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(54) Title: NPTX2 AS TUMOR MARKER AND THERAPEUTIC TARGET FOR CANCER

(57) Abstract: The present invention arises from the discovery that the NPTX2 gene is both specifically overexpressed in cancer and involved in cancer cell survival. The present invention features methods for detecting or diagnosing cancer, as well as monitoring, determining or assessing the prognosis of a subject with cancer, using the NPTX2 gene as a diagnostic or prognostic marker. The present invention also features a double-stranded molecule against NPTX2 gene that find utility in the context of methods and compositions for the treatment and/or prevention of cancer.



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Description

Title of Invention: NPTX2 AS TUMOR MARKER AND THERAPEUTIC TARGET FOR CANCER

Technical Field

[0001] The present invention relates to the field of biological science, more specifically to the field of cancer research, cancer diagnosis and cancer therapy. In particular, the present invention relates to methods for detecting and diagnosing cancer, as well as methods of screening for subjects with cancer and kits associated with such methods. Further, the present invention relates to methods of screening for a substance for the treatment and prevention of cancer. Furthermore, the present invention relates to methods of treating and/or preventing cancer as well as pharmaceutical compositions therefore.

PRIORITY

The present application claims the benefit of U.S. Provisional Applications No. 61/483,514, filed on May 6, 2011, the entire contents of which are incorporated by reference herein.

Background Art

[0002] Lung cancer is the leading cause of cancer mortality and non-small cell lung carcinoma (NSCLC) accounts for nearly 80% of the cases. The majority of patients have advanced stage disease at the diagnosis. Despite improvements in diagnostic imaging, surgery, radiotherapy and chemotherapy, the overall survival for lung cancer remains poor. This has emphasized the importance of early diagnosis, prevention and treatment of this disease and reaffirmed the urgent need for the development of practical prognostic and diagnostic biomarkers suitable for early detection of lung cancer.

[0003] A better understanding of the molecular pathogenesis of lung cancer is a necessary prerequisite to the development of effective diagnostic approaches and molecular-targeted therapies. While several tumor markers for lung cancer, including NSE, CEA, CYFRA21-1, and ProGRP, are clinically available (M. Seike, et al., Proteomics 4 (2004), pp. 2776-2788., G.A. Chen, et al., Proc Natl Acad Sci 100 (2003), pp. 13537-13542., B.K. Shin, et al., J Biol Chem 278 (2003), pp. 7607-16.), their usefulness in early detection of cancers and prediction of clinical outcome is still very limited, mainly due to the low sensitivity and/or specificity. Therefore, there is a significant need in the art to discover highly sensitive and specific cancer biomarkers that can assist clinicians in the diagnosis and monitoring of the various manifestations of lung cancer.

[0004] Evidence to date indicates that tumor cells express cell-surface and/or secretory markers unique to each histological type at particular stages of differentiation. Since cell-surface and secretory proteins are considered more accessible to immune mechanisms and drug-delivery systems, identification of these types of proteins is an effective approach to develop novel diagnostic and therapeutic strategies. Recent advances in microarray technology have enabled new approaches to screen cancer biomarkers. To that end, genome wide-expression profiles of 101 lung tissue samples were analyzed using cDNA microarrays and tumor cells purified by laser microdissection and genes encoding transmembrane/secretory proteins as well as cancer-testis and onco-fetal antigens that are up-regulated were screened as potential molecular targets for cancer diagnosis and/or treatment (PTL 1-2, NPL 1-5). To verify the biological and clinicopathological significance of the respective gene products, tumor-tissue microarray analysis of clinical lung-cancer materials as well as RNA interference (RNAi) systems was performed (NPL 6-22).

Through this systematic approach, a novel rat neuronal member of the pentraxin family (neuronal pentraxin) that may mediate the uptake of synaptic material and the presynaptic snake venom toxin, taipoxin was identified (NPL 23).

[0005] The neuronal pentraxin 2 (NPTX2) gene encodes a secretory protein of 431 amino acids with a N-terminal signal peptide and C-terminal pentraxin domain. NPTX2 shows 54% amino acid identity to rat neuronal pentraxin (NPTX1) with 69% identity over the carboxyl-terminal half of NPTX1. Like NPTX1, NPTX2 has potential N-linked glycosylation sites. The human NPTX2 gene is 11 kb in length, contains four introns, and is localized to chromosome 7q21.3-q22.1. Together, this data suggests the existence of a family of pentraxin proteins that are expressed in the brain and other tissues and that may play important roles in the uptake of extracellular material.

[0006] NPTX2 was found to be correlated with edema in all glioma patients (NPL 24). In addition, increased NPTX2 was associated with poorer survival in tumors with the highest levels of edema. Despite the recent evidence linking NPTX2 to cancer, the biological significance of NPTX2 activation in human cancer progression and its clinical potential as a therapeutic target has not yet been fully understood and described.

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[PTL 2] WO2007/013665

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Summary of Invention

- [0009] The present invention relates to NPTX2, and the roles it plays in carcinogenesis. As such, the present invention relates to novel kits, compositions and methods for detecting, diagnosing, monitoring, treating and/or preventing cancer, as well as methods of screening for candidate substances for cancer prevention and treatment.
- [0010] Central to the present invention is the identification, through genome-wide expression profile analyses, of NPTX2 as a potential target for cancer diagnosis. Subsequent study confirmed that NPTX2 is expressed in the great majority of lung, colon, breast and cervical cancer specimens, while scarcely expressed in normal tissues. In addition, a higher level of NPTX2 expression level was found to be associated with a shorter cancer specific survival periods. Together, these results suggest that NPTX2 has utility as a biomarker for cancer diagnosis and prognosis.
- [0011] Higher levels of NPTX2 were also detected in serum samples from cancer patients as compared to those samples taken from healthy volunteers. The higher serum levels of NPTX2 were detected in even earlier-stage cancer patients and serum levels of NPTX2

demonstrated a good correlation with the expression levels of NPTX2 in tumor tissue samples. Together, these results suggest that NPTX2 has utility as a serum marker for diagnosis of cancers, including earlier-stage cancers, and for monitoring the efficacy of cancer treatment and surveillance of disease relapse.

As demonstrated herein, RNA interference assay and matrigel invasion assay showed that NPTX2 was associated with cancer cell growth and invasion. Thus, the data herein support the utility of NPTX2 as a molecular target for novel cancer treatments.

[0012] Thus, it is object of the present invention to provide methods of diagnosing or detecting cancer in a subject by determining the expression level of the NPTX2 gene in a subject-derived biological sample, wherein an increase in the NPTX2 expression level as compared to a normal control level of the NPTX2 gene indicates the presence of cancer in said subject or that said subject suffers from cancer. Such methods may also be applicable to the assessment of a subject's predisposition for developing cancer, wherein a relatively increased NPTX2 expression level in a subject-derived sample as compared to a normal control level indicates that said subject is at risk of developing cancer.

[0013] It is another object of the present invention to provide methods of monitoring, assessing or predicting a prognosis of a subject with cancer, such methods including the step of determining the expression level of the NPTX2 gene in a cancerous tissue sample from the subject, wherein an increase in expression of the NPTX2 gene as compared to a selected control is indicative of a poor prognosis.

[0014] It is yet another object of the present invention to provide kits for use in diagnosing or detecting cancer, screening a subject suspected of suffering from cancer, or assessing or predicting the prognosis of a subject with cancer, such kits including at least one reagent for determining the expression level of the NPTX2 gene in a subject-derived biological sample.

[0015] It is yet another object of the present invention to provide reagents for use in diagnosing or detecting cancer, screening a subject suspected of suffering from cancer, or assessing or predicting the prognosis of a subject with cancer, such reagents including an oligonucleotide having a sequence complementary to a part of the NPTX2 mRNA that specifically binds to the NPTX2 mRNA, or an antibody against the NPTX2 polypeptide. In another aspect, the present invention provides for the use of an oligonucleotide having a sequence complementary to a part of the NPTX2 mRNA that specifically binds to the NPTX2 mRNA, or an antibody against the NPTX2 polypeptide in the manufacture of a reagent for diagnosing or detecting cancer, screening a subject suspected of suffering from cancer, or assessing or predicting the prognosis of a subject with cancer. In yet another aspect, the present invention provides an oligonucleotide having a sequence complementary to a part of the NPTX2

mRNA that specifically binds to the NPTX2 mRNA, or an antibody against the NPTX2 polypeptide for use in diagnosing or detecting cancer, screening a subject suspected of suffering from cancer, or assessing or predicting the prognosis of a subject with cancer.

It is yet another object of the present invention to provide methods of screening for a candidate substance for the treatment and/or prevention of cancer, or the inhibition of the proliferation of cancer cells, wherein the expression of the NPTX2 gene or an activity of the NPTX2 polypeptide is used as an index.

[0016] It is yet another object of the present invention to provide double-stranded molecules against the NPTX2 gene capable of inhibiting the expression of the NPTX2 gene as well as the proliferation of a cell expressing the NPTX2 gene when introduced into the cell. It is a further object of the present invention to provide vectors encoding such double-stranded molecules. The double-stranded molecules of the present invention are composed of a sense strand and an antisense strand, wherein the sense strand includes a nucleotide sequence corresponding to a target sequence selected from the NPTX2 gene sequence, and the antisense strand includes a nucleotide sequence complementary to the target sequence, further wherein the sense strand and the antisense strand hybridize to each other to form the double-stranded molecule.

[0017] It is yet another object of the present invention to provide methods for the treatment and/or prevention of, or the inhibition of the proliferation of cancer cells, such methods including the step of administering a double-stranded molecule or a vector encoding a double-stranded molecule against the NPTX2 gene that inhibits the expression of the NPTX2 gene as well as the proliferation of a cell expressing the NPTX2 gene when introduced into the cell.

[0018] It is yet another object of the present invention to provide pharmaceutical compositions for the treatment and/or prevention of cancer, or the inhibition of the proliferation of cancer cells, such compositions containing a pharmaceutically effective amount of a double-stranded molecule or vector encoding a double-stranded molecule against the NPTX2 gene that inhibits the expression of the NPTX2 gene as well as the proliferation of a cell expressing the NPTX2 gene when introduced into the cell, and a pharmaceutically acceptable carrier. In another aspect, the present invention provides uses of double-stranded molecule against the NPTX2 gene that inhibits the expression of the NPTX2 gene as well as the proliferation of a cell expressing the NPTX2 gene when introduced into the cell, or a vector encoding such double-stranded molecule, for the manufacture of a medicament for either or both of treating and preventing cancer. In yet another aspect, the present invention provides double-stranded molecules against the NPTX2 gene that inhibits the expression of the NPTX2 gene as well as the proliferation of a cell expressing the NPTX2 gene when introduced into the cell, or a

vector encoding such double-stranded molecule, for use in either or both of treating and preventing cancer.

[0019] More specifically, the present invention provides the following [1] to [29]:

[1] A method of diagnosing or detecting cancer in a subject, comprising the steps of:

(1) determining an expression level of an NPTX2 gene in a subject-derived biological sample by a method selected from the group consisting of:

- (a) detecting an mRNA of the NPTX2 gene;
- (b) detecting an NPTX2 polypeptide; and
- (c) detecting a biological activity of an NPTX2 protein;

(2) comparing the NPTX2 expression level determined in step (1) with a normal control level of the NPTX2 gene; and

(3) diagnosing said subject with cancer or determining the presence of the cancer in a subject when the NPTX2 expression level determined in step (1) is higher than said normal control level;

[2] The method of [1], wherein the subject-derived biological sample is a bodily tissue sample or blood sample;

[3] The method of [2], wherein the expression level of the NPTX2 gene is determined by detecting an NPTX2 polypeptide in a subject-derived blood sample;

[4] The method of [3], wherein the NPTX2 polypeptide is detected by the method comprising steps of:

(i) contacting an antibody against an NPTX2 polypeptide with the subject-derived blood sample; and

(ii) detecting the binding between said antibody and the NPTX2 polypeptide in said subject-derived blood sample;

[5] The method of [4], wherein the NPTX2 polypeptide is detected by Enzyme-Linked ImmunoSorbent Assay (ELISA);

[6] The method of any one of [3] to [5], wherein the subject-derived blood sample is a subject-derived serum sample;

[7] The method of any one of [3] to [6], wherein the method further comprises a step of detecting at least one other serum tumor marker in the subject-derived blood sample, wherein cancer is judged to be present or said subject is judged to be suffering from cancer when either (a) the level of the NPTX2 polypeptide in said subject derived blood sample is higher than a normal control level of the NPTX2 polypeptide, or (b) the level of said serum tumor marker in said subject derived blood sample is higher than a normal control level of said serum tumor marker, or (c) both;

[8] The method of any one of [1] to [7], wherein the cancer is lung cancer, breast cancer, cervical cancer or colon cancer;

[9] A kit for use in diagnosis or detection of cancer in a subject, wherein said kit

comprises at least one reagent selected from the group consisting of:

- (a) a reagent for detecting an mRNA of the NPTX2 gene;
- (b) a reagent for detecting an NPTX2 polypeptide; and
- (c) a reagent for detecting a biological activity of an NPTX2 polypeptide;

[10] The kit of [9], wherein the reagent comprises an oligonucleotide that has a sequence complementary to a part of an mRNA of the NPTX2 gene and that specifically binds to said mRNA; or an antibody against the NPTX2 polypeptide;

[11] The kit of [10], wherein the kit is an ELISA kit comprising at least one antibody against the NPTX2 polypeptide;

[12] The kit of any one of [9] to [11], wherein the cancer is lung cancer, breast cancer, cervical cancer or colon cancer;

[13] A method for assessing or predicting a prognosis of a subject with cancer, comprising the steps of:

(1) determining an expression level of the NPTX2 gene in a subject-derived biological sample, by a method selected from the group consisting of:

- (a) detecting an mRNA of the NPTX2 gene;
- (b) detecting an NPTX2 polypeptide; and
- (c) detecting a biological activity of an NPTX2 polypeptide;

(2) comparing the NPTX2 expression level determined in step (1) with a good prognosis control level of the NPTX2 gene; and

(3) predicting a poor prognosis for said subject when the NPTX2 expression level determined in step (1) is higher than said good prognosis control level;

[14] The method of [13], wherein the cancer is lung cancer;

[15] A kit for use in assessing or predicting a prognosis of a subject with cancer, wherein said kit comprises at least one reagent selected from the group consisting of:

- (a) a reagent for detecting an mRNA of the NPTX2 gene;
- (b) a reagent for detecting an NPTX2 polypeptide; and
- (c) a reagent for detecting a biological activity of an NPTX2 polypeptide;

[16] The kit of [15], wherein the reagent comprises an oligonucleotide that has a sequence complementary to a part of an mRNA of the NPTX2 gene and specifically binds to said mRNA; or an antibody against the NPTX2 polypeptide;

[17] The kit of [15] or [16], wherein the cancer is lung cancer;

[18] A method of screening for a candidate substance for either or both of treating and preventing cancer, wherein said method comprises steps of:

- (a) contacting a test substance with an NPTX2 polypeptide, or functional equivalent thereof;
- (b) detecting the binding between the NPTX2 polypeptide or functional equivalent thereof, and the test substance; and

(c) selecting the test substance that binds to the NPTX2 polypeptide or functional equivalent thereof;

[19] A method of screening for a candidate substance for either or both of treating and preventing cancer, wherein said method comprises steps of:

- (a) contacting a test substance with a cell expressing the NPTX2 gene;
- (b) determining an expression level of the NPTX2 gene in the cell of step (a); and
- (c) selecting the test substance that reduces the expression level of NPTX2 gene as compared to the expression level determined in the absence of the test substance;

[20] A method of screening for a candidate substance for either or both of treating and preventing cancer, wherein said method comprises steps of:

- (a) contacting a test substance with an NPTX2 polypeptide or functional equivalent thereof;
- (b) detecting a biological activity of the NPTX2 polypeptide or functional equivalent thereof of step (a); and
- (c) selecting the test substance that suppresses a biological activity of the NPTX2 polypeptide or functional equivalent thereof as compared to the biological activity detected in the absence of the test substance;

[21] The method of [20], wherein the biological activity is cell proliferation enhancing activity;

[22] A method of screening for a candidate substance for either or both of treating and preventing cancer, wherein said method comprises steps of:

- (a) contacting a test substance with a cell into which a vector comprising a transcriptional regulatory region of the NPTX2 gene and a reporter gene that is expressed under control of transcriptional regulatory region has been introduced,
- (b) measuring an expression or activity level of said reporter gene; and
- (c) selecting the test substance that reduces the expression or activity level of said reporter gene, as compared to the expression or activity level detected in the absence of the test substance;

[23] The method of any one [18] to [22], wherein the cancer is lung cancer, breast cancer, cervical cancer or colon cancer;

[24] A method of either or both of treating and preventing cancer in a subject, wherein said method comprises a step of administering to said subject a pharmaceutically effective amount of a double-stranded molecule against the NPTX2 gene, or a vector encoding said double-stranded molecule, wherein said double-stranded molecule comprises a sense strand and an antisense strand, wherein the sense strand comprises a nucleotide sequence corresponding to a target sequence selected from the NPTX2 gene sequence, and the antisense strand comprises a nucleotide sequence complementary to said target sequence, wherein said sense strand and said antisense strand hybridize to

each other to form the double-stranded molecule, and wherein said double stranded molecule, when introduced into a cell expressing the NPTX2 gene, inhibits cell proliferation as well as the expression of the NPTX2 gene;

[25] The method of [24], wherein the sense strand hybridizes with the antisense strand at the target sequence to form the double-stranded molecule having between 19 and 25 nucleotide pair in length;

[26] The method of [24] or [25], wherein the double-stranded molecule is a single polynucleotide comprising the sense strand and the antisense strand linked via a single-stranded nucleotide sequence;

[27] A composition for either or both of treating and preventing cancer, wherein said composition comprises a pharmaceutically effective amount of a double-stranded molecule against the NPTX2 gene, or a vector encoding said double-stranded molecule, and a pharmaceutically acceptable carrier, wherein said double-stranded molecules comprises a sense strand and an antisense strand, wherein the sense strand comprises a nucleotide sequence corresponding to a target sequence selected from the NPTX2 gene sequence, and the antisense strand comprises a nucleotide sequence complementary to said target sequence, wherein said sense strand and said antisense strand hybridize to each other to form the double-stranded molecule, and wherein said double stranded molecule, when introduced into a cell expressing the NPTX2 gene, inhibits cell proliferation as well as the expression of the NPTX2 gene;

[28] The composition of [27], wherein the sense strand hybridizes with the antisense strand at the target sequence to form the double-stranded molecule having between 19 and 25 nucleotide pair in length; and

[29] The composition of [27] or [28], wherein the double-stranded molecule is a single polynucleotide comprising the sense strand and the antisense strand linked via a single-stranded nucleotide sequence.

[0020] It will be understood by those skilled in the art that one or more aspects of this invention can meet certain objectives, while one or more other aspects can meet certain other objectives. Each objective may not apply equally, in all its respects, to every aspect of this invention. As such, the preceding objects can be viewed in the alternative with respect to any one aspect of this invention.

[0021] It will also be understood that both the foregoing summary of the present invention and the following detailed description are of exemplified embodiments, and not restrictive of the present invention or other alternate embodiments of the present invention. Other objects and features of the invention will become more fully apparent when the following detailed description is read in conjunction with the accompanying figures and examples. In particular, while the invention is described herein with reference to a number of specific embodiments, it will be appreciated that the de-

scription is illustrative of the invention and is not constructed as limiting of the invention. Various modifications and applications may occur to those who are skilled in the art, without departing from the spirit and the scope of the invention, as described by the appended claims. Likewise, other objects, features, benefits and advantages of the present invention will be apparent from this summary and certain embodiments described below, and will be readily apparent to those skilled in the art. Such objects, features, benefits and advantages will be apparent from the above in conjunction with the accompanying examples, data, figures and all reasonable inferences to be drawn therefrom, alone or with consideration of the references incorporated herein.

Brief Description of Drawings

[0022] Various aspects and applications of the present invention will become apparent to the skilled artisan upon consideration of the brief description of the figures and the detailed description of the present invention and its preferred embodiments which follows:

[fig.1A-B]Figure 1 demonstrates the expressions of NPTX2 in lung tumors. Part A depicts the expressions of NPTX2 in 15 clinical samples of lung cancer (10 NSCLC and 5 SCLC) (T) and their corresponding normal lung tissues (N), examined by semi-quantitative RT-PCR. Appropriate dilutions of each single-stranded cDNA prepared from mRNAs of clinical lung cancer samples were prepared, taking the level of beta-actin (ACTB) expression as a quantitative control. Part B depicts the expressions of NPTX2 in 23 lung cancer cell lines, examined by semiquantitative RT-PCR.

[0023] [fig.1C-E]Part C depicts the expressions of NPTX2 protein in 4 lung cancer cell lines and airway epithelia cell, examined by Western blot analysis. Part D depicts the sub-cellular localization of endogenous NPTX2 protein in the 4 lung cancer cell lines. NPTX2 was stained at the cytoplasm of the cell with granular appearance in NCI-H226, NCI-H520, and SBC-5 cells, but not in NCI-H2170 cells. Part E depicts the detection of secreted NPTX2 protein with ELISA in conditioned medium from NPTX2-expressing NCI-H520 and SBC-5 cells as well as NPTX2-non-expressing NCI-H2170 and A549 cells.

[0024] [fig.2A-C]Figure 2 demonstrates the expression of NPTX2 in normal tissues and lung cancer tissues. Part A depicts the expression of NPTX2 in normal human tissues detected by Northern blot analysis. Part B depicts the immunohistochemical evaluation of NPTX2 protein in representative lung adenocarcinoma (ADC) tissue and five normal tissues; heart, liver, kidney, lung, testis. Part C depicts the immunohistochemical staining of NPTX2 in representative lung adenocarcinoma (ADC), lung squamous cell carcinoma (SCC), and small cell lung cancer (SCLC), using anti-NPTX2 antibody on tissue microarrays (original magnification x100 and x200).

- [0025] [fig.2D-E]Part D depicts the examples of strong, weak, and absent NPTX2 expression in lung ADCs. Part E depicts the Kaplan-Meier analysis of tumor-specific survival in patients with NSCLC according to NPTX2 expression ($P = 0.0002$; Log-rank test).
- [0026] [fig.3A]Figure 3 demonstrates the serologic concentration of NPTX2 determined by ELISA in patients with lung cancers and in healthy donors or non-neoplastic lung disease patients with COPD. Part A depicts the distribution of NPTX2 in sera from patients with lung ADC, lung SCC, or SCLC. Differences were significant between ADC patients and healthy/COPD individuals ($P < 0.001$, Mann-Whitney U test), between SCC patients and healthy/COPD individuals ($P = 0.005$) and between SCLC patients and healthy/COPD individuals ($P = 0.0051$). The difference between healthy individuals and COPD was not significant.
- [0027] [fig.3B-C]Part B depicts the ROC curve analysis of NPTX2 as serum markers for lung cancers. X axis, 1-specificity; Y axis, sensitivity. Part C depicts the serologic concentration of NPTX2 before and after surgery (postoperative days at two months) in patients with NSCLC.
- [0028] [fig.3D]Part D depicts the serum NPTX2 levels (U/ml) and the expression levels of NPTX2 in primary tumor tissues in the same NSCLC patients.
- [0029] [fig.3E]Part E depicts the distribution of NPTX2 in sera from patients with colon cancer, breast cancer and cervical cancer.
- [0030] [fig.4]Figure 4 demonstrates the expression of NPTX2 in clinical samples of colon cancer, breast cancer and cervical cancer, examined by semiquantitative RT-PCR. "N" indicates a corresponding normal tissue sample.
- [0031] [fig.5]Figure 5 demonstrates the role of NPTX2 in cancer cell growth and cellular invasion. Part A depicts the inhibition of growth of lung cancer cells that over-expressed NPTX2 by siRNA against NPTX2. Upper panels depicts viability of the SBC-5 cells evaluated by MTT assay in response to si-NPTX2s, -LUC, or -EGFP. All assays were performed three times, and in triplicate wells. Bottom panels depicts expression of NPTX2 in response to si-NPTX2s (si-1, -2) or control siRNAs (LUC or EGFP) in SBC-5 cells, analyzed by RT-PCR analysis. Part B depicts the enhanced invasiveness of mammalian cells transfected with NPTX2-expressing plasmids. Assays demonstrating the invasive nature of COS-7 cells in Matrigel matrix after transfection with expression plasmids for human NPTX2. Upper panels depict the transient expression of NPTX2 in the COS-7 cells, detected by western-blot analysis. Middle and Lower panels depict the number of cells migrating through the Matrigel-coated filters and Giemsa staining (X200). Assays were performed three times, and each in triplicate wells.

Description of Embodiments

[0032] Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of embodiments of the present invention, the preferred methods, devices, and materials are now described. However, before the present materials and methods are described, it is to be understood that the present invention is not limited to the particular sizes, shapes, dimensions, materials, methodologies, protocols, etc. described herein, as these may vary in accordance with routine experimentation and optimization. It is also to be understood that the terminology used in the description is for the purpose of describing the particular versions or embodiments only, and is not intended to limit the scope of the present invention which will be limited only by the appended claims.

[0033] The disclosure of each publication, patent or patent application mentioned in this specification is specifically incorporated by reference herein in its entirety. However, nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the present invention belongs. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

[0034] Definitions

The words "a", "an", and "the" as used herein mean "at least one" unless otherwise specifically indicated.

The terms "isolated" and "purified" used in relation with a substance (e.g., polypeptide, antibody, polynucleotide, etc.) indicates that the substance is substantially free from at least one substance that can be included in the natural source. Thus, an isolated or purified antibody refers to antibodies that are substantially free of cellular material for example, carbohydrate, lipid, or other contaminating proteins from the cell or tissue source from which the protein (antibody) is derived, or substantially free of chemical precursors or other chemicals when chemically synthesized. The term "substantially free of cellular material" includes preparations of a polypeptide in which the polypeptide is separated from cellular components of the cells from which it is isolated or recombinantly produced.

[0035] Thus, a polypeptide that is substantially free of cellular material includes preparations of polypeptide having less than about 30%, 20%, 10%, or 5% (by dry weight) of heterologous protein (also referred to herein as a "contaminating protein"). When the polypeptide is recombinantly produced, in some embodiments it is also substantially free of culture medium, which includes preparations of polypeptide with culture medium less than about 20%, 10%, or 5% of the volume of the protein

preparation. When the polypeptide is produced by chemical synthesis, in some embodiments it is substantially free of chemical precursors or other chemicals, which includes preparations of polypeptide with chemical precursors or other chemicals involved in the synthesis of the protein less than about 30%, 20%, 10%, 5% (by dry weight) of the volume of the protein preparation. That a particular protein preparation contains an isolated or purified polypeptide can be shown, for example, by the appearance of a single band following sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of the protein preparation and Coomassie Brilliant Blue staining or the like of the gel. In one embodiment, proteins including antibodies of the present invention are isolated or purified.

[0036] As used herein, the term "biological sample" refers to a whole organism or a subset of its tissues, cells or component parts (e.g., body fluids, including but not limited to blood, mucus, lymphatic fluid, synovial fluid, cerebrospinal fluid, saliva, amniotic fluid, amniotic cord blood, urine, vaginal fluid and semen). "Biological sample" further refers to a homogenate, lysate, extract, cell culture or tissue culture prepared from a whole organism or a subset of its cells, tissues or component parts, or a fraction or portion thereof. Lastly, "biological sample" refers to a medium, for example, a nutrient broth or gel in which an organism has been propagated, which contains cellular components, for example, proteins or polynucleotides. In the context of the present invention, a biological sample may be a tissue sample, such as biopsy specimen, tumor biopsy specimen or collection of lung cells, breast cells, cervical cells or colon cells or, alternatively, a blood or serum sample.

[0037] The terms "polypeptide", "peptide", and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is a modified residue, or a non-naturally occurring residue, for example, an artificial chemical mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers.

[0038] The term "amino acid" refers to naturally occurring and synthetic amino acids, as well as amino acid analogs and amino acid mimetics that similarly functions to the naturally occurring amino acids. Naturally occurring amino acids are those encoded by the genetic code, as well as those modified after translation in cells (e.g., hydroxyproline, gamma-carboxyglutamate, and O-phosphoserine). The phrase "amino acid analog" refers to substances that have the same basic chemical structure (an alpha carbon bound to a hydrogen, a carboxy group, an amino group, and an R group) as a naturally occurring amino acid but have a modified R group or modified backbones (e.g., homoserine, norleucine, methionine, sulfoxide, methionine methyl sulfonium). The phrase "amino acid mimetic" refers to chemical substances that have different structures but similar functions to general amino acids.

Amino acids can be referred to herein by their commonly known three letter symbols or the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission.

[0039] The terms "gene", "polynucleotide", "oligonucleotide", "nucleic acid", and "nucleic acid molecule" are used interchangeably unless otherwise specifically indicated and are similarly to the amino acids referred to by their commonly accepted single-letter codes. Similar to the amino acids, they encompass both naturally-occurring and non-naturally occurring nucleic acid polymers. The gene, polynucleotide, oligonucleotide, nucleic acid, or nucleic acid molecule can be composed of DNA, RNA or a combination thereof.

[0040] In the context of the present invention, the phrase "NPTX2 gene" encompasses polynucleotides that encode human NPTX2 gene or any of the functional equivalents of the human NPTX2 gene. In the context of the present invention, the NPTX2 gene or its functional equivalent can be obtained from nature as naturally occurring proteins via conventional cloning methods or through chemical synthesis based on the selected nucleotide sequence. Methods for cloning genes using cDNA libraries and such are well known in the art.

[0041] Unless otherwise defined, the terms "cancer" refers to cancers over-expressing the NPTX2 gene, such as lung cancer, breast cancer, cervical cancer and colon cancer.

To the extent that the methods and compositions of the present invention find utility in the context of "prevention" and "prophylaxis", such terms are interchangeably used herein to refer to any activity that reduces the burden of mortality or morbidity from disease. Prevention and prophylaxis can occur "at primary, secondary and tertiary prevention levels". While primary prevention and prophylaxis avoid the development of a disease, secondary and tertiary levels of prevention and prophylaxis encompass activities aimed at the prevention and prophylaxis of the progression of a disease and the emergence of symptoms as well as reducing the negative impact of an already established disease by restoring function and reducing disease-related complications. Alternatively, prevention and prophylaxis can include a wide range of prophylactic therapies aimed at alleviating the severity of the particular disorder, e.g. reducing the proliferation and metastasis of tumors.

[0042] To the extent that certain embodiments of the present invention encompass the treatment and/or prophylaxis of cancer and/or the prevention of postoperative recurrence, such methods may include any of the following steps: the surgical removal of cancer cells, the inhibition of the growth of cancerous cells, the involution or regression of a tumor, the induction of remission and suppression of occurrence of cancer, the tumor regression, and the reduction or inhibition of metastasis. Effective treatment and/or the prophylaxis of cancer decreases mortality and improves the

prognosis of individuals having cancer, decreases the levels of tumor markers in the blood, and alleviates detectable symptoms accompanying cancer.

[0043] (1) Gene and Polypeptide

The NPTX2 gene encodes a member of the family of neuronal petraxins, synaptic proteins that are related to C-reactive protein. This protein is involved in excitatory synapse formation.

The nucleic acid and polypeptide sequences of the NPTX2 gene in the present invention are known to those skilled in the art, and obtained, for example, from gene databases on the web site such as GenBank™. An exemplified nucleotide sequence of the human NPTX2 gene is shown in SEQ ID NO: 1 (GenBank accession No. NM_002523.2), and an exemplified amino acid sequence of the human NPTX2 polypeptide is shown in SEQ ID NO: 2 (GenBank accession No. NP_002514.1). One skilled in the art will recognize that NPTX2 sequences need not be limited to these sequences and that variants (e.g., functional equivalents and allelic variants) can be used in the present invention as described below.

[0044] Herein, the polypeptide encoded by the NPTX2 gene is referred to as "NPTX2 polypeptide", and sometimes as "NPTX2" or "NPTX2 protein". In the context of the present invention, the phrase "NPTX2 gene" encompasses not only polynucleotides that encode the human NPTX2 polypeptide but also polynucleotides that encode functional equivalents of the human NPTX2 gene. The NPTX2 gene can be obtained from nature as naturally occurring polynucleotides via conventional cloning methods or through chemical synthesis based on the selected nucleotide sequence. Methods for cloning genes using cDNA libraries and such are well known in the art. As noted above, the present invention extends to "functional equivalents" and deems such to be NPTX2 polypeptides in the context. Herein, a "functional equivalent" of a protein is a polypeptide that has a biological activity equivalent to that of the original reference protein. Namely, any polypeptides that retain at least one biological activity of the NPTX2 polypeptide can be used as functional equivalents of the NPTX2 polypeptide in the present invention. For example, functional equivalents of the NPTX2 polypeptide retain cell proliferation enhancing activity of the NPTX2 polypeptide. In the context of the present invention, functional equivalents of the NPTX2 polypeptide include polymorphic variants, interspecies homologues, and those encoded by alleles of these polypeptides.

[0045] Examples of functional equivalents of the NPTX2 polypeptide include those wherein one or more amino acids, e.g., 1-5 amino acids, e.g., up to 5% of amino acids, are substituted, deleted, added, or inserted to the natural occurring amino acid sequence of the NPTX2 polypeptide.

It is generally known that modifications of one, two or more amino acid in a protein

will not significantly impact or influence the function of the protein. In some cases, it may even enhance the desired function of the original protein. In fact, mutated or modified proteins (i.e., peptides composed of an amino acid sequence in which one, two, or several amino acid residues have been modified through substitution, deletion, insertion and/or addition) have been known to retain the original biological activity (Mark DF, et al., *Proc Natl Acad Sci U S A*. 1984 Sep;81(18):5662-6; Zoller MJ & Smith M. *Nucleic Acids Res*. 1982 Oct 25;10(20):6487-500; Wang A, et al., *Science*. 1984 Jun 29;224(4656):1431-3; Dalbadie-McFarland G, et al., *Proc Natl Acad Sci U S A*. 1982 Nov;79(21):6409-13).

- [0046] Accordingly, one skilled in the art will recognize that individual additions, deletions, insertions, or substitutions to an amino acid sequence which alters a single amino acid or a small percentage of amino acids (i.e., less than 5%, more preferably less than 3%, even more preferably less than 1%) or those considered to be a "conservative modification" wherein the alteration of a protein results in a protein with similar functions are acceptable in the context of the instant invention. Thus, the peptides of the present invention may have an amino acid sequence wherein one, two or even more amino acids are added, inserted, deleted, and/or substituted in an originally disclosed reference sequence.
- [0047] So long as the activity the protein is maintained, the number of amino acid mutations or modifications is not particularly limited. However, it is generally preferred to alter a single amino acid or small percentage of amino acids, i.e., 5% or less of the amino acid sequence, more preferably less than 3%, even more preferably less than 1%. Accordingly, in a preferred embodiment, the number of amino acids to be mutated in such a mutant is generally 30 amino acids or less, preferably 20 amino acids or less, more preferably 10 amino acids or less, more preferably 5 or 6 amino acids or less, and even more preferably 2, 3 or 4 amino acids or less.
- [0048] An amino acid residue to be mutated is preferably mutated into a different amino acid in which the properties of the amino acid side-chain are conserved (a process known as conservative amino acid substitution). Examples of properties of amino acid side chains are hydrophobic amino acids (alanine, isoleucine, leucine, methionine, phenylalanine, proline, tryptophan, tyrosine, valine), hydrophilic amino acids (arginine, aspartic acid, asparagine, cysteine, glutamic acid, glutamine, glycine, histidine, lysine, serine, threonine), and side chains having the following functional groups or characteristics in common: an aliphatic side-chain (glycine, alanine, valine, leucine, isoleucine, proline); a hydroxyl group containing side-chain (serine, threonine, tyrosine); a sulfur atom containing side-chain (C, M); a carboxylic acid and amide containing side-chain (aspartic acid, asparagine, glutamic acid, glutamine); a base containing side-chain (arginine, lysine, histidine); and an aromatic containing side-

chain (histidine, phenylalanine, tyrosine, tryptophan). Furthermore, conservative substitution tables providing functionally similar amino acids are well known in the art. For example, the following eight groups each contain amino acids that are conservative substitutions for one another:

- (1) Alanine (A), Glycine (G);
- (2) Aspartic acid (D), Glutamic acid (E);
- (3) Asparagine (N), Glutamine (Q);
- (4) Arginine (R), Lysine (K);
- (5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V);
- (6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W);
- (7) Serine (S), Threonine (T); and
- (8) Cysteine (C), Methionine (M)

(see, e.g., Thomas E. Creighton, *Proteins* Publisher: New York: W.H. Freeman, c1984).

[0049] Such conservatively modified polypeptides are included in functional equivalents of the NPTX2 polypeptide. However, the present invention is not restricted thereto and functional equivalents of the NPTX2 polypeptide can include non-conservative modifications so long as the resulting modified peptide retains at least one of the biological activities of the original polypeptide, namely the NPTX2 polypeptide. In the context of the present invention, the modified proteins do not exclude polymorphic variants, interspecies homologues, and those encoded by alleles of these proteins.

[0050] An example of a functional equivalent of the NPTX2 polypeptide modified by addition of several amino acid residues is a fusion protein of the NPTX2 polypeptide and other polypeptides or peptides. Such fusion proteins can be made by techniques well known to a person skilled in the art, for example, by linking the DNA encoding the NPTX2 gene with a DNA encoding another polypeptides or peptides, so that the frames match, inserting the fusion DNA into an expression vector and expressing it in a host. The "other" component of the fusion protein is typically a small epitope composed of several to a dozen amino acids. There is no restriction as to polypeptides or peptides fused to the NPTX2 polypeptide so long as the resulting the fusion protein retains any one of the objective biological activity of the NPTX2 polypeptide.

[0051] Exemplary fusion proteins contemplated by the instant invention include fusions of the NPTX2 peptide and other small peptides or proteins such FLAG (Hopp TP, et al., *Biotechnology* 6: 1204-10 (1988)), 6xHis containing six His (histidine) residues, 10xHis, Influenza agglutinin (HA), human c-myc fragment, VSP-GP fragment, p18HIV fragment, T7-tag, HSV-tag, E-tag, SV40T antigen fragment, lck tag, alpha-tubulin fragment, B-tag, Protein C fragment, and the like. Examples of proteins that can be fused to a protein of the invention include GST (glutathione-S-transferase),

Influenza agglutinin (HA), immunoglobulin constant region, beta-galactosidase, MBP (maltose-binding protein), and such.

- [0052] In other embodiments, functional equivalents of above polypeptides can be encoded by a polynucleotide that hybridizes under stringent conditions to the natural occurring nucleotide sequence of the NPTX2 gene. Methods known in the art to isolate functional equivalents include, for example, hybridization techniques (Sambrook and Russell, *Molecular Cloning: A Laboratory Manual*, 3rd ed., Cold Spring Harbor Lab. Press, 2001). One skilled in the art can readily isolate a DNA having high homology (i.e., sequence identity) with a whole or part of the human NPTX2 DNA sequences (e.g., SEQ ID NO: 1) encoding the human NPTX2 polypeptide, and isolate functional equivalents of the human NPTX2 polypeptide from the isolated DNA.
- [0053] Thus, functional equivalents of the NPTX2 polypeptide used in the present invention include polypeptides encoded by DNAs that hybridize under stringent conditions with a whole or part of the DNA sequence encoding the human NPTX2 polypeptide. These functional equivalents include mammal homologues corresponding to the human NPTX2 polypeptide (for example, polypeptides encoded by monkey, mouse, rat, rabbit or bovine NPTX2 genes).
- [0054] The hybridization conditions for isolating a DNA encoding a functional equivalent of the human NPTX2 gene can be routinely selected by a person skilled in the art. The phrase "stringent (hybridization) conditions" refers to conditions under which a nucleic acid molecule will hybridize to its target sequence, typically in a complex mixture of nucleic acids, but not detectably to other sequences. Stringent conditions are sequence-dependent and will differ under different circumstances. Longer sequences hybridize specifically at higher temperatures. An extensive guide to the hybridization of nucleic acids is found in Tijssen, *Techniques in Biochemistry and Molecular Biology-Hybridization with Nucleic Probes*, "Overview of principles of hybridization and the strategy of nucleic acid assays" (1993). Generally, stringent conditions are selected to be about 5-10 degrees C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength, pH, and nucleic concentration) at which 50% of the probes complementary to the target hybridize to the target sequence at equilibrium (as the target sequences are present in excess, at T_m , 50% of the probes are occupied at equilibrium). Stringent conditions can also be achieved with the addition of destabilizing agents for example, formamide. For selective or specific hybridization, a positive signal is at least two times of background, for example, 10 times of background hybridization.
- [0055] In the context of the present invention, the optimal condition of hybridization for isolating a DNA encoding a polypeptide functionally equivalent to the above human

protein can be routinely selected by a person skilled in the art. For example, hybridization can be performed by conducting prehybridization at 68 degrees C for 30 min or longer using "Rapid-hyb buffer" (Amersham LIFE SCIENCE), adding a labeled probe, and warming at 68 degrees C for 1 hour or longer. The following washing step can be conducted, for example, in a low stringent condition. A low stringent condition is, for example, 42 degrees C, 2x SSC, 0.1% SDS, for example, 50 degrees C, 2x SSC, 0.1% SDS. In some embodiments, high stringent condition is used. A high stringent condition is, for example, washing 3 times in 2x SSC, 0.01% SDS at room temperature for 20 min, then washing 3 times in 1x SSC, 0.1% SDS at 37 degrees C for 20 min, and washing twice in 1x SSC, 0.1% SDS at 50 degrees C for 20 min. However, several factors for example, temperature and salt concentration can influence the stringency of hybridization and one skilled in the art can routinely adjust these and other factors to arrive at the desired stringency.

- [0056] In place of hybridization, a gene amplification method, for example, the polymerase chain reaction (PCR) method, can be utilized to isolate a DNA encoding a functional equivalent of the human NPTX2 polypeptide, using a primer synthesized based on the sequence information of the DNA (SEQ ID NO: 1) encoding the human NPTX2 polypeptide (SEQ ID NO: 2), examples of primer sequences are pointed out in Semi-quantitative RT-PCR in [EXAMPLE].
- [0057] Functional equivalents of the human NPTX2 polypeptide encoded by the DNA isolated through the above hybridization techniques or gene amplification techniques, normally have a high homology (also referred to as sequence identity) to the amino acid sequence of the human NPTX2 polypeptide. "High homology" (also referred to as "high sequence identity") typically refers to the degree of identity between two optimally aligned sequences (either polypeptide or polynucleotide sequences). Typically, high homology or sequence identity refers to homology of 40% or higher, for example, 60% or higher, for example, 80% or higher, for example, 85%, 90%, 95%, 98%, 99%, or higher. The degree of homology or identity between two polypeptide or polynucleotide sequences can be determined by following the algorithm (Wilbur WJ & Lipman DJ. Proc Natl Acad Sci U S A. 1983 Feb; 80 (3):726-30).
- [0058] Percent sequence identity and sequence similarity can be readily determined using conventional techniques such as the BLAST and BLAST 2.0 algorithms, which are described (Altschul SF, et al., J Mol Biol. 1990 Oct 5; 215 (3):403-10; Nucleic Acids Res. 1997 Sep 1;25(17):3389-402). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (on the worldwide web at ncbi.nlm.nih.gov/). The algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when

aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul et al, supra). These initial neighborhood word hits acts as seeds for initiating searches to find longer HSPs containing them.

[0059] The word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always >0) and N (penalty score for mismatching residues; always <0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached.

[0060] The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a word size (W) of 28, an expectation (E) of 10, M=1, N=-2, and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a word size (W) of 3, an expectation (E) of 10, and the BLOSUM62 scoring matrix (Henikoff S & Henikoff JG. Proc Natl Acad Sci U S A. 1992 Nov 15;89(22):10915-9).

[0061] Polypeptides useful in the context of the present invention can have variations in amino acid sequence, molecular weight, isoelectric point, the presence or absence of sugar chains, or form, depending on the cell or host used to produce it or the purification method utilized. Nevertheless, so long as it has any one of biological activities of the NPTX2 polypeptide (SEQ ID NO: 2), it is useful in the present invention.

[0062] The present invention also encompasses partial peptides of the NPTX2 polypeptide and their use in screening methods. A partial peptide of the NPTX2 polypeptide has an amino acid sequence specific to the NPTX2 polypeptide and is preferably composed of less than about 400 amino acids, usually less than about 200 and often less than about 100 amino acids, and at least 7 amino acids, preferably, 8 amino acids or more, 9 amino acids or more, 10 amino acids or more, 15 amino acids or more, or 20 amino acids or more.

[0063] The NPTX2 polypeptide and functional equivalent thereof used in the present invention can be obtained from nature as naturally occurring proteins via conventional purification methods or through chemical synthesis based on the selected amino acid sequence. For example, conventional peptide synthesis methods that can be adopted for the synthesis include:

- (1) Peptide Synthesis, Interscience, New York, 1966;
- (2) The Proteins, Vol. 2, Academic Press, New York, 1976;

- (3) Peptide Synthesis (in Japanese), Maruzen Co., 1975;
- (4) Basics and Experiment of Peptide Synthesis (in Japanese), Maruzen Co., 1985;
- (5) Development of Pharmaceuticals (second volume) (in Japanese), Vol. 14 (peptide synthesis), Hirokawa, 1991;
- (6) WO99/67288; and
- (7) Barany G. & Merrifield R.B., Peptides Vol. 2, "Solid Phase Peptide Synthesis", Academic Press, New York, 1980, 100-118.

[0064] Alternatively, the NPTX2 polypeptide and functional equivalent thereof can be obtained adopting any known genetic engineering methods for producing polypeptides (e.g., Morrison DA., et al., J Bacteriol. 1977 Oct;132(1):349-51; Clark-Curtiss JE & Curtiss R 3rd. Methods Enzymol. 1983;101:347-62). For example, first, a suitable vector comprising a polynucleotide encoding the objective polypeptide in an expressible form (e.g., downstream of a regulatory sequence comprising a promoter) is prepared, transformed into a suitable host cell, and then the host cell is cultured to produce the polypeptide. More specifically, a gene encoding the NPTX2, polypeptide or functional equivalent thereof is expressed in host (e.g., animal) cells and such by inserting the gene into a vector for expressing foreign genes, for example, pSV2neo, pcDNA I, pcDNA3.1, pCAGGS, or pCD8.

[0065] In addition to the protein of interest, the vector may also contain a promoter to include protein expression. Any commonly used promoters can be employed including, for example, the SV40 early promoter (Rigby in Williamson (ed.), Genetic engineering, vol. 3. Academic Press, London, 1982, 83-141), the EF- α promoter (Kim DW, et al. Gene. 1990 Jul 16;91(2):217-23), the CAG promoter (Niwa H, et al., Gene. 1991 Dec 15;108(2):193-9), the RSV LTR promoter (Cullen BR. Methods Enzymol. 1987;152:684-704), the SR α promoter (Takebe Y, et al., Mol Cell Biol. 1988 Jan;8(1):466-72), the CMV immediate early promoter (Seed B & Aruffo A. Proc Natl Acad Sci U S A. 1987 May;84(10):3365-9), the SV40 late promoter (Gheysen D & Fiers W. J Mol Appl Genet. 1982;1(5):385-94), the Adenovirus late promoter (Kaufman RJ, et al., Mol Cell Biol. 1989 Mar;9(3):946-58), the HSV TK promoter, and the like.

[0066] The introduction of the vector into host cells to express the gene encoding the NPTX2 polypeptide or functional equivalent thereof can be performed according to any methods, for example, the electroporation method (Chu G, et al., Nucleic Acids Res. 1987 Feb 11;15(3):1311-26), the calcium phosphate method (Chen C & Okayama H. Mol Cell Biol. 1987 Aug;7(8):2745-52), the DEAE dextran method (Lopata MA, et al., Nucleic Acids Res. 1984 Jul 25;12(14):5707-17; Sussman DJ & Milman G. Mol Cell Biol. 1984 Aug;4(8):1641-3), the Lipofectin method (Derijard B, et al., Cell. 1994 Mar 25;76(6):1025-37; Lamb BT, et al., Nat Genet. 1993 Sep;5(1):22-30; Rabindran

SK, et al., Science. 1993 Jan 8;259(5092):230-4), and the like.

The NPTX2 polypeptide and functional equivalent thereof can also be produced in vitro by using an in vitro translation system.

[0067] (2) Method for Diagnosing or Detecting Cancer

The present invention relates to the discovery that NPTX2 can serve as a diagnostic marker of cancer, finding utility in the detection and prognosis of cancers related thereto as well as in assessing and/or monitoring the efficacy or applicability of a cancer immunotherapy. As demonstrated herein, the expression level of the NPTX2 gene is significantly and specifically elevated in cancer tissues compared with corresponding normal tissues (Fig. 1A, Fig. 4). These results demonstrate the diagnostic utility of the NPTX2 gene as a tumor marker. Further, the serum levels of the NPTX2 polypeptide in subjects with cancer were significantly higher than those in healthy subjects (Fig. 3). Such high serum levels of the NPTX2 polypeptide can be detected even in subjects with earlier-stage cancers. Therefore, the NPTX2 polypeptide finds utility as a serum marker for cancer, including earlier-stage cancer.

[0068] In the context of the present invention, the term "diagnosing" is intended to encompass both predictions and likelihood analyses. Accordingly, it is an object of the present invention to provide a method for diagnosing or detecting cancer using the expression level of the NPTX2 gene as an index of cancer in a subject-derived biological sample, wherein an increased or elevated NPTX2 expression level in said sample as compared to a control level indicates the presence or suspicion of cancer cells in the tissue.

[0069] According to the present invention, an intermediate result for examining the condition of a subject can be provided. Such intermediate result can be combined with additional information to assist a doctor, nurse, or other practitioner to diagnose that a subject suffers from the disease. Alternatively, the present invention can be used to detect cancerous cells in a subject-derived tissue, and provide a doctor with useful information to diagnose that the subject suffers from the disease. In other words, the present invention provides a method of screening or identifying a subject that has a high probability of suffering from cancer by determining the expression level of the NPTX2 gene in a subject-derived biological sample.

[0070] Particularly preferred embodiments of the present invention are set forth below as items [1] to [15]:

[1] A method of diagnosing or detecting cancer in a subject, wherein the method comprises a step of determining an expression level of the NPTX2 gene in a subject-derived biological sample, wherein an increase of the expression level compared to a normal control level of the NPTX2 gene indicates that the subject suffers from cancer, or the presence of cancer in said subject, wherein said expression level is determined

by a method selected from the group consisting of:

(a) detecting an mRNA of the NPTX2 gene;

(b) detecting an NPTX2 polypeptide; and

(c) detecting a biological activity of an NPTX2 protein;

[2] The method of [1], wherein the expression level is at least 10 % greater than normal control level;

[3] The method of [1] or [2], wherein the expression level is determined by detecting the mRNA of the NPTX2 gene using an oligonucleotide that has a sequence complementary to a whole length or a part of the mRNA of the NPTX2 gene as a probe;

[4] The method of [1] or [2], wherein the expression level is determined by detecting the mRNA of the NPTX2 gene using oligonucleotides that has a sequence complementary to a part of the mRNA of the NPTX2 gene as a primer set;

[5] The method of [1] or [2], wherein the expression level is determined by detecting the NPTX2 polypeptide using an antibody against the NPTX2 polypeptide;

[6] The method of any one of [1] to [5], wherein the subject-derived biological sample is a bodily tissue sample or blood sample;

[7] The method of [6], wherein the bodily tissue sample is collected from a suspicious area that might be cancerous;

[8] The method of [7], wherein the bodily tissue sample is a lung tissue sample, breast tissue sample, cervical tissue sample or colon tissue sample;

[9] The method of [6], wherein the expression level is determined by detecting the NPTX2 polypeptide in a subject-derived blood sample;

[10] The method of [9], wherein the NPTX2 polypeptide is detected by the method comprising steps of:

(i) contacting an antibody against the NPTX2 polypeptide with the subject-derived blood sample; and

(ii) detecting the binding between the antibody and the NPTX2 polypeptide in the subject-derived blood sample;

[11] The method of [10], wherein the NPTX2 polypeptide is detected by Enzyme-Linked ImmunoSorbent Assay (ELISA);

[12] The method of any one of [9] to [11], wherein the subject-derived blood sample is a subject-derived serum sample;

[13] The method of any one of [9] to [12], wherein the method further comprises a step of detecting at least one another serum tumor marker in the subject-derived blood sample, wherein the cancer is judged to be present or the subject is judged to suffer from cancer when either (a) the level of the NPTX2 polypeptide is higher than a normal control level of the NPTX2 polypeptide, or (b) the level of the serum tumor marker is higher than a normal control level of the serum tumor marker, or (c) both;

[14] The method of [13], wherein the another serum tumor marker is selected from the group consisting of CEA (carcinoembryonic antigen), CYFRA (cytokeratin 19 fragment) and proGRP (progastrin releasing peptide);

[15] The method of any one of [1] to [13], wherein the cancer is lung cancer, breast cancer, cervical cancer or colon cancer; and

[16] The method of [14], wherein the cancer is lung cancer.

[0071] The method of diagnosing cancers is described in more detail below.

A subject to be diagnosed is preferably a mammal. Exemplary mammals include, but are not limited to, e.g., human, non-human primate, mouse, rat, dog, cat, horse, and cow.

The method of the present invention preferably utilizes a biological sample collected from a subject to be diagnosed cancer or assessed the presence of cancer. Any biological materials can be used as such subject-derived biological samples so long as they comprise either or both of the transcription product and translation product of the NPTX2 gene. Examples of suitable subject-derived biological samples include, but are not limited to, bodily tissues and fluids, for example, blood (e.g. serum, whole blood or plasma), sputum, urine and pleural effusion. In some embodiments, the subject-derived biological sample contains a cell population comprising an epithelial cell, for example, a cancerous epithelial cell or an epithelial cell derived from tissue suspected to be cancerous. Alternatively, a subject-derived biological sample may be a bodily tissue sample collected from a suspicious area that might be cancerous. Further, if necessary, cells can be purified from the obtained bodily tissues and fluids, and then used as subject-derived biological samples.

[0072] Any cancer that overexpresses the NPTX2 gene can be diagnosed or detected by the method of the present invention. Preferred cancers to be diagnosed or detected are lung cancer, breast cancer, cervical cancer or colon cancer. In the context of the present invention, lung cancer includes small-cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). NSCLC includes squamous cell carcinoma (SCC), adenocarcinoma (ADC) and large cell carcinoma (LCC). In order to diagnose or detect such cancers, a subject-derived biological sample is preferably collected from the following organs: lung for lung cancer; breast for breast cancer; uterine cervix for cervical cancer; and colon for colon cancer.

[0073] Preferably, a subject-derived biological sample is collected from an area suspected to be cancerous in the aforementioned organs. Therefore, a lung tissue sample, breast tissue sample, cervical tissue sample or colon tissue sample collected from a suspicious area can be preferably used as a subject-derived biological sample for diagnosis or detection of lung cancer, breast cancer, cervical cancer or colon cancer, respectively. Such tissue samples can be obtained by biopsy or surgical resection.

[0074] According to the present invention, the expression level of the NPTX2 gene in the subject-derived biological sample is determined and then correlated to a particular healthy or disease state by comparison to a control sample. The expression level of the NPTX2 gene can be determined at the transcription product (i.e., mRNA) level, using methods known in the art. For example, the mRNA of the NPTX2 gene can be quantified using a probe by hybridization method (e.g. Northern blot analysis). The detection can be carried out on a chip or an array. The use of an array is suitable to detect the expression levels of a plurality of genes (e.g., various cancer specific genes) including the NPTX2 gene. Those skilled in the art can prepare such probes utilizing the known sequence information for the NPTX2 gene (e.g., SEQ ID NO: 1). For example, the cDNA of NPTX2 gene can be used as a probe. If necessary, the probe can be labeled with a suitable label, for example, dyes, fluorescent and isotopes, and the expression level of the NPTX2 gene can be detected as the intensity of the hybridized labels.

[0075] Alternatively, the transcription product of the NPTX2 gene can be quantified using primers by amplification-based detection methods (e.g., RT-PCR). Such primers can also be prepared based on the available sequence information of the NPTX2 gene. For example, the primers (SEQ ID NO: 3 and 4) used in the Examples can be employed for the detection by RT-PCR or Northern blot analysis, but the present invention is not restricted thereto.

A probe or primer for use in the context of the method of the present invention will hybridize under stringent, moderately stringent, or low stringent conditions to the mRNA of the NPTX2 gene. Details of "stringent conditions" are described in the section entitled "(1) Gene and Polypeptide".

[0076] Alternatively, diagnosis may involve detection of an NPTX2 translation product (i.e., polypeptide or protein), using methods known in the art. For example, the quantity of the NPTX2 polypeptide can be determined using an antibody against the NPTX2 polypeptide and correlated to a disease or normal state. Herein, "antibody against the NPTX2 polypeptide" refers to an antibody that is raised against the NPTX2 polypeptide or fragment thereof and specifically binds to the NPTX2 polypeptide. Herein, "specifically bind to the NPTX2 polypeptide" means that an antibody binds to the NPTX2 polypeptide, but not detectably to other polypeptides. The quantity of the translation products/proteins may be determined using, for example immunoassay methods that use an antibody against the NPTX2 polypeptide.

[0077] Antibodies against the NPTX2 polypeptide for use in the context of the methods of the present invention can be monoclonal or polyclonal. Furthermore, any immunogenic fragments or modification (e.g., chimeric antibody, scFv, Fab, F(ab')₂, Fv, etc.) of such antibody can be used for the detection of the NPTX2 polypeptide, so long as the

fragment retains the binding ability to the NPTX2 polypeptide. Methods to prepare these kinds of antibodies are well known in the art, and any method can be employed in the present invention to prepare such antibodies and fragments thereof.

[0078] Alternatively, one may determine the expression level of the NPTX2 gene based on the translation product, for example through study of the intensity of staining observed via immunohistochemical analysis using an antibody against the NPTX2 polypeptide. More particularly, the observation of strong staining indicates increased presence of the NPTX2 polypeptide and at the same time high expression level of the NPTX2 gene (see, "Immunohistochemistry and Tissue Microarray" in "EXAMPLES").

[0079] Alternatively, the NPTX2 gene expression level may be correlated to cell proliferation activity. As discovered herein, inhibiting the expression of NPTX2 gene leads to suppression of cell growth in lung cancer cells; as such, the NPTX2 protein is presumed to promote cell proliferation. Thus, to determine the cell proliferation enhancing activity of NPTX2 protein, a cell is first cultured in the presence of a biological sample. Then, by detecting the speed of proliferation, or by measuring the cell cycle or the colony forming ability, the cell NPTX2 enhancing activity of the biological sample can be determined and the relative NPTX2 expression correlated thereto.

[0080] In addition to the expression level of the NPTX2 gene, the expression levels of other cancer-associated genes, for example genes known to be differentially expressed in cancers, such as CEA, CYFRA and proGRP for lung cancer, can also be determined to improve the accuracy of the diagnosis

In the context of the present invention, gene expression levels are deemed to be "altered" or "increased" when the gene expression changes or increases by, for example, 10%, 25%, or 50% from, or at least 0.1 fold, at least 0.2 fold, at least 0.5 fold, at least 2 fold, at least 5 fold, or at least 10 fold or more compared to a control level. Accordingly, the expression level of cancer marker genes including NPTX2 gene in a biological sample can be considered to be increased if it increases from a control level of the corresponding lung cancer marker gene by, for example, 10%, 25%, or 50%; or increases to more than 1.1 fold, more than 1.5 fold, more than 2.0 fold, more than 5.0 fold, more than 10.0 fold, or more.

[0081] The control level can be determined at the same time with the test subject-derived biological sample by using a sample(s) previously collected and stored from a subject/ subjects whose disease state (cancerous or non-cancerous) is/are known. Alternatively, the control level can be determined by a statistical method based on the results obtained by analyzing previously determined expression level(s) of the NPTX2 gene in samples from subjects whose disease state are known. Furthermore, the control level can be a database of expression patterns from previously tested cells. Moreover,

according to an aspect of the present invention, the expression level of the NPTX2 gene in a subject-derived biological sample can be compared to multiple control levels, which control levels are determined from multiple reference samples. In some embodiments, a control level determined from a reference sample derived from a tissue type similar to that of the subject-derived biological sample is used. In some embodiments, the standard value of the expression levels of the NPTX2 gene in a population with a known disease state is used. The standard value can be obtained by any method known in the art. For example, a range of mean $\pm$ 2 S.D. or mean $\pm$ 3 S.D. can be used as standard value.

In the context of the present invention, methods for detecting or identifying cancer in a subject or cancer cells in a subject-derived sample begin with a determination of NPTX2 gene expression level. The expression level may be determined by any of the aforementioned techniques. Once determined, then, this value may be compared to a control level.

[0082] In the context of the present invention, the phrase "control level" refers to the expression level of a test gene detected in a control sample and encompasses both a normal control level and a cancer control level. The phrase "normal control level" refers to a level of gene expression detected in a normal healthy individual or in a population of individuals known not to be suffering from cancer. A normal individual is one with no clinical symptom of cancer. A normal control level can be determined using a normal cell obtained from a non-cancerous tissue. A "normal control level" may also be the expression level of a test gene detected in a normal healthy tissue or cell of an individual or population known not to be suffering from lung cancer or esophageal cancer. On the other hand, the phrase "cancer control level" refers to an expression level of a test gene detected in the cancerous tissue or cell of an individual or population suffering from cancer.

[0083] When the expression level of the NPTX2 gene in a subject-derived biological sample is increased compared to the normal control level or is similar to the cancerous control level, the subject can be diagnosed to be suffering from cancer. Furthermore, in case where the expression levels of the NPTX2 gene are compared, a similarity in the expression level between the subject-derived biological sample and the reference which is cancerous indicates that the subject is suffering from cancer.

[0084] Difference between the expression levels of cancer marker genes including NPTX2 gene in a subject-derived biological sample and the control level of these genes can be normalized to the expression level of control nucleic acids, e.g., housekeeping genes, whose expression levels are known not to differ depending on the cancerous or non-cancerous state of the cell. Exemplary control genes include, but are not limited to, beta-actin, glyceraldehyde 3 phosphate dehydrogenase, and ribosomal protein P1.

Moreover, present invention also provides the NPTX2 polypeptide as a novel serum tumor marker. In cases where the NPTX2 polypeptide is used as a serum tumor marker, a subject-derived blood sample is prepared as a subject-derived biological sample.

[0085] Accordingly, the present invention also provides a method of diagnosing or detecting cancer in a subject, including a step of detecting the NPTX2 polypeptide in a subject-derived blood sample, wherein an increase of the level of the NPTX2 polypeptide detected in the subject-derived blood sample compared to a normal control level of the NPTX2 polypeptide indicates that the subject suffers from cancer, or the presence of cancer in said subject.

In the present invention, any blood sample can be used as a subject-derived blood sample. For example, a whole blood sample, a serum sample and a plasma sample may be used as a subject-derived blood sample. In preferred embodiments, a subject-derived blood sample can be a subject-derived serum sample.

In the present invention, the level of the NPTX2 polypeptide detected in a subject-derived blood sample is usually shown as the concentration of the NPTX2 polypeptide in the subject-derived blood sample after correcting the corpuscular volume.

[0086] One of skilled in the art will recognize that the percentage of corpuscular volume in blood varies greatly between individuals. For example, the percentage of erythrocytes in the whole blood is very different between men and women. Furthermore, differences between individuals cannot be ignored. Therefore, the apparent concentration of a substance in a whole blood sample including corpuscular components varies greatly depending on the percentage of corpuscular volume. For example, even if the concentration in serum is the same, the measured value for a sample with a large amount of corpuscular component will be lower than the value for a sample with a small amount of corpuscular component. Therefore, to compare the measured values of components in a blood sample, values for which the corpuscular volume has been corrected are usually used.

[0087] Accordingly, where a whole blood sample is used as a subject-derived blood sample, one must correct for the effect from corpuscular volume after the detection (or measurement) of the NPTX2 polypeptide in the whole blood sample. Methods for measuring a corpuscular volume and correcting the effect from corpuscular volume are known in the art. For example, the value determined in whole blood can be corrected by determining the percentage of corpuscular volume in the whole blood sample.

[0088] On the other hand, in cases where a blood sample obtained by removing corpuscular components from a whole blood sample, such as serum or plasma, is used as a subject-derived blood sample, it is not necessary to correct the effect from corpuscular volume because such samples do not contain corpuscular components. Methods for obtaining a

serum sample and a plasma sample from a whole blood sample are well-known in the art.

A subject-derived blood sample may be diluted before the detection of the NPTX2 polypeptide.

The detection of the NPTX2 polypeptide in a subject-derived blood sample can be performed by any methods well-known in the art.

[0089] For example, immunoassay, liquid chromatography, surface plasmon resonance (SPR), mass spectrometry, or the like can be used. In mass spectrometry, proteins can be quantitated by using a suitable internal standard. For example, an isotope-labeled NPTX2 polypeptide can be used as the internal standard. The concentration of the NPTX2 polypeptide in a subject-derived blood sample can be determined from the peak intensity of the NPTX2 polypeptide in the subject-derived blood sample and that of the internal standard. Generally, the matrix-assisted laser desorption/ionization (MALDI) method is used for mass spectrometry of proteins. With an analysis method that uses mass spectrometry or liquid chromatography, the NPTX2 polypeptide in a subject-derived blood sample can be analyzed simultaneously with other serum tumor markers (e.g. CEA, CYFRA or proGRP).

[0090] A preferred method for detection of the NPTX2 polypeptide in a subject-derived blood sample is an immunoassay method. Antibodies against the NPTX2 polypeptide to be used in immunoassay methods can be prepared by methods well-known in the art. Exemplary methods for preparation of antibodies against the NPTX2 polypeptide are described below, but the methods are not restricted thereto.

[0091] The NPTX2 polypeptide or immunogenic fragment thereof as an immunogen can be prepared based on the amino acid sequence data of the NPTX2 polypeptide (e.g., SEQ ID NO: 2). For example, the fragment peptide of the NPTX2 polypeptide used as an immunogen can be easily synthesized using a peptide synthesizer. The synthesized peptide may be linked to a carrier protein.

[0092] Keyhole limpet hemocyanin, myoglobin, albumin, and the like can be used as the carrier protein. Preferred carrier proteins are KLH, bovine serum albumin, and such. The maleimidobenzoyl-N-hydroxysuccinimide ester method (hereinafter abbreviated as the MBS method) and the like may generally be used to link synthesized peptides to carrier proteins.

Specifically, a cysteine is introduced into the synthesized peptide and the peptide is crosslinked to KLH by MBS using the cysteine's SH group. The cysteine residue may be introduced at the N-terminus or C-terminus of the synthesized peptide.

[0093] Alternatively, the NPTX2 polypeptide or immunogenic fragment thereof as an immunogen can be prepared based on the nucleotide sequence data of the NPTX2 gene (e.g., SEQ ID NO:1). DNA encoding the NPTX2 polypeptide or fragment thereof can

be prepared from mRNAs extracted from cells expressing the NPTX2 gene using a primer set or a probe designed based on the nucleotide sequence data of the NPTX2 gene. Alternatively, commercially available cDNA libraries can be used as the cloning source. DNAs having the necessary nucleotide sequence for the production of the NPTX2 polypeptide or fragment thereof can be cloned into an appropriate expression vector. And then, by introducing the vector into an appropriate host cell and culturing the host cell, the NPTX2 polypeptide or the fragment thereof can be produced by the host cell. Purification of the NPTX2 polypeptide or fragment thereof from the host cell can be conducted by methods well-known in the art.

[0094] In the context of the present invention, immunologically active fragments originated from the complete NPTX2 polypeptide, may also be used as immunogens, as well as the complete NPTX2 polypeptide. Any immunologically active fragments of the NPTX2 polypeptide can be used as immunogens so long as such fragments retain the ability to raise binding antibodies.

[0095] The NPTX2 polypeptide or immunogenic fragment thereof prepared as an immunogen is mixed with a suitable adjuvant and used to immunize an animal. Examples of adjuvants include, but are not limited to, Freund's complete adjuvant and incomplete adjuvant. The immunization procedure is repeated at appropriate intervals until an increase in the antibody titer is confirmed. There are no particular limitations on the immunized animals. Preferably, animals commonly used for immunization such as mice, rats, or rabbits can be used.

[0096] When obtaining the antibodies as monoclonal antibodies, animals that are advantageous for their production may be used. For example, many mice myeloma cell lines are known for cell fusion, as are techniques for establishing hybridomas with a high probability. Accordingly, mice are a particularly preferred animal for use in immunization processes to obtain monoclonal antibodies.

[0097] In addition to immunization of animals, immunological sensitization of cultured immunocompetent cells may be used in order to obtain antibody-producing cells. Antibody-producing cells obtained by these methods are transformed and cloned. Methods for transforming antibody-producing cells to obtain monoclonal antibodies are not limited to cell fusion. For example, clonable transformants may be obtained by known means using virus infection.

[0098] Antibody-producing cells (e.g., hybridomas) that produce the monoclonal antibodies can be screened based on their reactivity to the NPTX2 polypeptide or fragment thereof. Specifically, antibody-producing cells are selected by using as an index the binding activity to the NPTX2 polypeptide or fragment thereof.

Monoclonal antibodies against the NPTX2 polypeptide can be obtained by culturing the established hybridomas under suitable conditions and collecting the produced an-

tibodies. When the hybridomas are homohybridomas, they can be cultured in vivo by inoculating them intraperitoneally in syngeneic animals. In this case, monoclonal antibodies are collected as ascites fluid. When heterohybridomas are used, they can be cultured in vivo using nude mice as a host.

[0099] In addition to in vivo cultures, hybridomas can be also cultured ex vivo, in a suitable culture environment. For example, basal media such as RPMI 1640 and DMEM are generally used as the medium for hybridomas. Additives such as animal sera can be added to these media to maintain the antibody-producing ability to a high level. When hybridomas are cultured ex vivo, monoclonal antibodies can be collected as a culture supernatant. Culture supernatants can be collected by separating from cells after culturing, or by continuously collecting while culturing using a culture apparatus that uses a hollow fiber.

[0100] Monoclonal antibodies against the NPTX2 polypeptide can be prepared from monoclonal antibodies collected as ascites fluid or culture supernatants, by separating immunoglobulin fractions by saturated ammonium sulfate precipitation and further purifying by gel filtration, ion exchange chromatography, or such. In addition, if the monoclonal antibodies are IgGs, purification methods based on affinity chromatography with a protein A or protein G column are effective.

[0101] On the other hand, to obtain antibodies as polyclonal antibodies, blood may be drawn from animals whose antibody titer increased after immunization, and the serum may then be separated to obtain an anti-serum. Antibodies against the NPTX2 polypeptide can be prepared from the obtained anti-serum by combining immunoaffinity chromatography using the NPTX2 polypeptide or fragment thereof as a ligand with immunoglobulin purification.

[0102] In immunoassay methods, a subject-derived blood sample is contacted with an antibody against the NPTX2 polypeptide. When an antibody against the NPTX2 polypeptide contact the NPTX2 polypeptide in the subject-derived blood sample, the antibody bind to the antigenic determinant (epitope) of the NPTX2 polypeptide. In immunoassay methods, such binding of the antibody against the NPTX2 polypeptide to the NPTX2 polypeptide is detected as an index of the NPTX2 polypeptide in the subject-derived blood sample.

[0103] The binding of antibodies to antigens can be detected by various immunoassay principles. Immunoassays can be broadly categorized into heterogeneous analysis methods and homogeneous analysis methods. To maintain the sensitivity and specificity of immunoassays to a high level, the use of monoclonal antibodies is desirable. Further details of immunoassay methods are described bellow.

[0104] First, heterogeneous immunoassays are described. In heterogeneous immunoassays, the step of separating antibodies that bind to the NPTX2 polypeptide from antibodies

that do not bind to the NPTX2 polypeptide is required. To facilitate the separation, immobilized reagents are generally used. For example, a solid phase onto which antibodies against the NPTX2 polypeptide have been immobilized is prepared (immobilized antibodies). By contacting a subject-derived blood sample with the immobilized antibodies, the NPTX2 polypeptide in the subject-derived blood sample binds to the immobilized antibodies. After removing the liquid phase and washing the solid phase as necessary, secondary antibodies are further reacted thereto.

[0105] When the solid phase is separated from the liquid phase and further washed, as necessary, secondary antibodies remain on the solid phase in proportion to the concentration of the NPTX2 polypeptide. By labeling the secondary antibodies, the NPTX2 polypeptide can be quantitated by measuring the signal derived from the label.

[0106] Any method may be used to bind the antibodies to the solid phase. For example, antibodies can be physically adsorbed to a hydrophobic material such as polystyrene. Alternatively, antibodies can be chemically bound to a variety of materials having functional groups on their surfaces. Furthermore, antibodies labeled with a binding ligand can be bound to a solid phase by trapping them using a binding partner of the ligand. Combinations of a binding ligand and its binding partner include avidin-biotin and such. The solid phase and antibodies can be conjugated at the same time or before the reaction between the primary antibodies and the NPTX2 polypeptide.

Similarly, the secondary antibodies need not be directly labeled. That is, they can be indirectly labeled using antibodies against the secondary antibodies or using binding reactions such as that of avidin-biotin.

The concentration of the NPTX2 polypeptide in a subject-derived blood sample may be determined based on the signal intensities obtained using standard samples with known concentrations of the NPTX2 polypeptide.

[0107] Any antibody can be used as an immobilized antibody and a secondary antibody for heterogeneous immunoassays, so long as it retains an antigen-binding site that recognizes the NPTX2 polypeptide. Thus, in addition to naturally occurring antibodies, modified antibodies and immunogenic antibody fragments can be also used as immobilized antibodies and secondary antibodies. Further, the antibodies may be monoclonal antibodies, polyclonal antibodies, or a mixture or combination of both. For example, a combination of monoclonal antibodies and polyclonal antibodies may be used as a combination of an immobilized antibody and a secondary antibody. Alternatively, two kinds of monoclonal antibodies that recognize different epitopes of the NPTX2 polypeptide may be used as a combination of an immobilized antibody and a secondary antibody.

[0108] Since the antigens to be measured are sandwiched by antibodies, heterogeneous immunoassays described above are commonly referred to as "sandwich" methods. Since

sandwich methods excel in the measurement sensitivity and the reproducibility, they are preferably used in the method of the present invention.

- [0109] The principle of competitive inhibition reactions can also be applied to heterogeneous immunoassays. Specifically, competitive inhibition reaction systems are immunoassays based on the phenomenon where the antigen in a sample competitively inhibits the binding between the antigen with a known concentration and an antibody. The concentration of the antigen in the sample can be determined by detecting the labeled antigen with a known concentration that binds to the antibody or the labeled antibody that directly or indirectly binds to the antigen with a known concentration.
- [0110] In competitive reaction systems, an antigen in a sample and the antigen with a known concentration are simultaneously reacted to the antibody. Alternatively, an antigen in a sample and the antigen with a known concentration may be sequentially reacted to the antibody. In both types of reaction systems, reaction systems that excel in the operability can be constructed by setting either one of the antigens with a known concentration used as a reagent component or the antibody as the labeled component, and the other one as the immobilized reagent.
- [0111] Radioisotopes, fluorescent substances, luminescent substances, substances having an enzymatic activity, macroscopically observable substances, magnetically observable substances, and such are used as labeling substances in these heterogeneous immunoassays. Specific examples of those labeling substances are shown below.
- [0112] Substances having an enzymatic activity:
peroxidase,
alkaline phosphatase,
urease, catalase,
glucose oxidase,
lactate dehydrogenase, or
amylase, etc.
Fluorescent substances:
fluorescein isothiocyanate,
tetramethylrhodamine isothiocyanate,
substituted rhodamine isothiocyanate, or
dichlorotriazine isothiocyanate, etc.
Radioisotopes:
tritium,
¹²⁵I, or
¹³¹I, etc.
- [0113] Among labeling substances, non-radioactive labels such as enzymes are an advantageous label in terms of safety, operability, sensitivity, and such. Enzymatic labels

can be linked to the NPTX2 polypeptide or the fragment thereof, or antibodies against the NPTX2 polypeptide, by known methods such as the periodic acid method or maleimide method.

- [0114] As the solid phase for heterogeneous immunoassays, beads, inner walls of a container, fine particles, porous carriers, magnetic particles, or such can be used. For example, solid phases formed using materials such as polystyrene, polycarbonate, polyvinyl toluene, polypropylene, polyethylene, polyvinyl chloride, nylon, poly-methacrylate, latex, gelatin, agarose, glass, metal, ceramic, or such can be used. Solid materials in which functional groups to chemically bind antibodies and such have been introduced onto the surface of the above solid materials are also known. Known binding methods, including chemical binding such as poly-L-lysine or glutaraldehyde treatment and physical adsorption, can be applied for binding of solid phases and antibodies (or antigens).

Although the steps of separating the solid phase from the liquid phase and the washing steps are required in all heterogeneous immunoassays exemplified herein, these steps can easily be performed using the immunochromatography method, which is a variation of the sandwich method.

- [0115] Specifically, antibodies to be immobilized can be immobilized onto porous carriers capable of transporting a sample solution by the capillary phenomenon, then a mixture of a sample and labeled antibodies is deployed therein by this capillary phenomenon. During deployment, the antigen in the sample reacts with the labeled antibodies, and when it further contacts the immobilized antibodies, it is trapped at that location. The labeled antibodies that do not react with the antigen pass through, without being trapped by the immobilized antibodies.

- [0116] As a result, the presence of the antigen can be detected using, as an index, the signals of the labeled antibodies that remain at the location of the immobilized antibodies. If the labeled antibodies are maintained upstream in the porous carrier in advance, all reactions can be initiated and completed by just dripping in the sample solutions, and an extremely simple reaction system can be constructed. In the immunochromatography method, labeled components that can be distinguished macroscopically, such as colored particles, can be combined to construct an analytical device that does not even require a special reader.

- [0117] Furthermore, in the immunochromatography method, the detection sensitivity for the antigen can be adjusted. For example, by adjusting the detection sensitivity near the cutoff value described below, the aforementioned labeled components can be detected when the cutoff value is exceeded. By using such a device, whether a subject is positive or negative can be judged very simply. By adopting a constitution that allows a macroscopic distinction of the labels, necessary examination results can be obtained

by simply applying blood samples to the device for immunochromatography.

[0118] Various methods for adjusting the detection sensitivity of the immunochromatography method are known in the art. For example, a second immobilized antibody for adjusting the detection sensitivity can be placed between the position where samples are applied and the immobilized antibodies (Japanese Patent Application Kokai Publication No. (JP-A) H06-341989 (unexamined, published Japanese patent application)). The antigen in the sample is trapped by the second immobilized antibody while deploying from the position where the sample was applied to the position of the first immobilized antibody for label detection. After the second immobilized antibody is saturated, the antigen can reach the position of the first immobilized antibody located downstream. As a result, when the concentration of the antigen in the sample exceeds a predetermined concentration, the antigen bound to the labeled antibody is detected at the position of the first immobilized antibody.

[0119] Next, homogeneous immunoassays are described. As opposed to heterogeneous immunoassays that require a separation of the reaction solutions as described above, homogeneous immunoassays not require such separation step. Homogeneous analysis methods allow the detection of antigen-antibody reaction products without their separation from the reaction solutions.

[0120] A representative homogeneous analysis method is the immunoprecipitation reaction, in which an antigen is quantitatively analyzed by examining precipitates produced following an antigen-antibody reaction. Polyclonal antibodies are generally used for the immunoprecipitation reactions. When monoclonal antibodies are applied, multiple types of monoclonal antibodies that bind to different epitopes of the antigen are preferably used. The products of precipitation reactions that follow the immunological reactions can be macroscopically observed or can be optically measured for conversion into numerical data.

[0121] The immunological particle agglutination reaction, which uses as an index the agglutination by antigens of antibody-sensitized fine particles, is a common homogeneous analysis method. As in the aforementioned immunoprecipitation reaction, polyclonal antibodies or a combination of multiple types of monoclonal antibodies can be used in this method as well. Fine particles can be sensitized with antibodies through sensitization with a mixture of antibodies, or they can be prepared by mixing particles sensitized separately with each antibody. Fine particles obtained in this manner gives matrix-like reaction products upon contact with the antigen. The reaction products can be detected as particle aggregation. Particle aggregation may be macroscopically observed or can be optically measured for conversion into numerical data.

[0122] Immunological analysis methods based on energy transfer and enzyme channeling are also known as homogeneous immunoassays. In methods utilizing energy transfer,

different optical labels having a donor/acceptor relationship are linked to multiple antibodies that recognize adjacent epitopes on an antigen. When an immunological reaction takes place, the two parts approach and an energy transfer phenomenon occurs, resulting in a signal such as quenching or a change in the fluorescence wavelength. On the other hand, enzyme channeling utilizes labels for multiple antibodies that bind to adjacent epitopes, in which the labels are a combination of enzymes having a relationship such that the reaction product of one enzyme is the substrate of another enzyme. When the two parts approach due to an immunological reaction, the enzyme reactions are promoted; therefore, their binding can be detected as a change in the enzyme reaction rate.

[0123] Among immunoassays described above, ELISA methods can preferably used for the detection of the NPTX2 polypeptide in a subject-derived blood sample. ELISA methods for the detection of the NPTX2 polypeptide may be sandwich ELISA methods or competitive ELISA methods. The principles of those ELISA methods are described above in the description of heterogeneous immunoassays. Further, those ELISA methods are well-known in the art.

[0124] According to the present invention, a concentration of the NPTX2 polypeptide detected in a subject-derived blood sample is compared with a normal control level of the NPTX2 polypeptide. This "normal control level" is the same meaning as described above. In cases where the NPTX2 polypeptide in a subject-derived blood sample is detected as a tumor marker, a normal control level is preferably a concentration of the NPTX2 polypeptide found in a blood sample obtained from an individual or population not suffering from cancer. As described above, reference samples are samples similar in nature to a test sample. Thus, if a test sample is a subject-derived serum sample, the reference sample is preferably a serum sample obtained from an individual or population not suffering from cancer. The concentrations of NPTX2 polypeptide in a subject-derived blood sample and in a reference sample may be determined at the same time.

[0125] Alternatively, the standard value of the blood concentration of the NPTX2 polypeptide may be used as a normal control level. Such standard value can be determined statistically using samples previously collected from population whose disease state are known as described above.

The standard value may also be set based on the actual blood concentration of the NPTX2 polypeptide in blood samples derived from subjects with cancer and subjects without cancer. Generally, standard values set this way minimize the percentage of false positives, and are selected from a range of values satisfying conditions that can maximize detection sensitivity. In this case, the standard values are usually referred to as "cut off value". Herein, the percentage of false positives refers to a percentage of

subjects whose blood concentration of the NPTX2 polypeptide is higher than a standard value (cut off value) in healthy subjects. On the contrary, the percentage of subjects whose blood concentration of the NPTX2 polypeptide is lower than a standard value (cut off value) in a healthy population indicates specificity. That is, the sum of the false positive percentage and the specificity is always 1. The sensitivity refers to the percentage of subjects whose blood concentration of the NPTX2 polypeptide is higher than a standard value (cut off value) in subjects with cancer.

[0126] The percentage of subjects with cancer in subjects whose the NPTX2 polypeptide concentration is higher than a standard value (cut off value) represents the positive predictive value. On the other hand, the percentage of healthy subjects in subjects whose the NPTX2 polypeptide concentration is lower than a standard value (cut off value) represents the negative predictive value. The relationship between these values is summarized in Table 1. As the relationship shown below indicates, each of the values for sensitivity, specificity, positive predictive value, and negative predictive value, which are indexes for evaluating the diagnostic accuracy for cancer, varies depending on the standard value (cut off value) for judging the level of the blood concentration of NPTX2 polypeptide in a blood sample.

[0127] [Table 1]

Blood concentration of NPTX2 polypeptide	subjects with cancer	healthy subjects	
High	a: True positive	b: False positive	Positive predictive value $a/(a+b)$
Low	c: False negative	d: True negative	Negative predictive value $d/(c+d)$
	Sensitivity $a/(a+c)$	Specificity $d/(b+d)$	

[0128] As mentioned above, a standard value (cut off value) is usually set such that the false positive ratio is low and the sensitivity is high. However, as also apparent from the relationship shown above, there is a trade-off between the false positive ratio and sensitivity. That is, if the standard value (cut off value) is decreased, the sensitivity increases. However, since the false positive ratio also increases, it is difficult to satisfy the conditions to have a "low false positive ratio". Considering this situation, for example, values that give the following predicted results may be selected as the preferred standard values (cut off values).

Standard values (cut off values) for which the false positive ratio is 50% or less (that is, standard values (cut off values) for which the specificity is not less than 50%).
Standard values (cut off values) for which the sensitivity is not less than 20%.

[0129] Alternatively, the standard values (cut off values) can be set using a receiver operating characteristic (ROC) curve. An ROC curve is a graph that shows the sensitivity on the vertical axis and the false positive ratio (that is, "1 - specificity") on the

horizontal axis. In the present invention, an ROC curve can be obtained by plotting the changes in the sensitivity and the false positive ratio, which were obtained after continuously varying the standard value (cut off value) for determining the high/low degree of the blood concentration of the NPTX2 polypeptide.

[0130] The "standard value (cut off value)" for obtaining the ROC curve is a value temporarily used for the statistical analyses. The "standard value (cut off value)" for obtaining the ROC curve can be generally continuously varied within a range that is allowed to cover all selectable standard values (cut off value). For example, the standard value (cut off value) can be varied between the smallest and largest measured NPTX2 polypeptide values in an analyzed population.

[0131] Based on the obtained ROC curve, a preferred standard value (cut off value) can be selected from a range that satisfies the above-mentioned conditions. In the present invention, the standard value (cut off value) of the blood concentration of the NPTX2 polypeptide may be set at, for example, 3 to 11 U/ml, preferably 4 to 10 U/ml, more preferably 5 to 9 U/ml, further more preferably 6 to 8 U/ml. Further more preferably, the standard value (cut off value) of the blood concentration of the NPTX2 polypeptide may be set at 7 to 7.5 U/ml (e.g., 7.3 U/ml).

[0132] In the methods of the present invention, the blood concentrations of other serum tumor markers (e.g., CEA, CYFRA, proGRP etc.) may be determined, in addition to the blood concentration of the NPTX2 polypeptide.

For example, carcinoembryonic antigen (CEA) is a frequently studied tumor marker of cancer including lung cancer, especially adenocarcinoma. Cytokeratin 19-fragment (CYFRA) is a useful marker in lung carcinomas, especially non-small cell lung cancer (NSCLC). Progastrin releasing peptide (proGRP) is also a useful marker in lung carcinomas, especially small cell lung cancer (SCLC).

[0133] As shown in Examples, the NPTX2 polypeptide can be a better serum tumor marker than these conventional serum tumor markers (e.g., Fig. 3B). Furthermore, combination of the NPTX2 polypeptide and those conventional serum tumor markers could improve the accuracy of diagnosis for cancer (see "Combination assay of NPTX2, CEA, CYFRA and proGRP as tumor markers" in "Examples").

[0134] Specifically, the present invention also provides a method for diagnosing or detecting cancer in a subject, including steps of:

- (a) detecting the NPTX2 polypeptide in a subject-derived blood sample;
- (b) detecting the CEA in the subject-derived blood sample;
- (c) judging that the subject suffers from cancer or that cancer is present in the subject, when either that the level of the NPTX2 polypeptide is higher than a normal control level of the NPTX2 polypeptide, or that the CEA level is higher than a normal control level of CEA, or both.

[0135] As mentioned above, each of normal control levels of the NPTX2 polypeptide and CEA may be each of cut off values of the NPTX2 polypeptide and CEA.

The combination of the NPTX2 polypeptide and CEA is preferably applied to diagnosis or detection of lung cancer, especially adenocarcinoma (ADC). In the preferred embodiments, when the level of the NPTX2 polypeptide is higher than its normal control level or the CEA level is higher than its normal control level, the subject may be judged to have a high risk of cancer.

[0136] In another embodiment, the present invention also provides a method for diagnosing or detecting cancer in a subject, including steps of:

- (a) detecting the NPTX2 polypeptide in a subject-derived blood sample;
- (b) detecting the CYFRA in the subject-derived blood sample;
- (c) judging that the subject suffers from cancer or that cancer is present in the subject, when either that the level of the NPTX2 polypeptide is higher than a normal control level of the NPTX2 polypeptide, or that the CYFRA level is higher than a normal control level of CYFRA, or both.

[0137] As mentioned above, each of normal control levels of the NPTX2 polypeptide and CYFRA may be each of cut off values of the NPTX2 polypeptide and CYFRA.

The combination of the NPTX2 polypeptide and CYFRA is preferably applied to diagnosis or detection of lung cancer, especially squamous cell carcinoma (SCC). In the preferred embodiments, when the level of the NPTX2 polypeptide is higher than its normal control level or the CYFRA level is higher than its normal control level, the subject may be judged to have a high risk of cancer.

[0138] In another embodiment, the present invention also provides a method for diagnosing or detecting cancer in a subject, including steps of:

- (a) detecting the NPTX2 polypeptide in a subject-derived blood sample;
- (b) detecting the proGRP in the subject-derived blood sample;
- (c) judging that the subject suffers from cancer or that cancer is present in the subject, when either that the level of the NPTX2 polypeptide is higher than a normal control level of the NPTX2 polypeptide, or that the proGRP level is higher than a normal control level of proGRP, or both.

[0139] As mentioned above, each of normal control levels of the NPTX2 polypeptide and proGRP may be each of cut off values of the NPTX2 polypeptide and proGRP.

The combination of the NPTX2 polypeptide and proGRP is preferably applied to diagnosis or detection of lung cancer, especially small cell lung cancer (SCLC). In the preferred embodiments, when the level of the NPTX2 polypeptide is higher than its normal control level or the proGRP level is higher than its normal control level, the subject may be judged to have a high risk of cancer.

[0140] Moreover, the expression level of the NPTX2 polypeptide in a subject-derived bi-

ological sample may also be used to monitor the course of treatment of cancer. In this situation, a subject-derived biological sample is provided from a subject undergoing treatment of cancer. Preferably, multiple test samples are obtained from the subject at various time points, including before, during, and/or after the treatment. The level of the NPTX2 polypeptide in the post-treatment sample may then be compared with the level of the NPTX2 polypeptide in the pre-treatment sample or, alternatively, with a reference sample (e.g., a normal control level). For example, if the post-treatment level of the NPTX2 polypeptide is lower than the pre-treatment level of the NPTX2 polypeptide, one can conclude that the treatment is efficacious. Likewise, if the post-treatment level of the NPTX2 polypeptide is lower than or similar to the normal control level of the NPTX2 polypeptide, one can also conclude that the treatment is efficacious.

[0141] An "efficacious" treatment is the treatment that leads to a reduction in the level of NPTX2 polypeptide or a decrease in size, prevalence, or metastatic potential of cancer in a subject. When a treatment is applied prophylactically, "efficacious" means that the treatment retards or prevents occurrence of cancer or alleviates a clinical symptom of cancer. The assessment of cancer can be made using standard clinical protocols. Furthermore, the efficaciousness of a treatment can be determined in association with any known method for diagnosing cancer. For example, cancer is routinely diagnosed histopathologically or by identifying symptomatic anomalies.

[0142] In another aspect, the present invention provides a method of screening or identifying a subject that has high probability of developing or suffering from cancer, including a step of determining the expression level of the NPTX2 gene in a subject-derived biological sample, wherein an increase of the expression level compared to a normal control level of the NPTX2 gene indicates a probability that the subject is suffering from or will develop cancer, wherein the expression level is determined by a method selected from the group consisting of:

- (a) detecting an mRNA of the NPTX2 gene;
- (b) detecting an NPTX2 polypeptide; and
- (c) detecting a biological activity of an NPTX2 polypeptide.

[0143] In another aspect, the present invention provides a method of screening or identifying a subject-derived biological sample suspected of containing cancer cells, including a step of determining the expression level of the NPTX2 gene in a subject-derived biological sample, wherein an increase of the expression level compared to a normal control level of the NPTX2 gene indicates that the subject-derived biological sample is suspected to contain cancer cells, wherein the expression level is determined by a method selected from the group consisting of:

- (a) detecting an mRNA of the NPTX2 gene;

- (b) detecting an NPTX2 polypeptide; and
- (c) detecting a biological activity of an NPTX2 polypeptide.

[0144] Determining the expression level of the NPTX2 gene can be conducted by the methods described above. After identifying a candidate subject or a suspicious biological sample, such candidate subject or sample can be further examined, for example, by other tumor markers, imaging analysis, pathological observation, and so on.

In the course of the present invention, it was discovered that NPTX2 is not only a useful diagnostic and prognostic marker, but also suitable target for cancer therapy. Therefore, cancer treatment targeting NPTX2 can be achieved by the present invention. In the present invention, the cancer treatment targeting NPTX2 refers to suppression or inhibition of NPTX2 activity and/or expression in the cancer cells. Any anti- NPTX2 agents may be used for the cancer treatment targeting NPTX2. In the context of the present invention, the anti- NPTX2 agents may include any of the following substance(s) as active ingredient:

- (a) a double-stranded molecule of the present invention,
- (b) DNA encoding thereof, or
- (c) a vector encoding thereof.

[0145] Accordingly, in a preferred embodiment, the present invention provides a method of (i) diagnosing whether a subject has the cancer to be treated, and/or (ii) selecting a subject for cancer treatment, which method includes the steps of:

- (a) determining the expression level of NPTX2 in cancer cells or tissue(s) obtained from a subject who is suspected to have the cancer to be treated;
- (b) comparing the expression level of NPTX2 with a normal control level;
- (c) diagnosing the subject as having the cancer to be treated when the expression level of NPTX2 is increased as compared to the normal control level; and
- (d) selecting the subject for cancer treatment if the subject is diagnosed as having the cancer to be treated, in step (c).

[0146] Alternatively, such a method includes the steps of:

- (a) determining the expression level of NPTX2 in cancer cells or tissue(s) obtained from a subject who is suspected to have the cancer to be treated;
- (b) comparing the expression level of NPTX2 with a cancerous control level;
- (c) diagnosing the subject as having the cancer to be treated, when the expression level of NPTX2 is similar or equivalent to the cancerous control level; and
- (d) selecting the subject for cancer treatment, when the subject is diagnosed as having the cancer to be treated, in step (c).

[0147] (3) Method for Assessing the Prognosis of a Subject with Cancer

The present invention is based, in part, on the discovery that NPTX2

(over)expression is significantly associated with poorer prognosis of subjects with cancer. Thus, the present invention provides a method for predicting, monitoring or assessing the prognosis of a subject with cancer, by determining the expression level of the NPTX2 gene in a subject-derived biological sample, wherein an increase of said expression level as compared to a good prognosis control level of the NPTX2 gene is indicative of a poor prognosis (poor survival).

[0148] Herein, the term "prognosis" refers to a forecast as to the probable outcome of the disease as well as the prospect of recovery from the disease as indicated by the nature and symptoms of the case. Accordingly, a less favorable, negative or poor prognosis is defined by a lower post-treatment survival term or survival rate. Conversely, a positive, favorable, or good prognosis is defined by an elevated post-treatment survival term or survival rate.

[0149] The terms "assessing (or predicting) the prognosis" refer to predicting, forecasting or correlating a given detection or measurement with a future outcome of cancer of the subject (e.g., malignancy, likelihood of curing cancer, estimated time of survival, and the like). For example, a determination of the expression level of NPTX2 over time enables a predicting of an outcome for the subject (e.g., increase or decrease in malignancy, increase or decrease in grade of a cancer, likelihood of curing cancer, survival, and the like).

[0150] In the context of the present invention, the phrase "assessing (or predicting) the prognosis" is intended to encompass predictions and likelihood analysis of cancer, progression, particularly cancer recurrence, metastatic spread and disease relapse. The present method for predicting or assessing prognosis is intended to be used clinically in making decisions concerning treatment modalities, including therapeutic intervention, diagnostic criteria for example, disease staging, and disease monitoring and surveillance for metastasis or recurrence of neoplastic disease.

[0151] Specifically, the present invention provides the following methods [1] to [8]:

[1] A method for predicting or assessing a prognosis of a subject with cancer, wherein the method includes steps of:

(a) determining an expression level of the NPTX2 gene in a subject-derived biological sample;

(b) comparing the expression level determined in step (a) to a control level; and

(c) predicting the prognosis of the subject based on the comparison of (b);

[2] The method of [1], wherein the control level is a good prognosis control level and an increase of the expression level compared to the control level indicates poor prognosis;

[3] The method of [1], wherein the control level is a poor prognosis control level and a similar expression level to the control level indicates poor prognosis;

[4] The method of [2], wherein the increase is at least 10% greater than said control level; and

[5] The method of any one of [1] to [4], wherein the expression level is determined by a method selected from a group consisting of:

(a) detecting an mRNA of the NPTX2 gene;

(b) detecting an NPTX2 polypeptide; and

(c) detecting a biological activity of an NPTX2 polypeptide;

[6] The method of any one of [1] to [5], wherein the subject-derived biological sample is a sample containing a cancerous tissue, or cancerous cells, or blood sample;

[7] The method of any one of [1] to [6], wherein the cancer is lung cancer; and

[8] The method of [7], wherein the lung cancer is NSCLC.

[0152] The method of predicting or assessing the prognosis of a subject with cancers is described in more detail below.

The method of the present invention can be applied to any cancer that overexpresses the NPTX2 gene. Cancer is preferably lung cancer, more preferably NSCLC.

The subject-derived biological sample used for the method of the present invention can be any sample derived from the subject for predicting or assessing so long as a transcription product or translation product of the NPTX2 gene can be detected in the sample. For example, a subject-derived biological sample may be a bodily tissue sample or a bodily fluid sample. Examples of bodily fluid samples include sputum, blood, serum, plasma, pleural effusion, and so on. In preferred embodiments, a subject-derived biological sample is a tissue sample containing a cancerous area. For example, a lung cancer tissue sample is a preferred sample. In another preferred embodiment, a subject-derived biological sample is a subject-derived blood sample. Moreover, a subject-derived biological sample can be cells purified or obtained from a tissue. Subject-derived biological samples can be obtained from a patient at various time points, including before, during, and/or after a treatment. In preferred embodiments, for example, the biological sample for assessing a cancer prognosis is lung cancer cell(s) or tissue obtained from a subject to be assessed.

[0153] According to the present invention, it was shown that the higher expression level of the NPTX2 gene determined in a subject-derived biological sample, the poorer prognosis for post-treatment remission, recovery, and/or survival and the higher likelihood of poor clinical outcome. Thus, according to the present method, the "control level" used for comparison can be, for example, the expression level of the NPTX2 gene determined before any kind of treatment in an individual or a population of individuals who showed good or positive prognosis, after the treatment, which herein is referred to as "good prognosis control level". Alternatively, the "control level" can be the expression level of the NPTX2 gene determined before any kind of

treatment in an individual or a population of individuals who showed poor or negative prognosis, after the treatment, which herein will be referred to as "poor prognosis control level". The "control level" may be a single expression pattern derived from a single reference population or from a plurality of expression patterns. Thus, the control level can be determined based on the expression level of the NPTX2 gene determined before any kind of treatment in a subject with cancer, or a population of subjects whose prognosis are known. In some embodiments, the standard value of the expression levels of the NPTX2 gene in a subject group with known prognosis is used. The standard value can be obtained by any method known in the art. For example, a range of mean $\pm$ 2 S.D. or mean $\pm$ 3 S.D. can be used as standard value.

[0154] As noted above, the control level can be determined at the same time with the test sample by using a sample(s) previously collected and stored before any kind of treatment from cancer subject(s) (control or control group) whose prognosis are known.

[0155] Alternatively, the control level can be determined by a statistical method based on the results obtained by analyzing the expression level of the NPTX2 gene in samples previously collected and stored from a control group. Furthermore, the control level can be a database of expression patterns from previously tested cells or subjects. Moreover, the expression level of the NPTX2 gene determined in a subject-derived biological sample can be compared to multiple control levels, which control levels are determined from multiple reference samples. In some embodiments, a control level determined from a reference sample derived from a tissue type similar to that of the subject-derived biological sample is used.

[0156] According to the present invention, a similarity between a measured or calculated expression level of the NPTX2 gene and a level corresponding to a positive prognosis control level indicates a more favorable subject prognosis. Likewise, an increase in the expression level as compared to the positive prognosis control level indicates a less favorable, poorer prognosis for post-treatment remission, recovery, survival, and/or clinical outcome. On the other hand, a decrease in the expression level of the NPTX2 gene in comparison as compared to a negative prognosis control level indicates a more favorable prognosis of the subject, and a similarity between the two indicates a less favorable, poorer prognosis for post-treatment remission, recovery, survival, and/or clinical outcome. For example, a cancer cell(s) obtained from a subject who showed good or poor prognosis of cancer after treatment is a preferred subject-derived biological sample for good or poor prognosis control level, respectively. An expression level of the NPTX2 gene in a subject-derived biological sample can be considered altered (i.e., increased or decreased) when the expression level differs from the control level by more than 1.0, 1.5, 2.0, 5.0, 10.0, or more fold.

[0157] The difference in the expression level between the test sample and the control level can be normalized to a control, e.g., housekeeping gene. For example, polynucleotides whose expression levels are known not to differ between the cancerous and non-cancerous cells, including those coding for beta-actin, glyceraldehyde 3-phosphate dehydrogenase, and ribosomal protein P1, can be used to normalize the expression levels of the NPTX2 gene.

[0158] The expression level of the NPTX2 gene in a subject-derived sample can be determined by the methods described above in the section entitled "(2) Method for Diagnosing or Detecting Cancer".

Subjects to be predicted or assessed for the prognosis of cancer according to the method of the present invention can be a mammal including human, non-human primate, mouse, rat, dog, cat, horse, and cow.

[0159] Alternatively, according to the present invention, an intermediate result can also be provided in addition to other test results for assessing the prognosis of a subject with cancer. Such intermediate result can assist a doctor, nurse, or other practitioner to assess, determine, or estimate the prognosis of a subject and/or monitor the course of patient therapy. Additional information that can be considered, in combination with the intermediate result obtained by the present invention, to assess prognosis includes clinical symptoms and physical conditions of a subject.

[0160] In other words, the expression level of the NPTX2 gene is useful prognostic marker for assessing, predicting or determining the prognosis of a subject suffering from cancer including lung cancer. Therefore, the present invention also provides a method for detecting prognostic marker for assessing, predicting or determining the prognosis of a subject suffering from cancer including lung cancer, which includes steps of:

(a) detecting or determining an expression level of an NPTX2 gene in a subject-derived biological sample, and

(b) correlating the expression level detected or determined in step (a) with the prognosis of the subject.

According to the present invention, an increased expression level to the control level is indicative of potential or suspicion of poor prognosis (poor survival).

[0161] In another embodiment, the present invention provides a method for detecting a diagnostic or prognostic marker of cancer, said method comprising the step of detecting the expression level of the NPTX2 gene in a subject-derived biological sample as a diagnostic or prognostic marker of cancer including lung; breast; cervical; and colon cancer.

[0162] To facilitate the afore-mentioned utility, the present invention provides a reagent for determining, assessing, and/or monitoring prognosis of cancer. In some embodiments, the reagent is selected from the group consisting of:

- (a) a reagent for detecting an mRNA of the NPTX2 gene;
- (b) a reagent for detecting the NPTX2 polypeptide; and
- (c) a reagent for detecting the biological activity of the NPTX2 polypeptide.

Examples of such reagents include an oligonucleotide that hybridizes to the NPTX2 polynucleotide, or an antibody that binds to the NPTX2 polypeptide.

[0163] (4) Kits for Diagnosing Cancer or Assessing the Prognosis of a Subject with Cancer

In addition to assessing the prognosis of cancer, and/or monitoring the efficacy of a cancer therapy, the present invention also provides kits and methods for diagnosing or detecting cancer, or predicting or assessing the prognosis of a subject with cancer. The present invention also provides a kit for determining a subject suffering from cancer that can be treated with pharmaceutical compositions containing an inhibitor against the activity of the NPTX2 polypeptide or the expression of the NPTX2 gene, which may also be useful in assessing and/or monitoring the efficacy of a cancer treatment.

[0164] Specifically, the present invention provides the following kits [1] to [12]:

[1] A kit for use in diagnosis or detection of cancer in a subject, wherein the kit comprises at least one reagent selected from the group consisting of:

- (a) a reagent for detecting an mRNA of the NPTX2 gene;
- (b) a reagent for detecting an NPTX2 polypeptide; and
- (c) a reagent for detecting a biological activity of an NPTX2 polypeptide;

[2] The kit of [1], wherein the reagent comprises an oligonucleotide that has a sequence complementary to a part of an mRNA of the NPTX2 gene and that specifically binds to said mRNA; or an antibody against the NPTX2 polypeptide;

[3] The kit of [2], wherein the reagent comprises an antibody against the NPTX2 polypeptide;

[4] The kit of [3], wherein the kit is an ELISA kit comprising at least one antibody against the NPTX2 polypeptide;

[5] The kit of any one of [1] to [4], wherein the kit further comprises a control sample of the mRNA of the NPTX2 gene or the NPTX2 polypeptide;

[6] The kit of any one of [1] to [5], wherein the cancer is lung cancer, breast cancer, cervical cancer or colon cancer;

[7] A kit for use in predicting or assessing a prognosis of a subject with cancer, wherein the kit comprises at least one reagent selected from the group consisting of:

- (a) a reagent for detecting an mRNA of the NPTX2 gene;
- (b) a reagent for detecting an NPTX2 polypeptide or protein; and
- (c) a reagent for detecting a biological activity of an NPTX2 polypeptide or protein;

[8] The kit of [7], wherein the reagent comprises an oligonucleotide that has a sequence complementary to a part of an mRNA of the NPTX2 gene and specifically binds to said mRNA; or an antibody against the NPTX2 polypeptide or protein;

[9] The kit of [8], wherein the reagent comprises an antibody against the NPTX2 polypeptide;

[10] The kit of [9], wherein the kit is an ELISA kit comprising at least one antibody against the NPTX2 polypeptide;

[11] The kit of any one of [7] to [10], wherein the kit further comprises a control sample of the mRNA of the NPTX2 gene or the NPTX2 polypeptide; and

[12] The kit of any one of [7] or [11], wherein the cancer is lung cancer.

[0165] The kits for diagnosing or detecting cancer, or predicting or assessing a subject with cancer will be described in more detail below.

The kit of the present invention can be applied to any cancer. When diagnosing or detecting cancer, the cancer is preferably lung cancer, breast cancer, cervical cancer or colon cancer. When assessing or predicting a prognosis of a subject with cancer, the cancer is preferably lung cancer, especially NSCLC.

[0166] Suitable reagents for detecting an mRNA of the NPTX2 gene include nucleic acids that specifically bind to or identify the NPTX2 mRNA, for example, oligonucleotides that have a sequence complementary to a part of the NPTX2 mRNA. These kinds of oligonucleotides are exemplified by primers and probes that are specific to the NPTX2 mRNA. These kinds of oligonucleotides can be prepared based on methods well known in the art. If needed, the reagent for detecting the NPTX2 mRNA can be immobilized on a solid matrix. Moreover, more than one reagent for detecting the NPTX2 mRNA can be included in the kit.

[0167] The probes or primers may have specific sizes. The sizes are preferably at least 10 nucleotides, at least 12 nucleotides, at least 15 nucleotides, at least 20 nucleotides, at least 25 nucleotides or at least 30 nucleotides. The probes and primers may range in size from 5-50 nucleotides, 10-40 nucleotides, 15-30 nucleotides or 20-25 nucleotides. Alternatively, the probe for Northern hybridization analysis can have any sizes so long as the probe specifically hybridizes the NPTX2 mRNA. For example, the size of the probe may be 50 nucleotides or more, 75 nucleotide or more, 100 nucleotide or more, 200 nucleotide or more, 300 nucleotide or more, 400 nucleotide or more, or 500 nucleotide or more. Alternatively, cDNA of the NPTX2 gene may be used as a probe for the NPTX2 mRNA.

[0168] On the other hand, suitable reagents for detecting the NPTX2 polypeptide include antibodies against the NPTX2 polypeptide. The antibody can be monoclonal or polyclonal. Furthermore, any fragment or modification (e.g., chimeric antibody, scFv, Fab, F(ab')₂, Fv, etc.) of the antibody can be used as the reagent, so long as the fragment retains the binding ability to the NPTX2 polypeptide. Methods to prepare these kinds of antibodies for the detection of the NPTX2 polypeptide are well known in the art, and any method can be employed in the present invention to prepare such

antibodies and equivalents thereof. Examples of the methods for the preparation of such antibodies are described above. If needed, the reagent for detecting the NPTX2 polypeptide can be immobilized on a solid matrix.

- [0169] Furthermore, the antibody can be labeled with signal generating molecules via direct linkage or an indirect labeling technique. Labels and methods for labeling antibodies and detecting the binding of antibodies to their targets are well known in the art and any labels and methods can be employed for the present invention. Moreover, more than one reagent for detecting the NPTX2 polypeptide can be included in the kit.
- [0170] Furthermore, the biological activity of the NPTX2 polypeptide can be determined by, for example, measuring the cell proliferating activity due to the expressed NPTX2 polypeptide in a subject-derived biological sample. For example, the cell may be cultured in the presence of a subject-derived biological sample, and then by detecting the speed of proliferation, or by measuring the cell cycle or the colony forming ability the cell proliferating activity of the biological sample can be determined. Moreover, more than one reagent for detecting the biological activity of the NPTX2 polypeptide can be included in the kit.
- [0171] The kit can include more than one of the aforementioned reagents. Furthermore, the kit can include a solid matrix and reagent for binding a probe against the NPTX2 mRNA or antibody against the NPTX2 polypeptide, a medium and container for culturing cells, positive and negative control reagents, and a secondary antibody for detecting an antibody against the NPTX2 polypeptide.
- [0172] A kit of the present invention can further include other materials desirable from a commercial and user standpoint, including buffers, diluents, filters, needles, syringes, and package inserts (e.g., written, tape, CD-ROM, etc.) with instructions for use. These reagents and such can be included in a container with a label. Suitable containers include bottles, vials, and test tubes. The containers can be formed from a variety of materials, for example, glass or plastic.
- [0173] As an embodiment of the present invention, when the reagent is a probe against the NPTX2 mRNA, the reagent can be immobilized on a solid matrix, for example, a porous strip, to form at least one detection site. The measurement or detection region of the porous strip can include a plurality of sites, each containing a nucleic acid (probe). A test strip can also contain sites for negative and/or positive controls. Alternatively, control sites can be located on a strip separated from the test strip. Optionally, the different detection sites can contain different amounts of immobilized nucleic acids, i.e., a higher amount in the first detection site and lesser amounts in subsequent sites. Upon the addition of test sample, the number of sites displaying a detectable signal provides a quantitative indication of the amount of NPTX2 mRNA present in the sample. The detection sites can be configured in any suitably detectable shape and

are typically in the shape of a bar or dot spanning the width of a test strip.

[0174] The kit of the present invention can further include a positive control sample or a standard sample. The positive control sample can be prepared by collecting tissue or blood samples from a subject with cancer. Alternatively, purified NPTX2 polypeptide or polynucleotide can be added to appropriate carrier to form the positive sample or the standard sample. The NPTX2 level of the positive control sample may be, for example, more than cut off value.

[0175] According to an aspect of the present invention, the kit of the present invention for diagnosing cancer may further include either of a positive or negative control sample, or both. The positive control sample of the present invention may be an established lung cancer cell line(s), breast cancer cell line(s), cervical cancer cell line(s) or colon cancer cell line(s).

[0176] Alternatively, the NPTX2 positive samples may also be a clinical lung cancer tissue(s) obtained from a lung cancer patient(s), including small-cell lung cancer and non-small cell lung cancer (e.g., squamous cell carcinoma, adenocarcinoma and large cell carcinoma), a clinical breast cancer tissue(s) obtained from a breast cancer patient(s), a clinical cervical cancer tissue(s) obtained from a cervical cancer patient(s), and a clinical colon cancer tissue(s) obtained from a colon cancer patient(s). Alternatively, positive control samples may be prepared by determined a cut-off value and preparing a sample containing an amount of an NPTX2 mRNA or protein more than the cut-off value. Herein, the phrase "cut-off value" refers to the value dividing between a normal range and a cancerous range. For example, one skilled in the art may be determine a cut-off value using a receiver operating characteristic (ROC) curve. The kit of the present invention may include an NPTX2 standard sample providing a cut-off value amount of an NPTX2 mRNA or polypeptide. On the contrary, negative control samples may be prepared from non-cancerous cell lines or non-cancerous tissues such as normal lung, breast, cervical, or colon tissues, or may be prepared by preparing a sample containing an NPTX2 mRNA or protein less than cut-off value. The negative control sample may be non- NPTX2 expressing cells or tissue, or a blood sample derived from a subject without cancer.

[0177] In another embodiment, the kit, in the case for assessing or predicting the prognosis, may further include a good prognosis control sample and/or a poor prognosis control sample. The good prognosis control sample may be prepared from biological samples derived from subjects before any kind of treatment, wherein the subjects is known to have showed good or positive prognosis after the treatment. On the other hand, the poor prognosis control sample may be prepared from biological samples derived from subjects before any kind of treatment wherein the subjects is known to have showed poor or negative prognosis after treatment. The biological samples to be prepared

control samples are not limited to, and preferably lung tissue samples or blood samples such as serum.

[0178] Likewise, the kit of the present invention for assessing the prognosis of cancer may further includes either of a good prognosis control sample or a poor prognosis control sample, or both. As described above in the section marked (3) Method for Assessing the Prognosis of a Subject with Cancer, a good prognosis control sample may be tissues or cells obtained from an individual or a population of individuals who showed good or positive prognosis of cancer, after the treatment. Meanwhile, a poor prognosis control sample may be tissues or cells obtained from an individual or a population of individuals who showed poor or negative prognosis of cancer, after the treatment.

[0179] In a preferred embodiment, a good or positive prognosis control sample may also be a clinical lung cancer tissue(s) obtained from a lung cancer patient(s) who showed good or positive prognosis of lung cancer, after treatment. Similarly, a breast cancer tissue(s), cervical cancer tissue(s), or colon cancer tissue(s) obtained from a respective cancer patient(s) who showed good or positive prognosis of cancer, after the treatment may also be a preferred good or positive prognosis control sample for respective cancer.

[0180] In a preferred embodiment, such lung cancer tissue may be an NSCLC tissue(s) obtained from a lung cancer patient(s). In a more preferred embodiment, such NSCLC tissue may be a lung adenocarcinoma tissue(s), a lung squamous cell carcinoma tissue(s), and/or a large cell carcinoma tissue(s).

[0181] Alternatively, a good prognosis control sample may be prepared by determined a cut-off value and preparing a sample containing an amount of an NPTX2 mRNA or protein less than the cut-off value. Herein, the phrase "cut-off value" refers to the value dividing between a good prognosis range and a poor prognosis range. For example, one skilled in the art may be determine a cut-off value using a receiver operating characteristic (ROC) curve. The present kit may include an NPTX2 standard sample providing a cut-off value amount of an NPTX2 mRNA or polypeptide.

[0182] On the contrary, a poor or negative prognosis control sample may be a clinical lung cancer tissue(s) obtained from a lung cancer patient(s) who showed poor or negative prognosis of lung cancer, after the treatment. Similarly, a breast cancer tissue(s), cervical cancer tissue(s), or colon cancer tissue(s) obtained from a respective cancer patient(s) who showed poor or negative prognosis of cancer, after the treatment may also be a preferred poor or negative prognosis control sample for respective cancer.

In a preferred embodiment, such lung cancer tissue may be an NSCLC tissue(s) obtained from a lung cancer patient(s). In a more preferred embodiment, such NSCLC tissue may be a lung adenocarcinoma tissue(s), a lung squamous cell carcinoma tissue(s), and/or a large cell carcinoma tissue(s).

[0183] Alternatively, a poor prognosis control sample may be prepared by determined a cut-off value and preparing a sample containing an amount of an NPTX2 mRNA or polypeptide more than the cut-off value.

Alternatively, samples which contain the standard value of the transcription or translation product of the NPTX2 gene may preferably be used as control samples. The standard value may be obtained by any method known in the art. For example, a range of mean $\pm$ 2 S.D. or mean $\pm$ 3 S.D. may be used as standard value. Alternatively, the standard values can be obtained based on ROC curves, and the standard values obtained by this manner, are usually referred to as "cut off value". In cases where the expression level of the NPTX2 gene is detected as a level of the NPTX2 polypeptide in a blood sample, the cut off value may be set at, for example, 3 to 11 U/ml, preferably 4 to 10 U/ml, more preferably 5 to 9 U/ml, further more preferably 6 to 8 U/ml. Further more preferably, the cut off value may be set at 7 to 7.5 U/ml (e.g., 7.3 U/ml).

[0184] In cases where the expression level of the NPTX2 polypeptide is determined as a blood concentration of the NPTX2 polypeptide, the kit of the present invention is preferably an immunoassay kit.

The immunoassay kit of the present invention can include an immunoassay reagent for detecting the NPTX2 polypeptide in a subject-derived blood sample. In the preferred embodiments, the kit of the present invention may further include a positive control sample or standard sample of the NPTX2 polypeptide.

[0185] The kit of the present invention may further include an immunoassay reagent for detecting other serum tumor markers (e.g., CEA, CYFRA or proGRP) in a subject-derived blood sample. In the preferred embodiments, the kit of the present invention further includes a positive control sample or a standard sample for such serum tumor markers.

[0186] The reagents for the immunoassays that constitute a kit of the present invention may further include reagents necessary for the various immunoassays described above. Specifically, the reagents for the immunoassays include an antibody against the NPTX2 polypeptide. The antibody can be modified depending on the assay format of the immunoassay. ELISA can be used as a preferred assay format of the present invention. In ELISA, for example, a first antibody immobilized onto a solid phase and a second antibody having a label is generally used.

[0187] Therefore, the immunoassay reagents for ELISA can include a first antibody immobilized onto a solid phase carrier. Fine particles or the inner walls of a reaction container can be used as the solid phase carrier. Magnetic particles can be used as the fine particles. Alternatively, multi-well plates such as 96-well microplates are often used as the reaction containers. Containers for processing a large number of samples, which are equipped with wells having a smaller volume than in 96-well microplates at

a high density, are also known. In the present invention, the inner walls of these reaction containers can be used as the solid phase carriers.

[0188] The immunoassay reagents for ELISA may further include a second antibody having a label. The second antibody for ELISA may be an antibody onto which an enzyme is directly or indirectly linked. Methods for chemically linking an enzyme to an antibody are known. For example, immunoglobulins can be enzymatically cleaved to obtain fragments comprising the variable regions. By reducing the -SS- bonds comprised in these fragments to -SH groups, bifunctional linkers can be attached. By linking an enzyme to the bifunctional linkers in advance, enzymes can be linked to the antibody fragments.

[0189] Alternatively, to indirectly link an enzyme, for example, the avidin-biotin binding can be used. That is, an enzyme can be indirectly linked to an antibody by contacting a biotinylated antibody with an enzyme to which avidin has been attached. In addition, an enzyme can be indirectly linked to a second antibody using a third antibody which is an enzyme-labeled antibody recognizing the second antibody. For example, enzymes such as those exemplified above can be used as the enzymes to label the antibodies.

[0190] The immunoassay kits of the present invention may include a positive control sample of the NPTX2 polypeptide. A positive control sample of the NPTX2 polypeptide can contain a known concentration of NPTX2 polypeptide. The concentration of the NPTX2 polypeptide in a positive control sample are, for example, a concentration set as the standard value (cut off value) of the NPTX2 polypeptide. Alternatively, a positive control sample may have a higher concentration than the standard value. The positive control sample for the NPTX2 polypeptide may additionally contain a known concentration of other serum tumor marker (e.g., CEA, CYFRA, or proGRP).

[0191] In the immunoassay kit of the present invention, the positive control samples are preferably in a liquid form. For example, by dissolving a dried positive control with a predefined amount of liquid at the time of use, a control sample that gives the tested concentration can be prepared. By packaging, together with a dried positive control, an amount of liquid necessary to dissolve it, the user can obtain the necessary positive control sample by just mixing them. The NPTX2 polypeptide used as the positive control can be a naturally-derived polypeptide or it may be a recombinant polypeptide. Not only positive controls, but also negative controls can be combined in the kits of the present invention. The positive controls or negative controls are used to verify that the results indicated by the immunoassays are correct.

[0192] In another embodiment, the present invention also provides reagents for use in diagnosing or detecting cancer, or assessing, monitoring, determining, or predicting the prognosis of a subject with cancer, containing an oligonucleotide that has a sequence complementary to a part of the NPTX2 mRNA and that specifically binds to the

NPTX2 mRNA, or an antibody against the NPTX2 polypeptide.

- [0193] In another embodiment, the present invention also provides uses of an oligonucleotide that has a sequence complementary to a part of the NPTX2 mRNA and that specifically binds to the NPTX2 mRNA, or an antibody against the NPTX2 polypeptide, for manufacture of a reagent for diagnosing or detecting cancer, or assessing or predicting the prognosis of a subject with cancer.

In another embodiment, the present invention also provides an oligonucleotide that has a sequence complementary to a part of the NPTX2 mRNA and that specifically binds to the NPTX2 mRNA, or an antibody against the NPTX2 polypeptide, for use in diagnosing or detecting cancer, or assessing or predicting the prognosis of a subject with cancer.

- [0194] (5) Screening Methods

Using the NPTX2 gene, polypeptide encoded by the gene or functional equivalent thereof, or transcriptional regulatory region of the gene, it is possible to screen for substances that alter the expression of the NPTX2 gene or the biological activities of the NPTX2 polypeptide. Such substances may be used as candidate pharmaceuticals for treating or preventing cancer. Thus, the present invention provides methods of screening for candidate substances for the treatment or prevention of cancer or a post-operative recurrence thereof, or inhibiting cancer cell growth using the NPTX2 gene, an NPTX2 polypeptide or functional equivalent thereof, or a transcriptional regulatory region of the NPTX2 gene.

- [0195] Substances isolated by the screening method of the present invention are expected to inhibit the expression of the NPTX2 gene, or the activity of the translation product of the NPTX2 gene, and thus, is a candidate for the treatment or prevention of cancer or a post-operative recurrence thereof, or inhibiting cancer cell growth (in particular, lung cancer, breast cancer, cervical cancer and colon cancer). Namely, the substances screened through the screening method of the present invention are deemed to have a clinical benefit and can be further tested for its ability to prevent cancer cell growth in animal models or test subjects.

- [0196] (I) Test substances for screening

Substances to be identified through the screening method of the present invention can be any substance or composition that may include several substances in combination. Furthermore, the test substance exposed to a cell or protein according to the screening methods of the present invention can be a single substance or a combination of substances. When a combination of substances is used in the methods, the substances can be contacted sequentially or simultaneously.

- [0197] Any test substance, for example, cell extracts, cell culture supernatant, products of fermenting microorganism, extracts from marine organism, plant extracts, purified or

crude proteins, peptides, non-peptide compounds, synthetic micro-molecular compounds (including nucleic acid constructs, for example, antisense DNA, siRNA, ribozymes, etc.) and natural compounds can be used in the screening methods of the present invention. The test substance of the present invention can be also obtained using any of the numerous approaches in combinatorial library methods known in the art, including

- (1) biological libraries,
- (2) spatially addressable parallel solid phase or solution phase libraries,
- (3) synthetic library methods requiring deconvolution,
- (4) the "one-bead one-compound" library method and
- (5) synthetic library methods using affinity chromatography selection.

[0198] The biological library methods using affinity chromatography selection is limited to peptide libraries, while the other four approaches are applicable to peptide, non-peptide oligomer or small molecule libraries of compounds (Lam, *Anticancer Drug Des* 1997, 12: 145-67). Examples of methods for the synthesis of molecular libraries can be found in the art (DeWitt et al., *Proc Natl Acad Sci USA* 1993, 90: 6909-13; Erb et al., *Proc Natl Acad Sci USA* 1994, 91: 11422-6; Zuckermann et al., *J Med Chem* 37: 2678-85, 1994; Cho et al., *Science* 1993, 261: 1303-5; Carell et al., *Angew Chem Int Ed Engl* 1994, 33: 2059; Carell et al., *Angew Chem Int Ed Engl* 1994, 33: 2061; Gallop et al., *J Med Chem* 1994, 37: 1233-51). Libraries of compounds can be presented in solution (see Houghten, *Bio/Techniques* 1992, 13: 412-21) or on beads (Lam, *Nature* 1991, 354: 82-4), chips (Fodor, *Nature* 1993, 364: 555-6), bacteria (US Pat. No. 5,223,409), spores (US Pat. No. 5,571,698; 5,403,484 and 5,223,409), plasmids (Cull et al., *Proc Natl Acad Sci USA* 1992, 89: 1865-9) or phage (Scott and Smith, *Science* 1990, 249: 386-90; Devlin, *Science* 1990, 249: 404-6; Cwirla et al., *Proc Natl Acad Sci USA* 1990, 87: 6378-82; Felici, *J Mol Biol* 1991, 222: 301-10; US Pat. Application 2002-103360).

[0199] A substance in which a part of the structure of the substance screened by any of the present screening methods is converted by addition, deletion and/or replacement, is included in the substances obtained by the screening methods of the present invention.

Furthermore, when the screened test substance is a protein, for obtaining a DNA encoding the protein, either the whole amino acid sequence of the protein can be determined to deduce the nucleic acid sequence coding for the protein, or partial amino acid sequence of the obtained protein can be analyzed to prepare an oligo DNA as a probe based on the sequence, and screen cDNA libraries with the probe to obtain a DNA encoding the protein. The obtained DNA finds use in preparing a test substance that is a candidate for the treatment or prevention of cancer or a post-operative recurrence thereof, or the inhibition of cancer cell growth.

- [0200] Test substances useful in the screening described herein can also be antibodies or non-antibody binding proteins that specifically bind to the NPTX2 polypeptide or partial NPTX2 peptides that lack the activity to binding for partner. Such partial protein or antibody can be prepared by the methods described herein (see (1) Cancer-related genes and cancer-related protein, and functional equivalent thereof in Definition or Antibodies) and can be tested for their ability to block binding of the protein with its binding partners.
- [0201] (i) Molecular modeling
- Construction of test substance libraries is facilitated by knowledge of the molecular structure of substances known to have the properties sought, and/or the molecular structure of the target molecules to be inhibited. One approach to preliminary screening of test substances suitable for further evaluation is computer modeling of the interaction between the test substance and its target.
- [0202] Computer modeling technology allows the visualization of the three-dimensional atomic structure of a selected molecule and the rational design of new compounds that will interact with the molecule. The three-dimensional construct typically depends on data from x-ray crystallographic analysis or NMR imaging of the selected molecule. The molecular dynamics require force field data. The computer graphics systems enable prediction of how a new compound will link to the target molecule and allow experimental manipulation of the structures of the compound and target molecule to perfect binding specificity. Prediction of what the molecule-compound interaction will be when small changes are made in one or both requires molecular mechanics software and computationally intensive computers, usually coupled with user-friendly, menu-driven interfaces between the molecular design program and the user.
- [0203] An example of the molecular modeling system described generally above includes the CHARMM and QUANTA programs, Polygen Corporation, Waltham, Mass. CHARMM performs the energy minimization and molecular dynamics functions. QUANTA performs the construction, graphic modeling and analysis of molecular structure. QUANTA allows for interactive construction, modification, visualization, and analysis of the behavior of molecules with each other.
- [0204] A number of articles review computer modeling of drugs interactive with specific proteins, for example, Rotivinen et al. *Acta Pharmaceutica Fennica* 1988, 97: 159-66; Ripka, *New Scientist* 1988, 54-8; McKinlay & Rossmann, *Annu Rev Pharmacol Toxiciol* 1989, 29: 111-22; Perry & Davies, *Prog Clin Biol Res* 1989, 291: 189-93; Lewis & Dean, *Proc R Soc Lond* 1989, 236: 125-40, 141-62; and, with respect to a model receptor for nucleic acid components, Askew et al., *J Am Chem Soc* 1989, 111: 1082-90.
- [0205] Other computer programs that screen and graphically depict chemicals are available

from companies for example, BioDesign, Inc., Pasadena, Calif., Allelix, Inc, Mississauga, Ontario, Canada, and Hypercube, Inc., Cambridge, Ontario. See, e.g., DesJarlais et al., *J Med Chem* 1988, 31: 722-9; Meng et al., *J Computer Chem* 1992, 13: 505-24; Meng et al., *Proteins* 1993, 17: 266-78; Shoichet et al., *Science* 1993, 259: 1445-50.

[0206] Once an inhibitor of the activity of the NPTX2 polypeptide has been identified, combinatorial chemistry techniques can be employed to construct any number of variants based on the chemical structure of the identified inhibitor, as detailed below. The resulting library of candidate inhibitors, or "test substances" can be screened using the methods of the present invention to identify test substances of the library that disrupt the activity of the NPTX2 polypeptide for the treatment and/or prophylaxis of cancer and/or the prevention of post-operative recurrence of cancer.

[0207] (ii) Combinatorial chemical synthesis

Combinatorial libraries of test substances can be produced as part of a rational drug design program involving knowledge of core structures existing in known inhibitors of the activity of the NPTX2 polypeptide. This approach allows the library to be maintained at a reasonable size, facilitating high throughput screening. Alternatively, simple, particularly short, polymeric molecular libraries can be constructed by simply synthesizing all permutations of the molecular family making up the library. An example of this latter approach would be a library of all peptides six amino acids in length. Such a peptide library could include every 6 amino acid sequence permutation. This type of library is termed a linear combinatorial chemical library.

[0208] Preparation of combinatorial chemical libraries is well known to those of skill in the art, and can be generated by either chemical or biological synthesis. Combinatorial chemical libraries include, but are not limited to, peptide libraries (see, e.g., US Patent 5,010,175; Furka, *Int J Pept Prot Res* 1991, 37: 487-93; Houghten et al., *Nature* 1991, 354: 84-6). Other chemistries for generating chemical diversity libraries can also be used. Such chemistries include, but are not limited to: peptides (e.g., PCT Publication No. WO 91/19735), encoded peptides (e.g., WO 93/20242), random bio-oligomers (e.g., WO 92/00091), benzodiazepines (e.g., US Patent 5,288,514), diversomers for example, hydantoins, benzodiazepines and dipeptides (DeWitt et al., *Proc Natl Acad Sci USA* 1993, 90:6909-13), vinylogous polypeptides (Hagihara et al., *J Amer Chem Soc* 1992, 114: 6568), nonpeptidal peptidomimetics with glucose scaffolding (Hirschmann et al., *J Amer Chem Soc* 1992, 114: 9217-8), analogous organic syntheses of small compound libraries (Chen et al., *J. Amer Chem Soc* 1994, 116: 2661), oligocarbamates (Cho et al., *Science* 1993, 261: 1303), and/or peptidylphosphonates (Campbell et al., *J Org Chem* 1994, 59: 658), nucleic acid libraries (see Ausubel, *Current Protocols in Molecular Biology*, 1990-2008, John Wiley In-

terscience; Sambrook and Russell, *Molecular Cloning: A Laboratory Manual*, 3rd Ed., 2001, Cold Spring Harbor Laboratory, New York, USA), peptide nucleic acid libraries (see, e.g., US Patent 5,539,083), antibody libraries (see, e.g., Vaughan et al., *Nature Biotechnology* 1996, 14(3):309-14 and PCT/US96/10287), carbohydrate libraries (see, e.g., Liang et al., *Science* 1996, 274: 1520-22; US Patent 5,593,853), and small organic molecule libraries (see, e.g., benzodiazepines, Gordon EM. *Curr Opin Biotechnol.* 1995 Dec 1;6(6):624-31.; isoprenoids, US Patent 5,569,588; thiazolidinones and metathiazanones, US Patent 5,549,974; pyrrolidines, US Patents 5,525,735 and 5,519,134; morpholino compounds, US Patent 5,506,337; benzodiazepines, 5,288,514, and the like).

[0209] (iii) Other candidates

Another approach uses recombinant bacteriophage to produce libraries. Using the "phage method" (Scott & Smith, *Science* 1990, 249: 386-90; Cwirla et al., *Proc Natl Acad Sci USA* 1990, 87: 6378-82; Devlin et al., *Science* 1990, 249: 404-6), very large libraries can be constructed (e.g., 10^6 - 10^8 chemical entities). A second approach uses primarily chemical methods, of which the Geysen method (Geysen et al., *Molecular Immunology* 1986, 23: 709-15; Geysen et al., *J Immunologic Method* 1987, 102: 259-74); and the method of Fodor et al. (*Science* 1991, 251: 767-73) are examples. Furka et al. (14th International Congress of Biochemistry 1988, Volume #5, Abstract FR:013; Furka, *Int J Peptide Protein Res* 1991, 37: 487-93), Houghten (US Patent 4,631,211) and Rutter et al. (US Patent 5,010,175) describe methods to produce a mixture of peptides that can be tested as agonists or antagonists.

[0210] Aptamers are macromolecules composed of nucleic acid that bind tightly to a specific molecular target. TuERK and Gold (*Science*, 249:505-510 (1990)) discloses SELEX (Systematic Evolution of Ligands by Exponential Enrichment) method for selection of aptamers. In the SELEX method, a large library of nucleic acid molecules (e.g., 10^{15} different molecules) can be used for screening.

[0211] (II) Screening methods

(i) General screening Method

Using the NPTX2 gene, the NPTX2 polypeptide or transcriptional regulatory region of the NPTX2 gene, substances that alter the expression of the NPTX2 gene or the biological activity of the NPTX2 polypeptide can be identified. In the context of the present invention, the NPTX2 gene was found to be over-expressed in cancer, and demonstrated to be involved in cancer cell growth and/or survival. Therefore, substances that alter the expression level of the NPTX2 gene or its biological activity are potential therapeutics for cancer.

[0212] Antagonists that bind to the NPTX2 polypeptide may inhibit the biological activity to mediate cell proliferation of cancer, and thus, are candidates for treating the cancer.

Therefore, the present invention provides a method for identifying potential candidates for the treatment or prevention of cancer or a post-operative recurrence thereof, or the inhibition of cancer cell growth, particularly in cancers that express or overexpress the NPTX2 gene, by identifying substances that bind to the NPTX2 polypeptide.

- [0213] In the context of the present invention, the phrase "inhibition of binding" between two proteins refers to at least reducing binding between the proteins. Thus, in some cases, the percentage of binding pairs in a sample in the presence of a test substance will be decreased compared to an appropriate (e.g., not treated with test substance or from a non-cancer sample, or from a cancer sample) control. The reduction in the amount of proteins bound can be, e.g., less than 90%, 80%, 70%, 60%, 50%, 40%, 25%, 10%, 5%, 1% or less (e.g., 0%), than the pairs bound in a control sample.
- [0214] Examples of supports that can be used for binding proteins include, for example, insoluble polysaccharides, for example, agarose, cellulose and dextran; and synthetic resins, for example, polyacrylamide, polystyrene and silicon; for example, commercial available beads and plates (e.g., multi-well plates, biosensor chip, etc.) prepared from the above materials can be used. When using beads, they can be filled into a column. Alternatively, the use of magnetic beads is also known in the art, and enables one to readily isolate proteins bound on the beads via magnetism.
- [0215] The binding of a protein to a support can be conducted according to routine methods, for example, chemical bonding and physical adsorption, for example. Alternatively, a protein can be bound to a support via antibodies that specifically recognize the protein. Moreover, binding of a protein to a support can be also conducted by means of avidin and biotin.
- [0216] The methods of screening for molecules that bind when the immobilized polypeptide is exposed to synthetic chemical compounds, or natural substance banks, or a random phage peptide display library, and the methods of screening using high-throughput based on combinatorial chemistry techniques (Wrighton et al., *Science* 273: 458-63 (1996); Verdine, *Nature* 384: 11-3 (1996)) to isolate not only proteins but chemical compounds that bind to the protein (including agonist and antagonist) are well known to one skilled in the art.
- [0217] Furthermore, in the screening method of the present invention, substances that suppress the expression level of the NPTX2 gene can be also identified as candidate therapeutics for cancer. The expression level of a polypeptide or functional equivalent thereof can be detected according to any method known in the art. For example, a reporter assay can be used. Suitable reporter genes and host cells are well known in the art. The reporter construct required for the screening can be prepared by using the transcriptional regulatory region of the NPTX2 gene. When the transcriptional regulatory region of the gene has been known to those skilled in the art, a reporter construct can

be prepared by using the previous sequence information. When the transcriptional regulatory region remains unidentified, a nucleotide segment containing the transcriptional regulatory region can be isolated from a genome library based on the nucleotide sequence information of the gene. Specifically, the reporter construct required for the screening can be prepared by connecting reporter gene sequence to the transcriptional regulatory region of the NPTX2 gene. The transcriptional regulatory region of the NPTX2 gene is the region from a start codon to at least 500bp upstream, for example, 1000bp, for example, 5000 or 10000bp upstream. A nucleotide segment containing the transcriptional regulatory region can be isolated from a genome library or can be propagated by PCR. Methods for identifying a transcriptional regulatory region, and also assay protocol are well known (Sambrook and Russell, *Molecular Cloning: A Laboratory Manual*, 3rd Ed., Chapter 17, 2001, Cold Springs Harbor Laboratory Press).

Furthermore, in the screening method of the present invention, substances that inhibit a biological activity of the NPTX2 polypeptide can be also identified as candidate therapeutics for cancer.

[0218] Various low-throughput and high-throughput enzyme assay formats are known in the art and can be readily adapted for detection or measuring of a biological activity of the NPTX2 polypeptide. For high-throughput assays, a substrate can conveniently be immobilized on a solid support. Following the reaction, the substrate converted by the polypeptide can be detected on the solid support by the methods described above. Alternatively, the contact step can be performed in solution, after which a substrate can be immobilized on a solid support, and the substrate converted by the polypeptide can be detected. To facilitate such assays, the solid support can be coated with streptavidin and the substrate labeled with biotin, or the solid support can be coated with antibodies against the substrate. The skilled person can determine suitable assay formats depending on the desired throughput capacity of the screen.

[0219] The assays of the invention are also suitable for automated procedures that facilitate high-throughput screening. A number of well-known robotic systems have been developed for solution phase chemistries. These systems include automated workstations like the automated synthesis apparatus developed by Takeda Chemical Industries, Ltd. (Osaka, Japan) and many robotic systems utilizing robotic arms (Zymate II, Zymark Corporation, Hopkinton, Mass.; Orca, Hewlett Packard, Palo Alto, Calif.), which mimic the manual synthetic operations performed by a chemist. Any of the above devices are suitable for use with the present invention. The nature and implementation of modifications to these devices (if any) so that they can operate as discussed herein will be apparent to persons skilled in the relevant art. In addition, numerous combinatorial libraries are themselves commercially available (see, e.g.,

ComGenex, Princeton, N.J., Asinex, Moscow, Ru, Tripos, Inc., St. Louis, MO, ChemStar, Ltd, Moscow, RU, 3D Pharmaceuticals, Exton, PA, Martek Biosciences, Columbia, MD, etc.).

[0220] In the course of the present invention, it was revealed that suppression of the expression level of the NPTX2 gene or biological activity of the NPTX2 polypeptide led to suppression of the growth of cancer cells. Therefore, when a substance suppresses the expression of the NPTX2 gene or the activity of the NPTX2 polypeptide, the suppression is indicative of a potential therapeutic effect in a subject. In the context of the present invention, a potential therapeutic effect refers to a clinical benefit with a reasonable expectation. In the context of the present invention, such clinical benefit includes;

- (a) reduction in expression of the NPTX2 gene,
- (b) decrease in size, prevalence, or metastatic potential of the cancer in a subject, .
- (c) preventing cancers from forming, or
- (d) preventing or alleviating a clinical symptom of cancer.

[0221] (ii) Screening for Substances that Bind to NPTX2 polypeptide

In the course of the present invention, the over-expression of the NPTX2 gene was detected in cancer, but not in normal tissues. Further, the NPTX2 polypeptide was demonstrated to be involved in cancer cell growth. Therefore, the NPTX2 gene can be a good molecular target for cancer therapy and diagnosis. Substances that bind to the NPTX2 polypeptide may inhibit a biological activity of the NPTX2 polypeptide. Such substances are used as pharmaceuticals for either or both of treating and preventing cancer.

[0222] Specifically, the present invention provides a method of screening for a candidate substance for either or both of treating and preventing cancer, including steps of:

- (a) contacting a test substance with an NPTX2 polypeptide or functional equivalent thereof;
- (b) detecting the binding (level) between the NPTX2 polypeptide or functional equivalent thereof and the test substance;
- (c) selecting the test substance that binds to the NPTX2 polypeptide or functional equivalent thereof.

[0223] According to the present invention, the therapeutic effect of the test substance on inhibiting the cell growth or a candidate substance for either or both of treating and preventing cancer may be evaluated. Therefore, the present invention also provides a method of screening for a candidate substance that suppresses the proliferation of cancer cells, and a method of screening for a candidate substance for either or both of treating and preventing cancer.

[0224] More specifically, the method includes the steps of:

- (a) contacting a test substance with an NPTX2 polypeptide or functional equivalent thereof;
- (b) detecting the binding (level) between the NPTX2 polypeptide and the test substance;
- (c) correlating the binding level of (b) with the therapeutic effect of the test substance.

[0225] Alternatively, according to the present invention, the potential therapeutic effect of a test substance on either or both of treating and preventing cancer can also be evaluated or estimated. In some embodiments, the present invention provides a method for evaluating or estimating a therapeutic effect of a test substance on either or both of treating and preventing cancer or inhibiting cancer cell growth, the method including steps of:

- (a) contacting a test substance with an NPTX2 polypeptide;
- (b) detecting the binding activity (level) between the NPTX2 polypeptide and the test substance; and
- (c) correlating the potential therapeutic effect and the test substance, wherein the potential therapeutic effect is shown, when the substance binds to the NPTX2 polypeptide.

[0226] In the context of the present invention, the therapeutic effect may be correlated with the binding level of the NPTX2 polypeptide. For example, when the test substance binds to the NPTX2 polypeptide, the test substance may be identified or selected as the candidate substance having the therapeutic effect. Alternatively, when the test substance does not bind to the NPTX2 polypeptide, the test substance may be identified as the substance having no significant therapeutic effect.

[0227] The method of the present invention will be described in more detail below.

The NPTX2 polypeptide to be used for the screening method of the present invention can be a recombinant polypeptide or a protein derived from the nature or a partial peptide thereof. The NPTX2 polypeptide to be contacted with a test substance can be, for example, a purified polypeptide, a soluble protein, a form bound to a carrier or a fusion protein fused with other polypeptides. In preferred embodiments, the polypeptide is isolated from cells expressing NPTX2, or chemically synthesized to be contacted with a test substance in vitro.

[0228] As a method of screening for proteins, for example, that bind to the NPTX2 polypeptide, many methods well known by a person skilled in the art can be used. Such screening can be conducted by, for example, immunoprecipitation method. The gene encoding the NPTX2 polypeptide or functional equivalent thereof is expressed in host (e.g., animal) cells and so on by inserting the gene to an expression vector for foreign genes, for example, pSV2neo, pcDNA I, pcDNA3.1, pCAGGS and pCD8.

[0229] The promoter to be used for the expression can be any promoter that can be used

commonly and include, for example, the SV40 early promoter (Rigby in Williamson (ed.), Genetic Engineering, vol. 3. Academic Press, London, 83-141 (1982)), the EF-alpha promoter (Kim et al., Gene 91: 217-23 (1990)), the CAG promoter (Niwa et al., Gene 108: 193 (1991)), the RSV LTR promoter (Cullen, Methods in Enzymology 152: 684-704 (1987)) the SR alpha promoter (Takebe et al., Mol Cell Biol 8: 466 (1988)), the CMV immediate early promoter (Seed and Aruffo, Proc Natl Acad Sci USA 84: 3365-9 (1987)), the SV40 late promoter (Gheysen and Fiers, J Mol Appl Genet 1: 385-94 (1982)), the Adenovirus late promoter (Kaufman et al., Mol Cell Biol 9: 946 (1989)), the HSV TK promoter and so on.

[0230] The introduction of the NPTX2 gene into host cells to express a foreign gene can be performed according to any methods, for example, the electroporation method (Chu et al., Nucleic Acids Res 15: 1311-26 (1987)), the calcium phosphate method (Chen and Okayama, Mol Cell Biol 7: 2745-52 (1987)), the DEAE dextran method (Lopata et al., Nucleic Acids Res 12: 5707-17 (1984); Sussman and Milman, Mol Cell Biol 4: 1641-3 (1984)), the Lipofectin method (Derijard B., Cell 76: 1025-37 (1994); Lamb et al., Nature Genetics 5: 22-30 (1993); Rabindran et al., Science 259: 230-4 (1993)) and so on.

[0231] The NPTX2 polypeptide may be expressed as a fusion protein comprising a recognition site (epitope) of a monoclonal antibody by introducing the epitope of the monoclonal antibody, whose specificity has been revealed, to the N- or C- terminus of the polypeptide. A commercially available epitope-antibody system can be used (Experimental Medicine 13: 85-90 (1995)). Vectors that can express a fusion protein with, for example, beta-galactosidase, maltose binding protein, glutathione S-transferase, green fluorescence protein (GFP) and so on by the use of its multiple cloning sites are commercially available. A fusion protein prepared by introducing only small epitopes composed of several to a dozen amino acids so as not to change the property of the NPTX2 polypeptide by the fusion is also provided herein. Epitopes, for example, polyhistidine (His-tag), influenza aggregate HA, human c-myc, FLAG, Vesicular stomatitis virus glycoprotein (VSV-GP), T7 gene 10 protein (T7-tag), human simple herpes virus glycoprotein (HSV-tag), E-tag (an epitope on monoclonal phage) and such, and monoclonal antibodies recognizing them can be used as the epitope-antibody system for screening proteins binding to the NPTX2 polypeptide (Experimental Medicine 13: 85-90 (1995)).

[0232] In the context of immunoprecipitation, an immune complex is formed by adding these antibodies to cell lysate prepared using an appropriate detergent. The immune complex is composed of the NPTX2 polypeptide, a polypeptide comprising the binding ability with the polypeptide, and an antibody. Immunoprecipitation can be also conducted using antibodies against the NPTX2 polypeptide, besides using antibodies

against the above epitopes, which antibodies can be prepared as described above. An immune complex can be precipitated, for example by Protein A sepharose or Protein G sepharose when the antibody is a mouse IgG antibody. If the NPTX2 polypeptide is prepared as a fusion protein with an epitope, for example, GST, an immune complex can be formed in the same manner as in the use of the antibody against the NPTX2 polypeptide, using a substance specifically binding to these epitopes, for example, glutathione-Sepharose 4B.

- [0233] Immunoprecipitation can be performed by following or according to, for example, the methods in the literature (Harlow and Lane, *Antibodies*, 511-52, Cold Spring Harbor Laboratory publications, New York (1988)).
- [0234] SDS-PAGE is commonly used for analysis of immunoprecipitated proteins and the bound protein can be analyzed by the molecular weight of the protein using gels with an appropriate concentration. Since the protein bound to the NPTX2 polypeptide is difficult to detect by a common staining method, for example, Coomassie staining or silver staining, the detection sensitivity for the protein can be improved by culturing cells in culture medium containing radioactive isotope, ³⁵S-methionine or ³⁵S-cysteine, labeling proteins in the cells, and detecting the proteins. The target protein can be purified directly from the SDS-polyacrylamide gel and its sequence can be determined, when the molecular weight of a protein has been revealed.
- [0235] As a method of screening for proteins binding to the NPTX2 polypeptide, for example, West-Western blotting analysis (Skolnik et al., *Cell* 65: 83-90 (1991)) can be used. Specifically, a protein binding to the NPTX2 polypeptide can be obtained by preparing a cDNA library from cultured cells (e.g., lung cancer cell line) expected to express a protein binding to the NPTX2 polypeptide using a phage vector (e.g., ZAP), expressing the protein on LB-agarose, fixing the protein expressed on a filter, reacting the purified and labeled the NPTX2 polypeptide with the above filter, and detecting the plaques expressing proteins bound to the NPTX2 polypeptide according to the label. The polypeptide of the invention can be labeled by utilizing the binding between biotin and avidin, or by utilizing an antibody that specifically binds to the NPTX2 polypeptide, or a peptide or polypeptide (for example, GST) that is fused to the NPTX2 polypeptide. Methods using radioisotope or fluorescence and such can be also used.
- [0236] The terms "label" and "detectable label" are interchangeably used herein to refer to any composition detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical or chemical means. Such labels include biotin for staining with labeled streptavidin conjugate, magnetic beads (e.g., DYNABEADS™), fluorescent dyes (e.g., fluorescein, Texas red, rhodamine, green fluorescent protein, and the like), radiolabels (e.g., ³H, ¹²⁵I, ³⁵S, ¹⁴C, or ³²P), enzymes (e.g., horse radish

peroxidase, alkaline phosphatase and others commonly used in an ELISA), and calorimetric labels for example colloidal gold or colored glass or plastic (e.g., polystyrene, polypropylene, latex, etc.) beads. Patents teaching the use of such labels include U.S. Pat. Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,275,149; and 4,366,241. Means of detecting such labels are well known to those of skill in the art. Thus, for example, radiolabels can be detected using photographic film or scintillation counters, fluorescent markers can be detected using a photodetector to detect emitted light. Enzymatic labels are typically detected by providing the enzyme with a substrate and detecting, the reaction product produced by the action of the enzyme on the substrate, and calorimetric labels are detected by simply visualizing the colored label.

- [0237] In another embodiment, a two-hybrid system utilizing cells can be used ("MATCHMAKER Two-Hybrid system", "Mammalian MATCHMAKER Two-Hybrid Assay Kit", "MATCHMAKER one-Hybrid system" (Clontech); "HybriZAP Two-Hybrid Vector System" (Stratagene); the references "Dalton and Treisman, Cell 68: 597-612 (1992)", "Fields and Sternglanz, Trends Genet 10: 286-92 (1994)").
- [0238] In the two-hybrid system, the NPTX2 polypeptide is fused to the SRF-binding region or GAL4-binding region and expressed in yeast cells. A cDNA library is prepared from cells expected to express a protein binding to the NPTX2 polypeptide, such that the library, when expressed, is fused to the VP16 or GAL4 transcriptional activation region. The cDNA library is then introduced into the above yeast cells and the cDNA derived from the library is isolated from the positive clones detected (when a protein binding to the NPTX2 polypeptide is expressed in yeast cells, the binding of the two activates a reporter gene, making positive clones detectable). A protein encoded by the cDNA can be prepared by introducing the cDNA isolated above to E. coli and expressing the protein. . Examples of suitable reporter genes include, but are not limited to, the Ade2 gene, lacZ gene, CAT gene, luciferase gene and such can be used in addition to the HIS3 gene.
- [0239] A substance binding to the NPTX2 polypeptide can also be screened using affinity chromatography. For example, the NPTX2 polypeptide can be immobilized on a carrier of an affinity column, and a test substance, containing a protein capable of binding to the NPTX2 polypeptide, is applied to the column. A test substance herein can be, for example, cell extracts, cell lysates, etc. After loading the test substance, the column is washed, and substances bound to the NPTX2 polypeptide can be prepared. When the test substance is a protein, the amino acid sequence of the obtained protein is analyzed, an oligo DNA is synthesized based on the sequence, and cDNA libraries are screened using the oligo DNA as a probe to obtain a DNA encoding the protein.
- [0240] A biosensor using the surface plasmon resonance phenomenon can be used as a mean for detecting or quantifying the bound substance. When such a biosensor is used, the

interaction between the NPTX2 polypeptide and a test substance can be observed real-time as a surface plasmon resonance signal, using only a minute amount of polypeptide and without labeling (for example, BIAcore, Pharmacia). Therefore, it is possible to evaluate the binding between the NPTX2 polypeptide and a test substance using a biosensor for example, BIAcore.

[0241] The methods of screening for molecules that bind when the immobilized NPTX2 polypeptide is exposed to synthetic chemical substances, or natural substance banks or a random phage peptide display library, and the methods of screening using high-throughput based on combinatorial chemistry techniques (Wrighton et al., Science 273: 458-64 (1996); Verdine, Nature 384: 11-13 (1996); Hogan, Nature 384: 17-9 (1996)) to isolate not only proteins but chemical substances that bind to the NPTX2 polypeptide (including agonist and antagonist) are well known to those skilled in the art.

[0242] In the course of the present invention, it was revealed that suppressing the expression level of the NPTX2 gene, reduces cell growth. Thus, by screening for candidate substances that bind to the NPTX2 polypeptide, candidate substances that have the potential to treat or prevent cancer can be identified. Potential of these candidate substances to treat or prevent cancer may be evaluated by second and/or further screening to identify therapeutic agent for cancer. For example, when a substance binding to the NPTX2 polypeptide inhibits activities of cancer, it may be concluded that such substance has NPTX2 specific therapeutic effect.

[0243] (iii) Screening for Substance that Suppress the Biological Activity of NPTX2 Polypeptide

The present invention also provides a method for screening a candidate substance for either or both of treating and preventing cancer using a biological activity of the NPTX2 polypeptide, or functional equivalent thereof as an index.

[0244] Specifically, the present invention provides the following methods of [1] to [3]:

[1] A method of screening for a candidate substance for either or both of treating and preventing cancer, such method including steps of:

(a) contacting a test substance with an NPTX2 polynucleotide or functional equivalent thereof;

(b) detecting a biological activity of the NPTX2 polypeptide of step (a);

(c) comparing the biological activity detected in step (b) with that detected in the absence of the test substance;

(d) selecting the test substance that reduces or inhibits the biological activity of the NPTX2 polypeptide;

[2] The method of [1], wherein the biological activity is a cell proliferation promoting activity.

[0245] According to the present invention, the therapeutic effect of the test substance on inhibiting the cell growth or a candidate substance for either or both of treating and preventing cancer, may be evaluated. Therefore, the present invention also provides a method of screening for a candidate substance for inhibiting the cell growth or a candidate substance for either or both of treating and preventing cancer, using the NPTX2 polypeptide or functional equivalent thereof including steps as follows:

- (a) contacting a test substance with the NPTX2 polypeptide or a functional equivalent thereof; and
- (b) detecting the biological activity of the NPTX2 polypeptide or functional equivalent thereof of step (a), and
- (c) correlating the biological activity of (b) with the therapeutic effect of the test substance.

[0246] Alternatively, in some embodiments, the present invention provides a method for evaluating or estimating a therapeutic effect of a test substance on treating or preventing cancer or inhibiting cancer, the method including steps of:

- (a) contacting a test substance with a NPTX2 polypeptide or functional equivalent thereof;
- (b) detecting the biological activity of the NPTX2 polypeptide or functional equivalent thereof of step (a); and
- (c) correlating the potential therapeutic effect and the test substance, wherein the potential therapeutic effect is shown, when the test substance suppresses the biological activity of the NPTX2 polypeptide or functional equivalent thereof as compared to the biological activity of the NPTX2 polypeptide or functional equivalent thereof detected in the absence of the test substance.

[0247] In the present invention, the therapeutic effect may be correlated with the biological activity of the NPTX2 polypeptide or functional equivalent thereof. For example, when the test substance suppresses or inhibits the biological activity of the NPTX2 polypeptide or functional equivalent thereof as compared to the biological activity detected in the absence of the test substance, the test substance may be identified or selected as a candidate substance having the therapeutic effect. Alternatively, when the test substance does not suppress or inhibit the biological activity of the NPTX2 polypeptide or functional equivalent thereof as compared to the biological activity detected in the absence of the test substance, the test substance may be identified as the substance having no significant therapeutic effect.

[0248] The method of the present invention will be described in more detail below.

Any polypeptide can be used for screening, so long as it retains at least one biological activity of the NPTX2 polypeptide. Such biological activities include the cell proliferation enhancing activity. For example, the NPTX2 polypeptide can be used and

functionally equivalent of the NPTX2 polypeptide can also be used. These polypeptides can be expressed endogenously or exogenously by cells.

[0249] Substances isolated by the screening methods of the present invention are candidate antagonists of the NPTX2 polypeptide. The term "antagonist" refers to molecules that inhibit the function of the NPTX2 polypeptide by binding thereto. The term also refers to molecules that reduce or inhibit expression of the NPTX2 gene. Moreover, a substance isolated by this screening is a candidate substance which inhibits the in vivo interaction of the NPTX2 polypeptide with molecules (including DNAs and proteins).

[0250] In the context of the present invention, the NPTX2 polypeptide has the activity of promoting cell proliferation of cancer cells. Therefore, in the screening method of the present invention, using this biological activity, a substance which inhibits a biological activity of the NPTX2 polypeptide can be screened. Such substances would be potential candidates for treating cancer.

[0251] When the biological activity to be detected in the screening method of the present invention is cell proliferation promoting activity, it can be detected, for example, by preparing cells which express the NPTX2 polypeptide or functional equivalent thereof, culturing the cells in the presence of a test substance, and determining the speed of cell proliferation, measuring the cell cycle and such, as well as by measuring the colony formation activity, e.g. MTT assay, colony formation assay or FACS. In some embodiments, cells expressing NPTX2 gene are isolated and cultured cells exogenously or endogenously expressing NPTX2 gene in vitro.

[0252] The term of "suppress the biological activity" as defined herein refers to at least 10% suppression of the biological activity of the NPTX2 polypeptide in comparison with in absence of the substance, for example, at least 25%, 50% or 75% suppression, for example, at least 90% suppression.

[0253] In the preferred embodiments, control cells that do not express the NPTX2 gene are preferably used. Accordingly, the present invention also provides a method of screening for a candidate substance for inhibiting the cell growth or a candidate substance for treating or preventing cancer, using the NPTX2 polypeptide or functional equivalent thereof, including steps as follows:

(a) culturing cells which express an NPTX2 polypeptide or a functional equivalent thereof, and control cells that do not express an NPTX2 polypeptide or functional equivalent thereof in the presence of a test substance;

(b) detecting growth of the cell which express the NPTX2 polypeptide and control cells; and

(c) selecting the test substance that inhibits the growth of the cells that express the NPTX2 polypeptide as compared to the growth detected in the control cells and in the absence of the test substance.

[0254] In the course of the present invention, it was revealed that suppressing the biological activity of the NPTX2 polypeptide reduces cell growth. Thus, by screening for candidate substances that inhibits biological activity of the NPTX2 polypeptide, candidate substances that have the potential to treat or prevent cancers or inhibit cancer cell growth can be identified. Potential of these candidate substances to treat or prevent cancers may be evaluated by second and/or further screening to identify therapeutic agent for cancers. For example, when a substance inhibits the biological activity of NPTX2 polypeptide inhibits activities of cancer, it may be concluded that such substance has NPTX2 specific therapeutic effect.

[0255] (iv) Screening for Substances that Alter the Expression of the NPTX2 Gene

In the course of the present invention, a decrease in the expression of the NPTX2 gene by a double-stranded molecule specific for NPTX2 was demonstrated to inhibit cancer cell proliferation. Therefore, candidate substances for use in either or both of the treatment and prevention of cancer can be identified through screenings that use the expression level of the NPTX2 gene as indices. Accordingly, the present invention also provides a method of screening for a candidate substance for either or both of treating and preventing cancer, such method including steps of:

- (a) contacting a test substance with a cell expressing the NPTX2 gene ;
- (b) detecting the expression level of the NPTX2 gene; and
- (c) selecting the test substance that reduces the expression level of the NPTX2 gene as compared to that detected in the absence of the test substance.

[0256] According to the present invention, the therapeutic effect of the test substance on the inhibition of cell growth or a candidate substance for either or both of treating and preventing cancer may be evaluated. Therefore, the present invention also provides a method for screening a candidate substance that suppresses the proliferation of cancer cells, and a method for screening a candidate substance for either or both of treating and preventing cancer,.

[0257] In the context of the present invention, such screening may include, for example, the following steps:

- (a) contacting a test substance with a cell expressing the NPTX2 gene;
- (b) detecting the expression level of the NPTX2 gene; and
- (c) correlating the expression level of (b) with the therapeutic effect of the test substance.

[0258] Alternatively, according to the present invention, the potential therapeutic effect of a test substance on either or both of treating and preventing cancer can also be evaluated or estimated. In some embodiments, the present invention provides a method for evaluating or estimating a therapeutic effect of a test substance on treating or preventing cancer or inhibiting cancer, the method including steps of:

- (a) contacting a test substance with a cell expressing the NPTX2 gene;
- (b) detecting the expression level of the NPTX2 gene of step (a); and
- (c) correlating the potential therapeutic effect and the test substance, wherein the potential therapeutic effect is shown, when the test substance reduces the expression level of the NPTX2 gene.

[0259] In the context of the present invention, the therapeutic effect may be correlated with the expression level of the NPTX2 gene. For example, when the test substance reduces the expression level of the NPTX2 gene as compared to the expression level detected in the absence of the test substance, the test substance may be identified or selected as the candidate substance having the therapeutic effect. Alternatively, when the test substance does not reduce the expression level of the NPTX2 gene as compared to the expression level detected in the absence of the test substance, the test substance may be identified as the substance having no significant therapeutic effect.

[0260] The method of the present invention is described in more detail below.

Cells expressing the NPTX2 gene include, for example, cell lines established from lung cancer, breast cancer, cervical cancer or colon cancer; such cells can be used for the above screening of the present invention (e.g., SBC-5 and NCI-H520). The expression level can be estimated by methods well known to one skilled in the art, for example, RT-PCR, Northern blot assay, Western blot assay, immunostaining, ELISA or flow cytometry analysis. The term of "reduce the expression level" as defined herein refers to at least 10% reduction of the expression level of the NPTX2 gene in comparison to the expression level in absence of the substance, for example, at least 25%, 50% or 75% reduced level, for example, at least 95% reduced level. The substance herein includes chemical compound, double-strand nucleotide, and so on. The preparation of the double-strand nucleotide will be described below. In the method of screening, a substance that reduces the expression level of the NPTX2 gene can be selected as candidate substances to be used for either or both of the treatment and prevention of cancers. In some embodiments, cells expressing NPTX2 gene are isolated and cultured cells exogenously or endogenously expressing NPTX2 gene in vitro.

[0261] Alternatively, the screening method of the present invention can include the following steps:

- (a) contacting a test substance with a cell into which a vector, comprising the transcriptional regulatory region of the NPTX2 gene and a reporter gene that is expressed under the control of the transcriptional regulatory region, has been introduced;
- (b) measuring the expression level or activity of the reporter gene; and
- (c) selecting the test substance that reduces the expression level or activity of the reporter gene.

- [0262] According to the present invention, the therapeutic effect of the test substance on inhibiting the cell growth or a candidate substance for either or both of treating and preventing cancer may be evaluated. Therefore, the present invention also provides a method for screening a candidate substance that suppresses the proliferation of cancer cells, and a method for screening a candidate substance for treating or preventing cancer.
- [0263] According to another aspect, the present invention provides a method that includes the following steps of:
- (a) contacting a test substance with a cell into which a vector, composed of the transcriptional regulatory region of the NPTX2 gene and a reporter gene that is expressed under the control of the transcriptional regulatory region, has been introduced;
 - (b) detecting the expression level or activity of the reporter gene; and
 - (c) correlating the expression level or activity of (b) with the therapeutic effect of the test substance.
- [0264] Alternatively, in some embodiments, the present invention also provides a method for evaluating or estimating a therapeutic effect of a test substance on either or both of treating and preventing cancer or inhibiting cancer, the method including steps of:
- (a) contacting a test substance with a cell into which a vector, including the transcriptional regulatory region of the NPTX2 gene and a reporter gene that is expressed under the control of the transcriptional regulatory region, has been introduced;
 - (b) measuring the expression level or activity of the reporter gene; and
 - (c) correlating the potential therapeutic effect and the test substance, wherein the potential therapeutic effect is shown, when a test substance reduces the expression level or activity of the reporter gene.
- [0265] In the context of the present invention, the therapeutic effect may be correlated with the expression level or activity of the reporter gene. For example, when the test substance reduces the expression level or activity of the reporter gene as compared to a level detected in the absence of the test substance, the test substance may be identified or selected as the candidate substance having the therapeutic effect. Alternatively, when the test substance does not reduce the expression level or activity of the reporter gene as compared to a level detected in the absence of the test substance, the test substance may be identified as the substance having no significant therapeutic effect.
- [0266] Suitable reporter genes and host cells are well known in the art. For example, reporter genes are luciferase, green fluorescence protein (GFP), Discosoma sp. Red Fluorescent Protein (DsRed), Chrolamphenicol Acetyltransferase (CAT), lacZ and beta-glucuronidase (GUS), and host cell is COS7, HEK293, HeLa and so on. The reporter construct required for the screening can be prepared by connecting reporter

gene sequence to the transcriptional regulatory region of CX. The transcriptional regulatory region of CX herein is the region from start codon to at least 500bp upstream, for example, 1000bp, for example, 5000 or 10000bp upstream, but not restricted. A nucleotide segment containing the transcriptional regulatory region can be isolated from a genome library or can be propagated by PCR. Methods for identifying a transcriptional regulatory region, and also assay protocol are well known (Molecular Cloning third edition chapter 17, 2001, Cold Springs Harbor Laboratory Press).

[0267] The vector containing the reporter construct is infected to host cells and the expression or activity of the reporter gene is detected by method well known in the art (e.g., using luminometer, absorption spectrometer, flow cytometer and so on). In some embodiments, cells of the present invention are isolated and cultured cells into which a vector, composed of the transcriptional regulatory region of the NPTX2 gene and a reporter gene that is expressed under the control of the transcriptional regulatory region, has been introduced in vitro. "Reduces the expression or activity" as defined herein refers to at least 10% reduction of the expression or activity of the reporter gene in comparison with in absence of the substance, for example, at least 25%, 50% or 75% reduction, for example, at least 95% reduction.

[0268] The invention is further described in the following examples, which do not limit the scope of the invention described in the claims.

In the course of the present invention, it was revealed that suppressing the expression level of the NPTX2 gene, reduces cell growth. Thus, by screening for candidate substances that inhibits expression level of the NPTX2 gene, candidate substances that have the potential to treat or prevent cancers can be identified. Potential of these candidate substances to treat or prevent cancers may be evaluated by second and/or further screening to identify therapeutic agent for cancers. For example, when a substance inhibits the expression level of the NPTX2 polypeptide inhibits activities of cancer, it may be concluded that such substance has NPTX2 specific therapeutic effect.

[0269] (6) Double-Stranded Molecules:

As used herein, the term "double-stranded molecule" refers to a nucleic acid molecule that inhibits expression of a target gene and includes, for example, short interfering RNA (siRNA; e.g., double-stranded ribonucleic acid (dsRNA) or small hairpin RNA (shRNA)) and short interfering DNA/RNA (siD/R-NA; e.g. double-stranded chimera of DNA and RNA (dsD/R-NA) or small hairpin chimera of DNA and RNA (shD/R-NA)).

[0270] As used herein, the term "target sequence" refers to a nucleotide sequence within mRNA or cDNA sequence of a target gene, which will result in suppression of translation of the whole mRNA of the target gene if the double-stranded molecule is introduced within a cell expressing the gene. A nucleotide sequence within mRNA or

cDNA sequence of a target gene can be determined to be a target sequence when a double-stranded molecule having a sequence corresponding to the target sequence inhibits expression of the gene in a cell expressing the gene. When a target sequence is shown by cDNA sequence, a sense strand sequence of a double-stranded cDNA, i.e., a sequence that mRNA sequence is converted into DNA sequence, is used for defining a target sequence. A double-stranded molecule is composed of a sense strand that has a sequence corresponding to a target sequence and an antisense strand that has a sequence complementary to the target sequence, and the antisense strand hybridizes with the sense strand at the complementary sequence to form a double-stranded molecule.

[0271] A double-stranded molecule is composed of a sense strand having a sequence corresponding to a target sequence and an antisense strand having a complementary sequence to the target sequence, such that the antisense strand hybridizes with the sense strand at the complementary sequence to form a double-stranded molecule. Herein, the phrase "corresponding to" means converting a target sequence according to the kind of nucleic acid that constitutes a sense strand of a double-stranded molecule. For example, when a target sequence is shown in DNA sequence and a sense strand of a double-stranded molecule has an RNA region, base "t"s within the RNA region is replaced with base "u"s. On the other hand, when a target sequence is shown in RNA sequence and a sense strand of a double-stranded molecule has a DNA region, base "u"s within the DNA region is replaced with "t"s. For example, when a target sequence is the RNA sequence shown in SEQ ID NO: 7 or 8 and the sense strand of the double-stranded molecule is composed of RNA, "a sequence corresponding to a target sequence" is "GAAGCAGCACGACUUCUUC" (SEQ ID NO: 7) or "CGUACGCG-GAAUACUUCGA" (SEQ ID NO: 8).

[0272] Also, a complementary sequence to a target sequence for an antisense strand of a double-stranded molecule can be defined according to the kind of nucleic acid that constitutes the antisense strand. For example, when a target sequence is the RNA sequence shown in SEQ ID NO: 7 or 8 and the antisense strand of the double-stranded molecule is composed of RNA, "a complementary sequence to a target sequence" is "CUUCGUCGUGCUGAAGAAG" (for SEQ ID NO: 7) or "GCAUGCGCCU-UAUGAAGCU" (for SEQ ID NO: 8). A double-stranded molecule may have one or two 3'overhangs having 2 to 5 nucleotides in length (e.g., uu) and/or a loop sequence that links a sense strand and an antisense strand to form hairpin structure, in addition to a sequence corresponding to a target sequence and sequence complementary thereto.

[0273] As used herein, the term "siRNA" refers to a double-stranded RNA molecule which prevents translation of a target mRNA. Standard techniques of introducing siRNA into the cell are used, including those in which DNA is a template from which RNA is

transcribed. The siRNA includes an NPTX2 sense nucleic acid sequence (also referred to as "sense strand"), a NPTX2 antisense nucleic acid sequence (also referred to as "antisense strand") or both. The siRNA may be constructed such that a single transcript has both the sense and complementary antisense nucleic acid sequences of the target gene, e.g., a hairpin. The siRNA may either be a dsRNA or shRNA.

[0274] As used herein, the term "dsRNA" refers to a construct of two RNA molecules composed of sequences complementary to one another and that have annealed together via the complementary sequences to form a double-stranded RNA molecule. The nucleotide sequence of two strands may include not only the "sense" or "antisense" RNAs selected from a protein coding sequence of target gene sequence, but also RNA molecule having a nucleotide sequence selected from non-coding region of the target gene.

[0275] The term "shRNA", as used herein, refers to an siRNA having a stem-loop structure, composed of first and second regions complementary to one another, i.e., sense and antisense strands. The degree of complementarity and orientation of the regions are sufficient such that base pairing occurs between the regions, the first and second regions are joined by a loop region, the loop results from a lack of base pairing between nucleotides (or nucleotide analogs) within the loop region. The loop region of an shRNA is a single-stranded region intervening between the sense and antisense strands and may also be referred to as "intervening single-strand".

[0276] As use herein, the term "siD/R-NA" refers to a double-stranded polynucleotide molecule which is composed of both RNA and DNA, and includes hybrids and chimeras of RNA and DNA and prevents translation of a target mRNA. Herein, a hybrid indicates a molecule wherein a polynucleotide composed of DNA and a polynucleotide composed of RNA hybridize to each other to form the double-stranded molecule; whereas a chimera indicates that one or both of the strands composing the double stranded molecule may contain RNA and DNA. Standard techniques of introducing siD/R-NA into the cell are used. The siD/R-NA includes a NPTX2 sense nucleic acid sequence (also referred to as "sense strand"), a NPTX2 antisense nucleic acid sequence (also referred to as "antisense strand") or both. The siD/R-NA may be constructed such that a single transcript has both the sense and complementary antisense nucleic acid sequences from the target gene, e.g., a hairpin. The siD/R-NA may either be a dsD/R-NA or shD/R-NA.

[0277] As used herein, the term "dsD/R-NA" refers to a construct of two molecules composed of sequences complementary to one another and that have annealed together via the complementary sequences to form a double-stranded polynucleotide molecule. The nucleotide sequence of two strands may include not only the "sense" or "antisense" polynucleotides sequence selected from a protein coding sequence of target

gene sequence, but also polynucleotide having a nucleotide sequence selected from non-coding region of the target gene. One or both of the two molecules constructing the dsD/R-NA are composed of both RNA and DNA (chimeric molecule), or alternatively, one of the molecules is composed of RNA and the other is composed of DNA (hybrid double-strand).

- [0278] The term "shD/R-NA", as used herein, refers to an siD/R-NA having a stem-loop structure, composed of the first and second regions complementary to one another, i.e., sense and antisense strands. The degree of complementarity and orientation of the regions are sufficient such that base pairing occurs between the regions, the first and second regions are joined by a loop region, and the loop results from a lack of base pairing between nucleotides (or nucleotide analogs) within the loop region. The loop region of an shD/R-NA is a single-stranded region intervening between the sense and antisense strands and may also be referred to as "intervening single-strand".
- [0279] As used herein, an "isolated nucleic acid" is a nucleic acid removed from its original environment (e.g., the natural environment if naturally occurring) and thus, synthetically altered from its natural state. In the present invention, examples of isolated nucleic acid include DNA, RNA, and derivatives thereof.
- [0280] In the context of the present invention, a double-stranded molecule against the NPTX2 gene, which a molecule that hybridizes to target mRNA, decreases or inhibits production of the NPTX2 polypeptide encoded by the NPTX2 gene by associating with the normally single-stranded mRNA transcript of the gene, interferes with translation and thus, inhibits expression of the protein. As demonstrated herein, the expression of the NPTX2 gene in lung cancer cell lines was inhibited by dsRNA.
- [0281] Therefore the present invention provides isolated double-stranded molecules that are capable of inhibiting the expression of the NPTX2 gene when introduced into a cell expressing the gene. The target sequence of double-stranded molecule may be designed by an siRNA design algorithm such as that mentioned below.
- [0282] Methods for designing double-stranded molecules having the ability to inhibit target gene expression in cells are known. (See, for example, US Patent No. 6,506,559, herein incorporated by reference in its entirety). For example, a computer program for designing siRNAs is available from the Ambion website (http://www.ambion.com/techlib/misc/siRNA_finder.html).
- [0283] Such a computer program selects target nucleotide sequences for double-stranded molecules based on the following protocol.

Selection of Target Sites:

1.Beginning with the AUG start codon of the transcript, scan downstream for AA dinucleotide sequences. Record the occurrence of each AA and the 3' adjacent 19 nucleotides as potential siRNA target sites. Tuschl et al. recommend to avoid designing

siRNA to the 5' and 3' untranslated regions (UTRs) and regions near the start codon (within 75 bases) as these may be richer in regulatory protein binding sites, and UTR-binding proteins and/or translation initiation complexes may interfere with binding of the siRNA endonuclease complex.

2. Compare the potential target sites to the appropriate genome database (human, mouse, rat, etc.) and eliminate from consideration any target sequences with significant homology to other coding sequences. Basically, BLAST, which can be found on the NCBI server at: www.ncbi.nlm.nih.gov/BLAST/, is used (Altschul SF et al., *Nucleic Acids Res* 1997 Sep 1, 25(17): 3389-402).

3. Select qualifying target sequences for synthesis. Selecting several target sequences along the length of the gene to evaluate is typical.

[0284] Double-stranded molecules targeting the above-mentioned target sequences were respectively examined for their ability to suppress the growth of cells expressing the target genes.

The double-stranded molecule of the present invention may be directed to a single target NPTX2 gene sequence or may be directed to a plurality of target NPTX2 gene sequences. A double-stranded molecule of the present invention targeting the above-mentioned targeting sequence of the NPTX2 gene include isolated polynucleotides that contain any of the nucleic acid sequences of target sequences and/or sequences complementary to the target sequences.

[0285] In the context of the present invention, the term "several" as applies to nucleic acid substitutions, deletions, additions and/or insertions may mean 3 to 7, preferably 3 to 5, more preferably 3 to 4, even more preferably 3 nucleic acid residues.

According to the present invention, a double-stranded molecule of the present invention can be tested for its ability using the methods utilized in the Examples. In the Examples herein below, double-stranded molecules composed of sense strands of various portions of mRNA of the NPTX2 gene or antisense strands complementary thereto were tested in vitro for their ability to decrease production of the NPTX2 gene product in cancer cell lines (e.g., using NCI-H520 or SBC5) according to standard methods. Furthermore, for example, reduction in NPTX2 gene product in cells contacted with the candidate double-stranded molecule compared to cells cultured in the absence of the candidate molecule can be detected by, e.g. RT-PCR using primers for the NPTX2 mRNA mentioned under Example. Sequences which decrease the production of the NPTX2 gene product in vitro cell-based assays can then be tested for their inhibitory effects on cell growth. Sequences which inhibit cell growth in vitro cell-based assay can then be tested for their in vivo ability using animals with cancer, e.g. nude mouse xenograft models, to confirm decreased production of the NPTX2 gene product and decreased cancer cell growth.

- [0286] When the isolated polynucleotide is RNA or derivatives thereof, base "t" should be replaced with "u" in the nucleotide sequences. As used herein, the term "complementary" refers to Watson-Crick or Hoogsteen base pairing between nucleotides units of a polynucleotide, and the term "binding" means the physical or chemical interaction between two polynucleotides. When the polynucleotide includes modified nucleotides and/or non-phosphodiester linkages, these polynucleotides may also bind each other in the same manner. Generally, complementary polynucleotide sequences hybridize under appropriate conditions to form stable duplexes containing few or no mismatches. However, the present invention extends to complementary sequences that include mismatches of one or more nucleotides. In addition, the sense strand and antisense strand of the isolated polynucleotide of the present invention can form a double-stranded molecule or hairpin loop structure by the hybridization. In a preferred embodiment, such duplexes contain no more than 1 mismatch for every 10 matches. In an especially preferred embodiment, where the strands of the duplex are fully complementary, such duplexes contain no mismatches.
- [0287] For example, the complementary or antisense polynucleotide is less than 500, 200, 100, 75, 50, or 25 nucleotides in length. The isolated polynucleotides are useful for forming double-stranded molecules against the NPTX2 gene or preparing template DNAs encoding the double-stranded molecules. When the polynucleotides are used for forming double-stranded molecules, the polynucleotide may be longer than 19 nucleotides, preferably longer than 21 nucleotides, and more preferably has a length of between about 19 and 25 nucleotides. Accordingly, the present invention provides the double-stranded molecules comprising a sense strand and an antisense strand, wherein the sense strand comprises a nucleotide sequence corresponding to a target sequence. In preferred embodiments, the sense strand hybridizes with an antisense strand at the target sequence to form the double-stranded molecule having between 19 and 25 nucleotide pairs in length.
- [0288] The double-stranded molecules of the present invention may contain one or more modified nucleotides and/or non-phosphodiester linkages. It is well known in the art to introduce chemical modifications capable of increasing stability, availability, and/or cell uptake of the double-stranded molecule. A person skilled in the art will be aware of the wide array of chemical modifications that may be incorporated into the present molecules (WO03/070744; WO2005/045037). For example, in one embodiment, modifications can be used to provide improved resistance to degradation or improved uptake. Examples of such modifications include, but are not limited to, phosphorothioate linkages, 2'-O-methyl ribonucleotides (especially on the sense strand of a double-stranded molecule), 2'-deoxy-fluoro ribonucleotides, 2'-deoxy ribonucleotides, "universal base" nucleotides, 5'-C-methyl nucleotides, and inverted deoxybasic

residue incorporation (US20060122137).

[0289] In another embodiment, modifications can be used to enhance the stability or to increase targeting efficiency of the double-stranded molecule. Examples of such modifications include, but are not limited to, chemical cross linking between the two complementary strands of a double-stranded molecule, chemical modification of a 3' or 5' terminus of a strand of a double-stranded molecule, sugar modifications, nucleobase modifications and/or backbone modifications, 2'-fluoro modified ribonucleotides and 2'-deoxy ribonucleotides (WO2004/029212). In another embodiment, modifications can be used to increase or decrease affinity for the complementary nucleotides in the target mRNA and/or in the complementary double-stranded molecule strand (WO2005/044976). For example, an unmodified pyrimidine nucleotide can be substituted for a 2-thio, 5-alkynyl, 5-methyl, or 5-propynyl pyrimidine. Additionally, an unmodified purine can be substituted with a 7-diaza, 7-alkyl, or 7-alkenyl purine. In another embodiment, when the double-stranded molecule is a double-stranded molecule with a 3' overhang, the 3'-terminal nucleotide overhanging nucleotides may be replaced by deoxyribonucleotides (Elbashir SM et al., Genes Dev 2001 Jan 15, 15(2): 188-200). For further details, published documents such as US20060234970 are available. However, the present invention should not be construed as limited to these examples; any of a number of conventional chemical modifications may be employed for the double-stranded molecules of the present invention so long as the resulting molecule retains the ability to inhibit the expression of the target gene.

[0290] The double-stranded molecules of the present invention may include both DNA and RNA, e.g., dsD/R-NA or shD/R-NA. For example, a hybrid polynucleotide of a DNA strand and an RNA strand or a DNA-RNA chimera polynucleotide shows increased stability and are thus contemplated herein. Mixing of DNA and RNA, i.e., a hybrid type double-stranded molecule composed of a DNA strand (polynucleotide) and an RNA strand (polynucleotide), a chimera type double-stranded molecule containing both DNA and RNA on any or both of the single strands (polynucleotides), or the like may be formed for enhancing stability of the double-stranded molecule.

[0291] The hybrid of a DNA strand and an RNA strand may either have a DNA sense strand coupled to an RNA antisense strand, vice versa, so long as the resulting double stranded molecule can inhibit expression of the target gene when introduced into a cell expressing the gene. In a preferred embodiment, the sense strand polynucleotide is DNA and the antisense strand polynucleotide is RNA. Also, the chimera type double-stranded molecule may have either or both sense and antisense strands composed of DNA and RNA, so long as the resulting double-stranded molecule has an activity to inhibit the expression of the target gene when introduced into a cell expressing the gene. In order to enhance stability of the double-stranded molecule, the molecule

preferably contains as much DNA as possible, whereas to induce inhibition of the target gene expression, the molecule is required to be RNA within a range to induce sufficient inhibition of the expression.

[0292] A preferred example of the chimera type double-stranded molecule contains an upstream partial region (i.e., a region flanking to the target sequence or a complementary sequence thereof within the sense or antisense strands) composed of RNA. Preferably, the upstream partial region indicates the 5' side (5'-end) of the sense strand and the 3' side (3'-end) of the antisense strand. Alternatively, regions flanking to 5'-end of sense strand and/or 3'-end of antisense strand may be referred as the upstream partial region. That is, in preferred embodiments, a region flanking to the 3'-end of the antisense strand, or both of a region flanking to the 5'-end of sense strand and a region flanking to the 3'-end of antisense strand are composed of RNA. For instance, a chimera or hybrid type double-stranded molecule of the present invention may include following combinations.

sense strand:

5'-[---DNA---]-3'

3'-(RNA)-[DNA]-5'

:antisense strand,

sense strand:

5'-(RNA)-[DNA]-3'

3'-(RNA)-[DNA]-5'

:antisense strand, and

sense strand:

5'-(RNA)-[DNA]-3'

3'-(---RNA---)-5'

:antisense strand.

[0293] The upstream partial region preferably is a domain composed of 9 to 13 nucleotides counted from the terminus of the target sequence or sequence complementary thereto within the sense or antisense strands of the double-stranded molecules. Moreover, preferred examples of such chimera type double-stranded molecules include those having a strand length of 19 to 21 nucleotides in which at least the upstream half region (5' side region for the sense strand and 3' side region for the antisense strand) of the polynucleotide is RNA and the other half is DNA. In such a chimera type double-stranded molecule, the effect to inhibit expression of the target gene is much higher when the entire antisense strand is RNA (US20050004064).

[0294] In the context of the present invention, the double-stranded molecule may form a hairpin, such as a short hairpin RNA (shRNA) and short hairpin composed of DNA and RNA (shD/R-NA). The shRNA or shD/R-NA is a sequence of RNA or mixture of

RNA and DNA making a tight hairpin turn that can be used to silence gene expression via RNA interference. The shRNA or shD/R-NA comprises the sense target sequence and the antisense target sequence on a single strand wherein the sequences are separated by a loop sequence. Generally, the hairpin structure is cleaved by the cellular machinery into dsRNA or dsD/R-NA molecules, which are then bound to the RNA-induced silencing complex (RISC). This complex binds to and cleaves mRNAs that match the target sequence of the dsRNA or dsD/R-NA. A loop sequence composed of an arbitrary nucleotide sequence can be located between the sense and antisense sequence in order to form the hairpin loop structure. Thus, the present invention also provides a double-stranded molecule having the general formula 5'-[A]-[B]-[A']-3', wherein [A] is the sense strand containing a sequence corresponding to a target sequence, [B] is an intervening single-strand and [A'] is the antisense strand containing a sequence complementary to [A].

[0295] The present invention is not limited to these examples, and the target sequence in [A] may be modified sequences from these examples so long as the double-stranded molecule retains the ability to suppress the expression of the targeted NPTX2 gene. The region [A] hybridizes to [A'] to form a loop composed of the region [B]. The intervening single-stranded portion [B], i.e., loop sequence may be preferably 3 to 23 nucleotides in length. The loop sequence, for example, can be selected from among the following sequences (http://www.ambion.com/techlib/tb/tb_506.html). Furthermore, loop sequence composed of 23 nucleotides also provides active siRNA (Jacque JM et al., Nature 2002 Jul 25, 418(6896): 435-8, Epub 2002 Jun 26): CCC, CCACC, or CCACACC; Jacque JM et al., Nature 2002 Jul 25, 418(6896): 435-8, Epub 2002 Jun 26; UUCG: Lee NS et al., Nat Biotechnol 2002 May, 20(5): 500-5; Fruscoloni P et al., Proc Natl Acad Sci USA 2003 Feb 18, 100(4): 1639-44, Epub 2003 Feb 10; and UUCAAGAGA: Dykxhoorn DM et al., Nat Rev Mol Cell Biol 2003 Jun, 4(6): 457-67.

In preferred embodiments, the loop sequence can be selected from among AUG, CCC, UUCG, CCACC, CTCGAG, AAGCUU, CCACACC, and UUCAAGAGA, but are not limited to.

[0296] Additionally, nucleotide "u" can be added to 3' end of the sense strand and/or the antisense strand of the target sequence, as 3' overhangs in order to enhance the inhibition activity of the double-stranded molecules. The number of "u"s to be added is at least 2, generally 2 to 10, preferably 2 to 3. In cases where double-stranded molecules is composed of a single polynucleotide to a hairpin loop structure, a 3' overhang sequence may be added to the 3' end of the single polynucleotide.

[0297] The method for preparing the double-stranded molecule is not particularly limited though it is preferable to use one of the standard chemical synthetic method known in the art. According to one chemical synthesis method, sense and antisense single-

stranded polynucleotides are separately synthesized and then annealed together via an appropriate method to obtain a double-stranded molecule. In one specific annealing embodiment, the synthesized single-stranded polynucleotides are mixed in a molar ratio of preferably at least about 3:7, more preferably about 4:6, and most preferably substantially equimolar amount (i.e., a molar ratio of about 5:5). Next, the mixture is heated to a temperature at which double-stranded molecules dissociate and then is gradually cooled down. The annealed double-stranded polynucleotide can be purified by usually employed methods known in the art. Example of purification methods include methods utilizing agarose gel electrophoresis or wherein remaining single-stranded polynucleotides are optionally removed by, e.g., degradation with appropriate enzyme.

[0298] The regulatory sequences flanking the NPTX2 sequences may be identical or different, such that their expression can be modulated independently, or in a temporal or spatial manner. The double-stranded molecules can be transcribed intracellularly by cloning the NPTX2 gene templates into a vector containing, e.g., a RNA pol III transcription unit from the small nuclear RNA (snRNA) U6 or the human H1 RNA promoter.

[0299] (7) Vectors encoding a Double-Stranded Molecule:

The present invention also provides vectors encoding one or more of the double-stranded molecules described above, and a cell containing such a vector.

[0300] The vector preferably encodes the double-stranded molecule in an expressible form. Herein, the phrase "in an expressible form" indicates that the vector, when introduced into a cell, will express the molecule carried, contained or encoded. In a preferred embodiment, the vector includes one or more regulatory elements necessary for expression of the double-stranded molecule. Such vector may be used for producing the present double-stranded molecules, or directly as an active ingredient for treating cancer.

[0301] Vectors of the present invention can be produced, for example, by cloning the NPTX2 sequence into an expression vector so that regulatory sequences are operatively-linked to the NPTX2 sequence in a manner to allow expression (by transcription of the DNA molecule) of both strands (Lee NS et al., Nat Biotechnol 2002 May, 20(5): 500-5). For example, RNA molecule that is the antisense to mRNA is transcribed by a first promoter (e.g., a promoter sequence flanking to the 3' end of the cloned DNA) and RNA molecule that is the sense strand to the mRNA is transcribed by a second promoter (e.g., a promoter sequence flanking to the 5' end of the cloned DNA). The sense and antisense strands hybridize in vivo to generate a double-stranded molecule constructs for silencing of the gene. Alternatively, two vectors constructs respectively encoding the sense and antisense strands of the double-stranded molecule

are utilized to respectively express the sense and anti-sense strands and then forming a double-stranded molecule construct. Furthermore, the cloned sequence may encode a construct having a secondary structure (e.g., hairpin); accordingly, a single transcript of a vector may contain both the sense and antisense sequences of the target gene.

[0302] The vectors of the present invention may also be equipped so to achieve stable insertion into the genome of the target cell (see, e.g., Thomas KR & Capecchi MR, *Cell* 1987, 51: 503-12 for a description of homologous recombination cassette vectors). See, e.g., Wolff et al., *Science* 1990, 247: 1465-8; US Patent Nos. 5,580,859; 5,589,466; 5,804,566; 5,739,118; 5,736,524; 5,679,647; and WO 98/04720. Examples of DNA-based delivery technologies include "naked DNA", facilitated (bupivacaine, polymers, peptide-mediated) delivery, cationic lipid complexes, and particle-mediated ("gene gun") or pressure-mediated delivery (see, e.g., US Patent No. 5,922,687).

[0303] The vectors of the present invention include, for example, viral or bacterial vectors. Examples of expression vectors include attenuated viral hosts, such as vaccinia or fowlpox (see, e.g., US Patent No. 4,722,848). This approach involves the use of vaccinia virus, e.g., as a vector to express nucleotide sequences that encode the double-stranded molecule. Upon introduction into a cell expressing the target gene, the recombinant vaccinia virus expresses the molecule and thereby suppresses the proliferation of the cell. Another example of useable vector includes Bacille Calmette Guerin (BCG). BCG vectors are described in Stover et al., *Nature* 1991, 351: 456-60. A wide variety of other vectors are useful for therapeutic administration and production of the double-stranded molecules; examples include adeno and adeno-associated virus vectors, retroviral vectors, *Salmonella typhi* vectors, detoxified anthrax toxin vectors, and the like. See, e.g., Shata et al., *Mol Med Today* 2000, 6: 66-71; Shedlock et al., *J Leukoc Biol* 2000, 68: 793-806; and Hipp et al., *In Vivo* 2000, 14: 571-85.

[0304] (8) Methods of Treating cancer:

In the course of the present invention, two different dsRNA were tested for their ability to inhibit cell growth. The two dsRNA effectively knocked down the expression of the NPTX2 gene in lung cancer cell lines coincided with suppression of cell proliferation.

Therefore, the present invention also provides methods for inhibiting cancer cell growth, by inducing dysfunction of the NPTX2 gene via inhibiting the expression of the NPTX2 gene. The expression of the NPTX2 gene can be inhibited by any of the aforementioned double-stranded molecules which specifically target of the NPTX2 gene or the vectors encoding thereof that can express any of the double-stranded molecules.

[0305] Such ability of the present double-stranded molecules and vectors to inhibit cell

growth of cancerous cell indicates that they can be used for methods for treating primary cancer, as well as treating or preventing a post-operative, secondary, or metastatic recurrence thereof. Thus, the present invention provides methods to treat subjects with cancer by administering a double-stranded molecule against the NPTX2 gene or a vector expressing the molecule without adverse effect because that gene was hardly detected in normal organs. In the context of the present invention, the cancer is preferably lung cancer, breast cancer, cervical cancer or colon cancer, especially lung cancer.

[0306] Illustrative examples of present invention are set forth as items [1] to [20]:

[1] A method of either or both of treating and preventing cancer or inhibiting cancer cell growth in a subject, wherein said method comprises a step of administering to the subject a pharmaceutically effective amount of a double-stranded molecule against the NPTX2 gene, or a vector encoding the double-stranded molecule, wherein the double-stranded molecules comprises a sense strand and an antisense strand, wherein the sense strand comprises a nucleotide sequence corresponding to a target sequence selected from the NPTX2 gene sequence, and the antisense strand comprises a nucleotide sequence complementary to said target sequence, wherein the sense strand and the antisense strand hybridize to each other to form the double-stranded molecule, and wherein the double stranded molecule, when introduced into a cell expressing the NPTX2 gene, inhibits cell proliferation as well as the expression of the NPTX2 gene.;

[2] The method of [1], wherein the cancer is lung cancer, breast cancer, cervical cancer or colon cancer;

[3] The method of [2], wherein the lung cancer is SCLC;

[4] The method of any one of [1] to [3], wherein plural kinds of the double-stranded molecules are administered at the same time;

[5] The method of any one of [1] to [4], wherein the sense strand hybridize with antisense strand at the target sequence to form the double-stranded molecule having less than about 100 nucleotide pairs in length;

[6] The method of [5], wherein the sense strand hybridize with antisense strand at the target sequence to form the double-stranded molecule having less than about 75 nucleotide pairs in length;

[7] The method of [6], wherein the sense strand hybridize with antisense strand at the target sequence to form the double-stranded molecule having less than about 50 nucleotide pairs in length;

[8] The method of [7], wherein the sense strand hybridize with antisense strand at the target sequence to form the double-stranded molecule having less than about 25 nucleotide pairs in length;

[9] The method of [8], wherein the sense strand hybridize with antisense strand at the

target sequence to form the double-stranded molecule having between about 19 to 25 nucleotide pairs in length;

[10] The method of any one of [1] to [9], wherein the target sequence is a sequence of SEQ ID NO: 7 or 8;

[11] The method of any one of [1] to [10], wherein the double-stranded molecule is composed of a single polynucleotide containing both the sense strand and the antisense strand linked by an intervening single-strand;

[12] The method of [11], wherein the double-stranded molecule has the general formula 5'-[A]-[B]-[A']-3' or 5'-[A']-[B]-[A], wherein [A] is the sense strand containing a sequence corresponding to a target sequence of the NPTX2 gene, [B] is the intervening single strand composed of 3 to 23 nucleotides, and [A'] is the antisense strand containing a sequence complementary to the target sequence;

[13] The method of any one of [1] to [12], wherein the double-stranded molecule is an RNA;

[14] The method of any one of [1] to [12], wherein the double-stranded molecule contains both DNA and RNA;

[15] The method of [14], wherein the double-stranded molecule is a hybrid of a DNA polynucleotide and an RNA polynucleotide;

[16] The method of [15] wherein the sense and antisense strand polynucleotides are composed of DNA and RNA, respectively;

[17] The method of [15], wherein the double-stranded molecule is a chimera of DNA and RNA;

[18] The method of [17], wherein a region flanking to the 3'-end of the antisense strand, or both of a region flanking to the 5'-end of sense strand and a region flanking to the 3'-end of antisense strand are composed of RNA;

[19] The method of [18], wherein the flanking region is composed of 9 to 13 nucleotides;

[20] The method of any one of [1] to [19], wherein the double-stranded molecule contains 3' overhangs; and

[21] The method of any one of [1] to [20], wherein the double-stranded molecule or the vector is administered to the subject with a transfection-enhancing agent.

[0307] Therapeutic methods are described in more detail below.

The methods of the present invention may be preferably applicable to lung cancer, breast cancer, cervical cancer and colon cancer, more preferably lung cancer, further more preferably SCLC.

The growth of cells expressing the NPTX2 gene may be inhibited by contacting the cells with a double-stranded molecule against the NPTX2 gene, a vector expressing the molecule or a composition containing the same. The cell may be further contacted with

a transfection agent. Suitable transfection agents are known in the art. The phrase "inhibition of cell growth" indicates that the cell proliferates at a lower rate or has decreased viability as compared to a cell not exposed to the molecule. Cell growth may be measured by any of a number of methods known in the art, e.g., using the MTT cell proliferation assay.

[0308] Thus, subjects suffering from cancer may be treated by administering at least one of the double-stranded molecules against the NPTX2 gene, at least one vector expressing at least one of the molecules or at least one composition containing at least one of the molecules. Preferably, subjects treated by the methods of the present invention are selected by detecting the expression of the NPTX2 gene in a subject-derived biological sample from the subject.

[0309] For inhibiting cell growth, a double-stranded molecule against the NPTX2 gene may be directly introduced into the cells in a form to achieve binding of the molecule with corresponding mRNA transcripts. Alternatively, as described above, a DNA encoding the double-stranded molecule may be introduced into cells by means of a vector. For introducing the double-stranded molecules and vectors into the cells, transfection-enhancing agent, such as FuGENE (Roche diagnostics), Lipofectamine 2000 (Invitrogen), Oligofectamine (Invitrogen), and Nucleofector (Wako pure Chemical), may be employed.

[0310] A treatment is deemed "efficacious" if it leads to clinical benefit such as, reduction in the expression of the NPTX2 gene, or a decrease in size, prevalence, or metastatic potential of the cancer in the subject. When the treatment is applied prophylactically, "efficacious" means that it retards or prevents cancers from forming or prevents or alleviates a clinical symptom of cancer. Efficaciousness is determined in association with any known method for diagnosing or treating the particular tumor type.

[0311] It is understood that the double-stranded molecule against the NPTX2 gene degrades the NPTX2 mRNA in substoichiometric amounts. Without wishing to be bound by any theory, it is believed that the double-stranded molecule against the NPTX2 gene causes degradation of the target mRNA in a catalytic manner. Thus, compared to standard cancer therapies, significantly less a double-stranded molecule needs to be delivered at or near the site of cancer to exert therapeutic effect.

[0312] One skilled in the art can readily determine the optimal effective amount of the double-stranded molecule of the invention to be administered to a given subject, by taking into account factors such as body weight, age, sex, type of disease, symptoms and other conditions of the subject; the route of administration; and whether the administration is regional or systemic. Generally, an effective amount of the double-stranded molecule against the NPTX2 gene is an intercellular concentration at or near the cancer site of from about 1 nanomolar (nM) to about 100 nM, preferably from about 2 nM to

about 50 nM, more preferably from about 2.5 nM to about 10 nM. It is contemplated that greater or smaller amounts of the double-stranded molecule can be administered. The precise dosage required for a particular circumstance may be readily and routinely determined by one of skill in the art.

- [0313] For treating cancer, the double-stranded molecule against the NPTX2 gene can also be administered to a subject in combination with a pharmaceutical agent different from the double-stranded molecule. Alternatively, the double-stranded molecule against the NPTX2 gene can be administered to a subject in combination with another therapeutic method designed to treat cancer. For example, the double-stranded molecule against the NPTX2 gene can be administered in combination with therapeutic methods currently employed for treating cancer or preventing cancer metastasis (e.g., radiation therapy, surgery and treatment using chemotherapeutic agents).
- [0314] In the context of the present invention, the double-stranded molecule against the NPTX2 gene can be administered to the subject either as a naked double-stranded molecule, in conjunction with a delivery reagent, or as a recombinant plasmid or viral vector that expresses the double-stranded molecule.
- [0315] Suitable delivery reagents for administration in conjunction with the present a double-stranded molecule include the Mirus Transit TKO lipophilic reagent; lipofectin; lipofectamine; cellfectin; or polycations (e.g., polylysine), or liposomes. A preferred delivery reagent is a liposome.
- [0316] Liposomes can aid in the delivery of the double-stranded molecule to a particular tissue, such as retinal or tumor tissue, and can also increase the blood half-life of the double-stranded molecule. Liposomes suitable for use in the invention are formed from standard vesicle-forming lipids, which generally include neutral or negatively charged phospholipids and a sterol, such as cholesterol. The selection of lipids is generally guided by consideration of factors such as the desired liposome size and half-life of the liposomes in the blood stream. A variety of methods are known for preparing liposomes, for example as described in Szoka et al., *Ann Rev Biophys Bioeng* 1980, 9: 467; and US Pat. Nos. 4,235,871; 4,501,728; 4,837,028; and 5,019,369, the entire disclosures of which are herein incorporated by reference.
- [0317] Preferably, the liposomes encapsulating the present double-stranded molecule comprises a ligand molecule that can deliver the liposome to the cancer site. Ligands that bind to receptors prevalent in tumor or vascular endothelial cells, such as monoclonal antibodies that bind to tumor antigens or endothelial cell surface antigens, are preferred.
- [0318] Particularly preferably, the liposomes encapsulating the present double-stranded molecule are modified so as to avoid clearance by the mononuclear macrophage and reticuloendothelial systems, for example, by having opsonization-inhibition moieties

bound to the surface of the structure. In one embodiment, a liposome of the invention can comprise both opsonization-inhibition moieties and a ligand.

[0319] Opsonization-inhibiting moieties for use in preparing the liposomes of the invention are typically large hydrophilic polymers that are bound to the liposome membrane. As used herein, an opsonization inhibiting moiety is "bound" to a liposome membrane when it is chemically or physically attached to the membrane, e.g., by the intercalation of a lipid-soluble anchor into the membrane itself, or by binding directly to active groups of membrane lipids. These opsonization-inhibiting hydrophilic polymers form a protective surface layer which significantly decreases the uptake of the liposomes by the macrophage-monocyte system ("MMS") and reticuloendothelial system ("RES"); e.g., as described in US Pat. No. 4,920,016, the entire disclosure of which is herein incorporated by reference. Liposomes modified with opsonization-inhibition moieties thus remain in the circulation much longer than unmodified liposomes. For this reason, such liposomes are sometimes called "stealth" liposomes.

[0320] Stealth liposomes are known to accumulate in tissues fed by porous or "leaky" microvasculature. Thus, target tissue characterized by such microvasculature defects, for example, solid tumors, will efficiently accumulate these liposomes; see Gabizon et al., Proc Natl Acad Sci USA 1988, 18: 6949-53. In addition, the reduced uptake by the RES lowers the toxicity of stealth liposomes by preventing significant accumulation in liver and spleen. Thus, liposomes of the invention that are modified with opsonization-inhibition moieties can deliver the present double-stranded molecule to tumor cells.

[0321] Opsonization inhibiting moieties suitable for modifying liposomes are preferably water-soluble polymers with a molecular weight from about 500 to about 40,000 daltons, and more preferably from about 2,000 to about 20,000 daltons. Such polymers include polyethylene glycol (PEG) or polypropylene glycol (PPG) derivatives; e.g., methoxy PEG or PPG, and PEG or PPG stearate; synthetic polymers such as polyacrylamide or poly N-vinyl pyrrolidone; linear, branched, or dendrimeric polyamidoamines; polyacrylic acids; polyalcohols, e.g., polyvinylalcohol and polyxylitol to which carboxylic or amino groups are chemically linked, as well as gangliosides, such as ganglioside GM₁. Copolymers of PEG, methoxy PEG, or methoxy PPG, or derivatives thereof, are also suitable. In addition, the opsonization inhibiting polymer can be a block copolymer of PEG and either a polyamino acid, polysaccharide, polyamidoamine, polyethyleneamine, or polynucleotide. The opsonization inhibiting polymers can also be natural polysaccharides containing amino acids or carboxylic acids, e.g., galacturonic acid, glucuronic acid, mannuronic acid, hyaluronic acid, pectic acid, neuraminic acid, alginic acid, carrageenan; aminated polysaccharides or oligosaccharides (linear or branched); or carboxylated polysaccharides or oligosaccharides, e.g., reacted with derivatives of carbonic acids with resultant linking of carboxylic

groups. Preferably, the opsonization-inhibiting moiety is a PEG, PPG, or derivatives thereof. Liposomes modified with PEG or PEG-derivatives are sometimes called "PEGylated liposomes".

[0322] The opsonization inhibiting moiety can be bound to the liposome membrane by any one of numerous well-known techniques. For example, an N-hydroxysuccinimide ester of PEG can be bound to a phosphatidyl-ethanolamine lipid-soluble anchor, and then bound to a membrane. Similarly, a dextran polymer can be derivatized with a stearylamine lipid-soluble anchor via reductive amination using $\text{Na}(\text{CN})\text{BH}_3$ and a solvent mixture such as tetrahydrofuran and water in a 30:12 ratio at 60 degrees C.

[0323] Vectors expressing a double-stranded molecule against the NPTX2 gene are discussed above. Such vectors expressing at least one double-stranded molecule of the invention can also be administered directly or in conjunction with a suitable delivery reagent, including the Mirus Transit LT1 lipophilic reagent; lipofectin; lipofectamine; cellfectin; polycations (e.g., polylysine) or liposomes. Methods for delivering recombinant viral vectors, which express a double-stranded molecule of the invention, to an area of cancer in a subject are within the skill of the art.

[0324] The double-stranded molecule against the NPTX2 gene can be administered to the subject by any means suitable for delivering the double-stranded molecule into cancer sites. For example, the double-stranded molecule can be administered by gene gun, electroporation, or by other suitable parenteral or enteral administration routes.

[0325] Suitable enteral administration routes include oral, rectal, or intranasal delivery.

Suitable parenteral administration routes include intravascular administration (e.g., intravenous bolus injection, intravenous infusion, intra-arterial bolus injection, intra-arterial infusion and catheter instillation into the vasculature); peri- and intra-tissue injection (e.g., peri-tumoral and intra-tumoral injection); subcutaneous injection or deposition including subcutaneous infusion (such as by osmotic pumps); direct application to the area at or near the site of cancer, for example by a catheter or other placement device (e.g., a suppository or an implant comprising a porous, non-porous, or gelatinous material); and inhalation. It is preferred that injections or infusions of the double-stranded molecule or vector be given at or near the site of cancer.

[0326] The double-stranded molecule against the NPTX2 gene can be administered in a single dose or in multiple doses. Where the administration of the double-stranded molecule of the invention is by infusion, the infusion can be a single sustained dose or can be delivered by multiple infusions. Injection of the agent directly into the tissue is at or near the site of cancer preferred. Multiple injections of the agent into the tissue at or near the site of cancer are particularly preferred.

[0327] One skilled in the art can also readily determine an appropriate dosage regimen for administering the double-stranded molecule of the invention to a given subject. For

example, the double-stranded molecule can be administered to the subject once, for example, as a single injection or deposition at or near the cancer site. Alternatively, the double-stranded molecule can be administered once or twice daily to a subject for a period of from about three to about twenty-eight days, more preferably from about seven to about ten days. In a preferred dosage regimen, the double-stranded molecule is injected at or near the site of cancer once a day for seven days. Where a dosage regimen comprises multiple administrations, it is understood that the effective amount of a double-stranded molecule administered to the subject can comprise the total amount of a double-stranded molecule administered over the entire dosage regimen.

[0328] (9) Compositions for Treating Cancer:

The present invention also provides pharmaceutical compositions that include at least one double-stranded molecule against the NPTX2 gene or vector encoding thereof.

In the context of the present invention, the term "composition" is used to refer to a product including that include the specified ingredients in the specified amounts, as well as any product that results, directly or indirectly, from combination of the specified ingredients in the specified amounts. Such terms, when used in relation to the modifier "pharmaceutical" (as in "pharmaceutical composition"), are intended to encompass products including a product that includes the active ingredient(s), and any inert ingredient(s) that make up the carrier, as well as any product that results, directly or indirectly, from combination, complexation or aggregation of any two or more of the ingredients, or from dissociation of one or more of the ingredients, or from other types of reactions or interactions of one or more of the ingredients. Accordingly, in the context of the present invention, the term "pharmaceutical composition" refers to any product made by admixing a molecule or compound of the present invention and a pharmaceutically or physiologically acceptable carrier.

[0329] The phrase "pharmaceutically acceptable carrier" or "physiologically acceptable carrier", as used herein, means a pharmaceutically or physiologically acceptable material, composition, substance or vehicle, including but not limited to, a liquid or solid filler, diluent, excipient, solvent or encapsulating material.

[0330] The term "active ingredient" herein refers to a substance in composition that is biologically or physiologically active. Particularly, in the context of pharmaceutical composition, the term "active ingredient" refers to a substance that shows an objective pharmacological effect. For example, in case of pharmaceutical compositions for use in the treatment or prevention of cancer, active ingredients in the agents or compositions may lead to at least one biological or physiologically action on cancer cells and/or tissues directly or indirectly. Preferably, such action may include reducing or inhibiting cancer cell growth, damaging or killing cancer cells and/or tissues, and so on. Before being formulated, the "active ingredient" may also be referred to as "bulk",

"drug substance" or "technical product".

[0331] Of particular interest to the present invention are the following compositions [1] to [20]:

[1] A composition for either or both of treating and preventing cancer, wherein said composition comprises a pharmaceutically effective amount of a double-stranded molecule against the NPTX2 gene, or a vector encoding said double-stranded molecule, and a pharmaceutically acceptable carrier, wherein said double-stranded molecules comprises a sense strand and an antisense strand, wherein the sense strand comprises a nucleotide sequence corresponding to a target sequence selected from the NPTX2 gene sequence, and the antisense strand comprises a nucleotide sequence complementary to said target sequence, wherein said sense strand and said antisense strand hybridize to each other to form the double-stranded molecule, and wherein said double stranded molecule, when introduced into a cell expressing the NPTX2 gene, inhibits cell proliferation as well as the expression of the NPTX2 gene.;

[2] The composition of [1], wherein the cancer is lung cancer, breast cancer, cervical cancer or colon cancer;

[3] The composition of [2], wherein the lung cancer is SCLC;

[4] The composition of any one of [1] to [3], wherein plural kinds of the double-stranded molecules are administered at the same time;

[5] The composition of any one of [1] to [4], wherein the sense strand hybridize with antisense strand at the target sequence to form the double-stranded molecule having less than about 100 nucleotide pairs in length;

[6] The composition of [5], wherein the sense strand hybridize with antisense strand at the target sequence to form the double-stranded molecule having less than about 75 nucleotide pairs in length;

[7] The composition of [6], wherein the sense strand hybridize with antisense strand at the target sequence to form the double-stranded molecule having less than about 50 nucleotide pairs in length;

[8] The composition of [7], wherein the sense strand hybridize with antisense strand at the target sequence to form the double-stranded molecule having less than about 25 nucleotide pairs in length;

[9] The composition of [8], wherein the sense strand hybridize with antisense strand at the target sequence to form the double-stranded molecule having between about 19 to 25 nucleotide pairs in length;

[10] The composition of any one of [1] to [9], wherein the target sequence is a sequence of SEQ ID NO: 7 or 8;

[11] The composition of any one of [1] to [10], wherein the double-stranded molecule is composed of a single polynucleotide containing both the sense strand and

the antisense strand linked by an intervening single-strand;

[12] The composition of [11], wherein the double-stranded molecule has the general formula 5'-[A]-[B]-[A']-3' or 5'-[A']-[B]-[A], wherein [A] is the sense strand containing a sequence corresponding to a target sequence of the NPTX2 gene, [B] is the intervening single strand composed of 3 to 23 nucleotides, and [A'] is the antisense strand containing a sequence complementary to the target sequence;

[13] The composition of any one of [1] to [12], wherein the double-stranded molecule is an RNA;

[14] The composition of any one of [1] to [12], wherein the double-stranded molecule contains both DNA and RNA;

[15] The composition of [14], wherein the double-stranded molecule is a hybrid of a DNA polynucleotide and an RNA polynucleotide;

[16] The composition of [15] wherein the sense and antisense strand polynucleotides are composed of DNA and RNA, respectively;

[17] The composition of [15], wherein the double-stranded molecule is a chimera of DNA and RNA;

[18] The composition of [17], wherein a region flanking to the 3'-end of the antisense strand, or both of a region flanking to the 5'-end of sense strand and a region flanking to the 3'-end of antisense strand are composed of RNA;

[19] The composition of [18], wherein the flanking region is composed of 9 to 13 nucleotides;

[20] The composition of any one of [1] to [19], wherein the double-stranded molecule contains 3' overhangs; and

[21] The composition of any one of [1] to [20], wherein the composition contains a transfection-enhancing agent.

[0332] A composition of either or both of treating and preventing cancer is described in more detail below.

The composition of the present invention may be also applied to inhibiting cancer cell invasion. Preferably, the composition of the present invention may be applied to lung cancer, breast cancer, cervical cancer or colon cancer, more preferably SCLC.

[0333] The double-stranded molecules against the NPTX2 gene are preferably formulated as pharmaceutical compositions prior to administering to a subject, according to techniques known in the art. Pharmaceutical compositions of the present invention are characterized as being at least sterile and pyrogen-free. As used herein, "pharmaceutical compositions" include compositions for human and veterinary use. Thus, the compositions may be used as pharmaceuticals for humans and other mammals, such as mice, rats, guinea-pigs, rabbits, cats, dogs, sheep, pigs, cattle, monkeys, baboons, and chimpanzees.

- [0334] In the context of the present invention, suitable pharmaceutical formulations of the present invention include those suitable for oral, rectal, nasal, topical (including buccal, sub-lingual, and transdermal), vaginal or parenteral (including intramuscular, subcutaneous and intravenous) administration, or for administration by inhalation or insufflation. Other formulations include implantable devices and adhesive patches that release a therapeutic agent. When desired, the above-described formulations may be adapted to give sustained release of the active ingredient. Methods for preparing pharmaceutical compositions of the invention are within the skill in the art, for example as described in Remington's Pharmaceutical Science, 17th ed., Mack Publishing Company, Easton, Pa. (1985), the entire disclosure of which is herein incorporated by reference.
- [0335] The pharmaceutical compositions of the present invention contain at least one of the double-stranded molecules against the NPTX2 gene or vectors encoding them (e.g., 0.1 to 90% by weight), or a physiologically acceptable salt of the molecule, mixed with a physiologically acceptable carrier medium. Preferred physiologically acceptable carrier media are water, buffered water, normal saline, 0.4% saline, 0.3% glycine, hyaluronic acid and the like.
- [0336] According to the present invention, the composition may contain plural kinds of the double-stranded molecules, each of the molecules may be directed to NPTX2.
- Furthermore, the composition of the present invention may contain a vector encoding one or plural double-stranded molecules against the NPTX2 gene. For example, the vector may encode one or two kinds of the double-stranded molecules against the NPTX2 gene. Alternatively, the present composition may contain plural kinds of vectors, each of the vectors encoding a different double-stranded molecule.
- [0337] Moreover, the double-stranded molecules against the NPTX2 gene may be contained as liposomes in the composition of the present invention. The details of liposomes are described above.
- [0338] Pharmaceutical compositions of the present invention can also include conventional pharmaceutical excipients and/or additives. Examples of suitable pharmaceutical excipients include stabilizers, antioxidants, osmolality adjusting agents, buffers, and pH adjusting agents. Suitable additives include physiologically biocompatible buffers (e.g., tromethamine hydrochloride), additions of chelants (such as, for example, DTPA or DTPA-bisamide) or calcium chelate complexes (for example calcium DTPA, CaNaDTPA-bisamide), or, optionally, additions of calcium or sodium salts (for example, calcium chloride, calcium ascorbate, calcium gluconate or calcium lactate). Pharmaceutical compositions of the invention can be packaged for use in liquid form, or can be lyophilized.
- [0339] For solid compositions, conventional nontoxic solid carriers can be used; for

example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like.

[0340] For example, a solid pharmaceutical composition for oral administration can include any of the carriers and excipients listed above and 10-95%, preferably 25-75%, of one or more double-stranded molecule of the invention. A pharmaceutical composition for aerosol (inhalational) administration can comprise 0.01-20% by weight, preferably 1-10% by weight, of one or more double-stranded molecule of the invention encapsulated in a liposome as described above, and propellant. A carrier can also be included as desired; e.g., lecithin for intranasal delivery.

[0341] In addition to the above, the present composition may contain other pharmaceutical active ingredients so long as they do not inhibit the in vivo function of the present double-stranded molecules. For example, the composition may contain chemotherapeutic agents conventionally used for treating cancers. The pharmaceutical compositions may also contain other active ingredients such as antimicrobial agents, immunosuppressants or preservatives. Furthermore, it should be understood that, in addition to the ingredients particularly mentioned above, the formulations of this invention may include other agents conventional in the art having regard to the type of formulation in question; for example, those suitable for oral administration may include flavoring agents.

[0342] In another embodiment, the present invention also provides the use of the double-stranded nucleic acid molecules of the present invention or a vector(s) encoding the double-stranded nucleic acid molecule in manufacturing a pharmaceutical composition for treating cancer. For example, the present invention relates to a use of double-stranded molecule inhibiting the expression of the NPTX2 gene in a cell, which molecule includes a sense strand and an antisense strand complementary thereto, hybridized to each other to form the double-stranded molecule and targets to a sequence selected from among the nucleotide sequence of the NPTX2 gene, or a vector(s) encoding the double-stranded nucleic acid molecule for manufacturing a pharmaceutical composition for treating cancer.

[0343] Alternatively, the present invention further provides the double-stranded nucleic acid molecules of the present invention or a vector(s) encoding the double-stranded nucleic acid molecule for use in treating a cancer expressing the NPTX2 gene.

[0344] Alternatively, the present invention further provides a method or process for manufacturing a pharmaceutical composition for treating cancer, wherein the method or process includes a step for formulating a pharmaceutically or physiologically acceptable carrier with a double-stranded molecule capable of inhibiting the expression of the NPTX2 gene when introduced into a cell that over-expresses the NPTX2 gene.

and/or a vector(s) encoding the double-stranded nucleic acid molecule as an active ingredient, wherein such double-stranded molecule includes a sense strand and an antisense strand complementary thereto, hybridized to each other to form the double-stranded molecule and targets to a sequence selected from among the nucleotide sequence of the NPTX2 gene.

[0345] In another embodiment, the present invention also provides a method or process for manufacturing a pharmaceutical composition for inhibiting cancer cell growth or treating and/or preventing primary cancer or recurrence, wherein the method or process includes a step for admixing an active ingredient with a pharmaceutically or physiologically acceptable carrier, wherein the active ingredient is a double-stranded molecule capable of inhibiting the expression of the NPTX2 gene when introduced into a cell that over-expresses the NPTX2 gene or a vector(s) encoding the double-stranded nucleic acid molecule, wherein such double-stranded molecule includes a sense strand and an antisense strand complementary thereto, hybridized to each other to form the double-stranded molecule and targets to a sequence selected from among the nucleotide sequence of the NPTX2 gene.

[0346] Hereinafter, the present invention is described in more detail with reference to the Examples. However, the following materials, methods and examples only illustrate aspects of the invention and in no way are intended to limit the scope of the present invention. As such, methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention.

Examples

[0347] Materials and Methods

Cell lines and tissue samples.

[0348] The 23 human lung-cancer cell lines used in this study included nine adenocarcinomas (ADCs; A549, LC319, PC-3, PC-9, PC-14, A427, NCI-H1373, NCI-H1666, and NCI-H1781), nine squamous-cell carcinomas (SCCs; RERF-LC-AI, SK-MES-1, EBC-1, LU61, NCI-H520, NCI-H1703, NCI-H2170, NCI-H226, and NCI-H647), one large-cell carcinoma (LCC; LX1), and four small-cell lung cancers (SCLCs; DMS114, DMS273, SBC-3, and SBC-5). All cells were grown in monolayers in appropriate media supplemented with 10% fetal calf serum (FCS) and were maintained at 37 degrees C in an atmosphere of humidified air with 5% CO₂. Human small airway epithelial cells (SAEC) were grown in optimized medium (SAGM) purchased from Cambrex Bio Science Inc (Walkersville, MD). Primary lung-cancer tissue samples had been obtained with written informed consent as described previously (Kikuchi 2003; Taniwaki 2006). A total of 361 formalin-fixed samples of primary NSCLCs including 230 ADCs, 91 SCCs, 28 LCCs, and 13 ASCs, and adjacent

normal lung tissue, had been obtained earlier along with clinicopathological data from patients who had undergone surgery at Saitama Cancer Center (Saitama, Japan). Sixteen SCLCs were obtained from individuals who underwent autopsy at Hiroshima University (Hiroshima, Japan). Surgically resected primary cancers from six colon cancer patients, five breast cancer patients and five cervical cancer patients were obtained with informed consent at Kanagawa Cancer Center (Yokohama, Japan). The histological classification of the tumor specimens was based on WHO criteria (Travis WD). NSCLC specimen and five tissues (heart, liver, lung, kidney, and adrenal gland) from post-mortem materials (2 individuals with ADC) were also obtained from Hiroshima University. This study and the use of all clinical materials mentioned were approved by individual institutional Ethical Committees.

[0349] Serum samples.

Serum samples were obtained with informed consent from 109 healthy individuals as controls (90 males and 19 females; median age 49.1 $\pm$ 7.46 SD, range 31-60) and from 81 non-neoplastic lung disease patients with chronic obstructive pulmonary disease (COPD) enrolled as a part of the Japanese Project for Personalized Medicine (BioBank Japan) or admitted to Hiroshima University Hospital (69 males and 12 females; median age 66.4 $\pm$ 5.92 SD, range 54-73). All of these patients were current and/or former smokers (The mean [$\pm$ 1SD] of pack-year index (PYI) was 64.1 $\pm$ 41.6; PYI was defined as the number of cigarette packs [20 cigarette per pack] consumed a day multiplied by years). The healthy individuals showed no abnormalities in complete blood cell counts, C-reactive proteins (CRP), erythrocyte sedimentation rates, liver function tests, renal function tests, urinalyses, fecal examinations, chest X-rays, or electrocardiograms. Serum samples were also obtained with informed consent from 237 lung-cancer patients, 171 breast-cancer patients, 182 cervical-cancer patients and 100 colon-cancer patients admitted to Hiroshima University Hospital, as well as Kanagawa Cancer Center Hospital, and Japanese Project for Personalized Medicine BioBank Japan; (182 males and 55 females; median age 64.3 $\pm$ 11.2 SD, range 30-86). Samples were selected for the study on the basis of the following criteria: (1) patients were newly diagnosed and previously untreated and (2) their tumors were pathologically diagnosed as lung cancers (stages I - IV). These cases included 183 ADCs, 54 SCCs, and 83 SCLCs. Clinicopathological records were fully documented. Serum was obtained at the time of diagnosis and stored at -80 degrees C.

[0350] Semiquantitative RT-PCR analysis.

Total RNA was extracted from cultured cells and clinical tissues using Trizol reagent (Life Technologies, Inc. Gaithersburg, MD) according to the manufacturer's protocol. Extracted RNAs and normal human-tissue polyA RNAs were treated with DNase I (Roche Diagnostics, Basel, Switzerland) and then reverse-transcribed using oligo (dT)

¹²⁻¹⁸ primer and SuperScript II reverse transcriptase (Life Technologies, Inc.). Semi-quantitative RT-PCR experiments were carried out with synthesized NPTX2 gene-specific primers (5'- CCACTTGGTCCTACAAATGGA -3' (SEQ ID NO: 3) and 5'- CGACTTGGTCCTACAAATGGA -3' (SEQ ID NO: 4)), or with beta-actin (ACTB)-specific primers (5'-ATCAAGATCATTGCTCCTCCT-3' (SEQ ID NO: 5) and 5'-CTGCGCAAGTTAGGTTTTGT-3' (SEQ ID NO: 6)) as an internal control. All PCR reactions involved initial denaturation at 94 degrees C for 2 min followed by 22 (for ACTB) or 35 cycles (for NPTX2) of 94 degrees C 30 s, 54 or 60 degrees C for 30 s, and 72 degrees C for 60 s on a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA).

[0351] Northern-blot analysis.

Human multiple-tissue blots (BD Biosciences, Palo Alto, CA) were hybridized with ³²P-labeled PCR products. PCR product of NPTX2 was prepared as a probe by RT-PCR using the same primers above. Prehybridization, hybridization, and washing were performed according to the supplier's recommendations. The blots were autoradiographed with intensifying screens at -80 degrees C for one week.

[0352] Western blotting.

Cells were lysed with radioimmunoprecipitation assay buffer [50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 1% NP40, 0.5% deoxycholate-Na, 0.1% SDS] containing Protease Inhibitor Cocktail Set III (Calbiochem, Darmstadt, Germany). Protein samples were separated by SDS-polyacrylamide gels and electroblotted onto Hybond-ECL nitrocellulose membranes (GE Healthcare Bio-Sciences, Piscataway, NJ). Blots were incubated with a mouse monoclonal anti-NPTX2 antibody (Santa cruz). Antigen-antibody complexes were detected using secondary antibodies conjugated to horseradish peroxidase (GE Healthcare Bio-Sciences). Protein bands were visualized by enhanced chemiluminescence Western blotting detection reagents (GE Healthcare Bio-Sciences).

[0353] Immunofluorescence analysis.

Cells were plated on glass coverslips (Becton Dickinson Labware, Franklin Lakes, NJ), fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100 in PBS for three minutes at room temperature. Non-specific binding was blocked by CASBLOCK (ZYMED, South San Francisco, California) for 10 minutes at room temperature. Cells were then incubated for 60 minutes at room temperature with primary antibodies for human NPTX2 antibody (Santa cruz) diluted in PBS containing 3% BSA. After being washed with PBS, the cells were stained by Alexa Fluor 488-conjugated secondary antibody (Molecular Probes) for 60 minutes at room temperature. After another wash with PBS, each specimen was mounted with Vectashield (Vector Laboratories, Inc, Burlingame, CA) containing 4',

6'-diamidine-2'-phenylindoldihydrochloride (DAPI) and visualized with Spectral Confocal Scanning Systems (TSC SP2 AOBS: Leica Microsystems, Wetzlar, Germany).

[0354] Immunohistochemistry and Tissue Microarray.

To investigate the presence of NPTX2 protein in clinical samples embedded in paraffin blocks, the sections were stained in the following manner. Briefly, 20 microgram/ml of goat monoclonal anti-human NPTX2 antibody (Santa cruz) was added after blocking of endogenous peroxidase and proteins. The sections were incubated with HRP-labeled anti-mouse IgG as the secondary antibody. Substrate-chromogen was added and the specimens were counterstained with hematoxylin.

[0355] Tumor-tissue microarrays were constructed using 377 formalin-fixed primary lung cancers (361 NSCLCs and 16 SCLCs), as described elsewhere (Callagy, 2003, 2005; Chin). The tissue area for sampling was selected based on visual alignment with the corresponding HE-stained section on a slide. Three, four, or five tissue cores (diameter 0.6 micrometer; height 3-4 micrometer) taken from a donor tumor block were placed into a recipient paraffin block using a tissue microarrayer (Beecher Instruments, Sun Prairie, WI). A core of normal tissue was punched from each case, and 5-micrometer sections of the resulting microarray block were used for immunohistochemical analysis. Three independent investigators semi-quantitatively assessed NPTX2 positivity without prior knowledge of clinicopathological data. The intensity of NPTX2 staining was evaluated using following criteria: strong positive (scored as 2+), dark brown staining in more than 50% of tumor cells completely obscuring cytoplasm; weak positive (1+), any lesser degree of brown staining appreciable in tumor cell cytoplasm; absent (scored as 0), no appreciable staining in tumor cells. Cases were accepted as strongly positive only if reviewers independently defined them as such.

[0356] Statistical analysis.

Statistical analyses were performed using the StatView statistical program (SaS, Cary, NC). Associations between clinicopathological variables and positivity for NPTX2 were compared by Fisher's exact test. Tumor-specific survival was evaluated with the Kaplan-Meier method, and differences between the two groups were evaluated with the log-rank test. Risk factors associated with the prognosis were evaluated using Cox's proportional-hazard regression model with a step-down procedure. Proportional-hazard assumptions were checked and satisfied; only those variables with statistically significant results in univariate analysis were included in a multivariate analysis. The criterion for removing a variable from the model was the likelihood ratio statistic, which was based on the maximum partial likelihood estimate (default P value of 0.05 for removal).

[0357] ELISA.

Serum levels of NPTX2 were measured by ELISA system which had been originally constructed. First of all, 100 microliter per well of a goat polyclonal antibody specific to NPTX2 (sc12125 Santa Cruz Biotechnology, Santa Cruz, CA; 4 microgram/ml) was added to a 96-well microplate (Nunc Maxisorp Bioscience, Inc., Naperville, IL) as a capture antibody and incubated for two hours at room temperature. After washing away any unbound antibody using PBST (PBS containing 1% bovine serum albumin (BSA) and 0.05% Tween) at room temperature, 200 microliter per well of 5% BSA was added to the wells and incubated for two hours at room temperature for blocking. After three times wash, 100 microliter per well of 3-fold diluted sera in PBS with 1% BSA were added to the wells and incubated for two hours at room temperature. After washing away any unbound substances, 100 microliter per well of a goat polyclonal antibody specific for NPTX2 (sc12128 Santa Cruz Biotechnology, Santa Cruz, CA; 0.01 microgram/ml) biotinylated using Biotin Labeling Kit-NH₂ (DOJINDO, Kumamoto, Japan) was added to the wells as a detection antibody and incubated for two hours at room temperature. After three times wash to remove any unbound antibody-enzyme reagent, Streptavidin-Horseradish Peroxidase (SAv-HRP) was added to the wells and incubated for 20 minutes. After three times wash, 100 microliter per well of a substrate solution (R&D Systems, Inc., Minneapolis, MN) was added to the wells and allowed to react for 30 minutes. The reaction was stopped by adding 50 microliter of 2 N sulfuric acid. Color intensity was determined by a photometer at a wavelength of 450 nm, with a reference wavelength of 570 nm.

[0358] Levels of CEA in serum were measured by ELISA with a commercially available enzyme test kit (HOPE Laboratories, Belmont, CA), according to the supplier's recommendations. Levels of CYFRA in serum were measured by ELISA with a commercially available enzyme test kit (DRG International Inc USA, Mountainside, NJ), according to the supplier's recommendations. Levels of proGRP in serum were measured by ELISA with a commercially available enzyme test kit (TFB Tokyo Japan), according to the supplier's recommendations. Differences in the levels of NPTX2, CEA, CYFRA and proGRP between tumor groups and a healthy control group were analyzed by Mann-Whitney U tests. The levels of NPTX2, CEA, CYFRA and proGRP were evaluated by receiver-operating characteristic (ROC) curve analysis to determine cutoff levels with optimal diagnostic accuracy and likelihood ratios. The correlation coefficients between NPTX2 and CEA were calculated with Spearman rank correlation. Significance was defined as $P < 0.05$.

[0359] RNA interference assay.

To evaluate the biological functions of NPTX2 in lung cancer cells, small interfering RNAs (siRNAs) against the target genes (Dharmacon) were used. siRNA oligonucleotides (100 nM) was transfected, using 30 microliter of Lipofectamine 2000

(Invitrogen), into a SCLC cell line, SBC-5, which overexpressed NPTX2. The transfected cells were cultured for five days in the presence of appropriate concentrations of geneticin (G418), after which cell numbers and viability were measured by Giemsa staining and triplicate MTT assays; briefly, cell-counting kit-8 solution (DOJINDO) was added to each dish at a concentration of 1/10 volume, and the plates were incubated at 37 degrees C for additional two hours. Absorbance was then measured at 450 nm with a Microplate Reader 550 (BIO-RAD, Hercules, CA). To confirm suppression of NPTX2 mRNA expression, semiquantitative RT-PCR experiments were carried out with the synthesized NPTX2-specific primers. The target sequences of the synthetic oligonucleotides for RNAi were as follows: control 1 (EGFP, enhanced green fluorescent protein (GFP) gene, a mutant of *Aequorea victoria* GFP): 5'-GAAGCAGCACGACUUCUUC-3' (SEQ ID NO: 7); control 2 (Luciferase (LUC): *Photinus pyralis* luciferase gene); 5'-CGUACGCGGAUACUUCGA-3' (SEQ ID NO: 8), NPTX2 siRNA-1 (Dharmacon catalog no. D-012647-01 NPTX2 Target sequence: 5'-CAAUAGCGCCUUAAGUCA-3' (SEQ ID NO: 9)), NPTX2 siRNA-2 (Dharmacon catalog no. D-012647-03 NPTX2: 5'-ACAAUAACGUCGAUGUGUU-3' (SEQ ID NO: 10)).

[0360] Cell-growth assay.

The entire coding sequence of NPTX2 was cloned into the appropriate site of pcDNA3.1 myc-His plasmid vector (Invitrogen, Carlsbad, California). COS-7 cells transfected either with plasmids expressing myc-His-tagged NPTX2 or with mock plasmids were grown for eight days in DMEM containing 10% FCS in the presence of appropriate concentrations of geneticin (G418). Viability of cells was evaluated by MTT assay; briefly, cell-counting kit-8 solution (DOJINDO) was added to each dish at a concentration of 1/10 volume, and the plates were incubated at 37 degrees C for additional two hours. Absorbance was then measured at 450 nm as a reference, with a Microplate Reader 550 (BIO-RAD, Hercules, CA).

[0361] Matrigel invasion assay.

COS-7 cells transfected either with pcDNA3.1-myc/His plasmids expressing human NPTX2 or with mock plasmids were grown to near confluence in DMEM containing 10% FCS. The cells were harvested by trypsinization, washed in DMEM without addition of serum or proteinase inhibitor, and suspended in DMEM at concentration of 1×10^5 cells/ml. Before preparing the cell suspension, the dried layer of Matrigel matrix (Becton Dickinson Labware, Franklin Lakes, NJ) was rehydrated with DMEM for two hours at room temperature. DMEM (0.75 ml) containing 10% FCS was added to each lower chamber in 24-well Matrigel invasion chambers, and 0.5 ml (5×10^4 cells) of cell suspension was added to each insert of the upper chamber. The plates of inserts were incubated for 22 hours at 37 degrees C. After incubation the chambers were

processed; cells invading through the Matrigel were fixed and stained by Giemsa as directed by the supplier (Becton Dickinson Labware).

[0362] Results

NPTX2 expression in tumors and normal tissues.

To search for novel target molecules for development of therapeutic agents and/or diagnostic biomarkers for lung cancer, the present inventors first screened genes that showed more than a 3-fold higher level of expression in cancer cells than in normal cells, in half or more of 101 lung cancer samples analyzed by cDNA microarray (Kikuchi T, et al. *Oncogene* 2003;22:2192-205.; Kikuchi T, et al. *Int J Oncol* 2006; 28:799-805.; Kakiuchi S, et al. *Hum Mol Genet* 2004;13:3029-43.; Taniwaki M, et al. *Int J Oncol* 2006;29:567-75.). Among 27,648 genes screened, the present inventors identified the overexpression of NPTX2 in the great majority of lung cancers examined, and confirmed its transactivation by semiquantitative RT-PCR experiments in 8 of 15 additional lung-cancer tissues and in 11 of 23 lung-cancer cell lines (Figs. 1A and 1B). Subsequently, a mouse monoclonal antibody specific for human NPTX2 was generated, and an expression of endogenous NPTX2 protein in four lung-cancer cell lines (three NPTX2-positive cells: NCI-H520, and SBC-5 vs. one NPTX2-negative line, NCI-H2170 and A549) and small airway epithelia derived cells (SAEC) was confirmed by Western-blot analysis (Fig. 1C). Immunofluorescence analysis was performed to examine the subcellular localization of endogenous NPTX2 in these four lung-cancer cell lines. NPTX2 was detected at cytoplasm of tumor cells with granular appearance at a high level in NCI-H520 and SBC-5 cells, but not in NCI-H2170 and A549 cells, which was concordant with the result of western-blotting (Fig. 1D). Since the NPTX2 was a secretory protein (Schlimgen), ELISA method was applied to examine its presence in the culture media of these lung-cancer cell lines. NPTX2 protein was detected in media of NCI-H520 and SBC-5 cells, but not in medium of NCI-H2170 and A549 cells (Fig. 1E). The amounts of detectable NPTX2 in the cell lysate by Western blot and in the culture media by ELISA showed good correlation with those of NPTX2 detected by RT-PCR, indicating that the antibody specifically bound to NPTX2 protein.

[0363] Northern-blot analysis using human NPTX2 cDNA as a probe detected a very weak 2.8-kb band only in brain and adrenal gland; no expression was observed in any other tissues (Fig. 2A). The expression of NPTX2 protein was also examined with polyclonal antibody specific to NPTX2 on five normal tissues (liver, heart, kidney, lung, testis) and lung ADC tissues. NPTX2 staining was mainly observed at cytoplasm of tumor cells and cells (cortex) in adrenal gland, but not detected in normal cells (Fig. 2B). The expression levels of NPTX2 protein in lung cancer were significantly higher than those in adrenal gland.

[0364] Association of NPTX2 expression with poor prognosis.

To verify the biological and clinicopathological significance of NPTX2, the expression of NPTX2 protein was examined by means of tissue microarrays containing primary NSCLC tissues from 361 NSCLC patients as well as SCLC tissues from 16 patients. Strong positive cytoplasmic staining for NPTX2 was observed in 54.8% of surgically-resected NSCLCs (198/361) and in 62.5% of SCLCs (10/16), while no staining was observed in any of normal lung tissues examined (Fig. 2C). Then, the correlation of its positive staining with various clinicopathological parameters was examined in 361 NSCLC patients. A pattern of NPTX2 expression on the tissue array was classified ranging from absent (scored as 0) to weak/strong positive (scored as 1+ ~ 2+) (Fig. 2D; see "Materials and Methods") (Table 2A). In this study, tumor size (pT2-4 versus pT1; $P = 0.0183$ by Fisher's exact test) and lymph-node metastasis (pN1-2 versus pN0; $P = 0.0016$ by Fisher's exact test) were significantly associated with the NPTX2 status (Table 2A). Kaplan-Meier analysis indicated that the median survival time of patients with strong NPTX2-staining (scored 2+) was significantly shorter than that of NSCLC patients with absent/weak NPTX2-staining (scored 0, 1+) ($P = 0.0002$ by log-rank test; Fig. 2E). In multivariate analysis of the prognostic factors, pT stage, pN stage, and strong NPTX2 positivity were indicated to be an independent prognostic factor (Table 2B).

[0365]

[Table 2A]

Association between NPTX2-positivity in NSCLC tissues and patients' characteristics (n=361)

	Total n = 361	NPTX2 strong positive n = 198	NPTX2 weak positive/absent n = 163	P-value strong vs weak/absent
Gender				
Male	250	144	106	NS
Female	111	54	57	
Age (years)				
< 65	182	104	78	NS
>= 65	179	94	85	
Histological type				
ADC	230	118	112	NS
SCC	91	53	38	
Others	41	27	14	
pT factor				
T1	120	55	65	0.0183**
T2-T4	241	143	98	
pN factor				
N0	222	107	115	0.0016**
N1+N2	139	91	48	

ADC, adenocarcinoma; SCC, squamous-cell carcinoma

Others, large-cell carcinoma (LCC) plus adenosquamous-cell carcinoma (ASC)

*ADC versus non-ADC

** $P < 0.05$ (Fisher's exact test)

NS, no significance

[0366] [Table 2B]

Cox's proportional hazards model analysis of prognostic factors in patients with NSCLCs

Variables	Hazards ratio	95% CI	Unfavorable/Favorable	P-value
Univariate analysis				
NPTX2	1.563	1.162-2.101	Strong(2+) / Weak(1+) or (-)	0.0031*
Age (years)	1.335	0.998-1.786	65 >= / < 65	NS
Gender	1.717	1.227-2.403	Male / Female	0.0016*
Histological type	1.427	1.066-1.912	non-ADC / ADC ¹	0.0170*
pT factor	2.538	1.768-3.642	T2-T4 / T1	<0.0001*
pN factor	2.702	2.020-3.614	N1+N2 / N0	<0.0001*

Variables	Hazards ratio	95% CI	Unfavorable/Favorable	P-value
Multivariate analysis				
NPTX2	1.267	0.936-1.715	Strong(2+) / Weak(1+) or (-)	NS
Gender	1.392	0.960-2.017	Male / Female	NS
Histological type	1.035	0.751-1.427	non-ADC / ADC ¹	NS
pT factor	2.063	1.421-2.993	T2-T4 / T1	0.0001*
pN factor	2.349	1.740-3.170	N1+N2 / N0	<0.0001 *

¹ ADC, adenocarcinoma* $P < 0.05$

NS, no significance

[0367] Serum levels of NPTX2 in lung cancer patients.

Since NPTX2 encodes a secretory protein, it was investigated whether the NPTX2

protein was secreted into sera of patients with lung cancer. ELISA experiments detected NPTX2 in serologic samples from the majority of the 320 patients with lung cancer; serum levels of NPTX2 in lung cancer patients were 18.5 ± 25.2 U/ml (mean $\pm$ 1SD) and those in healthy individuals were 2.5 ± 2.94 U/ml (The difference was significant with P-value of < 0.001 by Mann-Whitney U test; Fig. 3A). According to histological types of lung cancer, the serum levels of NPTX2 were 20.0 ± 27.6 U/ml in ADC patients, 13.3 ± 13.3 U/ml in SCC patients, and 29.6 ± 59.8 U/ml in SCLC patients; the differences among the three histologic types were not significant. Serum levels of NPTX2 were 3.5 ± 5.23 U/ml in benign lung disease of COPD patients. Serum levels of NPTX2 in lung cancer patients were significantly higher than those of normal volunteers and COPD patients ($P < 0.0001$). High levels of serum NPTX2 were detected even in patients with earlier-stage tumors. Furthermore levels of NPTX2 were significantly more common in serum from patients with locally advanced lung cancer (stage IIIB) or distant organ metastasis (stage IV or ED) than in those with earlier stage diseases (stages I-III A or LD) (data not shown). Using receiver-operating characteristic (ROC) curves drawn with the data of these 320 cancer patients and 109 healthy controls (Fig. 3B Left Panel), the cut-off level in this assay was set to provide optimal diagnostic accuracy and likelihood ratios for NPTX2, i.e., 7.3 U/ml for NPTX2 (with a sensitivity of 63.3% and a specificity of 97.1% for lung cancer). Among the 81 patients with COPD, seven (8.6%) had a positive NPTX2 level. Then, ELISA experiments were performed using paired preoperative and postoperative (two months after the surgery) serum samples from four NSCLC patients to monitor the levels of serum NPTX2 in the same patients. The concentration of serum NPTX2 was dramatically reduced after surgical resection of primary tumors (Fig. 3C). The serum NPTX2 values were further compared with the expression levels of NPTX2 in primary tumors in the same set of eight NSCLC cases whose serum had been collected before surgery (four patients with NPTX2-positive tumors and four with NPTX2-negative tumors). The levels of serum NPTX2 showed good correlation with the expression levels of NPTX2 in primary tumor (Fig. 3D). The results independently support the high specificity and the great potentiality of serum NPTX2 as a biomarker for detection of cancer at an early stage and for monitoring of the relapse of the disease.

[0368] Combination assay of NPTX2, CEA, CYFRA and proGRP as tumor markers.

To evaluate the clinical usefulness of serum NPTX2 level as a tumor detection biomarker in clinic, serum levels of two conventional tumor markers (CEA for ADC patients, CYFRA for SCC patients and proGRP for SCLC patients) were also measured by ELISA, in the same set of serum samples from cancer patients and control individuals. Cut off levels in this assay determined by ROC analyses were set to result in optimal diagnostic accuracy and likelihood ratios for CEA, i.e., 2.5 ng/ml

(with a sensitivity of 37.9% and a specificity of 98.2% for ADC), CYFRA, i.e., 2.0 ng/ml (with a sensitivity of 53.7% and a specificity of 96.7% for SCC) and proGRP, i.e., 46.0 pg/ml (with a sensitivity of 65.1% and a specificity of 97.5% for SCLC). The correlation coefficient between serum NPTX2 and CEA values was not significant (Spearman rank correlation coefficient: $\rho = 0.109$, $P = 0.1474$). Measuring both NPTX2 and CEA in serum can improve overall sensitivity for detection of lung ADC patients to 82.0%. False-positive rates for either of the two tumor markers among normal volunteers (control group) amounted to 7.3%. The correlation coefficient between serum NPTX2 and CYFRA values was not significant (Spearman rank correlation coefficient: $\rho = 0.013$, $P = 0.9242$). Measuring both NPTX2 and CYFRA in serum can improve overall sensitivity for detection of lung SCC patients to 75.9%. False-positive rates for either of the two tumor markers among normal volunteers (control group) amounted to 8.8%. The correlation coefficient between serum NPTX2 and proGRP values was not significant (Spearman rank correlation coefficient: $\rho = 0.161$, $P = 0.1232$). Measuring both NPTX2 and proGRP in serum can improve overall sensitivity for detection of lung SCLC patients to 78.3%. False-positive rates for either of the two tumor markers among normal volunteers (control group) amounted to 8.8%.

[0369] Serum levels of NPTX2 in various cancer patients.

Since NPTX2 was frequently overexpressed in various cancers (Fig. 4), the serum NPTX2 level of patients with various cancer (colon cancer, breast cancer and cervical cancer) was investigated. ELISA experiments detected NPTX2 in serologic samples from the majority of the 100 colon cancer patients, 171 breast cancer patients and 182 cervical cancer patients; serum levels of NPTX2 in colon cancer patients were 28.3 ± 21.4 U/ml (mean $\pm$ 1SD), those in breast cancer patients were 26.5 ± 24.4 U/ml (mean $\pm$ 1SD), those in cervical cancer patients were 11.3 ± 9.8 U/ml (mean $\pm$ 1SD), and those in healthy individuals were 2.5 ± 2.94 U/ml (The difference was significant with P-value of < 0.001 by Mann-Whitney U test; Fig. 3E). Serum levels of NPTX2 in those cancer patients were significantly higher than those of normal volunteers and COPD patients ($P < 0.0001$). The cut-off level 7.3 U/ml for NPTX2 showed sensitivity of 65.0% and a specificity of 97.1% for colon cancer, sensitivity of 62.5% and a specificity of 97.1% for breast cancer and sensitivity of 51.1% and a specificity of 97.1% for cervical cancer.

[0370] Inhibition of growth of lung cancer cells by siRNA against NPTX2.

To assess whether NPTX2 is essential for growth or survival of lung cancer cells, commercial siRNAs against NPTX2 (si-NPTX2s) as well as control plasmids (siRNAs for luciferase and EGFP) were transfected into SBC-5 cells, which strongly expressed NPTX2. The NPTX2-mRNA levels in cells transfected with si-NPTX2-1 or si-NPTX2-2 were significantly decreased in comparison with cells transfected with

either control siRNAs. Significant decreases in the numbers of viable cells were observed (Fig. 5A).

[0371] Activation of cellular invasion by NPTX2.

As the immunohistochemical analysis on tissue microarray had indicated that NSCLC patients with NPTX2 strong-positive tumors showed shorter cancer-specific survival period than those with NPTX2-weak positive or -negative tumors, a possible role of NPTX2 in cellular invasion was examined using Matrigel assays, using COS-7 cells. Transfection of NPTX2 cDNA into COS-7 cells significantly enhanced its invasive activity through Matrigel, compared to cells transfected with mock vector (Fig. 5B).

[0372] Discussion

Despite the many advances in diagnostic imaging of tumors, combination chemotherapy, modern surgical techniques and radiation therapy, little improvement has been achieved within the last decade in terms of prognosis and quality of life for most patients with lung cancer. In fact, two thirds of the patients are diagnosed at an advanced stage in which curative surgical treatment is precluded. While the efficacy of new chemotherapeutic regimens for advanced NSCLC has been improved, the median survival for advanced NSCLC by conventional chemotherapy is still around 7-8 months (O.S. Breathnach, et al., J Clin Oncol 19 (2001), pp. 1734-1742., N. Hanna, et al., J Clin Oncol 22 (2004), pp. 1589-1597.). There is accordingly an urgent need to develop practical diagnostic biomarkers for early detection of cancer and new types of drugs targeting specific cell signals important for malignant nature of cancer cells. To that end, genome-wide expression profile analyses of 101 lung cancers after enrichment of cancer cells by laser microdissection has been performed using a cDNA microarray containing 27,648 genes. Through the analysis, several potential candidates for the development of novel diagnostic markers, therapeutic drugs, and/or immunotherapy were identified. Among those identified, the genes encoding putative tumor-specific transmembrane/secretory proteins are considered to have significant advantages because they are present on the cell surface or within the extracellular space, and/or in serum, making them easily accessible as molecular markers and therapeutic targets.

[0373] In the context of the present invention, NPTX2 was identified as a potential target for the development of novel tools for diagnosis and treatment of lung cancer. NPTX2 is a member of the newly recognized subfamily of "long pentraxin" (Goodman AR, et al., Cytokine Growth Factor Rev. 1996 Aug;7(2):191-202.). NPTX2 mediates uptake of synaptic macromolecules and is involved in both synaptogenesis and synaptic plasticity in developing and adult brain. However, to date, the relevance of NPTX2 to carcinogenesis has never been described.

[0374] The data presented herein demonstrate that NPTX2 protein is expressed in the great majority of lung, colon, breast and cervical cancer specimens, while only scarcely expressed in normal tissues. Furthermore, higher NPTX2 expression level was found to be associated with shorter cancer specific survival periods. Concordantly, induction of exogenous expression of NPTX2 enhanced the invasive activity of COS-7 cells; thus, secreted NPTX2 may function as an autocrine/paracrine cell growth/invasion factor. NPTX2 have previously identified to bind to Neuronal pentraxin receptor (NPTXR) (Goodman AR, et al., Cytokine Growth Factor Rev. 1996 Aug;7(2):191-202.). However, when mRNA expression of NPTXR was analyzed in lung cancer cell lines and cancer tissues using semiquantitative RT-PCR, the expression pattern of NPTXR was not perfectly concordant with that of NPTX2 (data not shown). Although the precise molecular mechanism underlying the observations remains to be elucidated by identification of the NPTX2 receptor in cancer cells, the results obtained by in vitro and in vivo assays clearly suggest that over-expressed NPTX2 is likely to be an autocrine/paracrine growth factor and might be associated with cancer cell growth and invasion, thereby inducing a highly malignant phenotype of lung cancer cells. Taken together, these results support the potential of NPTX2 as a molecular target for lung cancer treatment.

[0375] Interestingly, hypoxia induced a significant increase in NPTX2 expression in lung cancer cells (data not shown). Clinical studies have clearly shown that low pO₂ tension within a neoplastic lesion is an independent prognostic indicator of poor outcome and correlates with an increased risk to develop distant metastasis independently of therapeutic treatment (N. Hanna, et al., J Clin Oncol 22 (2004), pp. 1589-1597., Hossain MA, et al., J. Neurosci. 24:4187-96., Desheng Xu, et al., Neuron. 39:513-528). Hypoxia plays a key role in tumor cell survival, invasion, and metastasis. A series of genes and proteins that may increase the survival of tumor cells under hypoxic conditions, including vascular endothelial growth factor (VEGF), insulin-like growth factor, inducible nitric oxide synthase, platelet-derived endothelial growth factor, glucose transporter 1, erythropoietin and nitric oxide synthase gene, are regulated by Hypoxia Inducible Factor-1 α (Brown JM. et al., Cancer Res 1999;59:5863-70., Hockel M, et al., Cancer Res 1999;59:4525-8., Hockel M, et al., J Natl Cancer Inst 2001;93:266-76., Feldser D, et al., Cancer Res 1999; 59: 3915-3918). Other clinical studies have shown that reduced hypoxia in solid tumors adversely affects the outcome of radiotherapy. Thus, the data herein suggest that targeting NPTX2 is a promising therapeutic strategy for the treatment of invasive, metastatic, and radioresistant hypoxic lung cancers.

[0376] On the other hand, high levels of NPTX2 protein in serologic samples from lung, colon, breast and cervical cancer patients were also found. Serum markers could be

applied to the differential diagnoses, early detection of cancer, prognostic predictions, monitoring of treatment efficacy, and surveillance of disease relapse. In the present invention, it was revealed that high levels of serum NPTX2 were detected even in patients with earlier-stage tumors. Furthermore, serologic concentration of NPTX2 dramatically reduced after surgical resection of primary tumors. Furthermore, the levels of serum NPTX2 showed good correlation with the expression levels of NPTX2 in primary tumor tissue in the same patients. To validate the feasibility of applying NPTX2 as the diagnostic tool, serum levels of NPTX2 were compared with those of CEA, CYFRA and proGRP, a conventional diagnostic marker for ADC, SCC and SCLC, in terms of sensitivity and specificity for diagnosis. An assay combining both markers (NPTX2+CEA, NPTX2+CYFRA or NPTX2+proGRP) increased the sensitivity to about 76-82% for lung cancer (ADC, SCC or SCLC), higher than that of CEA, CYFRA or proGRP alone, whereas 7-8% of healthy volunteers were falsely diagnosed as positive. Thus, the data herein sufficiently demonstrate the utility of NPTX2 as a serologic biomarker for diagnosis of even early-stage lung cancers, monitoring of treatment efficacy and surveillance of disease relapse.

[0377] To summarize, NPTX2 was found to be overexpressed in the great majority of lung, colon, breast and cervical cancers and its serum levels were noted to be elevated in sera of a large proportion of the patients. Accordingly, NPTX2, particularly combined with other tumor marker(s), has the potential to significantly improve the sensitivity of cancer diagnosis, and further serve as an initial diagnostic, as an immunohistochemical marker identifying patients who might benefit from early systemic treatment. Since up-regulation of NPTX2 is a frequent and important feature of lung carcinogenesis, targeting NPTX2 represents a new strategy in the design of anti-cancer drugs specific for lung cancer.

Industrial Applicability

[0378] The gene-expression analysis of cancers described herein, using the combination of laser-capture dissection and genome-wide cDNA microarray, has identified a specific gene as a target for cancer prevention and therapy. Based on the expression of this differentially expressed gene, i.e., NPTX2, the present invention provides a novel molecular diagnostic marker for identifying and detecting cancers as well as assessing the prognosis. Therefore, the present invention also provides a novel diagnostic strategy using NPTX2.

[0379] Furthermore, as described herein, NPTX2 are involved in cancer cell survival. Therefore, the present invention also provides novel molecular targets for treating and preventing cancer. They may be useful for developing novel therapeutic drugs and preventative agents without adverse effects.

[0380] The methods described herein are also useful for the identification of additional molecular targets for prevention, diagnosis, and treatment of cancers. The data provided herein add to a comprehensive understanding of cancers, facilitate development of novel diagnostic strategies, and provide clues for identification of molecular targets for therapeutic drugs and preventative agents. Such information contributes to a more profound understanding of tumorigenesis, and provides indicators for developing novel strategies for diagnosis, treatment, and ultimately prevention of cancers.

[0381] All patents, patent applications, and publications cited herein are incorporated by reference in their entirety.

While the invention has been described in detail and with reference to specific embodiments thereof, it is to be understood that the foregoing description is exemplary and explanatory in nature and is intended to illustrate the invention and its preferred embodiments. Through routine experimentation, one skilled in the art will readily recognize that various changes and modifications can be made therein without departing from the spirit and scope of the invention. Thus, the invention is intended to be defined not by the above description, but by the following claims and their equivalents.

Claims

- [Claim 1] A method of diagnosing or detecting cancer in a subject, comprising the steps of:
- (1) determining an expression level of an NPTX2 gene in a subject-derived biological sample by a method selected from the group consisting of:
 - (a) detecting an mRNA of the NPTX2 gene;
 - (b) detecting an NPTX2 polypeptide; and
 - (c) detecting a biological activity of an NPTX2 protein;
 - (2) comparing the NPTX2 expression level determined in step (1) with a normal control level of the NPTX2 gene; and
 - (3) diagnosing said subject with cancer or determining the presence of the cancer in a subject when the NPTX2 expression level determined in step (1) is higher than said normal control level.
- [Claim 2] The method of claim 1, wherein the subject-derived biological sample is a bodily tissue sample or blood sample.
- [Claim 3] The method of claim 2, wherein the expression level of the NPTX2 gene is determined by detecting an NPTX2 polypeptide in a subject-derived blood sample.
- [Claim 4] The method of claim 3, wherein the NPTX2 polypeptide is detected by the method comprising steps of:
- (i) contacting an antibody against an NPTX2 polypeptide with the subject-derived blood sample; and
 - (ii) detecting the binding between said antibody and the NPTX2 polypeptide in said subject-derived blood sample.
- [Claim 5] The method of claim 4, wherein the NPTX2 polypeptide is detected by Enzyme-Linked ImmunoSorbent Assay (ELISA).
- [Claim 6] The method of any one of claims 3 to 5, wherein the subject-derived blood sample is a subject-derived serum sample.
- [Claim 7] The method of any one of claims 3 to 6, wherein the method further comprises a step of detecting at least one other serum tumor marker in the subject-derived blood sample, wherein cancer is judged to be present or said subject is judged to be suffering from cancer when either (a) the level of the NPTX2 polypeptide in said subject derived blood sample is higher than a normal control level of the NPTX2 polypeptide, or (b) the level of said serum tumor marker in said subject derived blood sample is higher than a normal control level of said

serum tumor marker, or (c) both.

- [Claim 8] The method of any one of claims 1 to 7, wherein the cancer is lung cancer, breast cancer, cervical cancer or colon cancer.
- [Claim 9] A kit for use in diagnosis or detection of cancer in a subject, wherein said kit comprises at least one reagent selected from the group consisting of:
- (a) a reagent for detecting an mRNA of the NPTX2 gene;
 - (b) a reagent for detecting an NPTX2 polypeptide; and
 - (c) a reagent for detecting a biological activity of an NPTX2 polypeptide.
- [Claim 10] The kit of claim 9, wherein the reagent comprises an oligonucleotide that has a sequence complementary to a part of an mRNA of the NPTX2 gene and that specifically binds to said mRNA; or an antibody against the NPTX2 polypeptide.
- [Claim 11] The kit of claim 10, wherein the kit is an ELISA kit comprising at least one antibody against the NPTX2 polypeptide.
- [Claim 12] The kit of any one of claims 8 to 11, wherein the cancer is lung cancer, breast cancer, cervical cancer or colon cancer.
- [Claim 13] A method for assessing or predicting a prognosis of a subject with cancer, comprising the steps of:
- (1) determining an expression level of the NPTX2 gene in a subject-derived biological sample, by a method selected from the group consisting of:
 - (a) detecting an mRNA of the NPTX2 gene;
 - (b) detecting an NPTX2 polypeptide; and
 - (c) detecting a biological activity of an NPTX2 polypeptide;
 - (2) comparing the NPTX2 expression level determined in step (1) with a good prognosis control level of the NPTX2 gene; and
 - (3) predicting a poor prognosis for said subject when the NPTX2 expression level determined in step (1) is higher than said good prognosis control level.
- [Claim 14] The method of claim 13, wherein the cancer is lung cancer.
- [Claim 15] A kit for use in assessing or predicting a prognosis of a subject with cancer, wherein said kit comprises at least one reagent selected from the group consisting of:
- (a) a reagent for detecting an mRNA of the NPTX2 gene;
 - (b) a reagent for detecting an NPTX2 polypeptide; and
 - (c) a reagent for detecting a biological activity of an NPTX2

polypeptide.

- [Claim 16] The kit of claim 15, wherein the reagent comprises an oligonucleotide that has a sequence complementary to a part of an mRNA of the NPTX2 gene and that specifically binds to said mRNA; or an antibody against the NPTX2 polypeptide.
- [Claim 17] The kit of claim 15 or 16, wherein the cancer is lung cancer.
- [Claim 18] A method of screening for a candidate substance for either or both of treating and preventing cancer, wherein said method comprises steps of:
- (a) contacting a test substance with an NPTX2 polypeptide, or functional equivalent thereof;
 - (b) detecting the binding between the NPTX2 polypeptide or functional equivalent thereof, and the test substance; and
 - (c) selecting the test substance that binds to the NPTX2 polypeptide or functional equivalent thereof.
- [Claim 19] A method of screening for a candidate substance for either or both of treating and preventing cancer, wherein said method comprises steps of:
- (a) contacting a test substance with a cell expressing the NPTX2 gene;
 - (b) determining an expression level of the NPTX2 gene in the cell of step (a); and
 - (c) selecting the test substance that reduces the expression level of NPTX2 gene as compared to the expression level determined in the absence of the test substance.
- [Claim 20] A method of screening for a candidate substance for either or both of treating and preventing cancer, wherein said method comprises steps of:
- (a) contacting a test substance with an NPTX2 polypeptide or functional equivalent thereof;
 - (b) detecting a biological activity of the NPTX2 polypeptide or functional equivalent thereof of step (a); and
 - (c) selecting the test substance that suppresses a biological activity of the NPTX2 polypeptide or functional equivalent thereof as compared to the biological activity detected in the absence of the test substance.
- [Claim 21] The method of claim 20, wherein the biological activity is cell proliferation enhancing activity.
- [Claim 22] A method of screening for a candidate substance for either or both of treating and preventing cancer, wherein said method comprises steps

of:

(a) contacting a test substance with a cell into which a vector comprising a transcriptional regulatory region of the NPTX2 gene and a reporter gene that is expressed under control of transcriptional regulatory region has been introduced,
(b) measuring an expression or activity level of said reporter gene; and
(c) selecting the test substance that reduces the expression or activity level of said reporter gene, as compared to the expression or activity level detected in the absence of the test substance.

[Claim 23] The method of any one of claims 18 to 22, wherein the cancer is lung cancer, breast cancer, cervical cancer or colon cancer.

[Claim 24] A method of either or both of treating and preventing cancer in a subject, wherein said method comprises a step of administering to said subject a pharmaceutically effective amount of a double-stranded molecule against the NPTX2 gene, or a vector encoding said double-stranded molecule, wherein said double-stranded molecule comprises a sense strand and an antisense strand, wherein the sense strand comprises a nucleotide sequence corresponding to a target sequence selected from the NPTX2 gene sequence, and the antisense strand comprises a nucleotide sequence complementary to said target sequence, wherein said sense strand and said antisense strand hybridize to each other to form the double-stranded molecule, and wherein said double stranded molecule, when introduced into a cell expressing the NPTX2 gene, inhibits cell proliferation as well as the expression of the NPTX2 gene.

[Claim 25] The method of claim 24, wherein the sense strand hybridizes with the antisense strand at the target sequence to form the double-stranded molecule having between 19 and 25 nucleotide pair in length.

[Claim 26] The method of claim 24 or 25, wherein the double-stranded molecule is a single polynucleotide comprising the sense strand and the antisense strand linked via a single-stranded nucleotide sequence.

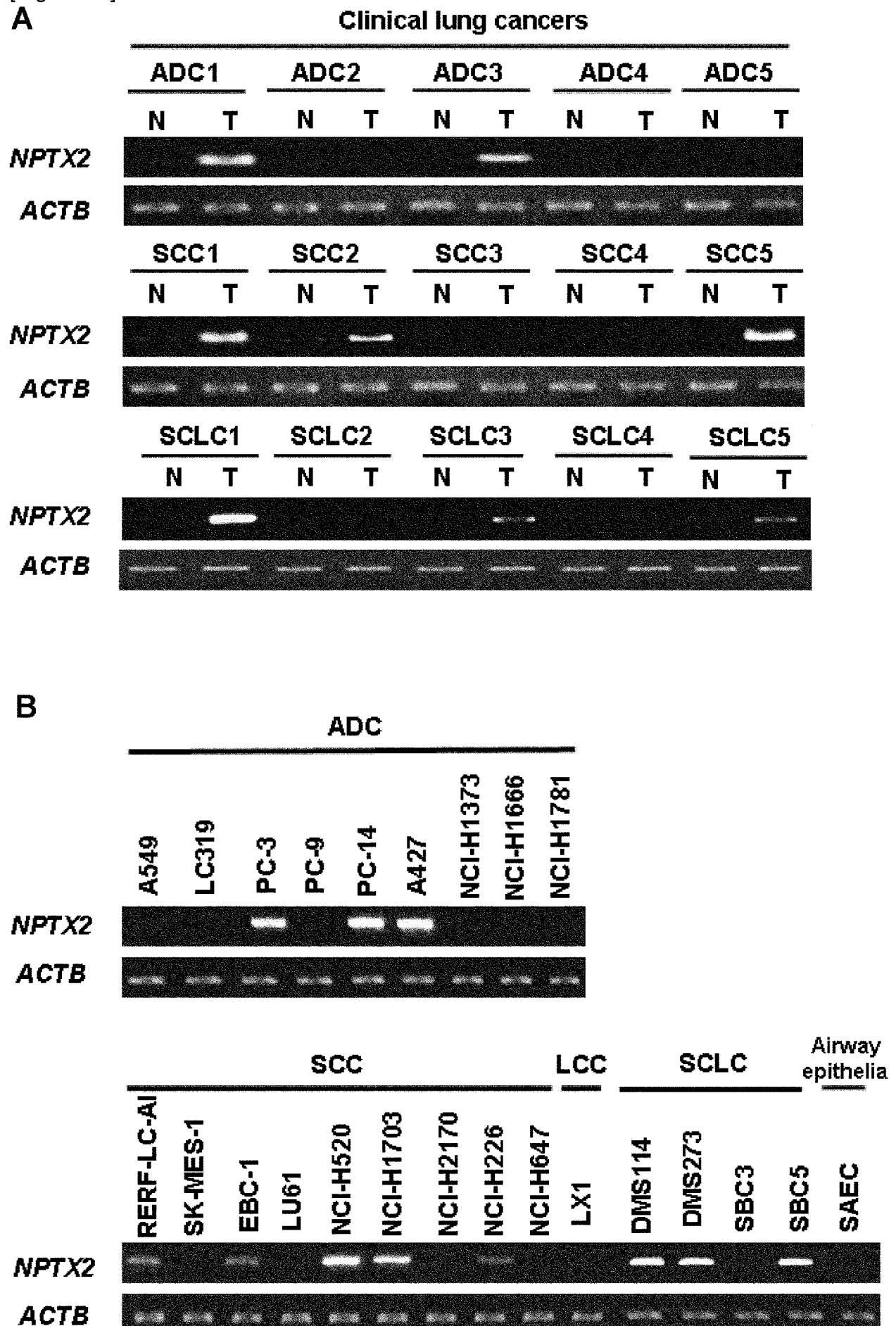
[Claim 27] A composition for either or both of treating and preventing cancer, wherein said composition comprises a pharmaceutically effective amount of a double-stranded molecule against the NPTX2 gene, or a vector encoding said double-stranded molecule, and a pharmaceutically acceptable carrier, wherein said double-stranded molecules comprises a sense strand and an antisense strand, wherein the sense strand comprises a nucleotide sequence corresponding to a target sequence

selected from the NPTX2 gene sequence, and the antisense strand comprises a nucleotide sequence complementary to said target sequence, wherein said sense strand and said antisense strand hybridize to each other to form the double-stranded molecule, and wherein said double stranded molecule, when introduced into a cell expressing the NPTX2 gene, inhibits cell proliferation as well as the expression of the NPTX2 gene.

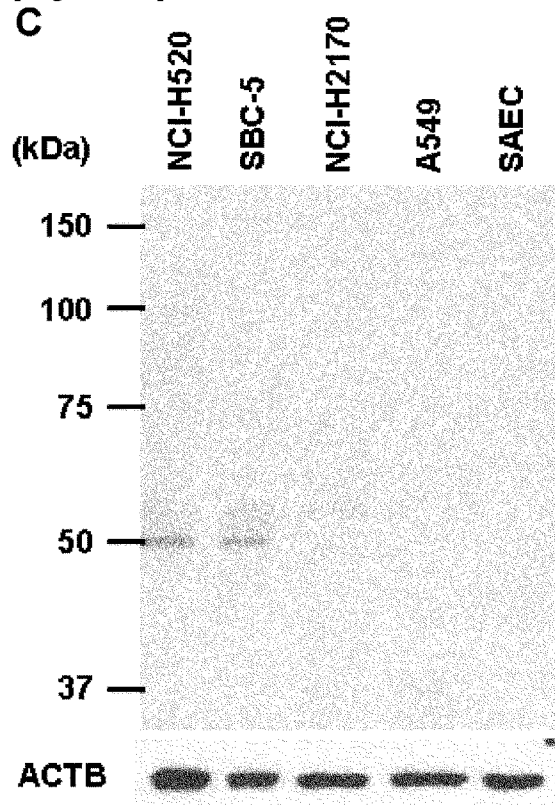
[Claim 28] The composition of claim 27, wherein the sense strand hybridizes with the antisense strand at the target sequence to form the double-stranded molecule having between 19 and 25 nucleotide pair in length.

[Claim 29] The composition of claim 27 or 28, wherein the double-stranded molecule is a single polynucleotide comprising the sense strand and the antisense strand linked via a single-stranded nucleotide sequence.

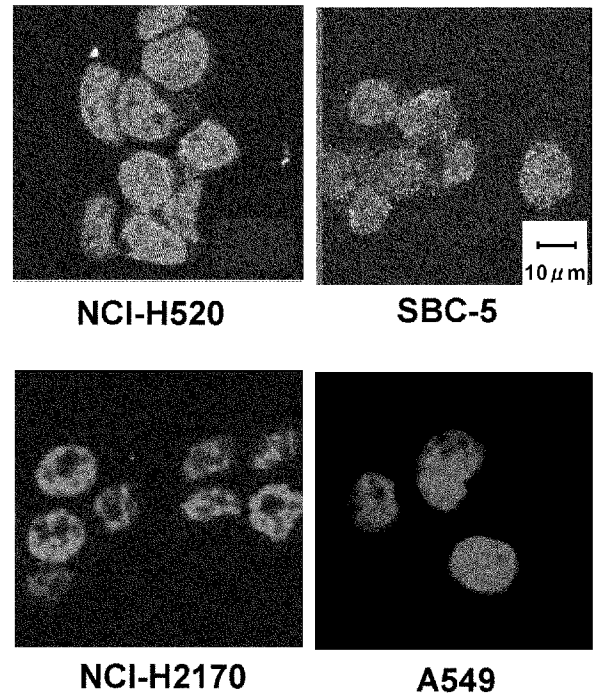
[Fig. 1A-B]



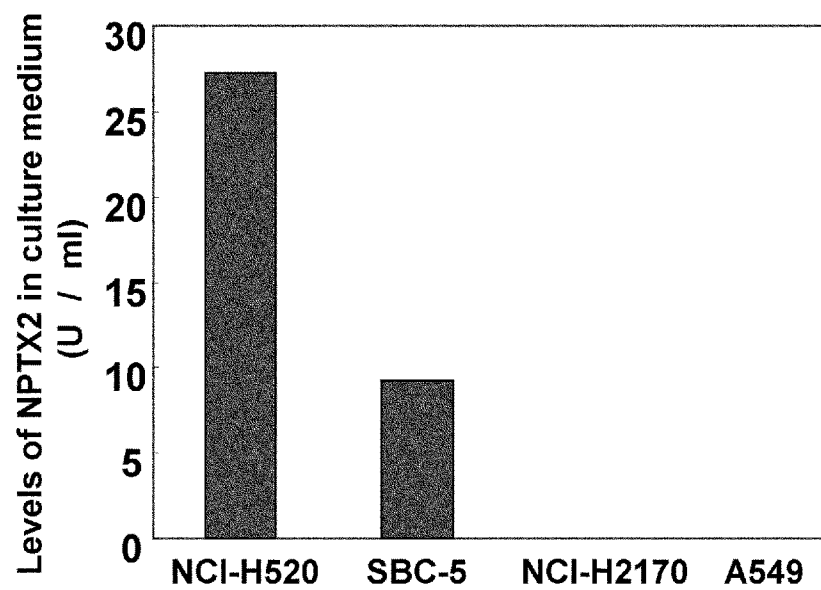
[Fig. 1C-E]



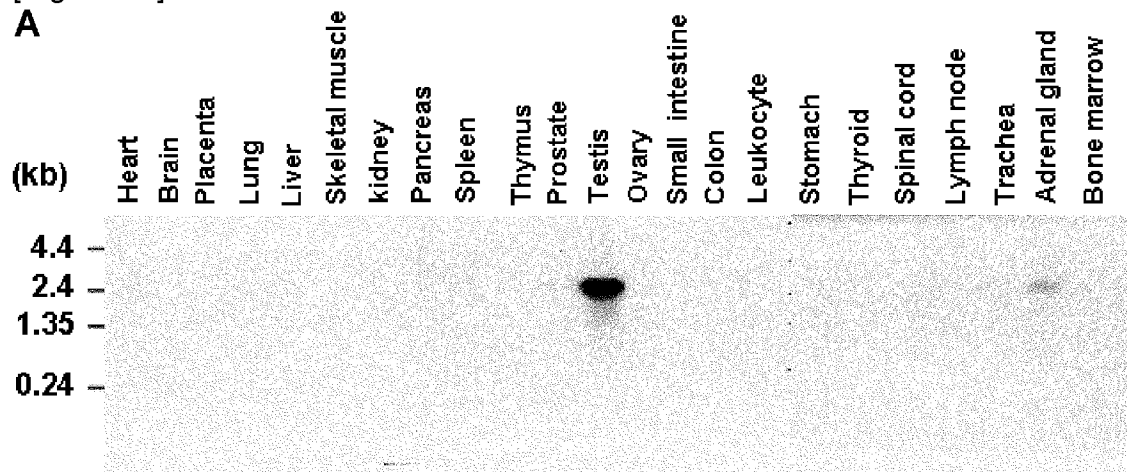
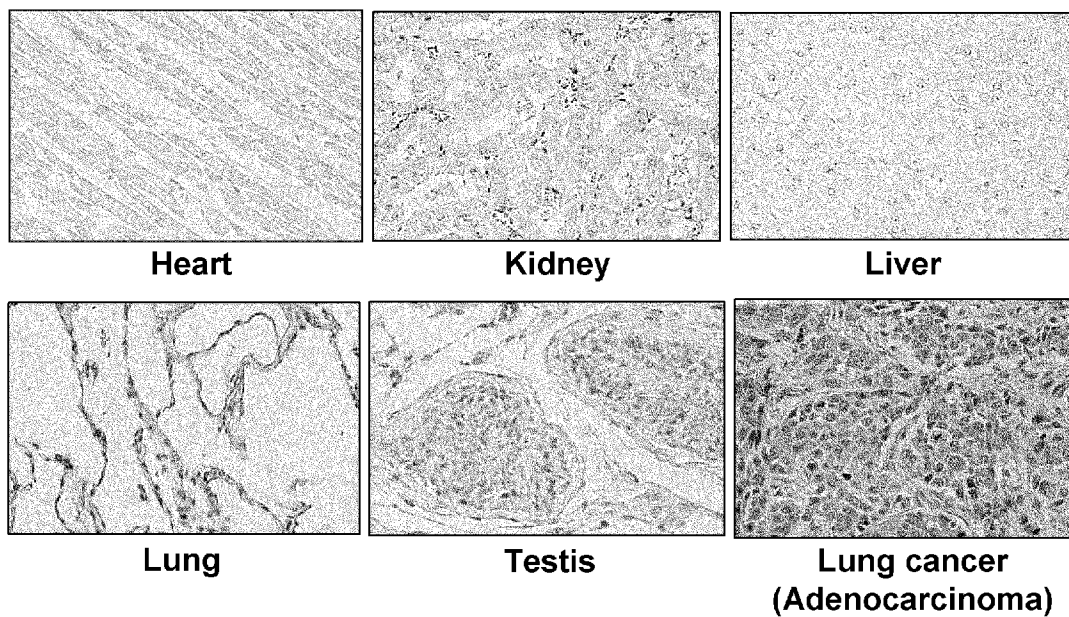
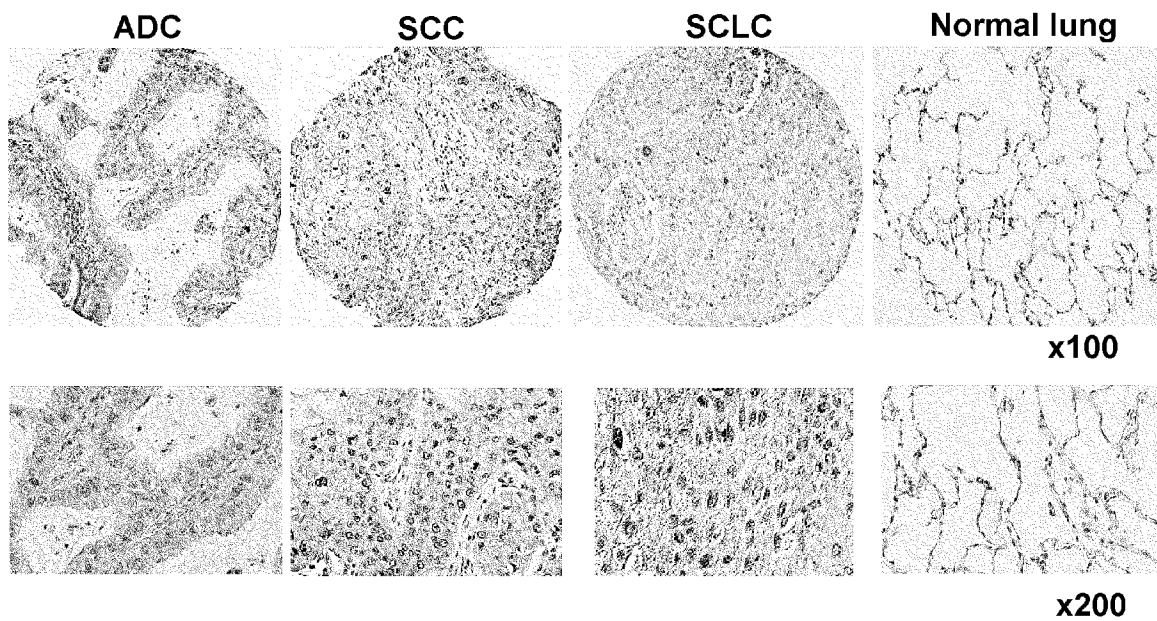
D



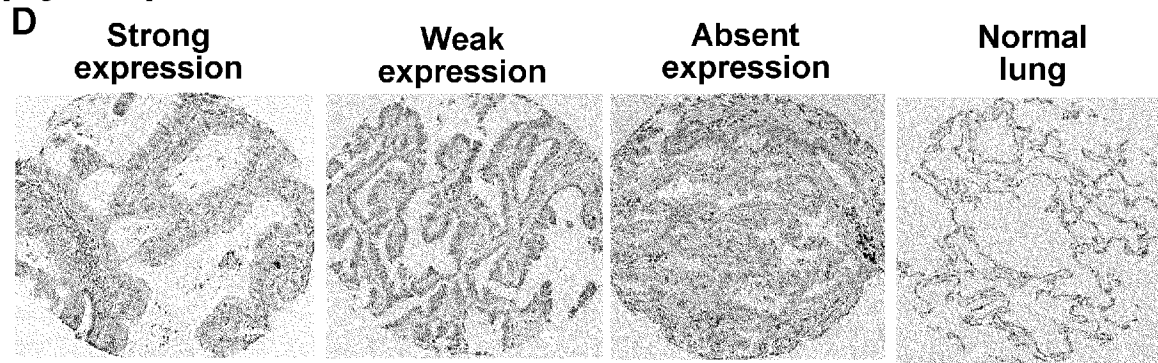
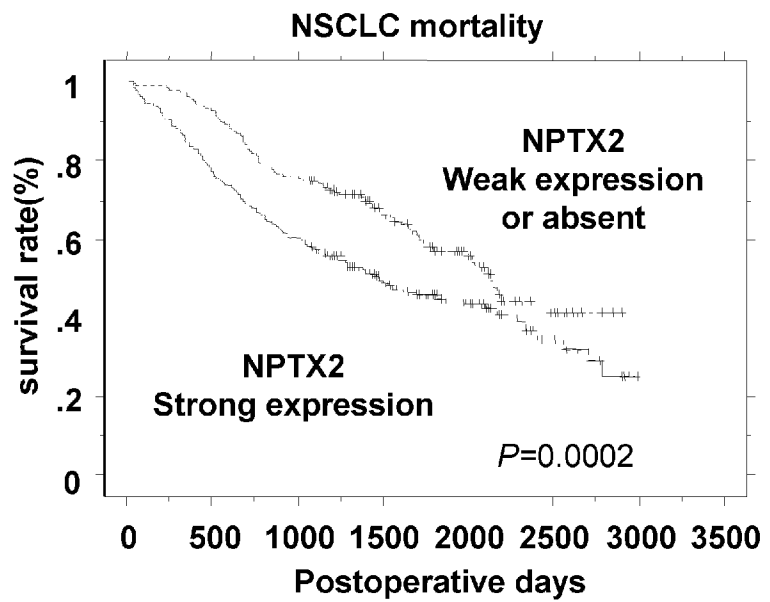
E



[Fig. 2A-C]

A**B****C**

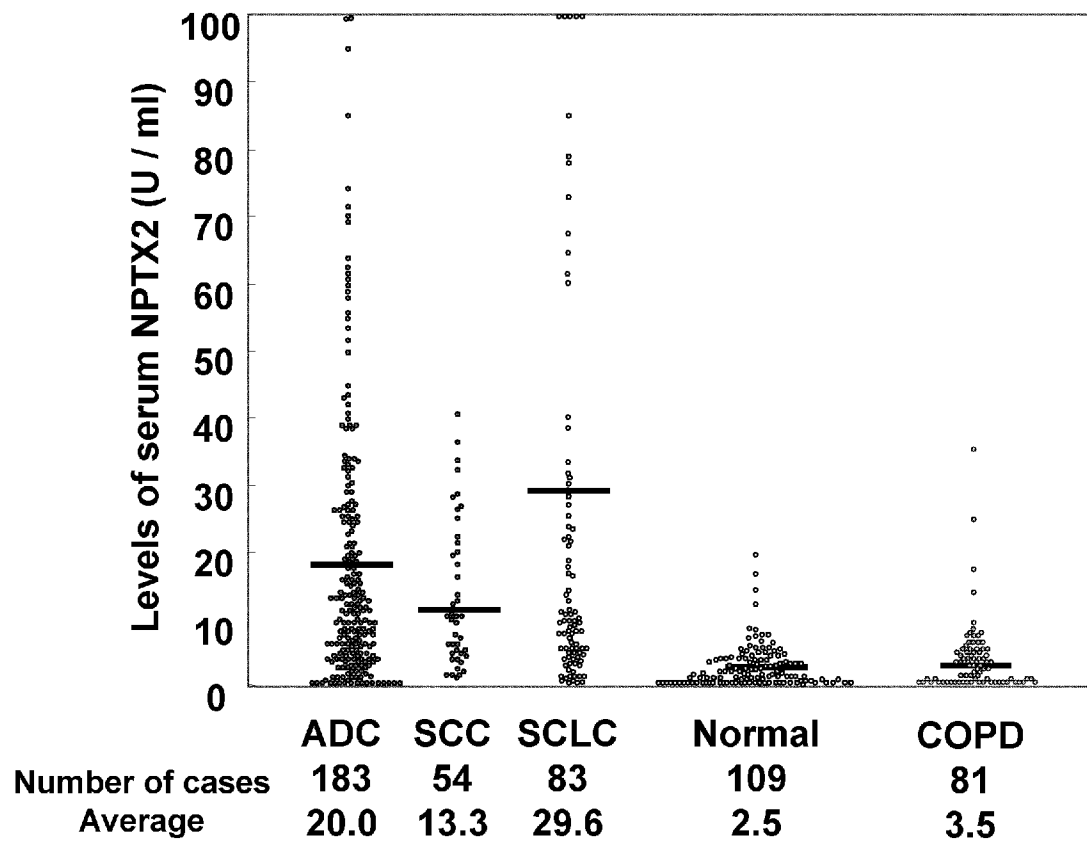
[Fig. 2D-E]

**E**

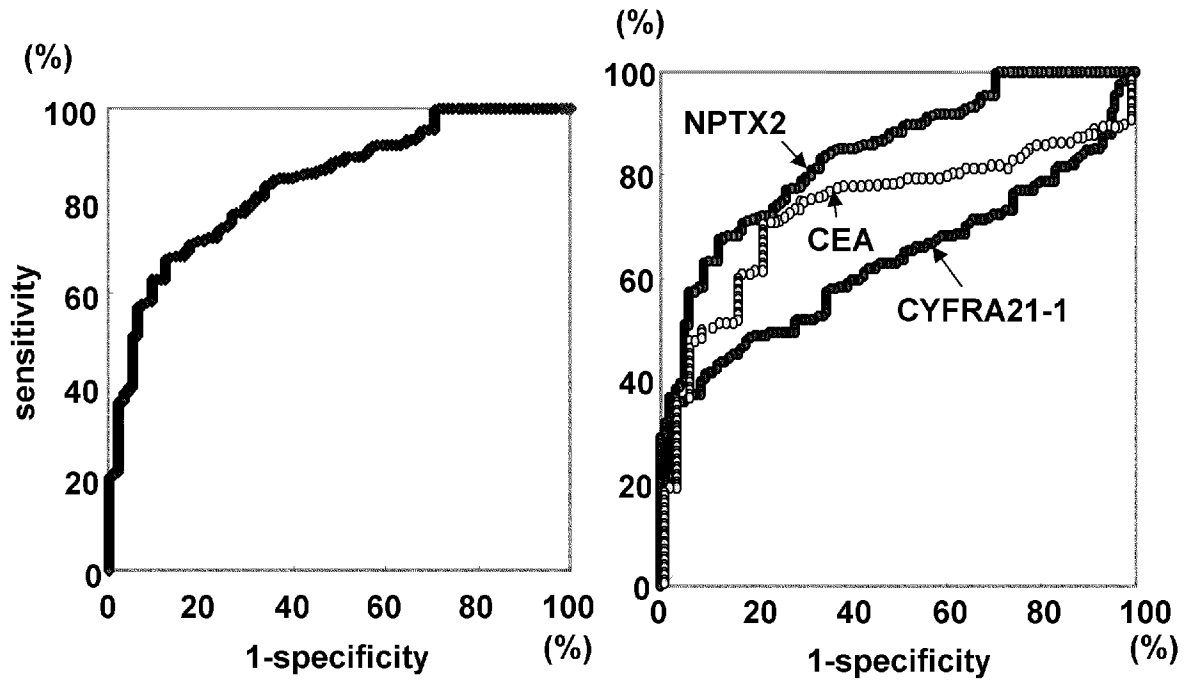
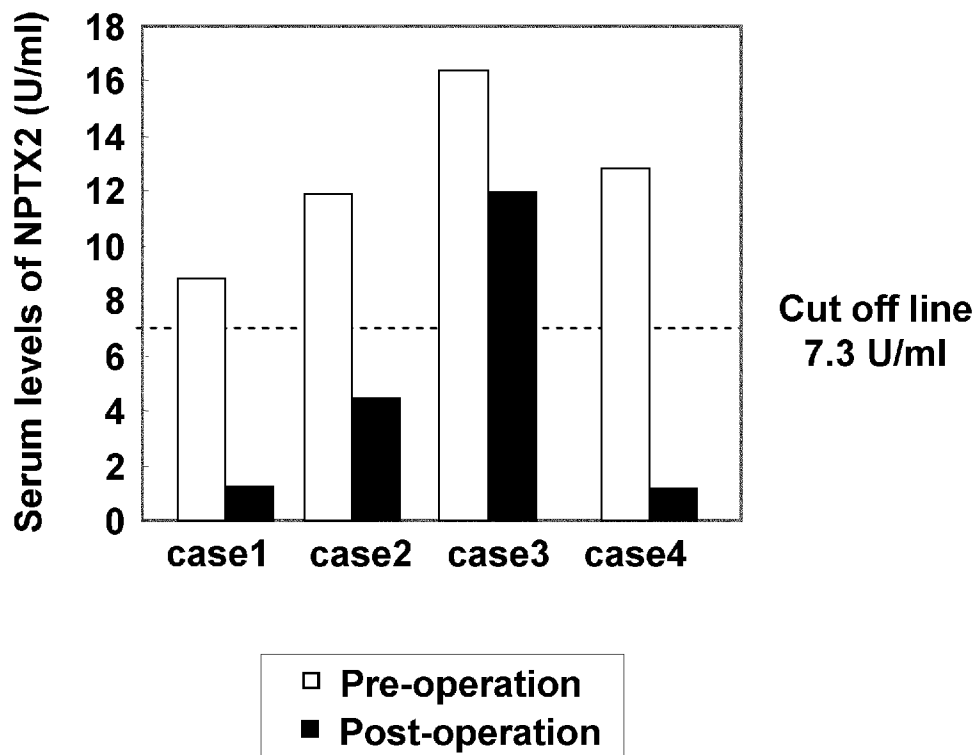
Number at risk

Absent or weak NPTX2 expression	163	152	123	78	42	13	4
Strong NPTX2 expression	198	155	118	65	38	14	6

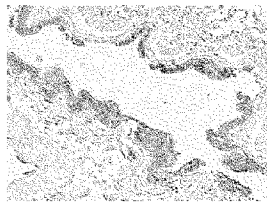
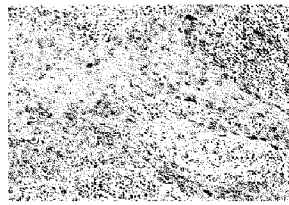
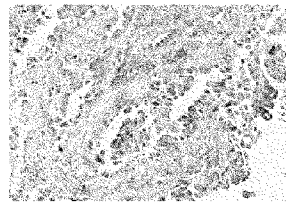
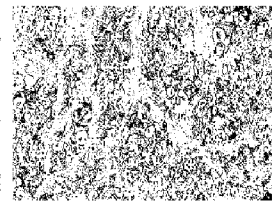
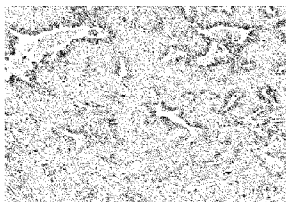
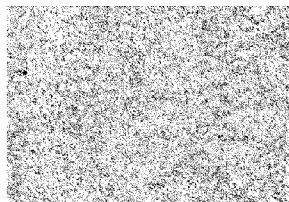
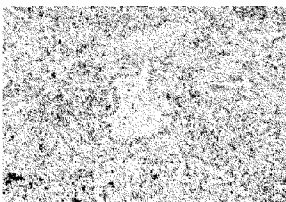
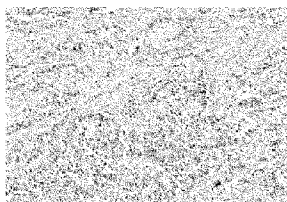
[Fig. 3A]

A

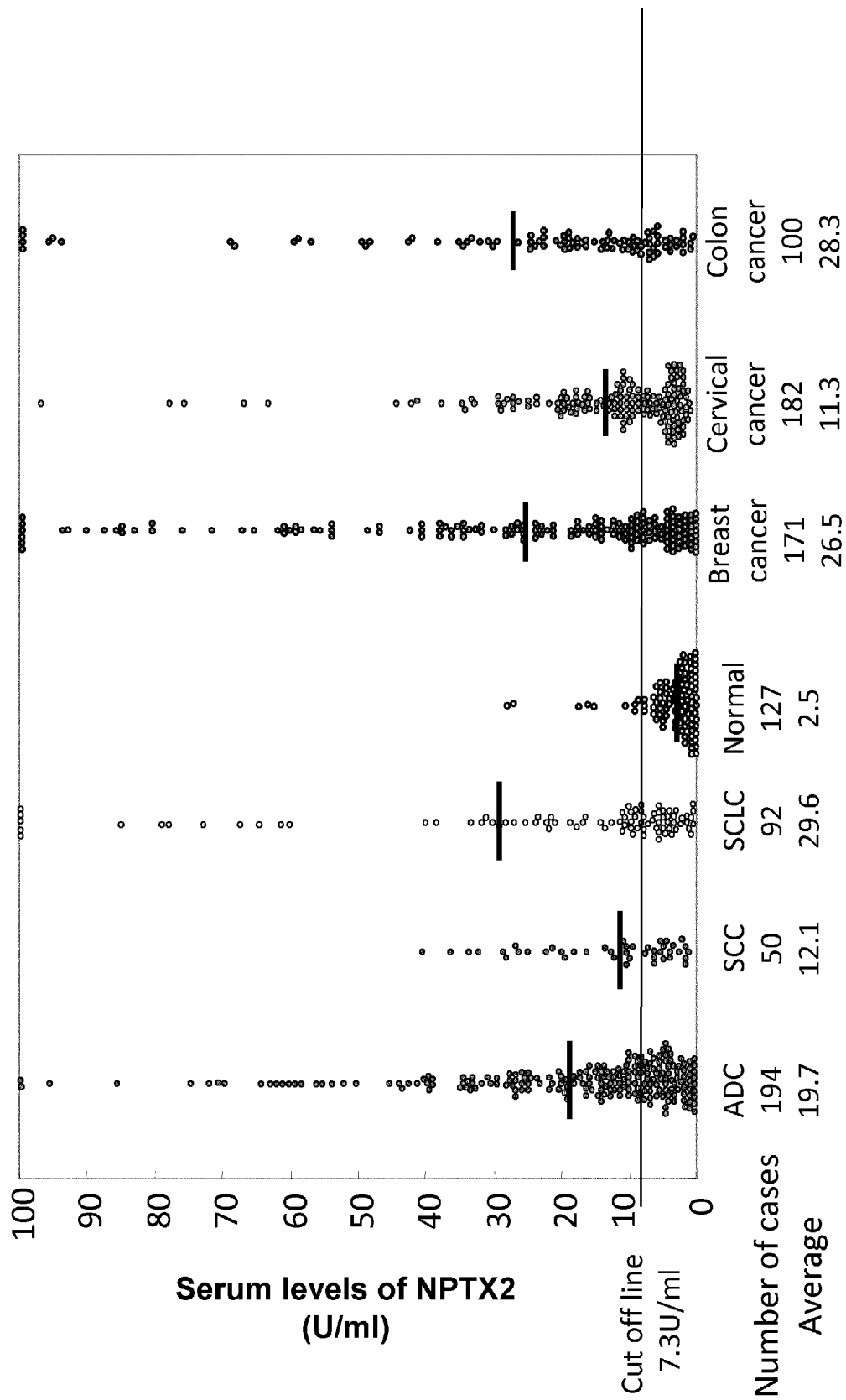
[Fig. 3B-C]

B**C**

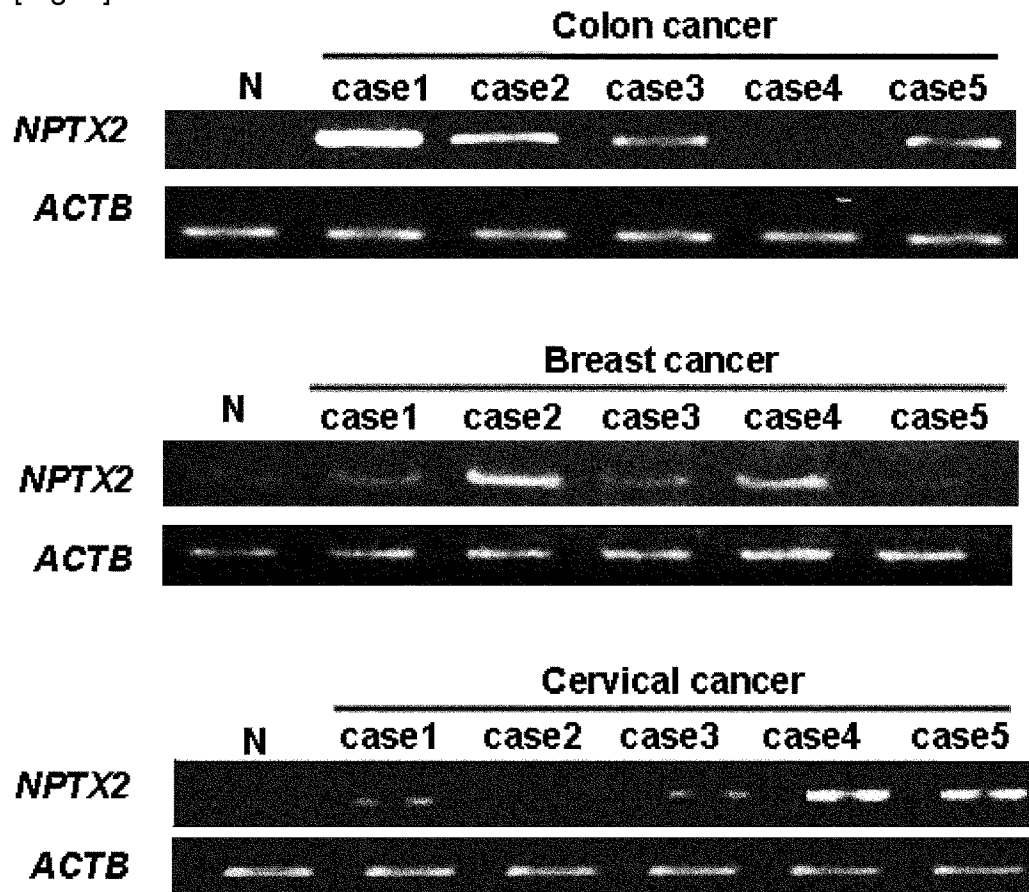
[Fig. 3D]

D**Serum level of NPTX2 positive****ADC IA 69.6 U/ml****SCC IB 21.0 U/ml****ADC IB 33.4 U/ml****ADC IB 19.8 U/ml****Serum level of NPTX2 negative****ADC IIB cut off>****SCC IIB cut off>****ADC IA cut off >****ADC IIB cut off >**

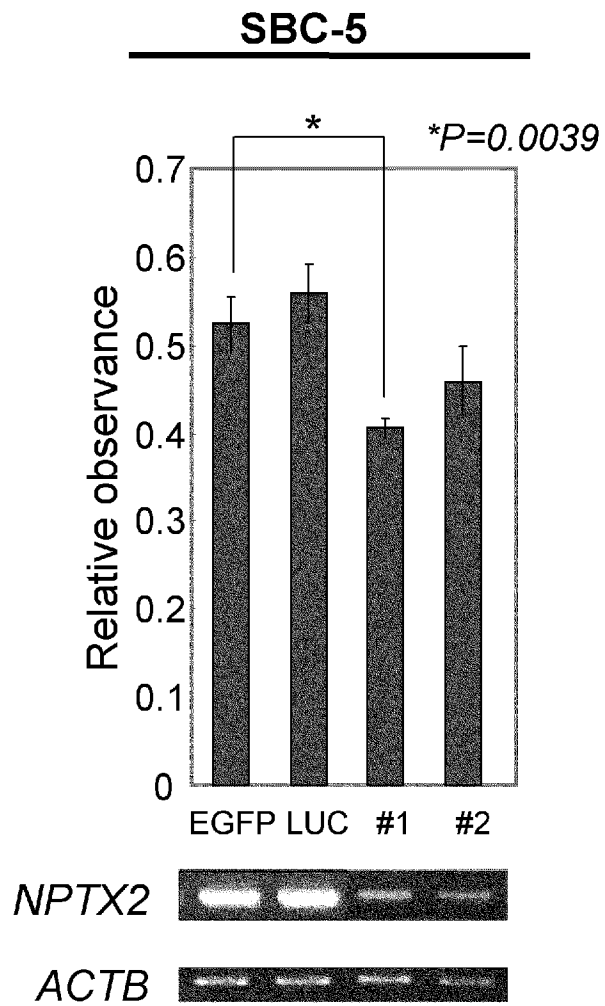
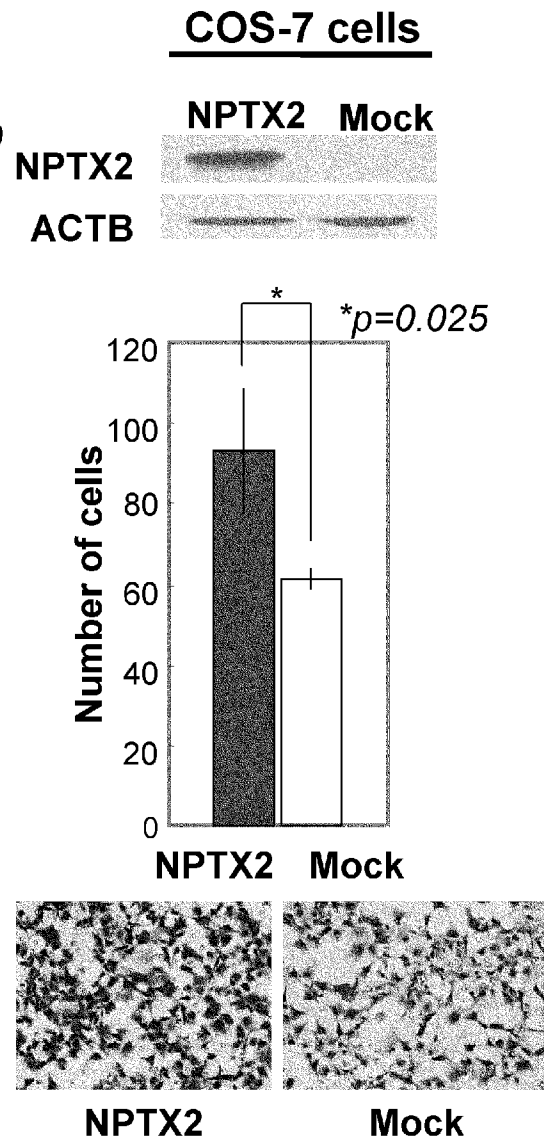
[Fig. 3E]

E

[Fig. 4]



[Fig. 5]

A**B**

INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP2012/002930

A. CLASSIFICATION OF SUBJECT MATTER			
Int.Cl. See extra sheet			
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)			
Int.Cl. C12Q1/68, A61K31/713, A61P35/00, C12N15/09, C12Q1/02, G01N33/15, G01N33/50, G01N33/53			
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched			
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)			
CAplus/MEDLINE/EMBASE/BIOSIS (STN), JSTPlus/JMEDPlus/JST/580 (JDreamII), GenBank/EMBL/DDBJ/GeneSeq, UniProt/GeneSeq			
C. DOCUMENTS CONSIDERED TO BE RELEVANT			
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.	
X/ Y	WO 2004/031413 A2 (ONCOTHERAPY SCIENCE, INC.) 2004.04.15, & US 2006/0024692 A1 & EP 1743947 A2	9-12, 15-23, 27-29/ 18-23, 27-29	
X/ Y	WO 2004/063355 A2 (PROTEIN DESIGN LABS, INC.) 2004.07.29, & US 2005/0181375 A1	9-12, 15-17/ 18-23, 27-29	
A	ZHANG, L. et al., The neuronal pentraxin II gene (NPTX2) inhibit proliferation and invasion of pancreatic cancer cells in vitro, Mol. Biol. Rep., 2010.12, Vol.38, pp.4903-4911	9-12, 15-23, 27-29	
☐ 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		Date of mailing of the international search report	
28.05.2012		05.06.2012	
Name and mailing address of the ISA/JP		Authorized officer	
Japan Patent Office		Miho Fujii	
3-4-3, Kasumigaseki, Chiyoda-ku, Tokyo 100-8915, Japan		4N 4 4 3 4	
		Telephone No. +81-3-3581-1101 Ext. 3488	

INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP2012/002930

CLASSIFICATION OF SUBJECT MATTER

C12Q1/68(2006.01) i, A61K31/713(2006.01) i, A61P35/00(2006.01) i,
C12N15/09(2006.01) i, C12Q1/02(2006.01) i, G01N33/15(2006.01) i,
G01N33/50(2006.01) i, G01N33/53(2006.01) i

INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP2012/002930

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.: 1-8, 13-14, 24-26
because they relate to subject matter not required to be searched by this Authority, namely:
Methods for treatment of the human body by therapy, and diagnostic methods practiced on the human body.
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.:
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.