MRM assay for the FASN protein. While not wishing to be bound by any theory, it is believed that some peptides might, for example, be difficult to detect by mass spectrometry because they do not ionize well or produce fragments that are not distinct from those generated from other proteins. Peptides may also fail to resolve well in separation (e.g., liquid chromatography), or may adhere to glass or plastic ware, which leads to erroneous results in the SRM/MRM assay.

FASN peptides found in various embodiments of this disclosure (e.g., Tables 1 and 2 below) were derived from the FASN protein by protease digestion of all the proteins within a complex Liquid Tissue™ lysate prepared from cells procured from formalin fixed cancer tissue. Unless noted otherwise, in each instance the protease was trypsin. The Liquid Tissue™ lysate was then analyzed by mass spectrometry to determine those peptides derived from the FASN protein that are detected and analyzed by mass spectrometry. Identification of a specific preferred subset of peptides for mass-spectrometric analysis is based on; 1) experimental determination of which peptide or peptides from a protein ionize in mass spectrometry analyses of Liquid Tissue™ lysates, and 2) the ability of the peptide to survive the protocol and experimental conditions used in preparing a Liquid Tissue™ lysate. This latter property extends not only to the amino acid sequence of the peptide but also to the ability of a modified amino acid residue within a peptide to survive in modified form during the sample preparation.

Protein lysates from cells procured directly from formalin (formaldehyde) fixed tissue were prepared using the Liquid Tissue™ reagents and protocol. This entails collecting cells into a sample tube via tissue microdissection followed by heating the cells in the Liquid Tissue™ buffer for an extended period of time. Once the formalin-induced cross linking has been negatively affected, the tissue/cells are then digested to completion in a predictable manner using a protease such as, trypsin. The skilled artisan will recognize that other proteases, and in particular, endoproteases may be used in place of, or in addition to, trypsin. Each protein lysate was used to prepare a collection of peptides by digestion of intact polypeptides with the protease or protease combination. Each Liquid Tissue™ lysate was analyzed (*e.g.*, by ion trap mass spectrometry) to perform multiple global proteomic surveys of the peptides where the data was presented as identification of as many peptides as could be identified by mass spectrometry from all cellular proteins present in each protein lysate. An ion trap mass spectrometer or another form of a mass spectrometer that is capable of performing global profiling for identification of as many peptides as possible from a single complex protein/peptide lysate may be employed. Ion trap mass spectrometers may, however, be the best type of mass spectrometer for conducting global profiling of peptides. Although SRM/MRM assays can be developed and performed on any type of mass spectrometer, including a MALDI, ion trap, or triple quadrupole, an instrument platform for SRM/MRM assay is often considered to be a triple quadrupole instrument platform.

Once as many peptides as possible were identified in a single mass spectrometric analysis of a single lysate under the conditions employed, then the list of identified peptides was collated and used to determine the proteins that were detected in that lysate. This process was repeated for multiple Liquid Tissue™ lysates, and the very large list of peptides was collated into a single dataset. The resulting dataset represents the peptides that can be detected in the type of biological sample that was analyzed (after protease digestion), and specifically in a Liquid Tissue™ lysate of the biological sample, and thus includes the peptides for specific proteins, such as for example the FASN protein.

In one embodiment, the FASN tryptic peptides identified as useful in the determination of absolute or relative amounts of the FASN receptor include one or more, two or more, three or more, four or more, five or more, six or more, eight or more, or ten or more of the peptides of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, and SEQ ID NO:11, each of which sequences are shown in Table 1. Each of those peptides was detected by mass spectrometry in Liquid Tissue™ lysates prepared from formalin fixed, paraffin embedded tissue. Thus, each of the peptides in Table 1, or any combination of those peptides (e.g., one or more, two or more, three or more, four or more, five or more, six or more, eight or more, or ten or more of those peptides recited in Table 1, and particularly combinations with the peptides found in Table 2) are candidates for use in quantitative SRM/MRM assay for the FASN protein in human biological samples, including directly in formalin fixed patient tissue. Table 2 shows additional information regarding three of the peptides shown in Table 1.

Table 1

SEQ ID	Peptide sequence
SEQ ID NO: 1	LPEDPLLSGLLDSPALK
SEQ ID NO: 2	VGDPQELNGITR
SEQ ID NO: 3	DLVEAVAHILGIR
SEQ ID NO: 4	LQVVDQPLPVR
SEQ ID NO: 5	GVDLVLNSLAEEK
SEQ ID NO: 6	VLEALLPLK
SEQ ID NO: 7	FDASFFGVHPK
SEQ ID NO: 8	HGLYLPTR
SEQ ID NO: 9	SEGVVAVLLTK
SEQ ID NO: 10	VYQWDDPDPR
SEQ ID NO: 11	AQVADVVVSR

Table 2

SEQ ID	Peptide sequence	Mono Isotopic Mass	Precursor Charge State	Precursor m/z	Transition m/z	Ion Type
SEQ ID NO: 4	LQVVDQPLPVR	1262.735	2	632.375	581.376	y5
			2	632.375	709.435	y6
			2	632.375	824.462	y7
			2	632.375	923.53	y8
			2	632.375	1022.599	y9
SEQ ID NO: 6	VLEALLPLK	994.643	2	498.329	583.417	y5
			2	498.329	654.454	y6
			2	498.329	783.497	y7
			2	498.329	896.581	y8
			2	498.329	995.649	y9
SEQ ID NO: 9	SEGVVAVLLTK	1114.66	2	558.337	573.397	y5
			2	558.337	644.434	y6
			2	558.337	743.502	y7
			2	558.337	842.57	y8
			2	558.337	899.592	y9
			2	558.337	1028.635	y10

The FASN tryptic peptides listed in Table 1 include those detected from multiple Liquid Tissue™ lysates of multiple different formalin fixed tissues of different human organs including prostate, colon, and breast. Each of those peptides is useful for quantitative SRM/MRM assay of the FASN protein in formalin fixed tissue. Further data analysis of these experiments indicated no preference is observed for any specific peptides from any specific organ site. Thus, each of these peptides is suitable for conducting SRM/MRM assays of the FASN protein on a Liquid Tissue™ lysate from any formalin fixed tissue originating from any biological sample or from any organ site in the body.

In one embodiment, the peptides in Table 1, or any combination of those peptides (e.g., one or more, two or more, three or more, four or more, five or more, six or more, eight or more, or ten or more of those peptides recited in Table 1, and particularly combinations with the peptides also found in Table 2) are assayed by methods that do not rely upon mass spectroscopy, including, but not limited to, immunological methods (e.g., Western blotting or ELISA). Regardless of how information directed to the amount of the peptide(s) (absolute or relative) is obtained, the information may be employed in any of the methods described herein, including indicating (diagnosing) the presence of cancer in a subject, determining the stage/grade/status of the cancer, providing a prognosis, or determining the therapeutics or treatment regimen for a subject/patient.

Embodiments of the present disclosure include compositions comprising one or more, two or more, three or more, four or more, five or more, six or more, eight or more, or ten or more of the peptides in Table 1. In some embodiments, the compositions comprise the peptides in Table 2. Compositions comprising peptides may include one or more, two or more, three or more, four or more, five or more, six or more, eight or more, or ten or more peptides that are isotopically labeled. Each of the peptides may be labeled with one or more isotopes selected independently from the group consisting of: ^{18}O, ^{17}O, ^{34}S, ^{15}N, ^{13}C, ^{2}H or combinations thereof. Compositions comprising peptides from the FASN protein, whether isotope labeled or not, need not contain all of the peptides from that protein (e.g., a complete set of tryptic peptides). In some embodiments the compositions do not contain one or more, two or more, three or more, four or more, five or more, six or more, eight or more, or ten or more peptides from FASN, and particularly peptides appearing in Table 1 or Table 2. Compositions comprising peptides may be in the form of dried or lyophized materials, liquid (e.g., aqueous) solutions or suspensions, arrays, or blots.

One consideration for conducting an SRM/MRM assay is the type of instrument that may be employed in the analysis of the peptides. Although SRM/MRM assays can be developed and performed on any type of mass spectrometer, including a MALDI, ion trap, or triple quadrupole, the most advantageous instrument platform for SRM/MRM assay is often considered to be a triple quadrupole instrument platform. That type of a mass spectrometer may be considered to be the most suitable instrument for analyzing a single isolated target peptide within a very complex protein lysate that may consist of hundreds of thousands to millions of individual peptides from all the proteins contained within a cell.

In order to most efficiently implement SRM/MRM assay for each peptide derived from the FASN protein it is desirable to utilize information in addition to the peptide sequence in the analysis. That additional information may be used in directing and instructing the mass spectrometer (e.g. a triple quadrupole mass spectrometer) to perform the correct and focused analysis of specific targeted peptide(s) such that the assay may be effectively performed.

The additional information about target peptides in general, and about specific FASN peptides, may include one or more of the mono isotopic mass of the peptide, its precursor charge state, the precursor m/z value, the m/z transition ions, and the ion type of each transition ion. Additional peptide information that may be used to develop an SRM/MRM assay for the FASN protein is shown by example for three (3) of the FASN peptides from the list in Table 1 and is shown in Table 2. Similar additional information described for these three (3) FASN peptides shown by example in Table 2 may be prepared, obtained, and applied to the analysis of the other peptides contained in Table 1.

The method described below was used to: 1) identify candidate peptides from the FASN protein that can be used for a mass spectrometry-based SRM/MRM assay for the FASN protein, 2) develop individual SRM/MRM assay, or assays, for target peptides from the FASN protein, and 3) apply quantitative assays to cancer diagnosis and/or choice of optimal therapy.

Assay Method

1. Identification of SRM/MRM candidate fragment peptides for the FASN protein
    a. Prepare a Liquid Tissue™ protein lysate from a formalin fixed biological sample using a protease or proteases, (that may or may not include trypsin), to digest proteins
    b. Analyze all protein fragments in the Liquid Tissue™ lysate on an ion trap tandem mass spectrometer and identify all fragment peptides from the FASN protein, where individual fragment peptides do not contain any peptide modifications such as phosphorylations or glycosylations
    c. Analyze all protein fragments in the Liquid Tissue™ lysate on an ion trap tandem mass spectrometer and identify all fragment peptides from the FASN protein that

carry peptide modifications such as for example phosphorylated or glycosylated residues

   d. All peptides generated by a specific digestion method from the entire, full length FASN protein potentially can be measured, but preferred peptides used for development of the SRM/MRM assay are those that are identified by mass spectrometry directly in a complex Liquid Tissue™ protein lysate prepared from a formalin fixed biological sample
   e. Peptides that are specifically modified (phosphorylated, glycosylated, etc.) in patient tissue and which ionize, and thus detected, in a mass spectrometer when analyzing a Liquid Tissue™ lysate from a formalin fixed biological sample are identified as candidate peptides for assaying peptide modifications of the FASN protein

2. Mass Spectrometry Assay for Fragment Peptides from FASN Protein
   a. SRM/MRM assay on a triple quadrupole mass spectrometer for individual fragment peptides identified in a Liquid Tissue™ lysate is applied to peptides from the FASN protein
      i. Determine optimal retention time for a fragment peptide for optimal chromatography conditions including but not limited to gel electrophoresis, liquid chromatography, capillary electrophoresis, nano-reversed phase liquid chromatography, high performance liquid chromatography, or reverse phase high performance liquid chromatography
      ii. Determine the mono isotopic mass of the peptide, the precursor charge state for each peptide, the precursor m/z value for each peptide, the m/z transition ions for each peptide, and the ion type of each transition ion for each fragment peptide in order to develop an SRM/MRM assay for each peptide.
      iii. SRM/MRM assay can then be conducted using the information from (i) and (ii) on a triple quadrupole mass spectrometer where each peptide has a characteristic and unique SRM/MRM signature peak that precisely defines the unique SRM/MRM assay as performed on a triple quadrupole mass spectrometer
   b. Perform SRM/MRM analysis so that the amount of the fragment peptide of the FASN protein that is detected, as a function of the unique SRM/MRM signature

peak area from an SRM/MRM mass spectrometry analysis, can indicate both the relative and absolute amount of the protein in a particular protein lysate.

i. Relative quantitation may be achieved by:

  1. Determining increased or decreased presence of the FASN protein by comparing the SRM/MRM signature peak area from a given FASN peptide detected in a Liquid Tissue™ lysate from one formalin fixed biological sample to the same SRM/MRM signature peak area of the same FASN fragment peptide in at least a second, third, fourth or more Liquid Tissue™ lysates from least a second, third, fourth or more formalin fixed biological samples
  2. Determining increased or decreased presence of the FASN protein by comparing the SRM/MRM signature peak area from a given FASN peptide detected in a Liquid Tissue™ lysate from one formalin fixed biological sample to SRM/MRM signature peak areas developed from fragment peptides from other proteins, in other samples derived from different and separate biological sources, where the SRM/MRM signature peak area comparison between the 2 samples for a peptide fragment are normalized to amount of protein analyzed in each sample.
  3. Determining increased or decreased presence of the FASN protein by comparing the SRM/MRM signature peak area for a given FASN peptide to the SRM/MRM signature peak areas from other fragment peptides derived from different proteins within the same Liquid Tissue™ lysate from the formalin fixed biological sample in order to normalize changing levels of FASN protein to levels of other proteins that do not change their levels of expression under various cellular conditions.
  4. These assays can be applied to both unmodified fragment peptides and for modified fragment peptides of the FASN protein, where the modifications include but are not limited to phosphorylation and/or glycosylation, and where the relative levels of modified peptides are determined in the same manner as determining relative amounts of unmodified peptides.

    ii. Absolute quantitation of a given peptide may be achieved by comparing the SRM/MRM signature peak area for a given fragment peptide from the FASN protein in an individual biological sample to the SRM/MRM signature peak area of an internal fragment peptide standard spiked into the protein lysate from the biological sample
        1. The internal standard is a labeled synthetic version of the fragment peptide from the FASN protein that is being interrogated. This standard is spiked into a sample in known amounts, and the SRM/MRM signature peak area can be determined for both the internal fragment peptide standard and the native fragment peptide in the biological sample separately, followed by comparison of both peak areas
        2. This can be applied to unmodified fragment peptides and modified fragment peptides, where the modifications include but are not limited to phosphorylation and/or glycosylation, and where the absolute levels of modified peptides can be determined in the same manner as determining absolute levels of unmodified peptides.

3. Apply Fragment Peptide Quantitation to Cancer Diagnosis and Treatment
    a. Perform relative and/or absolute quantitation of fragment peptide levels of the FASN protein and demonstrate that the previously-determined association, as well understood in the field of cancer, of FASN protein expression to the stage/grade/status of cancer in patient tumor tissue is confirmed
    b. Perform relative and/or absolute quantitation of fragment peptide levels of the FASN protein and demonstrate correlation with clinical outcomes from different treatment strategies, wherein this correlation has already been demonstrated in the field or can be demonstrated in the future through correlation studies across cohorts of patients and tissue from those patients. Once either previously established correlations or correlations derived in the future are confirmed by this assay then the assay method can be used to determine optimal treatment strategy

Assessment of FASN protein levels in tissues based on analysis of formalin fixed patient-derived tissue can provide diagnostic, prognostic, and therapeutically-relevant information about each particular patient. In one embodiment, this disclosure describes a method for measuring the level of the FASN protein in a biological sample, comprising detecting and/or quantifying the amount of one or more modified or unmodified FASN fragment peptides in a protein digest prepared from the biological sample using mass spectrometry; and calculating the level of modified or unmodified FASN protein in the sample; and where the level is a relative level or an absolute level. In a related embodiment, quantifying one or more FASN fragment peptides comprises determining the amount of each of the FASN fragment peptides in a biological sample by comparison to a known amount of an added internal standard peptide, where each of the FASN fragment peptides in the biological sample is compared to an internal standard peptide having the same amino acid sequence. In some embodiments the internal standard is an isotopically labeled internal standard peptide comprises one or more heavy stable isotopes selected from ^{18}O, ^{17}O, ^{34}S, ^{15}N, ^{13}C, ^{2}H or combinations thereof.

The method for measuring the level of the FASN protein in a biological sample described herein (or fragment peptides as surrogates thereof) may be used as a diagnostic indicator of cancer in a patient or subject. In one embodiment, the results from measurements of the level of the FASN protein may be employed to determine the diagnostic stage/grade/status of a cancer by correlating (e.g., comparing) the level of FASN protein found in a tissue with the level of that protein found in normal and/or cancerous or precancerous tissues.

EMBODIMENTS:

1. A method for measuring the level of the Fatty Acid Synthase (FASN) protein in a biological sample, comprising detecting and/or quantifying the amount of one or more modified or unmodified FASN fragment peptides in a protein digest prepared from said biological sample using mass spectrometry; and calculating the level of modified or unmodified FASN protein in said sample; and
wherein said amount is a relative amount or an absolute amount.
2. The method of embodiment 1, further comprising the step of fractionating said protein digest prior to detecting and/or quantifying the amount of one or more modified or unmodified FASN fragment peptides.
3. The method of embodiment 2, wherein said fractionating step is selected from the group consisting of gel electrophoresis, liquid chromatography, capillary electrophoresis, nano-reversed phase liquid chromatography, high performance liquid chromatography, or reverse phase high performance liquid chromatography.

4. The method of any of embodiments 1-3, wherein said protein digest of said biological sample is prepared by the Liquid Tissue™ protocol.
5. The method of any of embodiments 1-3, wherein said protein digest comprises a protease digest.
6. The method of embodiment 5, wherein said protein digest comprises a trypsin digest.
7. The method of any of embodiments 1-6, wherein said mass spectrometry comprises tandem mass spectrometry, ion trap mass spectrometry, triple quadrupole mass spectrometry, MALDI-TOF mass spectrometry, MALDI mass spectrometry, and/or time of flight mass spectrometry.
8. The method of embodiment 7, wherein the mode of mass spectrometry used is Selected Reaction Monitoring (SRM), Multiple Reaction Monitoring (MRM), and/or multiple Selected Reaction Monitoring (mSRM), or any combination thereof.
9. The method of any of embodiments 1 to 8, wherein the one or more modified or unmodified FASN fragment peptides comprise different amino acid sequences independently selected from those set forth in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, and SEQ ID NO:11.
10. The method of any of embodiments 1-9, wherein the biological sample is a blood sample, a urine sample, a serum sample, an ascites sample, a sputum sample, lymphatic fluid, a saliva sample, a cell, or a solid tissue.
11. The method of embodiment 10, wherein the tissue is formalin fixed tissue.
12. The method of embodiment 10 or 11, wherein the tissue is paraffin embedded tissue.
13. The method of embodiment 10, wherein the tissue is obtained from a tumor.
14. The method of embodiment 13, wherein the tumor is a primary tumor.
15. The method of embodiment 13, wherein the tumor is a secondary tumor.
16. The method of any of embodiments 1 to 15, further comprising quantifying a modified or unmodified FASN fragment peptide.
17. The method of embodiment 16, wherein quantifying the FASN fragment peptide comprises comparing an amount of one or more, two or more, three or more, four or more, or five or more FASN fragment peptides comprising an amino acid sequence of about 8 to about 45 amino acid residues of FASN as shown in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, or SEQ ID NO:11 in one biological sample to

the amount of the same FASN fragment peptide in a different and separate biological sample.

18. The method of embodiment 17, wherein quantifying one or more FASN fragment peptides comprises determining the amount of the each of the FASN fragment peptides in a biological sample by comparison to an added internal standard peptide of known amount, wherein each of the FASN fragment peptides in the biological sample is compared to an internal standard peptide having the same amino acid sequence.
19. The method of embodiment 18, wherein the internal standard peptide is an isotopically labeled peptide.
20. The method of embodiment 19, wherein the isotopically labeled internal standard peptide comprises one or more heavy stable isotopes selected from ^{18}O, ^{17}O, ^{34}S, ^{15}N, ^{13}C, ^{2}H or combinations thereof.
21. The method of any of embodiments 1 to 20, wherein detecting and/or quantifying the amount of one or more modified or unmodified FASN fragment peptides in the protein digest indicates the presence of modified or unmodified FASN protein and an association with cancer in the subject.
22. The method of embodiment 21, further comprising correlating the results of said detecting and/or quantifying the amount of one or more modified or unmodified FASN fragment peptides, or the amount of said FASN protein to the diagnostic stage/grade/status of the cancer.
23. The method of embodiment 22, wherein correlating the results of said detecting and/or quantifying the amount of one or more modified or unmodified FASN fragment peptides, or the amount of said FASN protein to the diagnostic stage/grade/status of the cancer is combined with detecting and/or quantifying the amount of other proteins or peptides from other proteins in a multiplex format to provide additional information about the diagnostic stage/grade/status of the cancer.
24. The method of any one of embodiments 1-23, further comprising selecting for the subject from which said biological sample was obtained a treatment based on the presence, absence, or amount of one or more FASN fragment peptides or the amount of FASN protein.
25. The method of any one of embodiments 1-24, further comprising administering to the patient from which said biological sample was obtained a therapeutically effective amount of a therapeutic agent, wherein the therapeutic agent and/or amount of the

therapeutic agent administered is based upon amount of one or more modified or unmodified FASN fragment peptides or the amount of FASN protein.

26. The method of embodiments 24 and 25, wherein the treatment or the therapeutic agent is directed to cancer cells expressing the FASN protein.
27. The method of embodiments 1 to 26, wherein the biological sample is formalin fixed tumor tissue that has been processed for quantifying the amount of one or more modified or unmodified FASN fragment peptides employing the Liquid Tissue™ protocol and reagents.
28. The method of any of embodiments 1-27, wherein said one or more modified or unmodified FASN fragment peptides is two or more, three or more, four or more, five or more, six or more, eight or more, or ten or more of the peptides in Table 1.
29. The method of any of embodiments 1-28, comprising quantifying the amount of one, two or more, three or more, four or more, five or more, six or more, eight or more, or ten or more of the peptides in Table 2.
30. A composition comprising one, two or more, three or more, four or more, five or more, six or more, eight or more, or ten or more of the peptides in Table 1 or antibodies thereto, said composition optionally excluding one, two, three or more other peptides of FASN.
31. The composition of embodiment 30, comprising one, two or three of the peptides of Table 2 or antibodies thereto.

It is to be understood that the description, specific examples, embodiments, and data, while indicating exemplary aspects, are given by way of illustration and are not intended to limit the present disclosure. Various changes and modifications within the present disclosure will become apparent to the skilled artisan from the discussion, detailed description and data contained herein, and thus are considered part of the subject matter of this application.

Claims:

1. A method for measuring the level of the Fatty Acid Synthase (FASN) protein in a biological sample, comprising detecting and/or quantifying the amount of one or more modified or unmodified FASN fragment peptides in a protein digest prepared from said biological sample using mass spectrometry; and calculating the level of modified or unmodified FASN protein in said sample; and

wherein said amount is a relative amount or an absolute amount.

2. The method of claim 1, further comprising the step of fractionating said protein digest prior to detecting and/or quantifying the amount of one or more modified or unmodified FASN fragment peptides.

3. The method of claim 2, wherein said fractionating step is selected from the group consisting of gel electrophoresis, liquid chromatography, capillary electrophoresis, nano-reversed phase liquid chromatography, high performance liquid chromatography, or reverse phase high performance liquid chromatography.

4. The method of claim 1, wherein said protein digest of said biological sample is prepared by the Liquid Tissue™ protocol.

5. The method of claim 1, wherein said protein digest comprises a protease digest.

6. The method of claim 5, wherein said protein digest comprises a trypsin digest.

7. The method of claim 1, wherein said mass spectrometry comprises tandem mass spectrometry, ion trap mass spectrometry, triple quadrupole mass spectrometry, MALDI-TOF mass spectrometry, MALDI mass spectrometry, and/or time of flight mass spectrometry.

8. The method of claim 7, wherein the mode of mass spectrometry used is Selected Reaction Monitoring (SRM), Multiple Reaction Monitoring (MRM), and/or multiple Selected Reaction Monitoring (mSRM), or any combination thereof.

9. The method of claim 1, wherein the one or more modified or unmodified FASN fragment peptides comprise different amino acid sequences independently selected from those set forth in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, and SEQ ID NO:11.

10. The method of claim 1, wherein the biological sample is a blood sample, a urine sample, a serum sample, an ascites sample, a sputum sample, lymphatic fluid, a saliva sample, a cell, or a solid tissue.

11. The method of claim 10, wherein the tissue is formalin fixed tissue.

12. The method of claim 10, wherein the tissue is paraffin embedded tissue.

13. The method of claim 10, wherein the tissue is obtained from a tumor.

14. The method of claim 13, wherein the tumor is a primary tumor.

15. The method of claim 13, wherein the tumor is a secondary tumor.

16. The method of claim 1, further comprising quantifying a modified or unmodified FASN fragment peptide.

17. The method of claim 16, wherein quantifying the FASN fragment peptide comprises comparing an amount of one or more FASN fragment peptides comprising an amino acid sequence of about 8 to about 45 amino acid residues of FASN as shown in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, or SEQ ID NO:11 in one biological sample to the amount of the same FASN fragment peptide in a different and separate biological sample.

18. The method of claim 17, wherein quantifying one or more FASN fragment peptides comprises determining the amount of the each of said one or more FASN fragment peptides in a biological sample by comparison to an added internal standard peptide of known amount, wherein each of the FASN fragment peptides in the biological sample is compared to an internal standard peptide having the same amino acid sequence.

19. The method of claim 18, wherein the internal standard peptide is an isotopically labeled peptide.

20. The method of claim 19, wherein the isotopically labeled internal standard peptide comprises one or more heavy stable isotopes selected from ^{18}O, ^{17}O, ^{34}S, ^{15}N, ^{13}C, ^{2}H or combinations thereof.

21. The method of claim 1, wherein detecting and/or quantifying the amount of one or more modified or unmodified FASN fragment peptides in the protein digest indicates the presence of modified or unmodified FASN protein and an association with cancer in the subject.

22. The method of claim 21, further comprising correlating the results of said detecting and/or quantifying the amount of one or more modified or unmodified FASN fragment peptides, or the amount of said FASN protein to the diagnostic stage/grade/status of the cancer.

23. The method of claim 22, wherein correlating the results of said detecting and/or quantifying the amount of one or more modified or unmodified FASN fragment peptides, or the amount of said FASN protein to the diagnostic stage/grade/status of the cancer is combined with detecting and/or quantifying the amount of other proteins or peptides from

other proteins in a multiplex format to provide additional information about the diagnostic stage/grade/status of the cancer.

24. The method of claim 1, further comprising selecting for the subject from which said biological sample was obtained a treatment based on the presence, absence, or amount of one or more FASN fragment peptides or the amount of FASN protein.

25. The method of claim 1, further comprising administering to the patient from which said biological sample was obtained a therapeutically effective amount of a therapeutic agent, wherein the therapeutic agent and/or amount of the therapeutic agent administered is based upon amount of one or more modified or unmodified FASN fragment peptides or the amount of FASN protein.

26. The method of claim 24, wherein the treatment or the therapeutic agent is directed to cancer cells expressing the FASN protein.

27. The method of claim 1, wherein the biological sample is formalin fixed tumor tissue that has been processed for quantifying the amount of one or more modified or unmodified FASN fragment peptides employing the Liquid Tissue™ protocol and reagents.

28. The method of claims 1, wherein said one or more modified or unmodified FASN fragment peptides is two or more, three or more, four or more, five or more, six or more, eight or more, or ten or more of the peptides in Table 1.

29. The method of claim 1, comprising quantifying the amount of the peptides in Table 2.

30. A composition comprising one, two or more, three or more, four or more, five or more, six or more, eight or more, or ten or more of the peptides in Table 1 or antibodies thereto.

31. The composition of claim 30 comprising one, two or three of the peptides of Table 2 or antibodies to any combination of those peptides.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 12/56959

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - G01N 24/00, 27/62, 30/72, 33/53, 33/68; C12Q 1/37; G01J 3/00 (2012.01)
USPC - 435/7.1, 23; 436/173, 86; 250/339.07, 339.08
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8) - G01N 24/00, 27/62, 30/72, 33/53, 33/68; C12Q 1/37; G01J 3/00 (2012.01)
USPC - 435/7.1, 23; 436/173, 86; 250/339.07, 339.08

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PatBase (EP, WO, US); Google Scholar; PubWEST (PGPB, USPT, USOC, EPAB, JPAB) and GenCore sequence search (AA).
Search terms: mass spectrometry, multiple reaction monitoring (MRM), selected reaction monitoring (SRM), absolute quantitation, fatty acid synthase (FASN, FAS); cancer, formalin fixed paraffin embedded (FFPE).

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- Y	US 2011/0028344 A1 (KRIZMAN et al.) 03 February 2011 (03.02.2011). Especially para [0008], [0022[, [0029], [0030], [0033], [0046] [0050], [0058], pg 10 table 1.	1-8, 10-16 and 21-27 ------------------------------ 9, 17-20, 28 and 29
X ---- Y	ZHANG, D. et al. Proteomic study reveals that proteins involved in metabolic and detoxification pathways are highly expressed in HER-2/neu-positive breast cancer. Mol Cell Proteomics. 26 July 2005, Vol 4.11, Pages 1686-1696: Especially pg 1690 table 1.	30 and 31 ------------------------ 9, 17-20, 28 and 29
Y	US 2010/0233815 A1 (HUNTER) 16 September 2010 (16.09.2010) Especially para [0067], [0068].	19 and 20
A	FLAVIN, R. et al. Fatty acid synthase as a potential therapeutic target in cancer. Future Oncol. April 2010, Vol 6, No 4, Pages 551-562: abstract.	25 and 26

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search 15 November 2012 (15.11.2012)	Date of mailing of the international search report 30 NOV 2012
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 12/56959

Box No. I **Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)**

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing filed or furnished:

a. (means)
☐ on paper
☒ in electronic form

b. (time)
☐ in the international application as filed
☒ together with the international application in electronic form
☐ subsequently to this Authority for the purposes of search

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

GenCore ver 6.4.1: SEQ ID NOs: 1, 2, 4, 6, 9

专利名称(译)	SRM / MRM测定脂肪酸合成酶蛋白		
公开(公告)号	EP2764350A1	公开(公告)日	2014-08-13
申请号	EP2012834405	申请日	2012-09-24
[标]申请(专利权)人(译)	爱科谱迅病理研究公司		
申请(专利权)人(译)	病理表达，INC.		
当前申请(专利权)人(译)	病理表达，INC.		
[标]发明人	KRIZMAN DAVID B HEMBROUGH TODD THYPARAMBIL SHEENO LIAO WEI LIAO		
发明人	KRIZMAN, DAVID, B. HEMBROUGH, TODD THYPARAMBIL, SHEENO LIAO, WEI-LIAO		
IPC分类号	G01N24/00 G01N27/62 G01N30/72 G01N33/53 G01N33/68 C12Q1/37 G01J3/00		
代理机构(译)	Bohmann，ARMIN K.		
优先权	61/538091 2011-09-22 US		
其他公开文献	EP2764350A4		
外部链接	Espacenet		

摘要(译)

提供了来自脂肪酸合酶（FASN）蛋白的肽的特定肽和衍生的电离特征，其特别有利于通过选择反应监测（SRM）方法在福尔马林中固定的生物样品中直接定量FASN蛋白。 ）质谱或多反应监测（MRM）质谱。