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(54) Title: BIOMARKERS FOR DIAGNOSIS OF LUNG CANCER

(57) Abstract: The present invention relates to biomarkers of lung cancer, particularly to markers that enable distinguishing between subtypes of non-small cell lung cancer (NSCLC), particularly between adenocarcinoma (AC) and squamous cell carcinoma (SCC). In particular, the present invention relates to means and methods for diagnosing, assessing the level of severity and selecting methods of treating NSCLC.



BIOMARKERS FOR DIAGNOSIS OF LUNG CANCER

FIELD OF THE INVENTION

The present invention relates to biomarkers of lung cancer, particularly to
5 markers that enable distinguishing between subtypes of non-small cell lung cancer (NSCLC), particularly between adenocarcinoma (AC) and squamous cell carcinoma (SCC). In particular, the present invention relates to compositions and methods for diagnosing, assessing the level of severity and treating of NSCLC.

10 BACKGROUND OF THE INVENTION

Non-small cell lung cancer (NSCLC) is the most prevalent form of lung cancer and represents the leading cause of cancer deaths worldwide in both men and women. Because the majority of diagnosed NSCLC patients are in advanced stages of the disease, overall survival after standard treatment with platinum-based chemotherapy,
15 radiation, and/or surgery remains less than 12 months. Median overall survival can, however, be increased by novel strategies implementing immunotherapies in different combinations; or if a driver mutation exists, then median overall survival can be increased to four years by targeted tyrosine kinase inhibitory therapy. NSCLC can be divided into a number of sub-types, with the two main sub-types being adenocarcinoma
20 (AC) and squamous cell carcinoma (SCC), together accounting for the vast majority of NSCLC cases (representing almost 80% of primary lung cancer cases) and being responsible for 30% of all cancer deaths. Specifically, AC is the most prevalent sub-type of lung cancer in non-smokers, and constitutes approximately 50% of all cases of lung cancer types. In AC, the tumor develops from glandular cells of the lungs that are
25 responsible for producing mucin and surfactants, located at the periphery of the lung. SCC, which constitutes approximately 30% of NSCLC cases, usually develops in central areas of the bronchi of the lung and is closely connected with smoking. Although these two NSCLC sub-types have both unique and shared clinical presentations and histopathological characteristics, the need for genetic investigations
30 and treatment strategy may differ significantly. To insure proper treatment strategy, it is therefore, crucial to be able to distinguish the two NSCLC sub-types during diagnosis (Janku F, et al. Nat Rev Clin Oncol 2010; 7:401-14; Kawase A, et al. Jpn J Clin Oncol

2012; 42:189-95). Current histological discrimination is based on tissue availability, wherein about 15-20% of the cases, tissue is exhausted before final histology can be defined, or as many as 7.2% are poorly differentiated and present not otherwise specified NSCLC. Lung cancer, as many other cancers, develops via a multistep process of tumor biogenesis involving accumulation of inherited or acquired genetic abnormalities (Tomasetti C, et al. Science. 2017; 355:1330-4). These can be detected by deep sequencing methods (Meldrum C, et al. Clin Biochem Rev 2011; 32:177-95), yet it is complicated by the heterogeneity and complexity of malignant tumors (Marusyk A, et al. - Biochim Biophys Acta. 2010; 1805:105-17). However, other cancer-associated changes are not mutation-related but rather appear as an increase or a decrease in protein expression or as differential post-translational modification of marker proteins (Tainsky MA. Biochim Biophys Acta 2009; 1796:176-93). Thus, biomarkers other than mutations should be identified and explored as early markers of the disease, as indicators of the disease state, and as predictive and prognostic measures of treatment effectiveness (Tainsky 2009, *ibid*).

Recent efforts have focused on changes that occur within the genome, epigenome, transcriptome, and proteome in lung AC and SCC that could serve to distinguish between these two NSCLC sub-types (Campbell JD, et al. Nat Genet 2016; 48:607-16). Currently about 17 biomarkers were reported to be differentially expressed in AC and SCC (Table 1 hereinbelow). Of these, 11 biomarkers are reported to detect AC while only 5 biomarkers are proposed for diagnosing SCC. Currently 4 markers are in use in the clinic to distinguish between the two subtypes and 6 are used to direct targeted therapy (Table 1). Among them are microRNAs, with miR21 being detected in AC while miR205 being associated with SCC (Campbell 2016, *ibid*). TTF1 (thyroid transcription factor 1), NAPS A (napsin A) and CD141 (Thrombomodulin) were found to be highly expressed in AC as compared to SCC, while high expression levels of TP63 (tumor protein 63) and its isoform p40 (Δ Np63) were reported as markers for SCC (Kim MJ, et al. Ann Diagn Pathol 2013; 17:85-90).

There remains an unmet need for adequate biomarkers that are suitable as diagnostic tools for assessing the presence or absence NSCLC, and, more importantly, for distinguishing between the major subtypes of this cancer, AC and SCC.

SUMMARY OF THE INVENTION

The present invention relates to novel biomarkers that are differentially expressed in non-small cell lung cancer (NSCLC) and to biomarkers that are differentially expressed in the NSCLC sub-types adenocarcinoma (AC) and squamous cell carcinoma (SCC), and thus can be used to distinguish between these NSCLC subtypes.

The present invention is based in part on the unexpected discovery that certain proteins show different expression patterns and/or levels of expression in SCC compared to AC.

According to certain aspects the present invention discloses that the expression of each of the proteins and/or mRNA encoding the proteins HAT1 (Histone acetyltransferase type B); LRRFIP2 (Leucine-rich repeat flightless- interacting protein 2); AKR1B10 (Aldo-keto reductase family 1 member B10, a secreted protein); WDR82 (WD repeat-containing protein 82); TTLL12 (Tubulin-tyrosine ligase-like protein 12); IGF2BP3 (Insulin-like growth factor 2 mRNA-binding protein); SMC2 (Structural maintenance of chromosomes protein 2); and ITGA7 (Integrin alpha-7) is higher in tumor samples obtained from patients diagnosed as having NSCLC subtype SCC compared to the expression in samples obtained from patients diagnosed to have the AC subtype.

According to certain aspects, the present invention further discloses that the expression of each of the proteins and/or mRNA encoding the proteins ACAD8 (Isobutyryl-CoA dehydrogenase); TSG101 (Tumor susceptibility gene 101 protein); RAB34 (Ras-related protein Rab-34); RSU1 (Ras suppressor protein); ACOT1 (Acyl-coenzyme A thioesterase 1); GALE (UDP-glucose 4-epimerase); and HYOU1 (Hypoxia up-regulated protein 1) is higher in tumor samples obtained from patients diagnosed as having AC compared to their expression in samples obtained from patients diagnosed for SCC.

According to other aspects, the protein SMAC/Diablo (second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI) has been found to be predominantly located in the mitochondria and cytosol in samples obtained from patient diagnosed with AC, while in those diagnosed for SCC, SMAC/Diablo was found to be located not only in the mitochondria and cytosol but

about 50% was located in the nucleus.

The present invention also provide newly identified biomarkers (proteins and/or mRNA) of NSCLC, that are highly expressed in samples obtained from cancerous lung tissues of patients diagnosed for NSCLC compared to healthy tissues obtained from the same subject. The novel biomarkers include, but are not limited to, APOOL (Apolipoprotein O-like); VPS29 (Vacuolar protein sorting-associated protein 29); and CAF17 (Iron-sulfur cluster assembly factor homolog), hitherto not known to be associated with cancer.

The present invention thus provides methods and kits for diagnosing NSCLC and for differentiating between the NSCLC subtypes SCC and AC. The present invention further provides markers and marker combinations assisting in determining the severity of NSCLC subtype AC. The markers of the invention, alone or in combination with additional markers, may assist in early diagnosis of the disease and/or its subtype, and enable selecting the proper therapy as early as possible. Several markers of the invention and additional markers

According to one aspect, the present invention provides a method for diagnosing a subtype of non small cell lung carcinoma (NSCLC) selected from adenocarcinoma (AC) and squamous cell carcinoma (SCC) in a subject suspected to have NSCLC, the method comprising:

- (a) determining the expression level of at least one biomarker selected from a protein and mRNA encoding said protein in a biological sample obtained from the subject, wherein the at least one biomarker is selected from the group consisting of HAT1, LRRFIP2, AKR1B10, WDR82, TTL12, IGF2BP3, SMC2, ACAD8, RSU1, ACOT1, HYOU1, GALE, ITGA7, TSG101, and RAB34;
- (b) comparing the expression level of said at least one biomarker to the expression level of said at least one biomarker in a healthy biological sample and/or a reference value representing healthy biological sample; optionally
- (c) computing a fold change of the expression level of said at least one biomarker in the sample obtained from said subject and the expression level in the healthy sample and/or reference value; and

(d) diagnosing said subject , wherein –

an elevated expression level in said sample obtained from said subject of at least one biomarker selected from the group consisting of HAT1, LRRFIP2, AKR1B10, WDR82, TTL12, IGF2BP3, and SMC2, and/or reduced expression of at least one biomarker selected from the group consisting of ACAD8, RSU1, ACOT1, HYOU1, and GALE compared to the expression level in said healthy biological sample and/or reference value indicates that said subject has NSCLC subtype SCC;

a reduced expression level in said sample obtained from said subject of at least one biomarker selected from the group consisting of HAT1, LRRFIP2, AKR1B10, WDR82, TTL12, IGF2BP3, and SMC2, and/or elevated expression of at least one biomarker selected from the group consisting of ACAD8, RSU1, ACOT1, HYOU1, and GALE compared to the expression level in said healthy biological sample and/or reference value indicates that said subject has NSCLC subtype AC;

an equal or elevated fold change of the biomarker ITGA7 compared to a reference value indicates that the subject has NSCLC subtype SCC, wherein the reference value is derived from the fold change of the expression of said ITGA7 biomarker in a plurality of samples obtained from SCC patients compared to its expression in a plurality of healthy biological samples;

an equal or elevated fold change of the biomarker TSG101 compared to a reference value indicates that the subject has NSCLC subtype AC, wherein the reference value is derived from the fold change of the expression of said TSG101 biomarker in a plurality of samples obtained from AC patients compared to its expression in a plurality of healthy biological samples;

an equal or reduced fold change of the biomarker RAB34 compared to a reference value indicates that the subject has NSCLC subtype AC, wherein the reference value is derived from a fold change of the expression of said RAB34 biomarker in a plurality of samples obtained from AC patients compared to its expression in a plurality of healthy biological samples.

According to certain embodiments, the method comprises determining the

expression level of a combination of biomarkers, the combination is selected from the group consisting of at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, and 15 biomarkers.

5 According to certain embodiments, the method comprises determining the expression level of at least one biomarker selected from the group consisting of HAT1, LRRFIP2, AKR1B10, WDR82, TTL12, IGF2BP3, and SMC2, and at least one biomarker selected from the group consisting of ACAD8, RSU1, ACOT1, HYOU1, and GALE. Each possibility represents a separate embodiment of the present invention.

10 According to certain embodiments, the method comprises determining the expression level of at least one biomarker selected from the group consisting of HAT1, LRRFIP2, AKR1B10, WDR82, TTL12, IGF2BP3, SMC2, and ITGA7 and at least one biomarker selected from the group consisting of ACAD8, RSU1, ACOT1, HYOU1, GALE, TSG101 and RAB34.

15 According to certain embodiments, the method comprises determining the expression level of a combination of markers, the combination comprises the biomarkers HAT1, LRRFIP2, AKR1B10, WDR82, TTL12, IGF2BP3, and SMC2.

 According to certain embodiments, the method comprises determining the expression level of a combination of markers, the combination comprises the
20 biomarkers ACAD8, RSU1, ACOT1, HYOU1, and GALE.

 According to certain embodiments, the method comprises determining the expression level of a combination of markers, the combination comprises the biomarkers HAT1, LRRFIP2, AKR1B10, WDR82, TTL12, IGF2BP3, SMC2, ITGA7, ACAD8, RSU1, ACOT1, HYOU1, GALE, TSG101 and RAB34.

25 According to certain embodiments, the method comprises determining the expression level of at least two biomarkers, said method further comprises determining the expression level of at least one of USP14 (Ubiquitin carboxyl terminal hydrolase 14), VDAC1 (voltage-dependent anion channel-1) and AIF (Apoptosis inducing factor), wherein an equal or elevated fold change of the at least one biomarker compared to a
30 reference value indicates that the subject has NSCLC subtype SCC, wherein the reference value is derived from the fold change of the expression of said at least one

biomarker in a plurality of samples obtained from SCC patients compared to the expression in a plurality of healthy biological samples.

According to certain embodiments, the expression level of the at least one biomarker is at least 2 fold, at least 3, fold, at least 4 fold, at least 5 fold, at least 10 fold,
5 at least 50 fold, at least 100 fold, at least 500 fold, at least 1,000 fold and more higher or lower compared to the expression of said biomarker in the healthy sample or to the reference value.

According to certain exemplary embodiments, expression level of the at least one biomarker is at least 4 fold higher compared to the expression of said biomarker in the
10 healthy sample or reference value.

According to certain embodiments, the biological marker is a protein.

According to certain exemplary embodiments, the biological sample is a lung tissue sample. According to these embodiments, the healthy biological sample is obtained from a healthy subject or from a healthy lung tissue of the subject suspected to
15 have NSCLC.

According to certain embodiments, the biomarker is a secreted protein and the biological sample is selected from the group consisting of blood sample, blood plasma sample and serum sample. According to some embodiments, the biological sample obtained from the subject is ascite.

20 According to certain embodiments, the method further comprises treating the subject diagnosed to have NSCLC subtype AC with a therapy suitable for treating AC.

Any therapy known to be effective in treating NSCLC subtype AC can be used according to the teachings of the present invention.

According to some embodiments, the therapy suitable for treating AC comprises
25 administering to the subject a therapeutically effective amount of at least one agent that reduces the expression or activity of at least one protein selected from the group consisting of TSG101, ACAD8, and GALE. Each possibility represents a separate embodiment of the present invention.

According to certain embodiments, the method further comprises treating the
30 subject diagnosed to have NSCLC subtype SCC with a therapy suitable for treating

SCC.

Any therapy known to be effective in treating NSCLC subtype SCC can be used according to the teachings of the present invention.

According to some embodiments, the therapy suitable for treating SCC comprises
5 administering to the subject a therapeutically effective amount of at least one agent that reduces the expression or activity of at least one protein selected from the group consisting of HAT1, LRRFIP2, AKR1B10, WDR82, TTL12, IGF2BP3, and ITGA7. Each possibility represents a separate embodiment of the present invention.

According to certain embodiments, the agent that reduces the expression or
10 activity of the at least one protein is selected from the group consisting of a chemical agent or moiety, a protein, a peptide, and a polynucleotide molecule. Each possibility represents a separate embodiment of the present invention.

According to certain embodiments, the agent is an antibody.

According to certain exemplary embodiments, the agent is an interfering RNA
15 (RNAi) molecule selected from the group consisting of shRNA, siRNA, and miRNA.

According to yet additional aspect, the present invention provides a method for diagnosing a subtype of non small cell lung carcinoma (NSCLC) selected from the group consisting of squamous cell carcinoma (SCC) and adenocarcinima (AC) in a subject suspected to have NSCLC, the method comprises (a) determining the presence
20 of SMAC/Diablo protein in a cell-comprising sample obtained from the subject and (b) diagnosing said subject as having NSCLC subtype SCC when a significant amount of the SMAC/Diablo protein is present in the cell nucleus and in the cell cytosol and as having NSCLC subtype AC when no significant amount of said SMAC/Diablo protein is present in the cell nucleus and a significant amount is present in the cytosol.

25 According to certain embodiments, the method further comprises treating the subject diagnosed to have NSCLC subtype AC with a therapy suitable for treating AC.

According to certain embodiments, the method further comprises treating the subject diagnosed to have NSCLC subtype SCC with a therapy suitable for treating SCC. The methods for treating AC or SCC are as known in the art and as described
30 hereinabove.

According to yet further aspect, the present invention provides a method for diagnosing NSCLC in a subject, the method comprising:

- 5 (a) comparing the expression level of at least one biomarker selected from a protein or mRNA encoding the protein in a biological sample of the subject to a control biological sample or reference value, wherein the at least one biomarker is selected from the group consisting of APOOL, VPS29, CAF17, and any combination thereof;
- 10 (b) diagnosing the subject as having NSCLC wherein the expression level of said at least one biomarker or of a combination of the biomarkers is increased compared to the expression in the control biological sample or to the reference value.

According to certain embodiments, the method for diagnosing NSCLC comprises comparing the expression level of at least two biomarkers or of the three biomarkers. According to certain embodiments, the method further comprises comparing the expression level of at least one additional biomarker selected from the biomarkers set
15 fort in Table 2 hereinbelow.

According to certain exemplary embodiments, the method for diagnosing NSCLC further comprises comparing the expression level at least one additional biomarker selected from the group consisting of VDAC1, AIF, ATP5B, HSp60, GADPH, PGK1,
20 ENO1, LDHA and Rab11B. Each possibility represents a separate embodiment of the present invention.

According to certain exemplary embodiments, the additional marker is selected from PGK1 and Rab11.

According to certain embodiments, the biological sample is a lung tissue.
25 According to these embodiments, the control sample is obtained from a healthy subject.

According to certain embodiments, the reference value represents a statistical measure representing the expression level of each of the biomarkers in a plurality of samples obtained from a plurality of healthy subjects.

According to certain embodiments, expression level of the at least one biomarker
30 is at least 2 fold, at least 3, fold, at least 4 fold, at least 5 fold, at least 10 fold, at least 50

fold, at least 100 fold, at least 500 fold, at least 1,000 fold and more higher compared to the expression of said biomarker in the healthy sample or reference value. According to certain exemplary embodiments, expression level of the at least one biomarker is at least 4 fold higher compared to the expression of said biomarker in the healthy sample or to the reference value.

According to certain embodiments, the at least one biomarker is a protein.

According to certain embodiments, the method of diagnosing a subject as having NSCLC further comprises treating said subject with a therapy suitable for treating NSCLC. Therapies for treating NSCLC are known in the art. According to some embodiments, treating the NSCLC comprises administering to the subject a therapeutically effective amount of at least one agent that reduces the expression or activity of at least one protein selected from the group consisting of APOOL, VPS29, and CAF17.

Agents that reduce the expression of the at least one biomarkers are as known in the art and as described hereinabove.

According to additional aspect, the present invention provides a method for predicting the severity of NSCLC subtype AC, the method comprising:

(a) comparing the expression level of at least one biomarker selected from a protein and mRNA encoding said protein in a biological sample obtained from a subject diagnosed to have NSCLC subtype AC to a reference value, wherein the at least one biomarker is selected from the group consisting of: VDAC1, SMAC, HYOU1, TTLL12, RAB34, ARL1, HAT1, p40, NAPSA LRRFIP2, AIF, TITF, WDR82 and TSG101;

(b) predicting the level of severity of the disease, wherein an increase in the level of at least one biomarker selected from the group consisting of VDAC1, SMAC, HYOU1, TTLL12, and RAB34 compared to the reference value characterizes said patient as having a severe form of the disease; and wherein an increase in the level of at least one biomarker selected from the group consisting of ARL1, HAT1, p40, NAPSA LRRFIP2, AIF, TITF, WDR82 and TSG101 compared to the reference value characterizes said patient as having a milder form of the disease.

According to certain embodiments, a milder form of the disease indicates a longer survival rate compared to the severe form.

According to certain embodiments, the biomarker is an mRNA marker.

According to certain embodiments of the present invention, comparing the expression level of at least one protein biomarker or mRNA encoding same in a biological sample of the subject to a reference value comprises determining the expression level of the at least one protein biomarker or mRNA encoding same in the sample and comparing said expression level to the reference value. According to additional embodiments, comparing the expression level of at least one protein or mRNA biomarker in a biological sample of the subject to a control sample comprises determining the expression level of the at least one protein or mRNA biomarker in the sample obtained from said subject and in the control sample and comparing said determined levels.

According to certain embodiments, the sample is a tissue sample. According to certain embodiments, the control sample is a tissue taken from a healthy subject or subject(s). According to certain exemplary embodiments, for differentiating between the NSCLC subtypes, the sample to be analyzed is a tumor tissue taken from a subject and the control tissue is a healthy tissue taken from the same subject. According to yet additional embodiments, the control tissue is taken from subject(s) diagnosed for NSCLC subtype SCC or subtype AC.

According to yet another aspect, the present invention provides a method for treating NSCLC subtype SCC, the method comprises administering to a subject in need thereof a therapeutically effective amount of at least one agent that reduces the expression or activity of at least one protein selected from the group consisting of HAT1, LRRFIP2, AKR1B10, WDR82, TTLL12, ITGA7, IGF2BP3, and USP14.

According to yet additional aspect, the present invention provides a method for treating NSCLC subtype AC, the method comprises administering to a subject in need thereof, a therapeutically effective amount of at least one agent that reduces the expression or activity of at least one protein selected from the group consisting of ACAD8, TSG101, and GALE.

According to yet further aspect, the present invention provides a method for

treating NSCLC, the method comprises administering to a subject in need thereof, a therapeutically effective amount of at least one agent that reduces the expression or activity of at least one protein selected from the group consisting of APOOL, VPS29, and CAF17.

- 5 Any agent as is known in the art and as described hereinabove that can reduce the expression or activity of the biomarker can be used according to the teachings of the invention.

 According to additional aspect, the present invention provides a kit for diagnosing a subtype of non- small cell lung carcinoma (NSCLC) selected from adenocarcinoma
10 (AC) and squamous cell carcinoma (SCC) in a biological sample obtained from a subject suspected to have NSCLC, the kit comprising:

- (a) at least one agent capable of detecting the expression level of at least one biomarker selected from a protein and mRNA encoding said protein, the biomarker is selected from the group consisting of HAT1, LRRFIP2, AKR1B10, WDR82, TTL12,
15 IGF2BP3, SMC2, ACAD8, RSU1, ACOT1, HYOU1, GALE, ITGA7, TSG101, and RAB34;

- (b) means for comparing the expression level of the at least one biomarker to a first reference value derived from the expression of the at least one biomarker in healthy biological sample and/or to a second reference value derived from the fold change of the
20 expression of said at least one biomarker in a plurality of samples obtained from SCC patients compared to the expression in a plurality of healthy biological samples; and/or to a third reference value derived from a fold change of the expression of the at least one biomarker in a plurality of samples obtained from AC patients compared to a plurality of healthy biological samples;

- 25 (c) instruction material providing guidance to the correlation of said expression level of said at least one biomarker with the NSCLC subtype, wherein:

 an increased expression level in said sample of at least one biomarker selected from the group consisting of HAT1, LRRFIP2, AKR1B10, WDR82, TTL12, IGF2BP3, and SMC2, and/or reduced expression of at least one
30 biomarker selected from the group consisting of ACAD8, RSU1, ACOT1, HYOU1, and GALE compared to the first reference value indicates that said

subject has NSCLC subtype SCC;

a reduced expression level in the sample of at least one biomarker selected from the group consisting of HAT1, LRRFIP2, AKR1B10, WDR82, TTL12, IGF2BP3, and SMC2, and/or elevated expression of at least one biomarker selected from the group consisting of ACAD8, RSU1, ACOT1, HYOU1, and GALE compared to the first reference value indicates that said subject has NSCLC subtype AC;

an equal or elevated fold change of the biomarker TGA7 compared to the second reference value indicates that the subject has NSCLC subtype SCC;

an equal or elevated fold change of the biomarker TSG101 compared to the third reference value indicates that the subject has NSCLC subtype AC; and/or

an equal or reduced fold change of the biomarker RAB34 compared to the third reference value indicates that the subject has NSCLC subtype AC.

According to certain embodiments, the kit further comprises at least one agent capable of detecting the expression of SMAC/Diablo protein within the nucleus of cells present within the biological sample and instruction material providing guidance to correlation of the amount of SMAC/Diablo within the cell nucleus and the cytosol and NSCLC subtype, wherein a significant amount of the SMAC/Diablo protein in the cell nucleus and cytosol diagnose the subject as having NSCLC subtype SCC and no significant amount of said SMAC/Diablo protein in the cell nucleus while a significant amount is present in the cytosol diagnose the subject as having NSCLC subtype AC.

According to yet additional aspect, the present invention provides a kit for diagnosing NSCLC, the kit comprising:

(a) at least one agent capable of detecting the expression level of at least one biomarker selected from a protein and mRNA encoding said protein, the biomarker is selected from the group consisting of APOOL, VPS29, and CAF17 in a biological sample of a subject suspected of having NSCLC;

(b) means for comparing the expression level of the at least one biomarker in a control sample obtained from a healthy subject or to a reference value; and

(c) instruction material providing guidance to the correlation of an increase in the

expression level of said at least one biomarker compared to the control sample or reference value with NSCLC.

It is to be understood that any combination of the aspects and the embodiments disclosed herein is explicitly encompassed within the disclosure of the present invention.

Further embodiments and the full scope of applicability of the present invention will become apparent from the detailed description given hereinafter. However, it should be understood that the detailed description and specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows statistical and functional analysis of protein expression in samples obtained from healthy and tumor samples of patients with lung cancer. **Fig. 1A** shows a Volcano plot representing the fold change (X axis) and fold discovery rate (FDR, Y axis) values for each identified protein. Vertical lines indicate fold change > 2 or < -2 and horizontal line indicates $p\text{-value} < 0.05$. 1,494 proteins passed these thresholds. **Fig. 1B**: significantly enriched functional groups in the proteins showing changed expression, based on the Gene Ontology system.

Figure 2 shows over-expression of VDAC1 and other apoptosis- and energy-related proteins in samples obtained from lung cancer patients. **Fig. 2A**: representative immunoblots of tissue lysates of tumor (T) and healthy (H) lung tissues derived from lung cancer patients probed with antibodies directed against VDAC1, SMAC, HK-I, MAVS, AIF and Bcl-2. **Fig. 2B**: quantitative analysis of VDAC1 (37 patients, fold change (FC)=6.2, $p\text{-value}=5 \times 10^{-5}$); SMAC (37 patients, FC=5, $p\text{-value}=3.4 \times 10^{-5}$); HK-I, (33 patients, FC=5.3, $p\text{-value}=5.3 \times 10^{-3}$); MAVS (22 patients, FC=2.6, $p\text{-value}=1.5 \times 10^{-4}$); AIF (35 patients, FC=3.5, $p\text{-value}=1.7 \times 10^{-2}$), and Bcl-2 (22 patients, FC=1.5, $p\text{-value}=1.4 \times 10^{-1}$) are presented as the mean $\pm$ SD. **Fig. 2C**: LC-HR MS/MS data for VDAC1, HK1 and SMAC. A difference between healthy and tumor tissues was considered statistically significant when $P < 0.001$ (***), $P < 0.01$ (**), $P < 0.05$ (*), as

determined by the Mann-Whitney test for the immunoblots and a two-way t-test for the LC-HR MS/MS data. Fig. 2D: quantitative analysis of gene expression based on RNAseq of VDAC1, HK-I, SMAC and AIF. The gene expression profiles was obtained from publicly available data (TCGA lung cancer dataset) for healthy (n=110) and tumor lung samples (n=1,017) of lung cancer patients. Fig. 2E: over-expression of VDAC1, SMAC, AIF, MAVS and Bcl-2 in lung cancer patients. Representative IHC staining for VDAC1, SMAC AIF, MAVS and Bcl-2 of normal (n=5) and lung cancer (n=20) tissue samples from tissue microarray slides (Biomax). The percentages of patient samples that stained at the indicated intensity are shown

- 10 **Figure 3** shows over-expression of known and newly identified proteins in samples obtained from lung cancer patients. Fig. 3A, B: representative immunoblots of tissue lysates of tumor (T) and healthy (H) lung tissues derived from lung cancer patients probed with antibodies directed against HYOU1 (ORP150), LDHA, HSPD1 (Hsp60), ATP5B, GAPDH and Rab11b. Fig. 3C: quantitative analysis of LC-HR MS/MS data. A difference between healthy and tumor tissues was considered statistically significant when $P < 0.001$ (***), $P < 0.01$ (**), as determined by two-way t-test for the LC-HR MS/MS data. Fig. 3D: quantitative analysis of gene expression based on RNAseq of GAPDH, PGK1, ENO1, LDHA and HYOU1. The gene expression profiles obtained from healthy (n=110) and tumor lung samples (n=1,017) of lung cancer patients.
- 20 **Figure 4** shows proteins differentially expressed in AC and SCC. Fig. 4A: IHC staining for VDAC1, AIF and SMAC of human normal lung tissue (n=10), lung SCC tissue (n=31) or lung AC tissue (n=17) in tissue array slides (Biomax), as described in material and methods. Percentages of sections stained at the intensity indicated are shown. Fig. 4B: LC-HR MS/MS data were used to identify proteins that can serve to distinguish between AC and SCC. A difference between AC and SCC groups was considered statistically significant when $P < 0.05$ (*), $P < 0.01$ (**) or $P < 0.001$ (***) as determined by the Mann-Whitney test.

30 **Figure 5** shows gene expression as determined by RNAseq of potential protein markers in lung cancer patients. Fig. 5A: RNAseq data imported from TCGA were subjected to quantitative analysis using t-test. The ratio of the expression of the proteins in SCC compared to AC is presented, and is considered statistically significant when $P < 0.001$

(***). The proteins were grouped according to function as: Apop, apoptosis; Metab, metabolism; HAR, histone activity regulation; Ubiqu, ubiquitination; Inflam, Inflammatory response; SP, Surfactant production; PT, protein transport. Fig. 5B: Quantitative analysis of RNAseq data of 24 selected genes showing differential expression between AC and SCC based on proteomics data, Functional groups are indicated: TS, tumor suppressor; Metab, galactose metabolism; LM, lipid metabolism; AAM, amino acid metabolism; StP, structural proteins; PI, proteinase inhibitor; SiP, signaling pathway; NA, nuclear activity; MT, mitochondrial translocase and IR, immune response.

10 **Figure 6** shows SMAC sub-cellular localization in lung cancer. IHC staining of SMAC (Fig. 6A) and AIF (Fig. 6B) in human SCC and AC lung cancer in tissue array slides (Biomax) with nuclear and cytosolic localization of SMAC shown. Fig. 6C: nuclear extracts were prepared from AC and SCC samples of lung cancer patient using a nuclear/cytosol fractionation kit (Biovision, Milpitas, CA) following the manufacturer's instructions. Following centrifugation (16,000 g, 10 min), the supernatant (cytosolic fraction), and pellet (nuclear fraction) were re-suspended in the original volume and subjected to immunoblotting for SMAC, VDAC1 and AIF. Fig. 6D: Quantitative analysis, presenting the results as mean $\pm$ SEM (n = 3).

20 DETAILED DESCRIPTION OF THE INVENTION

Several markers has been previously suggested to be associated with lung cancer, including non-small cell lung carcinoma (NSCLC) and its subtypes, adenocarcinoma (AC) and squamous cell carcinoma (SCC). Several markers proposed to be used in the diagnosis of lung cancer are listed in Table 1. The present invention answers the remaining need for accurate and efficient method for diagnosing NSCLC, particularly for distinguishing between NSCLC subtype SCC and NSCLC subtype AC, which enable selecting an appropriate treatment for each disease subtype based on the diagnosis.

The diagnosis of NSCLC and the differentiation between the NSCLC subtypes SCC and AC is based on differential expression of proteins and/or RNA encoding the proteins in cancerous lung tissue compared to healthy tissue and in SCC cancerous

tissues compared to AC cancerous tissues. The diagnosis can be assessed by measuring one or more of the biomarkers described herein. The correct diagnosis, particularly the precise diagnosis of the NSCLC subtype enables the selection and initiation of therapeutic interventions or treatment regimens that are suitable to the disease subtype, in order to delay, reduce, or treat the subject's disease. The diagnosis method of the invention may further provide for early diagnosis of the cancerous disease and/or its subtypes. An early diagnosis is of high importance in increasing the life expectancy of the patient.

The control samples to which the expression level of one or more biomarkers of the invention in a sample obtained from a subject suspected to have NSCLC is compared to are samples taken from healthy subjects or from healthy tissues of subjects suspected to have or affected with lung cancer. The control reference values are also based on samples taken from healthy subject or healthy tissue, or from subjects already diagnosed to have NSCLC, NSCLC subtype AC or NSCLC subtype SCC. Typically, the control reference value is an average or another statistical measure representing the expression level of each of the biomarkers in a plurality of samples. The control and cancerous level and cut-off points may vary based on whether a biomarker is used alone or in a formulae combining with other biomarkers into an index or indices. Alternatively, the normal or abnormal cancerous level can be a database of biomarker patterns or "signatures" from previously tested subjects who did or did not develop NSCLC, NSCLC subtype AC or NSCLC subtype SCC.

One or more clinical parameters may be used in combination with the biomarkers of the present invention as input to a formula or as pre-selection criteria defining a relevant population to be measured using a particular biomarker panel and formula. Clinical parameters may also be useful in the biomarker normalization and pre-processing, or in biomarker selection, formula type selection and derivation, and formula result post-processing.

Table 1: Biomarkers proposed for use in diagnosing lung cancer

	Protein/microRNAs (Uniprot)	Marker for:
1	miR21	AC (Campbell et al. 2016, <i>ibid</i>).
2	EGFR - Epidermal growth factor receptor (tyrosine kinase)	Over-expressed in NSCLC (Paez JG, et al. Science. 2004; 304: 1497-500; Mitsudomi T, Yatabe Y. Cancer Sci. 2007; 98: 1817-24) and AC (Saito M, et al. Surgery Today. 2017: 1-8).
3	ALK-EML4- Tyrosine-protein kinase receptor	AC (Plönes T, et al. Journal of Personalized Medicine. 2016; 6: 3; Mitsudomi T, Yatabe Y., 2017, <i>ibid</i>)
4	ROS1- Proto-oncogene tyrosine-protein kinase ROS	AC (Cao B, et al. OncoTargets and therapy. 2016; 9: 131-8)
5	RET- Proto-oncogene tyrosine-protein kinase receptor Ret	AC (Lee M-Set al. Oncotarget. 2016; 7: 36101-14).
6	c-MET -Hepatocyte growth factor receptor (tyrosine kinase)	Over-expressed in NSCLC (Benedettini E, et al. Met Am J Pathol. 2010; 177: 415-23; Nakamura Y, et al. Cancer Sci. 2007; 98: 1006-13).
7	ERBB2- Receptor tyrosine-protein kinase erbB-2	AC (Nakamura Y, et al. Cancer Sci. 2007; 98: 1006-13).
8	PPP3CA- Serine/threonine-protein phosphatase 2B catalytic subunit alpha isoform. (Mutation)	AC (Vargas AJ, et al. Nature Reviews Cancer. 2016; 16: 525-37).
9	DOT1L- Histone-lysine N-methyltransferase, H3 lysine-79 specific. (Mutated)	AC (Campbell JD, et al. 2016, <i>ibid</i>).
10	FTSJD1- cap-specific mRNA (nucleoside-2'-O-)-methyltransferase 2. (Mutation)	AC (Campbell JD, et al. 2016, <i>ibid</i>).
11	TTF1-thyroid transcription factor 1	AC (Ao MH, et al. Hum Pathol. 2014; 45: 926-34).
12	NAPSA- napsin A	AC (Ao MH, et al. 2014, <i>ibid</i>)
13	TP63- Tumor protein 63	SCC (Vogt AP, et al. Diagn Cytopathol. 2014; 42: 453-8).
14	p40- ΔNp63	SCC (Ao MH, et al. 2014, <i>ibid</i> ; Kim MJ, et al. Ann Diagn Pathol. 2013; 17: 85-90)
15	RASA1- Ras GTPase-activating protein-1	SCC (Paez JG, et al. 2004, <i>ibid</i> ; Mitsudomi T, Yatabe Y. 2007 <i>ibid</i>).
16	CD141- Thrombomodulin	SCC (Ogawa H, et al. Cancer Lett. 2000; 149: 95-103; Tolnay E, et al. Virchows Arch. 1997; 430: 209-12).
17	miR205	SCC (Campbell JD, et al. 2016, <i>ibid</i>).

Markers 2 to 6 are predictive markers used to direct targeted therapy and markers 11-14 serve in the clinic for diagnosis of AC or SCC.

Definitions

- 5 The term "biomarker" as used herein refers to a protein or gene (particularly RNA, more particularly mRNA) that is differentially expressed in a sample taken from a subject having NSCLC as compared to a sample taken from a healthy subject or in a

sample taken from subject having NSCLC subtype SCC in comparison to subject having NSCLC subtype AC or to a healthy subject, or in a sample taken from subject having NSCLC subtype AC in comparison to subject having NSCLC subtype SCC or to a healthy subject.

- 5 The term “diagnosing” as used herein means assessing whether a subject suffers from NSCLC or not, and/or whether a subject suffers from NSCLC subtype SCC or NSCLC subtype AC. As will be understood by those skilled in the art, such an assessment is usually not intended to be correct for all (i.e. 100%) of the subjects to be identified. The term, however, requires that a statistically significant portion of subjects
10 can be identified. The term diagnosis also refers, in some embodiments, to screening. Screening for cancer, in some embodiments, can lead to earlier diagnosis in specific cases and diagnosing the correct disease subtype can lead to adequate treatment.

As used herein, the term “level” refers to the degree of gene product expression in the biological sample.

- 15 As referred to herein, the term “treating” is directed to ameliorating symptoms associated with a disease, and lessening the severity or cure the disease.

The term “subject” refers to any mammalian subject. In some embodiments, the subject is a human subject.

- 20 The term “patient” as used herein refers to a subject that was diagnosed to have NSCLC, NSCLC subtype AC and NSCLC subtype AC.

- As used herein, the term “biological sample” refers to a sample obtained from a subject. According to certain typical embodiments, the sample is a biological tissue obtained in vivo or in vitro. Biological samples can be, without limitation, body fluid selected from blood, blood plasma, serum, organs, tissues, fractions and cells isolated
25 from the subject/patient. Biological samples also may include sections of the biological sample including tissues (e.g., sectional portions of an organ or tissue). Biological samples may be dispersed in solution or may be immobilized on a solid support, such as in blots, assays, arrays, glass slides, microtiter, or ELISA plates.

- 30 According to one aspect, the present invention provides a method for diagnosing a subtype of non small cell lung carcinoma (NSCLC) selected from adenocarcinoma (AC)

and squamous cell carcinoma (SCC) in a subject suspected to have NSCLC, the method comprising:

- 5 (a) determining the expression level of at least one biomarker selected from a protein and mRNA encoding said protein in a biological sample obtained from the subject, wherein the at least one biomarker is selected from the group consisting of HAT1, LRRFIP2, AKR1B10, WDR82, TTL12, IGF2BP3, SMC2, ACAD8, RSU1, ACOT1, HYOU1, GALE, ITGA7, TSG101, and RAB34;
- 10 (b) comparing the expression level of the at least one biomarker to the expression level of said at least one biomarker in a healthy biological sample and/or a reference value representing healthy biological sample; optionally
- (c) computing a fold change of the expression level of said at least one biomarker in the sample obtained from the subject and the expression level in the healthy sample and/or reference value; and
- 15 (d) diagnosing said subject, wherein –
 - an elevated expression level in said sample obtained from said subject of at least one biomarker selected from the group consisting of HAT1, LRRFIP2, AKR1B10, WDR82, TTL12, IGF2BP3, and SMC2, and/or reduced expression of at least one biomarker selected from the group consisting of ACAD8, RSU1, 20 ACOT1, HYOU1, and GALE compared to the expression level in said healthy biological sample and/or reference value indicates that said subject has NSCLC subtype SCC;
 - a reduced expression level in said sample obtained from said subject of at least one biomarker selected from the group consisting of HAT1, LRRFIP2, 25 AKR1B10, WDR82, TTL12, IGF2BP3, and SMC2, and/or elevated expression of at least one biomarker selected from the group consisting of ACAD8, RSU1, ACOT1, HYOU1, and GALE compared to the expression level in said healthy biological sample and/or reference value indicates that said subject has NSCLC subtype AC;
 - 30 an equal or elevated fold change of the biomarker ITGA7 compared to a

reference value indicates that the subject has NSCLC subtype SCC, wherein the reference value is derived from the fold change of the expression of said ITGA7 biomarker in a plurality of samples obtained from SCC patients compared to the expression in a plurality of healthy biological samples;

5 an equal or elevated fold change of the biomarker TSG101 compared to a reference value indicates that the subject has NSCLC subtype AC, wherein the reference value is derived from the fold change of the expression of said TSG101 biomarker in a plurality of cancerous samples obtained from AC patients compared to the expression in a plurality of healthy biological
10 samples;

 an equal or reduced fold change of the biomarker RAB34 compared to a reference value indicates that the subject has NSCLC subtype AC, wherein the reference value is derived from a fold change of the expression of said RAB34 biomarker in a plurality of cancerous samples obtained from AC patients
15 compared to a plurality of healthy biological samples.

 According to certain embodiments, each of the HAT1, LRRFIP2, AKR1B10, WDR82, TTL12, IGF2BP3, SMC2, ACAD8, RSU1, ACOT1, HYOU1, GALE, ITGA7, TSG101, and RAB34 biomarkers is a protein biomarker. According to certain
20 embodiments, each of the HAT1, AKR1B10, WDR82, TTL12, IGF2BP3, SMC2, ACAD8, RSU1, ACOT1, HYOU1, GALE, ITGA7, TSG101, and RAB34 biomarkers is an RNA biomarker.

 According to yet additional aspect, the present invention provides a method for diagnosing a subject suspected to have NSCLC for a subtype of NSCLC selected from the group consisting of SCC and AC, the method comprises determining the presence of
25 a SMAC/Diablo protein in a cell-comprising sample obtained from the subject, wherein said subject is diagnosed as having NSCLC subtype SCC when a significant amount of the SMAC/Diablo protein is present in the cell nucleus and in the cell cytosol and as having NSCLC subtype AC when no significant amount of said SMAC/Diablo protein is present in the cell nucleus and a significant amount is present in the cytosol.

30 According to yet further aspect, the present invention provides a method for diagnosing NSCLC in a subject, the method comprising:

- (a) comparing the expression level of at least one protein biomarker or mRNA encoding the protein in a biological sample of the subject to a reference value or a control sample, wherein said at least one biomarker is selected from the group consisting of APOOL; VPS29; CAF17; and any combination thereof;
- 5 (b) diagnosing the subject as having NSCLC wherein the expression level of the at least one biomarker or of combination thereof is increased compared to the reference value or control sample.

According to certain embodiments, the method further comprises comparing the expression level of at least one additional biomarker selected from the group presented
 10 in Table 2 or mRNA encoding same.

Table 2: NSCLC biomarkers

Protein name (Uniprot)	Fold change/ P value	Proposed function (cell localization)	Relation to cancer
RB11B/Rab11B - Ras-related protein	>1000 7.7×10^{-12}	Regulator of intracellular membrane trafficking (Extracellular space, Endosome)	Over-expressed in HL-60 leukemia cell line
PIGS - GPI transamidase component PIG-S	>1000 1.3×10^{-9}	Component of the GPI transamidase complex (ER)	Over-expressed in breast, ovary and uterus cancers
NICA - Nicastrin	>1000 5.8×10^{-9}	A subunit of the gamma-secretase complex (Melanosome)	Regulates breast cancer stem cell properties and tumor growth
NDKB - Nucleoside diphosphate kinase B	14.5 3.1×10^{-9}	Synthesis of nucleoside triphosphates other than ATP (Cytosol, Nucleus)	High expression reduce metastases in breast cancer, melanoma
HNRPL - Heterogeneous nuclear ribonucleoprotein L	7.3 1.3×10^{-8}	Splicing factor, acting as activator or repressor of exon inclusion (Cytosol, Nucleus)	Marker for secondary to brain ALL metastasis
STT3A - Dolichyl-diphospho-oligo saccharide-protein glycosyltransferase	8.3 1.2×10^{-7}	Catalytic subunit of the N-oligosaccharyl transferase (OST) complex (ER)	Marker for follicular thyroid carcinoma
COPA - Coatamer subunit alpha	14.6 1.3×10^{-7}	Part of a complex that mediates protein transport from the ER to the Golgi, (Cytosol, Golgi)	Associated with mouse mesothelioma progression
PDLI5 - PDZ and LIM domain protein 5	9.2 1.8×10^{-7}	Z-disc protein that interacts directly with α -actinin-2 (Cytosol, Cell junction)	Associated with gastric cancer. High deletion frequencies in oral squamous cell carcinoma.

Protein name (Uniprot)	Fold change/ P value	Proposed function (cell localization)	Relation to cancer
HINT1 - Histidine triad nucleotide-binding protein 1	5.4 2.0×10^{-7}	Hydrolyzes purine nucleotide phosphoramidates (Cytosol, Nucleus)	Over-expressed in prostate cancer
SEC11A - Signal peptidase complex catalytic subunit	>1000 2.3×10^{-7}	Component of a complex that removes signal peptides from proteins translocated into the ER (ER)	Contributes to malignant progression in gastric cancer
DDX6 - DEAD box protein 6	62.8 2.5×10^{-7}	Participates in mRNA degradation (Cytosol, Nucleus)	Chromosomal aberrations, DDX6 contribute to lymphomagenesis
PGK1 - Phosphoglycerate kinase 1	8.9 3.2×10^{-7}	Glycolytic enzyme, converting 3-phospho-D-glycerate to 3-phospho-D-glyceroyl phosphate (Cytosol)	Prognostic biomarker of poor survival and chemoresistance to paclitaxel treatment in breast cancer
IF4E - Eukaryotic translation initiation factor 4E	7.7 3.5×10^{-7}	Participates in the initiation of translation (Cytosol)	eIF4E over-expression can initiate malignant transformation
GDIB - Rab GDP dissociation inhibitor beta	4.5 3.9×10^{-7}	Regulates the GDP/GTP exchange of most Rab proteins (Cytosol, Plasma membrane)	Increased in metastatic gallbladder cancer cell line SD18H and in pancreatic carcinoma
RL9 - 60S ribosomal protein L9	21.1 4.5×10^{-7}	Translation. Component of the 60S subunit (Cytosol)	Over-expressed in colon adenoma and adenocarcinoma
NDUS7 - ADH dehydrogenase (ubiquinone) iron-sulfur protein 7	>1000 4.7×10^{-7}	Core subunit of the respiratory chain NADH dehydrogenase (Mitochondria)	Amplification in BRCA1-associated ovarian cancer
PTBP1 - Polypyrimidine tract-binding protein 1	8.4 5.1×10^{-7}	Plays a role in pre-mRNA splicing (Nucleus)	Over-expressed in colorectal cancer, gemcitabine resistance in pancreatic cancer, associated with breast tumorigenesis
PA1B2 - Platelet-activating factor acetylhydrolase 1B subunit beta	9.9 5.9×10^{-7}	Inactivates PAF (platelet-activating factor) (Cytosol)	Important in maintaining cancer pathogenicity across a wide spectrum of cancer types
PPOX - Protoporphyrinogen oxidase	>1000 6.6×10^{-7}	Catalyzes the oxidation of protoporphyrinogen-IX to form protoporphyrin-IX (Mitochondria)	Higher expression in faster growing cell lines and primary colorectal tumors
RL10 - 60S ribosomal protein L10a	7.8 7.1×10^{-7}	Translation. Component of the 60S subunit (Cytosol)	Mutated in T-cell acute lymphoblastic leukemia
ILF2 - Interleukin enhancer-binding factor 2	5.0 7.7×10^{-7}	Regulatory subunit of complexes involved in mitotic control, DNA break	Higher expression in esophageal squamous cell carcinoma

Protein name (Uniprot)	Fold change/ P value	Proposed function (cell localization)	Relation to cancer
		repair, and RNA splicing regulation (Cytosol Nucleus)	
UGPA - UTP-glucose- 1-phosphate uridylyltransferase	7.7 9.5x10- 7	Glucosyl donor in cellular metabolic pathways (Cytosol)	Biomarker for metastatic hepatocellular carcinoma
DDX17 - DEAD box protein 17	5.6 1.2x10- 6	RNA helicase, involved in transcription and splicing (Nucleus)	Increased expression in colon cancer
OSBL8 - Oxysterol- binding protein-related protein 8	>1000 1.2x10 ⁻⁶	Binds 25-hydroxycholesterol and cholesterol (ER membrane, Nucleus membrane)	Down-regulated in hepatoma tissues
TXD12 (ERp19) - Thioredoxin domain- containing protein 12	37.6 1.4x10 ⁻⁶	Involved in thiol-disulfide oxidase activity (ER)	A thioredoxin-like protein, implicated in development of breast, ovarian, gastrointestinal and gastric cancers
USO1 - General vesicular transport factor p115	8.7 1.4x10 ⁻⁶	General vesicular transport factor in Golgi (Cytosol, Golgi)	Promotes proliferation of gastric cancer cells
SMD3 - Small nuclear ribonucleoprotein Sm D3	9.0 1.4x10 ⁻⁶	Core component of the spliceosome (Cytosol, Nucleus)	Associated with metastatic behavior in soft tissue tumors
ITB2 - Integrin beta-2	5.9 1.5x10 ⁻⁶	Cell adhesion (Plasma membrane, Exosome)	Over-expressed in CLL patients harboring trisomy 12
COPB1 - Coatamer subunit beta 1	6.5 1.5x10 ⁻⁶	Involved in protein transport from the ER to the Golgi (Cytosol, Golgi)	Over-expressed in prostate cancer
MYH9 - myosin 9	6.5 1.7x10 ⁻⁶	Motor protein (Cytosol)	Highly expressed in CL16 breast cancer cell tumors in mice
PSME3 - Proteasome activator complex subunit 3	>1000 2.6x10 ⁻⁶	Subunit of the 11S REG proteasome regulator (Cytosol, Nucleus)	Serum tumor marker for colorectal cancer
TM9S3 - Transmembrane 9 superfamily member 3	11.3 2.6x10 ⁻⁶	Belongs to nonaspanin protein family. Function not known (Plasma membrane, Golgi)	Diagnostic and therapeutic target for scirrhous-type gastric cancer. Breast cancer chemoresistance factor.
ARPC3 - Actin-related protein 2/3 complex subunit 3	8.6 4.2x10 ⁻⁶	Component of the Arp2/3 complex involved in regulation of actin polymerization (Cytosol)	Associated with glioma.
RS15 - 40S ribosomal protein S15	15.9 4.3x10 ⁻⁶	Translation, component of the 40S subunit (Cytosol, Nucleus)	RS15 mutations are associated with increased cancer risk
PRKDC - DNA- dependent protein	10.1 4.5x10 ⁻⁶	Serine/threonine-protein kinase that acts as a	Highly expressed in advanced neuroblastoma,

Protein name (Uniprot)	Fold change/ P value	Proposed function (cell localization)	Relation to cancer
kinase catalytic subunit		molecular sensor for DNA damage (Nucleus)	associated with gastric carcinoma
RPN2 - Ribophorin II	8.8 4.5×10^{-6}	Protein glycosylation. Essential subunit of the N- oligosaccharyl transferase (OST) complex (ER Plasma membrane)	Breast cancer initiation and metastasis, associated with docetaxel response in oesophageal SCC

The identification of cancer biomarkers is a rapidly expanding field, with deep sequencing methods have become widely accepted as a means to detect and analyze cancer biomarkers. At the same time, other cancer-associated changes are not simply reflected as mutations in a gene but rather as increased or decreased expression or variations in post-translational modifications of marker proteins, as reported in some cancers. The present invention identified alterations in the expression levels of metabolic, apoptotic and other proteins in NSCLC as potential means for high sensitive platform that may allow better diagnosis of NSCLC and even early NSCLC diagnosis. Most importantly, the present invention now discloses proteins that allow for distinguishing between the AC and SCC subtypes, which is critical for accurate diagnosis and selection of treatment, particularly in unclear cases.

Over-expression of metabolism-related proteins in NSCLC - potential biomarkers

The inventors of the present invention have previously shown that the level of the mitochondrial gatekeeper protein, VDAC1, was substantially higher in different cancer types, in comparison to healthy tissue (WO 2013/035095). As such, its over-expression in NSCLC was also examined (Fig. 2, Fig. 4A and B and Fig. 5A). Previously, the VDAC1 gene expression level was reported to be increased in NSCLC, with this being associated with poor outcome. As the main transporter of ions, Ca^{2+} , ATP, and other metabolites across the outer mitochondrial membrane, VDAC1 over-expression could offer numerous advantages to highly energy-demanding cancer cells. Indeed, the requirement of VDAC1 for cancer development was demonstrated by silencing VDAC1 expression in cancer cells using specific siRNA, resulting in marked inhibition of cancer cells proliferation both *in vitro* and *in vivo*.

Other metabolism-related proteins that were also shown here to be over-

expressed in NSCLC include the glycolytic enzymes PGK1, LDHA, GAPDH, ENO1 and the oxidation phosphorylation (OXPHOS) protein ATP5B (Fig. 3C, Table 6). Of those, PGK1 is shown herein to be associated with NSCLC. Mitochondrial translocated PGK1 functions as a protein kinase, coordinating glycolysis and the TC cycle in
 5 tumorigenesis, and acting in tumor angiogenesis as disulphide reductase. PGK1 is activated by both hypoxia and EGFR signaling and was previously found to play a role in brain tumorigenicity (Li X, et al. Mol Cell 2016;61:705-19) and tumor angiogenesis (Lay AJ, et al. Nature. 2000; 408: 869-73). LDHA is over-expressed in several cancer types, including NSCLC (Miao P, et al. IUBMB Life. 2013; 65: 904-10). GAPDH and
 10 ENO1 expression or polymorphism is associated with poor prognosis in NSCLC (Puzone R, et al. Mol Cancer. 2013; 12: 97; Lee SY, et al. Sci Rep. 2016; 6: 35603). Finally, ATP5B, a constituent of the F_1F_0 ATP synthase, was identified as NSCLC tumor cellular membrane antigen (Lu ZJ, et al. BMC Cancer 2009;9:16).

Interestingly, network analysis demonstrated that most of these proteins are
 15 connected by direct physical interactions or co-expression and some are encoded by a gene cluster that is regulated by epigenetic modifications. Most pronounced is the group of proteins associated with cell metabolic processes. Furthermore, this cluster includes ATP5B associated with OXPHOS and VDAC1, a gatekeeper of mitochondria, suggesting a coupling between OXPHOS and glycolysis, an important factor in cancer
 20 cells energy homeostasis (Warburg effect).

These results point to the significance of reprogrammed metabolism in NSCLC, as in other cancers and that the listed proteins may serve as biomarkers.

Expression of the pro-apoptotic proteins SMAC/Diablo and AIF in NSCLC

SMAC/Diablo (second mitochondria-derived activator of caspases, also referred o
 25 herein as "SMAC") and AIF (apoptosis inducing factor) are normally located at the mitochondrial intermembrane space and released to the cytosol upon apoptotic signal (Kroemer G, et al. Physiol Rev 2007; 87:99-163). Unexpectedly, despite their pro-apoptotic function, SMAC and AIF were found to be over-expressed in NSCLC, as compared to healthy lung tissue (Fig. 2, Fig. 4 and Fig. 6). SMAC, as a pro-apoptotic
 30 protein, is released from mitochondria during apoptosis and counters the inhibitory activities of inhibitor of apoptosis proteins (IAPs) thus releasing their bound caspases.

SMAC was found to be over-expressed in some carcinomas and sarcomas, yet showed reduced expression levels in other cancers. This discrepancy between the increased SMAC expression level seen in many cancers and its pro-apoptotic activity may result from another unidentified function of SMAC (Paul, A et al. Mol. Therapy 2018; 26(3):680-694).

AIF is also over-expressed in NSCLC (Fig. 2). AIF, released to the cytosol upon apoptosis induction, translocates to the nucleus, where it triggers chromatin condensation and DNA degradation. As a pro-apoptotic protein, it is not clear why AIF is over-expressed in cancer cells. AIF, however, has emerged as a protein critical for cell survival, as homozygous AIF knockout in mice is embryonically lethal. The pro-survival activity of AIF was proposed to be related to oxidative phosphorylation, ROS detoxification, redox-sensing, mitochondrial morphology and cell cycle regulation. Thus, AIF over-expression in some cancers may offer an advantage to cancer cells via these additional functions.

Unexpectedly, the present invention demonstrates the cellular localization of SMAC/Diablo, being found not only in mitochondria but also in the nucleus, specifically in the nuclei of SCC samples (Fig. 6). Thus, the presence of SMAC/Diablo in the nucleus may be a clear signature for SCC.

Proteins with modified expression in NSCLC as potential biomarkers

Proteomics (LC-HR MS/MS) analysis of healthy and NSCLC tissues from the same lung revealed several proteins that were highly expressed in the cancer, some of which were previously reported to be associated with other cancers and others are reported as such for the first time here (Fig. 1, Fig. 3, Table 6). These proteins cover a spectrum of functional categories, such as tumor suppressors, protease inhibitors, structural proteins, RNA-binding factors, signaling of immune receptors, coordinators of mitochondrial peptide transmembrane transport, lipid or galactose metabolism or act as protein kinases.

Rab11b protein was over-expressed (~8000-fold) in the tumor tissues, yet was almost absent in the healthy lung tissues in all tested samples (Fig. 3, Table 6). The Rab11 family (Rab11a, Rab11b and Rab25) is associated with recycling endosomes, and Rab25 was previously reported as associated with cancer (Cheng KW, et al. Nat

Med 2004;10:1251-6). Vesicular trafficking in cancer has been suggested to regulate tumor invasion (Steffan JJ, et al. PLoS One 2014; 9:e87882).

HYOU1, also known as HSP12A, GRP170 or ORP150, is over-expressed (~60-fold) in lung cancer tissue (Fig. 3, Table 6). HYOU1 is proposed to play an important role in protein folding and secretion in the ER, and contributes to cytoprotection in hypoxia-induced cellular perturbation (Ozawa K, et al. J Biol Chem 1999; 274:6397-404). HYOU1 was shown to be up-regulated in breast and nasopharyngeal carcinomas, and was associated with tumor invasiveness and poor prognosis. It was also shown to be overexpressed in NSCLC (BC, Rom W, et al. Clin Proteomics. 2016; 13: 31).

EGFR and MEK1 were found to be over-expressed in the tumor, as compared to healthy lung tissues (Table 6). Hyper-activation of the EGFR-Ras-MAPK pathway, with the involvement of mutated protein versions, is the most common alteration in lung cancer (Campbell et al. 2016, *ibid*; Paez JG, et al. Science 2004; 304:1497-500; Mitsudomi T, et al. Cancer Sci 2007; 98:1817-24). Thus many of these proteins may serve as NSCLC biomarkers.

Biomarkers for SCC and AC diagnosis

The two main subtypes of NSCLC, AC and SCC, show differences in mutation within the genome, epigenome, transcriptome, and proteome (Campbell et al., 2016, *ibid*). Thyroid transcription factor-1 (TTF-1) is currently used in the clinic to distinguish between AC and SCC (Fujita J, et al. Lung Cancer 2003;39:31-6). Nevertheless, it is still challenging distinguishing between these two NSCLC sub-types (Zakowski MF, et al. Arch Pathol Lab Med 2016;140:1116-20). Precise diagnosis is essential for selecting the appropriate treatment and thus increasing a patient's life expectancy.

The present invention discloses newly identified proteins that allow for distinguishing between AC and SCC and also confirm the differential expression of several previously reported proteins (Tables 7 and 10). Compared to samples from healthy tissues, the expression of HAT1, LRRFIP2, AKR1B10, WDR82, TTLL12, IGF2BP3, and SMC2 was demonstrated to be upregulated in NSCLC subtype SCC and downregulated in NSCLC subtype AC. The expression level of ACAD8, RSU1, ACOT1, HYOU1 and GALE was upregulated in NSCLC subtype AC while it was downregulated in NSCLC subtype SCC. The expression level of ITGA7 was

upregulated in both SCC and AC subtypes, but with a significantly more pronounced upregulation in SCC. Same pattern was shown for USP14, known to be overexpressed in NSCLC. On the other hand, the expression of TSG10, while also upregulated in both subtype, was significantly higher in AC compared to SCC. The expression level of
5 RAB34 was downregulated compared to the healthy control in both AC and SCC, but the reduction was significantly lower in AC compared to SCC.

AKR1B10 has been previously reported as a potential diagnostic marker specific to smokers' NSCLCs; TSG101 was shown to be involved in lung cancer cell proliferation and IGF2BP3 was reported to be over-expressed in various types of
10 cancer, including NSCLC. Several of the proteins have been proposed to be associated with cancer, but not with NSCLC. TTL12 and HAT1 were previously reported to be associated with prostate cancer or lymphoma and esophageal squamous cell carcinoma progression, respectively (Table 7). ITGA7 has been shown to be associated with the occurrence and development of bladder cancer. RAB34 has been reported as a
15 progression- and prognosis-associated biomarker in gliomas and Ras-associated sarcomagenesis. LRRFIP2, WDR82, ACOT1, SMC2, ACAD8, GALE, and RSU1 were not identified previously as possible biomarkers for any type of cancer (Fig. 4B, Table 7). Finally, the expression levels of several of these proteins affected AC patient survival but had no effect on SCC survival (Table 9).

20 As demonstrated herein, proteins selected based on their differential expression levels in AC and SCC as revealed by LC-HR MS/MS (Fig. 4B) typically showed differential RNA levels in SCC and AC (Fig. 5A). Further analysis of RNAseq UCSC XENA data, selecting genes encoding proteins showing differential expression levels in AC and SCC (LC-HR MS/MS data) was performed. The mRNA levels encoding for
25 proteins associated with variety of functions were changed in AC and SCC (3-60-fold) (Fig. 5B). This analysis confirmed previous reports suggesting TP63 and Ck5, Ck13, Ck14, Ck17, CSTA and PFN2 as biomarkers for SCC. AKR7A3 and ACAD8 were identified here for the first time as being over-expressed in AC (2-6-fold), relative to their expression levels in SCC (Fig. 5B). Genes such as NPC2 (Niemann-Pick disease,
30 type C2), a secreted protein, and ARRB1, were previously reported as biomarkers for lung AC and confirmed here (Tables 8 and 10).

Another interesting group of genes that are highly expressed in AC, relative to SCC, are those associated with fatty acid/lipid metabolism and transport. Previously reported to be associated with AC is AZGP1 (zinc-alpha2-glycoprotein) (Albertus DL, et al. J Thorac Oncol 2008;3:1236-44), a secreted protein that stimulates lipid
5 degradation in adipocytes and causes the extensive fat losses associated with some advanced cancers (Bing C, et al. Proc Natl Acad Sci U S A 2004;101:2500-5). ACOT1 (acyl-CoA thioesterase 1) a secreted protein that is a regulator of peroxisomal lipid metabolism (Hunt MC, et al. J Biol Chem 2002;277:1128-38), and ACAD8 (isobutyryl-CoA dehydrogenase), a mitochondrial protein catalyzing the dehydrogenation of acyl-
10 CoA derivatives in the metabolism of fatty acids or branched- chain amino acids such as valine (Battaile KP, et al. J Biol Chem 2004;279:16526-34), are reported herein as markers for NSCLC subtype AC for the first time. In this respect, AC mostly originates from alveolar type 2 (AT2) cells, with lipid metabolism systems being part of surfactant production associated with these cells.

15 Collectively, based on the expression levels (fold change), specific expression in AC or SCC of protein/mRNA identified here for the first time, or in previous reports and confirmed here, we propose a list of proteins differentially expressed in SCC and AC, of which four are secreted proteins (Tables 7, 8 and 10) that can be used to clearly distinguish between SCC or AC. This is of high importance for guiding the appropriate
20 treatment for these two NSCLC sub-types. In summary, the present invention identified several proteins the expression levels of which are highly increased in lung cancer patients. Moreover, some of these biomarkers can be used as profiling platforms enable to distinguish between AC and SCC. The use of these molecules may facilitate accurate diagnosis and prognostic prediction and could contribute to individualized lung cancer
25 treatment. Finally, the search for drugs that target the biomarkers differentially expressed in NSCLC subtype AC and NSCLC subtype SCC may lead to new specific treatments for each of the lung cancer subtypes.

Methods of measuring expression level

30 Comparing an expression level of a biomarker of the invention to its expression in a control sample or to a reference value comprises measuring and determining the expression level of the biomarker in a biological sample. Any method for detecting the

marker expression as is known to a person skilled in the art may be used according to the teachings of the present invention. In some embodiments, the expression level can be measured by proteomic analysis methods as known in the art. Proteomics is the practice of identifying and quantifying the proteins, or the ratios of the amounts of
5 proteins expressed in cells and tissues.

Those skilled in the art will be familiar with numerous specific immunoassay formats and variations thereof which may be useful for carrying out the method disclosed herein including enzyme linked immunosorbent assays (ELISAs), enzyme linked immunospot assay (ELISPOT), LC-HR MS/MS analysis, radioimmunoassays
10 (RIA), radioimmune precipitation assays (RIPA), immunobead capture assays, Western blotting, dot blotting, gel-shift assays, flow cytometry, immunohistochemistry (IHC), fluorescence microscopy, protein arrays, multiplexed bead arrays, magnetic capture, and *in vivo* imaging. See generally E. Maggio, Enzyme-Immunoassay, (1980) (CRC Press, Inc., Boca Raton, Fla.).

15 "Immunoassay" is an assay that uses an antibody to specifically bind an antigen. The immunoassay is characterized by the use of specific binding properties of a particular antibody to isolate, target, and/or quantify the antigen.

The phrase "specifically (or selectively) binds" to an antibody or "specifically (or selectively) immunoreactive with," or "specifically interacts or binds" when referring to
20 a protein or peptide (or other epitope), refers, in some embodiments, to a binding reaction that is determinative of the presence of the protein in a heterogeneous population of proteins and other biologics. Thus, under designated immunoassay conditions, the specified antibodies bind to a particular protein at least two times greater than the background (non-specific signal) and do not substantially bind in a significant
25 amount to other proteins present in the sample. Specific binding to an antibody under such conditions may require an antibody that is selected for its specificity for a particular protein. For example, polyclonal antibodies raised to lung-specific protein from specific species such as rat, mouse, or human can be selected to obtain only those polyclonal antibodies that are specifically immunoreactive with lung-specific protein
30 and not with other proteins, except for polymorphic variants and alleles of the lung specific protein. This selection may be achieved by subtracting out antibodies that

cross-react with lung-specific protein molecules from other species. A variety of immunoassay formats may be used to select antibodies specifically immunoreactive with a particular protein. For example, solid-phase ELISA immunoassays are routinely used to select antibodies specifically immunoreactive with a protein (see, e.g., Harlow & Lane, Antibodies, A Laboratory Manual (1988), for a description of immunoassay formats and conditions that can be used to determine specific immunoreactivity). Typically, a specific or selective reaction will be at least twice the background signal or noise and more typically more than 10 to 100 times the background signal.

In some embodiments, the level of the biomarker is measured by contacting the biological sample with a specific antibody. A specific antibody may be for example a polyclonal antibody, a monoclonal antibody, a chimeric antibody, a human antibody, an affinity matured antibody or an antibody fragment. While monoclonal antibodies are highly specific to a marker/antigen, a polyclonal antibody can preferably be used as a capture antibody to immobilize as much of the marker/antigen as possible.

Polyclonal antibodies are raised by injecting (e.g., subcutaneous or intramuscular injection) an immunogen into a suitable non-human mammal (e.g., a mouse or a rabbit). Generally, the immunogen should induce production of high titers of antibody with relatively high affinity for the target antigen. If desired, the marker may be conjugated to a carrier protein by conjugation techniques that are well known in the art. Commonly used carriers include keyhole limpet hemocyanin (KLH), thyroglobulin, bovine serum albumin (BSA), and tetanus toxoid. The conjugate is then used to immunize the animal. The antibodies are then obtained from blood samples taken from the animal. The techniques used to produce polyclonal antibodies are extensively described in the literature (see, e.g., Methods of Enzymology, "Production of Antisera with Small Doses of Immunogen: Multiple Intradermal Injections," Langone, et al. eds. (Acad. Press, 1981)). Polyclonal antibodies produced by the animals can be further purified, for example, by binding to and elution from a matrix to which the target antigen is bound. Those of skill in the art will know of various techniques common in the immunology arts for purification and/or concentration of polyclonal, as well as monoclonal, antibodies.

Monoclonal antibodies (mAbs) may be readily prepared through use of well-

known techniques, such as those exemplified in U.S. Pat. No. 4,196,265. Typically, this technique involves immunizing a suitable animal with a selected immunogen composition, polypeptide or peptide. The immunizing composition is administered in a manner effective to stimulate antibody producing cells. Rodents such as mice and rats
5 are preferred animals, however, the use of rabbit, sheep, or frog cells is also possible. The use of rats may provide certain advantages but mice are preferred, with the BALB/c mouse being most preferred as this is most routinely used and generally gives a higher percentage of stable fusions.

Antibodies can be conjugated to a solid support suitable for a diagnostic assay
10 (e.g., beads such as protein A or protein G agarose, microspheres, plates, slides or wells formed from materials such as latex or polystyrene) in accordance with known techniques, such as passive binding. Antibodies as described herein may likewise be conjugated to detectable labels or groups such as radiolabels (e.g., ³⁵S, ¹²⁵I, ¹³¹I), enzyme labels (e.g., horseradish peroxidase, alkaline phosphatase), and fluorescent
15 labels (e.g., fluorescein, Alexa, green fluorescent protein, rhodamine) in accordance with known techniques.

Immunohistochemical staining may also be used to measure the differential expression of a biomarker or a plurality of biomarkers. This method enables the localization of a protein in the cells of a tissue section by interaction of the protein with
20 a specific antibody. For this, the tissue may be fixed in formaldehyde or another suitable fixative, embedded in wax or plastic, and cut into thin sections (from about 0.1 mm to several mm thick) using a microtome. Alternatively, the tissue may be frozen and cut into thin sections using a cryostat. The sections of tissue may be arrayed onto and affixed to a solid surface (i.e., a tissue microarray). The sections of tissue are incubated
25 with a primary antibody against the antigen of interest, followed by washes to remove the unbound antibodies. The primary antibody may be coupled to a detection system, or the primary antibody may be detected with a secondary antibody that is coupled to a detection system. The detection system may be a fluorophore or it may be an enzyme as described hereinabove. The stained tissue sections are generally scanned under a
30 microscope. Because a sample of tissue from a subject with cancer may be heterogeneous, i.e., some cells may be normal and other cells may be cancerous, the percentage of positively stained cells in the tissue may be determined. This

measurement, along with a quantification of the intensity of staining, may be used to generate an expression value for the biomarker. According to certain embodiments, the biomarker expression is measured by IHC.

According to some embodiments, the level of the biomarker is measured by proteomic analysis. According to certain embodiments, the biomarker expression is measured by LC-MS/MS.

Nucleic acid testing (NAT) Assays

According to some embodiments, the methods of the invention comprise the comparing and/or detecting the expression level of genes.

Detection of a nucleic acid of interest in a biological sample may be effected by NAT-based assays, which involve nucleic acid amplification technology, such as PCR or variations thereof e.g. real-time PCR, quantitative PCR (qPCR) and the like.

Amplification of a selected or target nucleic acid sequence may be carried out by a number of suitable methods. Numerous amplification techniques have been described and can be readily adapted to suit particular needs of a person of ordinary skill. Non-limiting examples of amplification techniques include polymerase chain reaction (PCR), ligase chain reaction (LCR), strand displacement amplification (SDA), transcription-based amplification, the ϕ 29 replicase system and Nucleic acid sequence-based amplification (NASBA).

Quantitative real-time PCR (QRT-PCR) may be used to measure the differential expression of a marker or a plurality of biomarkers. In QRT-PCR, the RNA template is generally reverse transcribed into cDNA, which is then amplified via a PCR reaction. The amount of PCR product is followed cycle-by-cycle in real time, which allows for determination of the initial concentrations of mRNA. To measure the amount of PCR product, the reaction may be performed in the presence of a fluorescent dye, such as SYBR Green, which binds to double-stranded DNA. The reaction may also be performed with a fluorescent reporter probe that is specific for the DNA being amplified. A non-limiting example of a fluorescent reporter probe is a TaqManTM probe (Applied Biosystems, Foster City, CA). The fluorescent reporter probe fluoresces when the quencher is removed during the PCR extension cycle. Multiplex QRT-PCR may be

performed by using multiple gene-specific reporter probes, each of which contains a different fluorophore. Fluorescence values are recorded during each cycle and represent the amount of product amplified to that point in the amplification reaction. To minimize errors and reduce any sample-to-sample variation, QRT-PCR is typically performed using a reference standard. The ideal reference standard is expressed at a constant level among different tissues, and is unaffected by the experimental treatment. Suitable reference standards include, but are not limited to, mRNAs for the housekeeping genes glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) and beta-actin. The level of mRNA in the original sample or the fold change in expression of each biomarker may be determined using calculations well known in the art.

A nucleic acid microarray may be used to quantify the differential expression of a plurality of biomarkers. Microarray analysis may be performed using commercially available equipment, following manufacturer's protocols. Typically, single-stranded nucleic acids (e.g., cDNAs or oligonucleotides) are plated, or arrayed, on a microchip substrate. The arrayed sequences are then hybridized with specific nucleic acid probes from the cells of interest. Fluorescently labeled cDNA probes may be generated through incorporation of fluorescently labeled deoxynucleotides by reverse transcription of RNA extracted from the cells of interest. Alternatively, the RNA may be amplified by *in vitro* transcription and labeled with a marker, such as biotin. The labeled probes are then hybridized to the immobilized nucleic acids on the microchip under highly stringent conditions. After stringent washing to remove the non-specifically bound probes, the chip is scanned by confocal laser microscopy or by another detection method, such as a CCD camera. The raw fluorescence intensity data in the hybridization files are generally preprocessed with the robust multichip average (RMA) algorithm to generate expression values.

In situ hybridization may also be used to measure the differential expression of a plurality of biomarkers. This method permits the localization of mRNAs of interest in the cells of a tissue section. For this method, the tissue may be frozen, or fixed and embedded, and then cut into thin sections, which are arrayed and affixed on a solid surface. The tissue sections are incubated with a labeled antisense probe that will hybridize with an mRNA of interest. The hybridization and washing steps are generally performed under highly stringent conditions. The probe may be labeled with a

fluorophore or a small tag (such as biotin or digoxigenin) that may be detected by another protein or antibody, such that the labeled hybrid may be detected and visualized under a microscope. Multiple mRNAs may be detected simultaneously, provided each antisense probe has a distinguishable label. The hybridized tissue array is generally scanned under a microscope. Because a sample of tissue from a subject with cancer may be heterogeneous, i.e., some cells may be normal and other cells may be cancerous, the percentage of positively stained cells in the tissue may be determined. This measurement, along with a quantification of the intensity of staining, may be used to generate an expression value for each biomarker.

10 Kits

In some embodiments, the present invention provides an article of manufacture e.g., kit, such as an FDA approved kit, which contains diagnostic or prognosis reagents and instructions for use. The kit, in some embodiments, is accommodated by a notice associated with the container in a form prescribed by a regulatory agency regarding the manufacture, use or sale of pharmaceuticals, which notice is reflective of approval by the agency of the form of the compositions or human use.

According to certain aspects, the present invention provides a kit for diagnosing a subtype of non- small cell lung carcinoma (NSCLC) selected from adenocarcinoma (AC) and squamous cell carcinoma (SCC) in a biological sample obtained from a subject suspected to have NSCLC, the kit comprising:

(a) at least one agent capable of detecting the expression level of at least biomarker selected from a protein and mRNA encoding the protein, the biomarker is selected from the group consisting of HAT1, LRRFIP2, AKR1B10, WDR82, TTL12, IGF2BP3, SMC2, ACAD8, RSU1, ACOT1, HYOU1, GALE, ITGA7, TSG101, and RAB34;

(b) means for comparing the expression level of the at least one biomarker to a first reference value derived from the expression of the at least one biomarker in healthy biological sample and/or to a second reference value derived from the fold change of the expression of said at least one biomarker in a plurality of samples obtained from SCC patients compared to the expression in a plurality of healthy biological samples; and/or to a third reference value derived from a fold change of the expression of the at least

one biomarker in a plurality of samples obtained from AC patients compared to a plurality of healthy biological samples;

(c) instruction material providing guidance to the correlation of said expression level of said at least one biomarker with the NSCLC subtype, wherein:

5 an increased expression level in said sample of at least one biomarker selected from the group consisting of HAT1, LRRFIP2, AKR1B10, WDR82, TTL12, IGF2BP3, and SMC2, and/or reduced expression of at least one biomarker selected from the group consisting of ACAD8, RSU1, ACOT1, HYOU1, and GALE compared to the first reference value indicates that said
10 subject has NSCLC subtype SCC;

 a reduced expression level in the sample of at least one biomarker selected from the group consisting of HAT1, LRRFIP2, AKR1B10, WDR82, TTL12, IGF2BP3, and SMC2, and/or elevated expression of at least one biomarker selected from the group consisting of ACAD8, RSU1, ACOT1, HYOU1, and
15 GALE compared to the first reference value indicates that said subject has NSCLC subtype AC;

 an equal or elevated fold change of the biomarker TGA7 compared to the second reference value indicates that the subject has NSCLC subtype SCC;

 an equal or elevated fold change of the biomarker TSG101 compared to the
20 third reference value indicates that the subject has NSCLC subtype AC; and/or

 an equal or reduced fold change of the biomarker RAB34 compared to the third reference value indicates that the subject has NSCLC subtype AC.

 According to certain embodiments, the kit further comprises at least one agent capable of detecting the expression of SMAC/Diablo protein within the nucleus of cells
25 present within the biological sample and instruction material providing guidance to correlation of the amount of SMAC/Diablo within the cell nucleus and the cytosol and NSCLC subtype, wherein a significant amount of the SMAC/Diablo protein in the cell nucleus and cytosol diagnose the subject as having NSCLC subtype SCC and no significant amount of said SMAC/Diablo protein in the cell nucleus while a significant
30 amount is present in the cytosol diagnose the subject as having NSCLC subtype AC.

According to certain additional aspects, the present invention provides a kit for diagnosing NSCLC, the kit comprising:

(a) at least one agent capable of detecting the expression level of at least one biomarker selected from a protein and mRNA encoding said protein, the biomarker is
5 selected from the group consisting of APOOL, VPS29, and CAF17 in a biological sample of a subject suspected of having NSCLC;

(b) means for comparing the expression level of the at least one biomarker in a control sample obtained from a healthy subject or to a reference value; and

(c) instruction material providing guidance to the correlation of an increase in the
10 expression level of said at least one biomarker compared to the control sample or to the reference value with NSCLC.

The kits may include antibodies, protein arrays, reagents for use in immunoassays, protein controls, RNA arrays, reagents for use in NAT-based assays, instruction sheets in addition to the guidance instruction material, gene expression database, and/or any
15 means for determining and analyzing the expression level of the protein or RNA biomarkers according to the teachings of the invention.

Method of treating NSCLC, NSCLC subtype AC and NSCLC sybtype AC

The diagnostic methods of the present invention may further comprise treating the subject according to the diagnosis, and the present invention further provides method of
20 treating a subject having NSCLC, NSCLC subtype AC or NSCLC subtype SCC. The principle underlying these methods is administering the subject and agent reducing the expression or activity of proteins highly expressed in each of these diseases.

According to certain aspects, the present invention provides an agent reducing the expression or activity of at least one protein selected from the group consisting of
25 HAT1, LRRFIP2, AKR1B10, WDR82, TTLL12, ITGA7, IGF2BP3, and USP14 for use in treating NSCLC subtype SCC.

According to certain additional aspects, the present invention provides an agent reducing the expression or activity of at least one protein selected from the group consisting of ACAD8, TSG101, and GALE for use in treating NSCLC subtype AC.

30 According to yet further aspects, the present invention provides an agent reducing

the expression or activity of at least one protein selected from the group consisting of APOOL, VPS29, and CAF17 for use in treating NSCLC.

According to some embodiments, the agent reducing the expression or activity of the at least one protein is selected from the group consisting of a chemical agent or moiety, a protein, a peptide, and a polynucleotide molecule.

According to some embodiments, the agent is an antibody. Methods for preparing antibodies specifically binding to the protein of interest are known in the art and described hereinabove.

According to some embodiments, the agent is an interfering RNA (RNAi) molecule. In certain embodiments, the interfering RNA molecule is selected from the group consisting of a shRNA, a siRNA, and a miRNA.

In certain aspects, an interfering RNA of the invention has a length of about 19 to about 49 nucleotides. In other aspects, the interfering RNA comprises a sense nucleotide strand and an antisense nucleotide strand.

RNA interference (RNAi) is a process by which double-stranded RNA (dsRNA) is used to silence gene expression. While not wishing to be bound by any theory or mechanism of action, RNAi begins with the cleavage of longer dsRNAs into small interfering RNAs (siRNAs) by an RNaseIII-like enzyme, dicer. SiRNAs are dsRNAs that are typically about 19 to 28 nucleotides, or 20 to 25 nucleotides, or 21 to 22 nucleotides in length and often contain 2-nucleotide 3' overhangs, and 5' phosphate and 3' hydroxyl termini. One strand of the siRNA is incorporated into a ribonucleoprotein complex known as the RNA-induced silencing complex (RISC). RISC uses this siRNA strand to identify mRNA molecules that are at least partially complementary to the incorporated siRNA strand, and then cleaves these target mRNAs or inhibits their translation. Therefore, the siRNA strand that is incorporated into RISC is known as the guide strand or the antisense strand. The other siRNA strand, known as the passenger strand or the sense strand, is eliminated from the siRNA and is at least partially homologous to the target mRNA.

Those of skill in the art will recognize that, in principle, either strand of siRNA molecule can be incorporated into RISC and function as a guide strand. However, siRNA design (e.g., decreased siRNA duplex stability at the 5' end of the desired guide

strand) can favor incorporation of the desired guide strand into RISC.

The antisense strand of an siRNA is the active guiding agent of the siRNA in that the antisense strand is incorporated into RISC, thus allowing RISC to identify target mRNAs with at least partial complementarity to the antisense siRNA strand for cleavage or translational repression. RISC-mediated cleavage of mRNAs having a sequence at least partially complementary to the guide strand leads to a decrease in the steady state level of that mRNA and of the corresponding protein encoded by this mRNA. Alternatively, RISC can also decrease expression of the corresponding protein via translational repression without cleavage of the target mRNA.

Interfering RNAs of the invention appear to act in a catalytic manner for cleavage of target mRNA, i.e., interfering RNA is able to effect inhibition of target mRNA in substoichiometric amounts. As compared to antisense therapies, significantly less interfering RNA is required to provide a therapeutic effect under such cleavage conditions.

Selection of appropriate oligonucleotides is facilitated by using computer programs that automatically align nucleic acid sequences and indicate regions of identity or homology. Such programs are used to compare nucleic acid sequences obtained, for example, by searching databases such as GenBank or by sequencing PCR products. Comparison of nucleic acid sequences from a range of species allows the selection of nucleic acid sequences that display an appropriate degree of identity between species. These procedures allow the selection of oligonucleotides that exhibit a high degree of complementarity to target nucleic acid sequences in a subject to be controlled and a lower degree of complementarity to corresponding nucleic acid sequences in other species. One skilled in the art will realize that there is considerable latitude in selecting appropriate regions of genes for use in the present invention.

Pharmaceutical compositions

The agents of the present invention can be administered to a subject per se, or in a pharmaceutical composition where they are mixed with suitable carriers or excipients. Examples of suitable pharmaceutically acceptable carriers may include water, saline, PBS (phosphate buffered saline), dextrin, glycerol, and ethanol. The pharmaceutically acceptable carrier may be formulated for administration to a human subject or patient.

The composition may be formulated into a dosage form which can release the active ingredient in a rapid or a sustained or delayed manner after administration.

According to some embodiments, the composition comprises as an active agent an interfering RNA molecule.

5 The interfering RNA molecule can be administered in a variety of methods as known in the art. Systemically administered RNA is rapidly cleared by the kidneys or liver due to its high solubility in water and negative charge. Therefore, according to some embodiments, the RNA is encapsulated. The encapsulation might enhance the circulation time of the RNA in the body and prevent degradation by extracellular
10 nucleases. According to some embodiments, the pharmaceutical composition comprises a siRNA component and lipid component. According to certain embodiments, the interfering RNA molecule is administered within liposome. For example, WO2006113679 provides methods for the delivery of RNA interfering molecules to a cell via a neutral (non-charged) liposome. WO201011317 describes the use of
15 amphoteric liposomal compositions for cellular delivery of small RNA molecules for use in RNA interference.

 According to other embodiments, the interfering RNA molecule is administered directly or via a nucleic acid delivery system. The system may comprise a compound that stabilizes the RNA, such as a lipid or a protein. For example, WO1995022618
20 discloses a delivery system that contains a fusion protein having a target moiety and a nucleic acid binding moiety.

 According to other embodiments, the composition comprises as an active agent at least one antibody specific to one biomarker according to the teachings of the invention.

 The actual dosage amount of a composition of the present invention administered
25 to a patient or subject can be determined by physical and physiological factors such as body weight, severity of condition, previous or concurrent therapeutic interventions, and on the route of administration. The practitioner responsible for administration will determine the concentration of active ingredient(s) in a composition and appropriate dose(s) for the individual subject.

30 The following examples are presented in order to more fully illustrate some embodiments of the invention. They should, in no way be construed, however, as

limiting the broad scope of the invention. One skilled in the art can readily devise many variations and modifications of the principles disclosed herein without departing from the scope of the invention.

5 EXAMPLES

Materials and Methods

Materials

Phenylmethylsulfonyl fluoride (PMSF), propidium iodide (PI), and trypan blue were purchased from Sigma (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM) and the supplements fetal calf serum, L-glutamine and penicillin-streptomycin were purchased from Biological Industries (Beit Haemek, Israel).. Horseradish peroxidase (HRP)-conjugated anti-mouse, anti-rabbit and anti-goat antibodies were from KPL (Gaithersburg, MD). 3,3-diaminobenzidine (DAB) was obtained from ImmPact-DAB (Burlingame, CA). Primary antibodies used in immunoblotting and immunohistochemistry (IHC), as well as their dilutions, are listed in Table 3.

Table 3: Antibodies used

Antibodies against the indicated protein, their catalogue number, source and the dilutions used in IHC and immunoblot experiments (Western blots, WB) are presented.

Antibody	Source and Catalogue Number	Dilution used	
		IHC	WB
Mouse monoclonal anti- β -Actin	Millipore, Billerica, MA, MAB1501	-	1:10,000
Mouse monoclonal anti-ATP5B	Abcam, Cambridge, UK, ab14730	-	1:10,000
Rabbit monoclonal anti-AIF	Abcam, ab32516	1:200	1:1000
Mouse monoclonal anti-Bcl-2	Calbiochem , Nottingham, UK, OP60	-	1:2000
Mouse monoclonal anti-HK-I	Abcam, ab105213	1:500	1:2000
Rabbit monoclonal anti-HK II	Santa Cruz Biotechnology Dallas, TX , sc-27230	-	1:1000
Goat polyclonal anti-LDHA	Epitomics, Cambridge, UK, 1980-1	1:300	1:1000
Rabbit polyclonal anti-MAVS	ALX-210-929-C100		1:2000
Rabbit monoclonal anti-HYOU1	Abcam, ab134944		1:3000

Antibody	Source and Catalogue Number	Dilution used	
		IHC	WB
Rabbit monoclonal anti- Hsp60	Abcam, ab46798	-	1:10,000
Mouse monoclonal anti - GAPDH	Abcam, ab9484	-	1:1000
Rabbit monoclonal anti- Rab11b	Santa Cruz Biotechnology, Dallas, TX, ab3612	-	1:1000
Rabbit monoclonal anti- SMAC/Diablo	Abcam, ab8115	1:300	1:2000
Rabbit monoclonal anti- VDAC1	Abcam, ab15895	1:500	1:5000
Goat anti-Rabbit- HRP	KPL, Gaithersburg, PA, 474-1506	1:250	1:15,000
Donkey anti-Goat- HRP	Abcam, ab97120	-	1:20,000

Patients

All the investigations presented in this study were conducted after informed consent was obtained and in accordance with an institutional review board protocol approved by the Ethics Committee of Soroka University Medical Center. All human tissues were collected with the understanding and written consent of each subject, and the study methodologies conformed to the standards set by the Declaration of Helsinki.

NSCLC specimens were obtained from 2010 to 2016 from 46 patients who underwent lung resection without any treatment at the time of surgery. The main clinical and pathologic variables of the patients are provided in Table 4.

Fresh paired healthy and cancer tissue specimens were obtained from the same lung cancer patient who underwent either pneumonectomy or pulmonary lobectomy to remove tumors tissue. The specimens were immediately frozen in liquid nitrogen and maintained at -80°C until analysis by immunoblotting or qPCR. Proteins were extracted from the tissue sample as described below. Cancer and normal lung tissue surrounding the tumor were validated by hospital pathologists.

Twenty-eight patients were males and twenty-seven were females, with an average age of 68 years (range, 36-86). Disease stage was staged according to the international tumor-node-metastasis system (TMM) and then classified to the ranging

from occult cancer, through stage 0, IA, IB, IIA, IIB, IIIA, IIIB to IV (grade I, n=30), (grade II, n=10), (grade III, n=5) (grade IV, n=1).

Table 4: Lung cancer patient characteristics

Patient No.	Age (years)	Gender	Type of Cancer	Stage of Disease
1	76	F	AC	2B
2	77	M	SCC	1A
3	54	M	AC	3A
4	58	M	SCC	2B
5	70	M	SCC	2B
6	69	F	AC	2A
7	36	M	AC	3A
8	62	M	AC	1A
9	82	M	AC	2A
10	48	M	AC	1A
11	65	F	AC	1A
12	78	M	SCC	1B
13	72	M	AC	1A
14	78	M	AC	1A
15	55	M	AC	1A
16	59	F	SCC	1A
17	74	F	SCC	2A
18	65	M	SCC	4
19	76	M	SCC	3A
20	65	F	SCC	1A
21	61	M	AC	1A
22	54	M	SCC	1B
23	56	F	AC	1A
24	58	M	AC	1A
25	55	F	AC	1A
26	76	F	AC	1A
27	85	M	AC	1B
28	55	F	AC	1A
29	62	M	AC	2A
30	79	M	AC	3A
31	81	M	AC	1A
32	62	F	AC	1B
33	83	F	SCC	1B
34	77	M	SCC	1A
35	86	M	SCC	1B
36	74	M	SCC	1B
37	74	M	AC	1A
38	67	F	AC	2A

39	71	M	AC	1A
40	75	F	AC	3A
41	85	M	SCC	1A
42	77	M	SCC	2A
43	59	F	SCC	2A
44	59	F	SCC	1B
45	58	M	AC	1B
46	68	F	AC	1A

5

Protein extraction from lung tissue

To extract proteins for immunoblotting, healthy and tumor lung tissues were solubilized in a lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1.5 mM MgCl₂, 10% glycerol, 1% Triton X-100, a protease inhibitor cocktail (Calbiochem)), followed by sonication and centrifugation (10 min, 600 g). The protein concentration of each lysate was determined using Lowry assay. Samples were stored in -80°C until analysis by gel electrophoresis and immunoblotting, as described hereinbelow. To extract proteins for LC-HR MS/MS, healthy and tumor lung tissues were solubilized in a different lysis buffer (100 mM Tris-HCl, pH 8.0, 5 mM DTT, 4% SDS and a protease inhibitor cocktail (Calbiochem; 100 µl/10 mg)), followed by homogenization, incubation for 3 min at 95°C and centrifugation (10 min, 15,000 g). The protein concentration of each lysate was determined using Lowry assay. Samples were stored in -80°C until MS/MS analysis, as described hereinbelow.

Gel electrophoresis and immunoblotting

Samples (10-40 µg of protein) were subjected to SDS-PAGE. Gels were stained with Coomassie Brilliant Blue or electro-transferred onto nitrocellulose membranes for immunostaining. Membranes containing the transferred proteins were blocked with 5% non-fat dry milk and 0.1% Tween-20 in Tris-buffered saline (TBS) and incubated overnight at 4°C with the different primary antibodies (sources and dilutions as detailed in Table 3), followed by incubation with the appropriate HRP-conjugated secondary antibodies for 1h. Enhanced chemiluminescence (Biological Industries) was used for detection of HRP activity. Band intensities were analyzed using FUSION-FX (Vilber Lourmat, France) and the values were normalized to the intensities of the appropriate α-actin signal that served as a loading control.

RNA isolation and qPCR

Total RNA was isolated from healthy and tumor lung samples using an RNeasy mini kit (Qiagen) according to the manufacturer's instructions. Total RNA quality was analyzed using the Agilent RNA 6000 nano kit. qPCR was performed using specific primers (KiCqStart Primers; Sigma Aldrich) in triplicate, using Power SYBER green master mix (Applied Biosystems, Foster City, CA). Levels of target genes were normalized relative to β -actin mRNA levels. Samples were amplified by a 7300 Real Time PCR System (Applied Biosystems) for 40 cycles using the following PCR parameters: 95°C for 15 seconds, 60°C for 1 minute, and 72°C for 1 minute. The copy numbers for each sample were calculated by the CT-based calibrated standard curve method. The mean fold changes ($\pm$ SEM) of the three replicates were calculated. Genes examined and primers used are listed in Table 5.

Table 5: qPCR primers used in this study

Gene	Primer sequences	SEQ ID NO
<i>β-Actin</i>	Forward 5'-ACTCTTCCAGCCTTCCTTCC-3'	1
	Reverse 5'- TGTTGGCGTACAGGTCTTTG-3'	2
<i>AKR1B10</i>	Forward 5'-GAGCAGGACGTGAGACTTCT-3'	3
	Reverse 5'-TTTGCCAAGAGGAGACTTCCAA-3'	4
<i>USP14</i>	Forward 5'-TGCCCTTAAAAGGTATGCAGGT-3'	5
	Reverse 5'- TCTCGGCAAACTGTGGGAAA-3'	6
<i>TLLI2</i>	Forward 5'-TGGAGCACGAGGTTTTCGAC-3'	7
	Reverse 5'- CGATGACCTTGTAGCACAGC-3'	8
<i>TSG101</i>	Forward 5'-GCCAGCTCAAGAAAATGGTGT-3'	9
	Reverse 5'- AGGTCTCTGTATTTGTACTGGGT-3'	10
<i>LRRF2</i>	Forward 5'-CCTCAGCAACAACCCCTCTA-3'	11
	Reverse 5'- GGT CATAGATATCCCGCAATTCA-3'	12
<i>WDR82</i>	Forward 5'-GCTTCGATTTTCAAGCCCAAC-3'	13
	Reverse 5'- TCTCTTTGGTTTGGCCTCCT-3'	14
<i>HAT1</i>	Forward 5'-ATGGCGGGATTTGGTGCTAT-3'	15
	Reverse 5'- GTTCAATTGCTGTGTTGGTGT-3'	16

LC-HR MS/MS analysis

Samples were subjected to in-solution tryptic digestion as follows: proteins were first reduced by incubation with 5 mM DTT for 30 min at 60°C, followed by alkylation with 10 mM iodoacetamide in the dark for 30 min at 21°C. Proteins were then subjected to digestion with trypsin (Promega, Madison, WI) at a 1:50 trypsin:protein ratio for 16 h at 37°C. Following digestion, detergents were cleared from the samples using commercial detergent removal columns (Pierce, Rockford, IL), and desalted using solid-phase extraction columns (Oasis HLB, Waters, Milford, MA). Digestion was stopped by addition of trifluoroacetic acid (1%). The samples were stored at -80°C until LC-HR MS/MS analysis.

For LC-HR MS/MS, ULC/MS grade solvents were used for all chromatographic steps. Each sample was separated using split-less nano-ultra performance liquid chromatography columns (10 kpsi nanoAcquity; Waters). The mobile phase was (A) H₂O and 0.1% formic acid, and (B) acetonitrile and 0.1% formic acid. Desalting of the samples was performed online using a reverse-phase C18 trapping column (180 µm internal diameter, 20 mm length, 5 µm particle size; Waters). The peptides were then separated using a T3 HSS nano-column (75 µm internal diameter, 250 mm length, 1.8 µm particle size; Waters) at 0.3 µL/min. Peptides were eluted from the column into the mass spectrometer using the following gradient: 4% to 35% (B) for 150 min, 35% to 90% (B) for 5 min, maintained at 90% for 5 min and then back to initial conditions. The nano-UPLC was coupled online through a nano-ESI emitter (10 µm tip; New Objective, Woburn, MA) to a quadrupole Orbitrap mass spectrometer (Q Executive, Thermo Scientific) using a FlexIon nanospray apparatus (Proxeon). Data were acquired in the DDA mode, using a Top12 method (Kelstrup CD, et al. J Proteome Res. 2012; 11: 3487-97). Raw data was imported into Expressionist software (Genedata) (Ueda K, et al. PLoS One. 2011; 6:e18567; Guryca V, et al. Proteomics. 2012; 12: 1207-1216). The software was used for retention time alignment and peak detection of precursor peptide intensities. A master peak list was generated from all MS/MS events and sent for database searching using Mascot v2.4 (Matrix Sciences). Data were searched against a database containing forward and reverse human protein sequences from UniprotKB/SwissProt, and 125 common laboratory contaminants, totaling 20,304 entries. Fixed modification was set to carbamidomethylation of cysteines, while

variable modification was set to oxidation of methionines. Search results were then imported back to Expressionist for annotation of detected peaks. Identifications were filtered such that the global false discovery rate was a maximum of 1%. Protein abundance was calculated based on the three most abundant peptides (D'Arena G, et al. Am J Hematol. 2006; 81: 598-602).

Proteins with less than 2 unique peptides were excluded from further analysis.

Samples from 9 AC patients were analyzed, with healthy and cancerous lung tissues being taken from the same patient lung. In additional assay, healthy and cancerous lung tissues were taken from 5 AC and 5 SCC patients. Proteins for which at least two unique peptides were identified were used for further analysis.

Immunohistochemistry (IHC) on tissue microarray (TMA) slides

Immunohistochemical staining was performed on formalin-fixed and paraffin-embedded tissue microarray slides obtained from Biomax US. The sections were deparaffinized using xylene and a graded ethanol series. Endogenous peroxidase activity was blocked by incubating the sections in 3% H₂O₂ for 10 minutes. Antigen retrieval was performed in 0.01M citrate buffer (pH 6.0) at 95°C-98°C for 20 min. After washing the sections in PBS (pH 7.4), non-specific antibody binding was reduced by incubating the sections in 10% normal goat serum for 2h. After decanting excess serum, sections were incubated overnight at 4°C with primary antibodies (Table 3). After washing with PBS, the sections were incubated for 2 h with the appropriate secondary antibodies conjugated to horseradish peroxidase (Table 3). Sections were washed three times in PBS and subsequently, the peroxidase-catalyzed reaction was visualized by incubation with 0.02% DAB. After rinsing in water, the sections were counterstained with hematoxylin, and mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Finally, the sections were observed under a microscope (DM2500, Leica) and images were taken at the indicated magnification with the same light intensity and exposure time. Controls were carried out with the same protocols but omitting the primary antibodies.

Biomax tissue arrays

Cancer tissue microarrays were purchased from Biomax US (US Biomax). These included arrays for lung cancer (LC807,) containing lung normal tissues (n=10) and various lung cancer types in different stages, including AC (n=21), adenosquamous carcinoma (n=1), squamous cell carcinoma (SCC, n=31), bronchioloalveolar carcinoma (BAC; n=6), small cell carcinoma (n=6) and large cell carcinoma (n=5). Second tissue array (BC041115c) contained normal lung tissue (n=10), and AC (n=51) and SCC (n=41) cancerous tissue samples.

RNAseq gene expression profiling

Data for the gene expression profile and for the heat map for healthy and tumor samples of lung cancer patients were obtained from XENA, TCGA [RNAseq using ployA+ Illumina HiSeq] (version 2016-08-16, TCGA hub, xena.ucsc.edu), with the unit being pan-cancer normalized (n=1,129). A linear fold of change and the statistical analysis were performed using a t-test.

Statistics and bioinformatics analysis

All descriptive statistics for data analysis were computed using the SPSS statistical package, version 17.0. Means $\pm$ SEM of results obtained from the indicated independent experiments are presented. The level of significance of differences between the control (healthy) and experimental (cancer) groups was determined by non-parametric Mann-Whitney U test. A difference was considered statistically significant when the P value was deemed <0.05 (*), <0.01 (**) or <0.001 (***).

LC-HR-MS/MS data were imported into Partek Genomics Suite software (Partek, St. Louis, MO) and differences between expression levels of the proteins in the different groups were calculated using a t-test. Functional enrichment analysis of differentially expressed proteins was performed using the DAVID and Gene Ontology (GO) bioinformatics resources, v6.7 (Nawarak J, et al. Biochim Biophys Acta. 2009; 1794: 159-67).

Example 1: Mass spectrometry analysis of the protein profiles of healthy and tumor tissues from NSCLC patients

To identify the proteins showing modified expression levels in NSCLC tumor tissues, relative to healthy tissues, nine samples of cancerous and healthy tissues were collected from the same lung of NSCLC patients and subjected to LC-HR MS/MS analysis. Hierarchical clustering based on the expression pattern of all detected proteins clearly allowed to distinguish between the healthy and tumor tissues (Fig. 1A), with the expression level of 1,494 proteins being changed (fold change (FC) ≥ 2 and false discovery rate (FDR) < 0.05 , of which 378 proteins showed a FC ≥ 100) (Fig. 1B). The up- and down-regulated proteins were further divided into two clusters, based on the combination of FC and p-value, due to some of the proteins being “absent” from some of the samples.

Next, functional analysis of the proteins differentially expressed between cancerous and healthy lung tissues was performed using the DAVID and Gene ontology databases (Ashburner M, et al. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nat Genet. 2000; 25: 25-9; Gene Ontology Consortium. Gene Ontology Consortium: going forward. Nucleic Acids Res. 2015; 43: D1049-56). Such analysis revealed enrichment of proteins related to protein synthesis and degradation, and in particular of proteins assigned roles in metabolism and to the mitochondria (Fig. 1C) (Table 6).

Table 6: Selected proteins differentially expressed in healthy donors and lung cancer patients identified by LC-HR MS/MS

No	Protein name (Uniprot)	Fold change/ P value	Proposed function (cell localization)	Relation to cancer
1	RB11B - Ras-related protein Rab-11B	>1000 7.7×10^{-12}	Regulator of intracellular membrane trafficking (Extracellular space, Endosome)	Over-expressed in HL-60 leukemia cell line
2	PIGS - GPI transamidase component PIG-S	>1000 1.3×10^{-9}	Component of the GPI transamidase complex (ER)	Over-expressed in breast, ovary and uterus cancers
3	APOOL - Apolipoprotein O-like	>1000 2.1×10^{-9}	Component of a large protein complex that functions in the maintenance of crista	No reported data

No	Protein name (Uniprot)	Fold change/ P value	Proposed function (cell localization)	Relation to cancer
			junctions (Mitochondria)	
4	NICA - Nicastrin	>1000 5.8x10 ⁻⁹	A subunit of the gamma-secretase complex (Melanosome)	Regulates breast cancer stem cell properties and tumor growth
5	NDKB - Nucleoside diphosphate kinase B	14.5 3.1x10 ⁻⁹	Synthesis of nucleoside triphosphates other than ATP (Cytosol, Nucleus)	High expression reduce metastases in breast cancer, melanoma
6	HNRPL - Heterogeneous nuclear ribonucleoprotein L	7.3 1.3x10 ⁻⁸	Splicing factor, acting as activator or repressor of exon inclusion (Cytosol, Nucleus)	Marker for secondary to brain ALL metastasis
7	LDHA - L-lactate dehydrogenase A chain	14.8 2.3x10 ⁻⁸	Catalyzes the conversion of pyruvate to lactate and back (Cytosol)	Over-expressed in NSCLC, pancreas, colorectal cancer and more
8	STT3A - Dolichyl-diphospho-oligo saccharide-protein glycosyltransferase	8.3 1.2x10 ⁻⁷	Catalytic subunit of the N-oligosaccharyl transferase (OST) complex (ER)	Marker for follicular thyroid carcinoma
9	COPA - Coatamer subunit alpha	14.6 1.3x10 ⁻⁷	Part of a complex that mediates protein transport from the ER to the Golgi, (Cytosol, Golgi)	Associated with mouse mesothelioma progression
10	PDL15 - PDZ and LIM domain protein 5	9.2 1.8x10 ⁻⁷	Z-disc protein that interacts directly with α -actinin-2 (Cytosol, Cell junction)	Associated with gastric cancer. High deletion frequencies in oral squamous cell carcinoma.
11	HINT1- Histidine triad nucleotide-binding protein 1	5.4 2.0x10 ⁻⁷	Hydrolyzes purine nucleotide phosphoramidates (Cytosol, Nucleus)	Over-expressed in prostate cancer
12	SEC11A - Signal peptidase complex catalytic subunit	>1000 2.3x10 ⁻⁷	Component of a complex that removes signal peptides from proteins translocated into the ER (ER)	Contributes to malignant progression in gastric cancer
13	DDX6 - DEAD box protein 6	62.8 2.5x10 ⁻⁷	Participates in mRNA degradation (Cytosol, Nucleus)	Chromosomal aberrations, DDX6 contribute to lymphomagenesis
14	PGK1 - Phosphoglycerate kinase 1	8.9 3.2x10 ⁻⁷	Glycolytic enzyme, converting 3-phospho-D-glycerate to 3-phospho-D-glyceroyl phosphate (Cytosol)	Prognostic biomarker of poor survival and chemoresistance to paclitaxel treatment in breast cancer
15	IF4E - Eukaryotic translation initiation factor 4E	7.7 3.5x10 ⁻⁷	Participates in the initiation of translation (Cytosol)	eIF4E over-expression can initiate malignant transformation
16	GDIB - Rab GDP dissociation inhibitor	4.5 3.9x10 ⁻⁷	Regulates the GDP/GTP exchange of most Rab	Increased in metastatic gallbladder cancer cell

No	Protein name (Uniprot)	Fold change/ P value	Proposed function (cell localization)	Relation to cancer
	beta		proteins (Cytosol, Plasma membrane)	line SD18H and in pancreatic carcinoma
17	RL9 - 60S ribosomal protein L9	21.1 4.5×10^{-7}	Translation. Component of the 60S subunit (Cytosol)	Over-expressed in colon adenoma and adenocarcinoma
18	NDUS7 - ADH dehydrogenase (ubiquinone) iron-sulfur protein 7	>1000 4.7×10^{-7}	Core subunit of the respiratory chain NADH dehydrogenase (Mitochondria)	Amplification in BRCA1-associated ovarian cancer
19	PTBP1 - Polypyrimidine tract-binding protein 1	8.4 5.1×10^{-7}	Plays a role in pre-mRNA splicing (Nucleus)	Over-expressed in colorectal cancer, gemcitabine resistance in pancreatic cancer, associated with breast tumorigenesis
20	CPNS1 - Calpain small subunit 1	11.8 5.7×10^{-7}	Regulatory subunit of the calcium-regulated thiol-protease (Cytosol, Plasma membrane)	Promotes NSCLC progression, over-expressed in liver cancer, marker of poor prognosis in nasopharyngeal carcinoma
21	PA1B2 - Platelet-activating factor acetyl-hydrolase 1B subunit beta	9.9 5.9×10^{-7}	Inactivates PAF (platelet-activating factor) (Cytosol)	Important in maintaining cancer pathogenicity across a wide spectrum of cancer types
22	PPOX - Protoporphyrinogen oxidase	>1000 6.6×10^{-7}	Catalyzes the oxidation of protoporphyrinogen-IX to form protoporphyrin-IX (Mitochondria)	Higher expression in faster growing cell lines and primary colorectal tumors
23	GBLP - Guanine nucleotide-binding protein subunit beta-2-like 1	5.8 7.0×10^{-7}	Intracellular receptor that binds activated PKC (Plasma membrane, Cytosol)	Over-expressed in NSCLC, breast cancer, hepatocellular carcinoma, esophageal squamous cell carcinoma
24	RL10 - 60S ribosomal protein L10a	7.8 7.1×10^{-7}	Translation. Component of the 60S subunit (Cytosol)	Mutated in T-cell acute lymphoblastic leukemia
25	ENO1 - Alpha-enolase	9.7 7.6×10^{-7}	Glycolytic enzyme (Cytosol)	Upregulated in lung, brain, breast, colon cancers
26	ILF2 - Interleukin enhancer-binding factor 2	5.0 7.7×10^{-7}	Regulatory subunit of complexes involved in mitotic control, DNA break repair, and RNA splicing regulation (Cytosol Nucleus)	Higher expression in esophageal squamous cell carcinoma
27	ROA1 (HNRNPA1) - Heterogeneous nuclear ribonucleo-	5.6 9.3×10^{-7}	Involved in the packaging of pre-mRNA into hnRNP particles (Cytosol,	Biomarker in cervical carcinoma, lung cancer progression

No	Protein name (Uniprot)	Fold change/ P value	Proposed function (cell localization)	Relation to cancer
	protein A1		Nucleus)	
28	VPS29- Vacuolar protein sorting-associated protein 29	14.7 9.3x10 ⁻⁷	Component of the retromer cargo-selective complex (CSC) (Cytosol, Cell membrane, Endosome membrane)	No reported data
29	UGPA - UTP-glucose-1-phosphate uridylyltransferase	7.7 9.5x10 ⁻⁷	Glucosyl donor in cellular metabolic pathways (Cytosol)	Biomarker for metastatic hepatocellular carcinoma
30	DDX17 - DEAD box protein 17	5.6 1.2x10 ⁻⁶	RNA helicase, involved in transcription and splicing (Nucleus)	Increased expression in colon cancer
31	HAT1- Histone acetyltransferase type B catalytic subunit	4.75 4.4x10 ⁻³	Acetylates soluble histone H4 (nucleus), HAT1 is one of type B HAT members and functions in DNA repair.	High expression in several types of lymphomas. proposed indicator for a poor prognosis (Min SK, et al. Korean J Pathol. 2012; 46: 142-50) and potential drug target in esophageal SCC (Xue L, et al. Int J Clin Exp Pathol. 2014; 7: 3898-907)
32	RS3 - 40S ribosomal protein S3	9.4 1.2x10 ⁻⁶	Translation. Component of the 40S subunit (Cytosol, Nucleus)	Proposed as an indicator of malignant tumors, over-expressed in colorectal cancer, under-expressed SCC
33	OSBL8 - Oxysterol-binding protein-related protein 8	>1000 1.2x10 ⁻⁶	Binds 25-hydroxycholesterol and cholesterol (ER membrane, Nucleus membrane)	Down-regulated in hepatoma tissues
34	TXD12 (ERp19) - Thioredoxin domain-containing protein 12	37.6 1.4x10 ⁻⁶	Involved in thiol-disulfide oxidase activity (ER)	A thioredoxin-like protein, implicated in development of breast, ovarian, gastrointestinal and gastric cancers
35	USO1 - General vesicular transport factor p115	8.7 1.4x10 ⁻⁶	General vesicular transport factor in Golgi (Cytosol, Golgi)	Promotes proliferation of gastric cancer cells
36	COPB2 - Coatomer subunit beta 2	12.0 1.4x10 ⁻⁶	Involved in protein transport from the ER to the Golgi (Cytosol, Golgi)	Over-expressed (mRNA) in lung adenocarcinoma tumors
37	SMD3 - Small nuclear ribonucleoprotein Sm D3	9.0 1.4x10 ⁻⁶	Core component of the spliceosome (Cytosol, Nucleus)	Associated with metastatic behavior is soft tissue tumors
38	ITB2 - Integrin beta-	5.9	Cell adhesion (Plasma	Over-expressed in CLL

No	Protein name (Uniprot)	Fold change/ P value	Proposed function (cell localization)	Relation to cancer
	2	1.5×10^{-6}	membrane, Exosome)	patients harboring trisomy 12
39	COPB1 - Coatamer subunit beta 1	6.5 1.5×10^{-6}	Involved in protein transport from the ER to the Golgi (Cytosol, Golgi)	Over-expressed in prostate cancer
40	MYH9 - myosin 9	6.5 1.7×10^{-6}	Motor protein (Cytosol)	Highly expressed in CL16 breast cancer cell tumors in mice
41	RAGE - Receptor for advanced glycolylation end products	-12.2 1.9×10^{-6}	Binds advanced glycation end products (Plasma membrane, Extracellular space)	Polymorphism associated with susceptibility to renal, lung and gastric cancers
42	VDAC1 - voltage dependent anion channel 1	6.3 2.2×10^{-6}	Channel transporting ions and metabolites, also involved in apoptosis (Mitochondria)	Over-expressed in CLL and lung cancer, predictor of poor outcome in early stage NSCLC
43	ENPL (HSP90B1) - Endoplasmic	7.3 2.3×10^{-6}	Chaperone that functions in the processing and transport of secreted proteins (ER, Melanosome)	Up-regulated (mRNA) in lung cancer. Down-regulated in non-cancer stroma cells from colon cancer tissues
44	CAF17 - Iron-sulfur cluster assembly factor homolog	>1000 2.5×10^{-6}	Involved in the maturation of mitochondrial 4Fe-4S proteins, (Mitochondria)	No reported data
45	PSME3 - Proteasome activator complex subunit 3	>1000 2.6×10^{-6}	Subunit of the 11S REG proteasome regulator (Cytosol, Nucleus)	Serum tumor marker for colorectal cancer
46	TM9S3 - Transmembrane 9 superfamily member 3	11.3 2.6×10^{-6}	Belongs to nonaspanin protein family. Function not known (Plasma membrane, Golgi)	Diagnostic and therapeutic target for scirrhus-type gastric cancer. Breast cancer chemoresistance factor
47	THY1 - Thy-1 membrane glycoprotein	8.5 2.9×10^{-6}	Proposed to function in cell-cell or cell-ligand interactions (Plasma membrane)	Marker for lung, liver, glioma and breast cancer stem cells
48	RS3A - 40S ribosomal protein S3a	11.8 3.3×10^{-6}	Translation, component of the 40S subunit (Cytosol, Nucleus)	Marker for human squamous cell lung cancer
49	MMP19 - Matrix metalloproteinase-19	>1000 3.3×10^{-6}	Endopeptidase that degrades various components of the extracellular matrix (ECM)	Involved in NSCLC metastasis and associated with increased mortality
50	ARPC3 - Actin-related protein 2/3 complex subunit 3	8.6 4.2×10^{-6}	Component of the Arp2/3 complex involved in regulation of actin polymerization (Cytosol)	Associated with glioma

No	Protein name (Uniprot)	Fold change/ P value	Proposed function (cell localization)	Relation to cancer
51	RS15 - 40S ribosomal protein S15	15.9 4.3x10 ⁻⁶	Translation, component of the 40S subunit (Cytosol, Nucleus)	RS15 mutations are associated with increased cancer risk
52	PRKDC - DNA-dependent protein kinase catalytic subunit	10.1 4.5x10 ⁻⁶	Serine/threonine-protein kinase that acts as a molecular sensor for DNA damage (Nucleus)	Highly expressed in advanced neuroblastoma, associated with gastric carcinoma
53	RPN2 - Ribophorin II	8.8 4.5x10 ⁻⁶	Protein glycosylation. Essential subunit of the N-oligosaccharyl transferase (OST) complex (ER Plasma membrane)	Breast cancer initiation and metastasis, associated with docetaxel response in oesophageal SCC
54	RBMX - RNA-binding motif protein, X chromosome	6.1 4.4x10 ⁻⁶	RNA-binding protein that plays several roles in the regulation of pre- and post-transcriptional processes (Nucleus)	Up-regulated in immortalized cells, cancer cells, and NSCLC tissues
55	ANM1 - Protein arginine N-methyltransferase 1	6.2 4.9x10 ⁻⁶	Arginine methyltransferase (Cytosol, Nucleus)	Over-expressed in NSCLC cell lines, proposed as a marker in breast cancer
56	MAP2K1 (MEK1) - Dual specificity mitogen-activated protein kinase 1	165.3 5.7x10 ⁻³	A component of the MAP kinase signal transduction pathway, binds extracellular ligands, activates RAS and RAF1 (Cytosol)	Over-expressed in NSCLC
57	EGFR - Epidermal growth factor receptor	92.6 1x 10 ⁻²	Receptor tyrosine kinase binding ligands of the EGF family (Cell membrane, ER, Golgi, Nucleus)	Over-expressed in NSCLC
58	HYOU1- Hypoxia up-regulated protein-1	-2.7 0.032	A chaperon molecule belongs to HSP70 family, induced by hypoxia, has cytoprotective activity (ER)	Over-expressed in NSCLC (Fahrman JF et al. Clin Proteomics. 2016; 13: 31)
59	LRRFIP2- leucine-rich repeat flightless-interacting protein 2	19.3 2.9x10 ⁻³	Positive regulator of the Toll-like receptor (TLR) signaling (cytoplasm)	No reported data
60	WDR82- WD repeat-containing protein 82	9.8 3.1x10 ⁻³	Component of histone methyl-transferase complex (nucleus)	No reported data
61	AKR1B10- aldo-keto reductase family 1 member B10	17.9 1.9x10 ⁻³	Regulates the balance of retinoic acid and lipid metabolism (lysosome, secreted)	Potential diagnostic marker specific to smokers' NSCLCs (Fukumoto S, et al. Clin Cancer Res. 2005; 11: 1776-85)

No	Protein name (Uniprot)	Fold change/ P value	Proposed function (cell localization)	Relation to cancer
62	TTL12- tubulin-tyrosine ligase-like protein 12	8.1 2.1×10^{-3}	Catalyze posttranslational modification of tubulins (cytoplasm)	Expression increases during cancer progression to metastasis of prostate cancer (Wasyluk C, et al. Int J Cancer. 2010; 127: 2542-53)
63	ACOT1- Acyl-co-enzyme A thioesterase 1	-2.8 5.6×10^{-3}	Long chain fatty acid metabolism (Cytoplasm)	Highly expressed in luminal breast tumors (Hill JJ, et al. J Proteome Res. 2015; 14: 1376-88)
64	TSG101- tumor susceptibility gene 101 protein	-38.3 2.6×10^{-3}	regulator of vesicular trafficking process (mainly cytoplasmic)	TSG101 splicing variant is linked to progressive tumor-stage and metastasis (Chua HH, et al. Oncotarget. 2016; 7: 8240-52)
65	RAB34- Ras-related protein Rab-34	-6.0 7.6×10^{-3}	GTPase involved in protein transport (Cytoplasm, Golgi)	RAB34 is a progression- and prognosis-associated biomarker in gliomas (Wang HJ, et al. Tumour Biol. 2015; 36: 1573-8); Ras association sarcomagenesis (Galoian K, et al. Tumour Biol. 2014; 35: 483-92)
66	ITGA7-integrin alpha-7	107.3 1.3×10^{-3}	Laminin receptor on skeletal myoblasts (plasma membrane)	Associated with the occurrence and development of bladder cancer (Jia Z, et al. Tumori. 2015; 101: 117-22)
67	GALE - UDP-galactose-4-epimerase	2.18	Catalyzes two distinct but analogous reactions: the epimerization of UDP-glucose to UDP-galactose, and the epimerization of UDP-N-acetylglucosamine to UDP-N-acetylgalactosamine	Overexpressed in thyroid papillary carcinoma (da Silveira Mitteldorf CA et al. Diagn Cytopathol. 2011 Aug;39(8):556-61)
68	ACAD8- acyl-CoA dehydrogenase family member 8	11.4	Catalyze the dehydrogenation of acyl-CoA derivatives in the metabolism of fatty acids or branch chained amino acids	No reported data

Table 6 above is based on two independent LC-HR MS/MS experiments that were performed as described hereinabove. From each experiment, differentially expressed

proteins (p-value <0.01, FC ≥ 2) were filtered and proteins differentially expressed in both experiments were selected. Proteins of relevance to lung cancer or with potential as biomarkers are listed. For each protein, the name, fold change and p-value as well as its function, sub-cellular localization and relevance to cancer are indicated.

5 **Example 2: Modified expression of metabolism- and apoptosis-related proteins**

Modified metabolism and the development of anti-apoptotic mechanism are hallmarks of cancer. As previously described (WO 2013/035095) several proteins associated with these hallmarks are overexpressed in certain types of cancer. Samples of tumor and healthy tissues from the same lung of NSCLC patients were analyzed by
10 immunoblotting using specific antibodies to assess levels of the voltage-dependent anion channel 1 (VDAC1), hexokinase I (HK-I), SMAC/Diablo (SMAC), Apoptosis inducing factor (AIF), mitochondrial anti-viral signaling (MAVS) and Bcl2 (Fig. 2). All of these proteins, with the exception of Bcl2, were significantly over-expressed (3- to 6-fold) in cancerous tissues as compared to a healthy tissues obtained from the same
15 NSCLC patient (Fig. 2A, B). LC-HR-MS/MS further confirmed that expression levels of VDAC1, HK-I and SMAC were highly increased in cancer tissue (Fig. 2C). The RNA expression levels of VDAC1, HK-I, SMAC and AIF showed a similar trend, although expression at the RNA level was lower, as revealed from the RNAseq gene expression profiling data (Fig. 2,E).

20 The expression levels of VDAC1, SMAC, AIF, HK-I, MAVS and Bcl2 was also analyzed by IHC in tissue microarrays comprising normal and NSCLC derived samples (Fig. 2F). All proteins were highly expressed in the tumor tissue. Thus, although SMAC, AIF are pro-apoptotic proteins, they are over-expressed in tumor tissue.

Other metabolism-related proteins, such as lactate dehydrogenase (LDHA), the
25 ATP synthase subunit 5B (ATP5B), the glycolysis enzyme glyceraldehyde 3 phosphate dehydrogenase (GAPDH), phosphoglycerate kinase 1, (PGK1) and enolase-1 (ENO1), were also highly expressed (up to 14-fold higher) in the tumor tissues, as determined by LC-HR-MS/MS analysis (Fig. 3A, , D ,E).

These results point to the significance of reprogrammed metabolism and apoptosis
30 avoidance in lung cancer.

Example 3: Identification of bio-markers of lung cancer

LC-HR-MS/MS analysis data revealed many other proteins that were differentially expressed in the NSCLC tumors (Table 6). The proteins with the most significant changes in expression in the tumors are presented along with their proposed
5 function and relation to cancer in Table 6. These include Ras-related protein Rab11B (Rab11B), a member of the Ras superfamily of small GTP-binding proteins, HYOU1 (ORP150), which plays a pivotal role in cytoprotective cellular mechanisms triggered by oxygen deprivation, and the heat-shock protein HSPD1 (HSP60). These findings were confirmed by immunoblot analysis, the RNAseq UCSC XENA data and qRT-PCR
10 (Fig. 3).

Network analysis of the proteins identified here by proteomics (and confirmed by the immunoblot analysis, the RNAseq gene expression profiling data and qRT-PCR) demonstrated that most of these proteins interact at several levels, with metabolic processes-related proteins being central. These interactions include common
15 functionality associated with cell metabolism, and involved direct physical interaction with each other. Many of these proteins are co-expressed and may therefore be defined as a cluster that is regulated by epigenetic modifications.

Example 4: Proteins differentially expressed in AC and SCC

Analysis of lung tissue microarrays for VDAC1 and AIF (from 10 healthy, 31
20 SCC and 17 AC samples) and for SMAC/Diablo (from 20 healthy, 72 SCC and 72 AC samples) expression levels by IHC staining using specific antibodies revealed high expression of these proteins in lung cancer, as compared to healthy tissue (Fig. 4A). Quantity analyses of the IHC results, presented as the number of patient samples showing staining at the indicated intensity and represented as a percentage of the total
25 number of section analyzed, showed that VDAC1, SMAC and AIF expression levels were higher in SCC than in AC (Fig. 4A).

Next, cancerous and healthy tissues samples from the lung of five of each AC and SCC patients were subjected to LC-HR-MS/MS analysis. The expression levels of 2,959 proteins were up- or down-regulated in the cancerous tissues relative to the
30 expression in the corresponding healthy tissue, with the change in expression of 1,513 proteins being significant. The proteins showing the highest change in the expression

levels (p-value < 0.01) between the two NSCLC sub-types were selected and the fold change of expression in the tumor relative to the healthy tissue was calculated and presented as the SCC/AC ratio for each protein (Fig 4B). Assessing the SCC/AC ratios revealed that HAT1, ITGA7, LRRFIP2, AKR1B10 (secreted protein), WDR82, TTLL12, and USP14 were highly over-expressed (up to 500-fold) in SCC, as were VDAC1 and SMAC to a lower extent, while HYOU1, ACOT1, RAB34, and TSG101 showed higher expression in AC. Table 7 presents the fold change in the expression of each of these and additional proteins in lung samples obtained from patients with NSCLC subtype AC or SCC compared to the expression in lung samples of healthy subject or healthy lung tissues. Further presented is the SCC to AC expression ratio, the proposed function of the protein and its relation to cancer.

Table 7: Biomarkers for differentiating between NSCLC subtype AC and SCC

Uniport Gene Name	AC Fold of change Tumor/Healthy	SCC Fold of change Tumor/Healthy	Ratio SCC/AC (p value)	Proposed function (cell localization)	Relation to cancer
HAT1- Histone acetyltransferase type B catalytic subunit (O14929)	-23.66	20.09	476 4.4×10^{-3}	Acetylates soluble histone H4, a type B HAT that functions in DNA repair (Nucleus)	High expression in several types of lymphomas. Proposed indicator for poor prognosis and potential drug target in esophageal SCC (Cho SJ, et al. Korean J Pathol. 2012; 46: 142-50; Xue L, et al. Int J Clin Exp Pathol. 2014; 7: 3898-907)
LRRFIP2- Leucine-rich repeat flightless-interacting protein 2 (Q9Y608)	-7.83	2.47	19.3 2.9×10^{-3}	Positive regulator of the Toll-like receptor (TLR) signaling (Cytosol)	No reported data
AKR1B10- Aldo-keto reductase family 1 member B10 (O60218)	-1.79	10.03	17.9 1.9×10^{-3}	Regulates the balance of retinoic acid and lipid metabolism (lysosome, Secreted)	Potential diagnostic marker specific to smokers' NSCLCs (Fukumoto S, et al. Clin Cancer Res. 2005; 11: 1776-85).
WDR82- WD repeat-containing	-2.15	4.56	9.81 3.1×10^{-3}	Component of histone methyl-transferase	No reported data

protein 82 (Q6UXN9)				complex (Nucleus)	
TTLL12- Tubulin- tyrosine ligase- like protein 12 (Q14166)	-1.69	4.82	8.14 2.1×10^{-3}	Catalyze post- translational modification of tubulins (Cytosol)	Expression increases during prostate cancer progression to metastasis (Wasylyk C, et al. Int J Cancer. 2010; 127: 2542-53)
IGF2BP3- Insulin-like growth factor 2 mRNA-binding protein (O00425)	-1.35	3.65	4.93	RNA-binding factor that may recruit target transcripts to cytoplasmic protein-RNA complexes (mRNPs)(Nucleus, Cytosol)	Associated with NSCLC (Shi R, et al. Tumour Biol. 2017; doi.org/10.1177/101042 8317695928)
SMC2 - Structural maintenance of chromosomes protein 2 (O95347)	-1.56	1.34	2.09	Involved in condensing chromatin complex (Cytosol, Nucleus)	No reported data
ACOT1- Acyl- co-enzyme A thioesterase 1 (Q86TX2)	1.34	-2.09	-2.8 5.6×10^{-3}	Long chain fatty acid metabolism (Cytosl)	Highly expressed in luminal breast tumors (Hill JJ, et al. J Proteome Res. 2015; 14: 1376-88)
ACAD8 - Isobutyryl-CoA dehydrogenase (Q9UKU7)	11.4	-1.42	-16.1	Acyl-CoA dehydrogenase, catabolism of valine (Mitochondria)	No reported data
GALE - UDP- glucose 4- epimerase (Q14376)	2.18	-1.33	-2.91	Galactose metabolism (Cytosol, Exosomes)	No reported data
RSU1 - Ras suppressor protein 1 (Q15404)	1.05	-3.98	- 4.29	Ras signal transduction pathway (Cytosol, Exosomes)	No reported data
HYOU1 Hypoxia up- regnlated protein 1	1.67	-1.59	-2.7	A chaperon molecule belongs to HSP70 family, induced by hypoxia, has cytoprotective activity (ER)	Over-expressed in NSCLC
USP14- Ubiquitin carboxyl- terminal	1.26	5.58	4.43 2.0×10^{-3}	Proteasome- associated deubiquitinase (Cytosol, Plasma	Over-expressed in various types of cancer including NSCLC (Zhu Y, et al. Cell Physiol

hydrolase 14 (P54578)				membrane)	Biochem. 2016; 38: 993-1002).
ITGA7- Integrin alpha-7 (Q13683)	4.17	447.21	107.2 1.3×10^{-3}	Laminin receptor on skeletal myoblasts (Plasma membrane)	Associated with the occurrence and development of bladder cancer (Jia Z, et al. Tumori. 2015; 101: 117-22
TSG101- Tumor susceptibility gene 101 protein (Q99816)	165.38	4.32	-38.5 2.6×10^{-3}	Regulator of vesicular trafficking process (Plasma membrane, Cytosol, Nucleus)	TSG101 splicing variant is linked to progressive tumor-stage and metastasis (Chua HH, et al.. Oncotarget. 2016; 7: 8240-52)
RAB34- Ras-related protein Rab-34 (Q9BZG1)	-1.16	-7.05	-6.08 7.6×10^{-3}	GTPase involved in protein transport (Cytosol, Golgi)	A progression- and prognosis-associated biomarker in gliomas (Wang HJ, et al. Tumour Biol. 2015; 36: 1573-8). Ras-associated sarcomagenesis (Galoian K, et al. Tumour Biol. 2014; 35: 483-92)

The expression of several of the proteins showing significant differential expression (MS/MS data, Fig. 4B), and of NAPSA (previously proposed for distinguishing between AC and SCC) was analyzed using RNAseq (UCSC XENA, n=1,129) on tissues obtained from healthy and lung cancer patients (Fig. 5A). The analysis revealed that ACOT1, RAB34, TSG101, and NAPSA RNA expression level was lower in SCC than in AC, while the RNA expression level of SMAC, AKR1B10 (a secreted protein), HAT1, USP14, and TTLL12, and to a lesser extent of WDR82 and VDAC1, was higher in SCC relative to the expression in AC. These results are in agreement with the proteomics data (Fig. 4B), and thus propose the use of these proteins and/or RNA encoding them as markers to distinguish between AC and SCC.

In an attempt to identify additional proteins having modified expression in NSCLC as revealed in the proteomics data, which can differentiate between AC and SCC, the RNA levels of several proteins was determined using RNAseq UCSC XENA data (Fig. 5B). The RNA level encoding for TP63, GGH (secreted protein), Ck5, Ck13, Ck14, Ck17, CSTA, RANBP1, TIMM44 FEN1, FEN2, SMC2, and IGF2BP3 were increased in SCC relative to AC, while the level of RNA encoding for RSU1, AKR7A3,

GALE, AZGP1 (secreted protein), ACOT1, ABCD3, NPC2 (secreted protein), ACAD8, RPS6KA3, ARRB1 and LRBA showed the opposite trend, namely higher expression in AC relative to SCC. The functions of the products of these genes and previously reported relation to AC or SCC are listed in Table 8.

5 Table 8: Proteins encoded by RNA differentially expressed in SCC and AC

Gene	Proposed function (cell localization)	Relation to NSCLC
Higher RNA expression levels in AC		
1. AZGP1 - Zinc-alpha-2-glycoprotein (P25311)	Lipid degradation in adipocytes, associated with fat losses in some advanced cancers (Plasma membrane, Secreted, Exosomes)	Associated with AC lung cancer (Falvella FS, et al. Oncogene. 2008; 27: 1650-6)
2. ACOT1 - Acyl-coenzyme A thioesterase 1 (Q86TX2)	Lipid metabolism , long chain fatty acid metabolism (Cytosol)	No reported data
3. ACAD8 - Isobutyryl-CoA dehydrogenase (Q9UKU7)	Acyl-CoA dehydrogenase, catabolism of valine (Mitochondria)	No reported data
4. NPC2 - Epididymal secretory protein E1 (P61916)	Cholesterol transporter (ER, Lysosome, Secreted)	Associated with lung AC (Pernemalm M, et al. Proteomics. 2009; 9: 3414-24)
5. ABCD3 - ATP-binding cassette sub-family D member 3 (P28288)	Involved in fatty acid transport (Peroxisome)	Associated with lung AC (Tran QN. BMC Med Genomics. 2013; 6: S11)
6. GALE - UDP-glucose 4-epimerase (Q14376)	Galactose metabolism (Cytosol, Exosomes)	No reported data
7. FEN1 - Flap endonuclease 1 (P39748)	Endonuclease involved in DNA replication and repair (Cytosol)	Associated with lung AC (Hwang JC, et al. PLoS One. 2015; 10: e0139435)
8. AKR7A3 - Aldo-Keto Reductase family 7A isoform 3 (O95154)	Involved in Aflatoxin B1 inactivation (Cytosol, Exosome)	No reported data
9. ARRB1 - Beta-arrestin-1 (P49407)	Signaling pathway: Functions in regulating agonist-mediated GPCR (Membrane, Cytosol, Nucleus)	Enhances chemosensitivity in NSCLC (Shen H, et al. Oncol Rep. 2017; 37: 761-7)
10. RSU1 - Ras suppressor protein 1 (Q15404)	Ras signal transduction pathway (Cytosol, Exosomes)	No reported data
11. LRBA - Lipopolysaccharide-responsive and beige-like anchor protein (P50851)	Coordinates signaling of immune receptors (Cell membrane, ER, Golgi, Lysosome)	No reported data

Gene	Proposed function (cell localization)	Relation to NSCLC
Higher expression RNA levels in SCC		
12. Ck5 - Keratin, type II cytoskeletal 5 (P13647)	Structural protein (Plasma membrane, Cytosol, Nucleus, Exosome)	Associated with lung SCC (Vogt AP, et al. Diagn Cytopathol. 2014; 42: 453-8; Chen Y, et al. Oncology. 2011; 80: 333-40)
13. Ck13 - Keratin, type I cytoskeletal 13 (P13646)	Structural protein (Cytosol, Nucleus, Exosome)	Associated with lung SCC (Lee M-S, et al. Oncotarget. 2016; 7: 36101-14)
14. Ck14 - Keratin, type I cytoskeletal 14 (P02533)	Structural protein (Cytosol, Nucleus)	Associated with lung SCC (Chen et al., 2011, <i>ibid</i> ; Nakanishi Y, et al. Acta Histochem Cytochem. 2013; 46: 85-96)
15. Ck17 - Keratin, type I cytoskeletal 17 (Q04695)	Structural protein (Cytosol)	Associated with lung SCC (Chen et al., 2011, <i>ibid</i>)
16. PFN2 - Profilin-2 (P35080)	Structural protein (Cytosol)	Associated with NSCLC (Tang YN, et al. Nat Commun. 2015; 6: 8230)
17. RANBP1 - Specific GTPase-activating protein (P43487)	Signaling pathway, Inhibits GTP exchange on Ran (Cytosol, Nucleus)	No reported data
18. CSTA - Cystatin-A (P01040)	Thiol proteinase inhibitor (Cytosol, Exosomes)	Associated with lung SCC (Butler MW, et al. Cancer Res. 2011; 71: 2572-81; Leinonen T, et al. J Clin Pathol. 2007; 60: 515-9)
19. GGH - Gamma-glutamyl hydrolase (Q92820)	Amino acid metabolism, Hydrolyzes polyglutamate (Lysosome, Secreted)	Associated with lung cancer (NSCLC) (Yoshida T, et al. Anticancer Res. 2016; 36: 6319-26)
20. TIMM44 - Mitochondrial IMM translocase subunit TIM44 (O43615)	Mitochondrial peptide transporter, essential component of the PAM complex, ATP binding (Mitochondria)	No reported data
21. TP63 - Tumor protein 63 (Q9H3D4)	Tumor suppressor (Nucleus)	Associated with lung SCC (Vogt et al. 2014, <i>ibid</i>)
22. SMC2 - Structural maintenance of chromosomes protein 2 (O95347)	Involved in condensing chromatin complex (Cytosol, Nucleus)	No reported data
23. IGF2BP3 Insulin-like growth factor 2 mRNA-binding protein	RNA-binding factor that may recruit target transcripts to cytoplasmic protein-RNA complexes (mRNPs)(Nucleus, Cytosol)	Associated with NSCLC (Shi R, et al. Tumour Biol. 2017; doi.org/10.1177/1010428317695928)
RPS6KA3- Ribosomal protein S6 kinase alpha-3 (P51812)	Serine/threonine-protein kinase acts downstream of ERK signaling (Nucleus, Cytosol)	Associated with NSCLC (Song R, et al. BMC Genomics. 2014; 15: S16; Tan Q, et al. Onco Targets Ther. 2013; 6: 1471-9)

Example 5: Expression of proteins associated with survival rates in AC and SCC

To further test the prognostic value of the proteins proposed to distinguish between AC and SCC, survival analysis was performed on public-available gene expression datasets of lung cancer patients. A Kaplan-Meier analysis assessing patient survival as a function of the relative indicated mRNA level (high and low) in AC and SCC was performed. The results show that in AC patients, high levels of VDAC1, SMAC, HYOU1, TTLL12, and RAB34 are associated with low survival rates, while high levels of AKR1B10, AIF (mitochondrial), ARL1, TSG101, HAT1, p40, NAPSA, LRRFIP2, TITF1, and WDR82 are associated with higher survival rates (Table 9). In contrast, the expression level of these proteins had no effect on SCC survival rates (Table 9). The data presented in Table 9 were obtained from KMplot.com. Total sample number was 2437, with initial number in each group presented in parenthesis. The Kaplan-Meier estimator used an earlier (2015) release of the database (Szasz AM, et al. Oncotarget. 2016; 7: 49322-33).

Table 9: The relationship between protein expression levels and survival in AC and SCC lung cancer subtypes

Higher survival associated with low mRNA level							
No	Gene symbol	Median Survival time (months)				P value	
		AC		SCC		AC	SCC
		High (No.)	low	high	low		
1.	VDAC1	75 (360)	150 (360)	40 (262)	50 (262)	0.0018	0.87
2.	SMAC	65 (360)	120 (360)	60 (262)	60 (262)	7.4×10^{-6}	0.9
3.	HYOU1	65 (360)	115 (360)	60 (262)	60 (262)	2.2×10^{-6}	0.76
4.	TTLL12	75 (337)	120 (336)	50 (135)	55 (136)	2.9×10^{-4}	0.63
5.	RAB34	90 (337)	122 (336)	65 (135)	45 (136)	0.013	0.087
6.	MAVS	90 (337)	115 (336)	65 (135)	50 (136)	0.13	0.25
Higher survival associated with high mRNA level							
7.	ARL1	175 (360)	60 (360)	50 (261)	60 (263)	3.4×10^{-14}	0.84
8.	TSG101	137.5 (360)	65 (360)	50 (262)	50 (262)	1.9×10^{-9}	0.18
9.	HAT1	118 (360)	70 (360)	51 (262)	60 (262)	0.0046	0.32
10.	p40	115	70	60	60	1.4×10^{-7}	0.84

		(360)	(360)	(262)	(262)		
11.	NAPSA	130 (337)	80 (336)	65 (135)	50 (136)	4.2×10^{-5}	0.2
12.	LRRFIP2	120 (361)	75 (359)	52 (261)	52 (263)	0.02	0.51
13.	AIF (Mitochondrial)	130 (360)	90 (360)	60 (262)	55 (262)	0.033	0.9
14.	TITF1	127 (360)	81.3 (360)	50 (262)	50 (262)	0.00051	0.11
15.	WDR82	120 (360)	90 (360)	50 (262)	50 (262)	2×10^{-4}	0.95

Example 5: SMAC/Diablo presence in the nucleus

Interestingly, analysis of SMAC/Diablo expression in a tissue array of lung cancer-derived samples revealed that although SMAC is a mitochondrial protein, high levels of the protein were found in the nucleus and cytosol of SCC but only to a lesser extent in AC tissue samples (Fig. 6A). No previous study has reported the presence of SMAC in the nucleus. The results further show that AIF, known to translocate to the nucleus upon apoptosis induction is not present in the nucleus (Fig. 6B).

To further demonstrate the presence of SMAC in the nucleus, the nuclear distribution of SMAC in AC and SCC lung cancer samples obtained from healthy and tumor tissues of the same lung was analyzed, after separating the nuclear and the cytosolic fractions (Fig. 6C, D). While in AC about 90% of SMAC was mitochondrial/cytosolic, in SCC about 50% was mitochondrial/cytosolic and 50% was found in the nuclear fraction (Fig. 6D). In the nuclear fraction containing SMAC, three other mitochondrial proteins, VDAC1, MAVS and AIF, were not found (Fig. 6C), indicating the specific nuclear localization of SMAC.

In summary, several biomarkers potentially enable for distinguishing between AC and SCC that are derived from published data, as confirmed here, and have been identified here for the first time were selected based on being differentially expressed in SCC or AC (Table 10).

Table 10: Selected biomarkers for distinguishing between AC and SCC

Proteins that can be used as biomarkers are presented, with their expression levels in SCC, relative to AC, as determined by proteomics, qPCR (in parenthesis) and RNASeq studies, listed. The source of the data is also indicated.

Protein Method:	SCC/AC		Marker for:
	Proteomics (qPCR)	RNA Seq	
HAT1 - Histone acetyltransferase type B	475 (1.8)	1.4	SCC, this study
AKR1B10 - Aldo-keto reductase family 1 member B10 (<i>secreted</i>)	17.9 (20)	4	SCC, this study
USP14 - Ubiquitin carboxyl-terminal hydrolase 14	4.4 (2)	1.3	SCC, this study
TTL12 - Tubulin-tyrosine ligase-like protein 12	8.1 (5)	2.5	SCC, this study
LRRFIP2 - Leucine-rich repeat flightless-interacting protein 2	19.3 (3)	1.1	SCC, this study
WDR82 - WD repeat-containing protein 82	9.8 (4.4)	1	SCC, this study
IGF2BP3 - Insulin-like growth factor 2 mRNA-binding protein	4.9	1.8	SCC, this study
ITGA7 - Integrin alpha-7 (Q13683)	107	-1.4	SCC, this study
PFN2 - Profilin-2	4.9	2.7	SCC, this study
TSG101 - Tumor susceptibility gene 101 protein	-38.3 (-1.6)	-1.1	AC, this study
ACOT1 - Acyl-coenzyme A thioesterase 1	-2.8	-1.7	AC, this study
RAB34 - Ras-related protein Rab-34	-6.0	-1.3	AC, this study
ACAD8 - Isobutyryl-CoA dehydrogenase, mitochondrial	-17	-2.4	AC, this study
SMAC - Second mitochondria-derived activator of caspases	Nuclear localization in SCC		SCC, this study
Ck5 - Keratin, type II cytoskeletal 5	4.6	67.5	SCC
Ck13 - Keratin, type I cytoskeletal 13	4.4	63.7	SCC
Ck14 - Keratin, type I cytoskeletal 14	3.2	59.2	SCC
Ck17- Keratin, type I cytoskeletal 17	2.1	19.5	SCC
GGH - Gamma-glutamyl hydrolase		1.9	SCC

Protein	SCC/AC		Marker for:
<i>(secreted)</i>			
NAPSA - Napsin A aspartic peptidase	-	-10	AC
FEN1 - Flap endonuclease 1	3.5	1.5	AC
AZGP1 - Zinc-alpha-2-glycoprotein	-2.7		AC
<i>(secreted)</i>		-4.1	
NPC2 Epididymal secretory protein E1		-2.1	AC
<i>(secreted)</i>			

Example 6: Silencing of APOOL, VPS29, and CAF17 for treating NSCLC

To verify the importance of the proteins identified to be overexpressed in NSCLC, the effects of their silencing by specific siRNA is examined. At least one siRNA, and typically two siRNAs are designed for silencing the RNA encoding each of the proteins APOOL, VPS29, and CAF17. In addition, a non-specific scrambled siRNA is designed.

In vitro assay

Cells of NSCLC cell line are transfected with scrambled siRNA or with the siRNA specific to each protein and cell growth is analyzed using the Sulforhodamine B (SRB) method. In this method, forty-eight or 96 h post-transfection with siRNA, cells are washed twice with PBS, fixed with 10% trichloroacetic acid for 1-2 h, and subsequently stained with SRB. SRB is extracted from the cells using 100 mM Tris-base and absorbance at 510 nm is determined using an Infinite M1000 plate reader (Tecan, Männedorf, Switzerland).

In vivo assay -xenograft experiments using nude mice.

A549 lung cancer cells (7×10^7) are injected s.c. into the hind leg flanks of Athymic 8-weekold male SCID nude mice. Eleven days after inoculation, the developing tumors are measured in two dimensions with a digital caliper and tumor volume is calculated as follows: volume = $X^2 \times Y/2$, where X and Y are the short and long tumor dimensions, respectively. The mice with xenografts reaching a volume of 65–100 mm³ are randomized for different treatments (eight or nine animals in each group): PBS, non-targeting (scrambled) siRNA or siRNA against the selected protein.

Each treatment substance is injected into the established s.c. tumors using the jetPEI delivery reagent (10µg siRNA/20-µl jetPEI). The xenografts are injected (20 µl per tumor) with PBS or the appropriate siRNA every 3 days. Beginning on the day of inoculation, mouse weight and tumor volume are monitored twice a week for a period of 33 days using a digital caliper. At the end point of the experiment, i.e., when tumor volume reached ~250 mm³, the mice are sacrificed using CO₂ gas; the tumors are excised and ex vivo weight is determined. Half of each tumor is fixed in 4% buffered formaldehyde, paraffin-embedded and processed for histological examination, while the second half is frozen in liquid nitrogen and stored in -80 °C for immunoblot analysis.

10 The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying current knowledge, readily modify and/or adapt for various applications such specific embodiments without undue experimentation and without departing from the generic concept, and, therefore, such adaptations and modifications should and are intended to be comprehended within the meaning and range of equivalents of the disclosed embodiments. It is to be understood that the phraseology or terminology employed herein is for the purpose of description and not of limitation. The means, materials, and steps for carrying out various disclosed functions may take a variety of alternative forms without departing from the invention.

CLAIMS

1. A method for diagnosing a subtype of non small cell lung carcinoma (NSCLC) selected from adenocarcinoma (AC) and squamous cell carcinoma (SCC) in a subject suspected to have NSCLC, the method comprising:
- 5
- a. determining the expression level of at least one biomarker selected from a protein and mRNA encoding said protein in a biological sample obtained from the subject, wherein the at least one biomarker is selected from the group consisting of HAT1, LRRFIP2, AKR1B10, WDR82, TTL12, IGF2BP3, SMC2, ACAD8, RSU1, ACOT1, HYOU1, GALE, ITGA7, TSG101, and RAB34;
- 10
- b. comparing the expression level of said at least one biomarker to the expression level of said at least one biomarker in a healthy biological sample and/or a reference value representing healthy biological sample; optionally
- 15
- c. computing a fold change of the expression level of said at least one biomarker in the sample obtained from said subject and the expression level in the healthy sample and/or reference value; and
- d. diagnosing said subject , wherein:
- 20
- an elevated expression level in said sample obtained from said subject of at least one biomarker selected from the group consisting of HAT1, LRRFIP2, AKR1B10, WDR82, TTL12, IGF2BP3, and SMC2, and/or reduced expression of at least one biomarker selected from the group consisting of ACAD8, RSU1, ACOT1, HYOU1, and GALE compared to the expression
- 25
- level in said healthy biological sample and/or reference value indicates that said subject has NSCLC subtype SCC;
- a reduced expression level in said sample obtained from said subject of at least one biomarker selected from the group consisting of HAT1, LRRFIP2, AKR1B10, WDR82, TTL12, IGF2BP3, and SMC2, and/or elevated
- 30
- expression of at least one biomarker selected from the group consisting of ACAD8, RSU1, ACOT1, HYOU1, and GALE compared to the expression

level in said healthy biological sample and/or reference value indicates that said subject has NSCLC subtype AC;

an equal or elevated fold change of the biomarker ITGA7 compared to a reference value indicates that the subject has NSCLC subtype SCC, wherein the reference value is derived from the fold change of the expression of said ITGA7 biomarker in a plurality of samples obtained from SCC patients compared to its expression in a plurality of healthy biological samples;

an equal or elevated fold change of the biomarker TSG101 compared to a reference value indicates that the subject has NSCLC subtype AC, wherein the reference value is derived from the fold change of the expression of said TSG101 biomarker in a plurality of samples obtained from AC patients compared to its expression in a plurality of healthy biological samples;

an equal or reduced fold change of the biomarker RAB34 compared to a reference value indicates that the subject has NSCLC subtype AC, wherein the reference value is derived from a fold change of the expression of said RAB34 biomarker in a plurality of samples obtained from AC patients compared to its expression in a plurality of healthy biological samples.

2. The method of claim 1, said method comprises determining the expression level of a combination of biomarkers, the combination is selected from the group consisting of at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, and 15 biomarkers.

3. The method of any one of claims 1-2, said method comprises determining the expression level of at least one biomarker selected from the group consisting of HAT1, LRRFIP2, AKR1B10, WDR82, TTL12, IGF2BP3, and SMC2, and at least one biomarker selected from the group consisting of ACAD8, RSU1, ACOT1, HYOU1, and GALE.

4. The method of any one of claims 1-2, said method comprises determining the expression level of at least one biomarker selected from the group consisting of HAT1, LRRFIP2, AKR1B10, WDR82, TTL12, IGF2BP3, SMC2, and ITGA7 and at least one biomarker selected from the group

consisting of ACAD8, RSU1, ACOT1, HYOU1, GALE, TSG101 and RAB34.

5. The method of any one of claims 1-2, said method comprises determining the expression level of a combination of markers, the combination comprises the biomarkers HAT1, LRRFIP2, AKR1B10, WDR82, TTL12, IGF2BP3, and SMC2.
6. The method of any one of claims 1-2, said method comprises determining the expression level of a combination of markers, the combination comprises the biomarkers ACAD8, RSU1, ACOT1, HYOU1, and GALE.
7. The method of any one of claims 1-6, said method comprises determining the expression level of a combination of markers, the combination comprises the biomarkers HAT1, LRRFIP2, AKR1B10, WDR82, TTL12, IGF2BP3, SMC2, ITGA7, ACAD8, RSU1, ACOT1, HYOU1, GALE, TSG101 and RAB34.
8. The method of any one of claims 1-4, said method further comprises determining the expression level of at least one biomarker selected from the group consisting of USP14, VDAC1 and AIF, wherein an equal or elevated fold change of the at least one biomarker compared to a reference value indicates that the subject has NSCLC subtype SCC, wherein the reference value is derived from the fold change of the expression of said at least one biomarker in a plurality of samples obtained from SCC patients compared to the expression of said biomarkers in a plurality of healthy biological samples.
9. The method of any one of claims 1-8, wherein the expression of the at least one biomarker in the sample obtained from the subject is increased or reduced by at least 2 fold compared to the expression level in the healthy sample or to the reference value.
10. The method of claim 9, wherein the expression of the at least one biomarker in the sample obtained from the subject is increased or reduced by at least 4 fold compared to the expression level in the healthy sample or to the reference value.

11. The method of any one of claims 1-10, wherein the biomarker is a protein.
12. The method of any one of claims 1-11, wherein the biological sample is a lung tissue sample.
13. The method of claim 12, wherein the healthy biological sample is obtained
5 from a healthy subject or from a healthy lung tissue of the subject suspected to have NSCLC.
14. The method of any one of claims 1-11, wherein the biological sample is selected from the group consisting of blood, blood plasma and serum sample.
15. The method of any one of claims 1-11, wherein the samples obtained from
10 the subject is ascite.
16. The method of any one of claims 1-15, said method further comprises treating the subject diagnosed to have NSCLC subtype AC with a therapy suitable for treating AC.
17. The method of claim 16, wherein the therapy suitable for treating AC
15 comprises administering to the subject a therapeutically effective amount of at least one agent that reduces the expression or activity of at least one protein selected from the group consisting of TSG101, ACAD8, and GALE.
18. The method of any one of claims 1-14, said method further comprises treating the subject diagnosed to have NSCLC subtype SCC with a therapy
20 suitable for treating SCC.
19. The method of claim 17, wherein the therapy suitable for treating SCC comprises administering to the subject a therapeutically effective amount of at least one agent that reduces the expression or activity of at least one protein selected from the group consisting of HAT1, LRRFIP2, AKR1B10, WDR82,
25 TTL12, IGF2BP3, and ITGA7.
20. The method of claim 17 or 19, wherein the agent that reduces the expression or activity of the at least one protein is selected from the group consisting of a chemical agent or moiety, a protein, a peptide, and a polynucleotide molecule.

21. The method of claim 20, wherein the agent is an interfering RNA (RNAi) molecule selected from the group consisting of shRNA, siRNA, and miRNA.
22. A method for diagnosing a subtype of NSCLC selected from the group consisting of SCC and AC a subject suspected to have NSCLC, the method comprises
- 5
- a. determining the presence of SMAC/Diablo protein in a cell-comprising sample obtained from the subject; and
- b. diagnosing said subject as having NSCLC subtype SCC when a significant amount of the SMAC/Diablo protein is present in the cell
- 10
- nucleus and in the cell cytosol and as having NSCLC subtype AC when no significant amount of said SMAC/Diablo protein is present in the cell nucleus and a significant amount is present in the cytosol.
23. The method of claim 22, said method further comprises treating the subject diagnosed to have NSCLC subtype SCC with a therapy suitable for treating
- 15
- SCC.
24. The method of claim 22, said method further comprises treating the subject diagnosed to have NSCLC subtype AC with a therapy suitable for treating AC.
25. A method for diagnosing NSCLC in a subject, the method comprising:
- 20
- a. comparing the expression level of at least one biomarker selected from a protein and mRNA encoding the protein in a biological sample of the subject to a control biological sample or reference value, wherein the at least one biomarker is selected from the group consisting of APOOL, VPS29, CAF17, and any combination thereof;
- 25
- b. diagnosing the subject as having NSCLC wherein the expression level of said at least one biomarker or of a combination of said biomarkers is increased compared to the expression in the control biological sample or to a reference value.
26. The method of claim 25, said method comprises comparing the expression
- 30
- level of at least two biomarkers.

27. The method of claim 26, said method comprises comparing the expression of a combination of biomarkers, the combination comprises APOOL, VPS29, and CAF17.
28. The method of any one of claims 25-27, said method further comprises comparing the expression level of at least one additional biomarker selected from the biomarkers set forth in Table 2.
29. The method of claim 28, said method further comprises comparing the expression level of at least one additional biomarker selected from the group consisting of VDAC1, AIF, ATP5B, HSp60, GADPH, PGK1, ENO1, LDHA and Rab11B.
30. The method of any one of claims 25-29, wherein the biological sample is lung tissue sample.
31. The method of claim 30, wherein the control sample is obtained from a healthy subject and the reference value is derived from the expression level in a plurality of samples obtained from healthy subjects.
32. The method of any one of claims 25-31, wherein the expression of the at least one biomarker in the sample obtained from the subject is increased by at least 2 fold compared to the expression level in the control sample or to the reference value.
33. The method of claim 32, wherein the expression of the at least one biomarker in the sample obtained from the subject is increased by at least 4 fold compared to the expression level in the control sample or to the reference value.
34. The method of any one of claims 25-33, wherein the biomarker is a protein.
35. The method of any one of claims 25-34, said method further comprises treating the subject with a therapy suitable for treating NSCLC.
36. The method of claim 35, wherein the therapy for treating NSCLC comprises administering to the subject a therapeutically effective amount of at least one agent that reduces the expression or activity of at least one protein selected from the group consisting of APOOL, VPS29, and CAF17.

37. The method of claim 36, wherein the agent that reduces the expression or activity of the at least one protein is selected from the group consisting of a chemical agent or moiety, a protein, a peptide, and a polynucleotide molecule.
38. The method of claim 37, wherein the agent is an antibody.
- 5 39. The method of claim 37, wherein the agent is an interfering RNA (RNAi) molecule selected from the group consisting of shRNA, siRNA, and miRNA.
40. A method for predicting the severity of NSCLC subtype AC, the method comprising:
- 10 a. comparing the expression level of at least one biomarker selected from a protein and mRNA encoding said protein in a biological sample obtained from a subject diagnosed to have NSCLC subtype AC to a reference value, wherein the at least one biomarker is selected from the group consisting of VDAC1, SMAC, HYOU1, TTLL12, RAB34, ARL1, HAT1, p40, NAPSA, LRRFIP2, AIF, TITF, WDR82 and TSG101;
- 15 b. predicting the level of severity of the disease, wherein an increase in the level of at least one biomarker selected from the group consisting of VDAC1, SMAC, HYOU1, TTLL12, and RAB34 compared to the reference value characterizes said patient as having a severe form of the disease; and wherein an increase in the level of at least one biomarker selected from the group consisting of ARL1, HAT1, p40, NAPSA LRRFIP2, AIF, TITF, WDR82 and TSG101 compared to the reference value characterizes said patient as having a milder form of the disease.
- 20
- 25 41. The method of claim 40, wherein the biological marker is RNA marker.
42. A method for treating NSCLC subtype SCC, the method comprises administering to a subject in need thereof a therapeutically effective amount of at least one agent that reduces the expression or activity of at least one protein selected from the group consisting of HAT1, LRRFIP2, AKR1B10, WDR82, TTLL12, ITGA7, IGF2BP3, and USP14.
- 30

43. A method for treating NSCLC subtype AC, the method comprises administering to a subject in need thereof a therapeutically effective amount of at least one agent that reduces the expression or activity of at least one protein selected from the group consisting of ACAD8, TSG101, and GALE.
- 5 44. A method for treating NSCLC, the method comprises administering to a subject in need thereof a therapeutically effective amount of at least one agent that reduces the expression or activity of at least one protein selected from the group consisting of APOOL, VPS29, and CAF17.
- 10 45. The method of any one of claims 42-44, wherein the agent reducing the expression or activity of the at least one protein is selected from the group consisting of a chemical agent or moiety, a protein, a peptide, and a polynucleotide molecule.
46. The method of claims 45, wherein the agent is an antibody.
- 15 47. The method of claim 45, wherein the agent is is an interfering RNA (RNAi) molecule selected from the group consisting of shRNA, siRNA, and miRNA.
- 20 48. A kit for diagnosing a subtype of non-small cell lung carcinoma (NSCLC) selected from adenocarcinoma (AC) and squamous cell carcinoma (SCC) in a biological sample obtained from a subject suspected to have NSCLC, the kit comprising:
- 25 a. at least one agent capable of detecting the expression level of at least one biomarker selected from a protein and mRNA encoding said protein, the biomarker is selected from the group consisting of HAT1, LRRFIP2, AKR1B10, WDR82, TTL12, IGF2BP3, SMC2, ACAD8, RSU1, ACOT1, HYOU1, GALE, ITGA7, TSG101, and RAB34;
- 30 b. means for comparing the expression level of the at least one biomarker to a first reference value derived from the expression of the at least one biomarker in healthy biological sample and/or to a second reference value derived from the fold change of the expression of said at least one biomarker in a plurality of samples obtained from SCC

5 patients compared to the expression in a plurality of healthy biological samples; and/or to a third reference value derived from a fold change of the expression of the at least one biomarker in a plurality of samples obtained from AC patients compared to a plurality of healthy biological samples;

c. instruction material providing guidance to the correlation of said expression level of said at least one biomarker with the NSCLC subtype, wherein:

10 an increased expression level in said sample of at least one biomarker selected from the group consisting of HAT1, LRRFIP2, AKR1B10, WDR82, TTL12, IGF2BP3, and SMC2, and/or reduced expression of at least one biomarker selected from the group consisting of ACAD8, RSU1, ACOT1, HYOU1, and GALE compared to the first reference value indicates that said subject has NSCLC subtype SCC;

15 a reduced expression level in the sample of at least one biomarker selected from the group consisting of HAT1, LRRFIP2, AKR1B10, WDR82, TTL12, IGF2BP3, and SMC2, and/or elevated expression of at least one biomarker selected from the group consisting of ACAD8, RSU1, ACOT1, HYOU1, and GALE compared to the first reference value indicates that said subject has NSCLC subtype AC;

20 an equal or elevated fold change of the biomarker TGA7 compared to the second reference value indicates that the subject has NSCLC subtype SCC;

25 an equal or elevated fold change of the biomarker TSG101 compared to the third reference value indicates that the subject has NSCLC subtype AC; and/or

an equal or reduced fold change of the biomarker RAB34 compared to the third reference value indicates that the subject has NSCLC subtype AC.

49. The kit of claim 48, said kit further comprises at least one agent capable of detecting the expression of SMAC/Diablo protein within the nucleus of cells present within the biological sample and instruction material providing

30

5 guidance to correlation of the amount of SMAC/Diablo within the cell nucleus and the cytosol and NSCLC subtype, wherein a significant amount of the SMAC/Diablo protein in the cell nucleus and cytosol diagnose the subject as having NSCLC subtype SCC and no significant amount of said SMAC/Diablo protein in the cell nucleus while a significant amount is present in the cytosol diagnose the subject as having NSCLC subtype AC.

50. A kit for diagnosing NSCLC, the kit comprising:

- 10 a. at least one agent capable of detecting the expression level of at least one biomarker selected from a protein and mRNA encoding said protein, the biomarker is selected from the group consisting of APOOL, VPS29, and CAF17 in a biological sample of a subject suspected of having NSCLC;
- 15 b. means for comparing the expression level of the at least one biomarker in a control sample obtained from a healthy subject or to a reference value; and
- c. instruction material providing guidance to the correlation of an increase in the expression level of said at least one biomarker compared to the control sample or to the reference value with NSCLC.

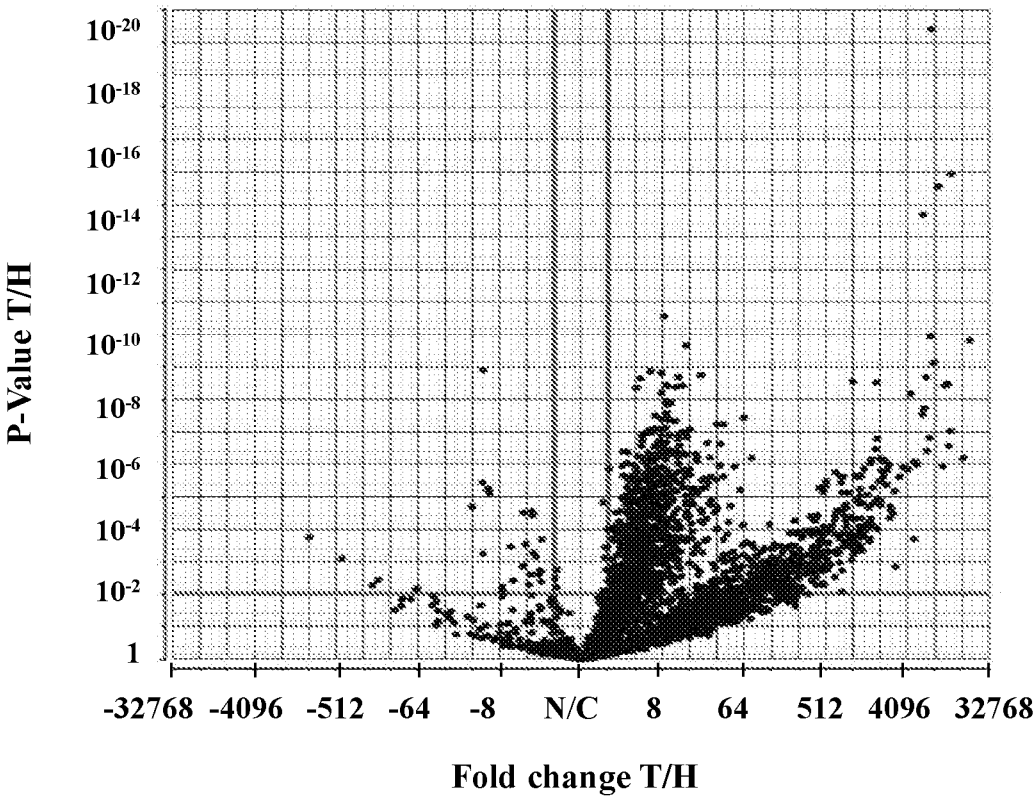


Figure 1A

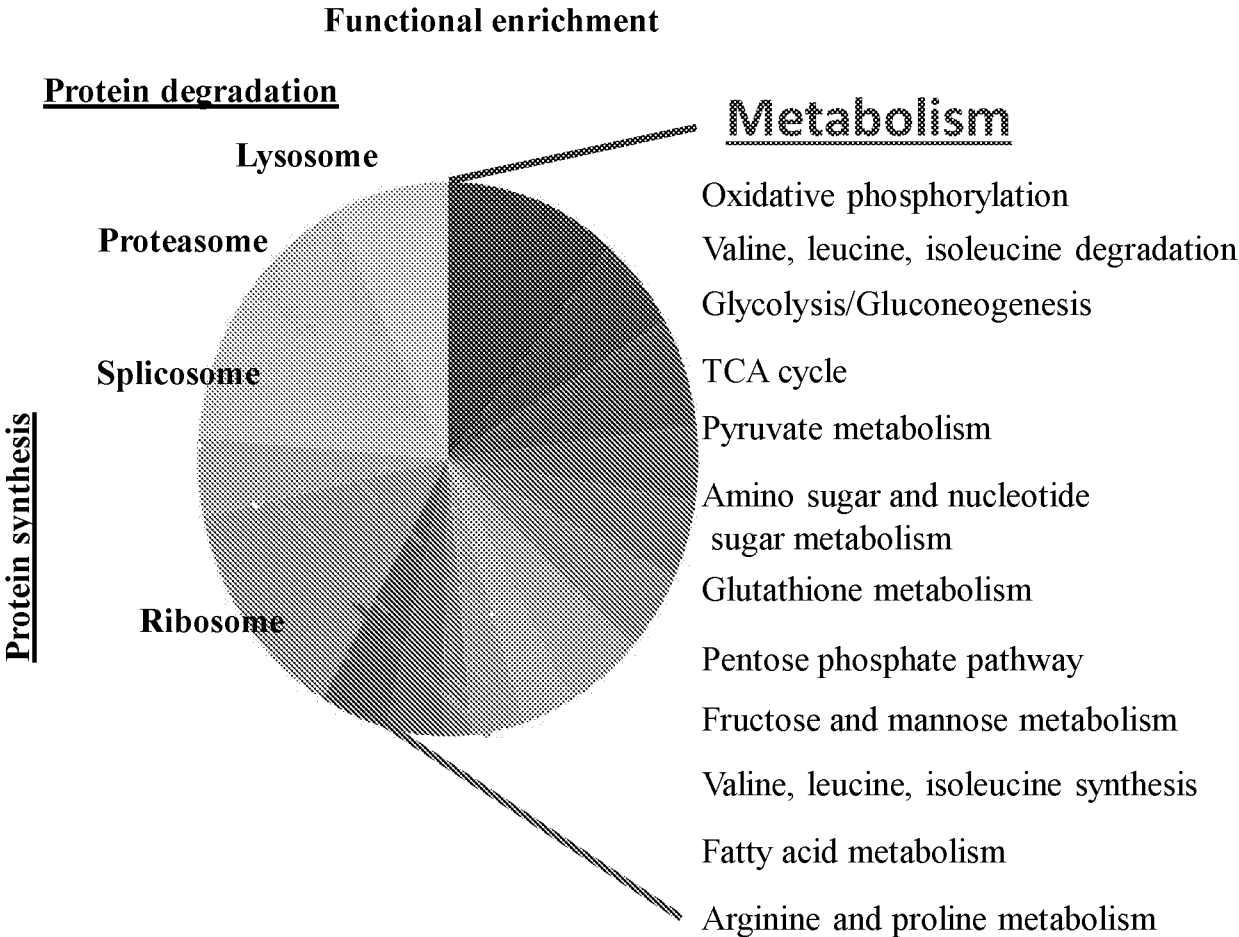


Figure 1B

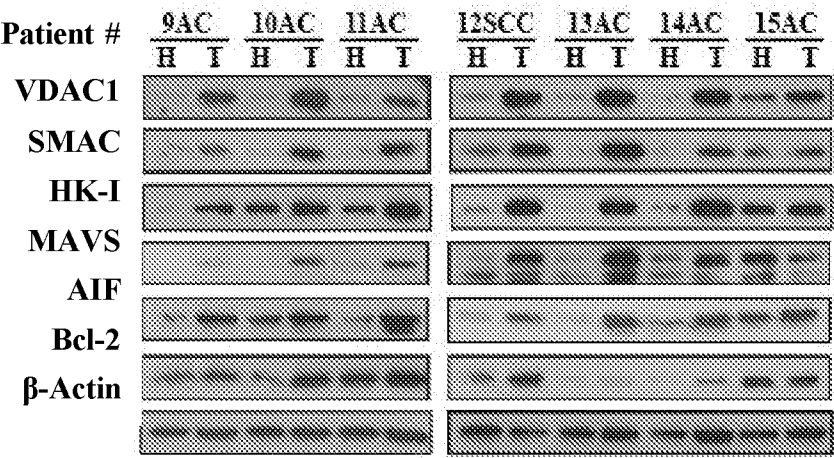


Figure 2A

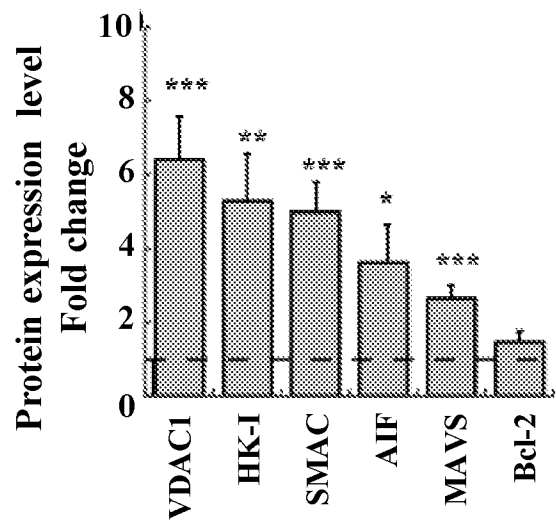


Figure 2B

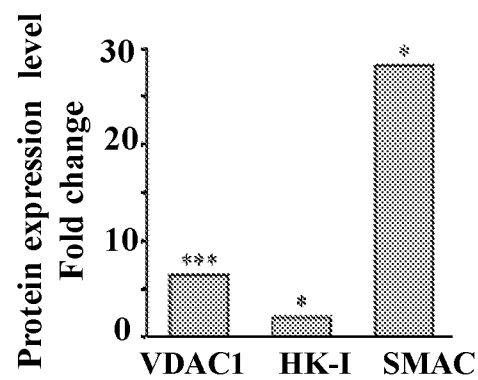


Figure 2C

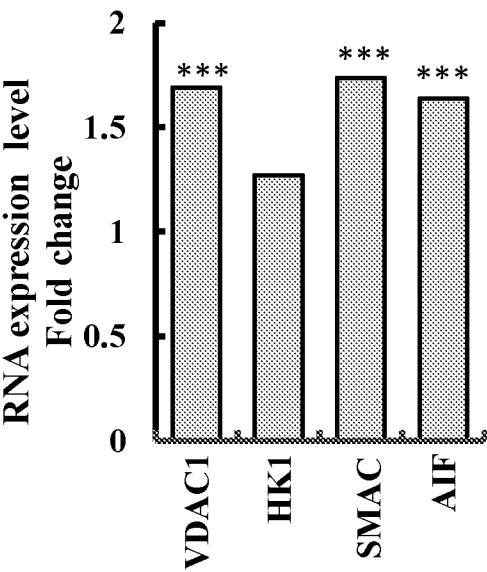


Figure 2D

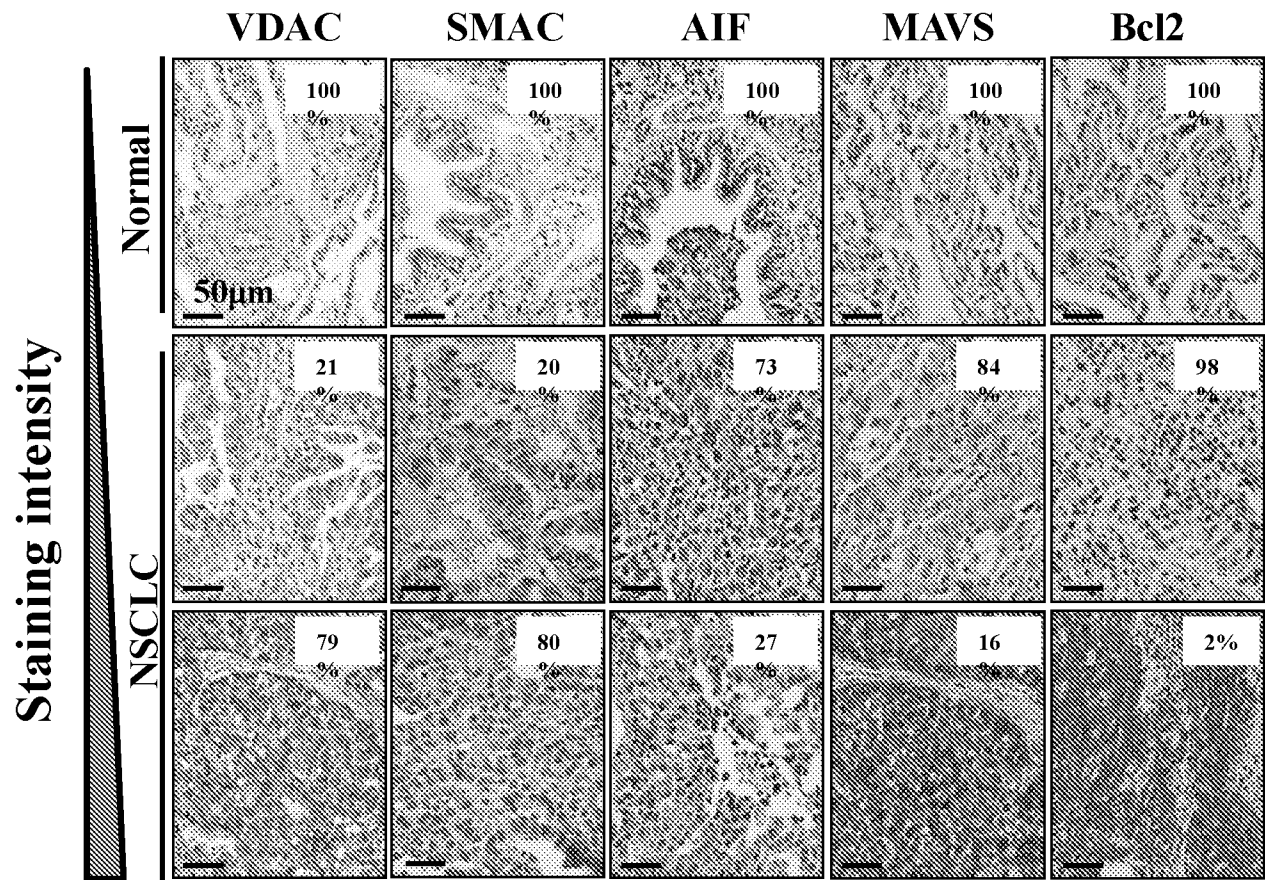


Figure 2E

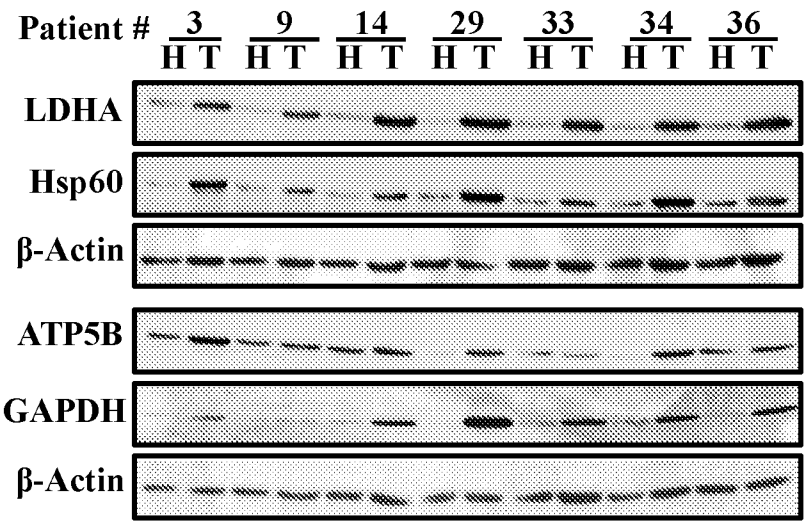


Figure 3A

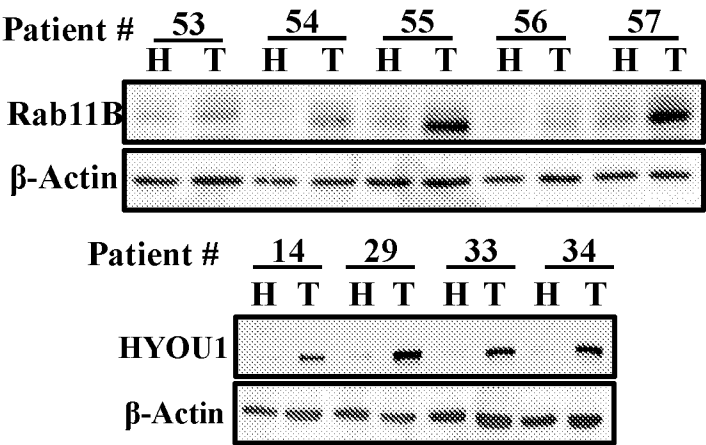


Figure 3B

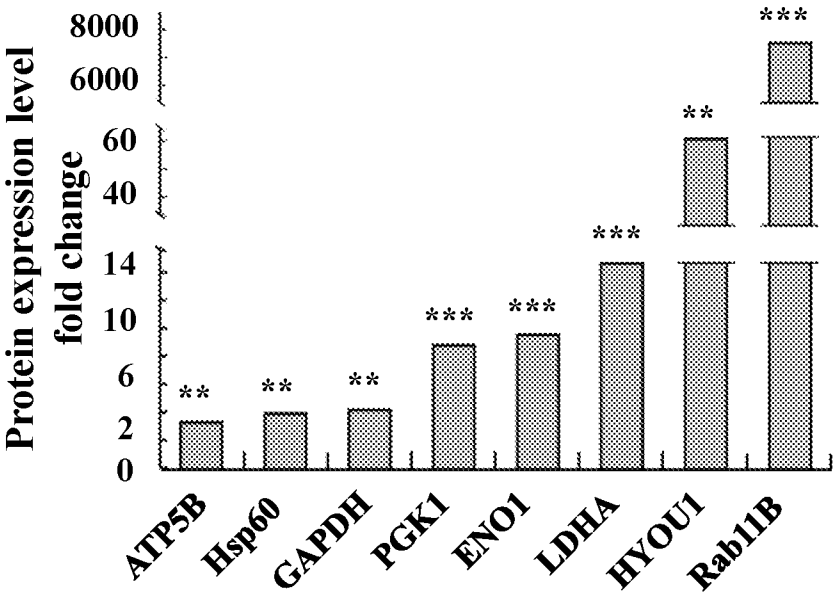


Figure 3C

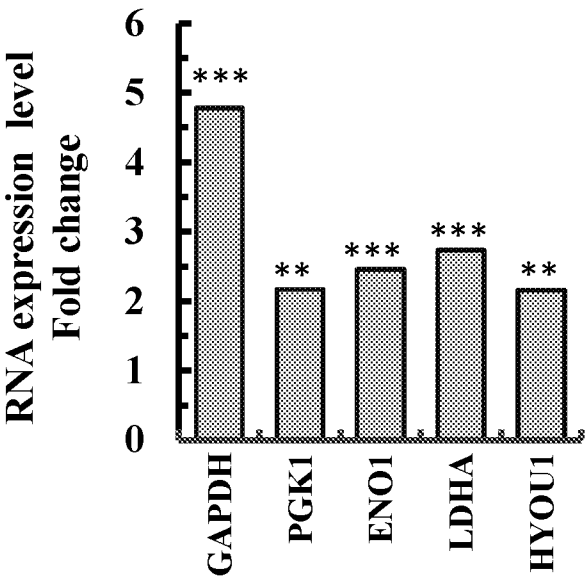


Figure 3D

Figure 4A

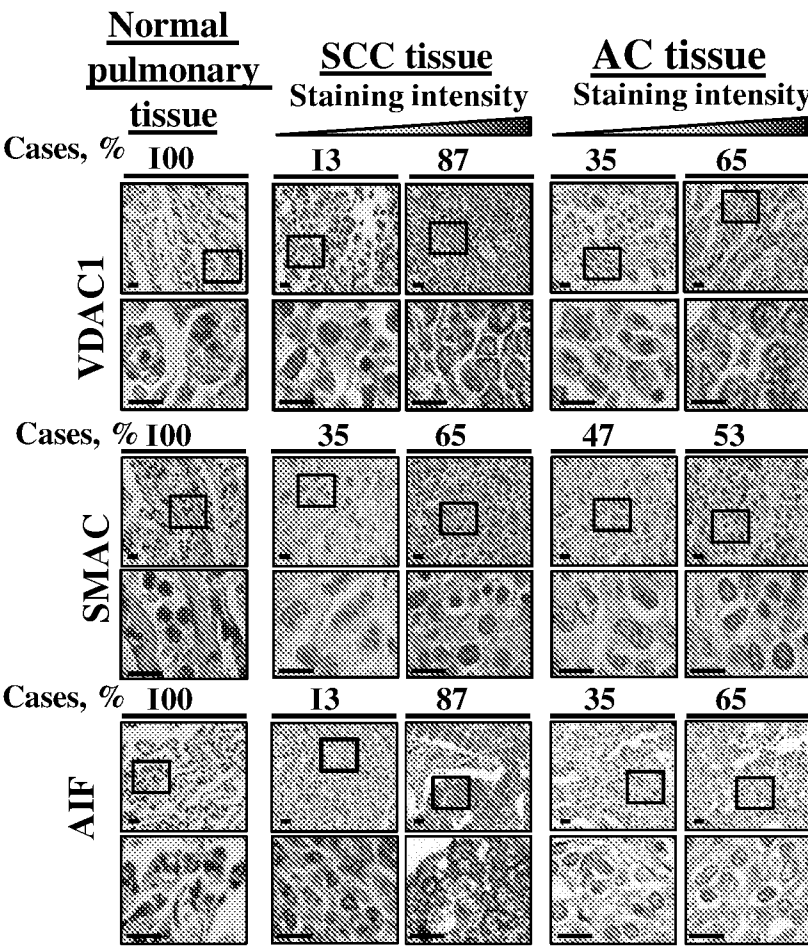
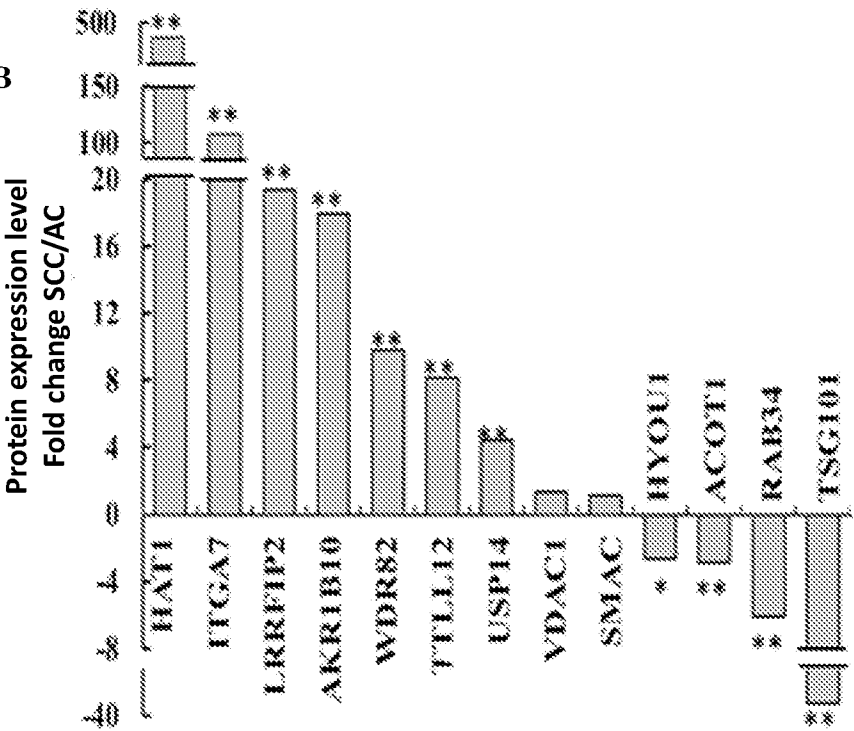


Figure 4B



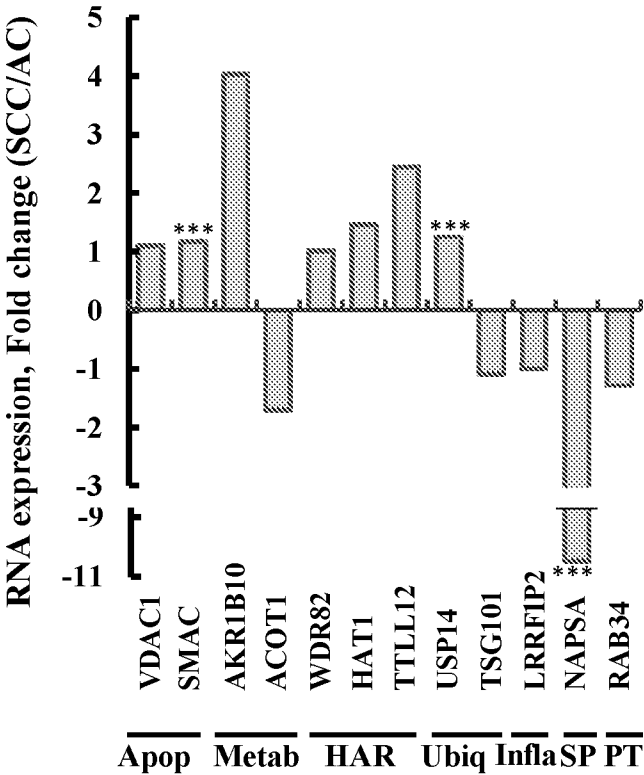


Figure 5A

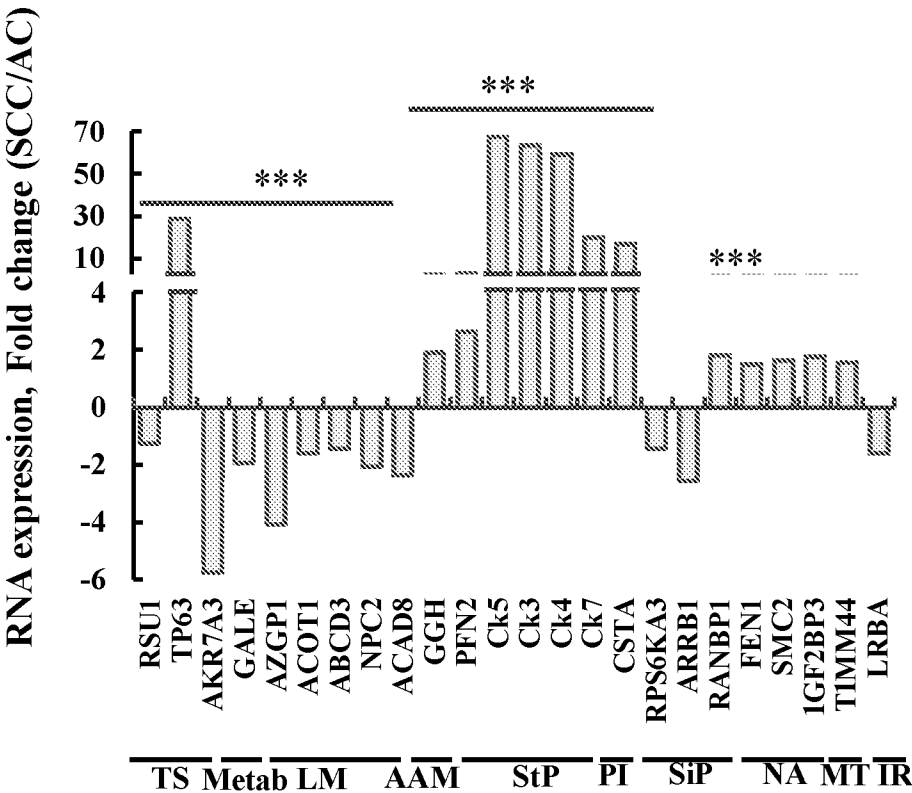


Figure 5B

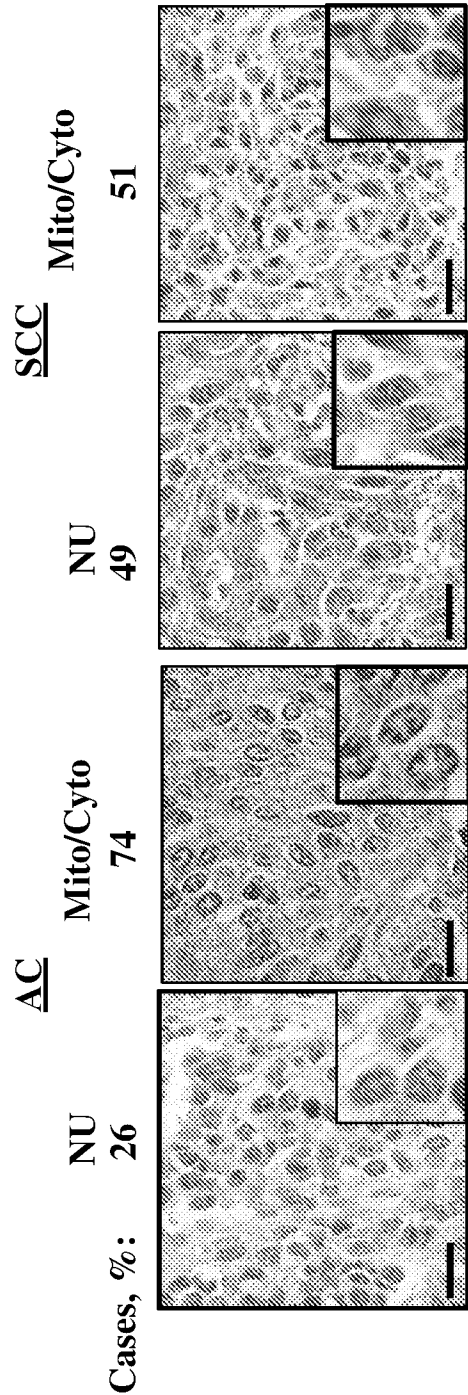


Figure 6A

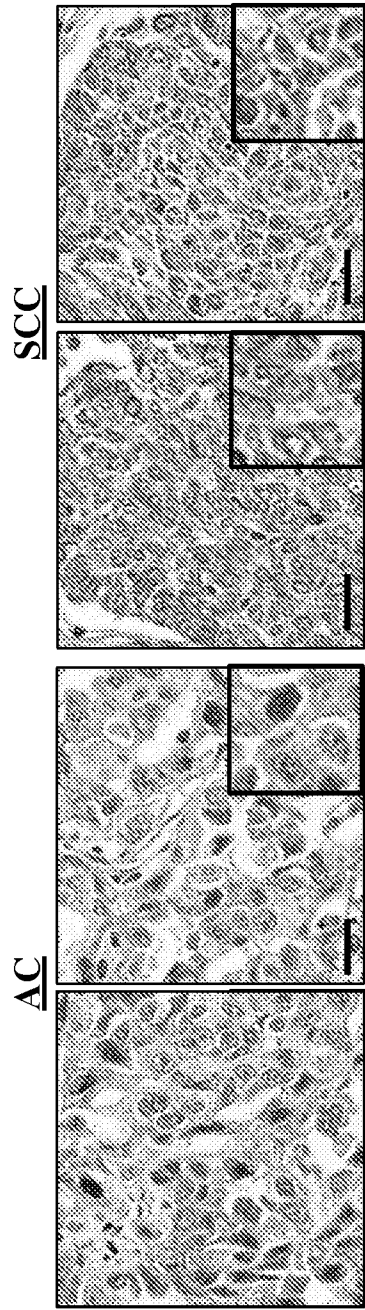


Figure 6B

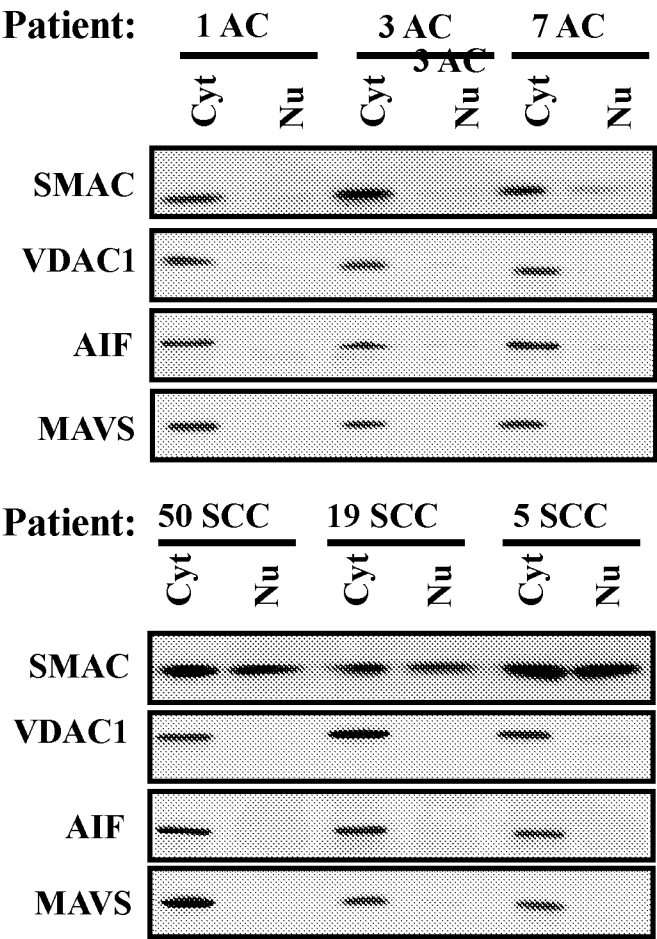


Figure 6C

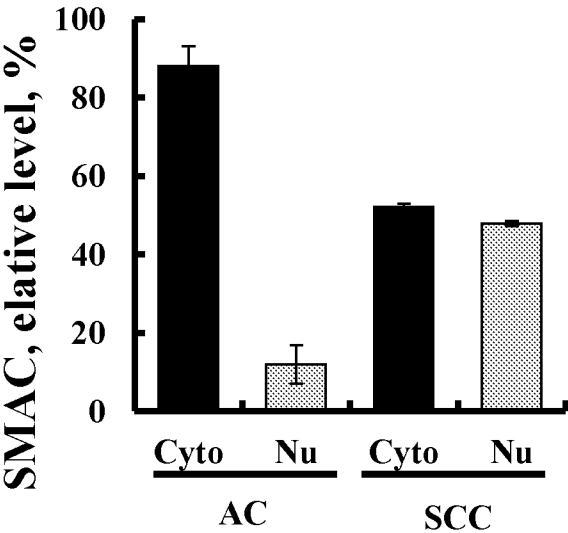


Figure 6D

INTERNATIONAL SEARCH REPORT

International application No.

PCT/IL2018/050554

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61P 35/00; C12Q 1/68; C12Q 1/6886; G01N 33/50; G01N 33/53; G01N 33/574 (2018.01)
 CPC - C12Q 1/68; C12Q 1/6886; G01N 33/574; G01N 33/57423; G01N 33/57484; G01N 2333/705;
 G01N 2800/00 (2018.08)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

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

USPC - 435/6; 435/375; 435/7.1; 435/91.2; 506/9 (keyword delimited)

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

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2012/0178111 A1 (DIAMANDIS et al) 12 July 2012 (12.07.2012) entire document	25, 28, 29, 50
P, X	SHOSHAN-BARMATZ et al. "A molecular signature of lung cancer: potential biomarkers for adenocarcinoma and squamous cell carcinoma," Oncotarget, 06 November 2017 (06.11.2017), Vol. 8, Pgs. 105492-105509 and Supplementary Materials, Pgs. 1-18. entire document	1-6, 22-29, 40-50
A	US 2016/0109453 A1 (AIT AUSTRIAN INSTITUTE OF TECHNOLOGY GMBH) 21 April 2016 (21.04.2016) entire document	1-6, 22-29, 40-50
A	US 2016/0130656 A1 (ALLEGRO DIAGNOSTICS CORP.) 12 May 2016 (12.05.2016) entire document	1-6, 22-29, 40-50
A	CAMPBELL et al. "Distinct patterns of somatic genome alterations in lung adenocarcinomas and squamous cell carcinomas," Nat Genet, 09 May 2016 (09.06.2016), Vol. 48, Pgs. 607-616. entire document	1-6, 22-29, 40-50
A	US 2016/0319361 A1 (CARIS LIFE SCIENCES SWITZERLAND HOLDINGS GMBH) 03 November 2016 (03.11.2016) entire document	1-6, 22-29, 40-50
A	FUKUMOTO et al. "Overexpression of the aldo-keto reductase family protein AKR1B10 is highly correlated with smokers' non-small cell lung carcinomas," Clin Cancer Res, 01 March 2005 (01.03.2005), Vol. 11, Pgs. 1776-1785. entire document	1-6, 22-29, 40-50
A	US 2013/0084287 A1 (GENENTECH, INC. et al) 04 April 2013 (04.04.2013) entire document	1-6, 22-29, 40-50

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

☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

29 August 2018

Date of mailing of the international search report

27 SEP 2018

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents

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

Facsimile No. 571-273-8300

Authorized officer

Blaine R. Copenheaver

PCT Helpdesk: 571-272-4300

PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/IL2018/050554

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

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

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 7-21, 30-39
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/IL2018/050554

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2016/0169900 A1 (INTEGRATED DIAGNOSTICS, INC.) 16 June 2016 (16.06.2016) entire document	1-6, 22-29, 40-50
A	US 2016/0263187 A1 (MOERAE MATRIX, INC.) 15 September 2016 (15.09.2016) entire document	1-6, 22-29, 40-50
A	SHI et al. "Expression profile, clinical significance, and biological function of insulin-like growth factor 2 messenger RNA-binding proteins in non-small cell lung cancer," Tumour Biol, 05 April 2017 (05.04.2017), Vol. 39, Pgs. 1-11. entire document	1-6, 22-29, 40-50
A	US 2016/0032396 A1 (THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY) 04 February 2016 (04.02.2016) entire document	1-6, 22-29, 40-50
A	US 2014/0186837 A1 (THE NATIONAL INSTITUTE FOR BIOTECHNOLOGY IN THE NEGEV LTD. et al) 03 July 2014 (03.07.2014) entire document	1-6, 22-29, 40-50
A	US 2012/0141603 A1 (TSAO et al) 07 June 2012 (07.06.2012) entire document	1-6, 22-29, 40-50

专利名称(译)	诊断肺癌的生物标志物		
公开(公告)号	EP3630293A1	公开(公告)日	2020-04-08
申请号	EP2018806242	申请日	2018-05-22
[标]申请(专利权)人(译)	国家生物技术研究公司		
申请(专利权)人(译)	研究所的生物技术在Negev有限公司.		
当前申请(专利权)人(译)	研究所的生物技术在Negev有限公司.		
[标]发明人	SHOSHAN BARMATZ VARDA		
发明人	SHOSHAN-BARMATZ, VARDA		
IPC分类号	A61P35/00 C12Q1/68 C12Q1/6886 G01N33/53 G01N33/574		
CPC分类号	A61P35/00 C12Q1/6886 C12Q2600/158 G01N33/57423 G01N2800/56 G01N2800/60 G01N33/6848 G01N2560/00		
代理机构(译)	斯特劳斯，亚历山大		
优先权	62/509214 2017-05-22 US		
外部链接	Espacenet		

摘要(译)

本发明涉及肺癌的生物标志物，尤其涉及能够区分非小细胞肺癌（NSCLC）的亚型，特别是腺癌（AC）和鳞状细胞癌（SCC）的标志物。特别地，本发明涉及用于诊断，评估严重性水平和选择治疗NSCLC的手段和方法。