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(54) Title: QUANTIFYING PROTEIN FOR OPTIMAL CANCER THERAPY

(57) Abstract: Methods are provided for identifying whether a lung tumor will be responsive to treatment with the therapeutic agents cisplatin and pemetrexed. Specific protein fragment peptides are precisely detected and quantitated by SRM-mass spectrometry directly in lung tumor cells collected from lung tumor tissue that was obtained from a cancer patient and compared to reference levels in order to determine if the lung cancer patient will positively respond to treatment with the combination of cisplatin and pemetrexed therapeutic agents.

Quantifying Proteins for Optimal Cancer Therapy

This application claims priority to U.S. Application Serial No. 62/274,935, filed January 5, 2016, the contents of which are hereby incorporated by reference in their entirety.

Introduction

New and improved methods for treating cancer patients are provided. The methods provide new assays for measuring the levels of specific proteins in tumor tissue from patients and then, based upon those levels, treating the patients with an optimized medication regimen. More specifically, new quantitative assays for the proteins XRCC1, ECAD, CAT and NQO1 are provided. Measurements from these assays, together with measurements of the levels of the proteins GART and KRAS, are used to identify those patients that will respond to, or are most likely to respond to, treatment with regimens that include the anti-cancer drug pemetrexed, and/or other anti-cancer drugs in the antifolate class of drugs, such as lometrexol, AG2034, LY309887 and pelitrexol, that function to kill tumor cells in a similar manner by attacking the GART protein.

Pemetrexed is a member of the antifolate class of drugs that enter the tumor cell via the Folate Receptor-alpha (FR-α), the Receptor-beta (FR-β), proton-coupled folate transporter (PCFT), and the Folate transporter 1 (RFC). Once inside the tumor cell the biochemical mode of action of the drug is inhibition of glycinamide ribonucleotide synthetase (GART) protein function, together with inhibition of other cellular proteins including thymidylate synthase (TS) and dihydrofolate reductase (DHFR). This results in the tumor cell being unable to synthesize nucleic acids, thereby preventing cell division and ultimately killing of the tumor cells. Reliance on the GART protein (and other proteins such as TS and DHFR) for tumor cell growth and division is interrupted by the biochemical function of pemetrexed.

The presence and/or quantitative levels of XRCC1, ECAD, CAT and NQO1 protein expression in the tumor tissue is determined by quantitating a specified fragment peptide derived from subsequences of each of the full-length proteins in digests prepared from formalin-fixed tissue samples. The presence and/or quantitative level of KRAS and GART also can be measured using specific fragment peptides derived from the proteins as described in PCT/US2015/040208 (filed July 13, 2015), PCT/U2016/45072 (filed August 1, 2016) and US application 62/199,202, (filed July 30, 2015), the contents of each of which are hereby incorporated by reference in their entireties. If expression of the KRAS protein is detected and specific levels of the GART protein is found below a specified quantitative level, the

patient is treated with a regimen that includes the pemetrexed therapeutic agent, and, optionally, other drugs that function similarly to pemetrexed. Alternatively, if the KRAS level is below the level of detection and levels of the GART protein are found to be above a specified quantitative level, the patient is treated with a regimen that does not include pemetrexed therapeutic agent, nor other drugs that function similarly such as lometrexol, AG2034, LY309887 and pelitrexol. Similarly, specified levels of XRCC1, ECAD, CAT and NQO1 are identified, such that when expression of the respective proteins in a tumor sample is observed to be below the specified level then the patient is treated with a regimen that includes the pemetrexed therapeutic agent, and, optionally, other drugs that function similarly to pemetrexed. If levels of the protein are found to be above a specified quantitative level, the patient is treated with a regimen that does not include pemetrexed therapeutic agent, nor other drugs that function similarly.

The specified XRCC1, ECAD, CAT, NQO1, KRAS and GART fragment peptides are detected using mass spectrometry-based Selected Reaction Monitoring (SRM), also referred to as Multiple Reaction Monitoring (MRM), and referred to herein as an SRM/MRM assay. An SRM/MRM assay is used to detect the presence and quantitatively measure the amount of the specified fragment peptides, directly in cells procured from cancer patient tissue, such as, for example formalin fixed cancer tissue. The amount of the specific peptides is then used to quantitate the amount of intact XRCC1, ECAD, CAT, NQO1, KRAS and GART proteins in the tumor sample. Specific and optimized therapeutic agents and treatment strategies are then determined and implemented to treat an individual cancer patient's disease based on how much of the XRCC1, ECAD, CAT, NQO1, KRAS and GART proteins are present in their cancer cells.

Summary of the invention

Methods are provided for treating a patient suffering from lung cancer comprising:

(a) detecting expression of a specified KRAS fragment peptide and quantifying the level of a specified GART fragment peptide in a protein digest prepared from a tumor sample obtained from the patient and calculating the level of the GART peptide in the sample by selected reaction monitoring using mass spectrometry;

(b) comparing the level of the GART fragment peptides to a reference level, and

(c) treating the patient with a therapeutic regimen that includes an effective amount of a combination of cisplatin and pemetrexed therapeutic agents when the level of the GART

fragment peptide is lower than the reference level and when the KRAS fragment peptide is not detected, and

(d) treating the patient with a therapeutic regimen that does not include an effective amount of the combination cisplatin and pemetrexed therapeutic agents when the level of the GART fragment peptide is above the reference level and the KRAS fragment peptide is detected.

In these methods the reference level of the GART fragment peptide may be, for example, 900 amol/µg., +/- 250 amol/µg, +/- 150 amol/µg, +/- 100 amol/µg, +/- 50 amol/µg, or +/- 25 amol/µg, of biological sample protein analyzed. The KRAS fragment peptide is either detected above the lower limit of detection or is not detected.

The protein digest may be a protease digest, such as a trypsin digest prepared, for example, using the liquid tissue protocol.

The mass spectrometry may be, for example, tandem mass spectrometry, ion trap mass spectrometry, triple quadrupole mass spectrometry, MALDI-TOF mass spectrometry, MALDI mass spectrometry, hybrid ion trap/quadrupole mass spectrometry and/or time of flight mass spectrometry. The mode of mass spectrometry used may be, for example, Selected Reaction Monitoring (SRM), Multiple Reaction Monitoring (MRM), Parallel Reaction Monitoring (PRM), intelligent Selected Reaction Monitoring (iSRM), and/or multiple Selected Reaction Monitoring (mSRM).

In these methods the specified KRAS peptide may have the amino acid sequence as set forth as SEQ ID NO:6. The specified GART peptide may have the amino acid sequence as set forth as SEQ ID NO:5. The tumor sample may be, for example, a cell, collection of cells, or a solid tissue. The tumor sample may be formalin fixed solid tissue, and may be paraffin embedded tissue.

In these methods quantifying the specified GART fragment peptide may include determining the amount of the GART peptide in the sample by comparing to a spiked internal standard peptide of known amount, where both the native peptide in the biological sample and the internal standard peptide corresponds to the same amino acid sequence of the GART fragment peptide as shown in SEQ ID NO:5. The internal standard peptide may be, for example, an isotopically labeled peptide. The isotopically labeled internal standard peptide may comprise one or more heavy stable isotopes selected from ^{18}O, ^{17}O, ^{15}N, ^{13}C, ^{2}H or combinations thereof.

Detecting and quantitating the specified GART fragment peptide and/or detecting the KRAS peptide can be combined with detecting and quantitating other peptides from other

proteins in a multiplex format so that the treatment decision about which agent used for treatment is based upon specific levels of the specified GART fragment peptide, or detection of the KRAS peptide, in combination with other peptides/proteins in the biological sample. When the level of the specified GART peptide is higher than the reference level and when the KRAS fragment peptide is detected, then the therapeutic strategy may include the combination of pemetrexed and cisplatin agents.

Methods are also provided for measuring the level of the human XRCC1 protein in a human biological sample of formalin-fixed tissue, comprising detecting and/or quantifying the amount of an XRCC1 fragment peptide in a protein digest prepared from the human biological sample using mass spectrometry; and calculating the level of XRCC1 protein in the sample; where the XRCC1 fragment peptide is SEQ ID NO:1, and where the level is a relative level or an absolute level.

Methods are also provided for measuring the level of the human CAT protein in a human biological sample of formalin-fixed tissue, comprising detecting and/or quantifying the amount of an CAT fragment peptide in a protein digest prepared from the human biological sample using mass spectrometry; and calculating the level of CAT protein in the sample; where the CATT fragment peptide is SEQ ID NO:2, and where the level is a relative level or an absolute level. Quantifying the specified CAT fragment peptide may include determining the amount of the CAT peptide in the sample by comparing to a spiked internal standard peptide of known amount, where both the native peptide in the biological sample and the internal standard peptide corresponds to the same amino acid sequence of the CAT fragment peptide as shown in SEQ ID NO:2.

Methods also are provided for measuring the level of the human NQO1 protein in a human biological sample of formalin-fixed tissue, comprising detecting and/or quantifying the amount of an NQO1 fragment peptide in a protein digest prepared from the human biological sample using mass spectrometry; and calculating the level of NQO1protein in the sample; where the NQO1fragment peptide is SEQ ID NO:3, and where the level is a relative level or an absolute level. Quantifying the specified NQO1 fragment peptide may include determining the amount of the NQO1 peptide in the sample by comparing to a spiked internal standard peptide of known amount, where both the native peptide in the biological sample and the internal standard peptide corresponds to the same amino acid sequence of the NQO1 fragment peptide as shown in SEQ ID NO:3.

Methods are also provided for measuring the level of the human ECAD protein in a human biological sample of formalin-fixed tissue, for example, paraffin-embedded tissue,

comprising detecting and/or quantifying the amount of an ECAD fragment peptide in a protein digest, such as a protease digest, prepared from the human biological sample using mass spectrometry; and calculating the level of ECAD protein in the sample; where the ECAD fragment peptide is SEQ ID NO:4, and where the level is a relative level or an absolute level. The tissue may be obtained from a tumor.

In the methods for detecting XRCC1, CAT, NQO1 and/or ECAD, the method may further include the step of fractionating the protein digest prior to detecting and/or quantifying the amount of the fragment peptide. Quantifying the fragment peptide may include comparing the amount of the fragment peptide in one biological sample to the amount of the same fragment peptide in a different and separate biological sample, or may include determining the amount of the fragment peptide in a biological sample by comparison to an added internal standard peptide of known amount, where the fragment peptide in the biological sample is compared to an internal standard peptide having the same amino acid sequence; and where the internal standard peptide is an isotopically labeled peptide.

In these methods detecting and/or quantifying the amount of the fragment peptide in the protein digest indicates the presence of the corresponding modified or unmodified protein and an association with cancer in the subject, and the results may be correlated to the diagnostic stage/grade/status of the cancer. The correlating step may be 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.

After the measurement and, optionally, the correlating step, the patient from which the biological sample was obtained is administered a therapeutically effective amount of a therapeutic agent, where the therapeutic agent and/or amount of the therapeutic agent administered is based upon the amount of the fragment peptide or the level of the protein.

Also provides are methods of treating a patient suffering from lung cancer comprising:

(a) quantifying expression of a specified CAT fragment peptide and quantifying the level of a specified NQO1 fragment peptide in a protein digest, for example a protease digest, such as a trypsin digest prepared, for example, using the liquid tissue protocol, where the protein digest is prepared from a tumor sample obtained from the patient, and calculating the level of the CAT and NQO1 peptides in the sample by selected reaction monitoring using mass spectrometry;

(b) comparing the level of each of the fragment peptides to a respective reference level, and

(c) treating the patient with a therapeutic regimen comprising an effective amount of a combination of cisplatin and pemetrexed therapeutic agents when the level of the CAT fragment peptide is higher than the CAT reference level and when the level of the NQO1 fragment peptide is below the NQO1 reference level, and

(d) treating the patient with a therapeutic regimen that does not comprise an effective amount of the combination cisplatin and pemetrexed therapeutic agents when the level of the CAT fragment peptide is below the reference level and the level of the NQO1 fragment peptide is above the reference level.

The reference level of the CAT fragment peptide may be 960 amol/µg, +/- 250 amol/µg, +/- 150 amol/µg, +/- 190 amol/µg, +/- 50 amol/µg, or +/- 25 amol/µg of biological sample protein analyzed and the reference level of the NQO1 fragment peptide may be 24000 amol/µg, +/- 250 amol/µg, +/- 150 amol/µg, +/- 190 amol/µg, +/- 50 amol/µg, or +/- 25 amol/µg of biological sample protein analyzed.

The mass spectrometry may be, for example, tandem mass spectrometry, ion trap mass spectrometry, triple quadrupole mass spectrometry, MALDI-TOF mass spectrometry, MALDI mass spectrometry, hybrid ion trap/quadrupole mass spectrometry and/or time of flight mass spectrometry. The mode of mass spectrometry used may be, for example, Selected Reaction Monitoring (SRM), Multiple Reaction Monitoring (MRM), Parallel Reaction Monitoring (PRM), intelligent Selected Reaction Monitoring (iSRM), and/or multiple Selected Reaction Monitoring (mSRM).

The tumor sample may be a cell, collection of cells, or a solid tissue, and may be formalin fixed solid tissue, which may be paraffin embedded tissue.

In these methods the internal standard peptide may be an isotopically labeled peptide, containing, for example, one or more heavy stable isotopes selected from ^{18}O, ^{17}O, ^{15}N, ^{13}C, ^{2}H or combinations thereof.

Detecting and quantitating the specified CAT and/or NQO1 fragment peptides can be combined with detecting and quantitating other peptides from other proteins in a multiplex format so that the treatment decision about which agent used for treatment is based upon specific levels of the specified CAT and/or NQO1 fragment peptide in combination with other peptides/proteins in the biological sample.

When the level of the specified CAT peptide is higher than the CAT reference level and where the level of the specified NQO1 fragment peptide is below than the NQO1

reference level, then the therapeutic strategy may include the combination of pemetrexed and cisplatin agents.

Also provided are methods of treating a patient suffering from lung cancer comprising:

(a) quantifying expression of a specified ECAD fragment peptide in a protein digest prepared from a tumor sample obtained from the patient and calculating the level of the ECAD peptide in the sample by selected reaction monitoring using mass spectrometry;

(b) comparing the level of the fragment peptide to a reference level, and

(c) treating the patient with a therapeutic regimen comprising an effective amount of a combination of cisplatin and pemetrexed therapeutic agents when the level of the ECAD fragment peptide is higher than the reference level, and

(d) treating the patient with a therapeutic regimen that does not comprise an effective amount of the combination cisplatin and pemetrexed therapeutic agents when the level of the ECAD fragment peptide is below the reference level.

The reference level of the ECAD fragment peptide may be 2450 amol/µg, +/- 250 amol/µg, +/- 150 amol/µg, +/- 190 amol/µg, +/- 50 amol/µg, or +/- 25 amol/µg, of biological sample protein analyzed. The protein digest of the biological sample may be prepared by the liquid tissue protocol and may include a protease digest, such as a trypsin digest.

The specified CAT peptide has the amino acid sequence as set forth as SEQ ID NO:4.

The mass spectrometry may be tandem mass spectrometry, ion trap mass spectrometry, triple quadrupole mass spectrometry, MALDI-TOF mass spectrometry, MALDI mass spectrometry, hybrid ion trap/quadrupole mass spectrometry and/or time of flight mass spectrometry and the the mode of mass spectrometry used may be Selected Reaction Monitoring (SRM), Multiple Reaction Monitoring (MRM), Parallel Reaction Monitoring (PRM), intelligent Selected Reaction Monitoring (iSRM), and/or multiple Selected Reaction Monitoring (mSRM).

Brief Description of the Drawings

Figure 1 shows an overall survival curve showing that patients whose tumor cells express levels of the GART protein below 900 amol/ug of protein analyzed have much greater probability of longer overall survival than patients whose tumor cells express above 900amol/ug of protein analyzed when treated with a therapeutic combination of pemetrexed and cisplatin.

Figure 2 shows an overall survival curve showing that patients whose tumor cells express detectable levels of KRAS and whose tumor cells also express greater than 900amol/ug GART analyzed have a much lower probability of longer overall survival than patients whose tumor cells do not express detectable levels of KRAS and also express less than 900amol/ug GART protein analyzed when treated with a therapeutic combination of pemetrexed and cisplatin.

Figure 3 is an overall survival curve that shows that patients whose tumor cells express levels of the ECAD protein over 2450 amol/μg have a lower probability of longer survival than patients whose tumor cells express lower levels of ECAD when treated with the combination of pemetrexed and cisplatin.

Figure 4 provides an overall survival curve that shows that patients whose tumor cells express NQO1 protein levels below 2400 amol/ug protein analyzed have a slightly greater probability of longer survival than patients whose tumor cells express NQO1 protein levels above 2400amol/ug when treated with the combination of pemetrexed and cisplatin

Figure 5 provides an overall survival curve that shows that patients whose tumor cells express XRCC1 protein levels below 521amol/ug protein analyzed have a slightly greater probability of longer survival than patients whose tumor cells express XRCC1 protein levels above 521amol/ug when treated with the combination of pemetrexed and cisplatin.

Figure 6 provides an overall survival curve that shows that patients whose tumor cells express CAT protein levels above 960 amol/ug protein analyzed have a greater probability of longer survival than patients whose tumor cells express CAT protein levels below 960 amol/ug when treated with the combination of pemetrexed and cisplatin.

Figure 7 provides an overall survival curve that shows that patients whose tumor cells exhibit high CAT expression (>960 amol/μg) and low NQ01 expression (≤24,000 amol/μg) have a significantly higher OS compared to the rest of the patients (20.3 vs 11.2 months).

Detailed Description

Methods are provided for identifying and optimizing treatment strategies for a cancer patient by determining whether or not a cancer patient will clinically respond in a favorable manner to the therapeutic cancer agent pemetrexed and therapeutic regimens containing pemetrexed. Pemetrexed is also referred to as Alimta. Specifically, diagnostic methods for measuring expression of the combination of the KRAS and GART proteins in a tumor sample or samples from the patient are provided. Methods of measuring expression of XRCC1,

ECAD, CAT and NQO1 in a tumor sample also are provided, together with methods of measuring combinations of these proteins, including the combination of CAT and NQO1. The tumor sample is advantageously a formalin-fixed sample.

Using an SRM/MRM assay that measures specific peptide fragments, and particular characteristics about the peptides, the amount of the KRAS, GART, XRCC1, ECAD, CAT and/or NQO1 proteins in cells derived from formalin fixed paraffin embedded (FFPE) tissue is determined. The peptide fragments derive from the full-length proteins; the specific peptide sequence that is used for KRAS is SFEDIHHYR and the peptide sequence used for detecting and quantitating the GART protein is VLAVTAIR. The fragment used to detect and quantify XRCC1 is TPATAPVPAR, for CAT it is LNVITVGPR, for NQO1 it is ALIVLAHSER, and for ECAD it is NTGVISVVTTGLDR.

Detection and accurate quantitation of specific peptides from any of the proteins in digests of FFPE tissue is highly unpredictable, due to the random protein crosslinking that occurs during formalin fixation of proteins. Surprisingly, however, it has been found that these specific KRAS, GART, XRCC1, CAT, NQO1, and ECAD peptides can be reliably detected and quantitated simultaneously in digests prepared from FFPE samples of tumor tissue. See, for example, U.S. Pat. App. No. 13/993,045, the contents of which are hereby incorporated by reference in their entirety.

More specifically, this 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. Pat. No. 7,473,532, the contents of which are hereby incorporated by reference in their entirety. The methods described in U.S. Pat. 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 tissue, and cancer tissue, from cancer patients is formalin fixed, paraffin embedded tissue. Formaldehyde/formalin fixation of surgically removed tissue is by far the most common method of preserving cancer tissue samples worldwide and is the accepted convention in 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. A small amount of stabilizer, usually methanol, is added 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.

Results from the SRM/MRM assay(s) can be used to correlate accurate and precise quantitative levels of the KRAS, GART, XRCC1, CAT, NQO1, and/or ECAD proteins within the specific cancer of the patient from whom the tissue was collected and preserved, including lung cancer tissue. 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. In this case, utilizing this assay can provide information about specific levels of KRAS, GART, XRCC1, CAT, NQO1, and/or ECAD protein expression in cancer tissue from a patient and makes it possible to determine whether or not the patient will respond favorably to therapy with the anti-cancer therapeutic agent pemetrexed, and potentially with other similar drugs in the antifolate class, designed to specifically inhibit the function of the GART, TS, and/or DHFR proteins.

Treating cancer patients with pemetrexed is one of the most common and effective strategies for preventing cancer from growing and thus prolonging the lives of cancer patients, especially lung cancer patients. The KRAS protein is a GTPase that performs an essential function in normal tissue signaling, and mutation of the KRAS gene is an essential step in the development of many cancers. The GART protein is a multifunctional transferase/ligase protein that is involved in purine metabolism and thus is an integral part of the nucleic acid synthesis function of the cell, and without which the cell cannot synthesize nucleic acids and grow/divide. Accordingly, inhibiting the function of the GART protein with pemetrexed prevents the tumor cell from synthesizing nucleic acids and leads to tumor cell death. It therefore is useful for a clinician to know the level of the GART protein in a patient's tumor cells to determine if pemetrexed will have a toxic effect on the tumor cells.

XRCC1, also known as X-ray repair cross-complementing protein 1, is involved in DNA repair where it complexes with DNA ligase III. XRCC1 is involved in repair of DNA single-strand breaks formed by exposure to ionizing radiation and alkylating agents. XRCC1 is over-expressed in non-small-cell lung carcinoma (NSCLC).

CAT (catalase) catalyzes the decomposition of hydrogen peroxide to water and oxygen, and is involved in protecting cells from oxidative damage by reactive oxygen species (ROS). It has been proposed that catalase may regulate cathepsin activity by controlling the production of ROS, leading to variation in migration and invasion ability of lung cancer cells.

NQO1 (NAD(P)H dehydrogenase [quinone] 1) is an enzyme that is a member of the NAD(P)H dehydrogenase (quinone) family. It is a flavoenzyme that protects against

endogenous and exogenous quinones by catalyzing two- or four-electron reductions of these substrates and protecting cells from unwanted oxidative damage. NQO1 has been shown to be overexpressed in non-small-cell lung carcinoma (NSCLC).

ECAD (E-cadherin) is a calcium-dependent cell-cell adhesion glycoprotein. Mutations of the ECAD gene are associated with gastric, breast, colorectal, thyroid, and ovarian cancers. Loss of function is thought to contribute to progression in cancer by increasing proliferation, invasion, and/or metastasis.

The most widely-used methodology presently applied to determine protein presence in cancer patient tissue, especially FFPE tissue, is immunohistochemistry (IHC). IHC methodology uses an antibody to detect the protein of interest. The results of an IHC test are most often interpreted by a pathologist or histotechnologist. This interpretation is subjective and does not provide quantitative data that are predictive of sensitivity to therapeutic agents that target specific oncoprotein targets. Thus, an IHC test cannot determine whether or not a tumor cell population will be sensitive to treatment with pemetrexed.

Studies involving other IHC assays, such as the Her2 IHC test, suggest the results obtained from such tests may be wrong or misleading. This is likely because different laboratories use different rules for classifying positive and negative IHC status. Each pathologist running a test also may use different criteria to decide whether the results are positive or negative. In most cases, this happens when the test results are borderline, *i.e.* the results are neither strongly positive nor strongly negative. In other cases, tissue from one area of cancer tissue can test positive while tissue from a different area of the cancer tests negative.

Inaccurate IHC test results may mean that patients diagnosed with cancer do not receive the best possible care. If all or part of a cancer is positive for a specific target oncoprotein but test results classify it as negative, physicians are unlikely to implement the correct therapeutic treatment, even though the patient could potentially benefit from agents that target the oncoprotein. If a cancer is oncoprotein target negative but test results classify it as positive, physicians may use a specific therapeutic treatment, even though the patient is not only unlikely to receive any benefit but also is exposed to the agent's secondary risks.

Accordingly, there is great clinical value in the ability to correctly evaluate quantitative levels of the KRAS, GART, CAT, NQO1, XRCC1 and ECAD proteins in tumors, especially lung tumors, so that the patient will have the greatest chance of receiving a successful treatment regimen while reducing unnecessary toxicity and other side effects.

Detection of peptides and determining quantitative levels of specified KRAS, GART, CAT, NQO1, XRCC1 and/or ECAD fragment peptides are determined in a mass spectrometer by the SRM/MRM methodology, in which the SRM/MRM signature chromatographic peak area of each peptide is determined within a complex peptide mixture present in a liquid tissue lysate (see U.S. Pat. No. 7,473,532, as described above). Quantitative levels of the analyzed proteins are then determined by the SRM/MRM methodology whereby the SRM/MRM signature chromatographic peak area of an individual specified peptide from each of the proteins in one biological sample is compared to the SRM/MRM signature chromatographic peak area of a known amount of a "spiked" internal standard for each of the individual specified fragment peptides.

In one embodiment, the internal standard is a synthetic version of the same fragment peptides where the synthetic peptides contain one or more amino acid residues labeled with one or more heavy isotopes, such as ^{2}H, ^{18}O, ^{17}O, ^{15}N, ^{13}C, or combinations thereof. Such isotope labeled internal standards are synthesized so that mass spectrometry analysis generates a predictable and consistent SRM/MRM signature chromatographic peak that is different and distinct from the native fragment peptide chromatographic signature peaks and which can be used as comparator peaks. Thus when the internal standard is "spiked" in known amounts into a protein or peptide preparation from a biological sample and analyzed by mass spectrometry, the SRM/MRM signature chromatographic peak area of the native peptide is compared to the SRM/MRM signature chromatographic 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. Quantitative data for fragment peptides are displayed according to the amount of protein analyzed per sample.

In order to develop the SRM/MRM assay for the fragment peptides additional information beyond simply the peptide sequence needs to be utilized by the mass spectrometer. That additional information is used to direct and instruct the mass spectrometer, (e.g., a triple quadrupole mass spectrometer) to perform the correct and focused analysis of the specified fragment peptides. An SRM/MRM assay may be effectively performed on a triple quadrupole mass spectrometer. That type of a mass spectrometer may be considered to be one of the most suitable instruments 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. The additional information provides the mass spectrometer, such as a triple quadrupole mass spectrometer, with the

correct directives to allow analysis of a single isolated target peptide within a very complex protein lysate. SRM/MRM assays also can be developed and performed on other types of mass spectrometer, including MALDI, ion trap, ion trap/quadrupole hybrid, or triple quadrupole instruments, but presently the most advantageous instrument platform for SRM/MRM assay is often considered to be a triple quadrupole instrument platform. The additional information about target peptides in general, and in particular about the specified fragment peptides, may include one or more of the mono isotopic mass of each peptide, its precursor charge state, the precursor m/z value, the m/z transition ions, and the ion type of each transition ion. The peptide sequence of the specified KRAS, GART, CAT, NQO1, XRCC1 and ECAD fragment peptides and the necessary additional information as described for these specified fragment peptides is shown in Table 1 below.

To determine an appropriate reference level for protein quantitation, tumor samples were obtained from a cohort of patients suffering from cancer, in this case lung cancer. The lung tumor samples were formalin-fixed using standard methods and the level of KRAS, GART, CAT, NQO1, XRCC1 and ECAD in the samples was measured using the methods as described above. The tissue samples may also be examined using IHC and FISH using methods that are well known in the art. The patients in the cohort were treated with the pemetrexed therapeutic agent and the response of the patients was measured using methods that are well known in the art, for example by recording the overall survival (OS) of the patients at time intervals after treatment. A suitable reference level was determined using statistical methods that are well known in the art, for example by determining the lowest p value of a log rank test. Once a reference level was determined it was used to identify those patients whose protein expression levels indicate that they may likely benefit from a pemetrexed therapeutic regimen as measured by extending the life of the patient.

The skilled artisan will recognize that pemetrexed may also be used as part of a treatment regimen that includes additional drugs or combinations of drugs, such as a combination with the drug cisplatin. Therapeutic regimens for treating lung cancer are known in the art and a very common and widely-used drug combination is cisplatin combined with pemetrexed. Levels of KRAS, GART, CAT, NQO1, XRCC1 and ECAD proteins in patient tumor samples are typically expressed in amol/µg, although other units can be used. The skilled artisan will recognize that a reference level can be expressed as a range around a central value, for example, +/- 250, 150, 100, 50 or 25 amol/ µg. In the specific example described in detail below a suitable reference level for the GART protein was found to be

900amol/µg. However, the skilled artisan will recognize that levels higher or lower than these reference levels can be selected based on clinical results and experience.

Table 1

SEQ ID	Peptide sequence	Mono Isotopic Mass	Precursor Charge State	Precursor m/z	Transition m/z	Ion Type
XRCC1						
SEQ ID NO: 1	TPATAPVPAR	979.545	2	490.78	539.33	y5
			2	490.78	610.367	y6
			2	490.78	711.414	y7
			2	490.78	782.451	y8
CAT						
SEQ ID NO: 2	LNVITVGPR	967.5814	2	484.798	428.261	y4
			2	484.798	529.3087	y5
			2	484.798	642.3928	y6
			2	484.798	741.4612	y7
			2	484.798	855.5042	y8
NQO1						
SEQ ID NO: 3	ALIVLAHSER	1107.64	3	370.2206	406.2244	y7
			3	370.2206	462.7664	y8
			3	370.2206	528.252	y4
			3	370.2206	599.2891	y5
			3	370.2206	712.3731	y6
ECAD						
SEQ ID NO: 4	NTGVISVVTTGLDR	1430.7728	2	716.394	761.415	y7
			2	716.394	947.515	y9
			2	716.394	1060.599	y10
GART						
SEQ ID NO:5	VLAVTAIR	841.5385	2	421.7765	359.2396	y3
			2	421.7765	460.2873	y4
			2	421.7765	559.3557	y5
			2	421.7765	630.3928	y6
			2	421.7765	743.4769	y7
KRAS						
SEQ ID NO:6	SFEDIHHYR	1202.5468	2	602.281	475.241	y3
			2	602.281	612.3	y4
			2	602.281	725.384	y5
			2	602.281	740.411	y6
			2	602.281	969.453	y7

5

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 KRAS, GART, CAT, NQO1, XRCC1 and ECAD proteins are 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, choice of optimal therapy, and potential drug resistance. At the same time, information about the status of 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. Nucleic acids can be assessed simultaneously to the SRM analysis of proteins, including the KRAS, GART, CAT, NQO1, XRCC1 and ECAD proteins. In one embodiment, information about one or more of the KRAS, GART, CAT, NQO1, XRCC1 and ECAD proteins 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.

Example: Determination of a predictive value of protein expression levels for pemetrexed sensitivity in a population of lung cancer patients.

Patients

37 patients were identified with non-small cell lung cancer (NSCLC). Tumors were surgically removed prior to treatment and archived as formalin-fixed, paraffin-embedded (FFPE) tissue and all were histologically confirmed as adenocarcinoma. All 37 patients were subsequently treated with the standard combination chemotherapy regimen of cisplatin and pemetrexed.

Methods

Tumor cells from FFPE tumor tissue were procured and isolated from the tumor tissue by tissue microdissection and solubilized for downstream mass spectrometry analysis using the Liquid Tissue® reagents as described above. Protein levels were quantitated using selected reaction monitoring mass spectrometry (SRM-MS). Overall survival curves of the patients in this study as related to levels of various proteins, including KRAS, GART, CAT, NQO1, XRCC1 and ECAD proteins, were developed.

Results

Quantitative SRM/MRM data across a number of proteins including those proteins specifically targeted by the therapeutic drug pemetrexed indicate a strong and significant correlation of the expression levels of KRAS, GART, CAT, NQO1, XRCC1 and ECAD with positive therapeutic outcome by treatment of the cancer patient with pemetrexed, as evidenced by extended survival after initial diagnosis and initiation of treatment regime with pemetrexed. The GART protein alone showed correlation (p=0.007) with increased overall survival to treatment with pemetrexed and cisplatin. A cutoff level of 900 amol/µg GART was established. Expression of GART above this level was associated with lower progression-free survival (PFS) and lower overall survival (OS). Detection of KRAS expression together with GART expression above the 900 amol/µg was strongly associated with reduced OS (p=0.002). Expression of XRCC1 above a cutoff level of 521 amol/µg was strongly associated with reduced OS (p=0.033). Expression of ECAD above a cutoff level of 2450 amol/µg was strongly associated with reduced OS (p=0.003). Expression of CAT below a cutoff level of 960 amol/µg was strongly associated with reduced OS (p=0.011). Expression of NQO1 above a cutoff level of 24000 amol/µg was strongly associated with reduced OS (p=0.043). Patients with both high CAT expression (>960 amol/µg) and low NQ01 expression (≤24,000 amol/µg) had a significantly higher OS compared the rest of the patients (20.3 vs 11.2 months), with P = 0.0015. Data from the analyses are shown in Figures 1-7.

Claims

1. A method of treating a patient suffering from lung cancer comprising:

(a) detecting expression of a specified KRAS fragment peptide and quantifying the level of a specified GART fragment peptide in a protein digest prepared from a tumor sample obtained from the patient and calculating the level of said GART peptide in said sample by selected reaction monitoring using mass spectrometry;

(b) comparing the level of said GART fragment peptides to a reference level, and

(c) treating the patient with a therapeutic regimen comprising an effective amount of a combination of cisplatin and pemetrexed therapeutic agents when the level of the GART fragment peptide is lower than said reference level and when the KRAS fragment peptide is not detected, and

(d) treating the patient with a therapeutic regimen that does not comprise an effective amount of the combination cisplatin and pemetrexed therapeutic agents when the level of the GART fragment peptide is above said reference level and the KRAS fragment peptide is detected.

2. The method of claim 1 wherein said reference level of the GART fragment peptide is 900 amol/µg., +/- 250 amol/µg, of biological sample protein analyzed and wherein said KRAS fragment peptide is detected above the lower limit of detection or not detected.

3. The method of claim 1 wherein said reference level is 900 amol/µg., +/- 150 amol/µg, of biological sample protein analyzed and wherein said KRAS fragment peptide is detected above the lower limit of detection or not detected.

4. The method of claim 1 wherein said reference level is 900 amol/µg., +/- 100 amol/µg, of biological sample protein analyzed and wherein said KRAS fragment peptide is detected above the lower limit of detection or not detected.

5. The method of claim 1 wherein said reference level is 900 amol/µg., +/- 50 amol/µg, of biological sample protein analyzed and wherein said KRAS fragment peptide is detected above the lower limit of detection or not detected.

6. The method of claim 1 wherein said reference level is 900 amol/µg., +/- 25 amol/µg, of biological sample protein analyzed and wherein said KRAS fragment peptide is detected above the lower limit of detection or not detected.

7. The method of any preceding claim, wherein said protein digest of said biological sample is prepared by the Liquid Tissue protocol.

8. The method of any preceding claim, wherein said protein digest comprises a protease digest.

9. The method of claim 8, wherein said protein digest comprises a trypsin digest.

10. The method of any preceding claim, wherein mass spectrometry comprises tandem mass spectrometry, ion trap mass spectrometry, triple quadrupole mass spectrometry, MALDI-TOF mass spectrometry, MALDI mass spectrometry, hybrid ion trap/quadrupole mass spectrometry and/or time of flight mass spectrometry.

11. The method of claim 10, wherein the mode of mass spectrometry used is Selected Reaction Monitoring (SRM), Multiple Reaction Monitoring (MRM), Parallel Reaction Monitoring (PRM), intelligent Selected Reaction Monitoring (iSRM), and/or multiple Selected Reaction Monitoring (mSRM).

12. The method of any preceding claim, wherein the specified KRAS peptide has the amino acid sequence as set forth as SEQ ID NO:6.

13. The method of any preceding claim, wherein the specified GART peptide has the amino acid sequence as set forth as SEQ ID NO:5.

14. The method of any preceding claim, wherein the tumor sample is a cell, collection of cells, or a solid tissue.

15. The method of claim 14, wherein the tumor sample is formalin fixed solid tissue.

16. The method of claim 15, wherein the tissue is paraffin embedded tissue.

17. The method of any preceding claim, wherein quantifying the specified GART fragment peptide comprises determining the amount of the GART peptide in said sample by comparing to a spiked internal standard peptide of known amount, wherein both the native

peptide in the biological sample and the internal standard peptide corresponds to the same amino acid sequence of the GART fragment peptide as shown in SEQ ID NO:5.

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

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

21. The method of claim 17, wherein detecting and quantitating the specified GART fragment peptide can be combined with detecting and quantitating other peptides from other proteins in multiplex so that the treatment decision about which agent used for treatment is based upon specific levels of the specified GART fragment peptide in combination with other peptides/proteins in the biological sample.

22. The method of any preceding claim wherein when said level of said specified GART peptide is higher than said reference level and wherein the KRAS fragment peptide is detected, then said therapeutic strategy comprises a combination of pemetrexed and cisplatin agents.

23. A method f/or measuring the level of the human XRCC1 protein in a human biological sample of formalin-fixed tissue, comprising detecting and/or quantifying the amount of an XRCC1 fragment peptide in a protein digest prepared from said human biological sample using mass spectrometry; and calculating the level of XRCC1 protein in said sample; wherein the XRCC1 fragment peptide is SEQ ID NO:1, and wherein said level is a relative level or an absolute level.

24. A method for measuring the level of the human CAT protein in a human biological sample of formalin-fixed tissue, comprising detecting and/or quantifying the amount of an CAT fragment peptide in a protein digest prepared from said human biological sample using mass spectrometry; and calculating the level of CAT protein in said sample; wherein the CATT fragment peptide is SEQ ID NO:2, and wherein said level is a relative level or an absolute level.

25. A method for measuring the level of the human NQO1 protein in a human biological sample of formalin-fixed tissue, comprising detecting and/or quantifying the

amount of an NQO1fragment peptide in a protein digest prepared from said human biological sample using mass spectrometry; and calculating the level of NQO1protein in said sample; wherein the NQO1fragment peptide is SEQ ID NO:3, and wherein said level is a relative level or an absolute level.

26. A method for measuring the level of the human ECAD protein in a human biological sample of formalin-fixed tissue, comprising detecting and/or quantifying the amount of an ECAD fragment peptide in a protein digest prepared from said human biological sample using mass spectrometry; and calculating the level of ECAD protein in said sample; wherein the ECAD fragment peptide is SEQ ID NO:4, and wherein said level is a relative level or an absolute level.

27. The method of any of claims 23-26, further comprising the step of fractionating said protein digest prior to detecting and/or quantifying the amount of said fragment peptide.

28. The method of any of claims 23-26, wherein said protein digest comprises a protease digest.

29. The method of any of claims 23-26, wherein the tissue is paraffin-embedded tissue.

30. The method of claim any of claims 23-26, wherein the tissue is obtained from a tumor.

31. The method of any of claims 23-26, further comprising quantifying said fragment peptide.

32. The method of claim 31, wherein quantifying said fragment peptide comprises comparing the amount of said fragment peptide in one biological sample to the amount of the same fragment peptide in a different and separate biological sample.

33. The method of claim 32, wherein quantifying said fragment peptide comprises determining the amount of said fragment peptide in a biological sample by comparison to an added internal standard peptide of known amount, wherein said fragment peptide in the biological sample is compared to an internal standard peptide having the same amino acid sequence; and wherein the internal standard peptide is an isotopically labeled peptide.

34. The method of any of claims 23-26, wherein detecting and/or quantifying the amount of said fragment peptide in the protein digest indicates the presence of the corresponding modified or unmodified protein and an association with cancer in the subject.

35. The method of claim 34, further comprising correlating the results of said detecting and/or quantifying the amount of said fragment peptide, or the level of said protein to the diagnostic stage/grade/status of the cancer.

36. The method of claim 35, wherein correlating the results of said detecting and/or quantifying the amount of said fragment peptide, or the level of said 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.

37. The method of any of claims 23-26, 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 the amount of said fragment peptide or the level of said protein.

38. A method of treating a patient suffering from lung cancer comprising:

(a) quantifying expression of a specified CAT fragment peptide and quantifying the level of a specified NQO1 fragment peptide in a protein digest prepared from a tumor sample obtained from the patient and calculating the level of said CAT and NQO1 peptides in said sample by selected reaction monitoring using mass spectrometry;

(b) comparing the level of each of said fragment peptides to a respective reference level, and

(c) treating the patient with a therapeutic regimen comprising an effective amount of a combination of cisplatin and pemetrexed therapeutic agents when the level of the CAT fragment peptide is higher than said reference level and when the level of the NQO1 fragment peptide is below said reference level, and

(d) treating the patient with a therapeutic regimen that does not comprise an effective amount of the combination cisplatin and pemetrexed therapeutic agents when the level of the CAT fragment peptide is below said reference level and the level of the NQO1 fragment peptide is above said reference level.

39. The method of claim 38 wherein said reference level of the CAT fragment peptide is 960 amol/µg, +/- 250 amol/µg, of biological sample protein analyzed and wherein said reference level of the NQO1 fragment peptide is 24000 amol/µg., +/- 250 amol/µg, of biological sample protein analyzed.

40. The method of claim 38 wherein said CAT reference level is 960 amol/µg., +/- 150 amol/µg, of biological sample protein analyzed and wherein said reference level of the NQO1 fragment peptide is 24000 amol/µg., +/- 150 amol/µg, of biological sample protein analyzed.

41. The method of claim 38 wherein said CAT reference level is 960 amol/µg., +/- 100 amol/µg, of biological sample protein analyzed and wherein said reference level of the NQO1 fragment peptide is 24000 amol/µg., +/- 100 amol/µg, of biological sample protein analyzed.

42. The method of claim 38 wherein said CAT reference level is 960 amol/µg, +/- 50 amol/µg, of biological sample protein analyzed and wherein said reference level of the NQO1 fragment peptide is 24000 amol/µg, +/- 50 amol/µg, of biological sample protein analyzed.

43. The method of claim 38 wherein said CAT reference level is 960 amol/µg, +/- 25 amol/µg, of biological sample protein analyzed and wherein said reference level of the NQO1 fragment peptide is 24000 amol/µg, +/- 25 amol/µg, of biological sample protein analyzed

44. The method of any of claims 38-43, wherein said protein digest of said biological sample is prepared by the liquid tissue protocol.

45. The method of any of claims 38-44, wherein said protein digest comprises a protease digest.

46. The method of claim 45, wherein said protein digest comprises a trypsin digest.

47. The method of any of claims 38-46, wherein mass spectrometry comprises tandem mass spectrometry, ion trap mass spectrometry, triple quadrupole mass spectrometry, MALDI-TOF mass spectrometry, MALDI mass spectrometry, hybrid ion trap/quadrupole mass spectrometry and/or time of flight mass spectrometry.

48. The method of claim 47, wherein the mode of mass spectrometry used is Selected Reaction Monitoring (SRM), Multiple Reaction Monitoring (MRM), Parallel Reaction Monitoring (PRM), intelligent Selected Reaction Monitoring (iSRM), and/or multiple Selected Reaction Monitoring (mSRM).

49. The method of any of claims 38-48, wherein the specified CAT peptide has the amino acid sequence as set forth as SEQ ID NO:2.

50. The method of any of claims 38-49, wherein the specified NQO1 peptide has the amino acid sequence as set forth as SEQ ID NO:3.

51. The method of any of claims 38-50, wherein the tumor sample is a cell, collection of cells, or a solid tissue.

52. The method of claim 51, wherein the tumor sample is formalin fixed solid tissue.

53. The method of claim 52, wherein the tissue is paraffin embedded tissue.

54. The method of any of claims 38-53, wherein quantifying the specified CAT fragment peptide comprises determining the amount of the CAT peptide in said sample by comparing to a spiked internal standard peptide of known amount, wherein both the native peptide in the biological sample and the internal standard peptide corresponds to the same amino acid sequence of the CAT fragment peptide as shown in SEQ ID NO:2.

55. The method of any of claims 38-54, wherein quantifying the specified NQO1 fragment peptide comprises determining the amount of the NQO1 peptide in said sample by comparing to a spiked internal standard peptide of known amount, wherein both the native peptide in the biological sample and the internal standard peptide corresponds to the same amino acid sequence of the NQO1 fragment peptide as shown in SEQ ID NO:3.

56. The method of any of claims 38-55, wherein the internal standard peptide is an isotopically labeled peptide.

57. The method of any of claims 38-56, wherein the isotopically labeled internal standard peptide comprises one or more heavy stable isotopes selected from ^{18}O, ^{17}O, ^{15}N, ^{13}C, ^{2}H or combinations thereof.

58. The method of claim 54, wherein detecting and quantitating the specified CAT and/or NQO1 fragment peptides can be combined with detecting and quantitating other peptides from other proteins in multiplex so that the treatment decision about which agent used for treatment is based upon specific levels of the specified CAT and/or NQO1 fragment peptide in combination with other peptides/proteins in the biological sample.

59. The method of any of claims 38-58 wherein when said level of said specified CAT peptide is higher than said CAT reference level and wherein said level of said specified NQO1 fragment peptide is below than said NQO1 reference level, then said therapeutic strategy comprises a combination of pemetrexed and cisplatin agents.

60. A method of treating a patient suffering from lung cancer comprising:

(a) quantifying expression of a specified ECAD fragment peptide in a protein digest prepared from a tumor sample obtained from the patient and calculating the level of said ECAD peptide in said sample by selected reaction monitoring using mass spectrometry;

(b) comparing the level of said fragment peptide to a reference level, and

(c) treating the patient with a therapeutic regimen comprising an effective amount of the combination of cisplatin and pemetrexed therapeutic agents when the level of the ECAD fragment peptide is higher than said reference level, and

(d) treating the patient with a therapeutic regimen that does not comprise an effective amount of a combination of cisplatin and pemetrexed therapeutic agents when the level of the ECAD fragment peptide is below said reference level.

61. The method of claim 60 wherein said reference level of the ECAD fragment peptide is 2450 amol/µg, +/- 250 amol/µg, of biological sample protein analyzed.

61. The method of claim 60 wherein said ECAD reference level is 2450 amol/µg., +/- 150 amol/µg, of biological sample protein analyzed.

62. The method of claim 60 wherein said ECAD reference level is 2450 amol/µg., +/- 100 amol/µg, of biological sample protein analyzed.

63. The method of claim 60 wherein said ECAD reference level is 2450 amol/µg, +/- 50 amol/µg, of biological sample protein analyzed.

64. The method of claim 60 wherein said ECAD reference level is 2450 amol/µg, +/- 25 amol/µg, of biological sample protein analyzed

65. The method of any of claims 60-64, wherein said protein digest of said biological sample is prepared by the liquid tissue protocol.

66. The method of any of claims 60-65, wherein said protein digest comprises a protease digest.

67. The method of claim 66, wherein said protein digest comprises a trypsin digest.

68. The method of any of claims 60-67, wherein mass spectrometry comprises tandem mass spectrometry, ion trap mass spectrometry, triple quadrupole mass spectrometry, MALDI-TOF mass spectrometry, MALDI mass spectrometry, hybrid ion trap/quadrupole mass spectrometry and/or time of flight mass spectrometry.

69. The method of claim 68, wherein the mode of mass spectrometry used is Selected Reaction Monitoring (SRM), Multiple Reaction Monitoring (MRM), Parallel Reaction Monitoring (PRM), intelligent Selected Reaction Monitoring (iSRM), and/or multiple Selected Reaction Monitoring (mSRM).

70. The method of any of claims 60-69, wherein the specified CAT peptide has the amino acid sequence as set forth as SEQ ID NO:4.

Figure 1: This overall survival curve shows that patients whose tumor cells express levels of the GART protein below 900amol/ug of protein analyzed have much greater probability of longer overall survival than patients whose tumor cells express above 900amol/ug of protein analyzed when treated with the therapeutic combination of pemetrexed and cisplatin. Results are statistically significant (p=0.007) for the GART protein alone.

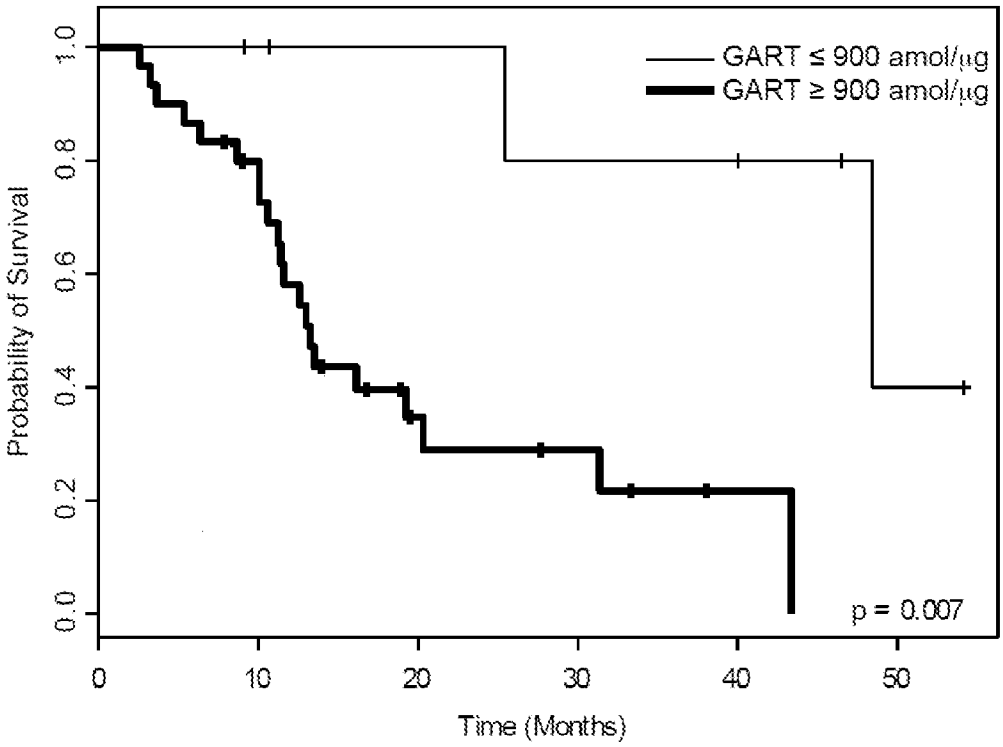
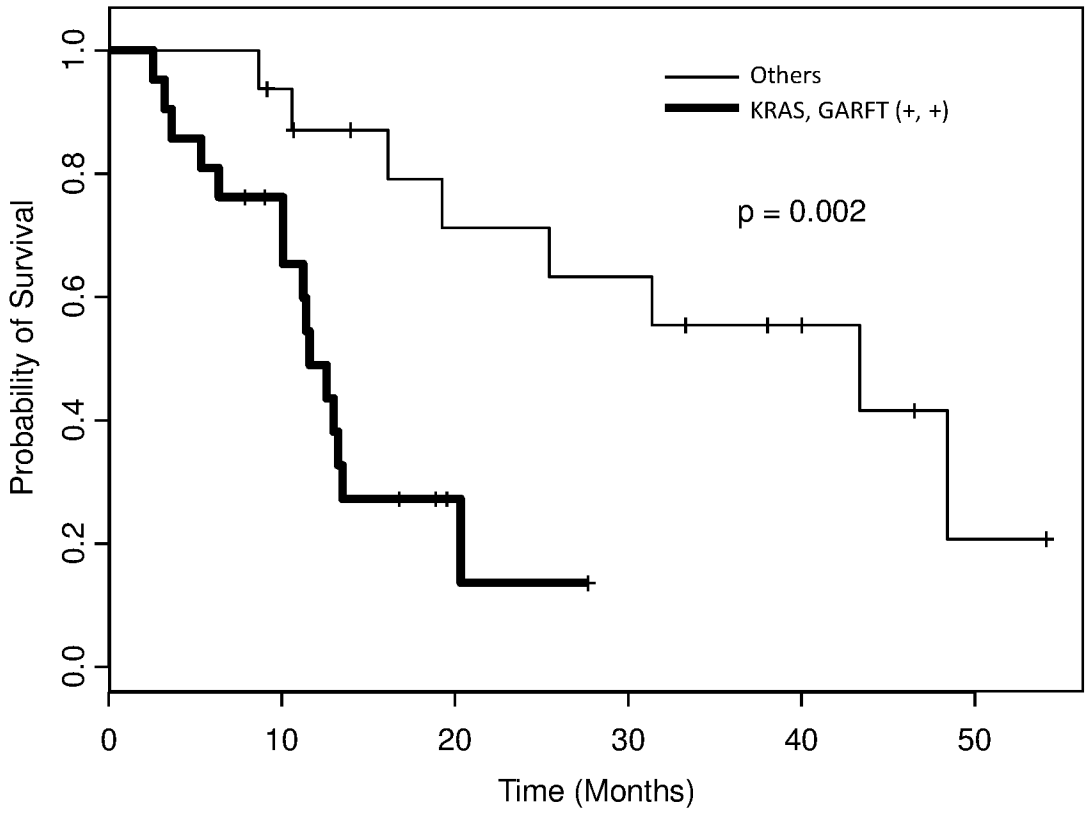


Figure 2: This overall survival curve shows that patients whose tumor cells express detectable levels of KRAS and whose tumor cells also express greater than 900amol/ug GART analyzed have much less probability of longer overall survival than patients whose tumor cells do not express detectable levels of KRAS and also express less than 900amol/ug GART protein analyzed when treated with the therapeutic combination of pemetrexed and cisplatin. Results are highly statistically significant for KRAS protein expression in combination with GART 900 > amol/ug (p=0.002) expression levels.

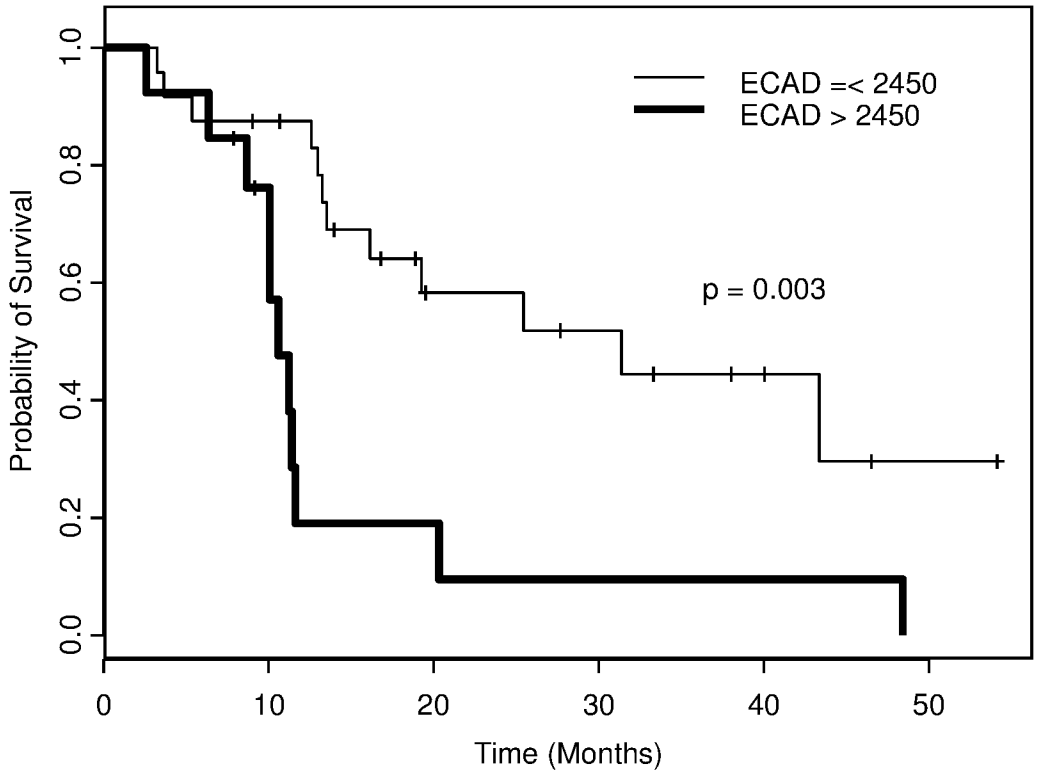
Overall survival:



KRAS+, GART > 900 amol/ug (n = 21): 11.6 months

KRAS, GART (others) (n = 16): 43.4 months

Figure 3: This overall survival curve shows that patients whose tumor cells express levels of the ECAD protein over 2450 amol/µg have a lower probability of longer survival than patients whose tumor cells express lower levels of ECAD when treated with the combination of pemetrexed and cisplatin. Results are statistically significant for overall survival and expression of the MRP1 protein (p=0.003).



Overall survival:

ECAD > 2450 (n = 13): 10.6 months

ECAD ≤ 2450 (n = 24): 31.4 months

Figure 4: This overall survival curve shows that patients whose tumor cells express NQO1 protein levels below 2400amol/ug protein analyzed have a slightly greater probability of longer survival than patients whose tumor cells express NQO1 protein levels above 2400amol/ug when treated with the combination of pemetrexed and cisplatin. Results are only slightly statistically significant for overall survival and expression of the NQO1 protein (p=0.043).

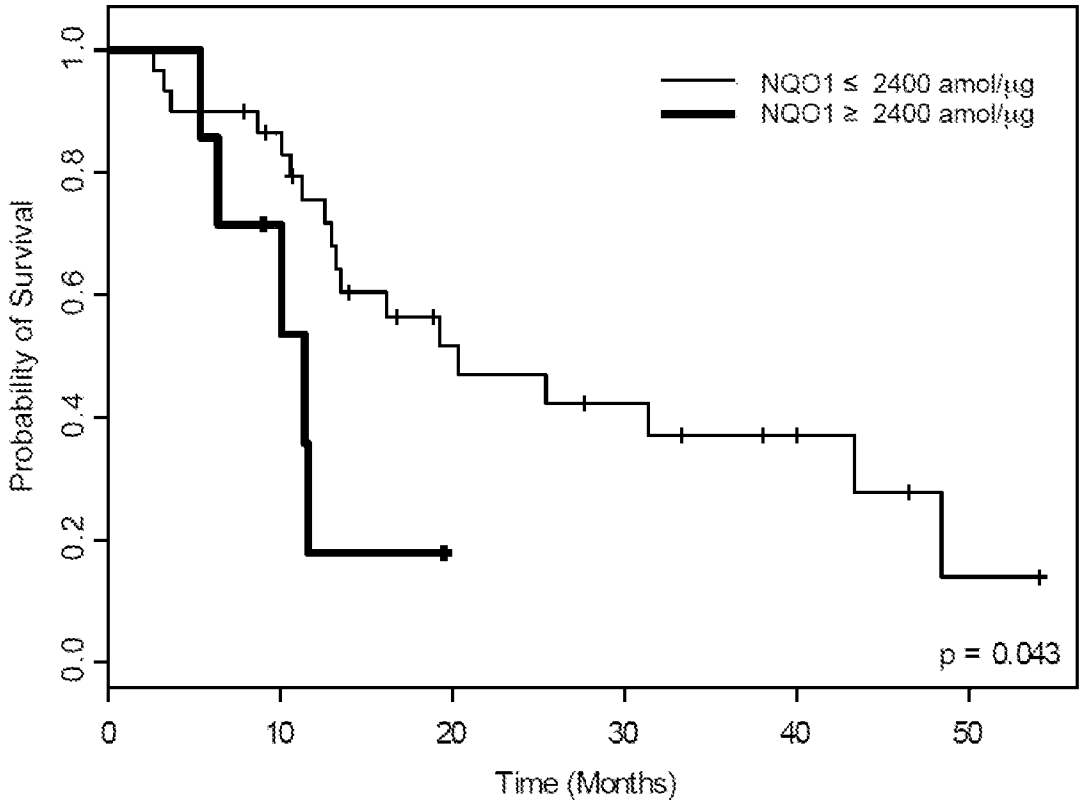


Figure 5: This overall survival curve shows that patients whose tumor cells express XRCC1 protein levels below 521amol/ug protein analyzed have a slightly greater probability of longer survival than patients whose tumor cells express XRCC1 protein levels above 521amol/ug when treated with the combination of pemetrexed and cisplatin. Results are only slightly statistically significant for overall survival and expression of the XRCC1 protein (p=0.033).

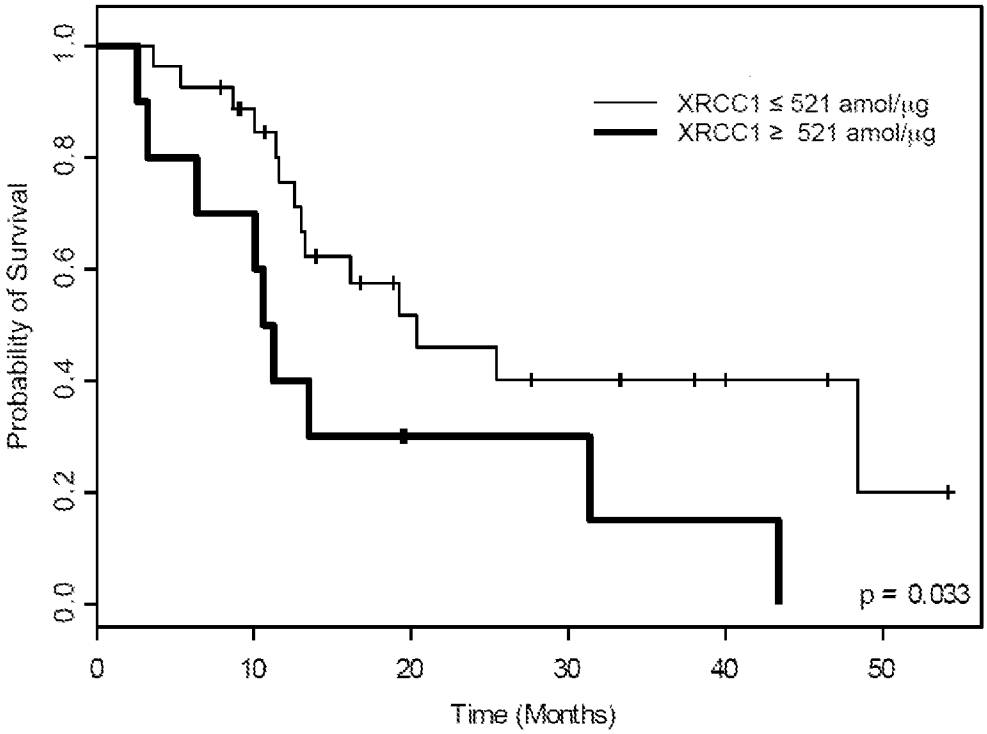


Figure 6: This overall survival curve shows that patients whose tumor cells express CAT protein levels above 960 amol/ug protein analyzed have a greater probability of longer survival than patients whose tumor cells express CAT protein levels below 960 amol/ug when treated with the combination of pemetrexed and cisplatin. Results are statistically significant for overall survival and expression of the CAT protein (p=0.011).

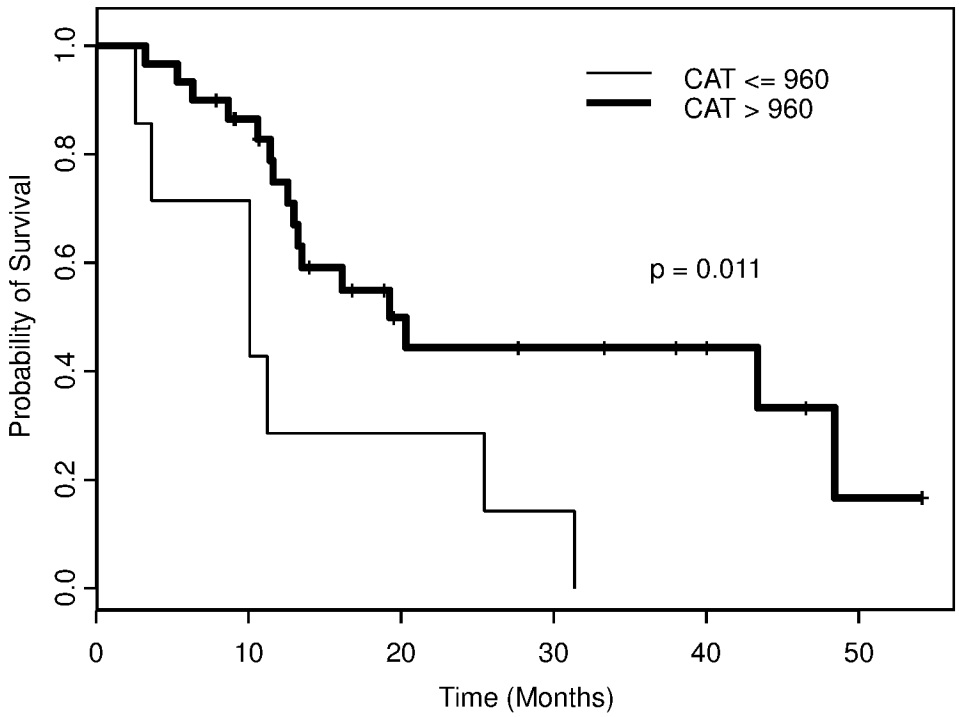
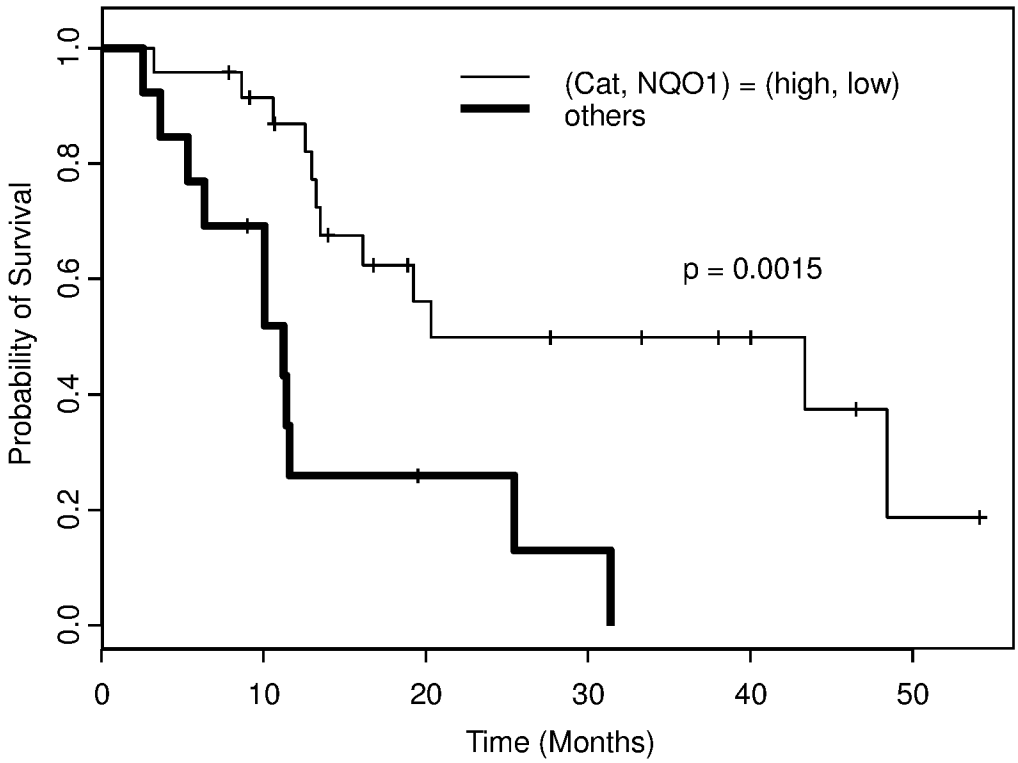


Figure 7: This overall survival curve shows that patients whose tumor cells exhibit high CAT expression (>960 amol/µg) and low NQ01 expression (≤24,000 amol/µg) have a significantly higher OS compared to the rest of the patients (20.3 vs 11.2 months), with P value = 0.0015.



Overall Survival

CAT, NQ01 (high, low) (n = 24): 20.3 months

CAT, NQ01 (others) (n = 13): 11.2

01152-8055.WO00/134069031.2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 17/12389

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - G01N 33/68, A61K 31/519 (2017.01)
CPC - G01N 33/6848, A61K 31/519

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)
See Search History Document

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History Document

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 2014/0018254 A1 (CARIS MPI, INC.) 16 January 2014 (16.01.2014) para [0013]; [0015]; [0028]; [0039]; [0111]; [0126]; [0262]-[0267]; Table 2.	1 ------ 2-7
Y	US 2015/0376678 A1 (EXPRESSION PATHOLOGY, INC.) 31 December 2015 (31.12.2015) abstract; para [0003]; [0008]; [0012]; [0015]-[0017]; [0020].	2-7
Y	US 2013/0108711 A1 (ASKARI et al.) 02 May 2013 (02.05.2013) claims 43, 44 and 50.	1

☐ 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
14 April 2017	05 MAY 2017
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. 571-273-8300	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 17/12389

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.: 8-22, 45-59 and 66-70
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:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I+: Claims 1-7, 23-44, 60-65, directed to a method for measuring the levels of human protein biomarkers in a human biological sample, and determining whether a patient suffering from lung cancer responds to therapeutic regimes based on the level of biomarkers. The method will be searched to the extent that the human protein biomarkers encompasses KRAS and GART. It is believed that claims 1-7 encompass this first named invention, and thus these claims will be searched without fee to the extent that they encompass KRAS and GART. Additional human protein biomarkers will be searched upon the payment of additional fees. Applicants must specify the claims that encompass any additionally elected human protein biomarkers. Applicants must further indicate, if applicable, the claims which encompass the first named invention, if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched. An exemplary election would be CAT and NQO1 (claims 24-25, (27-37)(in part), 38-44). Another exemplary election would be ECAD (claims 26, (27-37)(in part), 60-65). --continued on first extra sheet--

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.:
1-7 limited to KRAS and GART

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.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 17/12389

--continuation of Box No III: Observations where unity of invention is lacking--

The inventions listed as Groups I+ do not relate to a single special technical feature under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special technical features:

The special technical feature of the inventions listed as Group I+ is the specific human protein biomarkers, recited therein. Each of the inventions of Group I+ requires a unique human protein biomarker(s), not required by any of the other inventions.

Common technical features:

The inventions of Group I+ share the common technical feature of a method of treating a patient suffering from lung cancer comprising detecting expression of the first biomarker fragment peptide and quantifying the level of the second biomarker fragment peptide in a protein digest prepared from a tumor sample obtained from the patient and calculating the level of said second biomarker peptide in said sample by selected reaction monitoring using mass spectrometry; (b) comparing the level of each of said second biomarker fragment peptides to a respective reference level, and (c) treating the patient with a therapeutic regimen comprising an effective amount of a combination of cisplatin and pemetrexed therapeutic agents when the level of the second biomarker fragment peptides is below or higher than said reference level.

The inventions of Group I+ further share the common technical feature of a method for measuring the level of a protein in a human biological sample by detecting the amount of a peptide fragment of said protein in a protein digest prepared from said human biological sample using mass spectrometry, wherein said level is a relative level or an absolute level.

However, this shared technical feature does not represent a contribution over prior art, because this shared technical feature is made obvious over US 20150376678 A1 to Expression Pathology Inc., (hereinafter EP), in view of US 2013/0108711 A1 to Askari et al., (hereinafter Askari).

EP teaches a method of treating a patient suffering from lung cancer comprising:
(a) detecting expression of the first biomarker fragment peptide and quantifying the level of the second biomarker fragment peptide in a protein digest prepared from a tumor sample obtained from the patient and calculating the level of said peptides in said sample by selected reaction monitoring using mass spectrometry (para [0003] "The peptide sequence and fragmentation/transition ions for each peptide derived from proteins are potentially useful in a mass spectrometry-based Selected Reaction Monitoring (SRM) assay(s)"; [0008] "detection and/or quantitation of one or more, two or more, three or more, four or more, five or more of the ALK, Ros, Ron, Ret, TS, and/or FGFR1 proteins"; [0015] "To examine the levels of the proteins associated with lung cancer described herein, analysis can be conducted on cancerous tissue or tissue that is suspected of being cancerous that is removed from a patient or subject, either through surgical removal of partial or entire tumors"; [0020] "peptide samples suitable for mass spectroscopic analysis from formalin fixed paraffin embedded tissue by proteolytic digestion of the proteins in the tissue/biological sample");
(b) comparing the level of each of said second biomarker fragment peptides to a respective reference level (para [0015] "the expression level of one or more of those proteins can be determined and compared to a "normal" or reference level found in healthy tissue"), and
(c) treating the patient with a therapeutic regimen comprising an effective amount of a combination of therapeutic agents when the level of the second biomarker fragment peptides is below or higher than said reference level (para [0017] "comparing the level of the ALK, Ros, Ron, Ret, TS, and/or FGFR1 proteins, and their isoforms, or fragment peptides with the levels observed in normal tissues...represents a personalized medicine approach to treating cancer including lung cancer. The assay methods described herein form the foundation of a personalized medicine approach by using analysis of proteins from the patient's or subject's own tissue as a source for diagnostic and treatment decisions"). EP does not specifically teach the treatments are cisplatin and pemetrexed.

Askari teaches cisplatin and pemetrexed are anticancer agents that can be used to treat lung cancer (claims 43 and 44 "A method of treating a lung disease in a mammal...wherein the lung disease is a cancer of a lung"; claim 50 "The method of claim 44, wherein the anticancer agent is selected from the group consisting of docetaxel, paclitaxel, carboplatin, cisplatin, oxaliplatin, satraplatin, etoposide, gemcitabine, pemetrexed"). It would have been obvious to one of ordinary skill in the art to have considered known therapies for lung cancer when performing the method for determining optimal therapies for lung cancer taught by EP, in order to optimize therapy.

EP further teaches a method for measuring the level of a protein in a human biological sample by detecting the amount of a peptide fragment of said protein in a protein digest prepared from said human biological sample using mass spectrometry (para [0003] "The peptide sequence and fragmentation/transition ions for each peptide derived from proteins are potentially useful in a mass spectrometry"); [0008] "detection and/or quantitation of one or more, two or more, three or more, four or more, five or more of the ALK, Ros, Ron, Ret, TS, and/or FGFR1 proteins"; [0020] "peptide samples suitable for mass spectroscopic analysis...proteolytic digestion of the proteins in the tissue/biological sample"), wherein said level is a relative level or an absolute level (para [0015] "the expression level of one or more of those proteins can be determined and compared to a "normal" or reference level found in healthy tissue").

As the technical features were known in the art at the time of the invention, they cannot be considered special technical features that would otherwise unify the groups.

Therefore, Group I+ inventions lack unity under PCT Rule 13 because they do not share the same or corresponding special technical feature.