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(54) Title: USE OF NNMT AS A MARKER FOR LUNG CANCER

(57) Abstract: The present invention relates to the assessment of lung cancer. It discloses the use of protein NNMT in the assessment of lung cancer. It also relates to a method for assessing lung cancer by measuring NNMT in vitro in a liquid sample derived from an individual. Measurement of NNMT can, e.g., be used in the early detection or in the follow-up of patients with lung cancer.

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Use of NNMT as a marker for lung cancer

The present invention relates to a method aiding in the assessment of pulmonary or lung cancer (= LC) and in particular in the assessment of non-small cell lung carcinoma (NSCLC). It discloses the use of the protein nicotinamide N-methyl transferase (= NNMT) as a marker of LC, particularly NSCLC. Furthermore, it especially relates to a method for assessing lung cancer from a liquid sample, derived from an individual by measuring NNMT in said sample. Measurement of NNMT can, e.g., be used in the early detection of lung cancer or in the surveillance of patients who undergo surgery.

Cancer remains a major public health challenge despite progress in detection and therapy. Amongst the various types of cancer, LC is a frequent cancer in the Western world and among the most frequent cause of cancer-related mortality. This is in large part due to the diagnostic gap for early detection of the disease. LC is largely asymptomatic in its early stages. The majority of all lung cancers is detected at a late stage when the disease has already become inoperable.

The majority of LC tumors can be divided into small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC). SCLC accounts for about 20-25% of all lung cancer cases. SCLC is an aggressive neuroendocrine type of LC and has a very poor prognosis even if detected in early stages. SCLC is rarely amenable to curative treatment by resection. Because of the speed with which the disease progresses, SCLC is generally categorized using only two stages, i.e. limited and extensive disease, rather than the more complex TNM staging system (see below). About 75-80% of LC cases are grouped into the class of NSCLC including squamous cell carcinoma (carcinoma = CA), adeno-CA (comprising the subclasses of acinar CA, papillary CA, bronchoalveolar tumor, solid tumor, and mixed subtypes), and large cell carcinoma (comprising the subclasses of giant cell tumors, clear cell CA, adenosquamous CA, and undifferentiated CA).

NSCLC, if detected at late stages, also has a very poor prognosis. The staging of cancer is the classification of the disease in terms of extent, progression, and severity. It groups cancer patients so that generalizations can be made about prognosis and the choice of therapy.

Today, the TNM system is the most widely used classification system based on the anatomical extent of cancer. It represents an internationally accepted, uniform

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staging system. There are three basic variables: T (the extent of the primary tumor), N (the status of regional lymph nodes) and M (the presence or absence of distant metastases). The TNM criteria are published by the UICC (International Union Against Cancer), in: TNM Classification of Malignant Tumours, fifth edition, 5 Sobin, L.H. and Wittekind, Ch. (eds.), Wiley-Liss (1997), pp. 1803-1804)

Surgical resection of the primary tumor is widely accepted as the treatment of choice for early stage NSCLC. With the progression of NSCLC and, more specifically, the transition from stage IIIa (T3N1M0, T1N2M0, T2N2M0, T3N2M0) to IIb (T4N0M0, T4N1M0, T4N2M0), a significant shift in the physician's 10 approach is precipitated. However, if the cancer is detected during the more early stages (Ia – IIIa; preferably up to stage T3N1M0), the five-year survival rate varies between 35% and 80%. Detection at stage Ia ((T1N0M0); small tumor size, no metastasis) has evidently the best prognosis with a five-year survival of up to 80%.

Surgery is rarely, if ever, used in the management of stage IIb-IV of NSCLC. Stage 15 IV corresponds to distant metastasis, i.e. spread of the disease beyond the lungs and regional lymph nodes. The five-year survival rate in the later stages (III – IV) drops to between less than 1% and 15%.

What is especially important is that early diagnosis of NSCLC translates to a much better prognosis. Patients diagnosed as early as in stage Ia (T1N0M0), Ib 20 (T2N0M0), IIa (T1N1M0), IIb, (T3N0M0), and IIIa (T3N1M0), if treated properly have an up to 80% chance of survival 5 years after diagnosis. This has to be compared to a 5-years survival rate of less than 1% for patients diagnosed once distant metastases are already present.

In the sense of the present invention early assessment of LC refers to an assessment 25 at a tumor stage T1N0M0 or T1-3N0-1M0.

It is preferred that LC is assessed at stage T1-3N0-1M0 (=T1-3N0-1M0).

Most lung cancers are detected when they become symptomatic. Current detection methods include chest x-ray, spiral computer tomography, sputum cytology and bronchoscopy. However, there is controversy regarding the suitability of these 30 means for mass screenings.

A number of serum tumor markers for lung cancers are in clinical use. The soluble 30 kDa fragment of cytokeratin 19 (CYFRA 21-1), carcinoembryogenic antigen

(CEA), neuron-specific enolase (NSE), and squamous cell carcinoma antigen (SCC) are the most prominent LC markers. However, none of them meets the criteria for sensitivity and specificity required for a screening tool (Thomas, L., Labor und Diagnose, TH Books Verlagsgesellschaft, Frankfurt/Main, Germany (2000)).

- 5 In order to be of clinical utility, a new diagnostic marker as a single marker should be comparable to other markers known in the art, or better. Or, a new marker should lead to a progress in diagnostic sensitivity and/or specificity either if used alone or in combination with one or more other markers, respectively. The diagnostic sensitivity and/or specificity of a test is best assessed by its receiver-
10 operating characteristics, which will be described in detail below.

- Whole blood, serum or plasma are the most widely used sources of sample in clinical routine. The identification of an early LC tumor marker that would aid in the reliable cancer detection or provide early prognostic information could lead to a method that would greatly aid in the diagnosis and in the management of this
15 disease. Therefore, an urgent clinical need exists to improve the in vitro assessment of LC. It is especially important to improve the early diagnosis of LC, since for patients diagnosed early on chances of survival are much higher as compared to those diagnosed at a progressed stage of disease.

- The clinical utility of biochemical markers in lung cancer has recently been
20 reviewed (Duffy, M.J., Crit. Rev. Clin. Lab. Sci. 38 (2001) 225-262).

- CYFRA 21-1 is currently regarded to be the best of the presently known tumor markers for lung cancer. Even though not organ-specific it is predominantly found in lung tissue. Sensitivity of CYFRA 21-1 for lung cancer is described to be between 46-61% at a specificity of 95% towards other benign lung diseases. Increased serum
25 levels of CYFRA 21-1 are also associated with pronounced benign liver diseases, renal insufficiency and invasive bladder cancer. CYFRA 21-1 testing is recommended for postoperative therapy surveillance.

- CEA belongs to the group of carcinofoetal antigens, usually produced during embryogenesis. CEA is not organ-specific and predominantly used for monitoring
30 of colorectal cancer. Besides malignancies, also several benign diseases such as cirrhosis, bronchitis, pancreatitis and autoimmune diseases are associated with increased CEA serum levels. At 95% specificity towards benign lung diseases its sensitivity for lung cancer is reported to be 29-44%. A preferred use of CEA is therapy surveillance of lung cancer.

NSE is a tumor marker for SCLC. Generally, increased NSE serum levels are found in association with neuroectodermal and neuroendocrine tumors. Increased serum levels are also found in patients with benign lung diseases and cerebral diseases, such as meningitis or other inflammatory diseases of the brain, and traumatic
5 injuries to the head. While sensitivity for SCLC at 95% specificity is reported to be 60-87%, performance of NSE testing for NSCLC is poor (7-25%). NSE is recommended for therapy surveillance of SCLC.

SCC was originally identified in squamous cell CA of the cervix. The sensitivity of SCC for LC in general is low (18-27%). Therefore, SCC testing is regarded to be not
10 suitable for screening. However, due to a higher sensitivity for squamous cell CA, a preferred use for SCC is therapy surveillance, even though CYFRA 21-1 generally performs better.

With respect to marker profiles and aiming at improved diagnosis of lung cancer, a method was published (Schneider, J. et al., Int. J. Clin. Oncol. 7 (2002) 145-151)
15 using fuzzy logic based classification algorithms to combine serum levels of CYFRA 21-1, NSE and C-reactive protein (CRP) which is a general inflammation marker. The authors report a sensitivity of 92% at a specificity of 95%.

It was the task of the present invention to investigate whether a biochemical marker can be identified which may be used in assessing LC.

20 Surprisingly, it has been found that use of the marker NNMT can at least partially overcome some of the problems of the markers presently known in the state of the art.

The present invention relates to a method for assessing lung cancer in vitro comprising measuring in a sample the concentration of NNMT, and using the
25 concentration determined in the assessment of lung cancer. Surprisingly it could be established that an increased level of NNMT in a sample is for example indicative for the presence of lung cancer in an individual.

The present invention is also directed to a method for assessing LC in vitro by biochemical markers, comprising measuring in a sample the concentration of
30 NNMT and of one or more other marker of LC and using the concentrations determined in the assessment of LC. It is preferred that the one or more other marker of LC is selected from the group consisting of CYFRA 21-1, CEA, NSE, and SCC.

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The present invention, in a preferred embodiment, also relates to the use of a marker panel comprising at least NNMT and CYFRA 21-1 in the assessment of LC.

The present invention also relates to the use of a marker panel comprising at least NNMT and CEA in the assessment of LC.

- 5 The present invention also relates to the use of a marker panel comprising at least NNMT and SCC in the assessment of LC.

In a preferred embodiment the present invention relates to a method for assessing lung cancer in vitro comprising measuring in a sample the concentration of a) NNMT, b) optionally one or more other marker of lung cancer, and c) using the
10 concentrations determined in step (a) and optionally step (b) in the assessment of lung cancer.

NNMT (nicotinamide N-methyl transferase, EC 2.1.1.1) catalyzes the N-methylation of nicotinamide and other pyridines. The protein NNMT (Swiss-PROT: P40261) is characterized by the sequence given in SEQ ID NO:1. NNMT has
15 an apparent molecular weight of 29.6 kDa and an isoelectric point of 5.56.

NNMT activity is important for biotransformation of many drugs and xenobiotic compounds. The protein has been reported to be predominantly expressed in liver and is located in the cytoplasm. NNMT has been cloned from cDNA from human liver and contained a 792-nucleotide open reading frame that encoded a 264-amino
20 acid protein with a calculated molecular mass of 29.6 kDa (Aksoy, S. et al., J. Biol. Chem. 269 (1994) 14835-14840). Little is known in the literature about a potential role of the enzyme in human cancer. In one paper, increased hepatic NNMT enzymatic activity was reported as a marker for cancer cachexia in mice (Okamura, A. et al., Jpn. J. Cancer Res. 89 (1998) 649-656). In a recent report, down-regulation
25 of the NNMT gene in response to radiation in radiation sensitive cell lines was demonstrated (Kassem, H., et al., Int. J. Cancer 101 (2002) 454-460). In US 2006/0024692 the NNMT m-RNA-level was found to be lower in non-small cell lung carcinoma cells compared with non-cancerous tissue. WO 02/082076 describes NNMT protein as a marker for subtypes of renal carcinoma.

30 As used herein, each of the following terms has the meaning associated with it in this section.

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The articles "a" and "an" are used herein to refer to one or to more than one (i.e. to at least one) of the grammatical object of the article. By way of example, "a marker" means one marker or more than one marker.

5 The term "marker" or "biochemical marker" as used herein refers to a molecules to be used as a target in the analysis of a patient sample. Examples of such molecular targets are proteins or polypeptides themselves as well as antibodies to such targets as present in a sample. The expression "one or more" denotes 1 to 20, preferably 1 to 10, preferably 1 to 5, more preferred 3 or 4.

10 Proteins or polypeptides used as a marker in the present invention are contemplated to include naturally occurring variants of said protein as well as naturally occurring fragments or complexes of said protein or said variant, in particular immunologically detectable fragments or complexes, respectively.

As obvious to the skilled artisan, the present invention shall not be construed to be limited to the measurement of the full-length protein NNMT of SEQ ID NO: 1.
15 Physiological fragments of NNMT can also be measured and used as a marker for lung cancer while practicing the present invention. Immunologically detectable fragments preferably comprise at least 6, 7, 8, 10, 12, 15 or 20 contiguous amino acids of said marker polypeptide. One of skill in the art would recognize that proteins which are released by cells or present in the extracellular matrix may be
20 damaged, e.g., during inflammation, and could become degraded or cleaved into such fragments. As the skilled artisan will appreciate, NNMT or fragments thereof may also be present as part of a complex. Such complex also may be used as a marker in the sense of the present invention. In addition, or in the alternative the NNMT polypeptide may carry a post-translational modification, preferably a
25 glycosylation, acylation, and/or a phosphorylation, and such modified NNMT may also serve as a marker of LC.

The term "assessing lung cancer" is used to indicate that the method according to the present invention will (alone or together with other markers or variables, e.g., the criteria set forth by the UICC (see above)) e.g., aid the physician to establish or
30 confirm the absence or presence of LC or aid the physician in the prognosis, the detection of recurrence (follow-up of patients after surgery) and/or the monitoring of treatment, especially of chemotherapy.

The term "sample" as used herein refers to a biological sample obtained for the purpose of evaluation in vitro. In the methods of the present invention, the sample

or patient sample preferably may comprise any body fluid. Preferred samples are whole blood, serum, plasma, bronchial lavage or sputum, respectively, with plasma or serum being most preferred.

5 The inventors of the present invention have surprisingly been able to detect the marker NNMT in a significant percentage of samples derived from patients with LC. Even more surprising they have been able to demonstrate that the presence of NNMT in such liquid sample obtained from an individual can be used in the assessment of lung cancer.

10 In a preferred embodiment the present invention relates to a method for assessing LC by measuring in a sample the concentration of NNMT and using the concentration determined in the assessment of LC.

As the skilled artisan will appreciate, any such measurement is made in vitro. The patient sample is discarded afterwards. The patient sample is solely used for the in vitro diagnostic method of the invention and the material of the patient sample is not transferred back into the patient's body. Typically, the sample is a liquid sample, e.g., whole blood, serum, or plasma.

As the skilled artisan will appreciate from the examples described, NNMT has been identified by measurement in an immunoassay as a marker which is useful in the diagnosis of LC, alternative ways may be used to reach a result comparable to the achievements of the present invention. The marker protein NNMT may be detected by any appropriate means and used as a marker of LC. Such preferred appropriate means comprise the detection of NNMT by an immuno assay procedure, by any other form of binding assay, by measurement of its enzymatic activity, by liquid chromatography, especially high performance liquid chromatography, by electrophoresis, especially SDS-PAGE combined with Western Blotting and by mass spectroscopy.

30 The ideal scenario for diagnosis would be a situation wherein a single event or process would cause the respective disease as, e.g., in infectious diseases. In all other cases correct diagnosis can be very difficult, especially when the etiology of the disease is not fully understood as is the case for LC. As the skilled artisan will appreciate, no biochemical marker, for example in the field of LC, is diagnostic with 100% specificity and at the same time 100% sensitivity for a given disease. Rather, biochemical markers e.g. CYFRA 21-1, CEA, NSE, SCC, or as shown here NNMT can be used to assess with a certain likelihood or predictive value the presence or

absence of a disease. Therefore in routine clinical diagnosis, generally various clinical symptoms and biological markers are considered together in the diagnosis, treatment and management of the underlying disease.

Biochemical markers can either be determined individually or in a preferred embodiment of the invention they can be measured simultaneously using a chip or a bead based array technology. The concentrations of the biomarkers are then either interpreted independently using an individual cut-off for each marker or they are combined for interpretation.

In a further preferred embodiment the assessment of LC according to the present invention is performed in a method comprising measuring in a sample the concentration of a) NNMT, b) one or more other marker of lung cancer, and c) using the concentration determined in step (a) and step (b) in the assessment of lung cancer. As obvious to the skilled artisan the measurement of NNMT and the measurement of the one or more other marker of lung cancer can be made alternatively from the same aliquot of a sample or from different aliquots

According to the data shown in the Example section, the marker NNMT, both in the univariate analysis as well as in the multivariate analysis performed, has (at a specificity of about 95%) a remarkable sensitivity for LC of almost 50% and in this respect was found comparable or even superior as compared to other LC markers investigated. In the assessment of LC the marker NNMT will be of advantage in one or more of the following aspects: screening; diagnostic aid; prognosis; monitoring of therapy such as chemotherapy, radiotherapy, and immunotherapy.

Screening:

Screening is defined as the systematic application of a test to identify individuals at sufficient risk of suffering from LC to benefit from further investigation or direct preventive action, among persons who have not sought medical attention on account of symptoms of LC.

As the data given in the Examples section demonstrate NNMT alone will not suffice to allow for a general screening e.g. of the at risk population for LC. Most likely no single biochemical marker in the circulation will ever meet the sensitivity and specificity criteria required for screening purposes. Rather it has to be expected that a marker panel will have to be used in LC screening. The data established in the present invention indicate that the marker NNMT will form an integral part of a

marker panel appropriate for screening purposes. The present invention therefore relates to the use of NNMT as one marker of a LC marker panel for LC screening purposes. An increased level of NNMT is indicative for the presence of LC. The present data further indicate that certain combinations of markers will be advantageous in the screening for LC. Therefore the present invention also relates to the use of a marker panel comprising NNMT and CYFRA 21-1, or of a marker panel comprising NNMT and CEA, or of a marker panel comprising NNMT and NSE, or of a marker panel comprising NNMT and SCC or of a marker panel comprising NNMT and two markers selected from the group consisting of CYFRA 21-1, CEA, NSE, and SCC, for the purpose of screening for LC.

Diagnostic aid:

Markers may either aid the differential diagnosis of benign vs. malignant disease in a particular organ, or help distinguish between different histological types of a tumour. As reported by Molina, R. et al., Tumor Biol. 24 (2003) 209-218, CEA, CA 125, CYFRA 21-1, SSC and NSE were used as serum markers to aid histological diagnosis of LC. The results of that study also indicate that among the markers tested CYFRA 21-1 is the most sensitive marker in lung cancer but with no relationship to histology.

Since NNMT as a single marker according to the data of the present invention might be superior to other LC markers like CEA or NSE it has to be expected that NNMT will be used as a diagnostic aid, especially by establishing a baseline value before surgery. The present invention thus also relates to the use of NNMT for establishing a baseline value before surgery for LC.

Prognosis:

Prognostic indicators can be defined as clinical, pathological, or biochemical features of cancer patients and their tumours that forecast disease outcome. Their main use is to help rationally plan patient management, i.e. avoid undertreatment of aggressive disease and overtreatment of indolent disease. Molina R. et al., Tumor Biol. (2003) 24:209-218 evaluated the prognostic value of CEA, CA 125, CYFRA 21-1, SSC and NSE in NSCLC. E.g., overall survival of patients was assessed, whereby abnormal serum levels of the markers NSE, CEA, and LDH (lactate dehydrogenase) appeared to indicate shorter survival.

As NNMT alone significantly contributes to the differentiation of LC patients from healthy controls, it has to be expected that it will aid in assessing the prognosis of patients suffering from LC. The level of preoperative NNMT will most likely be combined with one or more other marker for LC and/or the TNM staging system.

- 5 In a preferred embodiment NNMT is used in the prognosis of patients with LC.

Monitoring of Chemotherapy:

- Merle, P. et al., Int. J. of Biological Markers 19 (2004) 310-315 have evaluated CYFRA 21-1 serum level variations in patients with locally advanced NSCLC treated with induction chemotherapy. They conclude that early monitoring of
10 CYFRA 21-1 serum levels may be a useful prognostic tool for tumor response and survival in stage III NSCLC patients. In addition, reports have described the use of CEA in monitoring the treatment of patients with LC (Fukasawa, T. et al., Gan to Kagaku Ryoho. Cancer & Chemotherapy 13 (1986) 1862-1867 (written in Japanese); Zhang, H. et al., Shandong Yike Daxue Xuebao 39 (2001) 537-538, 541
15 (written in Chinese)). Most of these were retrospective, non-randomized and contained small numbers of patients. As in the case of the studies with CYFRA 21-1 the CEA studies suggested: a) that patients with a decrease in CEA levels while receiving chemotherapy generally had a better outcome than those patients whose CEA levels failed to decrease and (b) for almost all patients, increases in CEA levels
20 were associated with disease progression.

Due to the data shown in the example section, it has to be expected that NNMT will be at least as good a marker for monitoring of chemotherapy as CYFRA 21-1 or CEA. The present invention therefore also relates to the use of NNMT in the monitoring of LC patients under chemotherapy.

25 Follow-up:

- A large portion of LC patients who undergo surgical resection aimed at complete removal of cancerous tissue, later develop recurrent or metastatic disease (Wagner, H., Chest 117 (2000) 110-116; Buccheri, G. et al., Ann. Thorac. Surg. 75 (2003) 973-980). Most of these relapses occur within the first 2-3 years after surgery. Since
30 recurrent/metastatic disease is invariably fatal, considerable research has focused on LC identification at an early and thus potentially treatable stage.

Consequently, many LC patients undergo a postoperative surveillance program which frequently includes regular monitoring with CEA. Serial monitoring with

CEA one year after surgical resection has been shown to detect an early postoperative recurrent/metastatic disease with a sensitivity of approximately of 29 %, specificity of approximately 97 %, even in the absence of suspicious symptoms or signs (Buccheri, G. et al., Ann. Thorac. Surg. 75 (2003) 973-980). Thus, the follow-up of patients with LC after surgery is one of the most important fields of use for an appropriate biochemical marker. Due to the high sensitivity of NNMT in the LC patients investigated it is expected that NNMT alone or in combination with one or more other marker will be of great help in the follow-up of LC patients, especially in LC patients after surgery. The use of a marker panel comprising NNMT and one or more other marker of LC in the follow-up of LC patients represents a further preferred embodiment of the present invention.

The present invention in a preferred embodiment relates to the use of NNMT in the diagnostic field of LC or in the assessment of LC, respectively.

In yet a further preferred embodiment the present invention relates to the use of NNMT as a marker molecule for lung cancer in combination with one or more marker molecules for lung cancer in the assessment of lung cancer from a liquid sample obtained from an individual.

Thus, a preferred embodiment of the present invention is the use of NNMT as a marker molecule for lung cancer in combination with one or more marker molecules for lung cancer in the assessment of lung cancer from a liquid sample obtained from an individual. Preferred selected other LC markers with which the measurement of NNMT may be combined are CYFRA 21-1, CEA, NSE, and SCC. Yet further preferred the marker panel used in the assessment of LC comprises NNMT and at least one other marker molecule selected from the group consisting of CYFRA 21-1 and CEA.

As the skilled artisan will appreciate there are many ways to use the measurements of two or more markers in order to improve the diagnostic question under investigation. In a quite simple, but nonetheless often effective approach, a positive result is assumed if a sample is positive for at least one of the markers investigated. This may e.g. the case when diagnosing an infectious disease, like AIDS.

Frequently, however, the combination of markers is evaluated. Preferably the values measured for markers of a marker panel, e.g. for CYFRA and CEA, are mathematically combined and the combined value is correlated to the underlying diagnostic question. Marker values may be combined by any appropriate state of

the art mathematical method. Well-known mathematical methods for correlating a marker combination to a disease employ methods like, discriminant analysis (DA) (i.e. linear-, quadratic-, regularized-DA), Kernel Methods (i.e. SVM), Nonparametric Methods (i.e. k-Nearest-Neighbor Classifiers), PLS (Partial Least Squares), Tree-Based Methods (i.e. Logic Regression, CART, Random Forest Methods, Boosting/Bagging Methods), Generalized Linear Models (i.e. Logistic Regression), Principal Components based Methods (i.e. SIMCA), Generalized Additive Models, Fuzzy Logic based Methods, Neural Networks and Genetic Algorithms based Methods. The skilled artisan will have no problem in selecting an appropriate method to evaluate a marker combination of the present invention. Preferably the method used in correlating the marker combination of the invention e.g. to the absence or presence of LC is selected from DA (i.e. Linear-, Quadratic-, Regularized Discriminant Analysis), Kernel Methods (i.e. SVM), Nonparametric Methods (i.e. k-Nearest-Neighbor Classifiers), PLS (Partial Least Squares), Tree-Based Methods (i.e. Logic Regression, CART, Random Forest Methods, Boosting Methods), or Generalized Linear Models (i.e. Logistic Regression). Details relating to these statistical methods are found in the following references: Ruczinski, I. et al., J. of Computational and Graphical Statistics 12 (2003) 475-511; Friedman, J. H., J. of the American Statistical Association 84 (1989) 165-175; Hastie, T. et al., The Elements of Statistical Learning, Springer Series in Statistics (2001); Breiman, L. et al., Classification and Regression Trees, Wadsworth International Group, Belmont, California (1984); Breiman, L., Random Forests, Machine Learning, 45 (2001) 5-32; Pepe, M.S., The Statistical Evaluation of Medical Tests for Classification and Prediction, Oxford Statistical Science Series, 28 (2003); and Duda, R.O. et al., Pattern Classification, Wiley Interscience, 2nd edition (2001).

It is a preferred embodiment of the invention to use an optimized multivariate cut-off for the underlying combination of biological markers and to discriminate state A from state B, e.g. diseased from healthy. In this type of analysis the markers are no longer independent but form a marker panel. It could be established that combining the measurements of NNMT with those of CYFRA 21-1 or CEA, respectively, does significantly improve the diagnostic accuracy for LC as compared to healthy controls.

Accuracy of a diagnostic method is best described by its receiver-operating characteristics (ROC) (see especially Zweig, M.H. and Campbell, G., Clin. Chem. 39 (1993) 561-577). The ROC graph is a plot of all of the sensitivity/specificity pairs

resulting from continuously varying the decision thresh-hold over the entire range of data observed.

The clinical performance of a laboratory test depends on its diagnostic accuracy, or the ability to correctly classify subjects into clinically relevant subgroups. Diagnostic accuracy measures the test's ability to correctly distinguish two different conditions of the subjects investigated. Such conditions are for example health and disease or benign versus malignant disease.

In each case, the ROC plot depicts the overlap between the two distributions by plotting the sensitivity versus 1 - specificity for the complete range of decision thresholds. On the y-axis is sensitivity, or the true-positive fraction [defined as (number of true-positive test results)/(number of true-positive + number of false-negative test results)]. This has also been referred to as positivity in the presence of a disease or condition. It is calculated solely from the affected subgroup. On the x-axis is the false-positive fraction, or 1 - specificity [defined as (number of false-positive results)/(number of true-negative + number of false-positive results)]. It is an index of specificity and is calculated entirely from the unaffected subgroup. Because the true- and false-positive fractions are calculated entirely separately, by using the test results from two different subgroups, the ROC plot is independent of the prevalence of disease in the sample. Each point on the ROC plot represents a sensitivity/1-specificity pair corresponding to a particular decision threshold. A test with perfect discrimination (no overlap in the two distributions of results) has an ROC plot that passes through the upper left corner, where the true-positive fraction is 1.0, or 100% (perfect sensitivity), and the false-positive fraction is 0 (perfect specificity). The theoretical plot for a test with no discrimination (identical distributions of results for the two groups) is a 45° diagonal line from the lower left corner to the upper right corner. Most plots fall in between these two extremes. (If the ROC plot falls completely below the 45° diagonal, this is easily remedied by reversing the criterion for "positivity" from "greater than" to "less than" or vice versa.) Qualitatively, the closer the plot is to the upper left corner, the higher the overall accuracy of the test.

One convenient goal to quantify the diagnostic accuracy of a laboratory test is to express its performance by a single number. Such an overall parameter e.g. is the so-called "total error" or alternatively the "area under the curve = AUC". The most common global measure is the area under the ROC plot. By convention, this area is always ≥ 0.5 (if it is not, one can reverse the decision rule to make it so). Values

range between 1.0 (perfect separation of the test values of the two groups) and 0.5 (no apparent distributional difference between the two groups of test values). The area does not depend only on a particular portion of the plot such as the point closest to the diagonal or the sensitivity at 90% specificity, but on the entire plot.

- 5 This is a quantitative, descriptive expression of how close the ROC plot is to the perfect one (area = 1.0).

Combining measurements of NNMT with other markers like CYFRA 21-1 or CEA, or with other markers of LC yet to be discovered, NNMT leads and will lead, respectively, to further improvements in assessment of LC.

- 10 The combination of the two markers NNMT and CYFRA 21-1 significantly improves the diagnostic accuracy for LC. The combination of the two markers NNMT and CEA also significantly improves the diagnostic accuracy for LC.

- 15 In a preferred embodiment the present invention relates to a method for improving the diagnostic accuracy for LC versus healthy controls by measuring in a sample the concentration of at least NNMT and CYFRA 21-1, CEA, NSE, or SCC, respectively and correlating the concentrations determined to the presence or absence of LC, the improvement resulting in more patients being correctly classified as suffering from LC versus healthy controls as compared to a classification based on any single marker investigated alone.

- 20 In a preferred method according to the present invention at least the concentration of the biomarkers NNMT and CYFRA 21-1, respectively, is determined and the marker combination is used in the assessment of LC.

- 25 In a further preferred method according to the present invention at least the concentration of the biomarkers NNMT and CEA, respectively, is determined and the marker combination is used in the assessment of LC.

In yet a further preferred method according to the present invention at least the concentration of the biomarkers NNMT, CYFRA 21-1, and CEA, respectively, is determined and the marker combination is used in the assessment of LC.

- 30 In yet a further preferred method according to the present invention at least the concentration of the biomarkers NNMT, CYFRA 21-1, and SCC, respectively, is determined and the marker combination is used in the assessment of LC.

The following examples are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

5 **Example 1**

Generation of antibodies to the lung cancer marker protein NNMT

Polyclonal antibody to the lung cancer marker protein NNMT was generated for further use of the antibody in the measurement of serum and plasma and blood levels of NNMT by immunodetection assays, e.g. Western Blotting and ELISA.

10 **Recombinant protein expression in E. coli**

In order to generate antibodies to NNMT, recombinant expression of the protein was performed for obtaining immunogens. The expression was done applying a combination of the RTS 100 expression system and expression in E.coli. In a first step, the DNA sequence was analyzed and recommendations for high yield cDNA
15 silent mutational variants and respective PCR-primer sequences were obtained using the "ProteoExpert RTS E.coli HY" system. This is a commercial web based service (www.proteoexpert.com). Using the recommended primer pairs, the "RTS 100 E. coli Linear Template Generation Set, His-tag" (Roche Diagnostics GmbH, Mannheim, Germany, Cat.No. 3186237) system to generate linear PCR templates
20 from the cDNA and for in-vitro transcription and expression of the nucleotide sequence coding for the NNMT protein was used. For Western-blot detection and later purification, the expressed protein contained a His-tag. The best expressing variant was identified. All steps from PCR to expression and detection were carried out according to the instructions of the manufacturer. The respective PCR product,
25 containing all necessary T7 regulatory regions (promoter, ribosomal binding site and T7 terminator) was cloned into the pBAD TOPO[®] vector (Invitrogen, Karlsruhe, Germany, Cat. No. K 4300/01) following the manufacturer's instructions. For expression using the T7 regulatory sequences, the construct was transformed into E. coli BL 21 (DE 3) (Studier, F.W. et al., Methods Enzymol. 185
30 (1990) 60-89) and the transformed bacteria were cultivated in a 1 l batch for protein expression.

Purification of His-NNMT fusion protein was done following standard procedures on a Ni-chelate column. Briefly, 1 l of bacteria culture containing the expression vector for the His-NNMT fusion protein was pelleted by centrifugation. The cell

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- pellet was resuspended in lysis buffer, containing phosphate, pH 8,0, 7 M guanidium chloride, imidazole and thioglycerole, followed by homogenization using a Ultra-Turrax®. Insoluble material was pelleted by high speed centrifugation and the supernatant was applied to a Ni-chelate chromatographic column. The column was washed with several bed volumes of lysis buffer followed by washes with buffer, containing phosphate, pH 8,0 and Urea. Finally, bound antigen was eluted using a phosphate buffer containing SDS under acid conditions.

Generation of polyclonal antibodies

a) Immunization

- For immunization, a fresh emulsion of the protein solution (100 µg/ml protein NNMT) and complete Freund's adjuvant at the ratio of 1:1 was prepared. Each rabbit was immunized with 1 ml of the emulsion at days 1, 7, 14 and 30, 60 and 90. Blood was drawn and resulting anti-NNMT serum used for further experiments as described in examples 3 and 4.

- b) Purification of IgG (immunoglobulin G) from rabbit serum by sequential precipitation with caprylic acid and ammonium sulfate

- One volume of rabbit serum was diluted with 4 volumes of acetate buffer (60 mM, pH 4.0). The pH was adjusted to 4.5 with 2 M Tris-base. Caprylic acid (25 µl/ml of diluted sample) was added drop-wise under vigorous stirring. After 30 min the sample was centrifuged (13 000 x g, 30 min, 4°C), the pellet discarded and the supernatant collected. The pH of the supernatant was adjusted to 7.5 by the addition of 2 M Tris-base and filtered (0.2 µm).

- The immunoglobulin in the supernatant was precipitated under vigorous stirring by the drop-wise addition of a 4 M ammonium sulfate solution to a final concentration of 2 M. The precipitated immunoglobulins were collected by centrifugation (8000 x g, 15 min, 4°C).

The supernatant was discarded. The pellet was dissolved in 10 mM NaH₂PO₄/NaOH, pH 7.5, 30 mM NaCl and exhaustively dialyzed. The dialysate was centrifuged (13 000 x g, 15 min, 4°C) and filtered (0.2 µm).

Biotinylation of polyclonal rabbit IgG

5 Polyclonal rabbit IgG was brought to 10 mg/ml in 10 mM NaH₂PO₄/NaOH, pH 7.5, 30 mM NaCl. Per ml IgG solution 50 µl Biotin -N-hydroxysuccinimide (3.6 mg/ml in DMSO) were added. After 30 min at room temperature, the sample was chromatographed on Superdex 200 (10 mM NaH₂PO₄/NaOH, pH 7.5, 30 mM NaCl). The fraction containing biotinylated IgG were collected. Monoclonal antibodies have been biotinylated according to the same procedure.

Digoxigenylation of polyclonal rabbit IgG

10 Polyclonal rabbit IgG was brought to 10 mg/ml in 10 mM NaH₂PO₄/NaOH, 30 mM NaCl, pH 7.5. Per ml IgG solution 50 µl digoxigenin-3-O-methylcarbonyl-ε-aminocaproic acid-N-hydroxysuccinimide ester (Roche Diagnostics, Mannheim, Germany, Cat. No. 1 333 054) (3.8 mg/ml in DMSO) were added. After 30 min at room temperature, the sample was chromatographed on Superdex® 200 (10 mM NaH₂PO₄/NaOH, pH 7.5, 30 mM NaCl). The fractions containing digoxigenylated
15 IgG were collected. Monoclonal antibodies have been labeled with digoxigenin according to the same procedure.

Example 2

ELISA for the measurement of NNMT in human serum and plasma samples.

20 For detection of NNMT in human serum or plasma, a sandwich ELISA was developed. For capture and detection of the antigen, aliquots of the anti-NNMT polyclonal antibody (see Example 2) were conjugated with biotin and digoxigenin, respectively.

25 Streptavidin-coated 96-well microtiter plates were incubated with 100 µl biotinylated anti-NNMT polyclonal antibody for 60 min at 10 µg/ml in 10 mM phosphate, pH 7.4, 1% BSA, 0.9% NaCl and 0.1% Tween 20. After incubation, plates were washed three times with 0.9% NaCl, 0.1% Tween 20. Wells were then incubated for 2 h with either a serial dilution of the recombinant protein (see Example 2) as standard antigen or with diluted plasma samples from patients. After binding of NNMT, plates were washed three times with 0.9% NaCl, 0.1% Tween
30 20. For specific detection of bound NNMT, wells were incubated with 100 µl of digoxigenylated anti-NNMT polyclonal antibody for 60 min at 10 µg/ml in 10 mM phosphate, pH 7.4, 1% BSA, 0.9% NaCl and 0.1% Tween 20. Thereafter, plates were washed three times to remove unbound antibody. In a next step, wells were

- incubated with 20 mU/ml anti-digoxigenin-POD conjugates (Roche Diagnostics GmbH, Mannheim, Germany, Catalog No. 1633716) for 60 min in 10 mM phosphate, pH 7.4, 1% BSA, 0.9% NaCl and 0.1% Tween 20. Plates were subsequently washed three times with the same buffer. For detection of antigen-antibody complexes, wells were incubated with 100 µl ABTS solution (Roche Diagnostics GmbH, Mannheim, Germany, Catalog No. 11685767) and OD was measured after 30-60 min at 405 nm with an ELISA reader.

Example 3

Study I: Study population

- 10 In a first study, samples derived from 56 well-characterized LC patients with the UICC classification given in Table 1 have been used. The focus of this cohort was on NSCLC (50 samples), since this form of LC has a better prognosis than the SC type, and thus its early detection is especially important.

Table 1

- 15 Study I: LC samples and corresponding UICC classification

Stage according to UICC	Number of samples
UICC I	7
UICC II	17
UICC III	14
UICC IV	14
without staging	4
total number of LC samples	56
thereof number of NSCLC samples	50
thereof number of SCLC samples	6

The LC samples of Table 1 have been evaluated in comparison to control samples obtained from 121 obviously healthy individuals without any known malignant lung disease (= control cohort).

Example 4**Study I: sensitivity of single markers**

Sensitivity for each marker has been calculated at a common specificity level of 95% for each individual marker tested. Table 2 gives the sensitivity in percent for each of

5 the most promising LC markers.

Table 2**Study I: Sensitivity of single markers**

marker	NNMT	CEA	NSE	CYFRA 21-1
sensitivity (%) at 95% specificity	50.0	42.9	12.5	69.6

As is readily obvious from Table 2, the marker NNMT has be found to have the

10 second highest sensitivity for LC of all the markers investigated, after CYFRA 21-1. The marker CEA appears to have a significantly lower sensitivity whereas NSE seems to perform rather poor.

Example 5**Study I: marker combinations**

15 An individual was classified as having LC if at least one of the markers of the respective combination exceeds a certain threshold. These cut-off values were defined such as to yield 95% specificity on the control cohort.

Table 3 presents the classification results of patients diagnosed with LC versus healthy controls.

Table 3

Study I: Classification results on the set of patients with LC versus healthy controls

No of Markers	Marker or marker panel	Cut-off	Sensitivity (%)
1	Cyfra 21-1	1.7 ng/ml	69.6
1	NNMT	884 pg/ml	50.0
1	CEA	5.5 ng/ml	42.9
1	NSE	17.7 ng/ml	12.5
2	Cyfra 21-1, CEA	1.9 ng/ml 5.8 ng/ml	73.0
2	Cyfra 21-1 NNMT	1.9 ng/ml 970 pg/ml	77.0
2	NNMT, CEA	1100 pg/ml 6 ng/ml	66.0
2	NNMT NSE	884 pg/ml 26 ng/ml	50.0
3	NNMT Cyfra 21-1 CEA	970 pg/ml 1.9 ng/ml 6 ng/ml	77.0

- 5 The combination of the markers NNMT and Cyfra 21-1 in this analysis yielded the highest sensitivity at a specificity level of 95%. Various other markers have been evaluated in combination with NNMT. As shown in Table 3, CEA in combination with NNMT also leads to a significant improvement in sensitivity.

Example 6

Study II: study population

- 10 A second study totally independent from the first one focused on the main types of NSCLC, adenocarcinoma and squamous cell carcinoma. Table 4a describes the type and stage distribution of the cancer cohort.

Table 4a**Study II: Type and staging of LC samples**

Type of Cancer	Number of samples	
	UICC I or II	UICC III or IV
Adenocarcinoma	12	18
Squamous cell carcinoma	12	18
total number of NSCLC samples	60	

The control cohort in this study was defined more specifically to contain samples from smokers and non-smokers as described in Table 4b. A lung function testing (spirometry, Miller, M.R., et al., Eur. Respir. J. 26 (2005) 319-338) was carried out with each individual. Samples were included in the control cohort only if the donor's result in the lung function test was within the normal range.

Table 4b**Study II: Composition of the control cohort**

Stage according to UICC	Number of samples
Smokers	30
Ex-smokers	6
Non-smokers	24

Example 7**Study II: sensitivity of single markers**

Sensitivity for each marker has been calculated as before based on the samples described in Table 4b. Sensitivity (in percent) is given at a common specificity level of 95% for each individual marker tested. Table 5 depicts the sensitivity for adenocarcinoma and squamous cell carcinoma, respectively, as well as the overall sensitivity of each of the markers.

Table 5**Study II: Sensitivity (%) of single markers at 95% specificity**

marker	NNMT	CEA	NSE	CYFRA 21-1
Adenocarcinoma	80	43	37	63
Squamous cell carcinoma	87	17	30	80
Lung cancer overall	83	30	33	72

- Clearly, in this case NNMT is superior to Cyfra 21-1 for two reasons: Firstly the overall sensitivity of NNMT is significantly better. Secondly and also very importantly, Cyfra 21-1 is indicating adenocarcinomas with less efficiency than squamous cell carcinomas, whereas NNMT performs similarly with both types of cancer.

Example 8**10 Study II: marker combinations**

Results with marker combinations in study II are shown in Table 6. The type of combination was the same as described above for study I.

Table 6

Study II: Performance of marker combinations on two major subtypes of lung cancer. Sensitivity given at 95% specificity

Marker or marker set #, respectively	Marker or marker panel	Cut-off	Sensitivity (%)		
			Adeno-carcinoma	Squamous cell carcinoma	Lung cancer overall
1	Cyfra 21-1	2.1 ng/ml	63	80	72
2	NNMT	438 pg/ml	80	87	83
3	CEA	5.6 ng/ml	43	17	30
4	NSE	14.6 ng/ml	37	30	33
5	Cyfra 21-1, CEA	2.1 ng/ml 9.2 ng/ml	80	80	80
6	Cyfra 21-1 NSE	2.1 ng/ml 17.3 ng/ml	70	80	75
7	Cyfra 21-1 NNMT	2.1 ng/ml 520 pg/ml	83	90	87
8	NNMT, CEA	438 pg/ml 9.2 ng/ml	93	87	90
9	NNMT NSE	438 pg/ml 17.3 ng/ml	83	90	87
10	NNMT Cyfra 21-1 CEA	520 pg/ml 2.1 ng/ml 9.2 ng/ml	97	90	93
11	NNMT Cyfra 21-1 NSE	520 pg/ml 2.1 ng/ml 17.3 ng/ml	87	90	88
12	NNMT CEA NSE	438 pg/ml 9.2 ng/ml 17.3 ng/m	93	90	92
13	Cyfra 21-1 CEA NSE	2.1 ng/ml 9.2 ng/ml 17.3 ng/m	83	80	82
14	NNMT Cyfra 21-1 CEA NSE	520 pg/ml 2.1 ng/ml 9.2 ng/ml 17.3 ng/m	97	90	93

Combining any two of the markers investigated leads to significant improvement in sensitivity of the marker combination as compared to the sensitivity obtained when using the markers individually (see marker sets #7 vs. #1 and #2 for illustration).

- 5 Some benefit is gained by adding a third marker (cf. sets #8 vs #10). The combination of all four markers (set #14), however, yields no higher performance than the best triple set (set #10).

Dual and triple combinations comprising NNMT are always better than the corresponding ones having NNMT replaced with any other marker.

- 10 In summary, NNMT alone demonstrates good sensitivity in detecting the two major types of NSC lung cancer. Very impressive results are obtained using a combination of NNMT with Cyfra 21-1 and/or CEA, respectively. Using a combination comprising all three markers 93% of cancer samples and 95% of controls are classified correctly.

Patent Claims

1. A method for assessing lung cancer in vitro comprising measuring in a sample the concentration of
 - a) nicotinamide N-methyl transferase (NNMT),
 - 5 b) optionally one or more other marker of lung cancer, and
 - c) using the concentration determined in step (a) and optionally step (b) in the assessment of lung cancer.
- 10 2. The method according to claim 1, wherein said one or more other marker is selected from the group consisting of CYFRA 21-1, CEA, NSE, and SCC.
3. The method according to claim 2, wherein said one or more other marker is CYFRA 21-1.
4. The method according to claim 2, wherein said one or more other marker is CEA.
- 15 5. The method according to claim 2, wherein said one or more other marker is SCC.
6. Use of NNMT in the assessment of lung cancer.
7. Use of a marker panel comprising NNMT and one or more other marker for lung cancer in the assessment of lung cancer.
- 20 8. Use of the marker panel according to claim 7, wherein the one or more other marker is selected from the group consisting of CYFRA 21-1, CEA, NSE, and SCC.
9. Use of a marker panel according to claim 8 comprising at least NNMT and CYFRA 21-1.

INTERNATIONAL SEARCH REPORT

International application No

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A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/574 C12Q1/48

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B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

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

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

EPO-Internal, WPI Data, EMBASE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2006/024692 A1 (NAKAMURA YUSUKE [JP] ET AL) 2 February 2006 (2006-02-02) claims 1-6,22 page 7, paragraph 66 table 1	1,2,5-8
Y	----- -/--	1-9

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

☒ See patent family annex.

* Special categories of cited documents:

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

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INTERNATIONAL SEARCH REPORT

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SCHNEIDER JOACHIM ET AL: "Fuzzy logic-based tumor-marker profiles improved sensitivity in the diagnosis of lung cancer." INTERNATIONAL JOURNAL OF CLINICAL ONCOLOGY / JAPAN SOCIETY OF CLINICAL ONCOLOGY. JUN 2002, vol. 7, no. 3, June 2002 (2002-06), pages 145-151, XP002403876 ISSN: 1341-9625 abstract page 145, right-hand column, paragraph 1 page 149, left-hand column, paragraph 2 - page 151, left-hand column, paragraph 2 figures 4,5 -----	1-9
Y	KELLER T ET AL: "Tumour markers in the diagnosis of bronchial carcinoma: new options using fuzzy logic-based tumour marker profiles" JOURNAL OF CANCER RESEARCH AND CLINICAL ONCOLOGY, SPRINGER INTERNATIONAL, BERLIN, DE, vol. 124, no. 10, 1998, pages 565-574, XP002307078 ISSN: 0171-5216 abstract page 566, right-hand column, paragraph 2 page 569, right-hand column, paragraph 2 - paragraph 4 table 6 figure 2 page 572, right-hand column, paragraph 2 - page 573, left-hand column, paragraph 3 -----	1-9

Information on patent family members

PCT/EP2007/006713

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权利要求书 1 页 说明书 24 页

[54] 发明名称

作为肺癌标记物的 NNMT 的应用

[57] 摘要

本发明涉及肺癌的评价。其公开了蛋白质 NNMT 在肺癌评价中的应用。本发明也涉及用于通过体外检测源自患者的液体样品中的 NNMT 评价肺癌的方法。NNMT 的检测可,如被用于肺癌患者的早期检测或随访。

1. 一种用于体外评价肺癌的方法，该方法包括检测样品中的
 - a) 烟酰胺 N-甲基转移酶(NNMT)的浓度，
 - b) 任选一种或多种其它肺癌标记物的浓度，和
 - c) 将在步骤(a)和任选的步骤(b)中测定的浓度用在肺癌的评价中。
2. 依据权利要求 1 的方法，其中所述一种或多种其它标记物选自 CYFRA 21-1、CEA、NSE 和 SCC。
3. 依据权利要求 2 的方法，其中所述一种或多种其它标记物是 CYFRA 21-1。
4. 依据权利要求 2 的方法，其中所述一种或多种其它标记物是 CEA。
5. 依据权利要求 2 的方法，其中所述一种或多种其它标记物是 SCC。
6. NNMT 在肺癌的评价中的应用。
7. 包含 NNMT 和一种或多种适于肺癌的其它标记物的标记板在肺癌的评价中的应用。
8. 依据权利要求 7 的标记板的应用，其中所述一种或多种其它标记物选自 CYFRA 21-1、CEA、NSE 和 SCC。
9. 依据权利要求 8 的标记板的应用，所述标记板至少包含 NNMT 和 CYFRA 21-1。

作为肺癌标记物的 NNMT 的应用

本发明涉及与肺有关的癌症或肺癌(=LC)的评价并且尤其是非小细胞肺癌(NSCLC)的评价的辅助方法。本文公开了作为LC,尤其是NSCLC的标记物的蛋白质烟酰胺N-甲基转移酶(=NNMT)的用途。再有,本文特别涉及通过检测源自患者的液体样品中的NNMT评价肺癌的方法。NNMT的检测可例如用于肺癌的早期探测或用于经受手术的患者监测。

尽管在探测和治疗方面取得进展,癌症仍然是主要的公共卫生挑战性问题。在多种类型的癌症中,LC是西方世界常常发生的癌症并且是癌症相关死亡率的最常死因一。这大部分是由于用于疾病的早期探测的诊断空白(diagnostic gap)。LC在其早期很大程度上是无症状的。所有肺癌中的大多数在晚期被探测到,此时该疾病已经变得不宜手术治愈。

大多数LC肿瘤可分为小细胞肺癌(SCLC)和非小细胞肺癌(NSCLC)。SCLC占有肺癌病例的约20-25%。SCLC是具侵袭性的神经内分泌型LC,并且即使被早期探测到预后也很差。SCLC是罕见的易于通过切除术根治的疾病。由于该疾病的发展迅速,通常仅用两期,即局限型(limited disease)和扩散型疾病(extensive disease)对SCLC进行分类,而不是更复杂的TNM分期系统(见下文)。约75-80%的LC病例被类分为包括鳞状细胞癌(癌=CA)、腺-CA(包括腺泡CA、乳头状CA、支气管肿瘤、实体瘤和混合的亚型的亚类)和大细胞癌(包括巨大细胞肿瘤、清亮细胞CA、腺鳞状CA和未分化CA的亚类)的NSCLC类别中。

如果在晚期探测到NSCLC,预后也很差。癌症的分期是根据扩散、侵袭性和严重性对该疾病的分类。将癌症患者分组以便对预后和

疗法的选择进行概括。

现今, TNM 系统是最广泛应用的基于癌症的解剖学范畴的分类系统。其代表国际上可接受的、统一的分期系统。有三个基本变化: T (原发性肿瘤的范围)、N (局部淋巴结的状况)和 M (存在或缺乏远程转移)。TNM 标准由 UICC (国际抗癌联合会(International Union Against Cancer))在: 恶性肿瘤的 TNM 分类(TNM Classification of Malignant Tumours), 第五版, Sobin, L.H.和 Wittekind, Ch. (编辑), Wiley-Liss (1997), 第 1803-1804 页)上公布。

原发性肿瘤的外科切除术是广泛可接受的用于早期 NSCLC 的治疗选择。随着 NSCLC 的进展, 特别是从 IIIa 期(T3N1M0、T1N2M0、T2N2M0、T3N2M0)转变至 IIIb 期(T4N0M0、T4N1M0、T4N2M0), 内科医师的重要的应急办法显著下降。然而, 如果在更早期(Ia - IIIa; 优选最早至 T3N1M0 期)探测癌症, 则五年生存率在 35%和 80%之间不等。在 Ia 期((T1N0M0); 肿瘤尺寸小, 未转移)探测, 明显具有五年生存率最高至 80%的最好预后。

即使有的话, 也几乎很少使用外科干预 NSCLC 的 IIIb-IV 期。IV 期相应于远程转移, 即疾病的蔓延远离肺和局部淋巴结。较晚期(III - IV)的五年生存率降至小于 1%和 15%之间。

特别重要的是, NSCLC 的早期诊断使预后转变得更好。早在 Ia (T1N0M0)、Ib (T2N0M0)、IIa (T1N1M0)、IIb, (T3N0M0)和 IIIa (T3N1M0)期诊断的患者, 如果治疗得当, 诊断后具有最高至 80%的 5 年生存机会。对于被诊断的患者而言, 一旦已经存在远程转移, 则不得不与小于 1%的 5 年生存率作比较。

在本发明看来, LC 的早期评价指在肿瘤 T1N0M0 期或 T1-3N0-1M0 期进行评价。

优选的是, 在 T1-3N0-1M0 (=T1-3N0-1M0)期评价 LC。

大多数肺癌在它们变得有症状时被探测到。现行的探测方法包括胸部 x-光透视、螺旋计算机 X 线断层摄影术、痰液细胞学检查和支气

管镜检。然而,有关将这些方法用于群体筛查的适宜性存在争论。

许多用于肺癌的血清肿瘤标记物在临床应用。细胞角蛋白(Cytokeratin) 19 (CYFRA 21-1)的可溶性的 30 kDa 片段、癌胚胎发生抗原(CEA)、神经元特异性烯醇化酶(NSE)和鳞状细胞癌抗原(SCC)是最突出的 LC 标记物。然而,它们中没有一个满足筛查工具所需要的灵敏度和特异性的标准(Thomas, L., Labor und Diagnose, TH Books Verlagsgesellschaft, Frankfurt/Main, Germany (2000))。

为了具有临床用途,作为单一标记物的新的诊断标记物应该与本领域已知的其它标记物具有可比性或更好。或者,即使单独使用或者与一种或多种其它标记物组合,新的标记物分别应该导致诊断的灵敏度和/或特异性有所提高。试验的诊断灵敏度和/或特异性最好由将在以下详细描述的其他受试者工作特征评价。

全血、血清或血浆是临床常规中最广泛使用的样品源。在可靠的癌症探测或提供早期预测信息中应该有所帮助的早期 LC 肿瘤标记物的鉴别,可导致产生在该疾病的诊断和处理中应该大有帮助的方法。因此,临床上存在急需的是改善 LC 的体外评价。特别重要的是改进 LC 的早期诊断,因为对于早期诊断的患者而言,其对于生存机会的要求比那些在疾病的进展阶段诊断的患者更高。

生化标记物在肺癌中的临床应用最近已有评述(Duffy, M.J., Crit. Rev. Clin. Lab. Sci. 38 (2001) 225-262)。

CYFRA 21-1 普遍被认为是目前已知最好的用于肺癌的肿瘤标记物。尽管不具器官特异性,其主要在肺组织中被发现。在对于其它良性肺部疾病的特异性为 95%时, CYFRA 21-1 对于肺癌的灵敏度经描述为 46-61%。升高的 CYFRA 21-1 的血清水平也与显著的良性肝病、肾功能不全和侵袭性膀胱癌相关。CYFRA 21-1 试验被推荐用于术后治疗监测。

CEA 属于通常在胚胎发生过程中产生的癌胚抗原的组别。CEA 不具器官特异性,其主要用于结肠直肠癌的监测。除了恶性肿瘤外,

几种良性疾病, 诸如硬化、支气管炎、胰腺炎和自身免疫疾病也与升高的 CEA 血清水平相关。在对于良性肺部疾病的特异性为 95% 时, 其对于肺癌的灵敏度报告为 29-44%。CEA 的优选的应用是肺癌的治疗监测。

NSE 是用于 SCLC 的肿瘤标记物。一般地, 升高的 NSE 血清水平被发现与神经外胚层和神经内分泌肿瘤相关。升高的血清水平也在良性肺部疾病和脑部疾病, 诸如脑膜炎或脑部其它炎症性疾病以及头部创伤的患者中发现。当对于 SCLC 的灵敏度为 95% 时, 报告的特异性是 60-87%, 对于 NSCLC, NSE 试验的表现差(7-25%)。NSE 被推荐用于 SCLC 的治疗监测。

最初在子宫颈鳞状细胞 CA 中鉴定 SCC。对于 LC 而言 SCC 的灵敏度通常低(18-27%)。因此, SCC 试验被认为不适宜筛查。然而, 由于对于鳞状细胞 CA 有比较高的灵敏度, SCC 的优选应用是治疗监测, 尽管 CYFRA 21-1 通常表现更好。

至于标记物的分布和针对改善的肺癌诊断, 公开了一种方法 (Schneider, J.等, Int. J. Clin. Oncol. 7 (2002) 145-151), 该方法采用基于模糊逻辑学的分类运算法则, 将 CYFRA 21-1、NSE 和为一般的炎症标记物的 C-反应性蛋白质(CRP)的血清水平合并。作者报告特异性为 95% 时的灵敏度为 92%。

本发明的任务是研究生化标记物是否可被鉴别, 可被用于评价 LC。

令人惊奇的是, 已经发现, 使用标记物 NNMT 可至少部分地克服目前现有技术已知的标记物的某些问题。

本发明涉及用于体外评价肺癌的方法, 该方法包括检测样品中的 NNMT 浓度, 并且将该测定的浓度用在肺癌评价中。令人惊奇的是, 可以确定的是, 升高的样品中的 NNMT 水平, 例如预示在患者中存在肺癌。

本发明也涉及通过生化标记物体外评价 LC 的方法, 该方法包括

检测样品中的 NNMT 和 LC 的一种或多种其它标记物的浓度,并且将该测定的浓度用在 LC 评价中。优选的是,LC 的一种或多种其它标记物选自 CYFRA 21-1、CEA、NSE 和 SCC。

在优选的实施方案中,本发明也涉及将包含至少 NNMT 和 CYFRA 21-1 的标记板(marker panel)用在 LC 的评价中。

本发明也涉及将包含至少 NNMT 和 CEA 的标记板用在 LC 的评价中。

本发明也涉及将包含至少 NNMT 和 SCC 的标记板用在 LC 的评价中。

在优选的实施方案中,本发明涉及用于体外评价肺癌的方法,该方法包括检测样品中的 a) NNMT 的浓度, b) 任选一种或多种其它肺癌标记物的浓度, 和 c) 将步骤(a)和任选的步骤(b)中测定的浓度用于肺癌的评价中。

NNMT (烟酰胺 N-甲基转移酶, EC 2.1.1.1)催化烟酰胺和其它吡啶的 N-甲基化。蛋白质 NNMT (Swiss-PROT: P40261)的特征是在已给出的序列表中为 SEQ ID NO:1。NNMT 的表观分子量为 29.6 kDa 和等电点为 5.56。

NNMT 活性对于许多药物和异种生物化合物的生物转化是重要的。已有报道,该蛋白质主要在肝中表达并且定位于细胞质中。NNMT 自人肝的 cDNA 克隆并且含编码 264-个氨基酸蛋白质的 792-个核苷开放读码框,其经计算的分子量为 29.6 kDa (Aksoy, S.等, J. Biol. Chem. 269 (1994) 14835-14840)。文献中有关该酶在人癌症中的潜在作用知之甚少。在一篇论文中,据报道,升高的肝 NNMT 酶活性作为小鼠中癌症恶病质的标记物(Okamura, A.等, Jpn. J. Cancer Res. 89 (1998) 649-656)。在近期的报道中, NNMT 基因响应射线的向下调节作用在在射线敏感细胞系中得到证实(Kassem, H.,等, Int. J. Cancer 101 (2002) 454-460)。在 US 2006/0024692 中,发现 NNMT m-RNA-水平在非小细胞肺癌细胞中比在非-癌症组织中低。WO 02/082076 描述了 NNMT

蛋白质作为肾癌亚型的标记物。

如在本文中使用的，以下每一个术语均有与其所在下文段节相关联的含义。

本文中使用的冠词“一”和“一个”指一个或多于一个(即指至少一个)的文章的语法上的宾语。例如，“标记物”指一个标记物或多于一个标记物。

如在本文中使用的，术语“标记物”或“生化标记物”指将要在患者样品的分析中用作靶标的分子。这样的分子靶标的实例为蛋白质或多肽本身以及针对如存在于样品中的此类靶标的抗体。表达“一个或多个”指1至20个，优选1至10个，优选1至5个，更优选3或4个。

在本发明中用作标记物的蛋白质或多肽期望包括所述蛋白质的天然存在的变量以及所述蛋白质或所述变量的天然存在的片段或复合物，尤其分别是可免疫探测的片段或复合物。

如对于专业技术人员而言明白的是，本发明应该不被解释为限制SEQ ID NO: 1的全长蛋白质NNMT的检测。在实施本发明时，NNMT的生理片段可也被检测到并且用作肺癌的标记物。可免疫探测的片段优选包含至少6、7、8、10、12、15或20个所述标记物多肽的邻近的氨基酸。本领域专业技术人员应该认识到，由细胞释放的或存在于细胞基质中的蛋白质可例如在炎症期间被破坏，并且可变得被降解或裂解为这样的片段。如专业技术人员应该认识的，NNMT或其片段也可作为复合物的部分存在。这样的复合物也可用作本发明意义上的标记物。另外，或作为选择，NNMT多肽可带有翻译后修饰，优选糖基化、酰基化和/或磷酸化作用，并且这样的经过修饰的NNMT也可作为LC的标记物。

术语“评价肺癌”用于指明，依据本发明的方法将(单独或与其它标记物或变量，如由UICC设立的标准(见上面)一起)如，辅助医师建立或确定LC的缺乏或存在或在预后中辅助医师，探测康复(手术后的患者随访)和/或治疗监测，尤其是化疗监测。

如在本文中使用的术语“样品”，指用于体外评价的目的而获得的生物样品。在本发明的方法中，样品或患者样品优选可包括任何体液。优选样品分别为全血、血清、血浆、支气管灌洗液或痰液，而血浆或血清最优选。

令人惊奇的是，本发明的发明人已经能够在源于 LC 的患者的具显著意义百分比的样品中探测标记物 NNMT。甚至更令人惊奇的是，他们已经能够证实，在得自患者的这样的液体样品中 NNMT 的存在可用于肺癌评价中。

在优选的实施方案中，本发明涉及通过检测样品的 NNMT 浓度评价 LC 和使用测定的浓度评价 LC 的方法。

如专业的技术人员认识到的，任何这样的检测都在体外进行。过后丢弃患者样品。患者样品单独用于本发明的体外诊断方法并且患者样品的材料并不转移进入患者身体中去。作为特色地，样品为液体样品，如全血、血清或血浆。

如专业的技术人员从描述的实施例认识到的，通过在免疫分析中检测已经鉴别 NNMT 为用于 LC 的诊断的标记物，可使用备选的途径实现与本发明的成就相比拟的结果。可通过任何适当的方法探测标记物蛋白质 NNMT 并将其用作 LC 的标记物。这样的优选的适当方法包括通过免疫分析程序探测 NNMT、通过任何其它形式的结合试验、通过检测其酶的活性、通过液相色谱，尤其是高效液相色谱、通过电泳，尤其是与蛋白质印迹结合的 SDS-PAGE 以及通过质谱进行。

用于诊断的理想方案应该是其中单个事件或过程应该引起各自的疾病的情形，如在感染性疾病中。在所有的其它病例中，正确的诊断可以是非常困难的，特别是当疾病的病因学没有完全被了解为 LC 的病例时。如专业的技术人员认识到的，没有生化标记物，例如在 LC 领域，对于给定的疾病被 100% 地特异性同时具 100% 的灵敏性地诊断。更合适地，生化标记物如，CYFRA 21-1、CEA、NSE、SCC 或如在本文所显示的 NNMT 可用于用某些可能的和预兆性的值评价疾病的存

在或缺乏。因此，在常规临床诊断中，通常多种临床症状和生物标记物在潜在的疾病的诊断、治疗和处理中被一同考虑。

生化标记物可或者被个别测定，或者在本发明的优选的实施方案中，它们可同时采用基于阵列技术的芯片或磁珠检测。然后或者使用对于每一标记物的个别的临界值(cut-off)对生物标记物的浓度进行独立解释，或者将它们合并用于解释。

在进一步的优选的实施方案中，在以下方法中实施依据本发明的LC的评价，所述方法包括检测样品中的 a) NNMT 的浓度，b) 一种或多种其它肺癌标记物的浓度和 c) 将在步骤(a)和步骤(b)中测定的浓度用于肺癌评价中。如为专业技术人员明白的，可备选地自等份样品或自不同等份的样品实施 NNMT 的检测和一种或多种其它肺癌标记物的检测。

依据在实施例部分显示的数据，在实施的单因素分析和在多因素分析中的标记物 NNMT，对于 LC 均具有(特异性为约 95%)显著的差不多为 50%的灵敏度，并且在这一方面发现是可比较的甚或在比较时高于所研究的其它 LC 标记物。在 LC 的评价中，标记物 NNMT 在以下一个或多个方面将是有优势的：筛查；诊断辅助；预后；治疗监测，诸如化疗、放疗法和免疫疗法的监测。

筛查：

筛查被定义为测试在没有由于 LC 的症状而就诊的人中的系统性应用以确定有罹患 LC 的足够的风险的个体，从进一步研究或直接的预防行动中得到益处。

如在实施例部分所给出的数据证实，NNMT 单独将不足以例如对 LC 的风险人群的一般性筛查。最可能的是，在循环中没有单一的生化标记物将在任何时候符合为筛查的目的所需的灵敏度和特异性标准。宁愿不得不期望，在 LC 筛查中将不得不使用标记板。本发明中确定的数据指出，标记物 NNMT 将形成适宜筛查目的的标记板的整体部分。因此，本发明涉及作为用于 LC 筛查目的的 LC 标记板的一个

标记物的 NNMT 的应用。NNMT 的升高的水平指示 LC 的存在。本文数据还指出,标记物的某些组合在 LC 的筛查中将是有优势的。因此,本发明还涉及包含 NNMT 和 CYFRA 21-1 的标记板,或包含 NNMT 和 CEA 的标记板,或包含 NNMT 和 NSE 的标记板,或包含 NNMT 和 SCC 的标记板,或包含 NNMT 和两个选自 CYFRA 21-1、CEA、NSE 和 SCC 的标记物的标记板于 LC 筛查目的中的应用。

诊断辅助:

标记物可或者辅助特定器官的良性与恶性疾病的鉴别诊断,或者帮助区别肿瘤的不同组织类型。如由 Molina, R.等, Tumor Biol. 24 (2003) 209-218 报告的, CEA、CA 125、CYFRA 21-1、SSC 和 NSE 被用作血清标记物以辅助 LC 的组织学诊断。该研究的结果还指出,在所测试的标记物中, CYFRA 21-1 是肺癌中最敏感的标记物,但与组织学无关。

依据本发明的资料,由于作为单一标记物的 NNMT 可优于其它 LC 标记物如 CEA 或 NSE,必须期望 NNMT 将被用作诊断辅助,特别在通过建立手术前的基线值中。因而,本发明也涉及使用 NNMT 建立 LC 手术前的基线值。

预后:

预后指标可被定义为癌症患者及其肿瘤的预测疾病转归的临床、病理学或生化特征。它们的主要应用是帮助合理计划患者干预,即避免对具发展性疾病的的治疗不足和对惰性不再发展的疾病的过度治疗。Molina R.等, Tumor Biol. (2003) 24:209-218 评价了 CEA、CA 125、CYFRA 21-1、SSC 和 NSE 在 NSCLC 中的预后值。如评价了患者的全部生存状况,籍此标记物 NSE、CEA 和 LDH (乳酸脱氢酶)的异常的血清水平似乎表示生存期更短。

由于 NNMT 单用对区分 LC 患者与健康对照者已有显著贡献,必须期望它将在评价罹患 LC 的患者的预后中起辅助作用。手术前的 NNMT 水平最有可能与 LC 的一种或多种其它标记物和/或 TNM 分期

系统联合使用。在优选的实施方案中，NNMT 被用于 LC 患者的预后。

化疗监测:

Merle, P.等, Int. J. of Biological Markers 19 (2004) 310-315 已经评价用诱导性化疗治疗的局部晚期 NSCLC 患者中的 CYFRA 21-1 血清水平变化。他们推断, CYFRA 21-1 血清水平的早期监测可以是用于 III 期 NSCLC 患者中的肿瘤应答和生存的有用的预测工具。另外, 报告描述了 CEA 在 LC 患者的治疗监测中的应用(Fukasawa, T.等, Gan to Kagaku Ryoho. Cancer & Chemotherapy 13 (1986) 1862-1867 (用日文写的); Zhang, H.等, Shandong Yike Daxue Xuebao 39 (2001) 537-538, 541 (用中文写的))。这些中的大多数是回顾性的、非随机的和包括少数的患者。如在用 CYFRA 21-1 研究的病例中那样, CEA 研究提示: a) 具有 CEA 水平降低的患者, 当其在接受化疗时, 与那些 CEA 水平未能下降的患者相比, 通常具有比较好的转归。和(b) 对于几乎所有患者而言, CEA 水平的升高与疾病的进展相关。

由于在实施例部分所显示的资料, 必须期望 NNMT 应该至少为与 CYFRA 21-1 或 CEA 同样好的用于化疗监测的标记物。因此本发明也涉及 NNMT 在监测进行化疗的 LC 患者中的应用。

随访:

大部分的经历外科切除术的 LC 患者, 其目标是完全去除癌症组织、后期发展的复发或转移性疾病(Wagner, H., Chest 117 (2000) 110-116; Buccheri, G.等, Ann. Thorac. Surg. 75 (2003) 973-980)。这些复发的大多数发生在手术后的最初 2-3 年内。因为复发/转移性疾病总是致命的, 相当多的研究集中在早期并且由此潜在可治疗阶段的 LC 鉴别。

因此, 许多 LC 患者经历手术后监测程序, 其常常包括用 CEA 的常规监测。外科切除术后使用 CEA 的连续监测一年已经显示, 用灵敏度约 29%的方法探测早期手术后的复发/转移性疾病, 其特异性为约 97%, 即使是在缺乏可疑的症状或体征的情况下(Buccheri, G.等, Ann.

Thorac. Surg. 75 (2003) 973-980)。因而，手术后对 LC 患者的随访是适宜的生化标记物应用中的一个最重要的领域。由于 NNMT 在被研究的 LC 患者中的高灵敏度，期望 NNMT 单独或与一种或多种其它标记物联合将在随访 LC 患者，尤其是手术后的 LC 患者中大有帮助。包含 NNMT 和 LC 的一种或多种其它标记物的标记板在随访 LC 患者中的应用代表本发明更优选的实施方案。

在优选的实施方案中本发明涉及 NNMT 分别在 LC 的诊断领域或在 LC 的评价中的应用。

还在更优选的实施方案中，本发明涉及 NNMT 作为用于肺癌的标记物分子在与用于肺癌的一种或多种标记物分子联合在从得自患者液体样品中评价肺癌中的应用。

因而，本发明优选的实施方案是 NNMT 作为用于肺癌的标记物分子在与用于肺癌的一种或多种标记物分子联合在从得自患者液体样品中评价肺癌中的应用。优选可与 NNMT 的检测组合的经选择的其它 LC 标记物是 CYFRA 21-1、CEA、NSE 和 SCC。仍然更优选的是，用于 LC 的评价的标记板包含 NNMT 和至少一种选自 CYFRA21-1 和 CEA 的其它标记物分子。

如专业技术人员将会认识到的，有许多方法应用两个或更多个标记物的检测，以改善研究中的诊断问题。在十分简单，但虽然如此经常是有效的手段中，如果样品对于至少一个被研究的标记物是阳性的，则假定阳性结果。这可能是例如当诊断感染性疾病如 AIDS 时的情形。

然而，经常地，评价标记物的组合。优选地，对于标记板的标记物，如，对于 CYFRA 和 CEA 的检测值是算术上合并，并且该合并值与潜在的诊断问题相关。可通过本领域数学方法的任何适宜的规定合并标记物值。用于将标记物组合与疾病关联的熟悉的数学方法使用方法，如判别分析(DA) (即线性-、二次方程式-、调整的-DA)、Kernel 方法(即 SVM)、非参数的方法(即 k-最近邻域分类器

(k-Nearest-Neighbor Classifiers)), PLS (偏最小二乘(Partial Least Squares)), 基于树状(tree-Based)的方法(即逻辑回归(Logic Regression)、CART、随机 Forest 方法、Boosting/Bagging 方法)、广义线性模型(Generalized Linear Models) (即 Logistic 回归)、基于主要成分的(Principal Components based)方法(即 SIMCA)、广义相加模型(Generalized Additive Models)、基于模糊逻辑的(Fuzzy Logic based)方法、基于神经网络和遗传算法的(Neural Networks and Genetic Algorithms based)方法。专业技术人员在选择适宜的评价本发明标记物组合的方法时将不存在问题。优选地, 使本发明的标记物组合, 如, 与缺乏或存在 LC 相关的所使用的方法选自 DA (即线性-、二次方程式-、调整的判别分析)、Kernel 方法(即 SVM)、非参数的方法(即 k-最近邻域分类器)、PLS (偏最小二乘)、基于树状的方法(即逻辑回归、CART、随机 Forest 方法、助推法(Boosting)方法)或广义线性模型(即逻辑回归)。有关这些统计方法的详细资料在以下参考资料中发现: Ruczinski, I.等, 计算和绘图统计学杂志(J. of Computational and Graphical Statistics) 12 (2003) 475-511; Friedman, J. H., 美国统计学学会杂志(J. of the American Statistical Association) 84 (1989) 165-175; Hastie, T.等, 统计学知识原理(The Elements of Statistical Learning), Springer 统计学系列丛书(Springer Series in Statistics) (2001); Breiman, L.等, 分类和回归树(Classification and Regression Trees), Wadsworth 国际集团, 贝尔蒙(Belmont), 加利福尼亚(1984); Breiman, L., 随机 Forests, 机械学习(Random Forests, Machine Learning), 45 (2001) 5-32; Pepe, M.S., 用于分类和预测的医学试验的统计评估(The Statistical Evaluation of Medical Tests for Classification and Prediction), 牛津统计学系列丛书(Oxford Statistical Science Series), 28 (2003); 和 Duda, R.O.等, 模式分类(Pattern Classification), Wiley Interscience, 第 2 版 (2001)。

本发明的优选的实施方案是, 使用对于潜在的生物标记物组合的

最优化多变量的临界值并区别状态 A 与状态 B, 如, 区别疾病与健康。在该类分析中, 标记物不再独立存在而形成标记板。可确定的是, 将 NNMT 的检测分别与 CYFRA 21-1 或 CEA 的检测联合, 与健康对照者比较, 显著改善对于 LC 的诊断的准确性。

诊断方法的准确性被其受试者工作特征(ROC)最优描述(特别参见 Zweig, M.H. 和 Campbell, G., Clin. Chem. 39 (1993) 561-577)。ROC 图是所有灵敏度/特异性对的绘图, 所述灵敏度/特异性对是由对所观察数据的整个范围连续地改变其决定阈值所得到的。

实验室试验的临床表现取决于其诊断的准确性或将患者准确地类分为临床有关的亚组的能力。诊断的准确性检测试验的准确地区别被研究的患者的两种不同病症的能力。这样的情况是例如健康和疾病或良性对恶性疾病。

在各种情况下, ROC 图通过以灵敏度对 1-特异性作图描述两个分布之间的交迭, 用于决定阈的全部范围。在 y-轴上是灵敏度或真阳性部分[被定义为(真阳性试验结果的数量)/(真阳性的数量+假阴性试验结果的数量)]。此也被称为疾病或病症存在下的阳性。从受侵袭的亚组单独计算。在 x-轴上是假阳性部分或 1-特异性[被定义为(假阳性结果的数量)/(真阴性的数量+假阳性结果的数量)]。其为特异性的指数并且完全从未受侵袭亚组计算。因为真阳性和假阳性部分完全分开计算, 通过使用来自两个不同亚组的试验结果, ROC 图独立于样本中的疾病的发病率。ROC 图上的每一点表示相应于具体决定阈的灵敏度/1-特异性对。具有完美区别的试验(在两个结果的分布中没有交迭)具有遍及左上角的 ROC 图, 在那里真阳性部分是 1.0 或 100% (准确的灵敏度)和假阳性部分是 0 (准确的特异性)。没有区别的试验的理论图(两组有同样的结果分布)是从左下角至右上角的 45°斜线。大多数图落在此两极之间。(如果 ROC 图完全落在 45°斜线之下, 易于矫正, 通过将用于“阳性”的标准倒转, 从“大于”至“小于”或反之亦然)。在质量上, 图越靠近左上角, 试验的整体精确度越高。

定量实验室试验的诊断准确性的一个便利目标是，用单个数字表达其表现。这样的完全参数，如，是所谓的“总误差”或作为选择地“曲线下面积=AUC”。最常见的全面测量是 ROC 图下的面积。按惯例，该面积总是 ≥ 0.5 (如果不是，任何人可倒转此决定规则以使其符合)。值的范围在 1.0 (两个组的试验值全然分开)和 0.5 (两组试验值之间没有明显的分布差异)之间。面积不仅取决于图的具体部分，诸如最靠近斜线的点或在 90%特异性时的灵敏度，但在全图上。此为定量的、描述性的表达，即该 ROC 图是最接近完美的一个(面积=1.0)。

将 NNMT 的检测与其它标记物如 CYFRA 21-1 或 CEA 的检测联合，或与至今仍待发现的 LC 的其它标记物的检测联合，NNMT 导致并且将会导致对 LC 的评价的进一步改善。

两个标记物 NNMT 和 CYFRA 21-1 的组合显著性地改善对 LC 的诊断的准确性。两个标记物 NNMT 和 CEA 的组合也显著性地改善对 LC 的诊断的准确性。

在优选的实施方案中，本发明涉及用于改善对 LC 相对于健康对照者的诊断准确性的方法，该方法通过检测样品中至少 NNMT 分别和 CYFRA 21-1、CEA、NSE 或 SCC 的浓度，和使测定的浓度与 LC 的存在或缺乏相关，与基于被单独研究的任何单个标记物的分类比较，该改善导致更多的患者被正确地类分为罹患 LC 与健康对照者。

在依据本发明的优选的方法中，分别测定至少生物标记物 NNMT 和 CYFRA 21-1 的浓度，以及该标记物组合用于 LC 的评价。

在依据本发明的更优选的方法中，分别测定至少生物标记物 NNMT 和 CEA 的浓度，以及该标记物组合用于 LC 的评价。

在依据本发明的又一个更优选的方法中，分别测定至少生物标记物 NNMT、CYFRA 21-1 和 CEA 的浓度，以及该标记物组合用于 LC 的评价。

在依据本发明的又一个更优选的方法中，分别测定至少生物标记物 NNMT、CYFRA 21-1 和 SCC 的浓度，以及该标记物组合用于 LC

的评价。

提供以下实施例以帮助理解本发明，在随附的权利要求书中列出其真实的范畴。应该理解，可在不背离本发明的精神下对列出的方法进行修饰。

实施例 1

对肺癌标记物蛋白质 NNMT 的抗体的产生

为了通过免疫探测分析，如蛋白质印迹和 ELISA，进一步将抗体应用在血清和血浆以及血液水平的 NNMT 的检测中，产生对肺癌标记物蛋白质 NNMT 的多克隆抗体。

在大肠杆菌(E. coli)中表达重组蛋白质

为了产生对 NNMT 的抗体，实施蛋白质的重组表达获得免疫原。应用 RTS 100 表达系统和在 E. coli 中表达的组合进行表达。在第一步中，使用“ProteoExpert RTS E. coli HY”系统分析 DNA 序列和得到推荐高产率的 cDNA 沉默突变变量以及各自的 PCR-引物序列。此为基于网络的商业服务(www.proteoexpert.com)。使用推荐的引物对，“RTS 100 E. coli 线性模板发生装置(Linear Template Generation Set, His-tag)”(Roche Diagnostics GmbH, 曼海姆, 德国, 目录号 3186237)系统，自 cDNA 产生线性 PCR 模板并且为了核苷的体外转录和表达，使用编码 NNMT 蛋白质的序列。对于蛋白质印迹(Western blot)探测和随后的纯化，表达的蛋白质含有 His-标记物(His-tag)。确定最佳表达变量。依据制造商的说明书实施从 PCR 至表达和探测的所有步骤。依据制造商的说明书，将各自的包含所有必需的 T7 调节区域的 PCR 产物(启动子、核糖体的结合位点和 T7 终止子)克隆进 pBAD TOPO[®] 载体(Invitrogen, 卡尔斯鲁厄, 德国, 目录号 K 4300/01)。为了表达，使用 T7 调节序列，将构造物转化为 E. coli BL 21 (DE 3) (Studier, F.W. 等, Methods Enzymol. 185 (1990) 60-89 并将转化的细菌在 11 批次中培养用于蛋白质表达。

按照标准程序在镍-螯合物柱上纯化 His-NNMT 融合蛋白。简要

地说, 经离心使含有表达用于 His-NNMT 融合蛋白的载体的 1 l 细菌培养液沉淀。将细胞沉淀再悬浮于含磷酸盐(pH 8.0)、7 M 氯化胍鎓、咪唑和巯基丙三醇的细胞裂解缓冲液中, 随后使用 Ultra-Turrax® 匀浆。通过高速离心使不可溶性物质沉淀并将上清液施用于镍-螯合物层析柱上。用几个床体积的细胞裂解缓冲液, 随后用含磷酸盐(pH 8.0)和尿素的缓冲液洗涤柱。最后, 用含 SDS 的磷酸盐缓冲液在酸性条件下洗脱结合的抗原。

多克隆抗体的产生

a) 免疫

为了免疫, 制备 1:1 比例的蛋白质溶液(100 µg/ml 蛋白质 NNMT)的新鲜乳液和完全弗氏佐剂(complete Freund's adjuvant)。用 1 ml 乳液在第 1、7、14 和 30、60 和 90 天免疫每一只兔。抽取血液和将得到的抗-NNMT 血清用于在实施例 3 和 4 中描述的下一步实验中。

b) 通过用己酸和硫酸铵连续沉淀从兔血清纯化 IgG (免疫球蛋白 G)

用 4 体积的乙酸盐缓冲液(60 mM, pH 4.0)稀释 1 体积的兔血清。用 2 M Tris-碱调节 pH 至 4.5。于剧烈搅拌下逐滴加入辛酸(25 µl/ml 的稀释样品)。30 min 后将样品离心(13 000 x g, 30 min, 4°C), 弃去沉淀物和收集上清液。通过加 2 M Tris-碱调节上清液的 pH 至 7.5 并且过滤(0.2 µm)。

通过于剧烈搅拌下逐滴加入 4 M 硫酸铵溶液至终浓度为 2 M, 使上清液中的免疫球蛋白沉淀。通过离心(8000 x g, 15 min, 4°C)收集沉淀的免疫球蛋白。

弃去上清液。将沉淀物溶解于 10 mM NaH₂PO₄/NaOH, pH 7.5, 30 mM NaCl 中并彻底透析。将透析液离心(13 000 x g, 15 min, 4°C)并过滤(0.2 µm)。

多克隆兔 IgG 的生物素化

将多克隆兔 IgG 溶于 10 mM NaH₂PO₄/NaOH, pH 7.5, 30 mM NaCl

中制成 10 mg/ml。向每 ml IgG 溶液中加入 50 μ l 生物素-N-羧基琥珀酰亚胺(3.6 mg/ml 于 DMSO 中)。室温下 30 min 后, 样品于 Superdex 200 上经层析(10 mM $\text{NaH}_2\text{PO}_4/\text{NaOH}$, pH 7.5, 30 mM NaCl)。收集含生物素化的 IgG 部分。依据同样的规程使单克隆抗体生物素化。

多克隆兔 IgG 的地高辛配基化(digoxigenylation)

将多克隆兔 IgG 溶于 10 mM $\text{NaH}_2\text{PO}_4/\text{NaOH}$ 、30 mM NaCl, pH 7.5 中制成 10 mg/ml。向每 ml IgG 溶液中加入 50 μ l 地高辛配基-3-O-甲基羧基- ϵ -氨基己酸-N-羧基琥珀酰亚胺酯(Roche Diagnostics, 曼海姆, 德国, 目录号 1 333 054) (3.8 mg/ml 于 DMSO 中)。室温下 30 min 后, 样品于 Superdex® 200 上经层析(10 mM $\text{NaH}_2\text{PO}_4/\text{NaOH}$, pH 7.5, 30 mM NaCl)。收集含地高辛配基化的 IgG 部分。依据同样的规程使单克隆抗体标记地高辛配基。

实施例 2

用于人血清和血浆样品中 NNMT 的检测的 ELISA。

为了探测人血清或血浆中的 NNMT, 开发了夹心 ELISA。为了捕获和探测抗原, 将抗-NNMT 多克隆抗体(见实施例 2)的等分试样分别与生物素和地高辛配基轭合。

将链亲合素包被的 96-孔微滴定板, 用在 10 mM 磷酸盐, pH 7.4, 1% BSA、0.9% NaCl 和 0.1% 吐温 20 中, 10 μ g/ml 下的 100 μ l 生物素化的抗-NNMT 多克隆抗体孵育 60 min。孵育后, 用 0.9% NaCl、0.1% 吐温 20 洗涤板三次。然后用或者作为标准抗原的系列稀释的重组蛋白质(见实施例 2)或者得自患者的修饰的血浆样品孵育孔 2 h。结合 NNMT 后, 用 0.9% NaCl、0.1% 吐温 20 洗涤板三次。为了特异性地探测结合的 NNMT, 用在 10 mM 磷酸盐, pH 7.4, 1% BSA、0.9% NaCl 和 0.1% 吐温 20 中, 10 μ g/ml 下的 100 μ l 的地高辛配基化的抗-NNMT 多克隆抗体孵育孔 60 min。此后, 洗涤板三次去除未结合的抗体。在下一步中, 用在 10 mM 磷酸盐, pH 7.4, 1% BSA、0.9% NaCl 和 0.1% 吐温 20 中, 20 mU/ml 的抗-地高辛配基-POD 共轭体(Roche Diagnostics

GmbH, 曼海姆, 德国, 目录号 1633716)孵育孔 60 min。随后用同样的缓冲液洗涤板三次。为了探测抗原-抗体复合物, 用 100 μ l ABTS 溶液(Roche Diagnostics GmbH, 曼海姆, 德国, 目录号 11685767)孵育孔, 并于 30-60 min 后用 ELISA 读板器在 405 nm 处检测 OD。

实施例 3

研究 I: 研究人群

在第一项研究中, 采用源自示于表 1 的按照 UICC 分类法的 56 个经充分特征鉴定的 LC 患者的样本。这组患者集中在 NSCLC (50 个样本), 因为该型的 LC 具有比 SC 型好的预后, 并且因而其早期探测特别重要。

表 1

研究 I: LC 样本和相应的 UICC 分类

依据 UICC 的分期	样本的数量
UICC I	7
UICC II	17
UICC III	14
UICC IV	14
没有分期	4
LC 样本的总数量	56
其中 NSCLC 样本的数量	50
其中 SCLC 样本的数量	6

对比从 121 个没有任何已知恶性肺病的明显健康的个体(=对照组)中获得的对照样本, 评价表 1 的 LC 样本。

实施例 4

研究 I: 单一标记物的灵敏度

在对于待测的每一单个标记物, 在共同的 95%的特异性水平上, 计算每一标记物的灵敏度。表 2 以百分比给出每一个最有前途的 LC 标记物的灵敏度。

表 2
研究 I: 单一标记物的灵敏度

标记物	NNMT	CEA	NSE	CYFRA 21-1
于 95%特异性的灵敏度(%)	50.0	42.9	12.5	69.6

如易于从表 2 中明白的, 已经发现标记物 NNMT 对于 LC 具有第二高的灵敏度, 在所有被研究的标记物中仅次于 CYFRA 21-1。标记物 CEA 似乎具有显著比较低的灵敏度, 而 NSE 似乎表现相当差。

实施例 5

研究 I: 标记物组合

如果各自的组合的标记物中至少一个超过某一阈值, 则将该个体分类为患有 LC。这些临界值被定义为, 诸如在对照组上得到 95%的特异性。

表 3 表示相对于健康对照者, 诊断为 LC 患者的分类结果。

表 3

研究 I: 相对健康对照者, 诊断为 LC 的一批患者的分类结果

标记物数	标记物或标记板	临界值	灵敏度(%)
1	Cyfra 21-1	1.7 ng/ml	69.6
1	NNMT	884 pg/ml	50.0
1	CEA	5.5 ng/ml	42.9
1	NSE	17.7 ng/ml	12.5
2	Cyfra 21-1, CEA	1.9 ng/ml 5.8 ng/ml	73.0
2	Cyfra 21-1 NNMT	1.9 ng/ml 970 pg/ml	77.0
2	NNMT, CEA	1100 pg/ml 6 ng/ml	66.0
2	NNMT NSE	884 pg/ml 26 ng/ml	50.0
3	NNMT Cyfra 21-1 CEA	970 pg/ml 1.9 ng/ml 6 ng/ml	77.0

标记物 NNMT 和 Cyfra 21-1 的组合在该分析中得到在 95% 特异性水平时最高的灵敏度。评价与 NNMT 组合的多种其它标记物。如在表 3 中所示的, 与 NNMT 组合的 CEA 也导致在灵敏度上的显著改善。

实施例 6

研究 II: 研究人群

第二项研究总体而言独立于第一项研究, 集中在主要类型的 NSCLC, 腺癌和鳞状细胞癌。表 4a 描述癌症组的类型和分期分布。

表 4a**研究 II: LC 样本的类型和分期**

癌症类型	样本数	
	UICC I 或 II	UICC III 或 IV
腺癌	12	18
鳞状细胞癌	12	18
NSCLC 样本的总数量	60	

如在表 4b 所描述的, 在该项研究中的对照组被定义为对吸烟者和非吸烟者的某些样本更具特异性。对每个患者实施肺功能试验(肺活量测定法, Miller, M.R., 等, Eur. Respir. J. 26 (2005) 319-338)。只有如果供体的肺功能试验的结果在正常范围内, 样本才被纳入对照组中。

表 4b**研究 II: 对照组的组成**

依据 UICC 的分期	样本数
吸烟者	30
已戒烟者	6
非吸烟者	24

实施例 7**研究 II: 单一标记物的灵敏度**

如前基于表 4b 中描述的样本, 计算每一标记物的灵敏度。在对于每一个体受试标记物的共同的 95%特异性水平上, 得出灵敏度(以百分比表示)。表 5 分别描述腺癌和鳞状细胞癌的灵敏度, 以及每一标记物的总的灵敏度。

表 5**研究 II: 单一标记物于 95%特异性时的灵敏度(%)**

标记物	NNMT	CEA	NSE	CYFRA 21-1
腺癌	80	43	37	63
鳞状细胞癌	87	17	30	80
全部肺癌	83	30	33	72

清楚地, 在这种情况下, NNMT 有两个理由优于 Cyfra 21-1: 首先 NNMT 的总灵敏度显著更好。第二也是非常重要的, Cyfra 21-1 适应腺癌的功效比鳞状细胞癌低, 而 NNMT 对此两种类型癌症的表现相似。

实施例 8**研究 II: 标记物组合**

研究 II 中标记物组合的结果示于表 6 中。组合的类型与在上文研究 I 中描述的相同。

表 6

研究 II: 标记物组合在肺癌的两个主要亚型上的表现。在 95% 特异性时给出的灵敏度

分别为标记物或标记物组的编号	标记物或标记板	临界值	灵敏度(%)		
			腺癌	鳞状细胞癌	所有肺癌
1	Cyfra 21-1	2.1 ng/ml	63	80	72
2	NNMT	438 pg/ml	80	87	83
3	CEA	5.6 ng/ml	43	17	30
4	NSE	14.6 ng/ml	37	30	33
5	Cyfra 21-1, CEA	2.1 ng/ml 9.2 ng/ml	80	80	80
6	Cyfra 21-1 NSE	2.1 ng/ml 17.3 ng/ml	70	80	75
7	Cyfra 21-1 NNMT	2.1 ng/ml 520 pg/ml	83	90	87
8	NNMT, CEA	438 pg/ml 9.2 ng/ml	93	87	90
9	NNMT NSE	438 pg/ml 17.3 ng/ml	83	90	87
10	NNMT Cyfra 21-1 CEA	520 pg/ml 2.1 ng/ml 9.2 ng/ml	97	90	93
11	NNMT Cyfra 21-1 NSE	520 pg/ml 2.1 ng/ml 17.3 ng/ml	87	90	88
12	NNMT CEA NSE	438 pg/ml 9.2 ng/ml 17.3 ng/m	93	90	92
13	Cyfra 21-1	2.1 ng/ml	83	80	82

	CEA	9.2 ng/ml			
	NSE	17.3 ng/m			
14	NNMT	520 pg/ml	97	90	93
	Cyfra 21-1	2.1 ng/ml			
	CEA	9.2 ng/ml			
	NSE	17.3 ng/m			

与单用标记物时得到的灵敏度相比, 将任何两个受试标记物组合导致标记物组合的灵敏度的显著改善(为了阐明, 见标记物组号#7对#1和#2)。通过加入第三个标记物得到某些利益(参考, 组号#8对#10)。然而, 所有四个标记物的组合(组号#14)得到不比最好的三重组合(组号#10)更高的灵敏度表现。

· 包含 NNMT 的二重组合和三重组合总是比相应的由任何其它标记物替代 NNMT 的标记物好。

概要地说, 证实 NNMT 单用在探测两个主要类型的 NSC 肺癌中具有好的灵敏度。使用 NNMT 分别与 Cyfra 21-1 和/或 CEA 组合得到给人非常深刻印象的结果。使用包含所有三个标记物的组合, 将 93% 的癌症样本和 95% 的对照样本正确分类。