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(54) Title: C-SRC SELECTED REACTION MONITORING ASSAY

(57) Abstract: Objective quantitation of the c-Src protein directly in cancer patient tissue can aid in determining die aggressiveness of an individual patient's tumor as well as help make more informed decisions about choice of therapy. However, fee c-Src protein is currently analyzed directly in formalin fixed patient tissue only by immunohistoehemistry methodology which is at best subjectively semi-quantitative. This invention describes an objective quantitative assay for the c-Sre protein using mass spectrometry as the analytical methodology, Specific peptides, experimentally discovered characteristics about the peptides, and experimentally established assay conditions based on those peptide characteristics are provided for use in a mass speetronieiry-based Selected Reaction Monitoring (SRM) assay in order to measure relative- or absolute quantitative levels of c-Src directly in a protein preparation obtained from a formalin fixed cancer patient tissue sample.

c-Src Selected Reaction Monitoring Assay

This application claims the benefit of U.S. Provisional Application No. 61/369,411, filed July 30, 2010, entitled "c-Src Selected Reaction Monitoring Assay" naming as an inventor David B. Krizman, the entirety of which is incorpoated by reference in its entirety.

Introduction

Specific peptides are provided that are derived from subsequences of the c-Src protein, also known as CSK and which will be referred to as Src. Specific characteristics about each peptide are provided, which includes the peptide sequence and fragmentation/transition ions for reliable, accurate and consistent analysis in mass spectrometric analysis. Also described is the use of those peptides in a mass spectrometry-based Selected Reaction Monitoring (SRM), which can also be referred to as a Multiple Reaction Monitoring (MRM) assay. This SRM assay can be used to measure *relative* or *absolute* quantitative levels of one or more of the specific peptides from the Src protein and therefore provide a means of measuring the amount of the Src protein by mass spectrometry in a given protein preparation obtained from a biological sample.

More specifically, the SRM 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 references in their entirety. The methods described in U.S. Patent No. 7,473,532 may conveniently be carried out using Liquid Tissue® reagents available from Expression Pathology, Inc. (Rockville, MD).

Results from the SRM assay can be used to correlate accurate and precise quantitative levels of the Src protein with the specific cancer of the patient from whom the tissue was collected. 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. Such an assay that provides diagnostically important information about levels of protein expression in a diseased tissue or other patient sample is termed a *companion diagnostic* assay. For example, such an assay can be designed to diagnose the stage or degree of a cancer and determine which therapeutic agent, or course of therapy, to which a patient is most likely to respond with a positive outcome.

The assays described herein measure *relative* or *absolute* levels of specific unmodified peptides from the Src protein and also can measure absolute or relative levels of specific modified peptides from the Src protein. Examples of modifications include phosphorylated amino acid residues and glycosylated amino acid residues that are present on the peptides.

Relative quantitative levels of the Src protein are determined by the SRM methodology whereby the chromatographic peak area (or the peak height if the peaks are sufficiently resolved) of an individual peptide, or multiple peptides, from the Src protein in one biological sample is compared to the chromatographic peak area determined for the same identical Src peptide, or peptides, using the same methodology in one or more additional and different biological samples. In this way, the amount of a particular peptide, or peptides, from the Src protein, and therefore the amount of the Src protein, is determined relative to the same Src 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 Src protein within a single sample by comparing the chromatographic peak area for that peptide by SRM methodology to the chromatographic 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 Src protein, and therefore the amount of the Src protein, is determined relative one to another within the same sample. These approaches generate quantitation of an individual peptide, or peptides, from the Src protein to the amount of another peptide, or peptides, between samples and within samples wherein the amounts as determined by chromatographic peak area are relative one to another, regardless of the absolute weight to volume or weight to weight amounts of the Src peptides in the protein preparation from the biological sample. Relative quantitative data about individual chromatographic peak areas between different samples are normalized to the amount of protein analyzed per sample. Relative quantitation can be performed across many peptides 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 Src protein are determined by the SRM methodology whereby the chromatographic peak area of an individual peptide from the Src protein in one biological sample is compared to the chromatographic peak area of a spiked internal standard, where the internal standard is a synthetic version of the same exact Src peptides that contains one or more amino acid residues labeled with one or more heavy isotopes. The internal standard is synthesized so that when analyzed by mass spectrometry it generates a predictable and consistent signature chromatographic peak that is different and distinct from the native Src peptide chromatographic signature peak. Thus when the internal standard is spiked into a protein preparation from a biological sample in known amounts and analyzed by mass spectrometry, the signature chromatographic peak area of the native peptide is compared to the signature chromatographic peak area of the internal standard peptide, and this numerical comparison

indicates either the absolute molarity 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 cohorts of individual samples.

The assay methods 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, and which therapeutic strategy, 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 the protein(s) can be determined and compared to a "normal" or reference level found in healthy tissue or tissue that shows a different stage/grade of cancer. This information can then be used to assign a stage or grade to a specific cancer and can be matched to a strategy for treating the patient based on the determined levels of specific proteins. Matching specific information about levels of the Src protein, as determined by an SRM assay, to a treatment strategy that is based on levels of these proteins in cancer cells derived from the patient 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 the Src protein, 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 the Src protein in a sample using a mass spectrometry-based SRM assay. Similarly, any predicted peptide sequence containing an amino acid residue at a site that is known to be potentially modified in the Src protein also might potentially be used to assay the extent of modification of the Src protein in a sample. Surprisingly, however, the present inventors have found that many potential peptide sequences are unsuitable or ineffective for use in mass spectrometry-based SRM assays. The peptides might, for example, be difficult to detect by mass spectrometry, or may be unstable to the conditions used to obtain the peptides from the

parent protein. This is especially found to be the case when interrogating protein lysates prepared from formalin fixed tissue using the Liquid Tissue® protocol provided in US Patent 7,473,532. Unexpectedly it was found to be advantageous to experimentally identify preferred modified and unmodified peptides in actual Liquid Tissue® lysates in order to develop a reliable and accurate SRM assay for the Src protein. Preferred modified and unmodified peptides for use in the mass spectrometric methods described herein (e.g., SRM), including identifying presence (or absence) and/or amount of proteins in formalin fixed tissues, are hereinafter known as optimized peptides.

In general, peptides were derived from the Src protein in the course of the protease digestion of the proteins within a complex Liquid Tissue® lysate prepared from cells procured from formalin fixed patient tissue. The Liquid Tissue® lysates were then analyzed by mass spectrometry to determine those peptides derived from the Src protein that are preferably detected and analyzed by mass spectrometry (*i.e.*, optimized preferred modified and unmodified peptides). The results are employed to identify a specific subset of preferred peptides selected for their suitability in mass spectrometric analysis. The procedure employed permits experimental determination of peptides or peptides fragments that ionize most effectively, and which provide suitable data for resulting peptide transition fragment ions that can be identified and quantitated in a Liquid Tissue® preparation from formalin fixed patient tissue. These results can then be compared to results obtained by mass spectrometry analysis of the recombinant protein that has been digested with the same protease, or proteases, in order to confirm the existence of preferred or optimized peptides and their resulting transition fragments.

In addition to their suitability in mass spectrometric analysis, the ability of labeled versions of preferred (or more specifically optimized) peptides to withstand the conditions used in Liquid Tissue® preparation protocols is an important determinant as to which peptides are preferred (or optimized where formalin fixed tissue is used) for qualitative or quantitative analyzing of tissues by mass spectrometry (e.g., SRM). This latter property depends not only on the amino acid sequence of the peptide but also to the ability of a modified residue within a peptide to survive in modified form during the sample preparation. The assay method described below can be used to identify the peptides from the Src protein that are preferred or optimized for identifying and quantitating protein expression or modification in patient samples, and more specifically patient samples derived from formalin fixed tissue, by mass spectrometry-based SRM assay.

Assay Method for the Identification of Peptides from the Src protein

1. Identification of a preferred (or optimized) fragment peptide, or preferred (or optimized) fragment peptides, for the Src protein
    a. Treat purified Src protein with the Liquid Tissue® reagents and protocol using a protease or proteases, (that may or may not include trypsin), to digest the Src protein. Analyze some (*e.g.*, 10, 20, 30 or 40%), most (*e.g.*, more than 50, 60, 70, 80, 90, 95, 98 or 99%), or all resulting protein fragments by tandem mass spectrometry and identify all fragment peptides from the Src protein, where individual fragment peptides do not contain any peptide modifications such as phosphorylations or glycosylations.
    b. Prepare a Liquid Tissue® protein lysate from a formalin fixed biological sample using the same protease or proteases as utilized when preparing the purified Src protein (that may or may not include trypsin), to digest most, or all proteins. Analyze some, most, or all resulting protein fragments from some, most, or all proteins in the mixture by tandem mass spectrometry and identify some, most, or all fragment peptides specifically from the Src protein, where individual fragment peptides do not contain any peptide modifications such as phosphorylations or glycosylations. Analyze some, most, or all resulting protein fragments from some, most, or all the proteins by tandem mass spectrometry and identify some, most, or all fragment peptides from the Src protein that carry peptide modifications such as for example phosphorylated or glycosylated residues.
    c. Some, most or all peptides generated by a specific digestion method from the entire, full length Src protein potentially can be measured, but preferred peptides are those that are identified by mass spectrometry from analysis of the purified proteins and that also are identified directly in a complex Liquid Tissue® protein lysate prepared from a histopathologically fixed biological sample (e.g., optimized peptides can be measured where the tissue is formalin fixed).

    Peptides that are post-translationally modified, and their specific fragment characteristics, can be considered preferred or optimized peptides and assayed where the relative levels of the modified peptides are determined in the same manner as determining relative amounts of unmodified peptides for the Src protein.
2. Mass Spectrometry Assay for Fragment Peptides from the Src protein
    a. A Selected Reaction Monitoring (SRM), or also known as a Multiple Reaction Monitoring (MRM), assay is conducted on a triple quadrupole mass spectrometer for

each individual preferred or optimized fragment peptides identified in a Liquid Tissue® lysate from the Src protein may be developed as follows:

i. Determine retention time for each fragment peptide of Src for at least one suitable fractioning method including but not limited to gel electrophoresis, liquid chromatography, capillary electrophoresis, isoelectric separation chromatography, nano-reversed phase liquid chromatography, high performance liquid chromatography, or reverse phase high performance liquid chromatography.

ii. Identify suitable fragment transition ions to monitor for one or more fragment peptides based on the highest signal to noise ratio and/or the lowest standard deviation between replicate analyses for use in Liquid Tissue® samples prepared from histopathologically fixed tissue (e.g., formalin fixed tissues).

b. Perform SRM/MRM analysis so that the amount of the fragment peptide, or peptides, of the Src protein that is detected, as a function of specific peak area (or height where suitable) from an SRM/MRM mass spectrometry analysis, reflects both the relative and absolute amount of the protein in a particular Liquid Tissue® lysate.

i. Relative quantitation is achieved by: comparing the (e.g., electrophoretic chromatographic, etc.) peak area for a fragment peptide to the peak area of the same fragment peptide, or other fragment peptides from other proteins, in other samples derived from different and separate biological sources, where the chromatographic peak area comparison between the samples for a peptide fragment are normalized to amount of protein analyzed in each sample.

Comparison of the separation peak area for a given fragment peptide to the separation peak areas from other fragment peptides derived from different proteins within the same sample can be performed to normalize changing levels of one protein to levels of other proteins that do not change their levels of expression under various conditions (e.g., cellular conditions).

Relative quantitation 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 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 is achieved by comparing the peak area for a given fragment peptide in an individual biological sample to the

peak area of an internal fragment peptide standard spiked into the protein lysate from the biological sample. The analysis of the given peptide and the standard spiked into the sample can be conducted simultaneously.

The internal standard can be a labeled version (e.g., a labeled synthetic version) consisting of the exact amino acid sequence of the fragment peptide that is being interrogated. The labeled standard is spiked into a sample in known amounts, and the chromatographic 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 and deriving the absolute amount of the native peptide as compared to the absolute amount of the spiked peptide standard.

Absolute quantitation 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. Associate Fragment Peptide Quantitation to Cancer Diagnosis and/or Treatment
    a. Perform relative and/or absolute quantitation of fragment peptide levels of the Src protein and associate results with the stage/grade/status of cancer in patient tumor tissue; and/or
    b. Perform relative and/or absolute quantitation of fragment peptide levels of the Src protein and correlate with specific and different treatment strategies, wherein this correlation has been, or can be demonstrated in the future, to correlate with outcome to various treatment strategies through correlation studies across cohorts of patients and tissue from those patients. Once either previously established correlations are confirmed by this assay, or new correlations established, the assay method can be used to associate quantitative results for the Src protein in patient tissue to more effective patient treatment strategy.

Table 1: c-Src Peptide Sequences and Specific Characteristics

SEQ ID	Peptide sequence	Monoisotopic Mass	Precursor charge state	Precursor m/z	Product Transition m/z	Ion Type
SEQ ID NO: 1	GPSAAFAPAAAEPK	1284.65828	2	642.83	1043.55	y11
			2	642.83	972.51	y10
			2	642.83	901.48	y9
			2	642.83	754.41	y8
			2	642.83	683.37	y7
SEQ ID NO: 2	AGPLAGGVTTFVALYDYESR	2087.0444	3	696.35	832.35	y6
			3	696.35	669.28	y5
			3	696.35	554.26	y4
			3	696.35	391.19	y3
			3	696.35	262.15	y2
			2	1044.03	1115.54	y9
			2	1044.03	1016.47	y8
			2	1044.03	945.43	y7
			2	1044.03	832.35	y6
			2	1044.03	669.28	y5
SEQ ID NO: 3	TETDLSFK	940.4622	2	470.73	839.41	y7
			2	470.73	710.37	y6
			2	470.73	609.32	y5
			2	470.73	494.30	y4
			2	470.73	381.21	y3
SEQ ID NO: 4	LLLNAENPR	1039.58947	2	520.30	813.42	y7
			2	520.30	700.34	y6
			2	520.30	586.29	y5
			2	520.30	386.21	y3
			2	520.30	272.17	y2
SEQ ID NO: 5	GTFLVR	692.40899	2	346.71	534.34	y4
			2	346.71	387.27	y3
			2	346.71	274.19	y2
			2	346.71	175.12	y1
SEQ ID NO: 6	GAYCLSVSDFDNAK	1489.66277	2	773.85	1095.53	y10
			2	773.85	982.45	y9
			2	773.85	895.42	y8
			2	773.85	796.35	y7
			2	773.85	709.32	y6
SEQ ID NO: 7	LDSGGFYITSR	1215.60043	2	608.30	987.49	y9
			2	608.30	786.41	y6
			2	608.30	639.35	y5
			2	608.30	476.28	y4
			2	608.30	363.20	y3

SEQ ID	Peptide sequence	Monoisotopic Mass	Precursor charge state	Precursor m/z	Product Transition m/z	Ion Type
SEQ ID NO: 8	TQFNSLQQLVAYYSK	1789.91193	3	597.31	843.46	y7
			3	597.31	730.38	y6
			3	597.31	631.31	y5
			3	597.31	560.27	y4
			3	597.31	397.21	y3
			2	895.46	1099.58	y9
			2	895.46	730.38	y6
			2	895.46	631.31	y5
			2	895.46	560.27	y4
			2	895.46	397.21	y3
SEQ ID NO: 9	HADGLCHR	908.41554	2	483.22	828.38	y7
			2	483.22	757.34	y6
			2	483.22	642.31	y5
			2	483.22	472.21	y3
			2	483.22	175.12	y1
SEQ ID NO: 10	GSLLIDFLK	892.51385	2	446.76	748.46	y6
			2	446.76	635.38	y5
			2	446.76	522.29	y4
			2	446.76	407.27	y3
			2	446.76	147.11	y1
SEQ ID NO: 11	AANILVGENLVCK	1343.73515	2	700.88	1031.56	y9
			2	700.88	918.47	y8
			2	700.88	819.40	y7
			2	700.88	406.21	y3
			2	700.88	307.14	y2
SEQ ID NO: 12	VADFGLAR	848.46248	2	424.73	749.39	y7
			2	424.73	678.36	y6
			2	424.73	563.33	y5
			2	424.73	416.26	y4
			2	424.73	246.16	y2
SEQ ID NO: 13	LIEDNEYTAR	1223.59026	2	612.30	997.42	y8
			2	612.30	868.38	y7
			2	612.30	753.35	y6
			2	612.30	510.27	y4
			2	612.30	347.20	y3
SEQ ID NO: 14	LIEDNE(pY)TAR	1303.55659	2	652.28	1077.39	y8
			2	652.28	948.35	y7
			2	652.28	833.32	y6
			2	652.28	590.23	y4
			2	652.28	347.20	y3

SEQ ID	Peptide sequence	Monoisotopic Mass	Precursor charge state	Precursor m/z	Product Transition m/z	Ion Type
SEQ ID NO: 15	WTAPEAALYGR	1234.6215	2	617.81	947.49	y9
			2	617.81	876.46	y8
			2	617.81	579.32	y5
			2	617.81	508.29	y4
			2	617.81	395.20	y3
SEQ ID NO: 16	SDVWSFGILLTELTTK	1809.96329	2	905.49	1088.66	y10
			2	905.49	918.55	y8
			2	905.49	805.47	y7
			2	905.49	692.38	y6
			2	905.49	591.33	y5

Claims

1. A method for measuring levels of the Src protein in a biological sample, comprising detecting at least one fragment peptide from Src in a protein digest prepared from said biological sample using mass spectrometry; and calculating the levels of the Src protein in said sample, wherein said measured levels of the Src protein are independently selected from a relative level or an absolute quantitative level.
2. The method of claim 1, further comprising the step of fractionating said protein digest prior to detecting said 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 performa nce liquid chromatography, isoelectric separation 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 and reagents.
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 5, wherein said protein digest comprises 2 or more proteases, where one of the proteases is, or is not, trypsin.
8. The method of claim 1, wherein mass spectrometry comprises tandem mass spectrometry.
9. The method of claims 1 and 8, wherein the mode of mass spectrometry used is Selected Reaction Monitoring (SRM), high resolution Selected Reaction Monitoring (hSRM), multiple Selected Reaction Monitoring (mSRM), Multiple Reaction Monitoring (MRM), and/or Selected Ion Monitoring (SIM).
10. The method of claim 1 or claim 9, wherein the Src fragment peptide comprises an amino acid sequence as shown in Table 1, and set forth as 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, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, and SEQ ID NO:16.
11. The method of claim 10, wherein Src peptide identification and characteristics are defined for optimized peptides as shown in Table 1 by its specified monoisotopic mass, specified precursor charge state, specified precursor mass over charge ratio (m/z), specified product transition ions (m/z), and specified ion type.

12. The method of claim 1, wherein the biological sample comprises blood, urine, serum, ascites, saliva, cells, or tissue.

13. The method of claim 12, wherein the tissue is formalin fixed tissue.

14. The method of claim 12 or claim 13, wherein the tissue is paraffin embedded tissue.

15. The method of claim 12, wherein the tissue is obtained from a tumor.

16. The method of claim 15, wherein the tumor is obtained from a primary tumor.

17. The method of claim 15, wherein the tumor is obtained from a secondary tumor.

18. The method of claim 1, further comprising quantifying the Src fragment peptide, or peptides.

19. The method of claim 18, wherein quantifying the Src fragment peptide comprises comparing an amount of the Src fragment peptide corresponding to an amino acid sequence having a length in a range selected from the group consisting of: from about 8 to about 15, from about 8 to about 25, from about 8 to about 35, from about 8 to about 45, from about 12 to about 15, from about 12 to about 25, from about 12 to about 35, from about 12 to about 45, from about 15 to about 20, from about 15 to about 25, from about 15to about 35, from about 15 to about 45, from about 20 to about 25, from about 20 to about 35,and from about 20 to about 45 amino acid residues, of Src, and the identified transition ions for analysis for each peptide 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, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, and SEQ ID NO:16 in one sample to the amount of the same Src fragment peptide in a different and separate biological sample from a different and separate primary and/or secondary tumor or tumors.

20. The method of claim 18, wherein quantifying the Src fragment peptide comprises comparing an amount of the Src fragment peptide to an internal standard peptide of known amount, wherein both the peptide in the biological sample and the internal standard peptide corresponds to the same amino acid sequence having a length in a range selected from the group consisting of: from about 8 to about 15, from about 8 to about 25, from about 8 to about 35, from about 8 to about 45, from about 12 to about 15, from about 12 to about 25, from about 12 to about 35, from about 12 to about 45, from about 15 to about 20, from about 15 to about 25, from about 15to about 35, from about 15 to about 45, from about 20 to about 25, from about 20 to about 35,and from about 20 to about 45 5 amino acid residues of Src, and the identified transition ions for analysis for each optimized peptide, 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, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, and SEQ ID NO:16.

21. The method of any of claim 20, wherein the internal standard peptide is an isotopically labeled peptide.

22. The method of claim 21, 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.

23. The method of claim 1, further comprising obtaining the biological sample from a subject, wherein detecting the Src fragment peptides in the protein digest indicates the presence of the Src protein and an association with cancer in the subject.

24. The method of claim 23, further comprising correlating detected and quantitated amounts of the Src fragment peptides to the diagnostic stage/grade/status of the cancer.

25. The method of claim 23, wherein detecting and quantitating the Src fragment peptides to indicate the diagnostic stage/grade/status of the cancer can be combined with detecting and quantitating other peptides from other proteins in a multiplexed fashion so that when combined provide more information about the diagnostic stage/grade/status of the cancer.

26. The method of any one of claims 18-23, further comprising selecting a therapeutic treatment for the subject based on the presence, absence, or quantified levels of the Src fragment peptides in the protein digest.

27. The method of any one of claims 18-23, further comprising administering a therapeutically effective amount of a therapeutic agent targeted specifically to the Src protein or the level of Src protein, wherein the treatment decision about which agent or agents, or the amount of said agent, or agents, used for treatment is based upon specific levels of the Src fragment peptides in the biological sample.

28. The method of claim 27, wherein therapeutic agents include those that specifically bind to the Src protein and inhibit the biological activity of Src.

29. The method of claims 26-28, wherein detecting and quantitating the Src fragment peptides can be combined with detecting and quantitating other peptides from other proteins in a multiplexed fashion so that the treatment decision about which agent or agents and/or the amount of said agent or agents used for treatment is based upon specific levels of the Src fragment peptides in combination with other peptides/proteins in the biological sample.

30. The method of claims 12, and 18, wherein the biological sample is formalin fixed tumor tissue that has been processed for quantitative analysis of the Src fragment peptides by the Liquid Tissue® protocol and reagents.

31. The method of claim 4, wherein said biological sample is a sample of formalin fixed tissue and the Liquid Tissue® protocol comprises the steps of (a) heating a composition comprising a formalin fixed biological sample and a reaction buffer at a temperature from 80° C to 100° C for a period of time from 10 minutes to 4 hours to reverse or release protein cross-linking in said biological sample, and (b) treating the resulting composition with an effective amount of a proteolytic enzyme selected from the group consisting of trypsin, chymotrypsin, and endoproteinase Lys-C for a period of time from 30 minutes to 24 hours at a temperature from 37° C to 65° C to disrupt the tissue and cellular structure of said biological sample and to liquefy said sample, thereby producing a liquid, soluble, dilutable biomolecule lysate that is suitable for protein analysis, wherein the protein content of said lysate is representative of the total protein content of said biological sample.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 11/45960

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - G01N 33/483; G01N 30/72 (20110.01)
USPC - 436/63, 436/173
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)
USPC: 436/63, 436/173

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC: 436/63, 436/173 (keyword limited; terms below)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWEST (USPT, PGPB, EPAB, JPAB), Google Patents/Scholar
Search Terms Used: C-src, reaction monitoring, liquid tissue, mass spectrometry, paraffin, tumor

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	Chen et al. "Quantification of beta-catenin signaling components in colon cancer cell lines, tissue sections, and microdissected tumor cells using reaction monitoring mass spectrometry" J Proteome Res vol 9 pg 4215-4227; 30 June 2010 (30.06.2010) abstract, pg 4215, col 2, para 2 to pg 4216, col 1, para 1, pg 4216, col 2, para 4 to pg 4217, col 1, para 3, pg 4220, col 1, para 1, pg 4221, col 1, para 3 to col 2, para 1, Fig. 2B, Table 1, 2	1-3, 5-6, 12, 15-25 --- 4, 7-8, 13-14, 26-28, 31
Y	US 2009/0136971 A1 (Krizman et al.) 28 May 2009 (28.05.2009) para [0007], [0015], [0019], [0040]-[0045], [0056]	4, 7-8, 13-14, 31
Y	US 6,503,914 B1 (Benish et al.) 07 January 2003 (07.01.2007) abstract, col 45, ln 15-25	26-28

☐ 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	Date of mailing of the international search report
20 December 2011 (20.12.2011)	05 JAN 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

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 11/45960

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 9-11, 29, 30
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.