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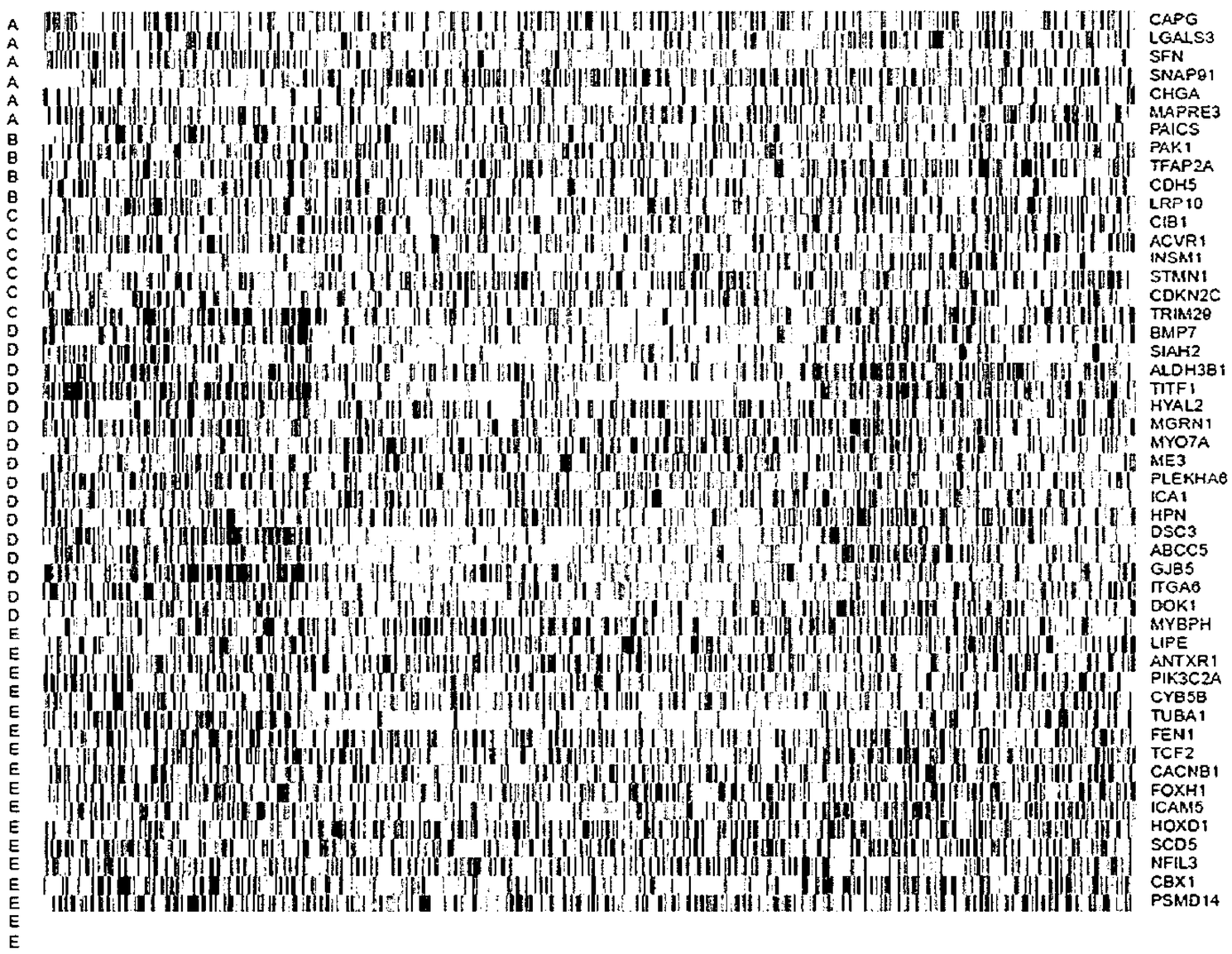
(71) **Demandeurs/Applicants:**
GENECENTRIC DIAGNOSTICS, INC., US;
THE UNIVERSITY OF NORTH CAROLINA AT CHAPEL HILL, US

(72) **Inventeurs/Inventors:**
LAI-GOLDMAN, MYLA, US;
PEROU, CHARLES, US;
HAYES, DAVID NEIL, US;
MAYHEW, GREG, US;
FAN, CHRIS, US;
MIGLARESE, MARK R., US;
FARUKI, HAWAZIN, US

(74) **Agent:** SMART & BIGGAR

(54) **Titre : PROCEDES DE TYPAGE DE CANCER DU POUMON**
(54) **Title: METHODS FOR TYPING OF LUNG CANCER**

Figure 1A GNCN-004/01WO



(57) **Abrégé/Abstract:**
Methods and compositions are provided for the molecular subtyping of lung cancer samples. Specifically, a method of assessing whether a patient's lung cancer subtype is adenocarcinoma, squamous cell carcinoma, or a neuroendocrine (encompassing both small cell carcinoma and carcinoid) is provided herein. A method for assessing whether a patient's lung cancer subtype is adenocarcinoma, squamous cell carcinoma, small cell carcinoma or carcinoid lung cancer is also provided. The methods provided herein entail probing the levels of the classifier biomarkers of Table 1-Table 6 or a subset thereof at the nucleic acid level, in a lung cancer sample obtained from the patient. Based in part on the levels of the classifier biomarkers, the lung cancer sample is classified as a particular lung cancer subtype.

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(72) Inventors; and

(71) Applicants : **FARUKI, Hawazin** [US/US]; c/o GeneCentric Diagnostics, Inc., 280 S. Mangum Street, Suite 350, Durham, North Carolina 27701 (US). **LAI-GOLDMAN, Myla** [US/US]; c/o GeneCentric Diagnostics, Inc., 280 S. Mangum Street, Suite 350, Durham, North Carolina 27701 (US). **PEROU, Charles** [US/US]; 103 Oak Spring Court, Carrboro, North Carolina 27510 (US). **HAYES, David Neil** [US/US]; 127 Glendale Drive, Chapel Hill, North Carolina 27514 (US). **MAYHEW, Greg** [US/US]; c/o GeneCentric Diagnostics, Inc., 280 S. Mangum Street, Suite 350, Durham, North Carolina 27701 (US). **FAN, Chris** [US/US]; c/o UNC-Chapel Hill, Department of Genetics, 120 Mason Farm Road, Chapel Hill, North Carolina 27599 (US). **MIGLARESE, Mark R.** [US/US]; c/o GeneCentric Diagnostics, Inc., 280 S. Mangum Street, Suite 350, Durham, North Carolina 27701 (US).

(74) Agents: **TUSCAN, Michael S.** et al.; Cooley LLP, 1299 Pennsylvania Avenue, NW, Suite 700, Washington, District of Columbia 20004 (US).

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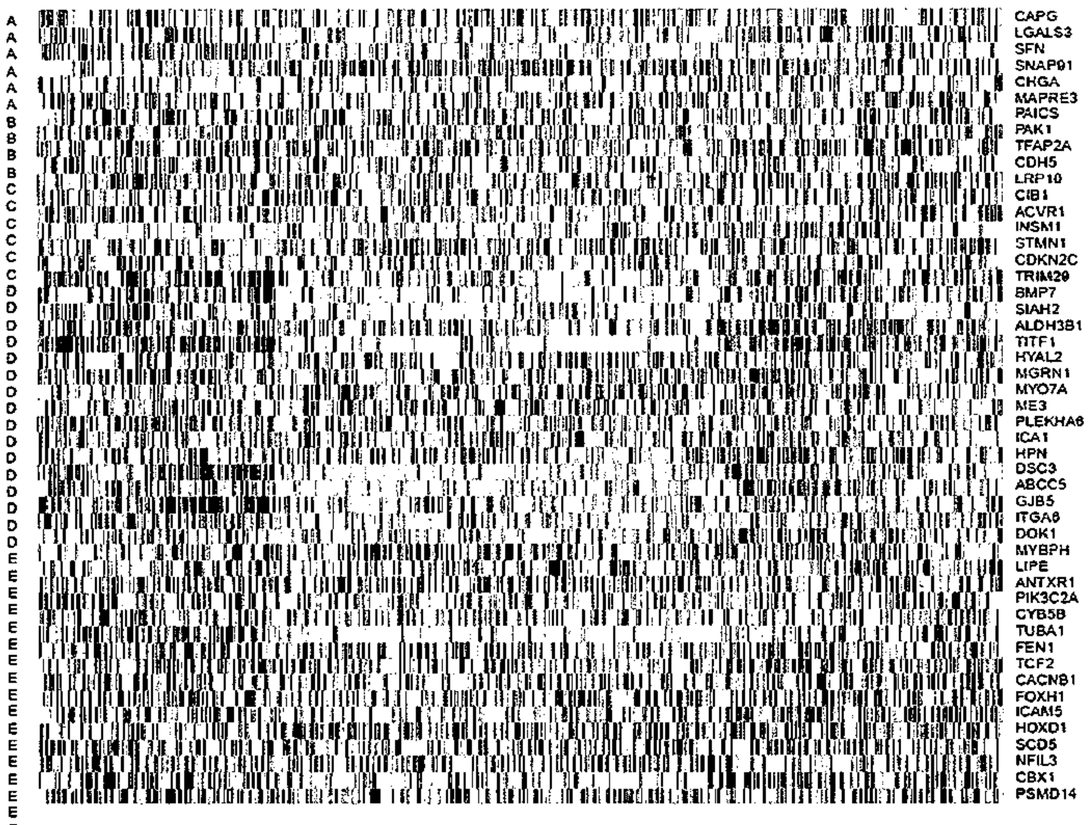
[Continued on next page]

(54) Title: METHODS FOR TYPING OF LUNG CANCER

Figure 1A GNCN-004/01WO



(57) Abstract: Methods and compositions are provided for the molecular subtyping of lung cancer samples. Specifically, a method of assessing whether a patient's lung cancer subtype is adenocarcinoma, squamous cell carcinoma, or a neuroendocrine (encompassing both small cell carcinoma and carcinoid) is provided herein. A method for assessing whether a patient's lung cancer subtype is adenocarcinoma, squamous cell carcinoma, small cell carcinoma or carcinoid lung cancer is also provided. The methods provided herein entail probing the levels of the classifier biomarkers of Table 1-Table 6 or a subset thereof at the nucleic acid level, in a lung cancer sample obtained from the patient. Based in part on the levels of the classifier biomarkers, the lung cancer sample is classified as a particular lung cancer subtype.

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METHODS FOR TYPING OF LUNG CANCER

CROSS REFERENCE TO U.S. NON-PROVISIONAL APPLICATONS

[0001] This application claims priority from U.S. Provisional Application Serial No. 62/005,229, filed May 30, 2014, which is incorporated by reference herein in its entirety for all purposes.

STATEMENT REGARDING SEQUENCE LISTING

[0002] The Sequence Listing associated with this application is provided in text format in lieu of a paper copy, and is hereby incorporated by reference into the specification. The name of the text file containing the Sequence Listing is GNCN_004_01WO.txt. The text file is 17 KB, was created on May 30, 2015, and is being submitted electronically via EFS-Web.

BACKGROUND OF THE INVENTION

[0003] Lung cancer is the leading cause of cancer death in the United States and over 220,000 new lung cancer cases are identified each year. Lung cancer is a heterogeneous disease with subtypes generally determined by histology (small cell, non-small cell, carcinoid, adenocarcinoma, and squamous cell carcinoma). Differentiation among various morphologic subtypes of lung cancer is essential in guiding patient management and additional molecular testing is used to identify specific therapeutic target markers. Variability in morphology, limited tissue samples, and the need for assessment of a growing list of therapeutically targeted markers pose challenges to the current diagnostic standard. Studies of histologic diagnosis reproducibility have shown limited intra- pathologist agreement and inter-pathologist agreement.

[0004] While new therapies are increasingly directed toward specific subtypes of lung cancer (bevacizumab and pemetrexed), studies of histologic diagnosis reproducibility have shown limited intra-pathologist agreement and even less inter-pathologist agreement. Poorly differentiated tumors, conflicting immunohistochemistry results, and small volume biopsies in which only a limited number of stains can be performed continue to pose challenges to the current diagnostic standard (Travis and Rekhtman Sem Resp and Crit Care Med 2011; 32(1): 22-31; Travis et al. Arch Pathol Lab Med 2013; 137(5):668-84; Tang et al. J Thorac Dis 2014; 6(S5):S489-S501).

[0005] A recent example involving expert pathology re-review of lung cancer samples submitted to the TCGA Lung Cancer genome project led to the reclassification of 15-20% of lung tumors submitted, confirming the ongoing challenge of morphology-based diagnoses. (Cancer Genome Atlas Research Network. "Comprehensive genomic characterization of squamous cell lung cancers." Nature 489.7417 (2012): 519-525; Cancer Genome Atlas Research Network. Comprehensive molecular profiling of lung adenocarcinoma. Nature 511.7511 (2014): 543-550, each of which is incorporated by reference herein in its entirety). Thus a need exists for a more reliable means for determining lung cancer subtype. The present invention addresses this and other needs.

SUMMARY OF THE INVENTION

[0006] In one aspect, a method of assessing whether a patient's lung cancer subtype is adenocarcinoma, squamous cell carcinoma, or a neuroendocrine (encompassing both small cell carcinoma and carcinoid). In one embodiment, the method comprises probing the levels of at least five of the classifier biomarkers of Table 1A, Table 1B, Table 1C, Table 2, Table 3, Table 4, Table 5 or Table 6 at the nucleic acid level, in a lung cancer sample obtained from the patient. The probing step, in one embodiment, comprises mixing the sample with five or more oligonucleotides that are substantially complementary to portions of nucleic acid molecules of the at least five classifier biomarkers of Table 1A, Table 1B, Table 1C, Table 2, Table 3, Table 4, Table 5 or Table 6 under conditions suitable for hybridization of the five or more oligonucleotides to their complements or substantial complements; detecting whether hybridization occurs between the five or more oligonucleotides to their complements or substantial complements; and obtaining hybridization values of the at least five classifier biomarkers based on the detecting step. The hybridization values of the at least five classifier biomarkers are then compared to reference hybridization value(s) from at least one sample training set, wherein the at least one sample training set comprises, hybridization values from a reference adenocarcinoma, squamous cell carcinoma, or a neuroendocrine sample. The lung cancer sample is classified as an adenocarcinoma, squamous cell carcinoma, or a neuroendocrine sample based on the results of the comparing step.

[0007] In another aspect, a method of assessing whether a patient's lung cancer subtype is adenocarcinoma, squamous cell carcinoma, small cell carcinoma or carcinoid is provided. In one embodiment, the method comprises probing the levels of at least five classifier biomarkers of Table 1A, Table 1B, Table 1C, Table 2, Table 3, Table 4, Table 5 or Table 6 at

the nucleic acid level, in a lung cancer sample obtained from the patient. The probing step, in one embodiment, comprises mixing the sample with five or more oligonucleotides that are substantially complementary to portions of nucleic molecules of the at least five classifier biomarkers of Table 1A, Table 1B, Table 1C, Table 2, Table 3, Table 4, Table 5 or Table 6 under conditions suitable for hybridization of the five or more oligonucleotides to their complements or substantial complements; detecting whether hybridization occurs between the five or more oligonucleotides to their complements or substantial complements; and obtaining hybridization values of the at least five classifier biomarkers based on the detecting step. The hybridization values of the at least five classifier biomarkers are then compared to reference hybridization value(s) from at least one sample training set, wherein the at least one sample training set comprises, hybridization values from a reference adenocarcinoma, squamous cell carcinoma, small cell carcinoma or carcinoid sample. The lung cancer sample is classified as an adenocarcinoma, squamous cell carcinoma, small cell carcinoma or carcinoid based on the results of the comparing step.

[0008] In yet another aspect, a method of assessing whether a patient's non-small cell lung carcinoma (NSCLC) subtype is adenocarcinoma or squamous cell carcinoma is provided. In one embodiment, the method comprises probing the levels of at least five of the classifier biomarkers of Table 1A, Table 1B, Table 1C, Table 2, Table 3, Table 4, Table 5 or Table 6 at the nucleic acid level, in a lung cancer sample obtained from the patient. The probing step, in one embodiment, comprises mixing the sample with five or more oligonucleotides that are substantially complementary to portions of cDNA molecules of the at least five classifier biomarkers of Table 1A, Table 1B, Table 1C, Table 2, Table 3, Table 4, Table 5 or Table 6 under conditions suitable for hybridization of the five or more oligonucleotides to their complements or substantial complements; detecting whether hybridization occurs between the five or more oligonucleotides to their complements or substantial complements; and obtaining hybridization values of the at least five classifier biomarkers based on the detecting step. The hybridization values of the at least five classifier biomarkers are then compared to reference hybridization value(s) from at least one sample training set, wherein the at least one sample training set comprises, hybridization values from a reference adenocarcinoma and/or squamous cell carcinoma sample. The NSCLC sample is classified as an adenocarcinoma or squamous cell carcinoma based on the results of the comparing step.

[0009] In one embodiment, the comparing step comprises determining a correlation between the hybridization values of the at least five classifier biomarkers and the reference hybridization values.

[0010] In one embodiment, the probing step comprises isolating the nucleic acid or portion thereof prior to the mixing step. In a further embodiment, the hybridization comprises hybridization of a cDNA to a cDNA, thereby forming a non-natural complex; or hybridization of a cDNA to an mRNA, thereby forming a non-natural complex. In even a further embodiment, the probing step comprises amplifying the nucleic acid in the sample.

[0011] In one embodiment, the at least five classifier biomarkers comprise from about 5 to about 50, from about 10 to about 50, from about 15 to about 50, from about 20 to about 50 or from about 25 to about 50 biomarkers. In another embodiment, the at least five classifier biomarkers comprise from about 5 to about 30, from about 10 to about 30, from about 15 to about 30, from about 20 to about 30 classifier biomarkers.

BRIEF DESCRIPTION OF THE DRAWINGS

[0012] Figures 1A-1D illustrate exemplary gene expression heatmaps for adenocarcinoma (Figure 1A), squamous cell carcinoma (Figure 1B), small cell carcinoma (Figure 1C), and carcinoid (Figure 1D).

[0013] Figure 2 is a heatmap of gene expression hierarchical clustering for FFPE RT-PCR gene expression dataset.

[0014] Figure 3 is a comparison of path review and LSP prediction for 77 FFPE samples. Each rectangle represents a single sample ordered by sample number. Arrows indicate 6 samples that disagreed with the original diagnosis by both pathology review and gene expression.

DETAILED DESCRIPTION OF THE INVENTION

[0015] As used herein, an "expression profile" comprises one or more values corresponding to a measurement of the relative abundance, level, presence, or absence of expression of a discriminative gene. An expression profile can be derived from a subject prior to or subsequent to a diagnosis of lung cancer, can be derived from a biological sample collected from a subject at one or more time points prior to or following treatment or therapy, can be

derived from a biological sample collected from a subject at one or more time points during which there is no treatment or therapy (e.g., to monitor progression of disease or to assess development of disease in a subject diagnosed with or at risk for lung cancer), or can be collected from a healthy subject.

[0016] The biomarker panels and methods provided herein are used in various aspects, to assess, (i) whether a patient's NSCLC subtype is adenocarcinoma or squamous cell carcinoma; (ii) whether a patient's lung cancer subtype is adenocarcinoma, squamous cell carcinoma, or a neuroendocrine (encompassing both small cell carcinoma and carcinoid) and/or (iii) whether a patient's lung cancer subtype is adenocarcinoma, squamous cell carcinoma, small cell carcinoma or carcinoid.

[0017] For example, the biomarker panels, or subsets thereof, as disclosed in Table 1A, Table 1B, Table 1C, Table 2, Table 3, Table 4, Table 5 and Table 6 are used in various embodiments to assess and classify a patient's lung cancer subtype. In one embodiment, the biomarker panel of Table 14 or Table 15, or a subset thereof is used to assess and classify a patient's lung cancer subtype.

[0018] In general, the methods provided herein are used to classify a lung cancer sample as a particular lung cancer subtype. In one embodiment, the method comprises probing the levels of at least five of the classifier biomarkers of Table 1A, Table 1B, Table 1C, Table 2, Table 3, Table 4, Table 5 or Table 6 at the nucleic acid level, in a lung cancer sample obtained from the patient. The probing step, in one embodiment, comprises mixing the sample with five or more oligonucleotides that are substantially complementary to portions of nucleic acid molecules, e.g., cDNA molecules or mRNA molecules, of the at least five classifier biomarkers of Table 1A, Table 1B, Table 1C, Table 2, Table 3, Table 4, Table 5 or Table 6 under conditions suitable for hybridization of the five or more oligonucleotides to their complements or substantial complements; detecting whether hybridization occurs between the five or more oligonucleotides to their complements or substantial complements; and obtaining hybridization values of the at least five classifier biomarkers based on the detecting step. The hybridization values of the at least five classifier biomarkers are then compared to reference hybridization value(s) from at least one sample training set. For example, the at least one sample training set comprises hybridization values from a reference adenocarcinoma, squamous cell carcinoma, a neuroendocrine sample, small cell carcinoma sample. The lung cancer sample is classified, for example, as an adenocarcinoma, squamous

cell carcinoma, a neuroendocrine or small cell carcinoma based on the results of the comparing step.

[0019] The lung tissue sample can be any sample isolated from a human subject. For example, in one embodiment, the analysis is performed on lung biopsies that are embedded in paraffin wax. This aspect of the invention provides a means to improve current diagnostics by accurately identifying the major histological types, even from small biopsies. The methods of the invention, including the RT-PCR methods, are sensitive, precise and have multianalyte capability for use with paraffin embedded samples. See, for example, Cronin et al. (2004) Am. J Pathol. 164(1):35-42, herein incorporated by reference.

[0020] Formalin fixation and tissue embedding in paraffin wax is a universal approach for tissue processing prior to light microscopic evaluation. A major advantage afforded by formalin-fixed paraffin-embedded (FFPE) specimens is the preservation of cellular and architectural morphologic detail in tissue sections. (Fox et al. (1985) J Histochem Cytochem 33:845-853). The standard buffered formalin fixative in which biopsy specimens are processed is typically an aqueous solution containing 37% formaldehyde and 10-15% methyl alcohol. Formaldehyde is a highly reactive dipolar compound that results in the formation of protein-nucleic acid and protein-protein crosslinks in vitro (Clark et al. (1986) J Histochem Cytochem 34:1509-1512; McGhee and von Hippel (1975) Biochemistry 14:1281-1296, each incorporated by reference herein).

[0021] In one embodiment, the sample used herein is obtained from an individual, and comprises fresh-frozen paraffin embedded (FFPE) tissue. However, other tissue and sample types are amenable for use herein.

[0022] Methods are known in the art for the isolation of RNA from FFPE tissue. In one embodiment, total RNA can be isolated from FFPE tissues as described by Bibikova et al. (2004) American Journal of Pathology 165:1799-1807, herein incorporated by reference. Likewise, the High Pure RNA Paraffin Kit (Roche) can be used. Paraffin is removed by xylene extraction followed by ethanol wash. RNA can be isolated from sectioned tissue blocks using the MasterPure Purification kit (Epicenter, Madison, Wis.); a DNase I treatment step is included. RNA can be extracted from frozen samples using Trizol reagent according to the supplier's instructions (Invitrogen Life Technologies, Carlsbad, Calif.). Samples with measurable residual genomic DNA can be resubjected to DNaseI treatment and assayed for

DNA contamination. All purification, DNase treatment, and other steps can be performed according to the manufacturer's protocol. After total RNA isolation, samples can be stored at -80 °C until use.

[0023] General methods for mRNA extraction are well known in the art and are disclosed in standard textbooks of molecular biology, including Ausubel et al., ed., Current Protocols in Molecular Biology, John Wiley & Sons, New York 1987-1999. Methods for RNA extraction from paraffin embedded tissues are disclosed, for example, in Rupp and Locker (Lab Invest. 56:A67, 1987) and De Andres et al. (Biotechniques 18:42-44, 1995). In particular, RNA isolation can be performed using a purification kit, a buffer set and protease from commercial manufacturers, such as Qiagen (Valencia, Calif.), according to the manufacturer's instructions. For example, total RNA from cells in culture can be isolated using Qiagen RNeasy mini-columns. Other commercially available RNA isolation kits include MasterPure.TM. Complete DNA and RNA Purification Kit (Epicentre, Madison, Wis.) and Paraffin Block RNA Isolation Kit (Ambion, Austin, Tex.). Total RNA from tissue samples can be isolated, for example, using RNA Stat-60 (Tel-Test, Friendswood, Tex.). RNA prepared from a tumor can be isolated, for example, by cesium chloride density gradient centrifugation. Additionally, large numbers of tissue samples can readily be processed using techniques well known to those of skill in the art, such as, for example, the single-step RNA isolation process of Chomczynski (U.S. Pat. No. 4,843,155, incorporated by reference in its entirety for all purposes).

[0024] In one embodiment, a sample comprises cells harvested from a lung tissue sample, for example, an adenocarcinoma sample. Cells can be harvested from a biological sample using standard techniques known in the art. For example, in one embodiment, cells are harvested by centrifuging a cell sample and resuspending the pelleted cells. The cells can be resuspended in a buffered solution such as phosphate-buffered saline (PBS). After centrifuging the cell suspension to obtain a cell pellet, the cells can be lysed to extract nucleic acid, *e.g*, messenger RNA. All samples obtained from a subject, including those subjected to any sort of further processing, are considered to be obtained from the subject.

[0025] The sample, in one embodiment, is further processed before the detection of the biomarker levels of the combination of biomarkers set forth herein. For example, mRNA in a cell or tissue sample can be separated from other components of the sample. The sample can be concentrated and/or purified to isolate mRNA in its non-natural state, as the mRNA is not

in its natural environment. For example, studies have indicated that the higher order structure of mRNA *in vivo* differs from the *in vitro* structure of the same sequence (*see*, *e.g.*, Rouskin *et al*. (2014). Nature 505, pp. 701-705, incorporated herein in its entirety for all purposes).

[0026] mRNA from the sample in one embodiment, is hybridized to a synthetic DNA probe, which in some embodiments, includes a detection moiety (e.g., detectable label, capture sequence, barcode reporting sequence). Accordingly, in these embodiments, a non-natural mRNA-cDNA complex is ultimately made and used for detection of the biomarker. In another embodiment, mRNA from the sample is directly labeled with a detectable label, *e.g.*, a fluorophore. In a further embodiment, the non-natural labeled-mRNA molecule is hybridized to a cDNA probe and the complex is detected.

[0027] In one embodiment, once the mRNA is obtained from a sample, it is converted to complementary DNA (cDNA) in a hybridization reaction or is used in a hybridization reaction together with one or more cDNA probes. cDNA does not exist *in vivo* and therefore is a non-natural molecule. Furthermore, cDNA-mRNA hybrids are synthetic and do not exist *in vivo*. Besides cDNA not existing *in vivo*, cDNA is necessarily different than mRNA, as it includes deoxyribonucleic acid and not ribonucleic acid. The cDNA is then amplified, for example, by the polymerase chain reaction (PCR) or other amplification method known to those of ordinary skill in the art. For example, other amplification methods that may be employed include the ligase chain reaction (LCR) (Wu and Wallace, Genomics, 4:560 (1989), Landegren *et al.*, Science, 241:1077 (1988), incorporated by reference in its entirety for all purposes, transcription amplification (Kwoh *et al.*, *Proc. Natl. Acad. Sci. USA*, 86:1173 (1989), incorporated by reference in its entirety for all purposes), self-sustained sequence replication (Guatelli *et al.*, *Proc. Nat. Acad. Sci. USA*, 87:1874 (1990), incorporated by reference in its entirety for all purposes), incorporated by reference in its entirety for all purposes, and nucleic acid based sequence amplification (NASBA). Guidelines for selecting primers for PCR amplification are known to those of ordinary skill in the art. *See*, *e.g.*, McPherson et al., PCR Basics: From Background to Bench, Springer-Verlag, 2000, incorporated by reference in its entirety for all purposes. The product of this amplification reaction, *i.e.*, amplified cDNA is also necessarily a non-natural product. First, as mentioned above, cDNA is a non-natural molecule. Second, in the case of PCR, the amplification process serves to create hundreds of millions of cDNA copies for every individual cDNA

molecule of starting material. The number of copies generated are far removed from the number of copies of mRNA that are present *in vivo*.

[0028] In one embodiment, cDNA is amplified with primers that introduce an additional DNA sequence (*e.g.*, adapter, reporter, capture sequence or moiety, barcode) onto the fragments (*e.g.*, with the use of adapter-specific primers), or mRNA or cDNA biomarker sequences are hybridized directly to a cDNA probe comprising the additional sequence (e.g., adapter, reporter, capture sequence or moiety, barcode). Amplification and/or hybridization of mRNA to a cDNA probe therefore serves to create non-natural double stranded molecules from the non-natural single stranded cDNA, or the mRNA, by introducing additional sequences and forming non-natural hybrids. Further, as known to those of ordinary skill in the art, amplification procedures have error rates associated with them. Therefore, amplification introduces further modifications into the cDNA molecules. In one embodiment, during amplification with the adapter-specific primers, a detectable label, *e.g.*, a fluorophore, is added to single strand cDNA molecules. Amplification therefore also serves to create DNA complexes that do not occur in nature, at least because (i) cDNA does not exist *in vivo*, (i) adapter sequences are added to the ends of cDNA molecules to make DNA sequences that do not exist *in vivo*, (ii) the error rate associated with amplification further creates DNA sequences that do not exist *in vivo*, (iii) the disparate structure of the cDNA molecules as compared to what exists in nature and (iv) the chemical addition of a detectable label to the cDNA molecules.

[0029] In some embodiments, the expression of a biomarker of interest is detected at the nucleic acid level via detection of non-natural cDNA molecules.

[0030] In some embodiments, the method for lung cancer subtyping includes detecting expression levels of a classifier biomarker set. In some embodiments, the detecting includes all of the classifier biomarkers of Table 1A, 1B or 1C, Table 2, Table 3, Table 4, Table 5 or Table 6 at the nucleic acid level. In another embodiment, a subset of the classifier biomarkers of Table 1A, 1B or 1C are probed for, for example, from about 5 to about 20. In one embodiment, a subset of biomarkers probed for comprises from about 5 to about 50, from about 5 to about 40, from about 5 to about 30, or from about 5 to about 25 of the classifier biomarkers of Table 1A, 1B or 1C, Table 2, Table 3, Table 4, Table 5 or Table 6. In another embodiment, a subset of biomarkers probed for comprises from about 10 to about 50, from about 10 to about 40, from about 10 to about 30, or from about 10 to about 25 of the classifier

biomarkers of Table 1A, 1B or 1C, Table 2, Table 3, Table 4, Table 5 or Table 6. In yet another embodiment, a subset of biomarkers probed for comprises 5, 10, 15, 20, 25, 30, 35, 40, 45 or 50 of the classifier biomarkers of Table 1A, 1B or 1C, Table 2, Table 3, Table 4, Table 5 or Table 6. In even another embodiment, a subset of biomarkers probed for comprises at least about 5, at least about 10, at least about 15, at least about 20, at least about 25, at least about 30, at least about 35, at least about 40, at least about 45 or at least about 50 of the classifier biomarkers of Table 1A, 1B or 1C, Table 2, Table 3, Table 4, Table 5 or Table 6. It should be understood that the number of biomarkers probed for will dictate the number of oligonucleotides initially used in a method provided herein. For example, where 5 or 10 of the biomarkers of Table 1A, 1B or 1C, Table 2, Table 3, Table 4, Table 5 or Table 6 are probed for, 5 oligonucleotides or 10 oligonucleotides, respectively, will be mixed with the sample under conditions suitable for hybridization of the oligonucleotides to the biomarkers, or fragments thereof.

[0031] In one embodiment, from about five to about 10, from about 5 to about 15, from about 5 to about 20, from about 5 to about 25, from about 5 to about 30, from about 5 to about 35, from about 5 to about 40, from about 5 to about 45, from about 5 to about 50 of the biomarkers in any of Table 1A, Table 1B, Table 1C, Table 2, Table 3, Table 4, Table 5 and Table 6 are probed for in a method to determine the lung cancer subtype. In another embodiment, each of the biomarkers from any one of Table 1A, Table 1B, Table 1C, Table 2, Table 3, Table 4, Table 5, or from Table 6 are probed for in a method to determine the lung cancer subtype of a patient's lung cancer sample.

[0032] The detecting can be performed by any suitable technique including, but not limited to, RNA-seq, a reverse transcriptase polymerase chain reaction (RT-PCR), a microarray hybridization assay, or another hybridization assay, *e.g.*, a NanoString assay for example, with primers and/or probes specific to the classifier biomarkers, and/or the like. It should be noted that the primers provided in Table 1A, Table 1B, Table 1C, Table 2, Table 3, Table 4, Table 5 and Table 6 are merely for illustrative purposes and should not be construed as limiting the invention.

[0033] The biomarkers described herein include RNA comprising the entire or partial sequence of any of the nucleic acid sequences of interest, or their non-natural cDNA product, obtained synthetically *in vitro* in a reverse transcription reaction. The term "fragment" is intended to refer to a portion of the polynucleotide that generally comprise at least 10, 15, 20,

50, 75, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 800, 900, 1,000, 1,200, or 1,500 contiguous nucleotides, or up to the number of nucleotides present in a full-length biomarker polynucleotide disclosed herein. A fragment of a biomarker polynucleotide will generally encode at least 15, 25, 30, 50, 100, 150, 200, or 250 contiguous amino acids, or up to the total number of amino acids present in a full-length biomarker protein of the invention.

[0034] In some embodiments, overexpression, such as of an RNA transcript or its expression product, is determined by normalization to the level of reference RNA transcripts or their expression products, which can be all measured transcripts (or their products) in the sample or a particular reference set of RNA transcripts (or their non-natural cDNA products). Normalization is performed to correct for or normalize away both differences in the amount of RNA or cDNA assayed and variability in the quality of the RNA or cDNA used. Therefore, an assay typically measures and incorporates the expression of certain normalizing genes, including well known housekeeping genes, such as, for example, GAPDH and/or β-Actin. Alternatively, normalization can be based on the mean or median signal of all of the assayed biomarkers or a large subset thereof (global normalization approach).

Table 1A					
Gene symbol	**Gene name**	**Forward primer**	**SEQ ID**	**Reverse primer**	**SEQ ID**
CDH5	cadherin 5, type 2, VE-cadherin (vascular epithelium)	AAGAGAGATTG GATTTGGAACC	1	TTCTTGCGACTCACGCT	58
CLEC3B	C-type lectin domain family 3, member B	CCAGAAGCCCA AGAAGATTGTA	2	GCTCCTCAAACAT CTTTGTGTTCA	59
PAICS	phosphoribosylaminoimidazole carboxylase, phosphoribosylaminoimidazole succinocarboxamide synthetase	AATCCTGGTGT CAAGGAAG	3	GACCACTGTGGG TCATTATT	60
PAK1	p21/Cdc42/Rac1-activated kinase 1 (STE20 homolog, yeast)	GGACCGATTTT ACCGATCC	4	GAAATCTCTGGC CGCTC	61
PECAM1	platelet/endothelial cell adhesion molecule (CD31 antigen)	ACAGTCCAGAT AGTCGTATGT	5	ACTGGGCATCAT AAGAAATCC	62
TFAP2A	transcription factor AP-2 alpha (activating enhancer binding protein 2 alpha)	GTCTCCGCCATC CCTAT	6	ACTGAACAGAAG ACTTCGT	63

Table 1A					
Gene symbol	Gene name	Forward primer	SEQ ID	Reverse primer	SEQ ID
ACVR1	activin A receptor, type 1	ACTGGTGTAAC AGGAACAT	7	AACCTCCAAGTG GAAATTCT	64
CDKN2C	cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)	TTTGGAAGGAC TGCGCT	8	TCGGTCTTTCAAA TCGGGATTA	65
CIB1	calcium and integrin binding 1 (calmyrin)	CACGTCATCTCC CGTTC	9	CTGCTGTCACAG GACAAT	66 66
INSM1	insulinoma-associated 1	ATTGAACTTCCC ACACGA	10	AAGGTAAAGCCA GACTCCA	67 67
LRP10	low density lipoprotein receptor-related protein 10	GGAACAGACTG TCACCAT	11	GGGAGCGTAGGG TTAAG	68
STMN1	stathmin 1/oncoprotein 18	TCAGAGTGTGTG G TCAGGC	12	CAGTGTATTCTGC ACAATCAAC	69
CAPG	capping protein (actin filament), gelsolin-like	GGGACAGCTTC AACACT	13	GTTCCAGGATGTT GGACTTTC	70
CHGA	chromogranin A (parathyroid secretory protein 1)	CCTGTGAACAG CCCTATG	14	GGAAAGTGTGTC GGAGAT	71
LGALS3	lectin, galactoside-binding, soluble, 3 (galectin 3)	TTCTGGGCACG GTGAAG	15	AGGCAACATCAT TCCCTC	72
MAPRE3	microtubule-associated protein, RP/EB family, member 3	GGCCAAACTAG AGCACGAATA	16	GTCAACACCCAT CTTCTTGAAA	73
SFN	stratifin	TCAGCAAGAAG GAGATGCC	17	CGTAGTGGAAGA CGGAAA	74
SNAP91	synaptosomal-associated protein, 91 kDa homolog (mouse)	GTGCTCCCTCTC CATTAAGTA	18	CTGGTGTAGAATT AGGAGACGTA	75
ABCC5	ATP-binding cassette, sub-family C(CFTR/MRP), member 5	CAAGTTCAGGA GAACTCGAC	19	GGCATCAAGAGA GAGGC	76
ALDH3B1	aldehyde dehydrogenase 3 family, member B1	GGCTGTGGTTA TGCGATAG	20	GATAAAGAGTTA CAAGCTCCTCTG	77
ANTXR1	Anthrax toxin receptor 1	ACCCGAGGAAC AACCTTA	21	TCTAGGCCTTGAC GGAT	78

Table 1A					
Gene symbol	**Gene name**	**Forward primer**	**SEQ ID**	**Reverse primer**	**SEQ ID**
BMP7	Bone morphogenetic protein 7 (osteogenic protein 1)	CCCTCTCCATTCCCTACA	22	TTTGGGCAAACCTCGGTAA	79
CACNB1	calcium channel, voltage-dependent, beta 1 subunit	CAGAGCGCCAGGCATTA	23	GCACAGCAAATGCCACT	80
CBX1	chromobox homolog 1 (HP1 beta homolog *Drosophila*)	CCACTGGCTGAGGTGTTA	24	CTTGTCTTTCCCTACTGTCTTAC	81
CYB5B	cytochrome b5 type B (outer mitochondrial membrane)	TGGGCGAGTCTACGATG	25	CTTGTTCCAGCAGAACCT	82
DOK1	docking protein 1, 62 kDa (downstream of tyrosine kinase 1)	CTTTCTGCCCTGGAGATG	26	CAGTCCTCTGCACCGTTA	83
DSC3	desmocollin 3	GCGCCATTTGCTAGAGATA	27	CATCCAGATCCCTCACAT	84
FEN1	flap structure-specific endonuclease 1	AGAGAAGATGGGCAGAAAG	28	CCAAGACACAGCCAGTAAT	85
FOXH1	forkhead box H1	GCCCAGATCATCCGTCA	29	TTTCCAGCCCTCGTAGTC	86
GJB5	gap junction protein, beta 5 (connexin 31.1)	ACCACAAGGACTTCGAC	30	GGGACACAGGGAAGAAC	87
HOXD1	homeobox D1	GCTCCGCTGCTATCTTT	31	GTCTGCCACTCTGCAAC	88
HPN	Hepsin (transmembrane protease, serine 1)	AGCGGCCAGGTGGATTA	32	GTCGGCTGACGCTTTGA	89
HYAL2	hyaluronoglucosaminidase 2	ATGGGCTTTGGGAGCATA	33	GAACAAGTCAGTCTAGGGAATAC	90
ICA1	islet cell autoantigen 1, 69 kDa	GACCTGGATGCCAAGCTA	34	TGCTTTCGATAAGTCCAGACA	91
ICAM5	intercellular adhesion molecule 5, telencephalin	CCGGCTCTTGGAAGTTG	35	CCTCTGAGGCTGGAAACA	92
ITGA6	integrin, alpha 6	ACGCGGATCGAGTTTGATAA	36	ATCCACTGATCTTCCTTGC	93
LIPE	lipase, hormone-sensitive	CGCAAGTCCCAGAAGAT	37	CAGTGCTGCTTCAGACACA	94
ME3	malic enzyme 3, NADP(+)-dependent, Mitochondrial	CGCGGATACGATGTCAC	38	CCTTTCTTCAAGGGTAAAGGC	95
MGRN1	mahogunin, ring finger 1	GAACTCGGCCTATCGCT	39	TCGAATTTCTCTCCTCCCAT	96

Table 1A					
Gene symbol	**Gene name**	**Forward primer**	**SEQ ID**	**Reverse primer**	**SEQ ID**
MYBPH	myosin binding protein H	TCTGACCTCATC ATCGGCAA	40	CTGAGTCCACAC AGGTTT	97
MYO7A	myosin VIIA	GAGGTGAAGCA AACTACGGA	41	CCCATACTTGTTG ATGGCAATTA	98
NFIL3	nuclear factor, interleukin 3 regulated	ACTCTCCACAA AGCTCG	42	TCCTGCGTGTGTT CTACT	99
PIK3C2A	phosphoinositide-3-kinase, class 2, alpha polypeptide	GGATTTCAGCT ACCAGTTACTT	43	AGTCATCATGTAC CCAGCA	100
PLEKHA6	pleckstrin homology domain containing, family A member 6	TTCGTCCTGGTG GATCG	44	CCCAGGATACTCT CTTCCTT	101
PSMD14	proteasome (prosome, macropain) 26S subunit, non-ATPase, 14	AGTGATTGATG TGTTTGCTATG	45	CACTGGATCAAC TGCCTC	102
SCD5	stearoyl-CoA desaturase 5	CAAAGCCAAGC CACTCACTC	46	CAGCTGTCACAC CCAGAGC	103
SIAH2	seven in absentia homolog 2 (*Drosophila*)	CTCGGCAGTCC TGTTTC	47	CGTATGGTGCAG GGTCA	104
TCF2	transcription factor 2, hepatic; LF-B3; variant hepatic nuclear factor	ACACCTGGTAC GTCAGAA	48	TCTGGACTGTCTG GTTGAAT	105
TCP1	t-complex 1	ATGCCCAAGAG AATCGTAAA	49	CCTGTACACCAA GCTTCAT	106
TTF1	thyroid transcription factor 1	ATGAGTCCAAA GCACACGA	50	CCATGCCCACTTT CTTGTA	107
TRIM29	tripartite motif-containing 29	TGAGATTGAGG ATGAAGCTGAG	51	CATTGGTGGTGA AGCTCTTG	108
TUBA1	tubulin, alpha 1	CCGACTCAACG TGAGAC	52	CGTGGACTGAGA TGCATT	109
CFL1	cofilin 1 (non-muscle)	GTGCCCTCTCCT TTTCG	53	TTCATGTCGTTGA ACACCTTG	110
EEF1A1	eukaryotic translation elongation factor 1 alpha 1	CGTTCTTTTTCG CAACGG	54	CATTTTGGCTTTT AGGGGTAG	111
RPL10	ribosomal protein L10	GGTGTGCCACT GAAGAT	55	GGCAGAAGCGAG ACTTT	112
RPL28	ribosomal protein L28	GTGTCGTGGTG GTCATT	56	GCACATAGGAGG TGGCA	113
RPL37A	ribosomal protein L37a	GCATGAAGACA GTGGCT	57	GCGGACTTTACC GTGAC	114

Table 1B					
Gene symbol	**Gene name**	**Forward primer**	**SEQ ID**	**Reverse primer**	**SEQ ID**
CDH5	cadherin 5, type 2, VE-cadherin (vascular epithelium)	AAGAGAGATTG GATTTGGAACC	1	TTCTTGCGACTCACGCT	58
CLEC3B	C-type lectin domain family 3, member B	CCAGAAGCCCA AGAAGATTGTA	2	GCTCCTCAAACAT CTTTGTGTTCA	59
PAICS	phosphoribosylaminoimidazole carboxylase, phosphoribosylaminoimidazole succinocarboxamide synthetase	AATCCTGGTGT CAAGGAAG	3	GACCACTGTGGG TCATTATT	60
PAK1	p21/Cdc42/Rac1-activated kinase 1 (STE20 homolog, yeast)	GGACCGATTTT ACCGATCC	4	GAAATCTCTGGC CGCTC	61
PECAM1	platelet/endothelial cell adhesion molecule (CD31 antigen)	ACAGTCCAGAT AGTCGTATGT	5	ACTGGGCATCAT AAGAAATCC	62
TFAP2A	transcription factor AP-2 alpha (activating enhancer binding protein 2 alpha)	GTCTCCGCCATC CCTAT	6	ACTGAACAGAAG ACTTCGT	63
ACVR1	activin A receptor, type 1	ACTGGTGTAAC AGGAACAT	7	AACCTCCAAGTG GAAATTCT	64
CDKN2C	cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)	TTTGGAAGGAC TGCGCT	8	TCGGTCTTTCAAA TCGGGATTA	65
CIB1	calcium and integrin binding 1 (calmyrin)	CACGTCATCTCC CGTTC	9	CTGCTGTCACAG GACAAT	66 66
INSM1	insulinoma-associated 1	ATTGAACTTCCC ACACGA	10	AAGGTAAAGCCA GACTCCA	67 67
LRP10	low density lipoprotein receptor-related protein 10	GGAACAGACTG TCACCAT	11	GGGAGCGTAGGG TTAAG	68
STMN1	stathmin 1/oncoprotein 18	TCAGAGTGTGTG G TCAGGC	12	CAGTGTATTCTGC ACAATCAAC	69
CAPG	capping protein (actin filament), gelsolin-like	GGGACAGCTTC AACACT	13	GTTCCAGGATGTT GGACTTTC	70
CHGA	chromogranin A (parathyroid secretory protein 1)	CCTGTGAACAG CCCTATG	14	GGAAAGTGTGTC GGAGAT	71
LGALS3	lectin, galactoside-binding, soluble, 3 (galectin 3)	TTCTGGGCACG GTGAAG	15	AGGCAACATCAT TCCCTC	72

Table 1B					
Gene symbol	**Gene name**	**Forward primer**	**SEQ ID**	**Reverse primer**	**SEQ ID**
MAPRE3	microtubule-associated protein, RP/EB family, member 3	GGCCAAACTAG AGCACGAATA	16	GTCAACACCCAT CTTCTTGAAA	73
SFN	stratifin	TCAGCAAGAAG GAGATGCC	17	CGTAGTGGAAGA CGGAAA	74
SNAP91	synaptosomal-associated protein, 91 kDa homolog (mouse)	GTGCTCCCTCTC CATTAAGTA	18	CTGGTGTAGAATT AGGAGACGTA	75
ABCC5	ATP-binding cassette, sub-family C(CFTR/MRP), member 5	CAAGTTCAGGA GAACTCGAC	19	GGCATCAAGAGA GAGGC	76
ALDH3B1	aldehyde dehydrogenase 3 family, member B1	GGCTGTGGTTA TGCGATAG	20	GATAAAGAGTTA CAAGCTCCTCTG	77
ANTXR1	Anthrax toxin receptor 1	ACCCGAGGAAC AACCTTA	21	TCTAGGCCTTGAC GGAT	78
CACNB1	calcium channel, voltage-dependent, beta 1 subunit	CAGAGCGCCAG GCATTA	23	GCACAGCAAATG CCACT	80
CBX1	chromobox homolog 1 (HP1 beta homolog *Drosophila*)	CCACTGGCTGA GGTGTTA	24	CTTGTCTTTCCCT ACTGTCTTAC	81
CYB5B	cytochrome b5 type B (outer mitochondrial membrane)	TGGGCGAGTCT ACGATG	25	CTTGTTCCAGCAG AACCT	82
DOK1	docking protein 1, 62 kDa (downstream of tyrosine kinase 1)	CTTTCTGCCCTG GAGATG	26	CAGTCCTCTGCAC CGTTA	83
DSC3	desmocollin 3	GCGCCATTTGCT AGAGATA	27	CATCCAGATCCCT CACAT	84
FEN1	flap structure-specific endonuclease 1	AGAGAAGATGG GCAGAAAG	28	CCAAGACACAGC CAGTAAT	85
FOXH1	forkhead box H1	GCCCAGATCAT CCGTCA	29	TTTCCAGCCCTCG TAGTC	86
GJB5	gap junction protein, beta 5 (connexin 31.1)	ACCACAAGGAC TTCGAC	30	GGGACACAGGGA AGAAC	87
HOXD1	homeobox D1	GCTCCGCTGCT ATCTTT	31	GTCTGCCACTCTG CAAC	88
HPN	Hepsin (transmembrane protease, serine 1)	AGCGGCCAGGT GGATTA	32	GTCGGCTGACGC TTTGA	89

Table 1B					
Gene symbol	**Gene name**	**Forward primer**	**SEQ ID**	**Reverse primer**	**SEQ ID**
HYAL2	hyaluronoglucosaminidase 2	ATGGGCTTTGGGAGCATA	33	GAACAAGTCAGTCTAGGGAATAC	90
ICA1	islet cell autoantigen 1, 69 kDa	GACCTGGATGCCAAGCTA	34	TGCTTTCGATAAGTCCAGACA	91
ICAM5	intercellular adhesion molecule 5, telencephalin	CCGGCTCTTGGAAGTTG	35	CCTCTGAGGCTGGAAACA	92
ITGA6	integrin, alpha 6	ACGCGGATCGAGTTTGATAA	36	ATCCACTGATCTTCCTTGC	93
LIPE	lipase, hormone-sensitive	CGCAAGTCCCAGAAGAT	37	CAGTGCTGCTTCAGACACA	94
ME3	malic enzyme 3, NADP(+)-dependent, Mitochondrial	CGCGGATACGATGTCAC	38	CCTTTCTTCAAGGGTAAAGGC	95
MGRN1	mahogunin, ring finger 1	GAACTCGGCCTATCGCT	39	TCGAATTTCTCTCCTCCCAT	96
MYBPH	myosin binding protein H	TCTGACCTCATCATCGGCAA	40	CTGAGTCCACACAGGTTT	97
MYO7A	myosin VIIA	GAGGTGAAGCAAACTACGGA	41	CCCATACTTGTTGATGGCAATTA	98
NFIL3	nuclear factor, interleukin 3 regulated	ACTCTCCACAAAGCTCG	42	TCCTGCGTGTGTTCTACT	99
PIK3C2A	phosphoinositide-3-kinase, class 2, alpha polypeptide	GGATTTCAGCTACCAGTTACTT	43	AGTCATCATGTACCCAGCA	100
PLEKHA6	pleckstrin homology domain containing, family A member 6	TTCGTCCTGGTGGATCG	44	CCCAGGATACTCTCTTCCTT	101
PSMD14	proteasome (prosome, macropain) 26S subunit, non-ATPase, 14	AGTGATTGATGTGTTTGCTATG	45	CACTGGATCAACTGCCTC	102
SCD5	stearoyl-CoA desaturase 5	CAAAGCCAAGCCACTCACTC	46	CAGCTGTCACACCCAGAGC	103
SIAH2	seven in absentia homolog 2 (*Drosophila*)	CTCGGCAGTCCTGTTTC	47	CGTATGGTGCAGGGTCA	104
TCF2	transcription factor 2, hepatic; LF-B3; variant hepatic nuclear factor	ACACCTGGTACGTCAGAA	48	TCTGGACTGTCTGGTTGAAT	105
TCP1	t-complex 1	ATGCCCAAGAGAATCGTAAA	49	CCTGTACACCAAGCTTCAT	106
TTF1	thyroid transcription factor 1	ATGAGTCCAAAGCACACGA	50	CCATGCCCACTTTCTTGTA	107
TRIM29	tripartite motif-containing 29	TGAGATTGAGGATGAAGCTGAG	51	CATTGGTGGTGAAGCTCTTG	108

Table 1B					
Gene symbol	**Gene name**	**Forward primer**	**SEQ ID**	**Reverse primer**	**SEQ ID**
TUBA1	tubulin, alpha 1	CCGACTCAACG TGAGAC	52	CGTGGACTGAGA TGCATT	109
CFL1	cofilin 1 (non-muscle)	GTGCCCTCTCCT TTTCG	53	TTCATGTCGTTGA ACACCTTG	110
EEF1A1	eukaryotic translation elongation factor 1 alpha 1	CGTTCTTTTTCG CAACGG	54	CATTTTGGCTTTT AGGGGTAG	111
RPL10	ribosomal protein L10	GGTGTGCCACT GAAGAT	55	GGCAGAAGCGAG ACTTT	112
RPL28	ribosomal protein L28	GTGTCGTGGTG GTCATT	56	GCACATAGGAGG TGGCA	113
RPL37A	ribosomal protein L37a	GCATGAAGACA GTGGCT	57	GCGGACTTTACC GTGAC	114

Table 1C					
Gene symbol	**Gene name**	**Forward primer**	**SEQ ID**	**Reverse primer**	**SEQ ID**
CDH5	cadherin 5, type 2, VE-cadherin (vascular epithelium)	AAGAGAGATTG GATTTGGAACC	1	TTCTTGCGACTCACGCT	58
CLEC3B	C-type lectin domain family 3, member B	CCAGAAGCCCA AGAAGATTGTA	2	GCTCCTCAAACAT CTTTGTGTTCA	59
PAICS	phosphoribosylaminoimidazole carboxylase, phosphoribosylaminoimidazole succinocarboxamide synthetase	AATCCTGGTGT CAAGGAAG	3	GACCACTGTGGG TCATTATT	60
PAK1	p21/Cdc42/Rac1-activated kinase 1 (STE20 homolog, yeast)	GGACCGATTTT ACCGATCC	4	GAAATCTCTGGC CGCTC	61
PECAM1	platelet/endothelial cell adhesion molecule (CD31 antigen)	ACAGTCCAGAT AGTCGTATGT	5	ACTGGGCATCAT AAGAAATCC	62
TFAP2A	transcription factor AP-2 alpha (activating enhancer binding protein 2 alpha)	GTCTCCGCCATC CCTAT	6	ACTGAACAGAAG ACTTCGT	63
ACVR1	activin A receptor, type 1	ACTGGTGTAAC AGGAACAT	7	AACCTCCAAGTG GAAATTCT	64
CDKN2C	cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)	TTTGGAAGGAC TGCGCT	8	TCGGTCTTTCAAA TCGGGATTA	65

Table 1C					
Gene symbol	Gene name	Forward primer	SEQ ID	Reverse primer	SEQ ID
CIB1	calcium and integrin binding 1 (calmyrin)	CACGTCATCTCC CGTTC	9	CTGCTGTCACAG GACAAT	66 66
INSM1	insulinoma-associated 1	ATTGAACTTCCC ACACGA	10	AAGGTAAAGCCA GACTCCA	67 67
LRP10	low density lipoprotein receptor-related protein 10	GGAACAGACTG TCACCAT	11	GGGAGCGTAGGG TTAAG	68
STMN1	stathmin 1/oncoprotein 18	TCAGAGTGTGTG G TCAGGC	12	CAGTGTATTCTGC ACAATCAAC	69
CAPG	capping protein (actin filament), gelsolin-like	GGGACAGCTTC AACACT	13	GTTCCAGGATGTT GGACTTTC	70
CHGA	chromogranin A (parathyroid secretory protein 1)	CCTGTGAACAG CCCTATG	14	GGAAAGTGTGTC GGAGAT	71
LGALS3	lectin, galactoside-binding, soluble, 3 (galectin 3)	TTCTGGGCACG GTGAAG	15	AGGCAACATCAT TCCCTC	72
MAPRE3	microtubule-associated protein, RP/EB family, member 3	GGCCAAACTAG AGCACGAATA	16	GTCAACACCCAT CTTCTTGAAA	73
SFN	stratifin	TCAGCAAGAAG GAGATGCC	17	CGTAGTGGAAGA CGGAAA	74
SNAP91	synaptosomal-associated protein, 91 kDa homolog (mouse)	GTGCTCCCTCTC CATTAAGTA	18	CTGGTGTAGAATT AGGAGACGTA	75
ABCC5	ATP-binding cassette, sub-family C(CFTR/MRP), member 5	CAAGTTCAGGA GAACTCGAC	19	GGCATCAAGAGA GAGGC	76
ALDH3B1	aldehyde dehydrogenase 3 family, member B1	GGCTGTGGTTA TGCGATAG	20	GATAAAGAGTTA CAAGCTCCTCTG	77
ANTXR1	Anthrax toxin receptor 1	ACCCGAGGAAC AACCTTA	21	TCTAGGCCTTGAC GGAT	78
BMP7	Bone morphogenetic protein 7 (osteogenic protein 1)	CCCTCTCCATTCC CTACA	22	TTTGGGCAAACCTCGGTA A	79
CACNB1	calcium channel, voltage-dependent, beta 1 subunit	CAGAGCGCCAG GCATTA	23	GCACAGCAAATG CCACT	80

Table 1C					
Gene symbol	**Gene name**	**Forward primer**	**SEQ ID**	**Reverse primer**	**SEQ ID**
CBX1	chromobox homolog 1 (HP1 beta homolog *Drosophila*)	CCACTGGCTGA GGTGTTA	24	CTTGTCTTTCCCT ACTGTCTTAC	81
CYB5B	cytochrome b5 type B (outer mitochondrial membrane)	TGGGCGAGTCT ACGATG	25	CTTGTTCCAGCAG AACCT	82
DOK1	docking protein 1, 62 kDa (downstream of tyrosine kinase 1)	CTTTCTGCCCTG GAGATG	26	CAGTCCTCTGCAC CGTTA	83
DSC3	desmocollin 3	GCGCCATTTGCT AGAGATA	27	CATCCAGATCCCT CACAT	84
FEN1	flap structure-specific endonuclease 1	AGAGAAGATGG GCAGAAAG	28	CCAAGACACAGC CAGTAAT	85
FOXH1	forkhead box H1	GCCCAGATCAT CCGTCA	29	TTTCCAGCCCTCG TAGTC	86
GJB5	gap junction protein, beta 5 (connexin 31.1)	ACCACAAGGAC TTCGAC	30	GGGACACAGGGA AGAAC	87
HOXD1	homeobox D1	GCTCCGCTGCT ATCTTT	31	GTCTGCCACTCTG CAAC	88
HPN	Hepsin (transmembrane protease, serine 1)	AGCGGCCAGGT GGATTA	32	GTCGGCTGACGC TTTGA	89
HYAL2	hyaluronoglucosam inidase 2	ATGGGCTTTGG GAGCATA	33	GAACAAGTCAGT CTAGGGAATAC	90
ICA1	islet cell autoantigen 1, 69 kDa	GACCTGGATGC CAAGCTA	34	TGCTTTCGATAAG TCCAGACA	91
ICAM5	intercellular adhesion molecule 5, telencephalin	CCGGCTCTTGG AAGTTG	35	CCTCTGAGGCTG GAAACA	92
ITGA6	integrin, alpha 6	ACGCGGATCGA GTTTGATAA	36	ATCCACTGATCTT CCTTGC	93
LIPE	lipase, hormone-sensitive	CGCAAGTCCCA GAAGAT	37	CAGTGCTGCTTCA GACACA	94
ME3	malic enzyme 3, NADP(+)-dependent, Mitochondrial	CGCGGATACGA TGTCAC	38	CCTTTCTTCAAGG GTAAAGGC	95
MGRN1	mahogunin, ring finger 1	GAACTCGGCCT ATCGCT	39	TCGAATTTCTCTC CTCCCAT	96
MYBPH	myosin binding protein H	TCTGACCTCATC ATCGGCAA	40	CTGAGTCCACAC AGGTTT	97
MYO7A	myosin VIIA	GAGGTGAAGCA AACTACGGA	41	CCCATACTTGTTG ATGGCAATTA	98
NFIL3	nuclear factor, interleukin 3 regulated	ACTCTCCACAA AGCTCG	42	TCCTGCGTGTGTT CTACT	99

Table 1C					
Gene symbol	**Gene name**	**Forward primer**	**SEQ ID**	**Reverse primer**	**SEQ ID**
PIK3C2A	phosphoinositide-3-kinase, class 2, alpha polypeptide	GGATTTCAGCT ACCAGTTACTT	43	AGTCATCATGTAC CCAGCA	100
PLEKHA6	pleckstrin homology domain containing, family A member 6	TTCGTCCTGGTG GATCG	44	CCCAGGATACTCT CTTCCTT	101
PSMD14	proteasome (prosome, macropain) 26S subunit, non-ATPase, 14	AGTGATTGATG TGTTTGCTATG	45	CACTGGATCAAC TGCCTC	102
SCD5	stearoyl-CoA desaturase 5	CAAAGCCAAGC CACTCACTC	46	CAGCTGTCACAC CCAGAGC	103
SIAH2	seven in absentia homolog 2 (*Drosophila*)	CTCGGCAGTCC TGTTTC	47	CGTATGGTGCAG GGTCA	104
TCF2	transcription factor 2, hepatic; LF-B3; variant hepatic nuclear factor	ACACCTGGTAC GTCAGAA	48	TCTGGACTGTCTG GTTGAAT	105
TCP1	t-complex 1	ATGCCCAAGAG AATCGTAAA	49	CCTGTACACCAA GCTTCAT	106
TTF1	thyroid transcription factor 1	ATGAGTCCAAA GCACACGA	50	CCATGCCCACTTT CTTGTA	107
TRIM29	tripartite motif-containing 29	TGAGATTGAGG ATGAAGCTGAG	51	CATTGGTGGTGA AGCTCTTG	108
TUBA1	tubulin, alpha 1	CCGACTCAACG TGAGAC	52	CGTGGACTGAGA TGCATT	109

Table 2					
Gene symbol	**Gene name**	**Forward primer**	**SEQ ID**	**Reverse primer**	**SEQ ID**
CDH5	cadherin 5, type 2, VE-cadherin (vascular epithelium)	AAGAGAGATTG GATTTGGAACC	1	TTCTTGCGACTCACGCT	58
PAICS	phosphoribosylaminoimidazole carboxylase, phosphoribosylaminoimidazole succinocarboxamide synthetase	AATCCTGGTGT CAAGGAAG	3	GACCACTGTGGG TCATTATT	60
PAK1	p21/Cdc42/Rac1-activated kinase 1 (STE20 homolog, yeast)	GGACCGATTTT ACCGATCC	4	GAAATCTCTGGC CGCTC	61
PECAM1	platelet/endothelial cell adhesion molecule (CD31 antigen)	ACAGTCCAGAT AGTCGTATGT	5	ACTGGGCATCAT AAGAAATCC	62

Table 2					
Gene symbol	Gene name	Forward primer	SEQ ID	Reverse primer	SEQ ID
TFAP2A	transcription factor AP-2 alpha (activating enhancer binding protein 2 alpha)	GTCTCCGCCATC CCTAT	6	ACTGAACAGAAG ACTTCGT	63
ACVR1	activin A receptor, type 1	ACTGGTGTAAC AGGAACAT	7	AACCTCCAAGTG GAAATTCT	64
CDKN2C	cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)	TTTGGAAGGAC TGCGCT	8	TCGGTCTTTCAAA TCGGGATTA	65
CIB1	calcium and integrin binding 1 (calmyrin)	CACGTCATCTCC CGTTC	9	CTGCTGTCACAG GACAAT	66 66
INSM1	insulinoma-associated 1	ATTGAACTTCCC ACACGA	10	AAGGTAAAGCCA GACTCCA	67 67
LRP10	low density lipoprotein receptor-related protein 10	GGAACAGACTG TCACCAT	11	GGGAGCGTAGGG TTAAG	68
STMN1	stathmin 1/oncoprotein 18	TCAGAGTGTGTG G TCAGGC	12	CAGTGTATTCTGC ACAATCAAC	69
CAPG	capping protein (actin filament), gelsolin-like	GGGACAGCTTC AACACT	13	GTTCCAGGATGTT GGACTTTC	70
CHGA	chromogranin A (parathyroid secretory protein 1)	CCTGTGAACAG CCCTATG	14	GGAAAGTGTGTC GGAGAT	71
LGALS3	lectin, galactoside-binding, soluble, 3 (galectin 3)	TTCTGGGCACG GTGAAG	15	AGGCAACATCAT TCCCTC	72
MAPRE3	microtubule-associated protein, RP/EB family, member 3	GGCCAAACTAG AGCACGAATA	16	GTCAACACCCAT CTTCTTGAAA	73
SFN	stratifin	TCAGCAAGAAG GAGATGCC	17	CGTAGTGGAAGA CGGAAA	74
SNAP91	synaptosomal-associated protein, 91 kDa homolog (mouse)	GTGCTCCCTCTC CATTAAGTA	18	CTGGTGTAGAATT AGGAGACGTA	75
ABCC5	ATP-binding cassette, sub-family C(CFTR/MRP), member 5	CAAGTTCAGGA GAACTCGAC	19	GGCATCAAGAGA GAGGC	76
ALDH3B1	aldehyde dehydrogenase 3 family, member B1	GGCTGTGGTTA TGCGATAG	20	GATAAAGAGTTA CAAGCTCCTCTG	77
ANTXR1	Anthrax toxin receptor 1	ACCCGAGGAAC AACCTTA	21	TCTAGGCCTTGAC GGAT	78

Table 2					
Gene symbol	Gene name	Forward primer	SEQ ID	Reverse primer	SEQ ID
CACNB1	calcium channel, voltage-dependent, beta 1 subunit	CAGAGCGCCAG GCATTA	23	GCACAGCAAATG CCACT	80
CBX1	chromobox homolog 1 (HP1 beta homolog *Drosophila*)	CCACTGGCTGA GGTGTTA	24	CTTGTCTTTCCCT ACTGTCTTAC	81
CYB5B	cytochrome b5 type B (outer mitochondrial membrane)	TGGGCGAGTCT ACGATG	25	CTTGTTCCAGCAG AACCT	82
DOK1	docking protein 1, 62 kDa (downstream of tyrosine kinase 1)	CTTTCTGCCCTG GAGATG	26	CAGTCCTCTGCAC CGTTA	83
DSC3	desmocollin 3	GCGCCATTTGCT AGAGATA	27	CATCCAGATCCCT CACAT	84
FEN1	flap structure-specific endonuclease 1	AGAGAAGATGG GCAGAAAG	28	CCAAGACACAGC CAGTAAT	85
FOXH1	forkhead box H1	GCCCAGATCAT CCGTCA	29	TTTCCAGCCCTCG TAGTC	86
GJB5	gap junction protein, beta 5 (connexin 31.1)	ACCACAAGGAC TTCGAC	30	GGGACACAGGGA AGAAC	87
HOXD1	homeobox D1	GCTCCGCTGCT ATCTTT	31	GTCTGCCACTCTG CAAC	88
HPN	Hepsin (transmembrane protease, serine 1)	AGCGGCCAGGT GGATTA	32	GTCGGCTGACGC TTTGA	89
HYAL2	hyaluronoglucosam inidase 2	ATGGGCTTTGG GAGCATA	33	GAACAAGTCAGT CTAGGGAATAC	90
ICA1	islet cell autoantigen 1, 69 kDa	GACCTGGATGC CAAGCTA	34	TGCTTTCGATAAG TCCAGACA	91
ICAM5	intercellular adhesion molecule 5, telencephalin	CCGGCTCTTGG AAGTTG	35	CCTCTGAGGCTG GAAACA	92
ITGA6	integrin, alpha 6	ACGCGGATCGA GTTTGATAA	36	ATCCACTGATCTT CCTTGC	93
LIPE	lipase, hormone-sensitive	CGCAAGTCCCA GAAGAT	37	CAGTGCTGCTTCA GACACA	94
ME3	malic enzyme 3, NADP(+)-dependent, Mitochondrial	CGCGGATACGA TGTCAC	38	CCTTTCTTCAAGG GTAAAGGC	95
MGRN1	mahogunin, ring finger 1	GAACTCGGCCT ATCGCT	39	TCGAATTTCTCTC CTCCCAT	96
MYBPH	myosin binding protein H	TCTGACCTCATC ATCGGCAA	40	CTGAGTCCACAC AGGTTT	97
MYO7A	myosin VIIA	GAGGTGAAGCA AACTACGGA	41	CCCATACTTGTTG ATGGCAATTA	98

Table 2					
Gene symbol	Gene name	Forward primer	SEQ ID	Reverse primer	SEQ ID
NFIL3	nuclear factor, interleukin 3 regulated	ACTCTCCACAA AGCTCG	42	TCCTGCGTGTGTT CTACT	99
PIK3C2A	phosphoinositide-3-kinase, class 2, alpha polypeptide	GGATTTCAGCT ACCAGTTACTT	43	AGTCATCATGTAC CCAGCA	100
PLEKHA6	pleckstrin homology domain containing, family A member 6	TTCGTCCTGGTG GATCG	44	CCCAGGATACTCT CTTCCTT	101
PSMD14	proteasome (prosome, macropain) 26S subunit, non-ATPase, 14	AGTGATTGATG TGTTTGCTATG	45	CACTGGATCAAC TGCCTC	102
SCD5	stearoyl-CoA desaturase 5	CAAAGCCAAGC CACTCACTC	46	CAGCTGTCACAC CCAGAGC	103
SIAH2	seven in absentia homolog 2 (*Drosophila*)	CTCGGCAGTCC TGTTTC	47	CGTATGGTGCAG GGTCA	104
TCF2	transcription factor 2, hepatic; LF-B3; variant hepatic nuclear factor	ACACCTGGTAC GTCAGAA	48	TCTGGACTGTCTG GTTGAAT	105
TTF1	thyroid transcription factor 1	ATGAGTCCAAA GCACACGA	50	CCATGCCCACTTT CTTGTA	107
TRIM29	tripartite motif-containing 29	TGAGATTGAGG ATGAAGCTGAG	51	CATTGGTGGTGA AGCTCTTG	108
TUBA1	tubulin, alpha 1	CCGACTCAACG TGAGAC	52	CGTGGACTGAGA TGCATT	109
CFL1	cofilin 1 (non-muscle)	GTGCCCTCTCCT TTTCG	53	TTCATGTCGTTGA ACACCTTG	110
EEF1A1	eukaryotic translation elongation factor 1 alpha 1	CGTTCTTTTTCG CAACGG	54	CATTTTGGCTTTT AGGGGTAG	111
RPL10	ribosomal protein L10	GGTGTGCCACT GAAGAT	55	GGCAGAAGCGAG ACTTT	112
RPL28	ribosomal protein L28	GTGTCGTGGTG GTCATT	56	GCACATAGGAGG TGGCA	113
RPL37A	ribosomal protein L37a	GCATGAAGACA GTGGCT	57	GCGGACTTTACC GTGAC	114

Table 3					
Gene symbol	Gene name	Forward primer	SEQ ID	Reverse primer	SEQ ID
CDH5	cadherin 5, type 2, VE-cadherin (vascular epithelium)	AAGAGAGATTG GATTTGGAACC	1	TTCTTGCGACTCACGCT	58

Table 3					
Gene symbol	Gene name	Forward primer	SEQ ID	Reverse primer	SEQ ID
CLEC3B	C-type lectin domain family 3, member B	CCAGAAGCCCA AGAAGATTGTA	2	GCTCCTCAAACAT CTTTGTGTTCA	59
PAICS	phosphoribosylaminoimidazole carboxylase, phosphoribosylaminoimidazole succinocarboxamide synthetase	AATCCTGGTGT CAAGGAAG	3	GACCACTGTGGG TCATTATT	60
PAK1	p21/Cdc42/Rac1-activated kinase 1 (STE20 homolog, yeast)	GGACCGATTTT ACCGATCC	4	GAAATCTCTGGC CGCTC	61
TFAP2A	transcription factor AP-2 alpha (activating enhancer binding protein 2 alpha)	GTCTCCGCCATC CCTAT	6	ACTGAACAGAAG ACTTCGT	63
ACVR1	activin A receptor, type 1	ACTGGTGTAAC AGGAACAT	7	AACCTCCAAGTG GAAATTCT	64
CDKN2C	cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)	TTTGGAAGGAC TGCGCT	8	TCGGTCTTTCAAA TCGGGATTA	65
INSM1	insulinoma-associated 1	ATTGAACTTCCC ACACGA	10	AAGGTAAAGCCA GACTCCA	67 67
LRP10	low density lipoprotein receptor-related protein 10	GGAACAGACTG TCACCAT	11	GGGAGCGTAGGG TTAAG	68
STMN1	stathmin 1/oncoprotein 18	TCAGAGTGTGTG G TCAGGC	12	CAGTGTATTCTGC ACAATCAAC	69
CAPG	capping protein (actin filament), gelsolin-like	GGGACAGCTTC AACACT	13	GTTCCAGGATGTT GGACTTTC	70
CHGA	chromogranin A (parathyroid secretory protein 1)	CCTGTGAACAG CCCTATG	14	GGAAAGTGTGTC GGAGAT	71
LGALS3	lectin, galactoside-binding, soluble, 3 (galectin 3)	TTCTGGGCACG GTGAAG	15	AGGCAACATCAT TCCCTC	72
MAPRE3	microtubule-associated protein, RP/EB family, member 3	GGCCAAACTAG AGCACGAATA	16	GTCAACACCCAT CTTCTTGAAA	73
SFN	stratifin	TCAGCAAGAAG GAGATGCC	17	CGTAGTGGAAGA CGGAAA	74
SNAP91	synaptosomal-associated protein, 91 kDa homolog (mouse)	GTGCTCCCTCTC CATTAAGTA	18	CTGGTGTAGAATT AGGAGACGTA	75

Table 3					
Gene symbol	Gene name	Forward primer	SEQ ID	Reverse primer	SEQ ID
ABCC5	ATP-binding cassette, sub-family C(CFTR/MRP), member 5	CAAGTTCAGGA GAACTCGAC	19	GGCATCAAGAGA GAGGC	76
ALDH3B1	aldehyde dehydrogenase 3 family, member B1	GGCTGTGGTTA TGCGATAG	20	GATAAAGAGTTA CAAGCTCCTCTG	77
ANTXR1	Anthrax toxin receptor 1	ACCCGAGGAAC AACCTTA	21	TCTAGGCCTTGAC GGAT	78
CACNB1	calcium channel, voltage-dependent, beta 1 subunit	CAGAGCGCCAG GCATTA	23	GCACAGCAAATG CCACT	80
CBX1	chromobox homolog 1 (HP1 beta homolog *Drosophila*)	CCACTGGCTGA GGTGTTA	24	CTTGTCTTTCCCT ACTGTCTTAC	81
CYB5B	cytochrome b5 type B (outer mitochondrial membrane)	TGGGCGAGTCT ACGATG	25	CTTGTTCCAGCAG AACCT	82
DOK1	docking protein 1, 62 kDa (downstream of tyrosine kinase 1)	CTTTCTGCCCTG GAGATG	26	CAGTCCTCTGCAC CGTTA	83
DSC3	desmocollin 3	GCGCCATTTGCT AGAGATA	27	CATCCAGATCCCT CACAT	84
FEN1	flap structure-specific endonuclease 1	AGAGAAGATGG GCAGAAAG	28	CCAAGACACAGC CAGTAAT	85
GJB5	gap junction protein, beta 5 (connexin 31.1)	ACCACAAGGAC TTCGAC	30	GGGACACAGGGA AGAAC	87
HOXD1	homeobox D1	GCTCCGCTGCT ATCTTT	31	GTCTGCCACTCTG CAAC	88
HPN	Hepsin (transmembrane protease, serine 1)	AGCGGCCAGGT GGATTA	32	GTCGGCTGACGC TTTGA	89
HYAL2	hyaluronoglucosam inidase 2	ATGGGCTTTGG GAGCATA	33	GAACAAGTCAGT CTAGGGAATAC	90
ICA1	islet cell autoantigen 1, 69 kDa	GACCTGGATGC CAAGCTA	34	TGCTTTCGATAAG TCCAGACA	91
ICAM5	intercellular adhesion molecule 5, telencephalin	CCGGCTCTTGG AAGTTG	35	CCTCTGAGGCTG GAAACA	92
ITGA6	integrin, alpha 6	ACGCGGATCGA GTTTGATAA	36	ATCCACTGATCTT CCTTGC	93
ME3	malic enzyme 3, NADP(+)-dependent, Mitochondrial	CGCGGATACGA TGTCAC	38	CCTTTCTTCAAGG GTAAAGGC	95

Table 3					
Gene symbol	Gene name	Forward primer	SEQ ID	Reverse primer	SEQ ID
MGRN1	mahogunin, ring finger 1	GAACTCGGCCT ATCGCT	39	TCGAATTTCTCTC CTCCCAT	96
MYBPH	myosin binding protein H	TCTGACCTCATC ATCGGCAA	40	CTGAGTCCACAC AGGTTT	97
MYO7A	myosin VIIA	GAGGTGAAGCA AACTACGGA	41	CCCATACTTGTTG ATGGCAATTA	98
NFIL3	nuclear factor, interleukin 3 regulated	ACTCTCCACAA AGCTCG	42	TCCTGCGTGTGTT CTACT	99
PIK3C2A	phosphoinositide-3-kinase, class 2, alpha polypeptide	GGATTTCAGCT ACCAGTTACTT	43	AGTCATCATGTAC CCAGCA	100
PLEKHA6	pleckstrin homology domain containing, family A member 6	TTCGTCCTGGTG GATCG	44	CCCAGGATACTCT CTTCCTT	101
PSMD14	proteasome (prosome, macropain) 26S subunit, non-ATPase, 14	AGTGATTGATG TGTTTGCTATG	45	CACTGGATCAAC TGCCTC	102
SCD5	stearoyl-CoA desaturase 5	CAAAGCCAAGC CACTCACTC	46	CAGCTGTCACAC CCAGAGC	103
SIAH2	seven in absentia homolog 2 (*Drosophila*)	CTCGGCAGTCC TGTTTC	47	CGTATGGTGCAG GGTCA	104
TCF2	transcription factor 2, hepatic; LF-B3; variant hepatic nuclear factor	ACACCTGGTAC GTCAGAA	48	TCTGGACTGTCTG GTTGAAT	105
TCP1	t-complex 1	ATGCCCAAGAG AATCGTAAA	49	CCTGTACACCAA GCTTCAT	106
TTF1	thyroid transcription factor 1	ATGAGTCCAAA GCACACGA	50	CCATGCCCACTTT CTTGTA	107
TRIM29	tripartite motif-containing 29	TGAGATTGAGG ATGAAGCTGAG	51	CATTGGTGGTGA AGCTCTTG	108
CFL1	cofilin 1 (non-muscle)	GTGCCCTCTCCT TTTCG	53	TTCATGTCGTTGA ACACCTTG	110
EEF1A1	eukaryotic translation elongation factor 1 alpha 1	CGTTCTTTTTCG CAACGG	54	CATTTTGGCTTTT AGGGGTAG	111
RPL10	ribosomal protein L10	GGTGTGCCACT GAAGAT	55	GGCAGAAGCGAG ACTTT	112
RPL28	ribosomal protein L28	GTGTCGTGGTG GTCATT	56	GCACATAGGAGG TGGCA	113
RPL37A	ribosomal protein L37a	GCATGAAGACA GTGGCT	57	GCGGACTTTACC GTGAC	114

Table 4

Gene symbol	Gene name	Forward primer	SEQ ID	Reverse primer	SEQ ID
ACVR1	activin A receptor, type 1	ACTGGTGTAAC AGGAACAT	7	AACCTCCAAGTG GAAATTCT	64
CDKN2C	cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)	TTTGGAAGGAC TGCGCT	8	TCGGTCTTTCAAA TCGGGATTA	65
CIB1	calcium and integrin binding 1 (calmyrin)	CACGTCATCTCC CGTTC	9	CTGCTGTCACAG GACAAT	66 66
INSM1	insulinoma-associated 1	ATTGAACTTCCC ACACGA	10	AAGGTAAAGCCA GACTCCA	67 67
LRP10	low density lipoprotein receptor-related protein 10	GGAACAGACTG TCACCAT	11	GGGAGCGTAGGG TTAAG	68
STMN1	stathmin 1/oncoprotein 18	TCAGAGTGTGTG G TCAGGC	12	CAGTGTATTCTGC ACAATCAAC	69

Table 5					
Gene symbol	**Gene name**	**Forward primer**	**SEQ ID**	**Reverse primer**	**SEQ ID**
CAPG	capping protein (actin filament), gelsolin-like	GGGACAGCTTC AACACT	13	GTTCCAGGATGTT GGACTTTC	70
CHGA	chromogranin A (parathyroid secretory protein 1)	CCTGTGAACAG CCCTATG	14	GGAAAGTGTGTC GGAGAT	71
LGALS3	lectin, galactoside-binding, soluble, 3 (galectin 3)	TTCTGGGCACG GTGAAG	15	AGGCAACATCAT TCCCTC	72
MAPRE3	microtubule-associated protein, RP/EB family, member 3	GGCCAAACTAG AGCACGAATA	16	GTCAACACCCAT CTTCTTGAAA	73
SFN	stratifin	TCAGCAAGAAG GAGATGCC	17	CGTAGTGGAAGA CGGAAA	74
SNAP91	synaptosomal-associated protein, 91 kDa homolog (mouse)	GTGCTCCCTCTC CATTAAGTA	18	CTGGTGTAGAATT AGGAGACGTA	75

Table 6					
Gene symbol	**Gene name**	**Forward primer**	**SEQ ID**	**Reverse primer**	**SEQ ID**
ABCC5	ATP-binding cassette, sub-family C(CFTR/MRP), member 5	CAAGTTCAGGA GAACTCGAC	19	GGCATCAAGAGA GAGGC	76

Table 6					
Gene symbol	**Gene name**	**Forward primer**	**SEQ ID**	**Reverse primer**	**SEQ ID**
ALDH3B1	aldehyde dehydrogenase 3 family, member B1	GGCTGTGGTTA TGCGATAG	20	GATAAAGAGTTA CAAGCTCCTCTG	77
ANTXR1	Anthrax toxin receptor 1	ACCCGAGGAAC AACCTTA	21	TCTAGGCCTTGAC GGAT	78
BMP7	bone morphogenetic protein 7 (osteogenic protein 1)	CCCTCTCCATTC CCTACA	22	TTTGGGCAAACCT CGGTAA	79
CACNB1	calcium channel, voltage-dependent, beta 1 subunit	CAGAGCGCCAG GCATTA	23	GCACAGCAAATG CCACT	80
CBX1	chromobox homolog 1 (HP1 beta homolog *Drosophila*)	CCACTGGCTGA GGTGTTA	24	CTTGTCTTTCCCT ACTGTCTTAC	81
CYB5B	cytochrome b5 type B (outer mitochondrial membrane)	TGGGCGAGTCT ACGATG	25	CTTGTTCCAGCAG AACCT	82
DOK1	docking protein 1, 62 kDa (downstream of tyrosine kinase 1)	CTTTCTGCCCTG GAGATG	26	CAGTCCTCTGCAC CGTTA	83
DSC3	desmocollin 3	GCGCCATTTGCT AGAGATA	27	CATCCAGATCCCT CACAT	84
FEN1	flap structure-specific endonuclease 1	AGAGAAGATGG GCAGAAAG	28	CCAAGACACAGC CAGTAAT	85
FOXH1	forkhead box H1	GCCCAGATCAT CCGTCA	29	TTTCCAGCCCTCG TAGTC	86
GJB5	gap junction protein, beta 5 (connexin 31.1)	ACCACAAGGAC TTCGAC	30	GGGACACAGGGA AGAAC	87
HOXD1	homeobox D1	GCTCCGCTGCT ATCTTT	31	GTCTGCCACTCTG CAAC	88
HPN	Hepsin (transmembrane protease, serine 1)	AGCGGCCAGGT GGATTA	32	GTCGGCTGACGC TTTGA	89
HYAL2	hyaluronoglucosaminidase 2	ATGGGCTTTGG GAGCATA	33	GAACAAGTCAGT CTAGGGAATAC	90
ICA1	islet cell autoantigen 1, 69 kDa	GACCTGGATGC CAAGCTA	34	TGCTTTCGATAAG TCCAGACA	91
ICAM5	intercellular adhesion molecule 5, telencephalin	CCGGCTCTTGG AAGTTG	35	CCTCTGAGGCTG GAAACA	92
ITGA6	integrin, alpha 6	ACGCGGATCGA GTTTGATAA	36	ATCCACTGATCTT CCTTGC	93
LIPE	lipase, hormone-sensitive	CGCAAGTCCCA GAAGAT	37	CAGTGCTGCTTCA GACACA	94

Table 6					
Gene symbol	**Gene name**	**Forward primer**	**SEQ ID**	**Reverse primer**	**SEQ ID**
ME3	malic enzyme 3, NADP(+)-dependent, Mitochondrial	CGCGGATACGA TGTCAC	38	CCTTTCTTCAAGG GTAAAGGC	95
MGRN1	mahogunin, ring finger 1	GAACTCGGCCT ATCGCT	39	TCGAATTTCTCTC CTCCCAT	96
MYBPH	myosin binding protein H	TCTGACCTCATC ATCGGCAA	40	CTGAGTCCACAC AGGTTT	97
MYO7A	myosin VIIA	GAGGTGAAGCA AACTACGGA	41	CCCATACTTGTTG ATGGCAATTA	98
NFIL3	nuclear factor, interleukin 3 regulated	ACTCTCCACAA AGCTCG	42	TCCTGCGTGTGTT CTACT	99
PIK3C2A	phosphoinositide-3-kinase, class 2, alpha polypeptide	GGATTTCAGCT ACCAGTTACTT	43	AGTCATCATGTAC CCAGCA	100
PLEKHA6	pleckstrin homology domain containing, family A member 6	TTCGTCCTGGTG GATCG	44	CCCAGGATACTCT CTTCCTT	101
PSMD14	proteasome (prosome, macropain) 26S subunit, non-ATPase, 14	AGTGATTGATG TGTTTGCTATG	45	CACTGGATCAAC TGCCTC	102
SCD5	stearoyl-CoA desaturase 5	CAAAGCCAAGC CACTCACTC	46	CAGCTGTCACAC CCAGAGC	103
SIAH2	seven in absentia homolog 2 (*Drosophila*)	CTCGGCAGTCC TGTTTC	47	CGTATGGTGCAG GGTCA	104
TCF2	transcription factor 2, hepatic; LF-B3; variant hepatic nuclear factor	ACACCTGGTAC GTCAGAA	48	TCTGGACTGTCTG GTTGAAT	105
TCP1	t-complex 1	ATGCCCAAGAG AATCGTAAA	49	CCTGTACACCAA GCTTCAT	106
TTF1	thyroid transcription factor 1	ATGAGTCCAAA GCACACGA	50	CCATGCCCACTTT CTTGTA	107
TRIM29	tripartite motif-containing 29	TGAGATTGAGG ATGAAGCTGAG	51	CATTGGTGGTGA AGCTCTTG	108
TUBA1	tubulin, alpha 1	CCGACTCAACG TGAGAC	52	CGTGGACTGAGA TGCATT	109

[0035] Isolated mRNA can be used in hybridization or amplification assays that include, but are not limited to, Southern or Northern analyses, PCR analyses and probe arrays, NanoString Assays. One method for the detection of mRNA levels involves contacting the isolated mRNA or synthesized cDNA with a nucleic acid molecule (probe) that can hybridize to the

mRNA encoded by the gene being detected. The nucleic acid probe can be, for example, a cDNA, or a portion thereof, such as an oligonucleotide of at least 7, 15, 30, 50, 100, 250, or 500 nucleotides in length and sufficient to specifically hybridize under stringent conditions to the non-natural cDNA or mRNA biomarker of the present invention.

[0036] As explained above, in one embodiment, once the mRNA is obtained from a sample, it is converted to complementary DNA (cDNA) in a hybridization reaction. cDNA does not exist *in vivo* and therefore is a non-natural molecule. In a further embodiment, the cDNA is then amplified, for example, by the polymerase chain reaction (PCR) or other amplification method known to those of ordinary skill in the art. The product of this amplification reaction, *i.e.*, amplified cDNA is necessarily a non-natural product. As mentioned above, cDNA is a non-natural molecule. Second, in the case of PCR, the amplification process serves to create hundreds of millions of cDNA copies for every individual cDNA molecule of starting material. The number of copies generated are far removed from the number of copies of mRNA that are present *in vivo*.

[0037] In one embodiment, cDNA is amplified with primers that introduce an additional DNA sequence (adapter sequence) onto the fragments (with the use of adapter-specific primers). Amplification therefore serves to create non-natural double stranded molecules from the non-natural single stranded cDNA, by introducing barcode, adapter and/or reporter sequences onto the already non-natural cDNA. In one embodiment, during amplification with the adapter-specific primers, a detectable label, *e.g.*, a fluorophore, is added to single strand cDNA molecules. Amplification therefore also serves to create DNA complexes that do not occur in nature, at least because (i) cDNA does not exist *in vivo*, (i) adapter sequences are added to the ends of cDNA molecules to make DNA sequences that do not exist *in vivo*, (ii) the error rate associated with amplification further creates DNA sequences that do not exist *in vivo*, (iii) the disparate structure of the cDNA molecules as compared to what exists in nature and (iv) the chemical addition of a detectable label to the cDNA molecules.

[0038] In one embodiment, the synthesized cDNA (for example, amplified cDNA) is immobilized on a solid surface via hybridization with a probe, e.g., via a microarray. In another embodiment, cDNA products are detected via real-time polymerase chain reaction (PCR) via the introduction of fluorescent probes that hybridize with the cDNA products. For example, in one embodiment, biomarker detection is assessed by quantitative fluorogenic

RT-PCR (*e.g.*, with TaqMan® probes). For PCR analysis, well known methods are available in the art for the determination of primer sequences for use in the analysis.

[0039] Biomarkers provided herein in one embodiment, are detected via a hybridization reaction that employs a capture probe and/or a reporter probe. For example, the hybridization probe is a probe derivatized to a solid surface such as a bead, glass or silicon substrate. In another embodiment, the capture probe is present in solution and mixed with the patient's sample, followed by attachment of the hybridization product to a surface, e.g., via a biotin-avidin interaction (*e.g.*, where biotin is a part of the capture probe and avidin is on the surface). The hybridization assay in one embodiment, employs both a capture probe and a reporter probe. The reporter probe can hybridize to either the capture probe or the biomarker nucleic acid. Reporter probes e.g., are then counted and detected to determine the level of biomarker(s) in the sample. The capture and/or reporter probe, in one embodiment contain a detectable label, and/or a group that allows functionalization to a surface.

[0040] For example, the nCounter gene analysis system (see, e.g., Geiss *et al.* (2008) Nat. Biotechnol. 26, pp. 317-325, incorporated by reference in its entirety for all purposes, is amenable for use with the methods provided herein.

[0041] Hybridization assays described in U.S. Patent Nos. 7,473,767 and 8,492,094, the disclosures of which are incorporated by reference in their entireties for all purposes, are amenable for use with the methods provided herein, *i.e.*, to detect the biomarkers and biomarker combinations described herein.

[0042] Biomarker levels may be monitored using a membrane blot (such as used in hybridization analysis such as Northern, Southern, dot, and the like), or microwells, sample tubes, gels, beads, or fibers (or any solid support comprising bound nucleic acids). See, for example, U.S. Pat. Nos. 5,770,722, 5,874,219, 5,744,305, 5,677,195 and 5,445,934, each incorporated by reference in their entireties.

[0043] In one embodiment, microarrays are used to detect biomarker levels. Microarrays are particularly well suited for this purpose because of the reproducibility between different experiments. DNA microarrays provide one method for the simultaneous measurement of the expression levels of large numbers of genes. Each array consists of a reproducible pattern of capture probes attached to a solid support. Labeled RNA or DNA is hybridized to

complementary probes on the array and then detected by laser scanning Hybridization intensities for each probe on the array are determined and converted to a quantitative value representing relative gene expression levels. See, for example, U.S. Pat. Nos. 6,040,138, 5,800,992 and 6,020,135, 6,033,860, and 6,344,316, each incorporated by reference in their entireties. High-density oligonucleotide arrays are particularly useful for determining the biomarker profile for a large number of RNAs in a sample.

[0044] Techniques for the synthesis of these arrays using mechanical synthesis methods are described in, for example, U.S. Pat. No. 5,384,261. Although a planar array surface is generally used, the array can be fabricated on a surface of virtually any shape or even a multiplicity of surfaces. Arrays can be nucleic acids (or peptides) on beads, gels, polymeric surfaces, fibers (such as fiber optics), glass, or any other appropriate substrate. See, for example, U.S. Pat. Nos. 5,770,358, 5,789,162, 5,708,153, 6,040,193 and 5,800,992, each incorporated by reference in their entireties. Arrays can be packaged in such a manner as to allow for diagnostics or other manipulation of an all-inclusive device. See, for example, U.S. Pat. Nos. 5,856,174 and 5,922,591, each incorporated by reference in their entireties.

[0045] Serial analysis of gene expression (SAGE) in one embodiment is employed in the methods described herein. SAGE is a method that allows the simultaneous and quantitative analysis of a large number of gene transcripts, without the need of providing an individual hybridization probe for each transcript. First, a short sequence tag (about 10-14 bp) is generated that contains sufficient information to uniquely identify a transcript, provided that the tag is obtained from a unique position within each transcript. Then, many transcripts are linked together to form long serial molecules, that can be sequenced, revealing the identity of the multiple tags simultaneously. The expression pattern of any population of transcripts can be quantitatively evaluated by determining the abundance of individual tags, and identifying the gene corresponding to each tag. *See*, Velculescu et al. Science 270:484-87, 1995; Cell 88:243-51, 1997, incorporated by reference in its entirety.

[0046] An additional method of biomarker level analysis at the nucleic acid level is the use of a sequencing method, for example, RNAseq, next generation sequencing, and massively parallel signature sequencing (MPSS), as described by Brenner *et al.* (Nat. Biotech. 18:630-34, 2000, incorporated by reference in its entirety). This is a sequencing approach that combines non-gel-based signature sequencing with in vitro cloning of millions of templates on separate 5 μm diameter microbeads. First, a microbead library of DNA templates is

constructed by in vitro cloning. This is followed by the assembly of a planar array of the template-containing microbeads in a flow cell at a high density (typically greater than 3.0 X 10^6 microbeads/cm^2). The free ends of the cloned templates on each microbead are analyzed simultaneously, using a fluorescence-based signature sequencing method that does not require DNA fragment separation. This method has been shown to simultaneously and accurately provide, in a single operation, hundreds of thousands of gene signature sequences from a yeast cDNA library.

[0047] Immunohistochemistry methods are also suitable for detecting the levels of the biomarkers of the present invention. Samples can be frozen for later preparation or immediately placed in a fixative solution. Tissue samples can be fixed by treatment with a reagent, such as formalin, gluteraldehyde, methanol, or the like and embedded in paraffin. Methods for preparing slides for immunohistochemical analysis from formalin-fixed, paraffin-embedded tissue samples are well known in the art.

[0048] In one embodiment, the levels of the biomarkers of Table 1A, Table 1B, Table 1C, Table 2, Table 3, Table 4, Table 5 or Table 6 (or subsets thereof, for example 5 to 20, 5 to 30, 5 to 40 biomarkers), are normalized against the expression levels of all RNA transcripts or their non-natural cDNA expression products, or protein products in the sample, or of a reference set of RNA transcripts or a reference set of their non-natural cDNA expression products, or a reference set of their protein products in the sample.

[0049] As provided throughout, the methods set forth herein provide a method for determining the lung cancer subtype of a patient. Once the biomarker levels are determined, for example by measuring non natural cDNA biomarker levels or non-natural mRNA-cDNA biomarker complexes, the biomarker levels are compared to reference values or a reference sample, for example with the use of statistical methods or direct comparison of detected levels, to make a determination of the lung cancer molecular subtype. Based on the comparison, the patient's lung cancer sample is classified, e.g., as neuroendocrine, squamous cell carcinoma, adenocarcinoma. In another embodiment, based on the comparison, the patient's lung cancer sample is classified as squamous cell carcinoma, adenocarcinoma or small cell carcinoma. In another embodiment, based on the comparison, the patient's lung cancer sample is classified as squamous cell carcinoma, adenocarcinoma, small cell carcinoma or carcinoid lung cancer.

[0050] In one embodiment, hybridization values of the at least five classifier biomarkers of Table 1A, Table 1B, Table 1C, Table 2, Table 3, Table 4, Table 5 or Table 6 are compared to reference hybridization value(s) from at least one sample training set, wherein the at least one sample training set comprises hybridization values from a reference sample(s). In a further embodiment, the at least one sample training set comprises hybridization values of the at least five classifier biomarkers of Table 1A, Table 1B, Table 1C, Table 2, Table 3, Table 4, Table 5 or Table 6 from an adenocarcinoma sample, a squamous cell carcinoma sample, a neuroendocrine sample, a small cell lung carcinoma sample, a carcinoid lung cancer sample, or a combination thereof. In another embodiment, the at least one sample training set comprises hybridization values of the at least five classifier biomarkers of Table 1A, Table 1B, Table 1C, Table 2, Table 3, Table 4, Table 5, Table 6 from the reference samples provided in Table A below.

Table A. Various sample training set embodiments of the invention

At least one sample training set	**Origin of reference sample hybridization values**	**Lung cancer subtyping method**
Embodiment 1	Adenocarcinoma reference sample and/or squamous cell carcinoma reference sample	Assessing whether patient sample is adenocarcinoma or squamous cell carcinoma
Embodiment 2	Adenocarcinoma reference sample, squamous cell carcinoma reference sample and/or neuroendocrine reference sample	Assessing whether patient sample is adenocarcinoma, squamous cell carcinoma or neuroendocrine sample
Embodiment 3	Adenocarcinoma reference sample, squamous cell carcinoma reference sample, small cell carcinoma reference and/or carcinoid reference sample	Assessing whether patient sample is adenocarcinoma, squamous cell carcinoma, small cell carcinoma sample or carcinoid

[0051] Methods for comparing detected levels of biomarkers to reference values and/or reference samples are provided herein. Based on this comparison, in one embodiment a correlation between the biomarker levels obtained from the subject's sample and the reference values is obtained. An assessment of the lung cancer subtype is then made.

[0052] Various statistical methods can be used to aid in the comparison of the biomarker levels obtained from the patient and reference biomarker levels, for example, from at least one sample training set.

[0053] In one embodiment, a supervised pattern recognition method is employed. Examples of supervised pattern recognition methods can include, but are not limited to, the nearest centroid methods (Dabney (2005) Bioinformatics 21(22):4148-4154 and Tibshirani et al. (2002) Proc. Natl. Acad. Sci. USA 99(10):6576-6572); soft independent modeling of class analysis (SIMCA) (see, for example, Wold, 1976); partial least squares analysis (PLS) (see, for example, Wold, 1966; Joreskog, 1982; Frank, 1984; Bro, R., 1997); linear descriminant analysis (LDA) (see, for example, Nillson, 1965); K-nearest neighbour analysis (KNN) (sec, for example, Brown et al., 1996); artificial neural networks (ANN) (see, for example, Wasserman, 1989; Anker et al., 1992; Hare, 1994); probabilistic neural networks (PNNs) (see, for example, Parzen, 1962; Bishop, 1995; Speckt, 1990; Broomhead et al., 1988; Patterson, 1996); rule induction (RI) (see, for example, Quinlan, 1986); and, Bayesian methods (see, for example, Bretthorst, 1990a, 1990b, 1988). In one embodiment, the classifier for identifying tumor subtypes based on gene expression data is the centroid based method described in Mullins et al. (2007) Clin Chem. 53(7):1273-9, each of which is herein incorporated by reference in its entirety.

[0054] In other embodiments, an unsupervised training approach is employed, and therefore, no training set is used.

[0055] Referring to sample training sets for supervised learning approaches again, in some embodiments, a sample training set(s) can include expression data of all of the classifier biomarkers (e.g., all the classifier biomarkers of any of Table 1A, Table 1B, Table 1C, Table 2, Table 3, Table 4, Table 5, Table 6) from an adenocarcinoma sample. In some embodiments, a sample training set(s) can include expression data of all of the classifier biomarkers (e.g., all the classifier biomarkers of any of Table 1A, Table 1B, Table 1C, Table 2, Table 3, Table 4, Table 5, Table 6) from a squamous cell carcinoma sample, an adenocarcinoma sample and/or a neuroendocrine sample. In some embodiments, the sample training set(s) are normalized to remove sample-to-sample variation.

[0056] In some embodiments, comparing can include applying a statistical algorithm, such as, for example, any suitable multivariate statistical analysis model, which can be parametric or non-parametric. In some embodiments, applying the statistical algorithm can include determining a correlation between the expression data obtained from the human lung tissue sample and the expression data from the adenocarcinoma and squamous cell carcinoma training set(s). In some embodiments, cross-validation is performed, such as (for example),

leave-one-out cross-validation (LOOCV). In some embodiments, integrative correlation is performed. In some embodiments, a Spearman correlation is performed. In some embodiments, a centroid based method is employed for the statistical algorithm as described in Mullins *et al.* (2007) Clin Chem. 53(7):1273-9, and based on gene expression data, which is herein incorporated by reference in its entirety.

[0057] Results of the gene expression performed on a sample from a subject (test sample) may be compared to a biological sample(s) or data derived from a biological sample(s) that is known or suspected to be normal ("reference sample" or "normal sample", e.g., non-adenocarcinoma sample). In another embodiment, a reference sample or reference biomarker level data is obtained or derived from an individual known to have a lung cancer subtype, *e.g.*, adenocarcinoma, squamous cell carcinoma, neuroendocrine, small cell carcinoma and/or carcinoid.

[0058] The reference sample may be assayed at the same time, or at a different time from the test sample. Alternatively, the biomarker level information from a reference sample may be stored in a database or other means for access at a later date.

[0059] The biomarker level results of an assay on the test sample may be compared to the results of the same assay on a reference sample. In some cases, the results of the assay on the reference sample are from a database, or a reference value(s). In some cases, the results of the assay on the reference sample are a known or generally accepted value or range of values by those skilled in the art. In some cases the comparison is qualitative. In other cases the comparison is quantitative. In some cases, qualitative or quantitative comparisons may involve but are not limited to one or more of the following: comparing fluorescence values, spot intensities, absorbance values, chemiluminescent signals, histograms, critical threshold values, statistical significance values, expression levels of the genes described herein, mRNA copy numbers.

[0060] In one embodiment, an odds ratio (OR) is calculated for each biomarker level panel measurement. Here, the OR is a measure of association between the measured biomarker values for the patient and an outcome, *e.g.*, lung cancer subtype. For example, see, *J. Can. Acad. Child Adolesc. Psychiatry* 2010; 19(3): 227-229, which is incorporated by reference in its entirety for all purposes.

[0061] In one embodiment, a specified statistical confidence level may be determined in order to provide a confidence level regarding the lung cancer subtype. For example, it may be determined that a confidence level of greater than 90% may be a useful predictor of the lung cancer subtype. In other embodiments, more or less stringent confidence levels may be chosen. For example, a confidence level of about or at least about 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 97.5%, 99%, 99.5%, or 99.9% may be chosen. The confidence level provided may in some cases be related to the quality of the sample, the quality of the data, the quality of the analysis, the specific methods used, and/or the number of gene expression values (*i.e.*, the number of genes) analyzed. The specified confidence level for providing the likelihood of response may be chosen on the basis of the expected number of false positives or false negatives. Methods for choosing parameters for achieving a specified confidence level or for identifying markers with diagnostic power include but are not limited to Receiver Operating Characteristic (ROC) curve analysis, binormal ROC, principal component analysis, odds ratio analysis, partial least squares analysis, singular value decomposition, least absolute shrinkage and selection operator analysis, least angle regression, and the threshold gradient directed regularization method.

[0062] Determining the lung cancer subtype in some cases be improved through the application of algorithms designed to normalize and or improve the reliability of the biomarker level data. In some embodiments of the present invention, the data analysis utilizes a computer or other device, machine or apparatus for application of the various algorithms described herein due to the large number of individual data points that are processed. A "machine learning algorithm" refers to a computational-based prediction methodology, also known to persons skilled in the art as a "classifier," employed for characterizing a biomarker level profile or profiles, *e.g.*, to determine the lung cancer subtype. The biomarker levels, determined by, *e.g.*, microarray-based hybridization assays, sequencing assays, NanoString assays, etc., are in one embodiment subjected to the algorithm in order to classify the profile. Supervised learning generally involves "training" a classifier to recognize the distinctions among classes (*e.g.*, adenocarcinoma positive, adenocarcinoma negative, squamous positive, squamous negative, neuroendocrine positive, neuroendocrine negative, small cell positive, small cell negative, carcinoid positive, carcinoid negative), and then "testing" the accuracy of the classifier on an independent test set. For new, unknown samples the classifier can be used to predict, for example, the class (*e.g.*, (i) adenocarcinoma

vs. squamous cell carcinoma vs. neuroendocrine or (ii) adenocarcinoma vs. squamous cell carcinoma vs. small cell vs. carcinoid, etc.) in which a particular sample or samples belongs.

[0063] In some embodiments, a robust multi-array average (RMA) method may be used to normalize raw data. The RMA method begins by computing background-corrected intensities for each matched cell on a number of microarrays. In one embodiment, the background corrected values are restricted to positive values as described by Irizarry *et al.* (2003). Biostatistics April 4 (2): 249-64, incorporated by reference in its entirety for all purposes. After background correction, the base-2 logarithm of each background corrected matched-cell intensity is then obtained. The background corrected, log-transformed, matched intensity on each microarray is then normalized using the quantile normalization method in which for each input array and each probe value, the array percentile probe value is replaced with the average of all array percentile points, this method is more completely described by Bolstad *et al.* Bioinformatics 2003, incorporated by reference in its entirety. Following quantile normalization, the normalized data may then be fit to a linear model to obtain an intensity measure for each probe on each microarray. Tukey's median polish algorithm (Tukey, J. W., Exploratory Data Analysis. 1977, incorporated by reference in its entirety for all purposes) may then be used to determine the log-scale intensity level for the normalized probe set data.

[0064] Various other software programs may be implemented. In certain methods, feature selection and model estimation may be performed by logistic regression with *lasso* penalty using *glmnet* (Friedman *et al.* (2010). *Journal of statistical software* **33**(1): 1-22, incorporated by reference in its entirety). Raw reads may be aligned using TopHat (Trapnell *et al.* (2009). *Bioinformatics* **25**(9): 1105-11, incorporated by reference in its entirety). In methods, top features (N ranging from 10 to 200) are used to train a linear support vector machine (SVM) (Suykens JAK, Vandewalle J. Least Squares Support Vector Machine Classifiers. *Neural Processing Letters* 1999; **9**(3): 293-300, incorporated by reference in its entirety) using the *e1071* library (Meyer D. Support vector machines: the interface to libsvm in package e1071. 2014, incorporated by reference in its entirety). Confidence intervals, in one embodiment, are computed using the pROC package (Robin X, Turck N, Hainard A, et al. pROC: an open-source package for R and S+ to analyze and compare ROC curves. *BMC bioinformatics* 2011; **12**: 77, incorporated by reference in its entirety).

[0065] In addition, data may be filtered to remove data that may be considered suspect. In one embodiment, data derived from microarray probes that have fewer than about 4, 5, 6, 7 or 8 guanosine+cytosine nucleotides may be considered to be unreliable due to their aberrant hybridization propensity or secondary structure issues. Similarly, data deriving from microarray probes that have more than about 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, or 22 guanosine+cytosine nucleotides may in one embodiment be considered unreliable due to their aberrant hybridization propensity or secondary structure issues.

[0066] In some embodiments of the present invention, data from probe-sets may be excluded from analysis if they are not identified at a detectable level (above background).

[0067] In some embodiments of the present disclosure, probe-sets that exhibit no, or low variance may be excluded from further analysis. Low-variance probe-sets are excluded from the analysis via a Chi-Square test. In one embodiment, a probe-set is considered to be low-variance if its transformed variance is to the left of the 99 percent confidence interval of the Chi-Squared distribution with (N-l) degrees of freedom. (N-l)*Probe-set Variance/(Gene Probe-set Variance). about.Chi-Sq(N-l) where N is the number of input CEL files, (N-l) is the degrees of freedom for the Chi-Squared distribution, and the "probe-set variance for the gene" is the average of probe-set variances across the gene. In some embodiments of the present invention, probe-sets for a given mRNA or group of mRNAs may be excluded from further analysis if they contain less than a minimum number of probes that pass through the previously described filter steps for GC content, reliability, variance and the like. For example in some embodiments, probe-sets for a given gene or transcript cluster may be excluded from further analysis if they contain less than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or less than about 20 probes.

[0068] Methods of biomarker level data analysis in one embodiment, further include the use of a feature selection algorithm as provided herein. In some embodiments of the present invention, feature selection is provided by use of the LIMMA software package (Smyth, G. K. (2005). Limma: linear models for microarray data. In: Bioinformatics and Computational Biology Solutions using R and Bioconductor, R. Gentleman, V. Carey, S. Dudoit, R. Irizarry, W. Huber (eds.), Springer, New York, pages 397-420, incorporated by reference in its entirety for all purposes).

[0069] Methods of biomarker level data analysis, in one embodiment, include the use of a pre-classifier algorithm. For example, an algorithm may use a specific molecular fingerprint to pre-classify the samples according to their composition and then apply a correction/normalization factor. This data/information may then be fed in to a final classification algorithm which would incorporate that information to aid in the final diagnosis.

[0070] Methods of biomarker level data analysis, in one embodiment, further include the use of a classifier algorithm as provided herein. In one embodiment of the present invention, a diagonal linear discriminant analysis, k-nearest neighbor algorithm, support vector machine (SVM) algorithm, linear support vector machine, random forest algorithm, or a probabilistic model-based method or a combination thereof is provided for classification of microarray data. In some embodiments, identified markers that distinguish samples (*e.g.*, of varying biomarker level profiles, of varying lung cancer subtypes, and/or varying molecular subtypes of adenocarcinoma are selected based on statistical significance of the difference in biomarker levels between classes of interest. In some cases, the statistical significance is adjusted by applying a Benjamin Hochberg or another correction for false discovery rate (FDR).

[0071] In some cases, the classifier algorithm may be supplemented with a meta-analysis approach such as that described by Fishel and Kaufman et al. 2007 Bioinformatics 23(13): 1599-606, incorporated by reference in its entirety for all purposes. In some cases, the classifier algorithm may be supplemented with a meta-analysis approach such as a repeatability analysis.

[0072] Methods for deriving and applying posterior probabilities to the analysis of biomarker level data are known in the art and have been described for example in Smyth, G. K. 2004 *Stat. Appi. Genet. Mol. Biol.* 3: Article 3, incorporated by reference in its entirety for all purposes. In some cases, the posterior probabilities may be used in the methods of the present invention to rank the markers provided by the classifier algorithm.

[0073] A statistical evaluation of the results of the biomarker level profiling may provide a quantitative value or values indicative of the lung cancer subtype (*e.g.*, adenocarcinoma, squamous cell carcinoma, neuroendocrine, small cell, carcinoid). In one embodiment, the data is presented directly to the physician in its most useful form to guide patient care, or is

used to define patient populations in clinical trials or a patient population for a given medication. The results of the molecular profiling can be statistically evaluated using a number of methods known to the art including, but not limited to: the students T test, the two sided T test, Pearson rank sum analysis, hidden Markov model analysis, analysis of q-q plots, principal component analysis, one way ANOVA, two way ANOVA, LIMMA and the like.

[0074] In some cases, accuracy may be determined by tracking the subject over time to determine the accuracy of the original diagnosis. In other cases, accuracy may be established in a deterministic manner or using statistical methods. For example, receiver operator characteristic (ROC) analysis may be used to determine the optimal assay parameters to achieve a specific level of accuracy, specificity, positive predictive value, negative predictive value, and/or false discovery rate.

[0075] In some cases the results of the biomarker level profiling assays, are entered into a database for access by representatives or agents of a molecular profiling business, the individual, a medical provider, or insurance provider. In some cases, assay results include sample classification, identification, or diagnosis by a representative, agent or consultant of the business, such as a medical professional. In other cases, a computer or algorithmic analysis of the data is provided automatically. In some cases the molecular profiling business may bill the individual, insurance provider, medical provider, researcher, or government entity for one or more of the following: molecular profiling assays performed, consulting services, data analysis, reporting of results, or database access.

[0076] In some embodiments of the present invention, the results of the biomarker level profiling assays are presented as a report on a computer screen or as a paper record. In some embodiments, the report may include, but is not limited to, such information as one or more of the following: the levels of biomarkers (*e.g.*, as reported by copy number or fluorescence intensity, etc.) as compared to the reference sample or reference value(s); the lung cancer subtype, proposed therapies.

[0077] In one embodiment, the results of the classifier biomarker profiling may be classified into one or more of the following: adenocarcinoma positive, adenocarcinoma negative, squamous cell carcinoma positive, squamous cell carcinoma negative, neuroendocrine positive, neuroendocrine negative, small cell carcinoma positive, small cell carcinoma negative, carcinoid positive, carcinoid negative or a combination thereof.

[0078] In some embodiments of the present invention, results are classified using a trained algorithm. Trained algorithms of the present invention include algorithms that have been developed using a reference set of known gene expression values and/or normal samples, for example, samples from individuals diagnosed with a particular molecular subtype of adenocarcinoma. In some cases a reference set of known gene expression values are obtained from individuals who have been diagnosed with a particular molecular subtype of lung cancer.

[0079] Algorithms suitable for categorization of samples include but are not limited to k-nearest neighbor algorithms, support vector machines, linear discriminant analysis, diagonal linear discriminant analysis, updown, naive Bayesian algorithms, neural network algorithms, hidden Markov model algorithms, genetic algorithms, or any combination thereof.

[0080] When a binary classifier is compared with actual true values (*e.g.*, values from a biological sample), there are typically four possible outcomes. If the outcome from a prediction is p (where "p" is a positive classifier output, such as the presence of a deletion or duplication syndrome) and the actual value is also p, then it is called a true positive (TP); however if the actual value is n then it is said to be a false positive (FP). Conversely, a true negative has occurred when both the prediction outcome and the actual value are n (where "n" is a negative classifier output, such as no deletion or duplication syndrome), and false negative is when the prediction outcome is n while the actual value is p. In one embodiment, consider a test that seeks to determine a molecular subtype of lung cancer. A false positive in this case occurs when the person tests for a molecular subtype that he or she does not actually have. A false negative, on the other hand, occurs when the person tests negative, suggesting the sample is not a particular lung cancer subtype, when the sample is in fact the lung cancer sample should be characterized as the particular lung cancer subtype.

[0081] The positive predictive value (PPV), or precision rate, or post-test probability of disease, is the proportion of subjects diagnosed with the correct lung cancer subtype. It reflects the probability that a positive test reflects the underlying condition being tested for. Its value does however depend on the prevalence of the disease, which may vary. In one example the following characteristics are provided: FP (false positive); TN (true negative); TP (true positive); FN (false negative). False positive rate (α)=FP/(FP+TN)-specificity; False negative rate (β)=FN/(TP+FN)-sensitivity; Power= sensitivity = 1-β; Likelihood-ratio positive=sensitivity/(1-specificity); Likelihood-ratio negative=(1 -sensitivity)/specificity.

The negative predictive value (NPV) is the proportion of subjects with negative test results who are correctly diagnosed.

[0082] In some embodiments, the results of the biomarker level analysis of the subject methods provide a statistical confidence level that a given diagnosis is correct. In some embodiments, such statistical confidence level is at least about, or more than about 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% 99.5%, or more.

[0083] In some embodiments, the method further includes classifying the lung tissue sample as a particular lung cancer subtype based on the comparison of biomarker levels in the sample and reference biomarker levels, for example present in at least one training set. In some embodiments, the lung tissue sample is classified as a particular subtype if the results of the comparison meet one or more criterion such as, for example, a minimum percent agreement, a value of a statistic calculated based on the percentage agreement such as (for example) a kappa statistic, a minimum correlation (*e.g.*, Pearson's correlation) and/or the like.

[0084] It is intended that the methods described herein can be performed by software (stored in memory and/or executed on hardware), hardware, or a combination thereof. Hardware modules may include, for example, a general-purpose processor, a field programmable gate array (FPGA), and/or an application specific integrated circuit (ASIC). Software modules (executed on hardware) can be expressed in a variety of software languages (*e.g.*, computer code), including Unix utilities, C, C++, Java™, Ruby, SQL, SAS®, the R programming language/software environment, Visual Basic™, and other object-oriented, procedural, or other programming language and development tools. Examples of computer code include, but are not limited to, micro-code or micro-instructions, machine instructions, such as produced by a compiler, code used to produce a web service, and files containing higher-level instructions that are executed by a computer using an interpreter. Additional examples of computer code include, but are not limited to, control signals, encrypted code, and compressed code.

[0085] Some embodiments described herein relate to devices with a non-transitory computer-readable medium (also can be referred to as a non-transitory processor-readable medium or memory) having instructions or computer code thereon for performing various computer-implemented operations and/or methods disclosed herein. The computer-readable medium

(or processor-readable medium) is non-transitory in the sense that it does not include transitory propagating signals per se (*e.g.*, a propagating electromagnetic wave carrying information on a transmission medium such as space or a cable). The media and computer code (also can be referred to as code) may be those designed and constructed for the specific purpose or purposes. Examples of non-transitory computer-readable media include, but are not limited to: magnetic storage media such as hard disks, floppy disks, and magnetic tape; optical storage media such as Compact Disc/Digital Video Discs (CD/DVDs), Compact Disc-Read Only Memories (CD-ROMs), and holographic devices; magneto-optical storage media such as optical disks; carrier wave signal processing modules; and hardware devices that are specially configured to store and execute program code, such as Application-Specific Integrated Circuits (ASICs), Programmable Logic Devices (PLDs), Read-Only Memory (ROM) and Random-Access Memory (RAM) devices. Other embodiments described herein relate to a computer program product, which can include, for example, the instructions and/or computer code discussed herein.

[0086] In some embodiments, at least five biomarkers, from about 5 to about 20 biomarkers, from about 5 to about 50 biomarkers, from about 5 to about 40 biomarkers, or from about 5 to about 30 biomarkers (*e.g.*, as disclosed in Table 1A, Table 1B, Table 1C, Table 2, Table 3, Table 4, Table 5 and Table 6) is capable of classifying types and/or subtypes of lung cancer with a predictive success of at least about 70%, at least about 71%, at least about 72%, about 73%, about 74%, about 75%, about 76%, about 77%, about 78%, about 79%, about 80%, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, up to 100%, and all values in between. In some embodiments, any combination of biomarkers disclosed herein (*e.g.*, in Table 1A, Table 1B, Table 1C, Table 2, Table 3, Table 4, Table 5 and Table 6 and sub-combinations thereof) can used to obtain a predictive success of at least about 70%, at least about 71%, at least about 72%, about 73%, about 74%, about 75%, about 76%, about 77%, about 78%, about 79%, about 80%, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, up to 100%, and all values in between.

[0087] In some embodiments, at least five biomarkers, from about 5 to about 20 biomarkers, from about 5 to about 50 biomarkers, from about 5 to about 40 biomarkers, or from about 5 to about 30 biomarkers (*e.g.*, as disclosed in Table 1A, Table 1B, Table 1C, Table 2, Table 3, Table 4, Table 5 and Table 6) is capable of classifying lung cancer types and/or subtypes with a sensitivity or specificity of at least about 70%, at least about 71%, at least about 72%, about 73%, about 74%, about 75%, about 76%, about 77%, about 78%, about 79%, about 80%, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, up to 100%, and all values in between. In some embodiments, any combination of biomarkers disclosed herein can be used to obtain a sensitivity or specificity of at least about 70%, at least about 71%, at least about 72%, about 73%, about 74%, about 75%, about 76%, about 77%, about 78%, about 79%, about 80%, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, up to 100%, and all values in between.

[0088] In some embodiments, one or more kits for practicing the methods of the invention are further provided. The kit can encompass any manufacture (*e.g.*, a package or a container) including at least one reagent, *e.g.*, an antibody, a nucleic acid probe or primer, and/or the like, for detecting the biomarker level of a classifier biomarker. The kit can be promoted, distributed, or sold as a unit for performing the methods of the present invention. Additionally, the kits can contain a package insert describing the kit and methods for its use.

[0089] In one embodiment, upon determining a patient's lung cancer subtype, the patient is selected for suitable therapy, for example chemotherapy or drug therapy with an angiogenesis inhibitor. In one embodiment, the therapy is angiogenesis inhibitor therapy, and the angiogenesis inhibitor is a vascular endothelial growth factor (VEGF) inhibitor, a VEGF receptor inhibitor, a platelet derived growth factor (PDGF) inhibitor or a PDGF receptor inhibitor.

[0090] In another embodiment, the angiogenesis inhibitor is an integrin antagonist, a selectin antagonist, an adhesion molecule antagonist (*e.g.*, antagonist of intercellular adhesion molecule (ICAM)-1, ICAM-2, ICAM-3, platelet endothelial adhesion molecule (PCAM), vascular cell adhesion molecule (VCAM)), lymphocyte function-associated antigen 1 (LFA-

1)), a basic fibroblast growth factor antagonist, a vascular endothelial growth factor (VEGF) modulator, or a platelet derived growth factor (PDGF) modulator (*e.g.*, a PDGF antagonist).

[0091] In one embodiment, as provided above, upon determining a patient's lung cancer subtype, the patient is selected for suitable therapy, for example chemotherapy or drug therapy with an angiogenesis inhibitor. In one embodiment, the angiogenesis inhibitor is one or more of the following: interferon gamma 1β, interferon gamma 1β (Actimmune®) with pirfenidone, ACUHTR028, αVβ5, aminobenzoate potassium, amyloid P, ANG1122, ANG1170, ANG3062, ANG3281, ANG3298, ANG4011, anti-CTGF RNAi, Aplidin, astragalus membranaceus extract with salvia and schisandra chinensis, atherosclerotic plaque blocker, Azol, AZX100, BB3, connective tissue growth factor antibody, CT140, danazol, Esbriet, EXC001, EXC002, EXC003, EXC004, EXC005, F647, FG3019, Fibrocorin, Follistatin, FT011, a galectin-3 inhibitor, GKT137831, GMCT01, GMCT02, GRMD01, GRMD02, GRN510, Heberon Alfa R, interferon α-2β, ITMN520, JKB119, JKB121, JKB122, KRX168, LPA1 receptor antagonist, MGN4220, MIA2, microRNA 29a oligonucleotide, MMI0100, noscapine, PBI4050, PBI4419, PDGFR inhibitor, PF-06473871, PGN0052, Pirespa, Pirfenex, pirfenidone, plitidepsin, PRM151, Px102, PYN17, PYN22 with PYN17, Relivergen, rhPTX2 fusion protein, RXI109, secretin, STX100, TGF-β Inhibitor, transforming growth factor, β-receptor 2 oligonucleotide,VA999260, XV615, endostatin, a 20 kDa C-terminal fragment derived from type XVIII collagen, angiostatin (a 38 kDa fragment of plasmin), or a member of the thrombospondin (TSP) family of proteins. In a further embodiment, the angiogenesis inhibitor is a TSP-1, TSP-2, TSP-3, TSP-4 and TSP-5.

[0092] In one embodiment, the therapy is aa soluble VEGF receptor, *e.g.*, soluble VEGFR-1 and neuropilin 1 (NPR1), angiopoietin-1, angiopoietin-2, vasostatin, calreticulin, platelet factor-4, a tissue inhibitor of metalloproteinase (TIMP) (*e.g.*, TIMP1, TIMP2, TIMP3, TIMP4), cartilage-derived angiogenesis inhibitor (*e.g.*, peptide troponin I and chrondomodulin I), a disintegrin and metalloproteinase with thrombospondin motif 1, an interferon (IFN) (*e.g.*, IFN-α, IFN-β, IFN-γ), a chemokine, *e.g.*, a chemokine having the C-X-C motif (*e.g.*, CXCL10, also known as interferon gamma-induced protein 10 or small inducible cytokine B10), an interleukin cytokine (*e.g.*, IL-4, IL-12, IL-18), prothrombin, antithrombin III fragment, prolactin, the protein encoded by the *TNFSF15* gene, osteopontin, maspin, canstatin, proliferin-related protein, angiopoietin-1, angiopoietin-2, angiostatin, endostatin, vasostatin, thrombospondin, calreticulin, platelet factor-4, TIMP, CDAI,

interferon α, interferon β,vascular endothelial growth factor inhibitor (VEGI) meth-1, meth-2, prolactin, VEGI, SPARC, osteopontin, maspin, canstatin, proliferin-related protein (PRP), restin, TSP-1, TSP-2, interferon gamma 1β, ACUHTR028, αVβ5, aminobenzoate potassium, amyloid P, ANG1122, ANG1170, ANG3062, ANG3281, ANG3298, ANG4011, anti-CTGF RNAi, Aplidin, astragalus membranaceus extract with salvia and schisandra chinensis, atherosclerotic plaque blocker, Azol, AZX100, BB3, connective tissue growth factor antibody, CT140, danazol, Esbriet, EXC001, EXC002, EXC003, EXC004, EXC005, F647, FG3019, Fibrocorin, Follistatin, FT011, a galectin-3 inhibitor, GKT137831, GMCT01, GMCT02, GRMD01, GRMD02, GRN510, Heberon Alfa R, interferon α-2β, ITMN520, JKB119, JKB121, JKB122, KRX168, LPA1 receptor antagonist, MGN4220, MIA2, microRNA 29a oligonucleotide, MMI0100, noscapine, PBI4050, PBI4419, PDGFR inhibitor, PF-06473871, PGN0052, Pirespa, Pirfenex, pirfenidone, plitidepsin, PRM151, Px102, PYN17, PYN22 with PYN17, Relivergen, rhPTX2 fusion protein, RXI109, secretin, STX100, TGF-β Inhibitor, transforming growth factor, β-receptor 2 oligonucleotide,VA999260, XV615 or a combination thereof.

[0093] In yet another embodiment, upon determining a patient's lung cancer subtype, the patient is selected for suitable therapy with pazopanib (Votrient), sunitinib (Sutent), sorafenib (Nexavar), axitinib (Inlyta), ponatinib (Iclusig), vandetanib (Caprelsa), cabozantinib (Cometrig), ramucirumab (Cyramza), regorafenib (Stivarga), ziv-aflibercept (Zaltrap), or a combination thereof. In yet another embodiment, upon determining a patient's lung cancer subtype, the patient is selected for suitable therapy with a VEGF inhibitor. In a further embodiment, the VEGF inhibitor is axitinib, cabozantinib, aflibercept, brivanib, tivozanib, ramucirumab or motesanib. In yet a further embodiment, the VEGF inhibitor is motesanib.

[0094] In yet another embodiment, upon determining a patient's lung cancer subtype, the patient is selected for suitable therapy with a platelet derived growth factor (PDGF) antagonist. For example, the PDGF antagonist, in one embodiment, is an anti-PDGF aptamer, an anti-PDGF antibody or fragment thereof, an anti-PDGF receptor antibody or fragment thereof, or a small molecule antagonist. In one embodiment, the PDGF antagonist is an antagonist of the PDGFR-α or PDGFR-β. In one embodiment, the PDGF antagonist is the anti-PDGF-β aptamer E10030, sunitinib, axitinib, sorefenib, imatinib, imatinib mesylate, nintedanib, pazopanib HCl, ponatinib, MK-2461, dovitinib, pazopanib, crenolanib, PP-121,

telatinib, imatinib, KRN 633, CP 673451, TSU-68, Ki8751, amuvatinib, tivozanib, masitinib, motesanib diphosphate, dovitinib dilactic acid, linifanib (ABT-869).

EXAMPLES

[0095] The present invention is further illustrated by reference to the following Examples. However, it should be noted that these Examples, like the embodiments described above, is illustrative and is not to be construed as restricting the scope of the invention in any way.

Methods

[0096] Several publically available lung cancer gene expression data sets including 2,168 lung cancer samples (TCGA, NCI, UNC, Duke, Expo, Seoul, Tokyo, and France) were assembled to validate a 57 gene expression Lung Subtype Panel (LSP) developed to complement morphologic classification of lung tumors. LSP included 52 lung tumor classifying genes plus 5 housekeeping genes. Data sets with both gene expression data and lung tumor morphologic classification were selected. Three categories of genomic data were represented in the data sets: Affymetrix U133+2(n=883) (also referred to as "A-833"), Agilent 44K(n=334) (also referred to as "A-334"), and Illumina RNAseq(n=951) (also referred to as "I-951"). Data sources are provided in Table 7 and normalization methods in Table 8. Samples with a definitive diagnosis of adenocarcinoma, carcinoid, small cell, and squamous cell carcinoma were used in the analysis.

Table 7. Data sources for publicly available lung cancer gene expression data

Source	Platform(s)	N	Subtype	Ref
TCGA[1]	RNASeq (LUAD)	528	adenocarcinomas	TCGA-DCC
TCGA[2]	RNASeq (LUSC)	534	Squamous	TCGA-DCC
UNC[3]	Agilent_44K	56	56 squamous	CCR (2010) PMID: 20643781
UNC[4]	Agilent_44K	116	116 adenocarcinomas	PLoS One (2012) PMID: 22590557
NCI[5]	Agilent_44K	172	56 adenocarcinoma, 92 squamous, 10 large cell	CCR (2009)
Korea[6]	HG-U133+2	138	63 adenocarcinoma, 75 squamous	CCR (2008) PMID: 19010856

Table 7. Data sources for publicly available lung cancer gene expression data				
Source	**Platform(s)**	**N**	**Subtype**	**Ref**
Expo[7]	HG-U133+2	130	all histology subtypes	GSE2109
French[8]	HG-U133+2	307	all histology subtypes	Sci Transl Med (2013) PMID: 23698379
Duke[9]	HG-U133+2	118	adenocarcinoma and squamous	Nature (2006) PMID: 16273092
Tokyo[10]	HG-U133+2	246	adenocarcinomas	PLoS One (2012) PMID: 22080568, 23028479

[1]https://tcga-data.nci.nih.gov/tcgafiles/ftp_auth/distro_ftpusers/anonymous/tumor/luad/cgcc/unc.edu/illuminahiseq_rnaseqv2/rnaseqv2/?C=S;O=A
[2]https://tcga-data.nci.nih.gov/tcgafiles/ftp_auth/distro_ftpusers/anonymous/tumor/lusc/cgcc/unc.edu/illuminahiseq_rnaseqv2/rnaseqv2/
[3] http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE17710
[4]http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE26939
[5]http://research.agendia.com/
[6]http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE8894
[7]http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE2109
[8]http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE30219
[9]http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE3141
[10]http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE31210

Table 8. Normalization methods used for the 3 public gene expression datasets		
Source	**Platforms**	**Data Preprocessing / Normalization**
TCGA	RNASeq	RSEM expression estimates are normalized to set the upper quartile count at 1000 for gene level, 2 based log transformed, data matrix is row (gene) median centered, column (sample) standardized.
UNC+NKI	Agilent_44K	2 based log ratio of the two channel intensities are LOWESS normalized, data matrix is row (gene) median centered, column (sample) standardized.
Affy	HG-U133+2	MAS5 normalized one channel intensities are 2 based log transformed, data matrix is row (gene) median centered, column (sample) standardized.

[0097] The A-833 dataset was used as training for calculation of adenocarcinoma, carcinoid, small cell carcinoma, and squamous cell carcinoma gene centroids according to methods described previously. Gene centroids trained on the A-833 data were then applied to the

normalized TCGA and A-334 datasets to investigate LSP's ability to classify lung tumors using publicly available gene expression data. For the application of A-833 training centroids to the A-833 dataset, evaluation was performed using Leave One Out (LOO) cross validation. Spearman correlations were calculated for tumor sample gene expression results to the A-833 gene expression training centroids. Tumors were assigned a genomic-defined histologic type (carcinoid, small cell, adenocarcinoma and squamous cell carcinoma) corresponding to the maximally correlated centroids. A 2 class, 3 class, and 4 class prediction was explored. Correct predictions were defined as LSP calls matching the tumor's histologic diagnosis. Percent agreement was defined as the number of correct predictions divided by the number of all predictions and an agreement kappa statistic was calculated.

[0098] Ten lung tumor RNA expression datasets were combined into three platform specific data sets (A-833, A-334, and I-951). The patient population was diverse and included smokers and nonsmokers with tumors ranging from Stage 1 – Stage IV. Sample characteristics and lung cancer diagnoses of the three datasets are included in Table 9.

Table 9. Sample Characteristics

Characteristic	TCGA RNA seq	Agilent	Affymetrix
Total # of samples	1062	334	875
Tumor Specimen histology			
Adenocarcinoma	468	174	490
Carcinoid	0	0	23
Neuroendocrine (NOS)	0	0	6
Squamous cell carcinoma	483	148	227
Other (excluded from analysis)	111	12	105
Gender			
Female/Male/NA	285/366/300	87/85/150	272/491/7
Age at diagnosis			
Median/(Range)	67/(38-88)	66/(37-90)	63/(13-85)
Age not available	323	150	7
Stage			
I	355	NA	NA
II	146	NA	NA
III	119	NA	NA
IV	26	NA	NA
Stage not available	305	322	770
Smoking			
Smoker	386	NA	NA

Table 9. Sample Characteristics			
Characteristic	TCGA RNA seq	Agilent	Affymetrix
Non-smoker	39	NA	NA
Smoking status not available	526	322	770

[0099] Predicted tumor type for a 2 class, 3 class, and 4 class predictor were compared with tumor morphologic classification and percent agreement and Fleiss' kappa was calculated for each predictor (Tables 10a, 10b and 10c).

Table 10a. A-833 dataset training gene centroids applied to 2 other publicly available lung cancer gene expression databases (TCGA & A-334) for a 2 class prediction of lung tumor type. LOO cross validation was performed for the A-833 dataset.

	Prediction								
Histology Diagnosis	**TCGA RNAseq**			**Agilent**			**Affymetrix LOO**		
	AD	**SQ**	**Sum**	**AD**	**SQ**	**Sum**	**AD**	**SQ**	**Sum**
Adenocarcinoma (AD)	452	16	468	151	23	174	423	67	490
Squamous cell carcinoma (SQ)	37	446	483	39	109	148	41	186	227
Sum	489	462	951	190	132	322	464	253	717
% Agreement	**94%**			**81%**			**85%**		
kappa	**0.89**			**0.61**			**0.66**		

Table 10b. A-833 dataset training gene centroids applied to data from 2 other publicly available lung cancer gene expression databases (TCGA & A-334) for a 3 class prediction of lung tumor type. LOO cross validation was performed for the A-833 dataset.

	Prediction											
Histology Diagnosis	**TCGA RNAseq**				**Agilent**				**Affymetrix LOO**			
	AD	**NE**	**SQ**	**Sum**	**AD**	**NE**	**SQ**	**Sum**	**AD**	**NE**	**SQ**	**Sum**
Adenocarcinoma (AD)	419	29	29	468	141	6	27	174	399	3	88	490
Neuroendocrine (NE)	NA	NA	NA	NA	NA	NA	NA	NA	2	49	2	53
Squamous cell carcinoma (SQ)	23	15	445	483	28	3	117	148	25	7	195	227
Sum	442	44	465	951	169	9	144	322	426	59	285	770
% Agreement	**91%**				**80%**				**84%**			
kappa	**0.82**				**0.61**				**0.69**			

Table 10c. A-833 dataset training gene centroids applied to data from 2 other publicly available lung cancer gene expression databases (TCGA & A-334) for a 4 class prediction of lung tumor type. L00 cross validation was performed for the A-833 dataset.

	Prediction														
Histology Diagnosis	**TCGA RNAseq**					**Agilent**					**Affymetrix LOO**				
	AD	**CA**	**SC**	**SQ**	**Sum**	**AD**	**CA**	**SC**	**SQ**	**Sum**	**AD**	**CA**	**SC**	**SQ**	**Sum**
Adeno-carcinoma (AD)	428	2	20	18	468	138	2	5	29	174	389	1	3	97	490
Carcinoid (CA)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1	22	0	0	23
Small cell (SC)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	27	1	5	194	227
Squamous cell carcinoma (SQ)	23	2	15	443	483	27	0	3	118	148	27	1	5	194	227
Sum	451	4	35	461	951	165	2	8	147	322	418	25	28	293	764
% Agreement	**92%**					**80%**					**82%**				
kappa	**0.84**					**0.60**					**0.65**				

[00100] Evaluation of inter-observer reproducibility of lung cancer diagnosis based on morphologic classification alone has previously been published. Overall inter-observer agreement improved with simplification of the typing scheme. Using the comprehensive 2004 World Health Organization classification system inter-observer agreement was low (k = 0.25). Agreement improved with simplification of the diagnosis to the therapeutically relevant 2 type differentiation of squamous/non-squamous (k = 0.55). Agreement of inter-observer diagnosis is compared to agreement of 2, 3 and 4 class LSP diagnosis in this validation study (Table 11).

Table 11. Inter-observer agreement (3) measured using kappa statistic and LSP agreement with histologic diagnosis in multiple gene expression datasets.					
	WHO 2004 Classification	**2 class squamous/ nonsquamous cell carcinoma**		**3 class**	**4 class**
Agreement	Inter-observer agreement	Inter-observer agreement	LSP agreement w/ Hist Dx	LSP agreement w/ Hist Dx	LSP agreement w/ Hist Dx
Kappa	0.25	0.55	0.61-0.89	0.61-0.82	0.60-0.84

[00101] Differentiation among various morphologic subtypes of lung cancer is increasingly important as therapeutic development and patient management become more specifically targeted to unique features of each tumor. Histologic diagnosis can be challenging and several studies have demonstrated limited reproducibility of morphologic diagnoses. The addition of several immunohistochemistry markers, such as p63 and TTF-1 improves diagnostic precision but many lung cancer biopsies are limited in size and/or cellularity precluding full characterization using multiple IHC markers. Agreement was markedly better for all the classifiers (2, 3, and 4 type) in the TCGA RNAseq dataset (% agreement range 91%-94%) as compared to the other datasets possibly due to the greater accuracy of the histologic diagnosis and/or the greater precision of the RNA expression results. Despite several limitations described below, this study demonstrates that LSP, can be a valuable adjunct to histology in typing lung tumors.

[00102] In multiple datasets with hundreds of lung cancer samples, molecular profiling using the Lung Subtype Panel (LSP) compared favorably to light microscopic derived diagnoses, and showed a higher level of agreement than pathologist reassessments. RNA-based tumor subtyping can provide valuable information in the clinic, especially when tissue is limiting and the morphologic diagnosis remains unclear.

[00103] The disclosures of the following references are incorporated herein by reference in their entireties for all purposes:

a. American Cancer Society. Cancer Facts and Figures, 2014.

b. National Comprehensive Cancer Network (NCCN) Clinical Practice Guideline in Oncology. Non-Small Cell Lung Cancer. Version 2.2013.

c. Grilley Olson JE, Hayes DN, Moore DT, et al. Arch Pathol Lab Med 2013; 137: 32-40

d. Thunnissen E, Boers E, Heideman DA, et al. Virchows Arch 2012; 461:629-38.

e. Wilkerson MD, Schallheim JM, Hayes DN, et al. J Molec Diagn 2013; 15:485-497.

f. Li B, Dewey CN. BMC Bioinformatics 2011, 12:323 doi:10.1186/1471-2105-12-323

g. Yang YH, Dudoit S, Luu P, et al. Nucleic Acids Research 2002, 30:e15.

h. Hubbell E, Liu, W, Mei R. Bioinformatics (2002) 18 (12): 1585-1592. doi:10.1093/bioinformatics/18.12.1585.

i. Travis WD, Brambilla E, Muller-Hermelink HK, Harris CC. Pathology and Genetics of Tumors of the Lung, Pleura, Thymus, and Heart. 3rd ed. Lyon, France: IARC Press; 2004. World Health Organization Classification of Tumors: vol 10.

j. Travis WD and Rekhtman N.. Sem Resp and Crit Care Med 2011; 32(1): 22-31.

Example 2 – Lung Cancer Subtyping of Multiple Fresh Frozen and Formalin Fixed Paraffin Embedded Lung Tumor Gene Expression Datasets

[00104] Multiple datasets comprising 2,177 samples were assembled to evaluate a Lung Subtype Panel (LSP) gene expression classifier. The datasets included several publically available lung cancer gene expression data sets, including 2,099 Fresh Frozen lung cancer samples (TCGA, NCI, UNC, Duke, Expo, Seoul, and France) as well as newly collected gene expression data from 78 FFPE samples. Data sources are provided in the Table 12 below. The 78 FFPE samples were archived residual lung tumor samples collected at the University of North Carolina at Chapel Hill (UNC-CH) using an IRB approved protocol. Only samples with a definitive diagnosis of AD, carcinoid, Small Cell Carcinoma (SCC), or SQC were used in the analysis. A total of 4 categories of genomic data were available for analysis: Affymetrix U133+2 (n=693), Agilent 44K (n=344), Illumina® RNAseq (n=1,062) and newly collected qRT-PCR (n=78) data.

[00105] Archived FFPE lung tumor samples (n=78) were analyzed using a qRT-PCR gene expression assay as previously described (Wilkerson et al. J Molec Diagn 2013; 15:485-497,

incorporated by reference herein in its entirety for all purposes) with the following modifications. RNA was extracted from one 10 μm section of FFPE tissue using the High Pure RNA Paraffin Kit (Roche Applied Science, Indianapolis, IN). Extracted RNA was diluted to 5 ng/μL and first strand cDNA was synthesized using gene specific 3' primers in combination with random hexamers (Superscript III®, Invitrogen®, Thermo Fisher Scientific Corp, Waltham, MA). An ABI 7900 (Applied Biosystems, Thermo Fisher Scientific Corp, Waltham, MA) was used for qRT-PCR with continuous SYBR green fluorescence (530nm) monitoring. ABI 7900 quantitation software generated amplification curves and associated threshold cycle (Ct) values. Original clinical diagnoses gathered with the samples is in Table 13.

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Table 12

Source	Platforms	N	Subtype	Normalization Method Used	Data Source
TCGA	RNASeq (LUAD)	528	adenocarcinomas	RSEM expression estimates are normalized to set the upper quartile count at 1000 for gene level, 2 based log transformed, data matrix is row (gene) median centered, column (sample) standardized.[28]	Ref 16 TCGA
TCGA	RNASeq (LUSC)	534	squamous cell carcinoma		Ref 15 TCGA
UNC	Agilent_44K	56	squamous cell carcinoma	2 based log ratio of the two channel intensities are LOWESS normalized, data matrix is row (gene) median centered, column (sample) standardized [25]	Ref 19 GSE17710
UNC	Agilent_44K	116	adenocarcinomas		Ref 20 GSE36939
NCI	Agilent_44K	172	adenocarcinoma, squamous cell, & large cell		Ref 22 http://research.agendia.com/
Korea	HG-U133+2	132	adenocarcinoma, squamous cell carcinoma	MAS5 normalized one channel intensitis are 2 based log transformed, data matrix is row (gene) median centered, column (sample) standardized [30]	Ref 23 GSE8894
Expo	HG-U133+2	130	all histology subtypes		Ref 24 GSE2109
French	HG-U133+2	307	all histology subtypes		Ref 25 GSE30219
Duke	HG-U133+2	118	adenocarcinoma, squamous cell carcinoma		Ref 26 GSE3141
UNC	FFPE tissue RT-PCR	78	adenocarcinoma, squamous cell carcinoma, small cell, & carcinoid	FFPE sample gene expression data was scaled to align gene variance with Wilkerson et al. data[21]. A gene-specific scaling factor was calculated that took into account label frequency differences between the data sets.	Ref 27 Supplemental File # 1

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Table 13	
Sample	Label
VELO001	Squamous.Cell.Carcinoma
VELO002	Squamous.Cell.Carcinoma
VELO004	Adenocarcinoma
VELO006	Squamous.Cell.Carcinoma
VELO007	Squamous.Cell.Carcinoma
VELO008	Squamous.Cell.Carcinoma
VELO010	Squamous.Cell.Carcinoma
VELO011	Squamous.Cell.Carcinoma
VELO012	Squamous.Cell.Carcinoma
VELO013	Squamous.Cell.Carcinoma
VELO014	Squamous.Cell.Carcinoma
VELO015	Adenocarcinoma
VELO016	Squamous.Cell.Carcinoma
VELO017	Squamous.Cell.Carcinoma
VELO018	Squamous.Cell.Carcinoma
VELO019	Squamous.Cell.Carcinoma
VELO020	Adenocarcinoma
VELO021	Adenocarcinoma
VELO022	Adenocarcinoma
VELO023	Adenocarcinoma
VELO024	Adenocarcinoma
VELO025	Adenocarcinoma
VELO026	Adenocarcinoma
VELO027	Adenocarcinoma
VELO028	Adenocarcinoma
VELO029	Adenocarcinoma
VELO030	Adenocarcinoma
VELO031	Adenocarcinoma
VELO032	Adenocarcinoma
VELO033	Adenocarcinoma
VELO034	Adenocarcinoma
VELO035	Adenocarcinoma
VELO036	Adenocarcinoma
VELO037	Adenocarcinoma
VELO038	Squamous.Cell.Carcinoma
VELO039	Squamous.Cell.Carcinoma
VELO040	Squamous.Cell.Carcinoma
VELO042	Squamous.Cell.Carcinoma
VELO044	Squamous.Cell.Carcinoma
VELO046	Squamous.Cell.Carcinoma
VELO048	Squamous.Cell.Carcinoma
VELO049	Squamous.Cell.Carcinoma

Table 13	
VELO050	Adenocarcinoma
VELO041	Squamous.Cell.Carcinoma
VELO043	Squamous.Cell.Carcinoma
VELO045	Squamous.Cell.Carcinoma
VELO055	Neuroendocrine
VELO056	Neuroendocrine
VELO057	Neuroendocrine
VELO058	Neuroendocrine
VELO059	Neuroendocrine
VELO060	Neuroendocrine
VELO061	Neuroendocrine
VELO062	Neuroendocrine
VELO063	Neuroendocrine
VELO064	Neuroendocrine
VELO065	Neuroendocrine
VELO066	Neuroendocrine
VELO067	Neuroendocrine
VELO068	Neuroendocrine
VELO069	Neuroendocrine
VELO070	Neuroendocrine
VELO071	Neuroendocrine
VELO072	Neuroendocrine
VELO073	Neuroendocrine
VELO074	Neuroendocrine
VELO075	Neuroendocrine
VELO076	Neuroendocrine
VELO077	Neuroendocrine
VELO078	Neuroendocrine
VELO079	Neuroendocrine
VELO080	Neuroendocrine
VELO081	Neuroendocrine
VELO082	Neuroendocrine
VELO083	Neuroendocrine
VELO084	Neuroendocrine
VELO085	Neuroendocrine

[00106] Pathology review was only possible for the FFPE lung tumor cohort in which additional sections were collected and imaged. Two contiguous sections from each sample were Hematoxylin & Eosin (H&E) stained and scanned using an Aperio™ ScanScope® slide scanner (Aperio Technologies, Vista, CA). Virtual slides were viewable at magnifications equivalent to 32 to 320 objectives (340 magnifier). Pathologist review was blinded to the

original clinical diagnosis and to the gene expression-based subtype classification. Pathology review-based histological subtype calls were compared to the original diagnosis (n=78). Agreement of pathology review was defined as those samples for which both slides were assigned the same subtype as the original diagnosis.

[00107] All statistical analyses were conducted using R 3.0.2 software (http://cran.R-project.org). Data analyses were conducted separately for FF and for FFPE tumor samples.

[00108] Fresh Frozen Dataset Analysis: Datasets were normalized as described in Table 12. The Affymetrix dataset served as the training set for calculation of AD, carcinoid, SCC, and SQC gene centroids according to methods described previously (Wilkerson et al. PLoS ONE. 2012; 7(5) e36530. Doi:10.1371/journal.pone.0036530; Wilkerson et al. J Molec Diagn 2013; 15:485-497, each of which is incorporated by reference herein in its entirety for all purposes)

[00109] Affymetrix training gene centroids are provided in Table 14. The training set gene centroids were tested in normalized TCGA RNAseq gene expression and Agilent microarray gene expression data sets. Due to missing data from the public Agilent dataset, the Agilent evaluations were performed with a 47 gene classifier, rather than a 52 gene panel with exclusion of the following genes: *CIB1 FOXH1, LIPE, PCAM1, TUBA1*.

Table 14.

Gene	Adenocarcinoma	Neuroendocrine	Squamous.Cell.Carcinoma
ABCC5	-0.453	0.3715	1.1245
ACVR1	0.0475	0.3455	-0.0465
ALDH3B1	0.4025	-0.638	-0.401
ANTXR1	-0.0705	-0.478	0.014
BMP7	-0.532	-0.6265	0.6245
CACNB1	0.024	0.157	-0.039
CAPG	0.109	-1.9355	-0.0605
CBX1	-0.2045	0.745	0.187
CDH5	0.391	0.145	-0.352
CDKN2C	-0.0045	1.496	0.004
CHGA	-0.143	5.7285	0.1075
CIB1	0.1955	-0.261	-0.065
CLEC3B	0.449	0.6815	-0.3085
CYB5B	0.058	1.487	-0.03
DOK1	0.233	-0.355	-0.183
DSC3	-0.781	-0.8175	4.3445

Table 14.			
Gene	**Adenocarcinoma**	**Neuroendocrine**	**Squamous.Cell.Carcinoma**
FEN1	-0.5025	-0.0195	0.4035
FOXH1	-0.0405	0.1315	-0.0105
GJB5	-1.388	-1.5505	0.7685
HOXD1	0.17	-0.462	-0.288
HPN	0.5335	0.444	-0.736
HYAL2	0.1775	0.073	-0.143
ICA1	0.3455	1.048	-0.233
ICAM5	0.13	-0.145	-0.12
INSM1	0.0705	7.5695	-0.0245
ITGA6	-0.709	0.029	1.074
LGALS3	0.1805	-1.1435	-0.2305
LIPE	0.0065	0.5225	-0.0015
LRP10	0.2565	-0.087	-0.16
MAPRE3	-0.0245	0.6445	-0.0025
ME3	0.3085	0.3415	-0.2915
MGRN1	0.429	0.8075	-0.3775
MYBPH	0.04	-0.193	-0.054
MYO7A	0.083	-0.287	-0.109
NFIL3	-0.332	-1.0425	0.3095
PAICS	-0.2145	0.3915	0.2815
PAK1	-0.112	0.6095	0.0965
PCAM1	0.232	-0.256	-0.144
PIK3C2A	0.1505	0.597	-0.021
PLEKHA6	0.4465	2.0785	-0.2615
PSMD14	-0.251	0.5935	0.1635
SCD5	-0.1615	0.06	0.13
SFN	-0.789	-3.026	0.91
SIAH2	-0.5795	0.1895	0.7175
SNAP91	-0.0255	3.818	0.003
STMN1	-0.0995	1.2095	0.1405
TCF2	0.2835	-0.5175	-0.4665
TCP1	-0.1685	0.9815	0.1985
TFAP2A	-0.374	-0.5075	0.3645
TITF1	1.482	0.1525	-1.2755
TRIM29	-1.0485	-1.318	1.379
TUBA1	0.155	1.71	-0.07

Table 15.			
Gene	**Adenocarcinoma**	**Neuroendocrine**	**Squamous.Cell.Carcinoma**
ABCC5	-1.105993	0.53584995	0.28498017
ACVR1	-0.1780792	0.27746814	-0.1331305

Table 15.			
Gene	Adenocarcinoma	Neuroendocrine	Squamous.Cell.Carcinoma
ALDH3B1	2.21915126	-1.0930042	0.82709803
ANTXR1	0.14704523	-0.0027417	-0.1000265
CACNB1	-0.2032444	0.36015235	-0.7588385
CAPG	0.52784999	-0.6495988	-0.0218352
CBX1	-0.5905845	-0.0461076	-0.2776489
CDH5	-0.1546498	0.53564677	-0.9166437
CDKN2C	-1.8382992	-0.1614815	-0.7501799
CHGA	-6.2702431	8.18090411	-7.4497926
CIB1	0.29948877	-0.1804507	0.06141265
CLEC3B	0.1454466	0.86221597	-0.6686516
CYB5B	-0.1957799	0.13060667	-0.2393801
DOK1	0.03629227	0.03029676	-0.2861762
DSC3	0.76811006	-2.2230482	4.45353398
FEN1	-0.4100344	-0.774919	0.19244803
FOXH1	1.36365962	-1.1539159	1.86758359
GJB5	2.19942372	-3.2908475	4.00132739
HOXD1	-0.069692	-0.3296808	0.50430984
HPN	0.62232864	-0.0416111	-0.5391064
HYAL2	0.47459315	-0.2332929	-0.0080073
ICA1	-0.8108302	1.25305275	-2.1742476
ICAM5	2.12506546	-2.2078991	2.89691121
INSM1	-2.4346556	1.92393374	-1.9749654
ITGA6	-0.7881662	0.36443897	0.54978058
LGALS3	-0.8270046	0.79512054	-0.9453521
LIPE	-0.2519692	0.29291064	-0.2216243
LRP10	0.09504093	0.14082188	-0.4042101
MAPRE3	-0.6806204	1.2417945	-0.5496704
ME3	0.17668171	0.67674964	-1.581183
MGRN1	-0.0839601	0.35069923	-0.6885404
MYBPH	0.73519429	-0.9569161	1.14344753
MYO7A	0.58098661	-0.2096425	0.0488886
NFIL3	0.22274434	-0.337858	0.66234639
PAICS	-0.2423309	-0.1863934	0.39037381
PAK1	-0.3803406	0.15627507	0.0677904
PCAM1	0.03655586	0.32457357	-0.6957339
PIK3C2A	-0.3868824	0.56861416	-0.6629455
PLEKHA6	-0.4007847	1.31002812	-1.9802266
PSMD14	-0.5115938	0.27513479	-0.2847234
SCD5	-0.4770619	-0.4338812	0.56043153
SFN	0.35719248	-1.4361124	2.34498532
SIAH2	-0.4222382	-0.3853078	0.43237756
SNAP91	-5.5499562	4.65742276	-2.5441741

Table 15.			
Gene	Adenocarcinoma	Neuroendocrine	Squamous.Cell.Carcinoma
STMN1	-1.4075058	0.49776156	-1.017481
TCF2	1.96819785	-0.4121173	-0.6555613
TCP1	-2.9255287	2.322428	-2.3059797
TFAP2A	2.02528144	-2.9053184	3.62844763
TITF1	0.46476685	-9.82E-05	-1.7079242
TRIM29	-1.6554559	-0.6463626	2.94818107
TUBA1	1.77126501	-2.0395783	1.58902579

[00110] Evaluation of the Affymetrix data was performed using Leave One Out (LOO) cross validation. Spearman correlations were calculated for tumor test sample to the Affymetrix gene expression training centroids. Tumors were assigned a genomic-defined histologic type (AD, SQC, or NE) corresponding to the maximally correlated centroids. Correct predictions were defined as LSP calls matching the tumor's original histologic diagnosis. Percent agreement was defined as the number of correct predictions divided by the number of total predictions and an agreement kappa statistic was calculated.

[00111] qRT-PCR from FFPE sample analysis: Previously published training centroids (Wilkerson et al. J Molec Diagn 2013; 15:485-497, incorporated by reference herein), calculated from qRT-PCR data of FFPE lung tumor samples, were cross-validated in this new sample set of qRT-PCR gene expression from FFPE lung tumor tissue. Wilkerson et al. AD and SQC centroids were used as published (Wilkerson et al. J Molec Diagn 2013; 15:485-497, incorporated by reference herein). Neuroendocrine gene centroids were calculated similarly using published gene expression data (n=130) (Wilkerson et al. J Molec Diagn 2013; 15:485-497, incorporated by reference herein). The Wilkerson et al. gene centroids (Wilkerson et al. J Molec Diagn 2013; 15:485-497, incorporated by reference herein) for the FFPE tissue evaluation are included in Table 15. FFPE sample gene expression data was scaled to align gene variance with Wilkerson et al. data. A gene-specific scaling factor was calculated that took into account label frequency differences between the data sets. Gene expression data was then median centered, sign flipped (high Ct = low abundance), and scaled using the gene specific scaling factor. Subtype was predicted by correlating each sample with the 3 subtype centroids and assignment of the subtype with the highest correlation centroid (Spearman correlation).

[00112] Ten lung tumor gene expression datasets including nine FF plus one new FFPE qRT-PCR gene expression dataset were combined into four platform-specific data sets (Affymetrix, Agilent, Illumina RNAseq, and qRT-PCR). For the datasets where clinical information was available, the patient population was diverse and included smokers and nonsmokers with tumors ranging from Stage 1 – Stage IV. Sample characteristics and lung cancer diagnoses of the datasets used in this study are included in Table 16. After exclusion of samples without a definitive diagnosis of AD, SQC, SCC, or carcinoid, and exclusion of 1 FFPE sample that failed qRT-PCR analysis, the following samples were available for further data analysis: Affymetrix (n=538), Agilent (n=322), Illumina RNAseq (n=951) and qRT-PCR (n=77).

Table 16.

Characteristic	TCGA RNA seq	Agilent	Affymetrix	UNC FFPE
Total # of samples	1062	344	693	78
Tissue Preservation	Fresh Frozen	Fresh Frozen	Fresh Frozen	FFPE
Tumor specimen histology				
Adenocarcinoma	468	174	264	21
Carcinoid	0	0	23	15
Small Cell Carcinoma	0	0	24	16
Squamous Cell Carcinoma	483	148	227	25
Other(excluded from analysis)	111	22	155	01
Gender				
Female/Male/NA	285/366/300	87/85/150	151/386/1	NA
Age at Diagnosis				
Median/(Range)	67/(38-88)	66/(37-90)	65/(13-85)	NA
Age not available	323	0	2	NA
Stage				
I	355	NA	NA	NA
II	146	NA	NA	NA
III	119	NA	NA	NA
IV	26	NA	NA	NA
Stage not available	305	322	538	77
Smoking				
Smoker	386	NA	NA	NA
Nonsmoker	39	NA	NA	NA
Smoking status not available	526	322	538	77

[00113] As a means of de novo evaluation of the new FFPE data set, we performed hierarchical clustering of LSP gene expression from the FFPE archived samples (n=77); as

expected, this analysis demonstrated three clusters/subtypes corresponding to AD, SQC, and NE (Figure 2). The predetermined LSP 3-subtype centroid predictor was then applied to all 4 datasets, and results were compared with tumor morphologic classifications. Percent agreement and Fleiss' kappa were calculated for each dataset (Table 17). The percent agreement ranged from 78% - 91% and kappa's from 0.57 – 0.85.

[00114] As another means of assessing independent pathology agreement, the agreement of blinded pathology review of the 77 FFPE lung tumors with the original morphologic diagnosis was found to be 82% (63/77). In 12/77 cases, blinded duplicate slides provided conflicting results and in 10/77 cases, at least one of the duplicates had a non-definitive pathological subtype classification of "Adenosquamous", "Large Cell", or "High grade poorly differentiated carcinoma". Comparison of the original morphologic diagnosis, blinded pathology review, and gene expression LSP subtype call for each of the 77 samples is shown in Figure 3. Details of discordant sample overlap (*i.e.*, 6 samples where tumor subtype disagreed with original morphology diagnosis by both path review and gene expression LSP call) are provided in Table 18. Overall, these concordance values of LSP relative to the original pathology calls were at least as great as the concordance between any two pathologists (Grilley et al. Arch Pathol Lab Med 2013; 137: 32-40; Thunnissen et al. Virchows Arch 2012; 461(6):629-38. Doi: 10.1007/s00428-012-1234-x. Epub 2012 Oct 12; Thunnissen et al. Mod Pathol 2012; 25(12):1574-83. Doi: 10.1038/modpathol.2012.106; each of which is incorporated by reference herein for all purposes) thus suggesting that the assay described herein performs at least as well as a trained pathologist.

[00115] In this study, LSP provided reliable subtype classifications, validating its performance across multiple gene expression platforms, and even when using FFPE specimens. Hierarchical clustering of the newly assayed FFPE samples demonstrated good separation of the 3 subtypes (AC, SQC, and NE) based on the levels of 52 classifier biomarkers. Concordance with morphology diagnosis when using the LSP centroids was greatest in the TCGA RNAseq dataset (agreement = 91%), possibly due to the very extensive pathology review and accuracy of the histologic diagnosis associated with TCGA samples as compared to other datasets. Agreement was lowest (78%) in the Agilent dataset, which may have been affected by the reduced number of genes that were available for that analysis. Overall, the LSP assay displayed a higher concordance with the original morphology

diagnosis than the pathology review in all datasets except in the Agilent dataset, in which only 47 genes, rather than 52, were present for the analysis.

[00116] In the FFPE samples where blinded pathology re-review was possible, results suggested that pathology calls were not always consistent with the original diagnosis, nor were they necessarily consistent in the duplicate slides provided from each sample. For a subset of samples (n=6), both the pathology re-review and the LSP gene expression analysis suggested the same alternate diagnosis, leading one to question the accuracy of the original morphologic diagnosis, which was our "gold standard".

[00117] In this study, there were a low number of NE tumor samples in the Affymetrix dataset, and an absence of NE samples in both the Agilent and TCGA datasets. This was partially overcome by a relatively high number of NE samples in the FFPE sample set (31/77), thus providing a good test of the LSP signature's ability to identify NE samples. Another limitation of the study relates to the blinded pathology re-review. The blinded pathology review was based on two imaged sections and did not reflect usual histology standard practice where multiple sections/blocks and potentially IHC stains would have been available to make a diagnosis.

* * * * * * *

[00118] The various embodiments described above can be combined to provide further embodiments. All of the U.S. patents, U.S. patent application publications, U.S. patent application, foreign patents, foreign patent application and non-patent publications referred to in this specification and/or listed in the Application Data Sheet are incorporated herein by reference, in their entirety. Aspects of the embodiments can be modified, if necessary to employ concepts of the various patents, application and publications to provide yet further embodiments.

[00119] These and other changes can be made to the embodiments in light of the above-detailed description. In general, in the following claims, the terms used should not be construed to limit the claims to the specific embodiments disclosed in the specification and the claims, but should be construed to include all possible embodiments along with the full scope of equivalents to which such claims are entitled. Accordingly, the claims are not limited by the disclosure.

What is claimed is:

1. A method of assessing whether a patient's lung cancer subtype is adenocarcinoma, squamous cell carcinoma, or neuroendocrine (encompassing both small cell carcinoma and carcinoid), comprising,

(a) probing the levels of at least five classifier biomarkers of Table 1A, Table 1B, Table 1C, Table 2, Table 3, Table 4, Table 5 or Table 6 in a lung cancer sample obtained from the patient at the nucleic acid level, wherein the probing step comprises;

(i) mixing the sample with five or more oligonucleotides that are substantially complementary to portions of nucleic acid molecules of the at least five classifier biomarkers of Table 1A, Table 1B, Table 1C, Table 2, Table 3, Table 4, Table 5 or Table 6 under conditions suitable for hybridization of the five or more oligonucleotides to their complements or substantial complements;

(ii) detecting whether hybridization occurs between the five or more oligonucleotides to their complements or substantial complements;

(iii) obtaining hybridization values of the at least five classifier biomarkers based on the detecting step;

(b) comparing the hybridization values of the at least five classifier biomarkers to reference hybridization value(s) from at least one sample training set, wherein the at least one sample training set comprises hybridization values from a reference adenocarcinoma sample, hybridization values from a reference squamous cell carcinoma sample, hybridization values from a reference neuroendocrine sample, or a combination thereof;

classifying the lung cancer sample as a adenocarcinoma, squamous cell carcinoma, or a neuroendocrine subtype based on the results of the comparing step.

2. A method of assessing whether a patient's lung cancer subtype is adenocarcinoma, squamous cell carcinoma, small cell carcinoma or carcinoid, comprising,

(a) probing the levels of at least five classifier biomarkers of Table 1A, Table 1B, Table 1C, Table 2, Table 3, Table 4, Table 5 or Table 6 in a lung cancer sample obtained from the patient at the nucleic acid level, wherein the probing step comprises;

(i) mixing the sample with five or more oligonucleotides that are substantially complementary to portions of nucleic acid molecules of the at least five classifier biomarkers of Table 1A, Table 1B, Table 1C, Table 2, Table 3, Table 4,

Table 5 or Table 6 under conditions suitable for hybridization of the five or more oligonucleotides to their complements or substantial complements;

(ii) detecting whether hybridization occurs between the five or more oligonucleotides to their complements or substantial complements;

(iii) obtaining hybridization values of the at least five classifier biomarkers based on the detecting step;

(b) comparing the hybridization values of the at least five classifier biomarkers to reference hybridization value(s) from at least one sample training set, wherein the at least one sample training set comprises hybridization values from a reference adenocarcinoma sample, hybridization values from a reference squamous cell carcinoma sample, hybridization values from a reference small cell carcinoma sample, hybridization values from a reference carcinoid sample, or a combination thereof;

classifying the lung cancer sample as a adenocarcinoma, squamous cell carcinoma, small cell carcinoma or carcinoid subtype based on the results of the comparing step.

3. The method of claim 1 or 2, wherein the comparing step comprises determining a correlation between the hybridization values of the at least five classifier biomarkers and the reference hybridization values.

4. The method of claim 1 or 2, wherein the comparing step further comprises determining an average expression ratio of the at least five biomarkers and comparing the average expression ratio to an average expression ratio of the at least five biomarkers, obtained from the references values in the sample training set.

5. The method of any one of claims 1-4, wherein the probing step comprises isolating the nucleic acid or portion thereof prior to the mixing step.

6. The method of any one of claims 1-5, wherein the hybridization comprises hybridization of a cDNA probe to a cDNA biomarker, thereby forming a non-natural complex.

7. The method of any one of claims 1-5, wherein the hybridization comprises hybridization of a cDNA probe to an mRNA biomarker, thereby forming a non-natural complex.

8. The method of any one of claims 1-6, wherein the probing step comprises amplifying the nucleic acid in the sample.

9. The method of any one of claims 1-8, wherein the at least five classifier biomarkers comprise at least 10 biomarkers, at least 20 biomarkers or at least 30 biomarkers of Table 1A, Table 1B or Table 1C.

10. The method of any one of claims 1-8, wherein the at least five classifier biomarkers comprise at least 10 biomarkers, at least 20 biomarkers or at least 30 biomarkers of Table 2.

11. The method of any one of claims 1-8, wherein the at least five classifier biomarkers comprise at least 10 biomarkers, at least 20 biomarkers or at least 30 biomarkers of Table 3.

12. The method of any one of claims 1-8, wherein the at least five classifier biomarkers comprise the 6 biomarkers of Table 4.

13. The method of any one of claims 1-8, wherein the at least five classifier biomarkers comprise the 6 biomarkers of Table 5.

14. The method of any one of claims 1-8, wherein the at least five classifier biomarkers comprise at least 10 biomarkers, at least 20 biomarkers or at least 30 biomarkers of Table 6.

15. The method of any one of claims 1-8, wherein the at least five classifier biomarkers comprise from about 5 to about 20 classifier biomarkers, from about 10 to about 30 classifier biomarkers, from about 10 to about 40 classifier biomarkers, or from about 20 to about 40 classifier biomarkers of Table 1A, Table 1B or Table 1C.

16. The method of any one of claims 1-8, wherein the at least five classifier biomarkers comprise from about 5 to about 20 classifier biomarkers, from about 10 to about 30 classifier biomarkers, from about 10 to about 40 classifier biomarkers, or from about 20 to about 40 classifier biomarkers of Table 2.

17. The method of any one of claims 1-8, wherein the at least five classifier biomarkers comprise from about 5 to about 20 classifier biomarkers, from about 10 to about 30 classifier biomarkers, from about 10 to about 40 classifier biomarkers, or from about 20 to about 40 classifier biomarkers of Table 3.

18. The method of any one of claims 1-8, wherein the at least five classifier biomarkers comprise from about from about 5 to about 30 classifier biomarkers, from about 10 to about 30 classifier biomarkers, from about 50 to about 20 classifier biomarkers, or from about 20 to about 30 classifier biomarkers of Table 6.

19. The method of any one of claims 1-8, wherein the at least five classifier biomarkers comprise each of the classifier biomarkers set forth in Table 1A, Table 1B or Table 1C.

20. The method of any one of claims 1-8, wherein the at least five classifier biomarkers comprise each of the classifier biomarkers set forth in Table 2.

21. The method of any one of claims 1-8, wherein the at least five classifier biomarkers comprise each of the classifier biomarkers set forth in Table 3.

22. The method of any one of claims 1-8, wherein the at least five classifier biomarkers comprise each of the classifier biomarkers set forth in Table 6.

23. The method of any one of claims 1-22, wherein the sample comprises lung cells embedded in paraffin.

24. The method of any one of claims 1-22, wherein the sample is a fresh frozen sample.

25. The method according to any one of claims 1-22, wherein the lung tissue sample is selected from a formalin-fixed, paraffin-embedded (FFPE) lung tissue sample, fresh and a frozen tissue sample.

26. The method of claim 19, wherein the at least five classifier biomarkers comprise each of the classifier biomarkers set forth in Table 1A.

27. The method of claim 19, wherein the at least five classifier biomarkers comprise each of the classifier biomarkers set forth in Table 1B.

28. The method of claim 19, wherein the at least five classifier biomarkers comprise each of the classifier biomarkers set forth in Table 1C.

29. The method of any one of claims 1-8 and 23-25, wherein the at least five classifier biomarkers are the classifier biomarkers set forth in Table 14.

30. The method of any one of claims 1-8 and 23-25, wherein the at least five classifier biomarkers are the classifier biomarkers set forth in Table 15.

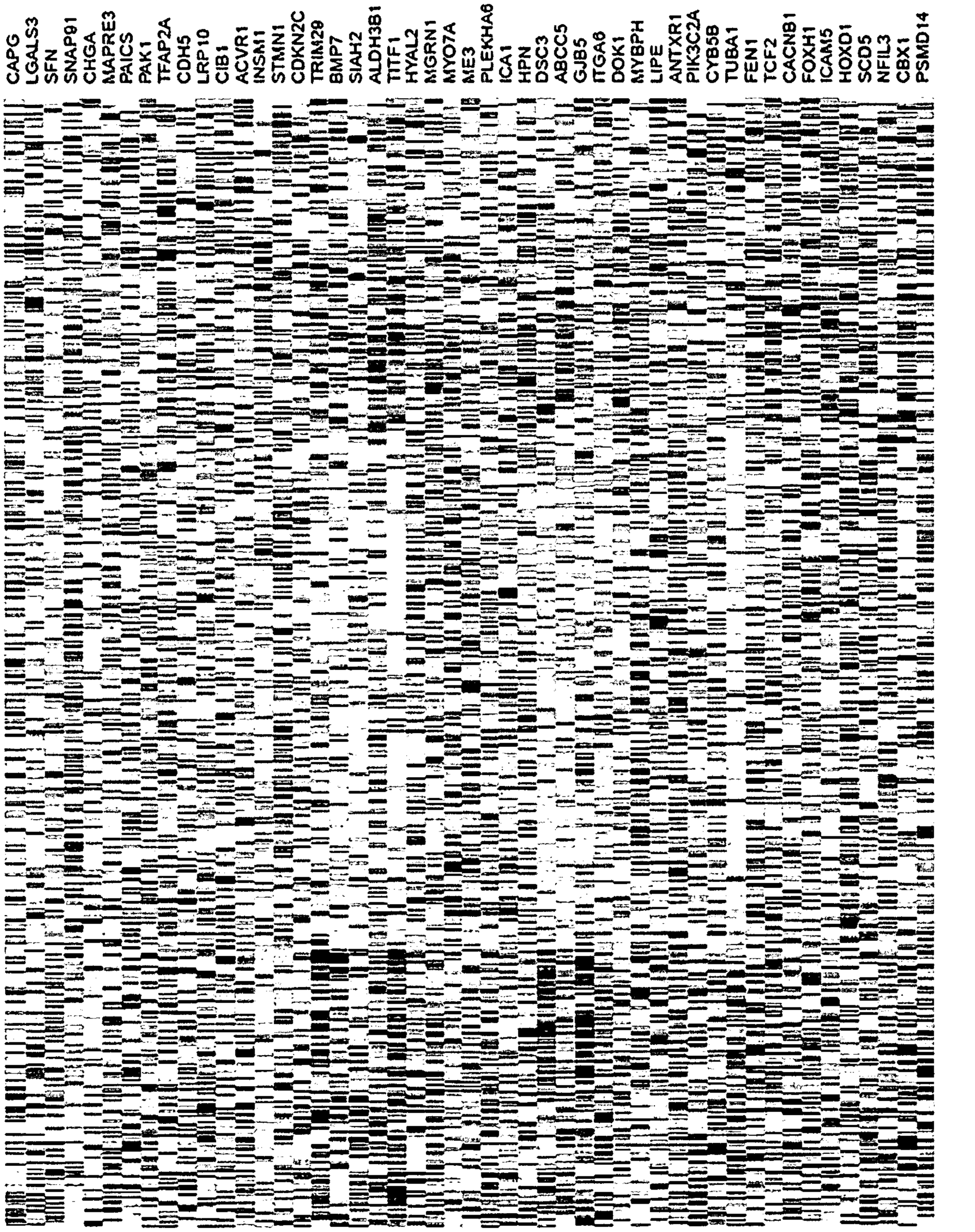
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Figure 1A
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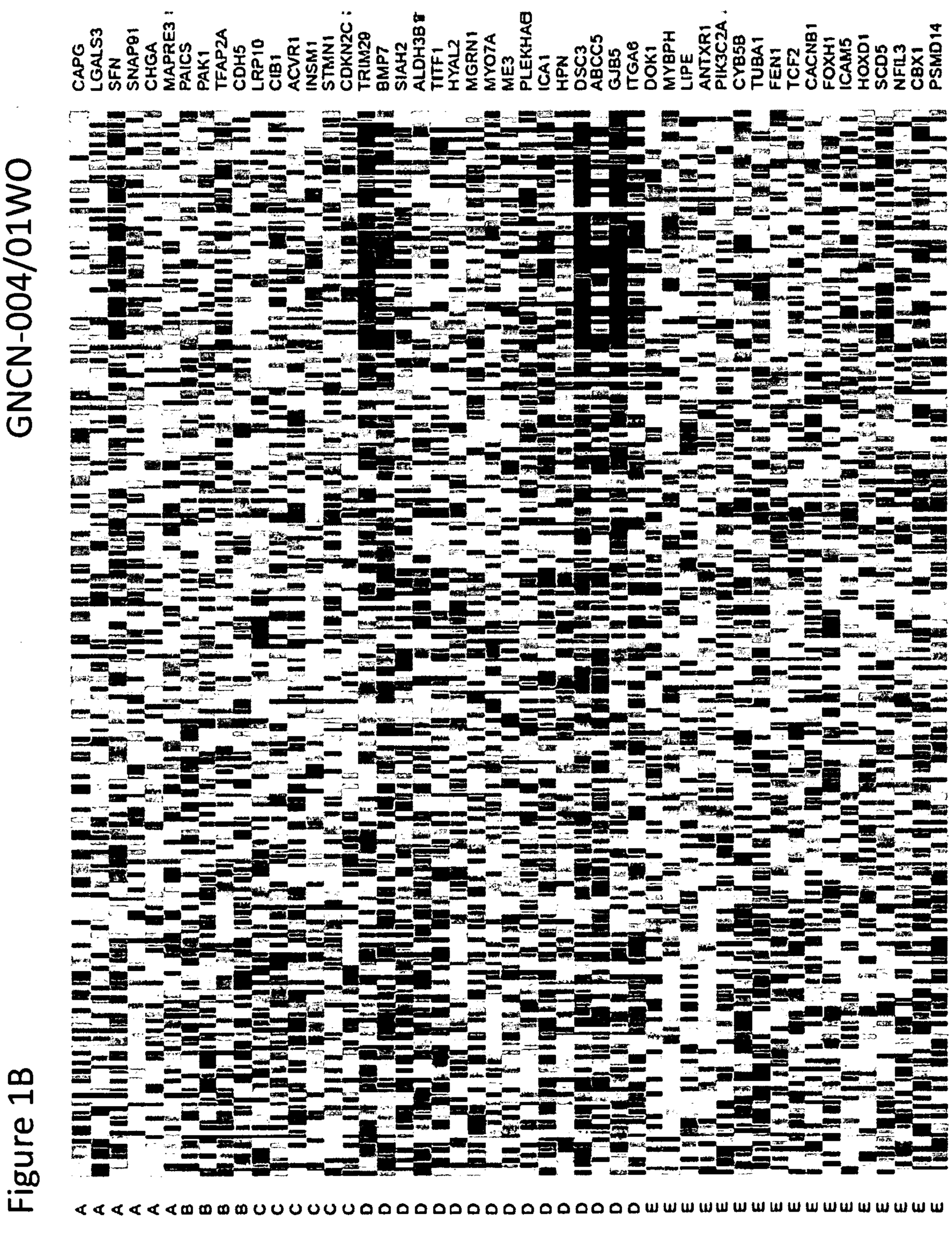
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Figure 1B

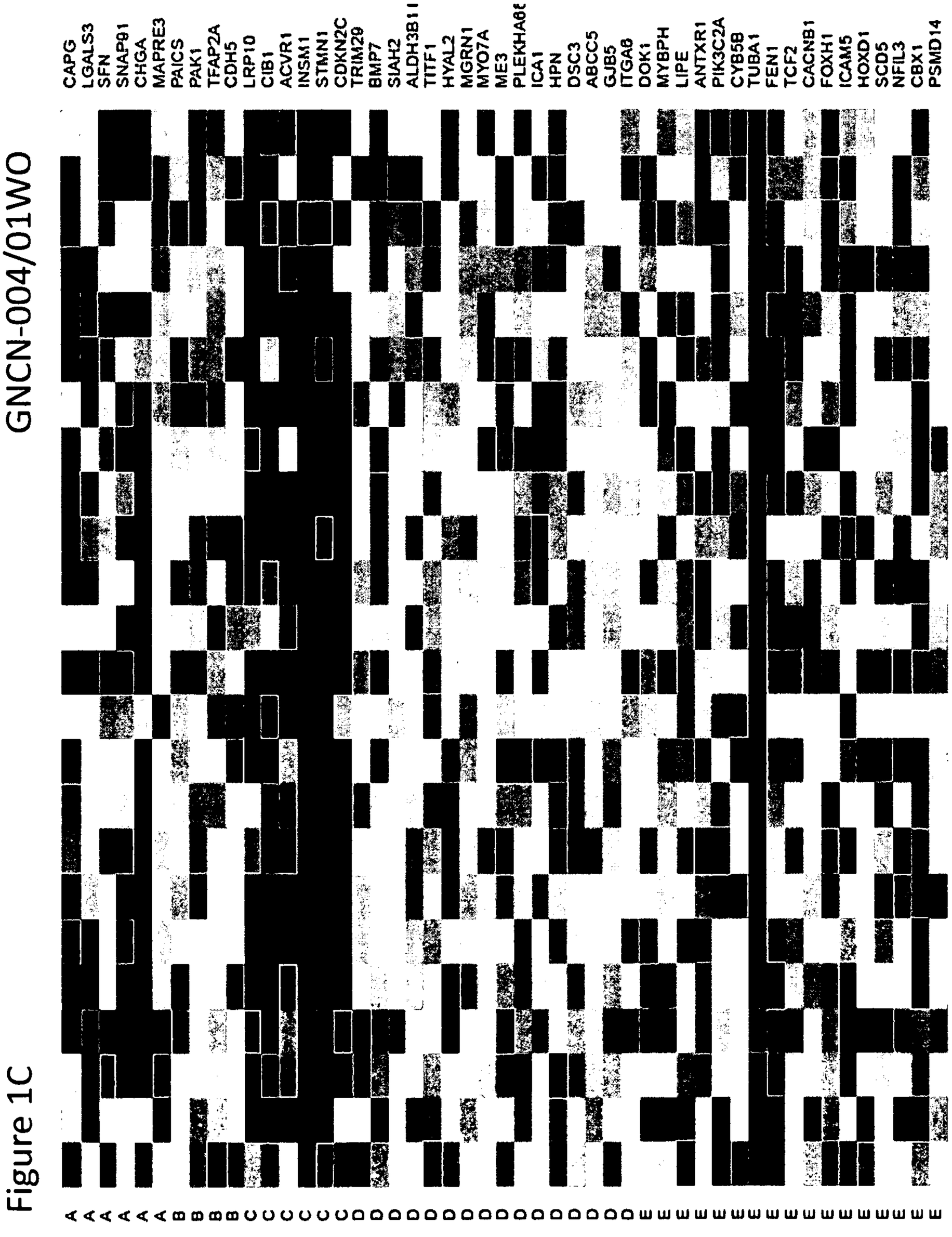
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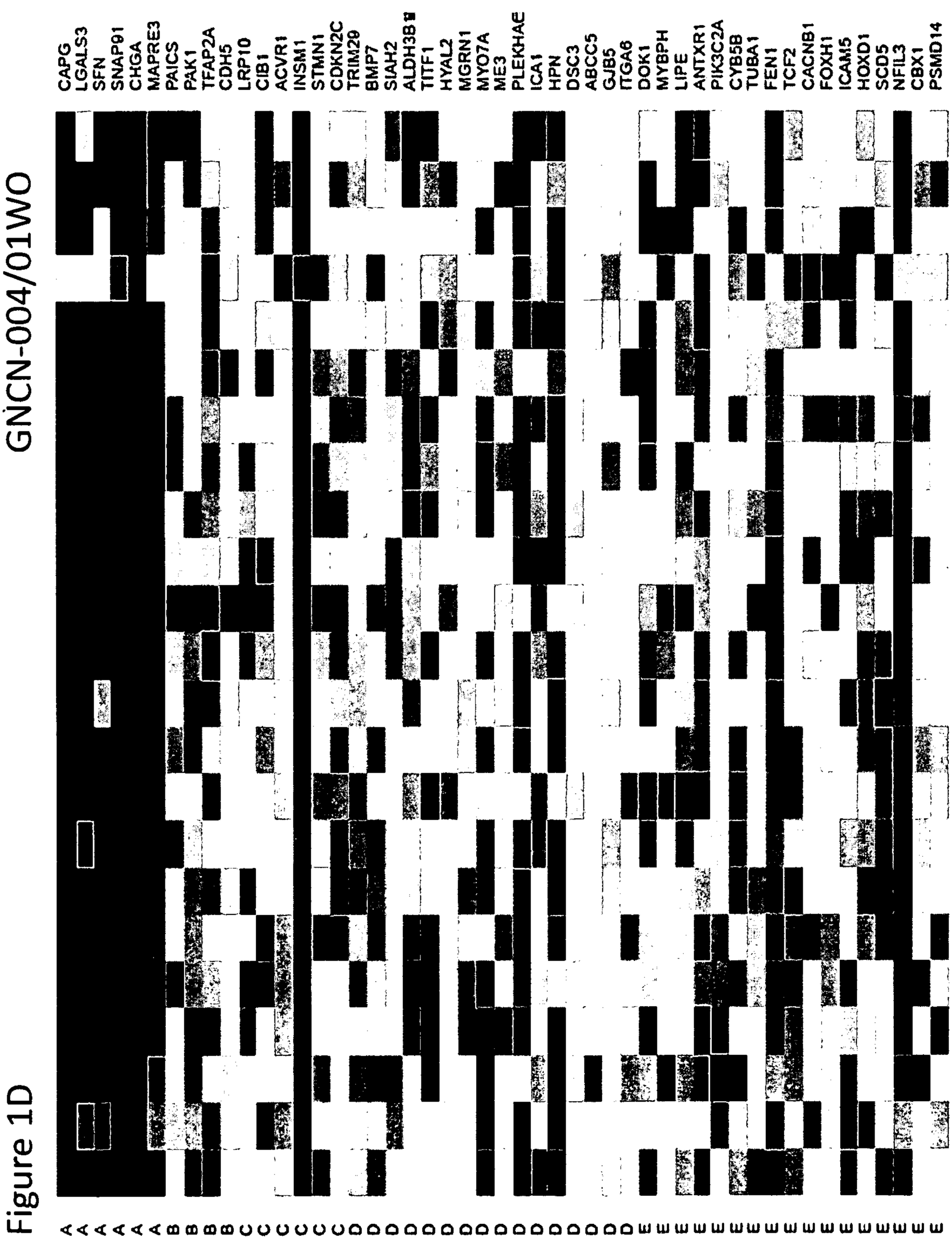
Figure 1C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MGRN1
MYO7A
ME3
PLEKHAE
ICA1
HPN
DSC3
ABCC5
GJB5
ITGA6
DOK1
MYBPH
LIPE
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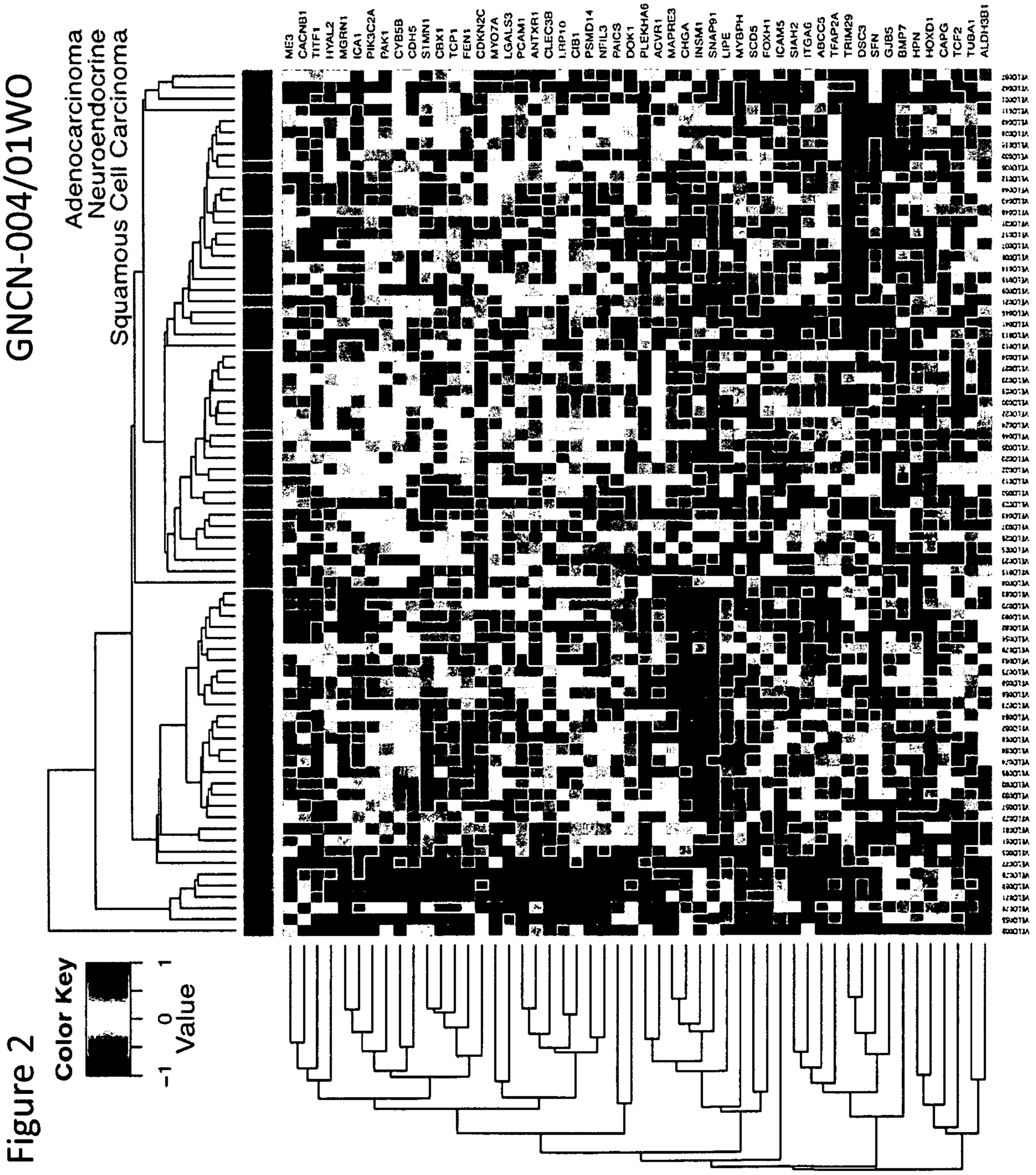
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Squamous Cell Carcinoma
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PAK1
CYB5B
CDH5
S1MN1
CBX1
TCP1
FEN1
CDKN2C
MYO7A
LGALS3
PCAM1
ANTXR1
CLEC3B
LRP10
CIB1
PSMD14
NFIL3
PAIC3
DOK1
PLEKHA6
ACVR1
MAPRE3
CHGA
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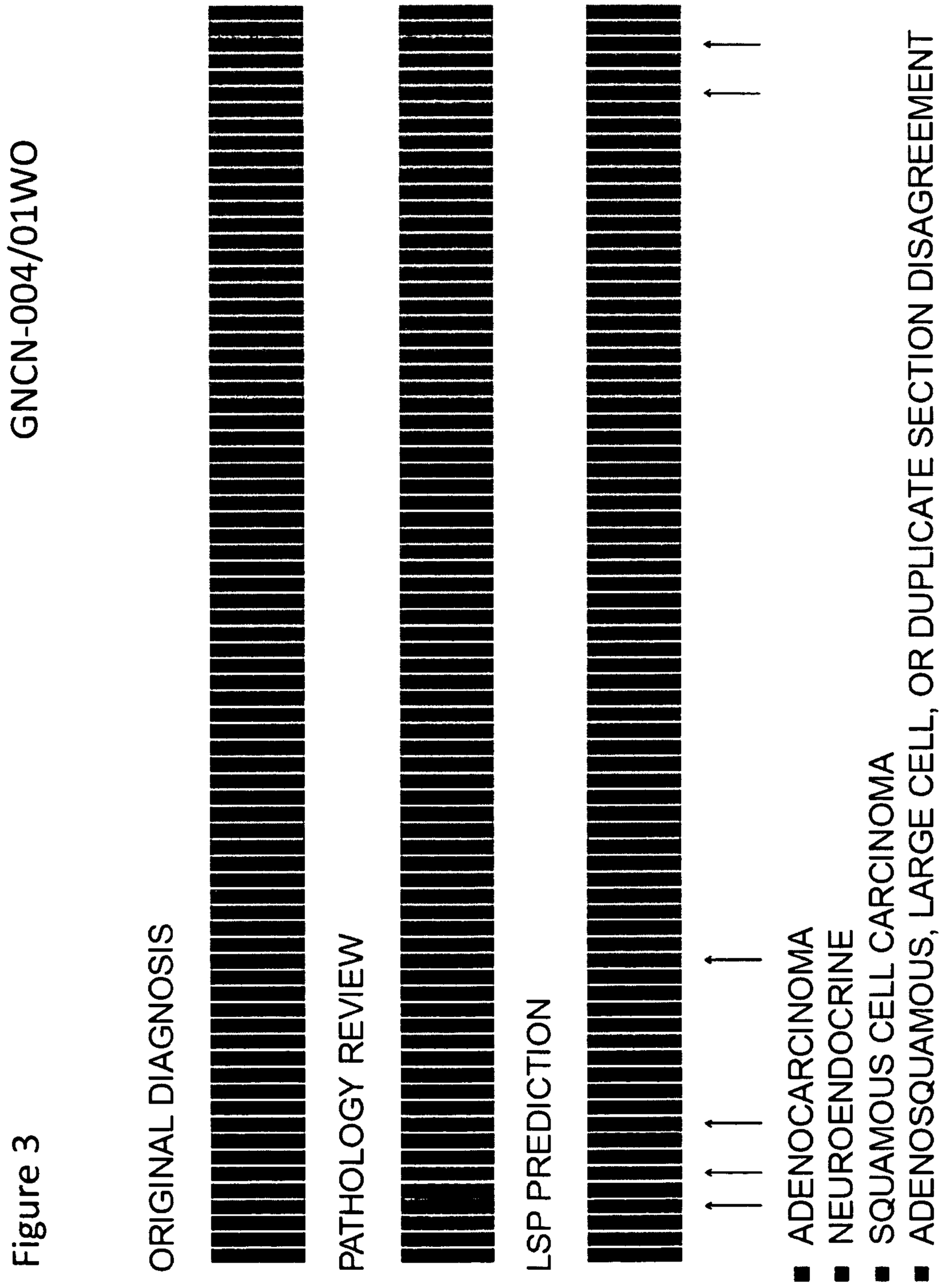
Figure 2


GNCN-004/01WO
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DUPLICATE SECTION DISAGREEMENT
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NEUROENDOCRINE
SQUAMOUS CELL CARCINOMA
ADENOSQUAMOUS, LARGE CELL, OR

Figure 3

Figure 1A

GNCN-004/01WO