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(54) Title: SRM/MRM ASSAY FOR THE FIBROBLAST GROWTH FACTOR RECEPTOR 2 (FGFR2) PROTEIN

(57) Abstract: Methods are provided for quantifying the fibroblast growth factor receptor 2 protein (FGFR2) directly in biological samples that have been fixed in formalin by the method of Selected Reaction Monitoring (SRM)/Multiple Reaction Monitoring (MRM) mass spectrometry. The biological sample may be selected from tissues and cells treated with formaldehyde containing agents/fixatives including formalin-fixed tissue/cells, formalin-fixed/paraffin embedded (FFPE) tissue/cells, FFPE tissue blocks and cells from those blocks, and tissue culture cells. A protein sample is prepared from the biological sample and the FGFR2 protein is quantitated in the sample using the method of SRM/MRM mass spectrometry by quantitating in the protein sample at least one frag-
ment peptide derived from FGFR2.



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SRM/MRM Assay for the Fibroblast Growth Factor Receptor 2 (FGFR2) protein

This application claims priority to provisional application serial no. 62/161,757, filed May 14, 2015, the contents of which are hereby incorporated by reference in their entirety.

5

Introduction

Cancer is treated with a collection of therapeutic agents that kill growing and dividing cells and that function in a variety of ways. A common collection of chemotherapeutic agents has been used for decades, either individually or in combinations, and this common collection of agents has become the traditional and routine cancer treatment in clinical oncology practice. These traditional chemotherapeutics agents act by killing all cells that divide rapidly, one of the main properties of most cancer cells. However, these agents also kill growing normal cells and thus these agents are not considered to be “targeted” approaches to killing cancer cells. In recent years a large group of cancer therapeutic agents has been developed that target only cancer cells where the therapeutic agent specifically attacks a protein that is only expressed by the cancer cells and not by normal cells. This approach is considered to be a “targeted” approach to cancer therapy. Most recently, another approach to killing cancer cells in a “targeted” fashion has been to specifically modulate the immune system to enhance the ability of the cancer patient’s immune system to kill cancer cells.

Therapeutic agents that target the fibroblast growth factor receptor 2 protein, which is also referred to as FGFR2, have shown promise in early clinical trials. However, only those patients whose cancer cells express high amounts of the FGFR2 protein are likely to benefit from treatment with such FGFR2-targeted therapeutic agents. The methods described below provide a quantitative proteomics-based assay that delivers a relevant measure of activation of the FGFR2 signal pathway as FGFR2 is not normally expressed in normal tissue and/or normal epithelial cells. In particular, the methods provide a mass spectrometry assay that quantifies FGFR2 in formalin fixed tissues from cancer patients and that enables improved treatment decisions for cancer therapy.

Specific peptides derived from subsequences of the fibroblast growth factor receptor 2 protein, also referred to as CD332, Keratinocyte growth factor receptor, and KGFR, and referred to herein as FGFR2, are provided. The peptide sequence and fragmentation/transition ions for each peptide are particularly useful in a mass spectrometry-based Selected Reaction Monitoring (SRM), which can also be referred to as a Multiple

Reaction Monitoring (MRM) assay, and is referred to herein as SRM/MRM. The use of peptides for SRM/MRM quantitative analysis of the FGFR2 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 FGFR2 protein and therefore provide a mass spectrometric methods of measuring the amount of the FGFR2 protein in a given protein preparation obtained from a biological sample.

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 references 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 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 by far 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 (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 FGFR2 protein within the specific tissue samples (e.g., cancer tissue sample) of the patient or subject from whom the tissue (biological sample) was collected and preserved. This not only provides diagnostic and prognostic information about the cancer, but also permits a physician or other medical professional to more accurately determine appropriate therapy for the patient. Such an assay that provides diagnostically, prognostically, and therapeutically 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 a therapeutic agent to which a patient is most likely to respond.

Summary

5 The assays described herein measure *relative* or *absolute* levels of specific unmodified peptides from the FGFR2 protein and also can measure absolute or relative levels of specific modified peptides from the FGFR2 protein. Examples of modifications include phosphorylated amino acid residues and glycosylated amino acid residues that may be present on the peptides.

10 *Relative* quantitative levels of the FGFR2 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 FGFR2 peptide in different samples. Alternatively, it is possible to compare multiple SRM/MRM signature peak areas for multiple FGFR2 signature peptides, where each peptide has its own specific SRM/MRM
15 signature peak, to determine the relative FGFR2 protein content in one biological sample with the FGFR2 protein content in one or more additional or different biological samples. In this way, the amount of a particular peptide, or peptides, from the FGFR2 protein, and therefore the amount of the FGFR2 protein, is determined relative to the same FGFR2 peptide, or peptides, across 2 or more biological samples under the same experimental
20 conditions. In addition, relative quantitation can be determined for a given peptide, or peptides, from the FGFR2 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
25 the FGFR2 protein, and therefore the amount of the FGFR2 protein, is determined relative one to another within the same sample. These approaches permit quantitation of an individual peptide, or peptides, from the FGFR2 protein to the amount of another peptide, or peptides, between samples and within samples wherein the amounts as determined by signature peak area are relative one to another, regardless of the absolute weight to volume or
30 weight to weight amounts of the FGFR2 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 FGFR2

protein simultaneously in a single sample and/or across many samples to gain insight into relative protein amounts of one peptide/protein with respect to other peptides/proteins.

Absolute quantitative levels of the FGFR2 protein are determined by, for example, the SRM/MRM methodology whereby the SRM/MRM signature peak area of an individual peptide from the FGFR2 protein in one biological sample is compared to the SRM/MRM signature peak area of a spiked internal standard. In one embodiment, the internal standard is a synthetic version of the same exact FGFR2 peptide that contains one or more amino acid residues labeled with one or more heavy isotopes. Such an isotope labeled internal standard is synthesized so that when analyzed by mass spectrometry it generates a predictable and consistent SRM/MRM signature peak that is different and distinct from the native FGFR2 peptide signature peak and which can be used as a comparator 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 SRM/MRM signature peak area of the native peptide is compared to the SRM/MRM signature peak area of the internal standard peptide, and this numerical comparison indicates 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, and/or the patient prognosis, 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., FGFR2 levels) can also be used to diagnose, and provide prognostic information about, the stage of cancer in a patient or subject diagnosed with cancer by employing the FGFR2 levels. The level of an individual FGFR2 peptide is defined as the molar amount of the peptide

5 determined by the SRM/MRM assay per total amount of protein lysate analyzed. Information regarding FGFR2 can thus be used to aid in determining the stage or grade of a cancer by correlating the level of the FGFR2 protein (or fragment peptides of the FGFR2 protein) with levels observed in normal tissues. Once the quantitative amount of the FGFR2 protein has been determined in the cancer cells, that information can be matched to a list of therapeutic
10 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., FGFR2) that were assayed. Matching information from an FGFR2 protein assay to a list of therapeutic agents that specifically targets, for example, the FGFR2 protein or cells/tissue expressing the protein, defines what has been termed a *personalized medicine* approach to
15 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

20 In principle, any predicted peptide derived from the FGFR2 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 FGFR2 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 the FGFR2 protein
25 also might potentially be used to assay the extent of modification of the FGFR2 protein in a sample.

FGFR2 fragment peptides may be generated by a variety of methods including by the use of the Liquid Tissue protocol provided in US Patent 7,473,532. The Liquid Tissue protocol and reagents are capable of producing peptide samples suitable for mass
30 spectroscopic analysis from formalin fixed paraffin embedded tissue by proteolytic digestion of the proteins in the tissue/biological sample. In the Liquid Tissue protocol the tissue/biological 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 (e.g. a period of time from 30 minutes to 24 hours at a temperature from 37° to 65°C). The result of the heating and proteolysis is a liquid, soluble, dilutable biomolecule lysate.

Surprisingly, it was found that many potential peptide sequences from the FGFR2 protein are unsuitable or ineffective for use in mass spectrometry-based SRM/MRM assays for reasons that are not immediately evident. This was especially true for peptides derived from formalin-fixed tissue. 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 FGFR2 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 either do not ionize well or they produce fragments that are not distinct from peptides derived from other proteins. Peptides may also fail to resolve well in separation (e.g., liquid chromatography), or may adhere to glass or plastic ware.

FGFR2 peptides found in various embodiments of this disclosure (e.g., Table 1) were derived from the FGFR2 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 FGFR2 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 that 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, as for example including but not limited to the protease trypsin. Each protein lysate is turned into a collection of peptides by digestion of intact polypeptides with the protease. 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 is typically employed. Ion trap mass spectrometers however may be the best type of mass spectrometer for conducting global profiling of peptides. Although an SRM/MRM assay can be developed and performed on any type of mass spectrometer, including a MALDI, ion trap, or triple quadrupole, advantageously the instrument platform for an SRM/MRM assay is a triple quadrupole instrument platform.

Once as many peptides as possible were identified in a single MS analysis of a single lysate under the conditions employed, then that list of peptides was collated and used to determine the proteins that were detected in that lysate. That process was repeated for multiple Liquid Tissue lysates, and the very large list of peptides was collated into a single dataset. That type of dataset can be considered to represent 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 FGFR2 protein.

In one embodiment, the FGFR2 tryptic peptides identified as useful in the determination of absolute or relative amounts of the FGFR2 protein include one or more of SEQ ID NO:1 and SEQ ID NO:2, both of which are listed in Table 1. Each of these peptides was detected by mass spectrometry in Liquid Tissue lysates prepared from formalin fixed, paraffin embedded tissue. Thus, each peptide is a candidate for use in developing a quantitative SRM/MRM assay for the FGFR2 protein in human biological samples, including directly in formalin fixed patient tissue.

Table 1

SEQ ID	Peptide sequence	Mono Isotopic Mass	Precursor Charge State	Precursor m/z	Transition m/z	Ion Type
SEQ ID NO: 1	EAVTVAVK	815.4752	2	408.745	317.218	y3
			2	408.745	416.286	y4
			2	408.745	517.334	y5
			2	408.745	616.402	y6
SEQ ID NO: 2	YGPDGLPYLK	1121.5756	2	561.795	451.753	y8
			2	561.795	520.312	y4
			2	561.795	690.418	y6
			2	561.795	805.445	y7
			2	561.795	902.498	y8

The FGFR2 tryptic peptides listed in Table 1 were 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 considered useful for quantitative SRM/MRM assay of the FGFR2 protein in formalin fixed tissue. Further data analysis of these experiments indicated no preference is observed for either of these peptides from any specific organ site. Thus, each of these peptides is believed to be suitable for conducting SRM/MRM assays of the FGFR2 protein on a Liquid Tissue lysate from any formalin fixed tissue originating from any biological sample or from any organ site in the body.

An important consideration when 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 an SRM/MRM assay is presently most likely considered to be a triple quadrupole instrument platform. That type of a mass spectrometer is presently the most suitable instrument for putative analysis of 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 an SRM/MRM assay for each peptide derived from the FGFR2 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 FGFR2 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 FGFR2 protein is shown for these FGFR2 peptides from Table 1.

The method described below was used to: 1) identify candidate peptides from the FGFR2 protein that can be used for a mass spectrometry-based SRM/MRM assay for the FGFR2 protein, 2) develop an individual SRM/MRM assay, or assays, for target peptides from the FGFR2 protein in order to correlate 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 FGFR2 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 FGFR2 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 FGFR2 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 FGFR2 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 FGFR2 protein

2. Mass Spectrometry Assay for Fragment Peptides from the FGFR2 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 FGFR2 protein

- 5 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
10 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
15 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
20 spectrometer
- b. Perform SRM/MRM analysis so that the amount of the fragment peptide of the FGFR2 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.
- 25 i. Relative quantitation may be achieved by:
 1. Determining increased or decreased presence of the FGFR2 protein by comparing the SRM/MRM signature peak area from a given FGFR2 peptide detected in a Liquid Tissue lysate from one formalin fixed biological sample to the same SRM/MRM signature
30 peak area of the same FGFR2 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 FGFR2 protein by comparing the SRM/MRM signature peak area from a given FGFR2 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 FGFR2 protein by comparing the SRM/MRM signature peak area for a given FGFR2 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 FGFR2 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 FGFR2 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 FGFR2 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 FGFR2 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 FGFR2 protein and demonstrate that the previously-determined association, as well understood in the field of cancer, of FGFR2 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 FGFR2 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

Specific and unique characteristics about specific FGFR2 peptides were developed by analysis of all FGFR2 peptides on both an ion trap and triple quadrupole mass spectrometers. That information includes the monoisotopic mass of the peptide, its precursor charge state, the precursor m/z value, the transition m/z values of the precursor, and the ion types of each of the identified transitions. That information must be determined experimentally for each and every candidate SRM/MRM peptide directly in Liquid Tissue lysates from formalin fixed samples/tissue; because, interestingly, not all peptides from the FGFR2 protein can be detected in such lysates using SRM/MRM as described herein, indicating that FGFR2 peptides not detected cannot be considered candidate peptides for developing an SRM/MRM assay for use in quantitating peptides/proteins directly in Liquid Tissue lysates from formalin fixed samples/tissue.

A particular SRM/MRM assay for a specific FGFR2 peptide is performed on a triple quadrupole mass spectrometer. An experimental sample analyzed by a particular FGFR2

SRM/MRM assay is for example a Liquid Tissue protein lysate prepared from a tissue that had been formalin fixed and paraffin embedded. Data from such as assay indicates the presence of the unique SRM/MRM signature peak for this FGFR2 peptide in the formalin fixed sample.

5 Specific transition ion characteristics for this peptide are used to quantitatively measure a particular FGFR2 peptide in formalin fixed biological samples. These data indicate absolute amounts of this FGFR2 peptide as a function of molar amount of the peptide per microgram of protein lysate analyzed. Assessment of FGFR2 protein levels in tissues based on analysis of formalin fixed patient-derived tissue can provide diagnostic, prognostic, and
10 therapeutically-relevant information about each particular patient. In one embodiment, this disclosure describes a method for measuring the level of the fibroblast growth factor receptor 2 protein (FGFR2) in a biological sample, comprising detecting and/or quantifying the amount of one or more modified or unmodified FGFR2 fragment peptides in a protein digest prepared from said biological sample using mass spectrometry; and calculating the level of
15 modified or unmodified FGFR2 protein in said sample; and wherein said level is a relative level or an absolute level. In a related embodiment, quantifying one or more FGFR2 fragment peptides comprises determining the amount of the each of the FGFR2 fragment peptides in a biological sample by comparison to an added internal standard peptide of known amount, wherein each of the FGFR2 fragment peptides in the biological sample is compared
20 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 , ^2H or combinations thereof.

 The method for measuring the level of the FGFR2 protein in a biological sample described herein (or fragment peptides as surrogates thereof) may be used as a diagnostic
25 and/or prognostic indicator of cancer in a patient or subject. In one embodiment, the results from measurements of the level of the FGFR2 protein may be employed to determine the diagnostic stage/grade/status of a cancer by correlating (e.g., comparing) the level of FGFR2 protein found in a tissue with the level of that protein found in normal and/or cancerous or precancerous tissues.

30 Because both nucleic acids and protein can be analyzed from the same Liquid Tissue™ biomolecular preparation it is possible to generate additional information about disease diagnosis and drug treatment decisions from the nucleic acids in same sample upon which proteins were analyzed. For example, if the FGFR2 protein is expressed by certain

cells at increased levels, when assayed by SRM the data can provide information about the state of the cells and their potential for uncontrolled growth, potential drug resistance and the development of cancers can be obtained. At the same time, information about the status of the FGFR2 genes and/or the nucleic acids and proteins they encode (*e.g.*, mRNA molecules and their expression levels or splice variations) can be obtained from nucleic acids present in the same Liquid Tissue™ biomolecular preparation can be assessed simultaneously to the SRM analysis of the FGFR2 protein. Any gene and/or nucleic acid not from the FGFR2 and which is present in the same biomolecular preparation can be assessed simultaneously to the SRM analysis of the FGFR2 protein. In one embodiment, information about the FGFR2 protein and/or one, two, three, four or more additional proteins may be assessed by examining the nucleic acids encoding those proteins. Those nucleic acids can be examined, for example, by one or more, two or more, or three or more of: sequencing methods, polymerase chain reaction methods, restriction fragment polymorphism analysis, identification of deletions, insertions, and/or determinations of the presence of mutations, including but not limited to, single base pair polymorphisms, transitions, transversions, or combinations thereof.

Claims

1. A method for measuring the level of the fibroblast growth factor receptor 2 protein (FGFR2) in a biological sample, comprising detecting and/or quantifying the amount of one or more modified or unmodified FGFR2 fragment peptides in a protein digest prepared from said biological sample using mass spectrometry; and calculating the level of modified or unmodified FGFR2 protein in said sample; and
wherein said level is a relative level or an absolute level.
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 FGFR2 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 any of claims 1-3, wherein said protein digest of said biological sample is prepared by the Liquid Tissue protocol.
5. The method of any of claims 1-3, 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 any of claims 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 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).
9. The method of any of claims 1 to 8, wherein the FGFR2 fragment peptide comprises an amino acid sequence as set forth as SEQ ID NO:1 and SEQ ID NO:2.

10. The method of any of claims 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 claim 10, wherein the tissue is formalin fixed tissue.
12. The method of claim 10 or 11, 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 any of claims 1 to 15, further comprising quantifying a modified or unmodified FGFR2 fragment peptide.
17. The method of claim 16, wherein quantifying the FGFR2 fragment peptide comprises comparing an amount of one or more FGFR2 fragment peptides comprising an amino acid sequence of about 8 to about 45 amino acid residues of FGFR2 as shown in SEQ ID NO:1 and SEQ ID NO:2 in one biological sample to the amount of the same FGFR2 fragment peptide in a different and separate biological sample.
18. The method of claim 17, wherein quantifying one or more FGFR2 fragment peptides comprises determining the amount of the each of the FGFR2 fragment peptides in a biological sample by comparison to an added internal standard peptide of known amount, wherein each of the FGFR2 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 , ^2H or combinations thereof.
21. The method of any of claims 1 to 20, wherein detecting and/or quantifying the amount of one or more modified or unmodified FGFR2 fragment peptides in the protein digest indicates the presence of modified or unmodified FGFR2 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 FGFR2 fragment peptides, or the level of said FGFR2 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 FGFR2 fragment peptides, or the level of said FGFR2 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 claims 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 FGFR2 fragment peptides or the level of FGFR2 protein.
25. The method any one of claims 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 FGFR2 fragment peptides or the level of FGFR2 protein.
26. The method of claims 24 and 25, wherein therapeutic agents bind the FGFR2 protein and/or inhibit its biological activity.
27. The method of claim 26, wherein the therapeutic agent is selected to specifically target FGFR2-expressing cancer cells.
28. The method of claims 1 to 27, wherein the biological sample is formalin fixed tumor tissue that has been processed for quantifying the amount of one or more modified or unmodified FGFR2 fragment peptides employing the Liquid Tissue protocol and reagents.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US16/32794

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - G01N 33/68, 33/48, 33/574 (2016.01)

CPC - G01N 33/00, 33/574, 33/57442, 33/68

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 33/68, 33/48, 33/574 (2016.01)

CPC: G01N 33/00, 33/574, 33/57442, 33/68, 2333/00, 2333/435, 2033/57403, 2033/57457

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)

PatSeer (US, EP, WO, JP, DE, GB, CN, FR, KR, ES, AU, IN, CA, INPADOC Data); Google Scholar, Pubmed, EBSCO; Fibroblast Growth Factor Receptor 2, FGFR2, CD332, KGFR, Keratinocyte growth factor receptor, BBDS, BEK, BFR-1, CEK3, CFD1, ECT1, JWS, K-SAM, TK14, TK25, Selected Reaction Monitoring, SRM/MRM, assay, method, detect, measure, spectroscopy*

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2013/0288233 A1 (MURRAY, R) October 31, 2013; paragraphs [0007], [0008], [0014], [0019]	1-3, 4/1-3, 5/1-3, 6/5/1-3
Y	(HATTORI, Y et al.) Immunohistochemical Detection of K-sam Protein in Stomach Cancer" by Clinical Cancer Research. 1996. Vol. 2. pages 1373-1381; abstract, pages 1375-1376; figure 4; page 1376	1-3, 4/1-3, 5/1-3, 6/5/1-3
Y	US 2005/0014203 A1 (DARFLER, MM et al.) 20 January 2005; paragraph [0021]	4/1-3
A	(KATOH, M) Cancer genomics and genetics of FGFR2 (Review). International Journal of Oncology. Vol. 33. Pages 233-237; entire document	1-6
A	(NOMURA, S et al.) FGF10/FGFR2 signal induces cell migration and invasion in pancreatic cancer. British Journal of Cancer. 2008. Vol. 99. Pages 305 - 313; entire document	1-6
A	(ZHAO, Q et al.) Tumor-Specific Isoform Switch of the Fibroblast Growth Factor Receptor 2 Underlies the Mesenchymal and Malignant Phenotypes of Clear Cell Renal Cell Carcinomas. Clin Cancer Res; 19(9) May 1, 2013; entire document	1-6

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* 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

02 August 2016 (02.08.2016)

Date of mailing of the international search report

07 SEP 2016

Name and mailing address of the ISA/

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P.O. Box 1450, Alexandria, Virginia 22313-1450

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US16/32794

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.: 7-28
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.