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(54) Title: LHX4 AS A TARGET GENE FOR CANCER THERAPY AND DIAGNOSIS

(55) Abstract: The present invention relates to the roles played by the LHX4 gene in lung cancer carcinogenesis and features a method for treating and/or preventing lung cancer by administering a double-stranded molecule against the LHX4 gene, Ku70 gene or Ku86 gene, or vector encoding the double-stranded molecule. The present invention also features methods for diagnosing lung cancer, using LHX4 gene. To that end, LHX4 may serve as a novel serological biomarker for lung cancer. Also, disclosed are methods of identifying substances for treating or/and preventing lung cancer, using as an index their effect on an expression of LHX4 gene or a biological activity of LHX4 polypeptide.

Description

Title of Invention: LHX4 AS A TARGET GENE FOR CANCER THERAPY AND DIAGNOSIS

Technical Field

- [0001] The present invention relates to the field of biological science, more specifically to the field of cancer diagnosis and cancer therapy. In particular, the present invention relates to methods for detecting and diagnosing lung cancer as well as methods for treating and/or preventing lung cancer. Moreover, the present invention relates to methods for screening agents for treating and/or preventing lung cancer.
- [0002] **PRIORITY**
The present invention claims the benefit of US Provisional Application No. 61/375,461, filed on August 20, 2010 and the entire contents of which are incorporated by reference herein.

Background Art

- [0003] Lung cancer is one of the most common and fatal cancers in the world (NPL 1). A number of genetic alterations associated with development and progression of lung cancer have been reported; however, its molecular mechanisms still largely remain unclear (NPL 2). Two major histologically distinct types of lung cancer, non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) have different pathophysiological and clinical features that suggest differences in the mechanisms of their carcinogenesis. NSCLC accounts for nearly 80% of lung cancers, whereas SCLC accounts for 20% of them and is categorized as neuroendocrine tumors of the lung with certain morphologic, ultrastructural, and immunohistochemical characteristics (NPLs 3, 4). In spite of applying surgical techniques combined with various treatment modalities such as radiotherapy and chemotherapy, the overall 5-year survival rate of lung cancer is still low at 15% (NPL 5). Patients with SCLC respond favorably to the 1st line multi-agent chemotherapy, but they often relapse in a short time. The only 20% of patients with limited-stage disease (LD) can be cured with combined modality therapy and less than 5% of those with extensive-disease (ED) can achieve 5-year survival after the initial diagnosis (NPL 5). Therefore, new therapeutic strategies focusing on SCLC as well as NSCLC such as molecular-targeted agents are eagerly awaited.
- [0004] Through screening for novel molecular targets for diagnosis, treatment and prevention of human cancers, we performed genome-wide expression profiles of 101 cases of lung cancers using cDNA microarray containing 27,648 genes or expressed sequence tags (ESTs), coupled with laser microdissection (NPLs 6-11), and found

several candidate molecular targets and biomarkers for lung cancer treatment (NPLs 12-37). Among them, a gene encoding LHX4 (LIM homeobox protein 4) was frequently over-expressed in the great majority of primary SCLC.

- [0005] The LHX4 protein exhibits features typical of transcription factors, including a DNA-binding HOX and two tandem LIM domains, each containing two zinc fingers. LIM HOX protein genes have been frequently found to be involved in cancer. Ectopic expression of the LHX1 and LHX2 genes was detected in various leukemia (NPLs 38-40) and stable expression of the LHX2 gene in hematopoietic stem cells, engrafted in stem cell-deficient mice, was shown to cause myeloproliferative disorders and acute leukemia (NPL 41). Aberrant expression of this gene was observed in cases of chronic myeloid leukemia in blast crisis and in cases of pre-B acute lymphoblastic leukemia. Both cases carried a t(1;14)(q25;q32) chromosome translocation, which resulted in juxtaposition of the respective LHX4 and IGH genes (NPLs 42, 43). During normal development, the LHX4 gene is predominantly expressed in the central nervous system (NPL 44), and it was proposed that its overexpression may be associated with cellular proliferation and/or anti-apoptosis (NPLs 42, 43). Indeed, knockout of LHX4 in mice leads to an increased apoptotic rate in Rathke's pouch, resulting in an impaired pituitary development reminiscent of human congenital pituitary hormone deficiency (CPHD; NPLs 45-47). In spite of these reports about aberrant LHX4 expression in leukemia, no report suggests a significance of LHX4 in human solid tumors.

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Summary of Invention

[0007] The present invention relates to the discovery, through microarray analysis and RT-PCR, that LHX4 gene is overexpressed in clinical lung cancer tissues. As demonstrated herein, functional knockdown of the endogenous LHX4 gene by siRNA in cancer cell lines results in drastic suppression of cancer cell growth, suggesting its essential role in maintaining viability of cancer cells. Since it is only scarcely expressed in adult normal organs, LHX4 gene is a useful molecular target for a novel therapeutic approach with minimal adverse effects. Furthermore, the results disclosed herein demonstrate that LHX4 protein interacts with Ku protein and such interaction is important for stability of LHX4 protein.

Accordingly, the present invention relates to LHX4, and its role in lung cancer carcinogenesis and other cancers that overexpress LHX4 gene. As such, the present invention relates to compositions and methods for detecting, diagnosing, treating and/or preventing cancers that overexpress LHX4 gene, e.g., lung cancer, particularly SCLC, as well as methods of screening for candidate substances for cancer prevention and treatment using LHX4 as a molecular target.

[0008] Central to the present invention is, in part, the discovery that double-stranded molecules composed of specific sequences (in particular, SEQ ID NOs: 11 and 12) that inhibit the expression of LHX4 gene are effective for inhibiting cellular growth of cancer cells that overexpress LHX4 gene, e.g., lung cancer cells. Specifically, small interfering RNAs (siRNAs) targeting LHX4 gene are provided by the present invention. These double-stranded molecules can be utilized in an isolated state or encoded in vectors and expressed from the vectors. Accordingly, it is an object of the present invention to provide such double-stranded molecules that inhibit the expression of LHX4 gene as well as vectors and host cells expressing them.

In another aspect, the present invention provides double-stranded molecules against Ku70 gene or Ku86 gene, and vector encoding such double-stranded molecule. These double-stranded molecules inhibit the expression of Ku70 gene or Ku86 gene, and leads to reduction of LHX4 protein by increasing instability of LHX4 protein.

In one aspect, the present invention provides methods for inhibiting cancer cell growth and treating lung cancer by administering the double-stranded molecule against LHX4 gene, Ku70 gene or Ku86 gene, or vectors encoding the double-stranded molecule to a subject in need thereof. Such methods encompass administering to a subject a composition containing one or more of such double-stranded molecules or vectors.

[0009] In another aspect, the present invention provides compositions for treating and preventing lung cancer, containing at least one of the double-stranded molecule against

LHX4 gene, Ku70 gene or Ku86 gene or a vector encoding the double-stranded molecule.

In yet another aspect, the present invention provides a method of detecting or diagnosing lung cancer or a predisposition for developing lung cancer in a subject by determining an expression level of LHX4 gene in a subject-derived biological sample. An increase in the expression level of LHX4 gene as compared to a normal control level of LHX4 gene indicates that the subject suffers from or is at risk of developing lung cancer.

In yet another aspect, the present invention provides a kit that includes a reagent for detecting an mRNA of a LHX4 gene or a protein encoded by a LHX4 gene. Typically, the reagent may be comprises an oligonucleotide that hybridizes to the mRNA of the LHX4 gene, or an antibody against the protein encoded by the LHX4 gene.

In yet another aspect, the present invention provides a reagent for diagnosis or detection of cancer, which comprises an oligonucleotide that hybridizes to the mRNA of the LHX4 gene, or an antibody against the protein encoded by the LHX4 gene.

In yet another aspect, the present invention provides use of a probe or primer to the mRNA of the LHX4 gene, or an antibody against the protein encoded by the LHX4 gene. for the manufacture of a reagent for diagnosis or detection of cancer.

[0010] In further another aspect, the present invention provides methods of screening for a candidate substance that inhibits cancer cell growth, such substances are useful in the treatment and/or prevention of cancer, such as lung cancer. The methods of the present invention can be carried out in vitro or in vivo and use as an index the binding activity to a LHX4 polypeptide, an expression level of a LHX4 gene, a biological activity of a LHX4 polypeptide, an expression level of a reporter gene or an activity of a reporter gene controlled under a transcriptional regulatory region of the LHX4 gene, or a binding between a LHX4 polypeptide and a Ku protein. Substances that bind to a LHX4 polypeptide, or suppress a LHX4 expression or activity, a reporter gene expression or activity, or binding between a LHX4 polypeptide and a Ku protein can be identified as candidate substances for treating and/or preventing cancer, or inhibiting cancer cell growth. The biological activity of the LHX4 polypeptide to be detected can be cell-proliferating activity or binding activity to a Ku protein. A decrease in the biological activity of the LHX4 polypeptide as compared to a control level in the absence of the test substance indicates that the test substance may be used to reduce symptoms of cancer, or treating and/or preventing cancer.

[0011] In addition, the present invention arises, in part, from the discovery that inhibition of the binding between a LHX4 polypeptide and a Ku protein by a LHX4-derived peptide, which corresponded to the binding domain to the Ku protein, effectively suppressed growth of lung cancer cells. Accordingly, the present invention also

provides a polypeptide including the amino acid sequence of SEQ ID NO: 21 or variant polypeptide thereof, wherein the polypeptide inhibits a biological activity of the LHX4 polypeptide. In some embodiments, the polypeptide is modified with a cell-membrane permeable substance.

In another aspect, the present invention provides methods for treating and/or preventing cancer, wherein the method including the step of administering the aforementioned polypeptide to a subject.

In another aspect, the present invention provides compositions for treating and/or preventing cancer, wherein the composition including the aforementioned polypeptide and a pharmaceutically acceptable carrier.

[0012] More specifically, the present invention provides the following [1] to [37]:

[1] A method for diagnosing lung cancer, said method comprising the steps of:

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

(i) detecting an mRNA of a LHX4 gene;

(ii) detecting a LHX4 protein;

(iii) detecting a biological activity of the LHX4 protein; and

(b) correlating an increase in the expression level determined in step (a) as compared to a normal control level of the gene to the presence of lung cancer;

[2]The method of [1], wherein the expression level determined in step (a) is at least 10% greater than the normal control level,;

[3]The method of [1], wherein the expression level determined in step (a) is determined by detecting the hybridization of the oligonucleotide that hybridizes to the mRNA of the LHX4 gene to the mRNA of the LHX4 gene or the binding of an antibody against the LHX4 protein to the LHX4 protein;

[4]The method of [1], wherein the subject-derived biological sample comprises a biopsy specimen, sputum, blood, pleural effusion or urine;

[5]A kit for diagnosing lung cancer, which comprises a reagent selected from the group consisting of:

(a) a reagent for detecting mRNA of a LHX4 gene;

(b) a reagent for detecting the LHX4 protein; and

(c) a reagent for detecting the biological activity of the LHX4 protein;

[6] The kit of [5], wherein the reagent comprises an oligonucleotide that hybridizes to the mRNA of the LHX4 gene;

[7] The kit of [5], wherein the reagent comprises an antibody against the LHX4 protein;

[8]An isolated double-stranded molecule that, when introduced into a cell, inhibits expression of LHX4 gene, Ku70 gene or Ku86 gene as well as cell proliferation, said

molecule comprising a sense strand and an antisense strand complementary thereto, said strands hybridized to each other to form the double-stranded molecule.

[9]The double-stranded molecule of [8], wherein the sense strand comprises the sequence corresponding to a target sequence selected from the group consisting of SEQ ID NOs: 11, 12, 13 and 14;

[10]The double-stranded molecule of [8] or [9], wherein the sense strand hybridizes with antisense strand at the target sequence to form the double-stranded molecule having between 19 and 25 nucleotide pairs in length.

[11]The double-stranded molecule of any one of [8] to [10], which consists of a single polynucleotide comprising both the sense and antisense strands linked by an intervening single-strand;

[12]The double-stranded molecule of [11], which has the general formula 5'-[A]-[B]-[A']-3' or 5'-[A']-[B]-[A]-3', wherein [A] is the sense strand comprising a sequence corresponding to a target sequence selected from the group consisting of SEQ ID NOs: 11, 12, 13, and 14, [B] is the intervening single-strand consisting of 3 to 23 nucleotides, and [A'] is the antisense strand comprising a complementary sequence to the target sequence;

[13]A vector encoding the double-stranded molecule of any one of [8] to [12];

[14]Vectors comprising each of a combination of polynucleotide comprising a sense strand nucleic acid and an antisense strand nucleic acid, wherein said sense strand nucleic acid comprises nucleotide sequence of SEQ ID NOs: 11, 12, 13 or 14, and said antisense strand nucleic acid consists of a sequence complementary to the sense strand, wherein the transcripts of said sense strand and said antisense strand hybridize to each other to form a double-stranded molecule, and wherein said vectors, when introduced into a cell expressing the LHX4 gene, Ku70 gene or Ku86 gene, inhibit expression of said gene;

[15]A method for treating and/or preventing cancer expressing a LHX4 gene, wherein the method comprises the step of administering at least one isolated double-stranded molecule against the LHX4 gene, Ku70 gene or Ku86 gene, or vector encoding the double-stranded molecule;

[16]The method of [15], wherein the double-stranded molecule is that of any one of [8] to [12].

[17]The method of [15], wherein the vector is that of [13] or [14];

[18]The method of any one of [15] to [18], wherein the cancer to be treated is lung cancer;

[19]A composition for treating and/or preventing cancer expressing a LHX4 gene, wherein the composition comprises at least one isolated double-stranded molecule against the LHX4 gene, Ku70 gene or Ku86 gene, or vector encoding the double-

stranded molecule;

[20]The composition of [19], wherein the double-stranded molecule is that of any one of [8] to [12];

[21]The composition of [19], wherein the vector is that of [13] or [14];

[22]The composition of any one of [19] to [21], wherein the cancer to be treated is lung cancer;

[23]A method of screening for a candidate substance for treating and/or preventing lung cancer, or inhibiting lung cancer cell growth, said method comprising the steps of:

(a) contacting a test substance with a LHX4 polypeptide or functional equivalent thereof;

(b) detecting the binding activity between the polypeptide or the functional equivalent and the test substance; and

(c) selecting a substance that binds to the polypeptide or the functional equivalent;

[24]A method of screening for a candidate substance for treating and/or preventing lung cancer, or inhibiting lung cancer cell growth, said method comprising the steps of:

(a) contacting a test substance with a LHX4 polypeptide or functional equivalent thereof ;

(b) detecting the biological activity of the polypeptide or the functional equivalent of step (a); and

(c) selecting the test substance that suppresses the biological activity of the polypeptide or the functional equivalent as compared to the biological activity detected in the absence of the test substance;

[25]The method of [24], wherein the biological activity is selected from the group consisting of the facilitation of the cell proliferation or binding activity to a Ku protein;

[26]A method of screening for a candidate substance for treating and/or preventing lung cancer or inhibiting lung cancer cell growth, said method comprising the steps of:

(a) contacting a test substance with a cell expressing a LHX4 gene;

(b) detecting an expression level of LHX4 gene in the cell of step (a);

(c) selecting the test substance that reduces the expression level of a LHX4 gene in comparison with the expression level detected in the absence of the test substance;

[27]A method of screening for a candidate substance for treating and/or preventing lung cancer or inhibiting lung cancer cell growth, said method comprising the steps of:

(a) contacting a test substance with a cell into which a vector, comprising the transcriptional regulatory region of a LHX4 gene and a reporter gene that is expressed under the control of the transcriptional regulatory region, has been introduced;

(b) measuring the expression or activity level of said reporter gene; and

(c) selecting a 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;

[28]A method of screening for a candidate substance for inhibiting a binding between a LHX4 polypeptide and a Ku protein, or treating and/or preventing lung cancer, said method comprising steps of:

- (a) contacting the LHX4 polypeptide or functional equivalent thereof with Ku protein or functional equivalent thereof in presence of a test substance;
- (b) detecting a binding level between the polypeptides;
- (c) comparing the binding level detected in the step (b) with the binding level detected in absence of the test substance; and
- (d) selecting the test substance that reduces the binding level;

[29]A method of screening for a candidate substance for inhibiting the phosphorylation of a LHX4 polypeptide, or treating and/or preventing lung cancer, comprising the steps of:

- (a) contacting the LHX4 polypeptide or functional equivalent thereof with a test substance under a condition that allows phosphorylation of the polypeptide;
- (b) detecting the phosphorylation level of the polypeptide described in (a);
- (c) comparing the phosphorylation level of the polypeptide with the phosphorylation level detected in the absence of the test substance; and
- (d) selecting the test substance that reduced the phosphorylation level of the polypeptide as the candidate substance;

[30]A polypeptide comprising the amino acid sequence of (a) or (b) below:

- (a) the amino acid sequence of SEQ ID NO: 21;
- (b) the amino acid sequence in which one, two or several amino acids are substituted, deleted, inserted and/or added in the amino acid sequence of SEQ ID NO: 21; wherein the polypeptide inhibits a biological activity of the LHX4 polypeptide;

[31]The polypeptide of claim 30, wherein the biological activity of the LHX4 polypeptide is a binding activity to the Ku protein;

[32]The polypeptide of claim 30 or 31, which is modified with a cell-membrane permeable substance;

[33]The polypeptide of claim 32, which has the following general formula:

[R]-[D];

wherein [R] represents the cell-membrane permeable substance; and [D] represents a polypeptide comprising the amino acid sequence of (a) or (b) below:

- (a) the amino acid sequence of SEQ ID NO: 21;
- (b) the amino acid sequence in which one, two or several amino acids are substituted, deleted, inserted and/or added in the amino acid sequence of SEQ ID NO: 21, wherein [R] and [D] are linked directly or indirectly through a linker;

[34]A composition for treating and/or preventing cancer expressing a LHX4 gene,

wherein the composition comprises the polypeptide of any one of claims 30 to 33 and a pharmaceutically acceptable carrier;

[35]The composition of claim 34, wherein the cancer to be treated is lung cancer;

[36]A method for treating and/or preventing cancer expressing a LHX4 gene, wherein the method comprises the step of administering the polypeptide of any one of claims 30 to 33 to a subject; and

[37]The method of [36], wherein the cancer to be treated is lung cancer.

- [0013] 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.

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 description 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

- [0014] [fig.1-1]Fig. 1 demonstrates LHX4 expression in lung cancers and normal tissues. A, Expression of LHX4 in clinical SCLC tissues, and normal lung tissues, analyzed by semiquantitative RT-PCR. Appropriate dilutions of each single-stranded cDNA were prepared from mRNAs of SCLC cancer samples, using the level of beta-actin (ACTB) expression as a quantitative control. B, Expression of LHX4 in SCLC cell lines, examined by semiquantitative RT-PCR. C, Expression of LHX4 in normal human tissues, detected by Northern blot analysis. D, Subcellular localization of exogenous LHX4 protein in COS-7 cells detected by anti-myc, which were co-stained with DAPI.

[fig.1-2]E, Expression of LHX4 in clinical NSCLC tissues, examined by semiquantitative RT-PCR, and F, Expression of LHX4 in NSCLC cell lines, examined by semi-quantitative RT-PCR. Appropriate dilutions of each single-stranded cDNA prepared from mRNAs of lung cancer samples were prepared, using the level of beta-actin (ACTB) expression as a quantitative control. G, Expression of LHX4 protein in lung cancer cell lines examined by Western blotting. H, Expression and subcellular localization of endogenous LHX4 protein in lung cancer DMS114 cells and normal human epithelial BEAS-2B cells.

[fig.2-1]Fig. 2 demonstrates inhibition of growth of lung cancer cells that over-expressed LHX4 by siRNA against LHX4. A, Expression of LHX4 in response to si-LHX4 (si-#A or si-#B) or control siRNAs (LUC or SCR) in SBC-3 and SBC-5 cells, analyzed by semiquantitative RT-PCR. B, Colony formation assays of SBC-3 and SBC-5 cells transfected with specific siRNAs for LHX4 or control plasmids.

[fig.2-2]C, Viability of SBC-3 and SBC-5 cells evaluated by MTT assay in response to si-LHX4 (si-#A or si-#B), si-LUC, or si-SCR. All assays were done thrice, and in triplicate wells.

[fig.3]Fig. 3 demonstrates enhancement of cell growth by LHX4 introduction into COS-7 cells, SBC-3 and HEK293 cells. A, Transient expression of LHX4 in COS-7 cells and SBC-3 cells, detected by Western blot analysis. B, Assays demonstrating the growth promoting effect of transient LHX4 expression on mammalian COS-7 cells and lung cancer SBC-3 cells. Assays were done thrice and in triplicate wells. C, Transient expression of LHX4 in COS-7 cells and HEK293 cells, detected by Western blot analysis. D, Assays demonstrating the growth promoting effect of transient LHX4 expression on mammalian COS-7 cells HEK293 cells. Assays were done thrice and in triplicate wells.

[fig.4]Fig. 4 demonstrates interaction of LHX4 and Ku70/Ku86 proteins. A, Interaction of exogenous LHX4 and endogenous Ku70/Ku86 proteins. SBC-5 cells were transfected with Flag-tagged mock and Flag-tagged LHX4 expression vector. Cell lysates were immunoprecipitated with anti-Flag M2 agarose; and immunoblotted with anti-Ku86 antibody (left panels) or anti-Ku70 antibody (right panels), respectively. B, Endogenous expression of Ku70 and Ku86 proteins in small cell lung cancer cells. Immunocytochemistry was performed in SBC-5 small cell lung cancer cells using an anti-Flag antibody (LHX4) (first from left panels), anti-Ku86 antibody (second from left upper panel), anti-Ku70 antibody (second from left lower panel) and DAPI (first from right panels). Exogenous LHX4 and endogenous Ku70/Ku86 proteins were colocalized in nucleus.

[fig.5]Fig. 5 demonstrates cell cycle-dependent expression of LHX4 protein and its interaction Ku proteins. A, Cell cycle dependent expression of LHX4 and Ku86 proteins

in SBC-5 cells synchronized by aphidicoline, detected by Western blot analysis. B, Cell cycle dependent expression of exogenous LHX4 and Ku86 mRNA in SBC-5 cells synchronized by aphidicoline, detected by semiquantitative RT-PCR. C, Flow cytometric analysis of cell cycle at every 3 hours from aphidicoline release.

[fig.6]Fig. 6 demonstrates effect of Ku expression on the levels of LHX4 gene and protein. A, The levels of Ku and LHX4 proteins (left panels) and transcripts (right panels), detected by Western blot analysis and semiquantitative RT-PCR analysis in SBC-5 cells transfected with si-Ku70 or si-Ku86. B, The levels of Ku and LHX4 proteins (left panels) and transcripts (right panels), detected by Western blot analysis and semiquantitative RT-PCR analysis in COS-7 cells transfected with LHX4 and Ku proteins expression vector.

[fig.7]Fig. 7 demonstrates LHX4 expression in tissues. A, Immunohistochemical evaluation of LHX4 protein using anti-LHX4 polyclonal antibody in lung ADC, lung SCC, lung LCC lung SCLC and five normal tissues. B, Immunohistochemical staining of LHX4 protein using anti-LHX4 polyclonal antibody in four representative paired lung tumors and adjacent normal lung tissues.

[fig.8-1]Fig. 8 demonstrates identification of Ku-interacting region in LHX4 and inhibition of growth of lung cancer cells by dominant-negative peptides of LHX4. A, Schematic drawing of three N-terminal Flag-tagged LHX4 partial protein constructs and three cell permeable peptides of LHX4 covering L2 157-219 that corresponds to the Ku-interacting region in LHX4. B, Identification of the region in LHX4 that binds to Ku proteins by immunoprecipitation experiments using SBC-3 cells.

[fig.8-2]C, MTT assay showing growth suppressive effect of 11R-P3 199-219 peptides that were introduced into DMS114 cells that expressed both LHX4 and Ku proteins. Bars, SD of triplicate assays. D, MTT assay showing no off-target effect of the 11R-P3 199-219 peptides on BEAS-2B cells that scarcely expressed LHX4 protein.

[fig.9]Fig. 9 demonstrates protein phosphatase assay indicating LHX4 phosphorylation.

[fig.10]Fig. 10 demonstrates confirmation of the specificity of the LHX4 signals in western blotting (A) and immunocytochemistry (B). The signals of LHX4 of DMS114 cells were decreased by siRNA against LHX4.

Description of Embodiments

- [0015] 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.

The disclosure of each publication, GenBank Accession or other sequence, 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.

[0016] **Definition**

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

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

[0017] As used herein, the term "biological sample" refers to a whole organism or a subset of its tissues (e.g., lung tissue), cells or component parts (e.g., body fluids, including but not limited to blood, serum, plasma, mucus, lymphatic fluid, synovial fluid, cerebrospinal fluid, saliva, sputum, 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, such as a nutrient broth or gel in which an organism has been propagated, which contains cellular components, such as proteins or polynucleotides.

[0018] The term "polynucleotide", "oligonucleotide", "nucleic acid", and "nucleic acid molecule" are used interchangeably herein to refer to a polymer of nucleic acid residues and, unless otherwise specifically indicated are referred to by their commonly accepted single-letter codes. The terms apply to nucleic acid (nucleotide) polymers in which one or more nucleic acids are linked by ester bonding. The nucleic acid polymers may be composed of DNA, RNA or a combination thereof and encompass both naturally-occurring and non-naturally occurring nucleic acid polymers.

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, such as an artificial chemical mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers.

The terms "cell-proliferating activity" refers to an activity of a polypeptide, which promote or enhance cell proliferation, when the polypeptide is contacted with the cell or the gene encoding the polypeptide is introduced into the cell.

[0019] 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 polypeptide refers to a polypeptide that are substantially free of cellular material for example, carbohydrate, lipid, or other contaminating proteins from the cell or tissue source from which the polypeptide 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.

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.

- [0020] In the context of the present invention, the phrase "LHX4 gene" encompasses polynucleotides that encode the human LHX4 or any of the functional equivalents of the human LHX4 gene. The LHX4 gene 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.
- [0021] 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.
- [0022] 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. A treatment may also be deemed "efficacious" if it leads to clinical benefit such as, reduction in expression of the LHX4 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 di-

agnosing or treating the particular tumor type.

- [0023] Unless otherwise defined, the term "cancer" refers to cancer over-expressing the LHX4 gene. Examples of cancers over-expressing LHX4 gene include, but are not limited to, lung cancers including SCLC and NSCLC. NSCLC includes, adenocarcinoma (ADC), large cell carcinoma (LCC) and squamous-cell carcinoma (SCC).
- [0024] The term "Ku protein" herein refers to a heterodimer protein composed of two subunits having the molecular weight of around 70 kDa and 86 kDa, respectively. Herein, the 70 kDa- subunit is referred to as "Ku70", "Ku70 protein" or "Ku70 polypeptide". Also, herein, the 86 kDa- subunit is referred to as "Ku86", "Ku86 protein" or "Ku86 polypeptide". Those two Ku subunits form a basket-shaped structure that threads onto the DNA end. Ku protein is the DNA-binding component of the DNA-dependent protein kinase, and it functions together with the DNA ligase IV-XRCC4 complex in the repair of DNA double-strand break by non-homologous end joining and the completion of V(D)J recombination events.
- [0025] The terms "antibody" as used herein is intended to include immunoglobulins and fragments thereof which are specifically reactive to the designated protein or peptide thereof. An antibody can include human antibodies, primatized antibodies, chimeric antibodies, bispecific antibodies, humanized antibodies, antibodies fused to other proteins or radiolabels, and antibody fragments. Furthermore, an antibody herein is used in the broadest sense and specifically covers intact monoclonal antibodies, polyclonal antibodies, multispecific antibodies (e.g. bispecific antibodies) formed from at least two intact antibodies, and antibody fragments so long as they exhibit the desired biological activity. An "antibody" indicates all classes (e.g. IgA, IgD, IgE, IgG and IgM).
- [0026] Genes and Polypeptides
Nucleotide sequences of LHX4, Ku70 and Ku86 polynucleotides and amino acid sequences of LHX4, Ku70 and Ku86 polypeptides are known to those skilled in the art, and can be obtained, for example, from gene databases on the web site such as GenBankTM. Exemplified nucleotide sequence of LHX4 polynucleotide is shown in SEQ ID NO: 1, and exemplified amino acid sequence of LHX4 polypeptide is shown in SEQ ID NO: 2. The sequence data are also available, for example, via GenBank accession NM_033343. Also, exemplified nucleotide sequences of Ku70 polynucleotide and Ku86 polynucleotide are shown in SEQ ID NO: 15 and 17, respectively. Exemplified amino acid sequences of Ku70 polypeptide and Ku86 polypeptide are shown in SEQ ID NO: 16 and 18, respectively. The sequence data are also available, for example, via GenBank accession No. NM_001469 and NM_021141, respectively. One of skill will recognize that LHX4 sequences, Ku76 sequences or Ku86 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. According to an aspect of the present invention, functional equivalents are also considered to be above "polypeptides". Herein, a "functional equivalent" of a protein is a polypeptide that has a biological activity equivalent to the protein. Namely, any polypeptide that retains the biological ability may be used as such a functional equivalent in the present invention. Such functional equivalents include those wherein one or more amino acids are substituted, deleted, added, or inserted to the natural occurring amino acid sequence of the protein. Alternatively, the polypeptide may be composed an amino acid sequence having at least about 80% homology (also referred to as sequence identity) to the sequence of the respective protein, more usually at least about 90%, 93%, 95%, 97%, 99% sequence identity to a reference sequence, e.g., a LHX4 polypeptide, e.g., SEQ ID NO: 2, as determined using a known sequence comparison algorithm, e.g., BLAST or ALIGN, set to default settings. In other embodiments, the polypeptide can be encoded by a polynucleotide that hybridizes under stringent conditions to the natural occurring nucleotide sequence of the gene. In some embodiments, the polypeptide is encoded by a polynucleotide that shares at least about 90%, 93%, 95%, 97%, 99% sequence identity to a reference sequence, e.g., a LHX4 polynucleotide, e.g., SEQ ID NO: 1, as determined using a known sequence comparison algorithm.

- [0027] A polypeptide of the present invention may 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 a functional equivalent to that of the human protein of the present invention, it is within the scope of the present invention.

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 be different in 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 (Tm) for the specific sequence at a defined ionic strength and pH. The Tm 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 Tm, 50% of the probes are occupied at equilibrium). Stringent conditions may also be achieved with the addition of destra-

bilizing agents such as formamide. For selective or specific hybridization, a positive signal is at least two times of background, preferably 10 times of background hybridization. Exemplary stringent hybridization conditions include the following: 50% formamide, 5x SSC, and 1% SDS, incubating at 42 degrees C, or, 5x SSC, 1% SDS, incubating at 65 degrees C, with wash in 0.2x SSC, and 0.1% SDS at 50 degrees C.

- [0028] In the context of the present invention, a 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 may be performed by conducting pre-hybridization 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. An exemplary low stringent condition may include 42 degrees C, 2x SSC, 0.1% SDS, typically 50 degrees C, 2x SSC, 0.1% SDS. High stringency conditions are often used. An exemplary high stringency condition may include 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, such as temperature and salt concentration, can influence the stringency of hybridization and one skilled in the art can suitably select the factors to achieve the requisite stringency.

Generally, it is known that modifications of one or more amino acid in a protein do not influence the function of the protein. In fact, mutated or modified proteins, proteins having amino acid sequences modified by substituting, deleting, inserting, and/or adding one or more amino acid residues of a certain amino acid sequence, have been known to retain the original biological activity (Mark et al., Proc Natl Acad Sci USA 81: 5662-6 (1984); Zoller and Smith, Nucleic Acids Res 10:6487-500 (1982); Dalbadie-McFarland et al., Proc Natl Acad Sci USA 79: 6409-13 (1982)). Accordingly, one of skill in the art will recognize that individual additions, deletions, insertions, or substitutions to an amino acid sequence which alter a single amino acid or a small percentage of amino acids or those considered to be a "conservative modifications", wherein the alteration of a protein results in a protein with similar functions, are acceptable in the context of the instant invention.

- [0029] So long as the activity of the protein is maintained, the number of amino acid mutations is not particularly limited. As demonstrated in Examples, the inhibition of endogenous expression of LHX4 by siRNA resulted in marked reduction of viability of lung cancer cells, and exogenous expression of LHX4 enhanced the cell growth in mammalian cells. Furthermore, it was revealed that LHX4 was phosphorylated and interacted with Ku protein. However, in most embodiments 5% or less of the amino acid

sequence is altered. Accordingly, in one embodiment, the number of amino acids to be mutated in such a mutant is generally 30 amino acids or less, usually 20 amino acids or less, more usually 10 amino acids or less, 6 amino acids or less, and even more commonly 3 amino acids or less.

An amino acid residue to be mutated is typically 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 (A, I, L, M, F, P, W, Y, V), hydrophilic amino acids (R, D, N, C, E, Q, G, H, K, S, T), and side chains having the following functional groups or characteristics in common: an aliphatic side-chain (G, A, V, L, I, P); a hydroxyl group containing side-chain (S, T, Y); a sulfur atom containing side-chain (C, M); a carboxylic acid and amide containing side-chain (D, N, E, Q); a base containing side-chain (R, K, H); and an aromatic containing side-chain (H, F, Y, W). 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) Aspargine (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., Creighton, Proteins 1984).

Such conservatively modified polypeptides are included in the present protein.

However, the present invention is not restricted thereto and the protein includes non-conservative modifications, so long as at least one biological activity of the protein is retained. Furthermore, the modified proteins do not exclude polymorphic variants, interspecies homologues, and those encoded by alleles of these proteins.

[0030] An example of a protein modified by addition of one or more amino acids residues is a fusion protein of the LHX4 protein. Fusion proteins can be made by techniques well known to a person skilled in the art, for example, by linking the DNA encoding the LHX4 gene with a DNA encoding another peptide or protein, 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 the peptides or proteins fused to the LHX4 protein so long as the resulting fusion protein retains any one of the objective biological activities of the LHX4 proteins. Exemplary fusion proteins con-

templated by the instant invention include fusions of the LHX4 protein and other small peptides or proteins such as FLAG (Hopp TP, et al., Biotechnology 6: 1204-10 (1988)), a polyhistidine (His-tag) such as 6xHis containing six His (histidine) residues or 10xHis containing 10 His residues, Influenza aggregate or agglutinin (HA), human c-myc fragment, Vesicular stomatitis virus glycoprotein (VSV-GP), p18HIV fragment, T7 gene 10 protein (T7-tag), human simple herpes virus glycoprotein (HSV-tag), E-tag (an epitope on monoclonal phage), SV40T antigen fragment, lck tag, alpha-tubulin fragment, B-tag, Protein C fragment, and the like. Other 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.

Other examples of modified proteins contemplated by the present invention include polymorphic variants, interspecies homologues, and those encoded by alleles of these proteins.

- [0031] Moreover, the gene of the present invention encompasses polynucleotides that encode such functional equivalents of the protein. In addition to hybridization, a gene amplification method, for example, the polymerase chain reaction (PCR) method, can be utilized to isolate a polynucleotide encoding a polypeptide functionally equivalent to the protein, using a primer synthesized based on the sequence above information. Polynucleotides and polypeptides that are functionally equivalent to the human gene and protein, respectively, normally have a high homology to the originating nucleotide or amino acid sequence of. "High homology" typically refers to a homology of 40% or higher, usually 60% or higher, more usually 80% or higher, even more usually 90% to 95% or higher. The homology of a particular polynucleotide or polypeptide can be determined by following the algorithm in "Wilbur and Lipman, Proc Natl Acad Sci USA 80: 726-30 (1983)".

The present invention also encompasses partial peptides of the LHX4 protein. A partial peptide having an amino acid sequence specific to the LHX4 protein 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 about 7 amino acids, for example, about 8 amino acids or more, for example, about 9 amino acids or more.

A partial LHX4 peptide of the present invention typically contains, at a minimum, at least one binding domain of LHX4, more preferably Ku protein binding domain. Such partial peptides are also encompassed by the phrase "functional equivalent" of the LHX4 protein.

- [0032] 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 in-

terfering 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)). In some embodiments, the double-stranded molecules are isolated or recombinant.

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 a double-stranded molecule targeting the sequence is introduced into a cell expressing the target gene. A nucleotide sequence within mRNA or cDNA sequence of a gene can be determined to be a target sequence when a double-stranded molecule comprising a sequence corresponding to the target sequence inhibits expression of the gene in a cell expressing the gene. The double stranded polynucleotide which suppresses the gene expression may consist of the target sequence and 3'overhang having 2 to 5 nucleotides in length (e.g., uu).

- [0033] 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 complementary sequence to the target sequence, and the antisense strand hybridizes with the sense strand at the complementary sequence to form a double-stranded molecule.
- [0034] 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 shown in the DNA sequence of SEQ ID NO: 11 and the sense strand of the double-stranded molecule is composed of RNA, "a sequence corresponding to a target sequence" is "5'-GCAGUGUAGGCUAUCCCGA-3'".
- [0035] 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 shown in the DNA sequence of SEQ ID NO: 11 and the antisense strand of the double-stranded molecule is composed of RNA, "a complementary sequence to a target sequence" is "3'- CGUCACAUCCGAUAGGGCU-5'".

- [0036] 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 complementary sequence thereto.
- [0037] 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 LHX4, Ku70 or Ku86 sense nucleic acid sequence (also referred to as "sense strand"), an LHX4, Ku70 or Ku86 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.
- [0038] As used herein, the term "dsRNA" refers to a construct of two RNA molecules composed of complementary sequences 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.
- [0039] 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 is 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".
- [0040] As used herein, the term "siD/R-NA" refers to a double-stranded 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 LHX4, Ku70 or Ku86 sense nucleic acid sequence (also referred to as "sense strand"), a LHX4, Ku70 or Ku86 antisense nucleic acid sequence (also referred to as "antisense strand") or both. The siD/R-NA may be con-

structed 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.

- [0041] As used herein, the term "dsD/R-NA" refers to a construct of two molecules composed of complementary sequences to one another and that have annealed together via the complementary sequences to form a double-stranded 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).
- [0042] 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 is 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 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".
- [0043] 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 includes DNA, RNA, and derivatives thereof.
- [0044] A double-stranded molecule against LHX4, Ku70 or Ku86, which molecule hybridizes to target mRNA, decreases or inhibits production of LHX4, Ku70 or Ku86 protein encoded by LHX4, Ku70 or Ku86 gene by associating with the normally single-stranded mRNA transcript of the gene, thereby interfering with translation and thus, inhibiting expression of the protein. As demonstrated herein, the expression of LHX4 in lung cancer cell lines was inhibited by dsRNA that specifically annealed to the LHX4 encoding gene (Fig. 2A). Therefore, the present invention provides isolated double-stranded molecules that inhibit the expression of LHX4 gene when introduced into a cell expressing the LHX4 gene. Furthermore, siRNAs against Ku70 gene and Ku86 gene induced the decrease of LHX4 polypeptide level in a cancer cell line. Such result indicates that double-stranded molecules against Ku70 gene or Ku86 gene also have ability of suppressing cancer cell growth. The target sequence of those double-stranded molecule may be designed by an siRNA design algorithm such as that

mentioned below.

Examples of LHX4 target sequences include, for example, the nucleotide sequences of SEQ ID NO: 11 (positions 1323-1341nt of SEQ ID NO: 1) and SEQ ID NO: 12 (positions 1350-1368nt of SEQ ID NO: 1).

Example of Ku70 target sequence includes, for example, the nucleotide sequence of SEQ ID NO: 13 (positions 1605-1623nt of SEQ ID NO: 15).

Example of Ku86 target sequence includes, for example, the nucleotide sequence of SEQ ID NO: 14 (positions 1379-1397nt of SEQ ID NO: 17)

[0045] Specifically, the present invention provides the following double-stranded molecules [1] to [18]:

- [1] An isolated double-stranded molecule that, when introduced into a cell, specifically inhibits expression of LHX4, Ku70 or Ku86, such molecule composed of a sense strand and an antisense strand complementary thereto, hybridized to each other to form the double-stranded molecule;
- [2] The double-stranded molecule of [1], wherein said double-stranded molecule acts on an mRNA of LHX4, Ku70 or Ku86, matching a target sequence selected from SEQ ID NOS: 11, 12, 13 and 14;
- [3] The double-stranded molecule of [1] or [2], wherein the sense strand contains a sequence corresponding to a target sequence selected from among SEQ ID NOS: 11, 12, 13 and 14;
- [4] The double-stranded molecule of any one of [1] to [3], wherein the sense strand hybridize with antisense strand at the target sequence to form the double-stranded molecule having a less than about 100 nucleotide pairs in length;
- [5] The double-stranded molecule of [4], 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;
- [6] The double-stranded molecule of [5], 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;
- [7] The double-stranded molecule of [6], 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;
- [8] The double-stranded molecule of [7], wherein the sense strand hybridize with antisense strand at the target sequence to form the double-stranded molecule having a between about 19 and about 25 nucleotide pairs in length;
- [9] The double-stranded molecule of any one of [1] to [8], composed of a single polynucleotide having both the sense and antisense strands linked by an intervening single-strand;

- [10] The double-stranded molecule of [9], having the general formula 5'-[A]-[B]-[A']-3' or 5'-[A']-[B]-[A]-3', wherein [A] is the sense strand containing a sequence corresponding to a target sequence selected from SEQ ID NOS: 11, 12, 13 and 14 , [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;
- [11] The double-stranded molecule of any one of [1] to [10], composed of RNA;
- [12] The double-stranded molecule of any one of [1] to [10], composed of both DNA and RNA;
- [13] The double-stranded molecule of [12], wherein the molecule is a hybrid of a DNA polynucleotide and an RNA polynucleotide;
- [14] The double-stranded molecule of [13] wherein the sense and the antisense strands are composed of DNA and RNA, respectively;
- [15] The double-stranded molecule of [12], wherein the molecule is a chimera of DNA and RNA;
- [16] The double-stranded molecule of [15], 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 RNA;
- [17] The double-stranded molecule of [16], wherein the flanking region is composed of 9 to 13 nucleotides; and
- [18] The double-stranded molecule of any one of [1] to [17], wherein the molecule contains one or more 3' overhangs;
- [0046] The double-stranded molecule of the present invention will be described in more detail below.

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). For example, a computer program for designing siRNAs is available from the Ambion website (on the worldwide web at ambion.com/techlib/misc/siRNA_finder.html).

The 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 di-nucleotide 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: ncbi.nlm.nih.gov/BLAST/, is used (Altschul SF et al., Nucleic Acids Res 1997, 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.

Using the above protocol, the target sequence of the isolated double-stranded molecules of the present invention were designed as the following nucleotide sequences:

SEQ ID NOs: 11 and 12 for LHX4 gene,

SEQ ID NO: 13 for Ku70 gene, and

SEQ ID NO: 14 for Ku86 gene.

[0047] 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. Therefore, the present invention provides double-stranded molecules targeting any of the sequences selected from:

SEQ ID NOs: 11 and 12 for LHX4 gene;

SEQ ID NO: 13 for Ku70 gene, and

SEQ ID NO: 14 for Ku86 gene.

Examples of double-stranded molecules that target the above-mentioned target sequence of the LHX4 gene, Ku70 gene or Ku86 gene include isolated polynucleotides that contain the nucleic acid sequences corresponding to target sequences and/or complementary sequences to the target sequences. Examples of polynucleotides targeting the LHX4 gene, Ku70 gene or Ku86 gene include those containing the sequence corresponding to SEQ ID NOs: 11, 12, 13 or 14 and/or complementary sequences to these sequences. In an embodiment, a double-stranded molecule is composed of two polynucleotides, one polynucleotide has a sequence corresponding to a target sequence, i.e., sense strand, and another polypeptide has a complementary sequence to the target sequence, i.e., antisense strand. The sense strand polynucleotide and the antisense strand polynucleotide hybridize to each other to form double-stranded molecule.

Examples of such double-stranded molecules include dsRNA and dsD/R-NA.

[0048] In an another embodiment, a double-stranded molecule is composed of a polynucleotide that has both a sequence corresponding to a target sequence, i.e., sense strand, and a complementary sequence to the target sequence, i.e., antisense strand. Generally, the sense strand and the antisense strand are linked by a intervening strand, and hybridize to each other to form a hairpin loop structure. Examples of such double-stranded molecule include shRNA and shD/R-NA.

In other words, a double-stranded molecule of the present invention comprises a

sense strand polynucleotide having a nucleotide sequence corresponding to the target sequence and antisense strand polynucleotide having a nucleotide sequence complementary to the target sequence, and both of polynucleotides hybridize to each other to form the double-stranded molecule. In the double-stranded molecule comprising the polynucleotides, a part of the polynucleotide of either or both of the strands may be RNA, and when the target sequence is defined with a DNA sequence, the nucleotide "t" within the target sequence and complementary sequence thereto is replaced with "u".

- [0049] In one embodiment of the present invention, such a double-stranded molecule of the present invention comprises a stem-loop structure, composed of the sense and antisense strands. The sense and antisense strands may be joined by a loop. Accordingly, the present invention also provides the double-stranded molecule comprising a single polynucleotide containing both the sense strand and the antisense strand linked or flanked by an intervening single-strand.

In the present invention, double-stranded molecules targeting the LHX4 gene may have a sequence selected from SEQ ID NOs: 11 and 12 as a target sequence. Accordingly, examples of the double-stranded molecule of the present invention include polynucleotides that hybridize to each other at a sequence corresponding to SEQ ID NOs: 11 or 12 and a complementary sequence thereto, and a polynucleotide that has a sequence corresponding to SEQ ID NOs: 11 or 12 and a complementary sequence thereto.

- [0050] Also, double-stranded molecules targeting the Ku70 gene may have a sequence of SEQ ID NO: 13 as a target sequence. Accordingly, examples of the double-stranded molecule of the present invention include polynucleotides that hybridize to each other at a sequence corresponding to SEQ ID NO: 13 and a complementary sequence thereto, and a polynucleotide that has a sequence corresponding to SEQ ID NO: 13 and a complementary sequence thereto.

Also, double-stranded molecules targeting the Ku86 gene may have a sequence of SEQ ID NO: 14 as a target sequence. Accordingly, examples of the double-stranded molecule of the present invention include polynucleotides that hybridize to each other at a sequence corresponding to SEQ ID NO: 14 and a complementary sequence thereto, and a polynucleotide that has a sequence corresponding to SEQ ID NO: 14 and a complementary sequence thereto.

The double-stranded molecule of the present invention may be directed to a single target gene sequence or may be directed to a plurality of target gene sequences.

A double-stranded molecule of the present invention targeting the above-mentioned targeting sequence of LHX4 gene, Ku70 gene or Ku86 gene include isolated polynucleotides that contain any of the nucleic acid sequences of target sequences and/or

complementary sequences to the target sequences. Examples of polynucleotides targeting LHX4 gene, Ku70 gene or Ku86 gene include those containing the sequence of SEQ ID NOS: 11, 12, 13 or 14 and/or complementary sequences to these nucleotide sequences; However, the present invention is not limited to these examples, and minor modifications in the aforementioned nucleic acid sequences are acceptable so long as the modified molecule retains the ability to suppress the expression of LHX4 gene, Ku70 gene or Ku86 gene. Herein, the phrase "minor modification" as used in connection with a nucleic acid sequence indicates one, two or several substitution, deletion, addition or insertion of nucleotides to the sequence.

In the context of the present invention, the term "several" as applies to nucleotide substitutions, deletions, additions and/or insertions may mean 3-7, preferably 3-5, more preferably 3-4, even more preferably 3 nucleotides.

- [0051] 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 LHX4 gene, Ku70 gene or Ku86 gene or antisense strands complementary thereto were tested in vitro for their ability to decrease production of LHX4 gene, Ku70 gene or Ku86 gene product in lung cancer cell lines (e.g., using A549 and SBC-5) according to standard methods. Furthermore, for example, reduction in LHX4 gene, Ku70 gene or Ku86 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 LHX4, Ku70 or Ku86 gene mRNA mentioned under Example 1 item "Semi-quantitative RT-PCR". Sequences which decrease the production of LHX4 gene, Ku70 gene or Ku86 gene product in vitro cell-based assays can then be tested for there inhibitory effects on lung cancer cell growth. Sequences which inhibit lung cancer cell growth in vitro cell-based assay can then be tested for their in vivo ability using animals with lung cancer, e.g. nude mouse xenograft models, to confirm decreased production of LHX4gene, Ku70 gene or Ku86 gene product and decreased lung cancer cell growth.
- [0052] 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 as same manner. Generally, complementary polynucleotide sequences hybridize under appropriate conditions to form stable duplexes containing few or no mismatches. Furthermore, the sense strand and antisense strand of the isolated polynu-

cleotide of the present invention can form double-stranded molecule or hairpin loop structure by the hybridization. In a typical 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.

- [0053] The polynucleotide is usually less than 1913 nucleotides in length for LHX4, 2156 nucleotides in length for Ku70, 3448 nucleotides in length for Ku86. For example, the polynucleotide is less than 500, 200, 100, 75, 50, or 25 nucleotides in length for all of the genes. The isolated polynucleotides of the present invention are useful for forming double-stranded molecules against LHX4 gene, Ku70 gene or Ku86 gene or preparing template DNAs encoding the double-stranded molecules. When the polynucleotides are used for forming double-stranded molecules, the sense strand of polynucleotide may be longer than 19 nucleotides, usually longer than 21 nucleotides, and more usually has a length of between about 19 and 25 nucleotides. Accordingly, the present invention provides the double-stranded molecules including a sense strand and an antisense strand, wherein the sense strand includes a nucleotide sequence corresponding to a target sequence. In some embodiments, the sense strand hybridizes with antisense strand at the target sequence to form the double-stranded molecule having between 19 and 25 nucleotide pairs in length.
- [0054] The double-stranded molecule serves as a guide for identifying homologous sequences in mRNA for the RNA-induced silencing complex (RISC), when the double-stranded molecule is introduced into cells. The identified target RNA is cleaved and degraded by the nuclease activity of Dicer, through which the double-stranded molecule eventually decreases or inhibits production (expression) of the polypeptide encoded by the RNA. Thus, a double-stranded molecule of the invention can be defined by its ability to generate a single-strand that specifically hybridizes to the mRNA of the LHX4 gene, Ku70 gene or Ku86 gene under stringent conditions. Herein, the portion of the mRNA that hybridizes with the single-strand generated from the double-stranded molecule is referred to as "target sequence" or "target nucleotide". In the present invention, nucleotide sequence of the "target sequence" can be shown using not only the RNA sequence of the mRNA, but also the DNA sequence of cDNA synthesized from the mRNA.
- [0055] The double-stranded molecules of the invention may contain one or more modified nucleotides and/or non-phosphodiester linkages. Chemical modifications well known in the art are capable of increasing stability, availability, and/or cell uptake of the double-stranded molecule. The skilled person will be aware of other types of chemical modification which may be incorporated into the present molecules (WO03/070744; WO2005/045037). 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).

[0056] 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 increased or decreased 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-deaza, 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, 15(2): 188-200). For further details, published documents such as US20060234970 are available. The present invention is not limited to these examples and any known 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.

[0057] Furthermore, the double-stranded molecules of the invention may include both DNA and RNA, e.g., dsD/R-NA or shD/R-NA. Specifically, a hybrid polynucleotide of a DNA strand and an RNA strand or a DNA-RNA chimera polynucleotide shows increased stability. 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.

The hybrid of a DNA strand and an RNA strand may be either where the sense strand is DNA and the antisense strand is RNA, or the opposite so long as it can inhibit expression of the target gene when introduced into a cell expressing the gene. Usually, the sense strand polynucleotide is DNA and the antisense strand polynucleotide is RNA. Also, the chimera type double-stranded molecule may be either where both of

the sense and antisense strands are composed of DNA and RNA, or where any one of the sense and antisense strands is composed of DNA and RNA so long as it has an activity to inhibit 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.

- [0058] As an example of the chimera type double-stranded molecule, an upstream partial region (i.e., a region flanking to the target sequence or complementary sequence thereof within the sense or antisense strands) of the double-stranded molecule is 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 are referred to upstream partial region. That is, in usual 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, the chimera or hybrid type double-stranded molecule of the present invention 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.

The upstream partial region preferably is a domain composed of 9 to 13 nucleotides counted from the terminus of the target sequence or complementary sequence thereto within the sense or antisense strands of the double-stranded molecules. Moreover, 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).

- [0059] In the present invention, the double-stranded molecule may form a hairpin, such as a short hairpin RNA (shRNA) and short hairpin consisting 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 includes 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, which is then bound to the RNA-induced silencing complex (RISC). This complex binds to and cleaves mRNAs which 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', or 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 complementary sequence to [A]. The target sequence may be selected from among, for example, nucleotide sequences of SEQ ID NOs: 11 and 12 for LHX4, SEQ ID NO: 13 for Ku70 and SEQ ID NO: 14 for Ku86.

- [0060] 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 LHX4 gene, Ku70 gene or Ku86 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 3 to 23 nucleotides in length. The loop sequence, for example, can be selected from among the following sequences (on the worldwide web at ambion.com/techlib/tb/tb_506.html). Furthermore, loop sequence consisting of 23 nucleotides also provides active siRNA (Jacque JM et al., Nature 2002, 418(6896): 435-8):

CCC, CCACC, or CCACACC: Jacque JM et al., Nature 2002, 418(6896): 435-8;
UUCG: Lee NS et al., Nat Biotechnol 2002, 20(5): 500-5; Fruscoloni P et al., Proc Natl Acad Sci USA 2003, 100(4): 1639-44; and

UUCAAGAGA: Dykxhoorn DM et al., Nat Rev Mol Cell Biol 2003, 4(6): 457-67.

Examples of double-stranded molecules of the present invention having hairpin loop structure are shown below. In the following structure, the loop sequence can be selected from among AUG, CCC, UUCG, CCACC, CTCGAG, AAGCUU, CCACACC, and UUCAAGAGA; however, the present invention is not limited

thereto:

GCAGUGUAGGCUAUCCGA-[B]-UCGGGAUAGCCUACACUGC

(for target sequence of SEQ ID NO: 11);

CUAGCCCAGGCUCUUGGCU-[B]-AGCCAAGAGCCUGGGCUAG

(for target sequence of SEQ ID NO: 12);

AAGCAAUGAAUAAAAGACU-[B]- AGUCUUUUAUCAUUGCUU

(for target sequence of SEQ ID NO: 13);

GCAUACUAUGAGUGUUUA-[B]- UAAACACUCAUAGUUAUGC

(for target sequence of SEQ ID NO: 14);

- [0061] Furthermore, in order to enhance the inhibition activity of the double-stranded molecules, several nucleotides can be added to 3'end of the sense strand and/or antisense strand of the target sequence, as 3' overhangs. The typical examples of nucleotides constituting a 3' overhang include "t" and "u", but are not limited to. The number of nucleotides to be added is at least 2, generally 2 to 10, preferably 2 to 5. The added nucleotides form single strand at the 3'end of the antisense strand of the double-stranded molecule. In cases where double-stranded molecules consists of a single polynucleotide to form a hairpin loop structure, a 3' overhang sequence may be added to the 3' end of the single polynucleotide.
- [0062] The method for preparing the double-stranded molecule is not particularly limited though it is preferable to use a chemical synthetic method known in the art. According to the 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. Specific example for the annealing includes wherein 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.

The regulatory sequences flanking LHX4, Ku70 or Ku86 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 LHX4, Ku70 or Ku86 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.

[0063] Alternatively, the double-stranded molecules may be transcribed intracellularly by cloning its coding sequence into a vector containing a regulatory sequence that directs the expression of the double-stranded molecule in an adequate cell (e.g., a RNA poly III transcription unit from the small nuclear RNA (snRNA) U6 or the human H1 RNA promoter) adjacent to the coding sequence. The regulatory sequences flanking the coding sequences of double-stranded molecule may be identical or different, such that their expression can be modulated independently, or in a temporal or spatial manner. Details of vectors which are capable of producing the double-stranded molecules will be described below.

[0064] **Vectors Encoding Double-Stranded Molecule:**

Also included in the present invention are vectors encoding one or more of the double-stranded molecules described herein, and a cell containing such a vector.

Specifically, the present invention provides the following vector of [1] to [11].

[1] A vector, encoding a double-stranded molecule that, when introduced into a cell, specifically inhibits expression of LHX4, Ku70 or Ku86, such molecule composed of a sense strand and an antisense strand complementary thereto, hybridized to each other to form the double-stranded molecule.

[2] The vector of [1], encoding the double-stranded molecule acts on mRNA, matching a target sequence selected from SEQ ID NOs: 11, 12, 13 and 14;

[3] The vector of [1] or [2], wherein the sense strand contains a sequence corresponding to a target sequence selected from among SEQ ID NOs: 11, 12, 13 and 14;

[4] The vector of any one of [1] to [3], encoding the double-stranded molecule, wherein the sense strand of the double-stranded molecule hybridizes with antisense strand at the target sequence to form the double-stranded molecule having a length of less than about 100 nucleotide pairs in length;

[5] The vector of [4], encoding the double-stranded molecule, wherein the sense strand of the double-stranded molecule hybridizes with antisense strand at the target sequence to form the double-stranded molecule having less than about 75 nucleotide pairs in length;

[6] The vector of [5], encoding the double-stranded molecule, wherein the sense strand of the double-stranded molecule hybridizes with antisense strand at the target sequence to form the double-stranded molecule having less than about 50 nucleotide pairs in length;

[7] The vector of [6] encoding the double-stranded molecule, wherein the sense strand of the double-stranded molecule hybridizes with antisense strand at the target sequence to form the double-stranded molecule having less than about 25 nucleotide pairs in length;

[8] The vector of [7], encoding the double-stranded molecule, wherein the sense

strand of the double-stranded molecule hybridizes with antisense strand at the target sequence to form the double-stranded molecule having between about 19 and about 25 nucleotide pairs in length;

[9] The vector of any one of [1] to [8], wherein the double-stranded molecule is composed of a single polynucleotide having both the sense and antisense strands linked by an intervening single-strand;

[10] The vector of [9], encoding the double-stranded molecule having the general formula 5'-[A]-[B]-[A']-3' or 5'-[A']-[B]-[A]-3', wherein [A] is the sense strand containing a sequence corresponding to a target sequence selected from among SEQ ID NOS: 11, 12, 13 and 14, [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; and

[11] The double-stranded molecule of any one of [1] to [10], wherein the double-stranded molecule contains one or two 3' overhangs.

[0065] A vector of the present invention encodes a double-stranded molecule of the present invention in an expressible form. Herein, the phrase "in an expressible form" indicates that the vector, when introduced into a cell, will express the molecule. In a preferred embodiment, the vector includes regulatory elements necessary for expression of the double-stranded molecule. Accordingly, in one embodiment, the expression vector encodes the nucleic acid sequence of the double-stranded molecule of the present invention and is adapted for expression of such double-stranded molecule. Such vectors of the present invention may be used for producing the double-stranded molecules of the present invention, or directly as an active ingredient for treating cancer.

Alternatively, the present invention provides vectors including each of a combination of polynucleotide including a sense strand nucleic acid and an antisense strand nucleic acid, wherein said sense strand nucleic acid includes nucleotide sequence of SEQ ID NOS: 11, 12, 13 and 14, and said antisense strand nucleic acid consists of a sequence complementary to the sense strand, wherein the transcripts of said sense strand and said antisense strand hybridize to each other to form a double-stranded molecule, and wherein said vectors, when introduced into a cell expressing the LHX4 gene, Ku70 gene or Ku86 gene, inhibits expression of said gene. Preferably, the polynucleotide is an oligonucleotide of between about 19 and 25 nucleotides in length (e.g., contiguous nucleotides from the nucleotide sequence of SEQ ID NO: 1, 15 or 17). More preferably, the combination of polynucleotide includes a single nucleotide transcript including the sense strand and the antisense strand linked via a single-stranded nucleotide sequence. More preferably, the combination of polynucleotide has the general formula 5'-[A]-[B]-[A']-3' or 5'-[A']-[B]-[A]-3', wherein [A] is a nucleotide sequence

including SEQ ID NOs: 11, 12, 13 or 14; [B] is a nucleotide sequence consisting of about 3 to about 23 nucleotide; and [A'] is a nucleotide sequence complementary to [A].

- [0066] Vectors of the present invention can be produced, for example, by cloning LHX4 sequence , Ku70 sequence or Ku86 sequence into an expression vector so that regulatory sequences are operatively-linked to LHX4 sequence, Ku70 sequence or Ku86 sequence in a manner to allow expression (by transcription of the DNA molecule) of both strands (Lee NS et al., Nat Biotechnol 2002, 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); namely, a single transcript of a vector contains both the sense and complementary antisense sequences of the target gene.

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).

- [0067] 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.

[0068] **Methods of Inhibiting or Reducing Cancer Cell Growth and Treating Cancer:**

In the present invention, two different dsRNA for LHX4 were tested for their ability to inhibit lung cancer cell growth. The two dsRNA for LHX4, effectively knocked down the expression of the gene in lung cancer cell lines coincided with suppression of cell proliferation (Figs. 2). Furthermore, the dsRNAs for Ku70 and Ku86 induced the decrease of LHX4 level in the cancer cell as well as suppression of the expressions of Ku70 and Ku86 (Figs. 6).

Therefore, the present invention provides methods for inhibiting lung cancer cell growth, by inhibiting the expression of LHX4, Ku70 or Ku86. LHX4 gene, Ku70 gene or Ku86 gene expression can be inhibited by any method known in the art, including use of the aforementioned double-stranded molecules which specifically target of LHX4 gene, Ku70 gene or Ku86 gene or the aforementioned vectors that express double-stranded molecules which specifically target the LHX4 gene, Ku70 gene or Ku86 gene.

Such ability of the present double-stranded molecules and vectors to inhibit cell growth of lung cancer cells indicates that they can be used for methods for treating and/or preventing lung cancer, as well as treating or preventing a post-operative, secondary, or metastatic recurrence thereof. Thus, the present invention provides methods to treat patients with lung cancer by administering a double-stranded molecule against LHX4 gene, Ku70 gene or Ku86 gene, or a vector expressing the double-stranded molecule to a subject. The treating methods of the present invention may be carried out without adverse side effects because the LHX4 gene is not over-expressed in normal tissues (Figs. 1, 7).

[0069] Specifically, the present invention provides the following methods [1] to [34]:

[1] A method for inhibiting cancer cell growth, or treating and/or preventing cancer in a subject, wherein the cancer cell or the cancer expresses LHX4 gene, such method including the step of administering to the subject a pharmaceutically effective amount of a double-stranded molecule against a LHX4 gene, Ku70 gene or Ku86 gene, or a vector encoding thereof, wherein the double-stranded molecule, when introduced into a cell expressing the LHX4 gene, inhibits an expression of the LHX4 gene, Ku70 gene or Ku86 as well as cell proliferation;

[2] The method of [1], wherein the double-stranded molecule acts at LHX4, Ku70 or Ku86 mRNA which matches a target sequence selected from SEQ ID NOS: 11, 12, 13 and 14;

[3] The method of [1] or [2], wherein the sense strand contains the sequence corre-

sponding to a target sequence selected from among SEQ ID NOs: 11, 12, 13 and 14;

[4] The method of any one of [1] to [3], wherein the cancer to be treated is lung cancer;

[5] The method of any one of [1] to [3], wherein a plurality of double-stranded molecules against a LHX4 gene, Ku70 gene or Ku86 gene or vectors encoding thereof are administered;

[6] The method of any one of [1] to [5], 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;

[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 75 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 50 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 less than about 25 nucleotide pairs in length;

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

[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]-3', wherein [A] is the sense strand containing a sequence corresponding to a target sequence selected from among SEQ ID NOs: 11, 12, 13 and 14, [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 [14], 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 one or two 3' overhangs;
- [21] The method of any one of [1] to [19], wherein the double-stranded molecule is contained in a composition which includes, in addition to the molecule, a transfection-enhancing agent and pharmaceutically acceptable carrier;
- [22] The method of [1], wherein the double-stranded molecule is encoded by a vector;
- [23] The method of [22], wherein the double-stranded molecule encoded by the vector acts at mRNA which matches a target sequence selected from SEQ ID NOs: 11, 12, 13 and 14;
- [24] The method of [22] or [23], wherein the sense strand of the double-stranded molecule encoded by the vector contains the sequence corresponding to a target sequence selected from among SEQ ID NOs: 11, 12, 13 and 14;
- [25] The method of any one of [22] to [24], wherein the cancer to be treated is lung cancer;
- [26] The method of any one of [22] to [25], wherein plural kinds of the double-stranded molecules are administered;
- [27] The method of any one of [22] to [26], wherein the sense strand of the double-stranded molecule encoded by the vector hybridize with antisense strand at the target sequence to form the double-stranded molecule having less than about 100 nucleotide pairs in length;
- [28] The method of [27], wherein the sense strand of the double-stranded molecule encoded by the vector hybridize with antisense strand at the target sequence to form the double-stranded molecule having less than about 75 nucleotide pairs in length;
- [29] The method of [28], wherein the sense strand of the double-stranded molecule encoded by the vector hybridize with antisense strand at the target sequence to form the double-stranded molecule having less than about 50 nucleotide pairs in length;
- [30] The method of [29], wherein the sense strand of the double-stranded molecule encoded by the vector hybridize with antisense strand at the target sequence to form the double-stranded molecule having less than about 25 nucleotide pairs in length;
- [31] The method of [30], wherein the sense strand of the double-stranded molecule encoded by the vector hybridize with antisense strand at the target sequence to form the double-stranded molecule having less than about 19 and about 25 nucleotide pairs

in length;

- [32] The method of any one of [22] to [31], wherein the double-stranded molecule encoded by the vector is composed of a single polynucleotide containing both the sense strand and the antisense strand linked by an intervening single-strand;
- [33] The method of [32], wherein the double-stranded molecule encoded by the vector has the general formula 5'-[A]-[B]-[A']-3' or 5'-[A']-[B]-[A]-3', wherein [A] is the sense strand containing a sequence corresponding to a target sequence selected from among SEQ ID NOS: 11, 12, 13 and 14, [B] is a intervening single-strand is composed of 3 to 23 nucleotides, and [A'] is the antisense strand containing a sequence complementary to the target sequence; and
- [34] The method of any one of [22] to [33], wherein the double-stranded molecule encoded by the vector is contained in a composition which includes, in addition to the molecule, a transfection-enhancing agent and pharmaceutically acceptable carrier.

[0070] The methods of the present invention will be described in more detail below.

The growth of cells expressing LHX4 gene may be inhibited by contacting the cells with a double-stranded molecule that specifically anneals to the LHX4 gene, Ku70 gene or Ku86 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 methods known in the art, including, e.g., using the MTT cell proliferation assay.

The growth of any kind of cell may be suppressed according to the present method so long as the cell expresses or over-expresses LHX4, the target gene of the double-stranded molecule of the present invention. Exemplary cells include lung cancer cells.

Thus, patients suffering from or at risk of developing disease caused or promoted in part by the overexpression of LHX4 are treated by administering at least one of the present double-stranded molecules, at least one vector expressing at least one of the molecules or at least one composition containing at least one of the molecules. For example, patients of lung cancer are treated according to the present methods. The type of cancer can be identified by standard methods according to the particular type of tumor to be diagnosed. Lung cancer may be diagnosed, for example, with Carcinoembryonic antigen (CEA), CYFRA, pro-GRP and so on, as lung cancer marker, or with Chest X-Ray and/or Sputum Cytology. In some embodiments, patients treated by the methods of the present invention are selected by detecting the expression of LHX4 in a biopsy from the patient by RT-PCR or immunoassay. Usually, before the treatment of the present invention, the biopsy specimen obtained from the subject is confirmed for LHX4 gene over-expression by methods known in the art, for example,

immunohistochemical analysis or RT-PCR.

- [0071] According to the present method to inhibit lung cancer cell growth and thereby treating lung cancer, when administering a plurality of double-stranded molecules (or vectors expressing or compositions containing the same), each of the molecules may have different structures but act at mRNA which matches the same target sequence of LHX4, Ku70 or Ku86. Alternatively, a plurality of double-stranded molecules may act at mRNA which matches different target sequences within the LHX4 gene, Ku70 gene or Ku86 gene or acts at mRNA which matches different target sequence of different gene. For example, the method may utilize double-stranded molecules directed to LHX4, Ku70 or Ku86. Alternatively, for example, the method may utilize double-stranded molecules directed to one, two or more target sequence of within the LHX4 coding sequence, Ku70 coding sequence or Ku86 coding sequence.

For inhibiting lung cancer cell growth, a double-stranded molecule of the present invention 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 as 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.

- [0072] The term "specifically inhibit" in the context of inhibitory polynucleotides and polypeptides refers to the ability of a substance or ligand to preferentially inhibit the expression or the biological function of LHX4 in comparison to the expression or biological function of polynucleotides and polypeptides other than LHX4. Specific inhibition typically results in at least about a 2-fold inhibition over background, preferably greater than about 10-fold and most preferably greater than 100-fold inhibition of the expression (e.g., transcription or translation) or the measured biological function (e.g., cell growth or proliferation, inhibition of apoptosis, intracellular signaling from LHX4). Inhibition of the expression or the biological function of LHX4 can be measured by comparing treated and untreated cells, or a cell population before and after treatment. In some embodiments, the expression or biological function of LHX4 is completely inhibited. Typically, specific inhibition is a statistically meaningful reduction in LHX4 expression or biological function (e.g., $p \leq 0.05$) using an appropriate statistical test.

A treatment is deemed "efficacious" if it leads to clinical benefit such as, reduction in expression of LHX4 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.

- [0073] 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.
- [0074] The treatment and/or prophylaxis of cancer and/or the prevention of postoperative recurrence thereof include any of the following steps, such as 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. Effectively treating 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. For example, reduction or improvement of symptoms constitutes effectively treating and/or the prophylaxis include 10%, 20%, 30% or more reduction, or stable disease.
- [0075] It is understood that the double-stranded molecules of the invention degrade the LHX4 mRNA in substoichiometric amounts. Without wishing to be bound by any theory, it is believed that the double-stranded molecules of the invention cause degradation of the target mRNA in a catalytic manner. Thus, compared to standard cancer therapies, significantly less double-stranded molecule needs to be delivered at or near the site of cancer to exert a therapeutic effect.
- [0076] One skilled in the art can readily determine an effective amount of the double-stranded molecules 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 molecules of the invention is an intracellular concentration at or near the cancer site of from about 1 nanomolar (nM) to about 100 nM, usually from about 2 nM to about 50 nM, more usually 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.

The present methods can be used to inhibit the growth or metastasis of a cancer expressing LHX4; for example lung cancer. In particular, a double-stranded molecule containing a target sequence of LHX4 (i.e., SEQ ID NO: 11 and 12), Ku70 (i.e., SEQ ID NO: 13) or Ku86 (i.e., SEQ ID NO: 14) is particularly useful for the treatment of lung cancer.

[0077] For treating cancer, the double-stranded molecules of the invention can also be administered to a subject in combination with a pharmaceutical agent different from the double-stranded molecule. Alternatively, the double-stranded molecules of the invention can be administered to a subject in combination with another therapeutic method designed to treat cancer. For example, the double-stranded molecules of the invention 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, such as cisplatin, carboplatin, cyclophosphamide, 5-fluorouracil, adriamycin, daunorubicin or tamoxifen).

In the present methods, the double-stranded molecule 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 which expresses the double-stranded molecule.

[0078] 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.

Liposomes can aid in the delivery of the double-stranded molecule to a particular tissue, such as lung 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.

Preferably, the liposomes encapsulating the present double-stranded molecule includes a ligand molecule that can deliver the liposome to the cancer site. Ligands which 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.

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 include both opsonization-inhibition moieties and a ligand.

- [0079] 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. 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.

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, 85: 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.

- [0080] 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".

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 Na(CN)BH₃ and a solvent mixture such as tetrahydrofuran and water in a 30:12 ratio at 60 degrees C.

- [0081] Vectors expressing a double-stranded molecule of the invention 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 patient are within the skill of the art.
- [0082] The double-stranded molecules of the invention 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.
Suitable enteral administration routes include oral, rectal, inhalational 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 including 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.
- [0083] The double-stranded molecules of the invention can be administered in a single dose or in multiple doses. Where the administration of the double-stranded molecules 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 at or near the site of cancer is typical. Multiple injections of the agent into the tissue at or near the site of cancer are particularly useful.

One skilled in the art can also readily determine an appropriate dosage regimen for administering the double-stranded molecules 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 typical 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 includes multiple administrations, it is understood that the effective amount of a double-stranded molecule administered to the subject can include the total amount of a double-stranded molecule administered over the entire dosage regimen.

[0084] In the present invention, cancer overexpressing LHX4 can be treated with at least one active ingredient selected from the group consisting of:

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

The cancer includes, but is not limited to, lung cancer. Accordingly, prior to the administration of double-stranded molecule of the present invention as an active ingredient, it is preferable to confirm whether the expression level of LHX4 in the cancer cells or tissues to be treated is enhanced as compared with normal cells of the same organ. Thus, in one embodiment, the present invention provides a method for treating a cancer (over)expressing LHX4, which method may include the steps of:

i) determining the expression level of LHX4 in cancer cells or tissue(s) obtained from a subject with the cancer to be treated;

ii) comparing the expression level of LHX4 with normal control; and

iii) administrating at least one component selected from the group consisting of:

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

to a subject with a cancer overexpressing LHX4 as compared with normal control.

Alternatively, the present invention also provides a pharmaceutical composition comprising at least one component selected from the group consisting of:

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

for use in administrating to a subject having a cancer overexpressing LHX4. In other words, the present invention further provides a method for identifying a subject to be treated with:

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

which method may include the step of determining an expression level of LHX4 in subject-derived cancer cells or tissue(s), wherein an increase of the level compared to a normal control level of the gene indicates that the subject has cancer which may be treated with:

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

[0085] The method of treating a cancer of the present invention will be described in more detail below. A subject to be treated by the present method 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.

According to the present invention, the expression level of LHX4 in cancer cells or tissues obtained from a subject is determined. The expression level can be determined at the transcription (nucleic acid) product level, using methods known in the art. For example, the mRNA of LHX4 may be quantified using probes by hybridization methods (e.g., Northern hybridization). The detection may be carried out on a chip or an array. The use of an array is suitable for detecting the expression level of LHX4. Those skilled in the art can prepare such probes utilizing the sequence information of LHX4. For example, the cDNA of LHX4 may be used as the probes. If necessary, the probes may be labeled with a suitable label, such as dyes, fluorescent substances and isotopes, and the expression level of the gene may be detected as the intensity of the hybridized labels.

[0086] Furthermore, the transcription product of LHX4 may be quantified using primers by amplification-based detection methods (e.g., RT-PCR). Such primers may be prepared based on the available sequence information of the gene.

Specifically, a probe or primer used for the present method hybridizes under stringent, moderately stringent, or low stringent conditions to the mRNA of LHX4. As used herein, the phrase "stringent (hybridization) conditions" refers to conditions under which a probe or primer will hybridize to its target sequence, but not to other sequences. Stringent conditions are sequence-dependent and will be different under different circumstances. Specific hybridization of longer sequences is observed at higher temperatures than shorter sequences. Generally, the temperature of a stringent

condition is selected to be about 5 degrees Centigrade lower than the thermal melting point (Tm) for a specific sequence at a defined ionic strength and pH. The Tm is the temperature (under a defined ionic strength, pH and nucleic acid concentration) at which 50% of the probes complementary to their target sequence hybridize to the target sequence at equilibrium. Since the target sequences are generally present at excess, at Tm, 50% of the probes are occupied at equilibrium. Typically, stringent conditions will be those in which the salt concentration is less than about 1.0 M sodium ion, typically about 0.01 to 1.0 M sodium ion (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30 degrees Centigrade for short probes or primers (e.g., 10 to 50 nucleotides) and at least about 60 degrees Centigrade for longer probes or primers. Stringent conditions may also be achieved with the addition of destabilizing agents, such as formamide.

[0087] Alternatively, the translation product may be detected for the diagnosis of the present invention. For example, the quantity of LHX4 protein (SEQ ID NO: 2) may be determined. Methods for determining the quantity of the protein as the translation product include immunoassay methods that use an antibody specifically recognizing the protein. The antibody may be monoclonal or polyclonal. Furthermore, any fragment or modification (e.g., chimeric antibody, scFv, Fab, F(ab')2, Fv, etc.) of the antibody may be used for the detection, so long as the fragment or modified antibody retains the binding ability to the LHX4 protein. Methods to prepare these kinds of antibodies for the detection of proteins are well known in the art, and any method may be employed in the present invention to prepare such antibodies and equivalents thereof.

As another method to detect the expression level of LHX4 gene based on its translation product, the intensity of staining may be measured via immunohistochemical analysis using an antibody against the LHX4 protein. Namely, in this measurement, strong staining indicates increased presence/level of the protein and, at the same time, high expression level of LHX4 gene.

The expression level of a target gene, e.g., the LHX4 gene, in cancer cells can be determined to be increased if the level increases from the control level (e.g., the level in normal cells) of the target 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.

[0088] The control level may be determined at the same time with the cancer cells by using a sample(s) previously collected and stored from a subject/subjects whose disease state(s) (cancerous or non-cancerous) is/are known. In addition, normal cells obtained from non-cancerous regions of an organ that has the cancer to be treated may be used as normal control. Alternatively, the control level may be determined by a statistical method based on the results obtained by analyzing previously determined expression

level(s) of LHX4 gene in samples from subjects whose disease states are known. Furthermore, the control level can be derived from a database of expression patterns from previously tested cells. Moreover, according to an aspect of the present invention, the expression level of LHX4 gene in a biological sample may be compared to multiple control levels, which are determined from multiple reference samples. It is common to use a control level determined from a reference sample derived from a tissue type similar to that of the subject-derived biological sample. Moreover, it is typical to use the standard value of the expression levels of LHX4 gene in a population with a known disease state. The standard value may be obtained by any method known in the art. For example, a range of mean +/- 2 S.D. or mean +/- 3 S.D. may be used as the standard value.

- [0089] In the context of the present invention, a control level determined from a biological sample that is known to be non-cancerous is referred to as a "normal control level". On the other hand, if the control level is determined from a cancerous biological sample, it is referred to as a "cancerous control level".

When the expression level of LHX4 gene is increased as compared to the normal control level, or is similar/equivalent to the cancerous control level, the subject may be diagnosed with cancer to be treated.

- [0090] Compositions Containing Double-Stranded Molecule:

In addition to the above, the present invention also provides pharmaceutical compositions that include at least one of the present double-stranded molecules or the vectors coding for the molecules.

In the context of the present invention, the term "composition" is used to refer to a product 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.

- [0091] 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.

- [0092] 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.
- [0093] Specifically, the present invention provides the following compositions [1] to [34]:
- [1] A composition for treating and/or preventing cancer, wherein the composition comprises a pharmaceutically effective amount of a double-stranded molecule against a LHX4 gene, Ku70 gene or Ku86 gene, or a vector encoding thereof, and a pharmaceutically acceptable carrier, wherein the double-stranded molecule, when introduced into a cell expressing the LHX4 gene, Ku70 gene or Ku86 gene, inhibits an expression of the LHX4 gene, Ku70 gene or Ku86 gene as well as cell proliferation;
 - [2] The composition of [1], wherein the double-stranded molecule acts at mRNA which matches a target sequence selected from SEQ ID NOs: 11,12, 13 and 14;
 - [3] The composition of [1] or [2], wherein the double-stranded molecule, wherein the sense strand contains a sequence corresponding to a target sequence selected from SEQ ID NOs: 11, 12, 13 and 14;
 - [4] The composition of any one of [1] to [3], wherein the cancer to be treated is lung cancer;
 - [5] The composition of any one of [1] to [4], wherein the composition contains plural kinds of the double-stranded molecules or vectors encoding thereof;
 - [6] The composition of any one of [1] to [5], wherein the sense strand of the double-stranded molecule hybridize with antisense strand at the target sequence to form the double-stranded molecule having less than about 100 nucleotide pairs in length;
 - [7] The composition of [6], wherein the sense strand of the double-stranded molecule hybridize with antisense strand at the target sequence to form the double-stranded molecule having less than about 75 nucleotide pairs in length;
 - [8] The composition of [7], wherein the sense strand of the double-stranded molecule hybridize with antisense strand at the target sequence to form the double-stranded molecule having less than about 50 nucleotide pairs in length;
 - [9] The composition of [8], wherein the sense strand of the double-stranded molecule hybridize with antisense strand at the target sequence to form the double-stranded molecule having less than about 25 nucleotide pairs in length;
 - [10] The composition of [9], wherein the sense strand of the double-stranded

molecule hybridize with antisense strand at the target sequence to form the double-stranded molecule having between about 19 and about 25 nucleotide pairs in length;

[11] The composition of any one of [1] to [10], wherein the double-stranded molecule is composed of a single polynucleotide containing 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]-3', wherein [A] is the sense strand sequence contains a sequence corresponding to a target sequence selected from SEQ ID NOs:11, 12, 13 and 14, [B] is the intervening single-strand consisting of 3 to 23 nucleotides, and [A'] is the antisense strand contains 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 is DNA and/or 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 [14], 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 one or two 3' overhangs;

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

[22] The composition of [1], wherein the double-stranded molecule is encoded by a vector and contained in the composition;

[23] The composition of [22], wherein the double-stranded molecule encoded by the vector acts at mRNA which matches a target sequence selected from SEQ ID NOs: 11, 12, 13 and 14.

[24] The composition of [22] or [23], wherein the sense strand of the double-stranded molecule encoded by the vector contains the sequence corresponding to a target sequence selected from SEQ ID NOs: 11 , 12, 13 and 14.

- [25] The composition of any one of [22] to [24], wherein the cancer to be treated is lung cancer;
- [26] The composition of any one of [22] to [24], wherein plural kinds of the double-stranded molecules are administered;
- [27] The composition of any one of [22] to [26], wherein the sense strand of the double-stranded molecule encoded by the vector hybridize with antisense strand at the target sequence to form the double-stranded molecule having less than about 100 nucleotide pairs in length;
- [28] The composition of [27], wherein the sense strand of the double-stranded molecule encoded by the vector hybridize with antisense strand at the target sequence to form the double-stranded molecule having less than about 75 nucleotide pairs in length;
- [29] The composition of [28], wherein the sense strand of the double-stranded molecule encoded by the vector hybridize with antisense strand at the target sequence to form the double-stranded molecule having less than about 50 nucleotide pairs in length;
- [30] The composition of [29], wherein the sense strand of the double-stranded molecule encoded by the vector hybridize with antisense strand at the target sequence to form the double-stranded molecule having less than about 25 nucleotide pairs in length;
- [31] The composition of [30], wherein the sense strand of the double-stranded molecule encoded by the vector hybridize with antisense strand at the target sequence to form the double-stranded molecule having between about 19 and about 25 nucleotide pairs in length;
- [32] The composition of any one of [22] to [31], wherein the double-stranded molecule encoded by the vector is composed of a single polynucleotide containing both the sense strand and the antisense strand linked by an intervening single-strand;
- [33] The composition of [32], wherein the double-stranded molecule has the general formula 5'-[A]-[B]-[A']-3' or 5'-[A']-[B]-[A]-3', wherein [A] is the sense strand containing a sequence corresponding to a target sequence selected from among SEQ ID NOs: 11, 12, 13 and 14, [B] is a intervening single-strand composed of 3 to 23 nucleotides, and [A'] is the antisense strand containing a sequence complementary to the target sequence; and
- [34] The composition of any one of [22] to [33], wherein the composition includes a transfection-enhancing agent.
- [0094] Suitable compositions of the present invention are described in additional detail below.
- The double-stranded molecules of the present invention are formulated as pharma-

ceutical 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 formulations" include formulations for human and veterinary use. The pharmaceutical compositions of the present invention 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.

- [0095] 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: The Science and Practice of Pharmacy, 21st ed., Lippincott, Williams and Wilkins. (2005).

The present pharmaceutical formulations contain at least one of the double-stranded molecules or vectors encoding them of the present invention (e.g., 0.1 to 90% by weight), or a physiologically acceptable salt of the molecule, mixed with a physiologically acceptable carrier medium. Typical physiologically acceptable carrier media are water, buffered water, normal saline, 0.4% saline, 0.3% glycine, hyaluronic acid and the like.

- [0096] According to the present invention, the composition may contain a plurality of double-stranded molecules, each of the molecules may be directed to the same target sequence, or different target sequences of LHX4, Ku70 or Ku86. For example, the composition may contain double-stranded molecules directed to LHX4, Ku70 or Ku86. Alternatively, for example, the composition may contain double-stranded molecules directed to one, two or more target sequences LHX4, Ku70 or Ku86.
- [0097] Furthermore, the present composition may contain a vector coding for one or a plurality of double-stranded molecules. For example, the vector may encode one, two or several kinds of the present double-stranded molecules. Alternatively, the present composition may contain a plurality of vectors, each of the vectors coding for a different double-stranded molecule.
- [0098] Moreover, the present double-stranded molecules may be contained as liposomes in the present composition. See under the item of "Methods of treating cancer using the double-stranded molecule" for details of liposomes.
- [0099] Pharmaceutical compositions of the invention can also include conventional pharma-

ceutical excipients and/or additives. 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. 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. 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 molecules of the invention. A pharmaceutical composition for aerosol (inhalational) administration can include 0.01-20% by weight, preferably 1-10% by weight, of one or more double-stranded molecules 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.

- [0100] In addition to the above, the present composition may contain other pharmaceutically 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.

- [0101] Alternatively, the present invention further provides the double-stranded nucleic acid molecules of the present invention for use in treating a cancer expressing the LHX4 gene.

- [0102] In another embodiment, the present invention also provides the use of the double-stranded molecules of the present invention in manufacturing a pharmaceutical composition for treating a lung cancer characterized by the over-expression of LHX4. For example, the present invention relates to a use of double-stranded molecule inhibiting the expression of LHX4 gene, Ku70 gene or Ku86 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 SEQ ID NOs: 11, 12, 13 and 14, for manufacturing a pharmaceutical composition for treating lung cancer over-expressing LHX4.

- [0103] Alternatively, the present invention further provides a method or process for manufacturing a pharmaceutical composition for treating a cancer caused or promoted in part by the overexpression of LHX4, e.g., a lung cancer characterized by the over-expression of LHX4, wherein the method or process includes a step for formulating a pharmaceutically or physiologically acceptable carrier with a double-stranded molecule inhibiting the expression of LHX4 gene, Ku70 gene or Ku86 gene in a cell, which over-expresses the gene, 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 SEQ ID NOs: 11, 12, 13 and 14 as active ingredients.
- [0104] In another embodiment, the present invention also provides a method or process for manufacturing a pharmaceutical composition for treating a cancer caused or promoted in part by the overexpression of LHX4, e.g., a lung cancer characterized by the expression of LHX4, 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 inhibiting the expression of LHX4 gene, Ku70 gene or Ku86 gene in a cell, which over-expresses the gene, 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 SEQ ID NOs: 11, 12, 13 and 14.
- [0105] Method of Detecting or Diagnosing Lung Cancer
The expression of LHX4 was found to be specifically elevated in lung cancer cells (Figs. 1). Therefore, the LHX4 gene identified herein as well as its transcription and translation products find diagnostic utility as markers for lung cancer and by measuring the expression of LHX4 gene in a lung tissue sample, lung cancer can be diagnosed. Specifically, the present invention provides a method for diagnosing lung cancer by determining the expression level of LHX4 gene in the subject.
According to the present invention, an intermediate result for examining the condition of a subject may be provided. Such intermediate result may 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 may 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.
- [0106] Alternatively, the present invention provides a method for detecting or identifying cancer cells in a subject-derived lung tissue sample, said method including the step of determining the expression level of the LHX4 gene in a subject-derived biological

sample, wherein an increase in said expression level as compared to a normal control level of said gene indicates the presence or suspicion of cancer cells in the lung tissue. Such results may be combined with additional information to assist a doctor, nurse, or other healthcare practitioner in diagnosing a subject as afflicted with the disease. In other words, the present invention may provide a doctor with useful information to diagnose a subject as afflicted with the disease. For example, according to the present invention, when there is doubt regarding the presence of cancer cells in the tissue obtained from a subject, clinical decisions can be reached by considering the expression level of the LHX4 gene, plus a different aspect of the disease including tissue pathology, levels of known tumor marker(s) in blood, and clinical course of the subject, etc. For example, some well-known diagnostic lung tumor markers in blood are IAP, ACT, BFP, CA19-9, CA50, CA72-4, CA130, CEA, KMO-1, NSE, SCC, SP1, Span-1, TPA, CSLEX, SLX, STN and CYFRA. Namely, in this particular embodiment of the present invention, the outcome of the gene expression analysis serves as an intermediate result for further diagnosis of a subject's disease state.

- [0107] In another embodiment, the present invention provides a method for detecting a diagnostic marker of cancer, said method including the step of detecting the expression of the LHX4 gene in a subject-derived biological sample as a diagnostic marker of lung cancer.
- [0108] Specifically, the present invention provides the following methods [1] to [10]:
 - [1] A method for detecting or diagnosing lung cancer, said method comprising the steps of:
 - (a) determining the expression level of the gene in a subject-derived biological sample; and
 - (b) correlating an increase in the expression level determined in step (a) as compared to a normal control level of the gene to the presence of lung cancer.
 - [2] The method of [1], wherein the expression level is at least 10% greater than the normal control level.
 - [3] The method of [1], wherein the expression level is detected by a method selected from among:
 - (a) detecting an mRNA of LHX4 gene,
 - (b) detecting a LHX4 protein, and
 - (c) detecting a biological activity of a LHX4 protein;
 - [4] The method of [3], wherein the expression level is determined by detecting hybridization of an oligonucleotide that hybridizes to the mRNA of the LHX4 gene, to the mRNA of the LHX4 gene;
 - [5] The method of [3], wherein the expression level is determined by detecting the binding of an antibody against the LHX4 protein to the LHX4 protein;

- [6] The method of [1], wherein the biological sample includes biopsy sample, sputum or blood;
 - [7] The method of [1], wherein the subject-derived biological sample includes an epithelial cell;
 - [8] The method of [1], wherein the subject-derived biological sample includes a cancer cell;
 - [9] The method of [1], wherein the subject-derived biological sample includes a cancerous epithelial cell;
 - [10] The method of any one of [1] to [9], wherein the subject-derived biological sample includes a lung tissue or lung cells.
- [0109] The method of diagnosing lung cancer will be described in more detail below. A subject to be diagnosed by the present method 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. It is preferred to collect a biological sample from the subject to be diagnosed to perform the diagnosis. Any biological material can be used as the biological sample for the determination so long as it includes the objective transcription or translation product of LHX4. The biological samples include, but are not limited to, bodily tissues which are desired for diagnosing or are suspicion of suffering from cancer, and fluids, such as biopsy, blood, serum, plasma, saliva, sputum, pleural effusion and urine. Preferably, the biological sample contains a cell population including an epithelial cell, more preferably a cancerous epithelial cell or an epithelial cell derived from tissue suspected to be cancerous, e.g., lung tissue. Further, if necessary, the cell may be purified from the obtained bodily tissues and fluids, and then used as the biological sample.
- [0110] According to the present invention, the expression level of LHX4 in a 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 can be determined at the transcription (nucleic acid) product level, using methods known in the art. For example, the mRNA of LHX4 may be quantified using probes by hybridization methods (e.g., Northern hybridization). The detection may be carried out on a chip or an array. The use of an array is typical for detecting the expression level of a plurality of genes (e.g., various cancer specific genes) including LHX4. Those skilled in the art can prepare such probes utilizing the sequence information of the LHX4 (e.g., SEQ ID NO 1; GenBank accession number: NM_033343). For example, the cDNA of LHX4 may be used as the probes. If necessary, the probe may be labeled with a suitable label, such as dyes, fluorescent and isotopes, and the expression level of the gene may be detected as the intensity of the hybridized labels.

- [0111] Furthermore, the transcription product of LHX4 may 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 gene. For example, the primers (SEQ ID NOS: 3 and 4, or 7 and 8) used in the Example may be employed for the detection by RT-PCR or Northern blot, but the present invention is not restricted thereto.
- [0112] Specifically, a probe or primer used for the present method hybridizes under stringent, moderately stringent, or low stringent conditions to the mRNA of LHX4. As used herein, the phrase "stringent (hybridization) conditions" refers to conditions under which a probe or primer will hybridize to its target sequence, but to no other sequences. Stringent conditions are sequence-dependent and will be different under different circumstances. Specific hybridization of longer sequences is observed at higher temperatures than shorter sequences. Generally, the temperature of a stringent condition is selected to be about 5 degrees Centigrade lower than the thermal melting point (Tm) for a specific sequence at a defined ionic strength and pH. The Tm is the temperature (under defined ionic strength, pH and nucleic acid concentration) at which 50% of the probes complementary to the target sequence hybridize to the target sequence at equilibrium. Since the target sequences are generally present at excess, at Tm, 50% of the probes are occupied at equilibrium. Typically, stringent conditions will be those in which the salt concentration is less than about 1.0 M sodium ion, typically about 0.01 to 1.0 M sodium ion (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30 degrees Centigrade for short probes or primers (e.g., 10 to 50 nucleotides) and at least about 60 degrees Centigrade for longer probes or primers. Stringent conditions may also be achieved with the addition of destabilizing agents, such as formamide.
- [0113] Alternatively, the translation product may be detected for the diagnosis of the present invention. For example, the quantity of LHX4 protein may be determined. A method for determining the quantity of the protein as the translation product includes immunoassay methods that use an antibody specifically recognizing the protein. The antibody may be monoclonal or polyclonal. Furthermore, any fragment or modification (e.g., chimeric antibody, scFv, Fab, F(ab')2, Fv, etc.) of the antibody may be used for the detection, so long as the fragment retains the binding ability to LHX4 protein. Methods to prepare these kinds of antibodies for the detection of proteins are well known in the art, and any method may be employed in the present invention to prepare such antibodies and equivalents thereof.
- [0114] As another method to detect the expression level of LHX4 gene based on its translation product, the intensity of staining may be observed via immunohistochemical analysis using an antibody against LHX4 protein. Namely, the observation of

strong staining indicates increased presence of the protein and at the same time high expression level of LHX4 gene.

- [0115] 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 LHX4 gene expression level. Once determined, using any of the aforementioned techniques, this value is as compared to a control level.
- [0116] 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 lung 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. 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 lung cancer. An increase in the expression level of LHX4 detected in a subject-derived sample as compared to a normal control level indicates that the subject (from which the sample has been obtained) suffers from or is at risk of developing lung cancer. In the context of the present invention, the subject-derived sample may be any tissues obtained from test subjects, e.g., patients suspected of having cancer. For example, tissues may include epithelial cells. More particularly, tissues may be epithelial cells collected from a suspected cancerous area.. Alternatively, the expression level of LHX4 in a sample can be compared to a cancer control level of LHX4 gene. A similarity between the expression level of a sample and the cancer control level indicates that the subject (from which the sample has been obtained) suffers from or is at risk of developing cancer. When the expression levels of other cancer-related genes are also measured and compared, a similarity in the gene expression pattern between the sample and the reference that is cancerous indicates that the subject is suffering from or at a risk of developing cancer.

The control level may be determined at the same time with the test 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 may be determined by a statistical method based on the results obtained by analyzing previously determined expression level(s) of LHX4 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 LHX4 gene in a biological sample may be compared to multiple control levels, which control levels are determined from multiple reference samples. It is common to use a control level determined from a reference sample derived from a tissue type similar to that of the patient-derived biological sample, e.g., lung tissue. Moreover, it is preferred, to use the standard value of the expression levels of LHX4 gene in a population with a known disease state. The standard value may be obtained by any method known in the art. For example, a range of mean +/- 2 S.D. or mean +/- 3 S.D. may be used as standard value.

- [0117] To improve the accuracy of the diagnosis, the expression level of other cancer-associated genes, for example, genes known to be differentially expressed in lung cancer may also be determined, in addition to the expression level of the LHX4 gene. Furthermore, in the case where the expression levels of multiple cancer-related genes are compared, a similarity in the gene expression pattern between the sample and the reference that is cancerous indicates that the subject is suffering from or at a risk of developing lung cancer.
- [0118] 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 lung cancer marker genes including LHX4 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.
- Differences between the expression levels of a test biological sample and the control level 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.
- [0119] In the present invention, it is revealed that LHX4 is not only a useful diagnostic marker, but is also suitable target for cancer therapy. Therefore, cancer treatment targeting LHX4 can be achieved by the present invention. In the present invention, the cancer treatment targeting LHX4 refers to suppression or inhibition of LHX4 activity and/or expression in the cancer cells. Any anti-LHX4 agents may be used for the cancer treatment targeting LHX4. In the present invention, the anti-LHX4 agents include following substance or active ingredient:
- (a) a double-stranded molecule of the present invention,

- (b) DNA encoding thereof, and
- (c) a vector encoding thereof.

Accordingly, in a one embodiment, the present invention provides a method of (i) diagnosing whether a subject has the cancer to be treated with anti- LHX4 agent, and/or (ii) selecting a subject for cancer treatment targeting LHX4, which method includes the steps of:

- a) determining the expression level of LHX4 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 LHX4 with a normal control level;
- c) diagnosing the subject as having the cancer to be treated, if the expression level of LHX4 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).

Alternatively, such a method includes the steps of:

- a) determining the expression level of LHX4 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 LHX4 with a cancerous control level;
- c) diagnosing the subject as having the cancer to be treated, if the expression level of LHX4 is similar or equivalent to the cancerous 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).

[0120] **A kit for Detecting or Diagnosing Cancer:**

The present invention also provides a kit for diagnosing cancer, which may also be useful in assessing and/or monitoring the efficacy of a cancer therapy. The present invention also provides a kit for determining a subject suffering from cancer that can be treated with the double-stranded molecule of the present invention or vector encoding thereof, which may also be useful in assessing and/or monitoring the efficacy of such cancer treatment. Usually, the cancer to be diagnosed by the present kit is lung cancer, including NSCLC and SCLC. The kit usually includes at least one reagent for detecting the expression level of the LHX4 gene in a subject-derived biological sample, which reagent may be selected from the group consisting of:

- (a) a reagent for detecting an mRNA of a LHX4 gene;
- (b) a reagent for detecting a protein encoded by a LHX4 gene; and
- (c) a reagent for detecting a biological activity of a protein encoded by a LHX4 gene.

Suitable reagents for detecting an mRNA of a LHX4 gene (LHX4 mRNA) include nucleic acids that specifically bind to or identify the LHX4 mRNA, such as oligonucleotides which have a complementary sequence to a part of the LHX4 mRNA. Such oligonucleotides specifically hybridize to the LHX4 mRNA and the hybridization level

correlate with the quantity of LHX4 mRNA. These kinds of oligonucleotides are exemplified by primers and probes that are specific to the LHX4 mRNA. These kinds of oligonucleotides may be prepared based on methods well known in the art. If needed, the reagent for detecting the LHX4 mRNA may be immobilized on a solid matrix. Moreover, more than one reagent for detecting the LHX4 mRNA may be included in the kit.

- [0121] A probe or primer of the present invention is typically a substantially purified oligonucleotide. The oligonucleotide typically includes a region of nucleotide sequence that hybridizes under stringent conditions to at least about 2000, 1000, 500, 400, 350, 300, 250, 200, 150, 100, 50, or 25 bases of consecutive sense strand nucleotide sequence of a nucleic acid including a LHX4 sequence, or an anti-sense strand nucleotide sequence of a nucleic acid including a LHX4 sequence, or of a naturally occurring mutant of these sequences. In particular, for example, in some embodiments, an oligonucleotide having 5-50 bases in length can be used as a primer for amplifying the genes, to be detected. More typically, mRNA or cDNA of a LHX4 gene can be detected with oligonucleotide probes or primers of a specific size, generally 15-30 bases in length. In other embodiments, the length of the oligonucleotide probe or primer can be selected from 15-25 bases. Assay procedures, devices, or reagents for the detection of gene by using such oligonucleotide probe or primer are well known (e.g. oligonucleotide microarray or PCR). In these assays, probes or primers can also include tag or linker sequences. Further, probes or primers can be modified with detectable label or affinity ligand to be captured. Alternatively, in hybridization based detection procedures, a polynucleotide having a few hundreds (e.g., about 100-200) bases to a few kilo (e.g., about 1000-2000) bases in length can also be used for a probe (e.g., northern blotting assay or cDNA microarray analysis).
- [0122] On the other hand, suitable reagents for detecting a LHX4 protein include antibodies against the LHX4 protein. The antibody may be monoclonal or polyclonal. Furthermore, any fragment or modification (e.g., chimeric antibody, scFv, Fab, F(ab')₂, Fv, etc.) of the antibody may be used as the reagent, so long as the fragment retains the binding ability to the LHX4 protein. Methods to prepare these kinds of antibodies for the detection of proteins are well known in the art, and any method may be employed in the present invention to prepare such antibodies and equivalents thereof. Furthermore, the antibody may 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 may be employed for the present invention. Moreover, more than one reagent for detecting the LHX4 protein may be included in the kit.
- [0123] Furthermore, the biological activity can be determined by, for example, measuring

the cell proliferating activity due to the expressed LHX4 protein in the biological sample. For example, the cell is 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. If needed, the reagent for detecting the LHX4 mRNA may be immobilized on a solid matrix. Moreover, more than one reagent for detecting the biological activity of the LHX4 protein may be included in the kit.

- [0124] The kit may contain more than one of the aforementioned reagents. Furthermore, the kit may include a solid matrix and reagent for binding a probe against the LHX4 gene or antibody against the LHX4 protein, a medium and container for culturing cells, positive and negative control reagents, and a secondary antibody for detecting an antibody against the LHX4 protein. For example, tissue samples obtained from subject suffering from cancer or not may serve as useful control reagents. A kit of the present invention may 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 may be provided in a container with a label. Suitable containers include bottles, vials, and test tubes. The containers may be formed from a variety of materials, such as glass or plastic.
- [0125] As an embodiment of the present invention, when the reagent is a probe against the LHX4 mRNA, the reagent may be immobilized on a solid matrix, such as a porous strip, to form at least one detection site. The measurement or detection region of the porous strip may include a plurality of sites, each containing a nucleic acid (probe). A test strip may also contain sites for negative and/or positive controls. Alternatively, control sites may be located on a strip separated from the test strip. Optionally, the different detection sites may 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 LHX4 mRNA present in the sample. The detection sites may 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.
- [0126] The kit of the present invention may further include positive and/or negative controls sample, and/or an LHX4 standard sample. The positive control sample of the present invention may be prepared by collecting LHX4 positive samples. Such LHX4 positive samples may be obtained, for example, from established lung cancer cell lines, including lung adenocarcinoma cell (ADC) lines such as A427, NCI-H1781, A549, LC319 and the like; lung squamous cell carcinoma (SCC) cell lines such as NCI-H26, EBC-1, NCI-H520, NCI-H2170 and the like; and SCLC cell lines such as DMS114,

DMS273, SBC-3, SBC-5, H196, H446 and the like. Alternatively, the LHX4 positive samples may be obtained from clinical lung cancer tissues, including lung adenocarcinoma tissues, lung squamous cell carcinoma tissues and SCLC tissues. Alternatively, positive control samples may be prepared by determining a cut-off value and preparing a sample containing an amount of an LHX4 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 present kit may include an LHX4 standard sample containing a cut-off value amount of an LHX4 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 tissues, or may be prepared by preparing a sample containing an LHX4 mRNA or protein less than cut-off value.

[0127] Alternatively, the present invention provides use of a reagent for preparing a diagnostic reagent for diagnosing cancer. In some embodiments, the reagent can be selected from the group consisting of:

- (a) a reagent for detecting mRNA of the LHX4 gene;
- (b) a reagent for detecting the LHX4 protein; and
- (c) a reagent for detecting the biological activity of the LHX4 protein.

Specifically, such reagent includes an oligonucleotide that hybridizes to the LHX4 mRNA, or an antibody that binds to the LHX4 protein. Furthermore, the present invention also provides a reagent for detecting or diagnosing cancer. Such reagent can include an oligonucleotide that hybridize to the mRNA of the LHX4 gene, or an antibody against the protein encoded by the LHX4 gene.

[0128] Screening for Anti-Lung Cancer Substances

In the context of the present invention, substances to be identified through the present screening methods may be any substance or composition including several substances. Furthermore, the test substance exposed to a cell or protein according to the screening methods of the present invention may be a single compound or a combination of compounds. When a combination of compounds is used in the methods, the compounds may be contacted sequentially or simultaneously.

Any test agent, 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 micromolecular compounds (including nucleic acid constructs, such as antisense RNA, siRNA, Ribozymes, and aptamer 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. 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 compound s (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 compound s may 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 2002103360).

A compound 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 agents obtained by the screening methods of the present invention.

- [0129] 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 may be determined to deduce the nucleic acid sequence coding for the protein, or a partial amino acid sequence of the obtained protein may 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 is confirmed for its usefulness in preparing the test agent which is a candidate for treating or preventing cancer.

Test substances useful in the screenings described herein can also be antibodies that specifically bind to LHX4 protein or partial peptides thereof that lack the biological activity of the original proteins in vivo.

Although the construction of test substance libraries is well known in the art, herein below, additional guidance in identifying test substances and construction libraries of such substances for the present screening methods are provided.

In the present invention, it is revealed that suppression of the expression level and/or biological activity of LHX4 lead to suppression of the growth of cancer cells.

Therefore, when a substance suppresses the expression and/or activity of LHX4, the

suppression is indicative of a potential therapeutic effect in a subject. In the present invention, a potential therapeutic effect refers to a clinical benefit with a reasonable expectation. In the present invention, such clinical benefit includes;

- (a) reduction in expression of the LHX4 gene,
- (b) a decrease in size, prevalence, or metastatic potential of the cancer in the subject,
- (c) preventing cancers from forming, or
- (d) preventing or alleviating a clinical symptom of cancer.

[0130] **(i) Molecular modeling:**

Construction of test substance libraries is facilitated by knowledge of the molecular structure of compounds known to have the properties sought, and/or the molecular structure of LHX4. One approach to preliminary screening of test agents suitable for further evaluation is computer modeling of the interaction between the test agent and its target.

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. 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.

[0131] 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 interactive construction, modification, visualization, and analysis of the behavior of molecules with each other.

A number of articles review computer modeling of drugs interactive with specific proteins, such as Rotivinen et al. Acta Pharmaceutica Fennica 1988, 97: 159-66; Ripka, New Scientist 1988, 54-8; McKinlay & Rossmann, Annu Rev Pharmacol Toxicol 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.

Other computer programs that screen and graphically depict chemicals are available

from companies such as 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.

- [0132] Once a putative inhibitor has been identified, combinatorial chemistry techniques can be employed to construct any number of variants based on the chemical structure of the identified putative inhibitor, as detailed below. The resulting library of putative inhibitors, or "test agents" may be screened using the methods of the present invention to identify test agents treating or preventing the lung cancer.
- [0133] (ii) Combinatorial chemical synthesis:
Combinatorial libraries of test substances may be produced as part of a rational drug design program involving knowledge of core structures existing in known inhibitors. This approach allows the library to be maintained at a reasonable size, facilitating high throughput screening. Alternatively, simple, particularly short, polymeric molecular libraries may 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.
- [0134] Preparation of combinatorial chemical libraries is well known to those of skill in the art, and may 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 such as 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 1995-2009 Wiley Interscience; Sambrook et al., 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;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).

Devices for the preparation of combinatorial libraries are commercially available (see, e.g., 357 MPS, 390 MPS, Advanced Chem Tech, Louisville KY, Symphony, Rainin, Woburn, MA, 433A Applied Biosystems, Foster City, CA, 9050 Plus, Millipore, Bedford, MA). In addition, numerous combinatorial libraries are themselves commercially available (see, e.g., ComGenex, Princeton, N.J., Tripos, Inc., St. Louis, MO, 3D Pharmaceuticals, Exton, PA, Martek Biosciences, Columbia, MD, etc.).

[0135] **(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., 106-108 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.

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.

[0136] **I. Protein based screening methods**

The present invention provides methods of screening for a candidate substance applicable to the treatment and/or prevention of cancer using an LHX4 polypeptide.

In the context of the present screening method, the LHX4 polypeptide to be used may be, for example, a purified polypeptide, a soluble protein, a form bound to a carrier or a fusion protein fused with other polypeptides. Further, the LHX4 polypeptide may be a recombinant polypeptide, a protein derived from the nature or a partial peptide thereof.

[0137] In addition to naturally-occurring LHX4 polypeptides, functional equivalents of the

polypeptides may be included in LHX4 polypeptides used for the present screening so long as the modified peptide retains at least one biological activity of the original polypeptide. Examples of the biological activity of the LHX4 polypeptide include, but are not limited to, cell proliferative activity, binding activity to Ku protein. Preferred examples of such functional equivalents are described above in the section entitled "The Genes and Polypeptides". For example, a preferred example of such functional equivalents includes a polypeptide containing a Ku protein-binding domain of LHX4 polypeptide.

The LHX4 protein may be produced in vitro by means of an in vitro translation system. The LHX4 polypeptide to be used in the screening method of the present invention can be, for example, a purified polypeptide, a soluble protein, or a fusion protein fused with other polypeptides.

[0138] The polypeptides or fragments used for the present method may 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.

Alternatively, the polypeptides may be obtained by adapting any known genetic engineering methods to the production of the instant polypeptides (e.g., Morrison J., J Bacteriology 1977, 132: 349-51; Clark-Curtiss & Curtiss, Methods in Enzymology (eds. Wu et al.) 1983, 101: 347-62). For example, first, a suitable vector including a polynucleotide encoding the objective protein in an expressible form (e.g., downstream of a regulatory sequence including a promoter) is prepared, transformed into a suitable host cell, and then the host cell is cultured to produce the protein. More specifically, a gene encoding a LHX4 polypeptide are expressed in host (e.g., animal) cells and such by inserting the gene into a vector for expressing foreign genes, such as pSV2neo, pcDNA I, pcDNA3.1, pCAGGS, or pCD8. A promoter may be used for the expression. Any commonly used promoters may 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-alpha promoter (Kim et al., Gene 1990,

91:217-23), the CAG promoter (Niwa et al., Gene 1991, 108:193), the RSV LTR promoter (Cullen, Methods in Enzymology 1987, 152:684-704), the SR-alpha promoter (Takebe et al., Mol Cell Biol 1988, 8:466), the CMV immediate early promoter (Seed et al., Proc Natl Acad Sci USA 1987, 84:3365-9), the SV40 late promoter (Gheysen et al., J Mol Appl Genet 1982, 1:385-94), the Adenovirus late promoter (Kaufman et al., Mol Cell Biol 1989, 9:946), the HSV TK promoter, and such. The introduction of the vector into host cells to express a LHX4 polypeptide may be performed according to any conventional methods, for example, the electroporation method (Chu et al., Nucleic Acids Res 1987, 15:1311-26), the calcium phosphate method (Chen et al., Mol Cell Biol 1987, 7:2745-52), the DEAE dextran method (Lopata et al., Nucleic Acids Res 1984, 12:5707-17; Sussman et al., Mol Cell Biol 1985, 4:1641-3), the Lipofectin method (Derijard B, Cell 1994, 7:1025-37; Lamb et al., Nature Genetics 1993, 5:22-30; Rabindran et al., Science 1993, 259:230-4), and such. A LHX4 polypeptide may be further linked to other substances, so long as the polypeptides and fragments retain at least one biological activity. Usable substances include: peptides, lipids, sugar and sugar chains, acetyl groups, natural and synthetic polymers, etc. Such modifications may be used to confer additional functions or to stabilize the polypeptide and fragments.

For example, the polypeptides may be expressed as a fusion protein including 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. For example, a commercially available epitope-antibody system may be used (Experimental Medicine 13: 85-90 (1995)). Vectors that are capable of expressing 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.

[0139] 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 original polypeptide by the fusion, is also provided herein. Epitopes, such as 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 antibodies recognizing them may be used as the epitope-antibody system for detecting the binding activity between the polypeptides (Experimental Medicine 13: 85-90 (1995)).

[0140] (i) Screening for a LHX4 Binding Substance:

In the context of the present invention, an over-expression of LHX4 gene was detected in lung cancer, in spite of no expression in normal organs (Fig. 1). Accordingly, using the LHX4 gene and proteins encoded thereby, the present invention

provides a method of screening for a substance that binds to LHX4 polypeptide. Due to the expression of LHX4 in cancer, a substance binds to LHX4 polypeptide is a candidate substance that suppresses the proliferation of cancer cells, and thus be useful for treating and/or preventing cancer. Therefore, the present invention also provides a method of screening for a candidate substance that suppresses the proliferation of cancer cells, and a method for screening a candidate substance for treating or preventing cancer using the LHX4 polypeptide. On particular, an embodiment of this screening method includes the steps of:

- (a) contacting a test substance with a LHX4 polypeptide or functional equivalent thereof;
- (b) detecting the binding activity between the polypeptide or the functional equivalent and the test substance; and
- (c) selecting the test substance that binds to the polypeptide or the functional equivalent.

[0141] Alternatively, according to the present invention, the potential therapeutic effect of a test substance for treating and/or 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 for treating and/or preventing cancer and/or inhibiting cancer associated with over-expression of LHX4 gene, the method including steps of:

- (a) contacting a test substance with a LHX4 polypeptide or functional equivalent thereof;
- (b) detecting the binding activity between the polypeptide or the functional equivalent and the test substance; and
- (c) correlating the potential therapeutic effect and the test substance, wherein the potential therapeutic effect is shown, when a substance binds to the polypeptide or the functional equivalent.

In the context of the present invention, the therapeutic effect may be correlated with the binding level of the test substance and LHX4 polypeptide (or functional equivalent thereof). For example, when the test substance binds to a LHX4 polypeptide, the test substance may identified or selected as a candidate substance having the requisite therapeutic effect. Alternatively, when the test substance does not bind to a LHX4 polypeptide, the test substance may characterized as having no significant therapeutic effect.

[0142] The method of the present invention will be described in more detail below. The LHX4 polypeptide to be used for screening may be a recombinant polypeptide or a protein derived from the nature or a partial peptide thereof. The polypeptide to be contacted with a test substance may 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 LHX4 or chemically synthesized to be contacted with a test substance in vitro.

- [0143] In one embodiment, test substances used by the present invention may be proteins such as antibodies or synthetic chemical compounds. As a method of screening substances that bind to a LHX4 polypeptide, many methods well known by a person skilled in the art may be used. Such a screening may be conducted by, for example, immunoprecipitation method.

When a immunoprecipitation method is used, it is preferred that a LHX4 polypeptide contains an antibody recognition site. LHX4 polypeptides to be used for the screening method of the present invention may be prepared as described above. Alternatively, a LHX4 polypeptide can be expressed as a fusion protein as described above.

In immunoprecipitation, an immune complex is formed by adding these antibodies to cell lysate prepared using an appropriate detergent. The immune complex consists of the LHX4 polypeptide, a polypeptide including the binding ability with the polypeptide, and an antibody. Immunoprecipitation can be also conducted using antibodies against the LHX4 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 a LHX4 polypeptide is prepared as a fusion protein with an epitope, such as GST, an immune complex can be formed in the same manner as in the use of the antibody against the LHX4 polypeptide, using a substance specifically binding to these epitopes, such as glutathione-Sepharose 4B.

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)).

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 LHX4 polypeptide is difficult to detect by a common staining method, such as 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-cystein, 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.

West-Western blotting analysis (Skolnik et al., Cell 65: 83-90 (1991)) can be used as a method of screening for proteins binding to the LHX4 polypeptide using the polypeptide. In particular, a protein binding to the LHX4 polypeptide can be obtained

by preparing a cDNA library from cultured cells expected to express a protein binding to the LHX4 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 LHX4 polypeptide with the above filter, and detecting the plaques expressing proteins bound to the LHX4 polypeptide according to the label. The polypeptide of the invention may be labeled by utilizing the binding between biotin and avidin, or by utilizing an antibody that specifically binds to the LHX4, or a peptide or polypeptide (for example, GST) that is fused to the LHX4 polypeptide. Methods using radioisotope or fluorescence and such may be also used.

- [0144] Alternatively, in another embodiment of the screening method of the present invention, a two-hybrid system utilizing cells may 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)").

In the two-hybrid system, the polypeptide of the invention 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 polypeptide of the invention, 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 polypeptide of the invention 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. As a reporter gene, for example, Ade2 gene, lacZ gene, CAT gene, luciferase gene and such can be used in addition to the HIS3 gene.

- [0145] A substance that binds to a LHX4 polypeptide may also be screened using affinity chromatography. For example, a LHX4 polypeptide may be immobilized on a carrier of an affinity column, and a composition containing test substances is applied to the column. A composition herein may be, for example, cell extracts, cell lysates, antibody libraries etc. After loading test substances, the column is washed, and substances bound to the LHX4 polypeptide can be collected. 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.

A biosensor using the surface plasmon resonance phenomenon may be used as a mean for detecting or quantifying the bound substance in the present invention. When

such a biosensor is used, the interaction between a LHX4 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 a LHX4 polypeptide and a test substance using a biosensor such as BIACore.

The methods of screening for molecules that bind when the immobilized LHX4 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 LHX4 protein (including agonist and antagonist) are well known to one skilled in the art.

[0146] **(ii) Screening for a Substance that Suppresses the Biological Activity of LHX4:**

In the context of present invention, the LHX4 polypeptide is characterized as having the activity of promoting cell proliferation of cancer cells (Fig. 2, 3). In the context of the present invention, the LHX4 polypeptide is also characterized as having the activity of binding to a Ku protein (Fig. 4). Using these biological activities as indices, the present invention provides a method for screening a substance that suppresses the proliferation of cancer cells expressing LHX4 gene, and a method of screening for a substance for treating and/or preventing the cancer, particular LHX4 associated cancers such as lung cancer. Thus, the present invention provides a method of screening for a candidate substance for treating and/or preventing cancer using a LHX4 polypeptide including the steps as follows:

- (a) contacting a test substance with a LHX4 polypeptide or functional equivalent thereof;
- (b) detecting the biological activity of the polypeptide or the functional equivalent of step (a); and
- (c) selecting the test substance that suppresses the biological activity of the polypeptide or the functional equivalent as compared to the biological activity detected in the absence of the test substance.

[0147] According to the present invention, the therapeutic effect of the test substance in suppressing the biological activity (e.g., the cell-proliferating activity) of LHX4 polypeptide (or functional equivalent thereof), or a candidate substance for treating and/or preventing cancer may be evaluated. Therefore, the present invention also provides a method of screening for a candidate substance that suppresses the biological activity of LHX4 polypeptide, or a candidate substance for treating and/or preventing cancer, using the LHX4 polypeptide or functional equivalent thereof, including the following steps:

- (a) contacting a test substance with the LHX4 polypeptide or a functional equivalent thereof; and
 - (b) detecting the biological activity of the polypeptide or the functional equivalent of step (a), and
 - (c) correlating the biological activity of (b) with the therapeutic effect of the test substance.
- [0148] Alternatively, in some embodiments, the present invention provides a method for evaluating or estimating a therapeutic effect of a test substance in the treatment and/or prevention of cancer and/or in the inhibition of the growth of a cancer associated with over-expression of LHX4 gene, the method including steps of:
- (a) contacting a test substance with the LHX4 polypeptide or a functional equivalent thereof;
 - (b) detecting the biological activity of the polypeptide or functional equivalent of step (a); and
 - (c) correlating the potential therapeutic effect and the test substance, wherein the potential therapeutic effect is shown, when a substance suppresses the biological activity of the LHX4 polypeptide or functional equivalent as compared to the biological activity of said polypeptide detected in the absence of the test substance. Such cancer includes lung cancer.
- [0149] In the context of the present invention, the therapeutic effect may be correlated with the biological activity of the LHX4 polypeptide or a functional equivalent thereof. For example, when the test substance suppresses or inhibits the biological activity of the LHX4 polypeptide or a functional equivalent thereof as compared to a level detected in the absence of the test substance, the test substance may identified or selected as the candidate substance having the therapeutic effect. Alternatively, when the test substance does not suppress or inhibit the biological activity of the LHX4 polypeptide or a functional equivalent thereof as compared to a level detected in the absence of the test substance, the test substance may identified as the substance having no significant therapeutic effect.
- [0150] The method of the present invention will be described in more detail below. Any polypeptides can be used for screening so long as they retain a biological activity of the LHX4 polypeptide. Such biological activity includes, but are not limited to, cell proliferation promoting activity, binding activity to Ku protein and nuclear localization activity. For example, naturally occurring human LHX4 polypeptides (e.g., polypeptide having an amino acid sequence of SEQ ID NO: 2) can be used and polypeptides functionally equivalent to these polypeptide can also be used (see "Genes and Polypeptides"). Such polypeptides may be expressed endogenously or exogenously by cells. Methods for preparing such polypeptides are described above.

In another aspect, the present invention also provides a screening method following the method described in the above "Screening" section, such method including the steps of:

- a) contacting a test substance with the LHX4 polypeptide or functional equivalent thereof;
- b) detecting the binding between the polypeptide or the functional equivalent and the test substance;
- c) selecting the test substance that binds to the polypeptide or the functional equivalent;
- d) contacting the test substance selected in step c) with the LHX4 polypeptide or the functional equivalent;
- e) comparing the biological activity of the polypeptide or the functional equivalent detected in the step d) with the biological activity detected in the absence of the test substance; and
- f) selecting the test substance that suppresses the biological activity of the polypeptide or the functional equivalent as a candidate substance for treating or preventing lung cancer.

The substance isolated by this screening is a candidate for antagonists of the polypeptide encoded by LHX4 gene. The term "antagonist" refers to molecules that inhibit the function of the polypeptide by binding thereto. This term also refers to molecules that reduce or inhibit expression of the gene encoding LHX4. Moreover, a substance isolated by this screening is a candidate for substances which inhibit the *in vivo* interaction of the LHX4 polypeptide with molecules (including DNAs and proteins).

[0151] When the biological activity to be detected in the present method is cell proliferation, it can be detected, for example, by preparing cells which express the LHX4 polypeptide, 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 survival cells or the colony forming activity, for example, shown in Fig. 2. The substances that reduce the speed of proliferation of the cells expressed LHX4 gene are selected as candidate substance for treating or preventing cancer. In some embodiments, cells expressing LHX4 gene may be isolated cells or cultured cells, which exogenously or endogenously express LHX4 gene *in vitro*.

More specifically, the method includes the step of:

- (a) contacting a test substance with cells overexpressing LHX4 gene;
- (b) measuring cell-proliferating activity; and
- (c) selecting the test substance that reduces the cell-proliferating activity in the comparison with the cell-proliferating activity in the absence of the test substance.

In some embodiments, the method of the present invention may further include the steps of:

(d) selecting the test substance that have no effect to the cells no or little expressing LHX4 gene.

The phrase "suppress the biological activity" as defined herein are preferably at least 10% suppression of the biological activity of LHX4 polypeptide in comparison with in absence of the substance, more preferably at least 25%, 50% or 75% suppression and most preferably at 90% suppression.

- [0152] In the some embodiments, control cells that do not express LHX4 gene are used. Accordingly, the present invention also provides a method of screening for a candidate substance that inhibits cell growth or a candidate substance for treating and/or preventing an LHX4- associated disease, using the LHX4 polypeptide or functional equivalent thereof including the steps as follows:
- a) culturing cells which express an LHX4 polypeptide or a functional equivalent thereof, and control cells that do not express an LHX4 polypeptide or a functional equivalent thereof in the presence of the test substance;
 - b) detecting the biological activity of the cells which express the LHX4 polypeptide and control cells; and
 - c) selecting the test substance that inhibits the biological activity in the cells which express the protein as compared to the proliferation detected in the control cells and in the absence of said test substance.
- [0153] When the biological activity to be detected in the screening method of the present invention is binding activity to Ku protein, it can be detected, for example, by detecting the binding between LHX4 polypeptide and Ku protein in the presence of a test substance. Details will be described under the following item "(iii) Screening for a Substance that Inhibits the Binding between LHX4 and Ku Protein".
- [0154] As revealed herein, suppressing the biological activity of LHX4 polypeptide reduces cell growth. Thus, by screening for a candidate substance that inhibits the biological activity of LHX4 polypeptide, candidate substance that have the potential to treat and/or prevent cancers can be identified. The potential of these candidate substances to treat or prevent cancers may be evaluated by second and/or further screening to identify therapeutic substance, compounds or agent for cancers. For example, when a substance that inhibits the biological activity of a LHX4 polypeptide also inhibits the activity of a cancer, it may be concluded that such a substance has a LHX4 specific therapeutic effect.
- [0155] (iii) Screening for a Substance that Inhibits the Binding between the LHX4 and the Ku protein:

In the present invention, the LHX4 polypeptide was confirmed to interact with the

Ku protein (Fig. 4). Accordingly, a substance that inhibits the binding between the LHX4 polypeptide and the Ku protein can be identified using the binding of the LHX4 polypeptide and the Ku protein as an index. In view thereof, it is an object of the present invention to provide a method of screening for a substance that inhibits the binding between the LHX4 polypeptide and the Ku protein. Furthermore, as demonstrated in Examples, the interaction with the Ku protein is involved in the stability of the LHX4 polypeptide. Accordingly, substances that inhibit the binding between the LHX4 polypeptide and the Ku protein are candidates for cancer therapeutic agents. Thus, the present invention also provides a method of screening for a candidate substance that inhibits or reduces the growth or adhesion of breast cancer cells, and a candidate substance for treating and/or preventing cancers, e.g. lung cancer.

- [0156] Accordingly, the present invention provides the following methods of [1] to [4]:
- [1] A method of screening for a substance that inhibits or reduces the binding between a LHX4 polypeptide and a Ku protein, such method including the steps of:
 - (a) contacting a LHX4 polypeptide or functional equivalent thereof with Ku protein in the presence of a test substance;
 - (b) detecting a binding level between the LHX4 polypeptide or the functional equivalent and Ku protein in the step (a);
 - (c) comparing the binding level detected in the step (b) with those detected in the absence of the test substance; and
 - (d) selecting the test substance that reduces or inhibits the binding level between the LHX4 polypeptide or the functional equivalent and Ku protein;
 - [2] A method of screening for a candidate substance suitable for the treatment and/or prevention of cancer or that inhibits cancer cell growth, such method including the steps of:
 - (a) contacting a LHX4 polypeptide or functional equivalent thereof with a Ku protein, in the presence of a test substance;
 - (b) detecting the binding level between the LHX4 polypeptide or the functional equivalent and Ku protein in the step (a);
 - (c) comparing the binding level detected in the step (b) with those detected in the absence of the test substance; and
 - (d) selecting the test substance that inhibits or reduces the binding level between the LHX4 polypeptide or the functional equivalent and Ku protein;
 - [3] The method of [1] or [2], wherein the functional equivalent of the LHX4 polypeptide includes an amino acid sequence of a Ku protein-binding domain of the LHX4 polypeptide;
 - [4] The method of any one of [2] to [3], wherein the cancer is lung cancer.

[0157] According to the present invention, the therapeutic effect of a candidate substance on the inhibition of the cell growth or a candidate substance in connection with the treatment and/or prevention of 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 suited to the treatment and/or prevention cancer.

An illustrative example of such a method includes the steps of:

- (a) contacting a LHX4 polypeptide or functional equivalent thereof with Ku protein in the presence of a test substance;
- (b) detecting the level of binding between the polypeptides in the step (a);
- (c) comparing the binding level detected in the step (b) with those detected in the absence of the test substance; and
- (d) correlating the binding level of (c) with the therapeutic effect of the test substance.

[0158] Alternatively, in other embodiments, the present invention may provide a method for evaluating or estimating the therapeutic effect of a test substance in connection with the treatment and/or prevention of cancer or the inhibition of cancer, the method including steps of:

- (a) contacting a LHX4 polypeptide or functional equivalent thereof with a Ku protein in the presence of a test substance;
- (b) detecting a binding level between the polypeptides in the step (a);
- (c) comparing the binding level detected in the step (b) with those detected in the absence of the test substance; and
- (d) correlating the potential therapeutic effect and the test substance, wherein the potential therapeutic effect is shown, when a test substance reduces the binding level.

[0159] In the context of the present invention, therapeutic effect may be correlated with the binding level of a LHX4 polypeptide and a Ku protein. For example, when a test substance reduces the binding level of LHX4 and Ku proteins as compared to a level detected in the absence of the test substance, the test substance may be identified or selected as a candidate substance having the desired therapeutic effect. Alternatively, when the test substance does not reduce the binding level of LHX4 polypeptide and Ku protein 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.

[0160] In the context of the present invention, a functional equivalent of a LHX4 polypeptide or Ku protein will have a biological activity equivalent to a LHX4 polypeptide or Ku protein (see, "Genes and Polypeptides"). In some embodiments, functional equivalents of a LHX4 polypeptide include a Ku protein-binding domain of the LHX4 polypeptide. In the present invention, it was revealed that the Ku-

protein-binding domain of the LHX4 polypeptide was located in the amino acid position from 199 to 219 of SEQ ID NO: 2 (Fig. 8). Accordingly, functional equivalents of a LHX4 polypeptide can be polypeptides that include the amino acid sequence from 199 to 219 of SEQ ID NO: 2 (i.e., the amino acid sequence of SEQ ID NO: 21). Examples of the such polypeptides include, but are not limited to, a polypeptide consisting of the amino acid sequence of SEQ ID NO: 21 or SEQ ID NO: 22. Alternatively, these polypeptides can be prepared as fusion proteins with one or more known epitopes such as 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. In preferred embodiments, the polypeptide is isolated from cells expressing LHX4 or chemically synthesized to be contacted with a test substance in vitro.

- [0161] In the context of screening for substances that inhibit or reduce the binding between LHX4 polypeptide and Ku protein, many methods well known by one skilled in the art can be used. Such a screening can be conducted via, for example, an immunoprecipitation, West-Western blotting analysis (Skolnik et al., Cell 65: 83-90 (1991)), a two-hybrid system utilizing cells ("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)"), affinity chromatography and a biosensor using the surface plasmon resonance phenomenon. Those methods can be conducted in a manner similar to the methods described above under the item "(i) Screening for a LHX4 Binding Substance".
- [0162] In some embodiments, the screening method of the present invention may be carried out in a cell-based assay using cells expressing both of a LHX4 polypeptide and a Ku protein. Cells expressing LHX4 polypeptide and Ku protein include, for example, cell lines established from cancer, e.g. lung cancer. Alternatively, the cells may be prepared through transformation with polynucleotides encoding a LHX4 polypeptide and a Ku protein. Such transformation may be carried out using an expression vector encoding both LHX4 polypeptide and Ku protein, or expression vectors encoding either LHX4 polypeptide or Ku protein. The screening method of the present invention can be conducted by incubating such cells in the presence of a test substance. The binding between LHX4 polypeptide to Ku protein can be detected by immunoprecipitation assay using an anti-LHX4 antibody or anti-Ku protein antibody.

When immunoprecipitation method is used, it is preferred that a LHX4 polypeptide and a Ku protein contains antibody recognition sites. For example, a LHX4 and/or Ku

protein may be prepared as fusion proteins that includes the polypeptide and a commercially available epitope. Methods for preparing such fusion proteins are described above.

In immunoprecipitation, an immune complex may be formed by adding antibodies against epitopes fused to LHX4 polypeptide and/or Ku protein to cell lysate prepared using an appropriate detergent. The immune complex consists of the LHX4 polypeptide, the Ku protein, and the antibody. Immunoprecipitation can be also conducted using antibodies against the LHX4 polypeptide and/or Ku protein, 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.

- [0163] In the present invention, it is revealed that suppression of the binding between LHX4 polypeptide and Ku protein lead to suppression of the growth of cancer cells. Therefore, when a substance inhibits the binding between LHX4 polypeptide and Ku protein, the inhibition is indicative of a potential therapeutic effect in a subject. In the present invention, a potential therapeutic effect refers to a clinical benefit with a reasonable expectation. In the present invention, such clinical benefit may include;
- (a) a reduction of the binding between LHX4 polypeptide and Ku protein,
 - (b) a decrease in size, prevalence, or metastatic potential of the cancer in the subject,
 - (c) the prevention of further cancer formation, or
 - (d) the prevention or alleviation of a clinical symptom of cancer.

[0164] II. Screening for a Substance that Alters the Expression of LHX4:

In the context of the present invention, a decrease in the expression of LHX4 gene by siRNA results in the inhibition of cancer cell proliferation (Fig.2). Accordingly, the present invention provides a method of screening for a substance that inhibits the expression of LHX4. A substance that inhibits the expression of LHX4 suppresses the proliferation of cancer cells, and thus is useful for treating or preventing cancer, particularly LHX4-associated cancers such as lung cancer. Therefore, the present invention also provides a method for screening a substance that suppresses the proliferation of cancer cells, and a method for screening a candidate substance for treating or preventing cancer. 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 LHX4 gene;
- (b) detecting an expression level of the LHX4 gene in the cell of the step (a); and
- (c) selecting the test substance that reduces the expression level of the LHX4 gene in the absence of the test substance.

- [0165] 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 LHX4 gene;
- b) detecting the expression level of the LHX4 gene; and
- c) correlating the expression level of b) with the therapeutic effect of the test substance.

In some embodiments, cells expressing LHX4 gene may be isolated cells or cultured cells, which exogenously or endogenously express LHX4 gene in vitro.

In the context of present invention, the therapeutic effect may be correlated with the expression level of the LHX4 gene. For example, when the test substance reduces the expression level of the LHX4 gene as compared to a level detected in the absence of the test substance, the test substance may identified or selected as the candidate substance having the therapeutic effect. Alternatively, when the test substance does not reduce the expression level of the LHX4 gene as compared to a level detected in the absence of the test substance, the test substance may identified as the substance having no significant therapeutic effect.

[0166] The method of the present invention will be described in more detail below.

Cells expressing a LHX4 gene include, for example, cell lines established from lung cancer or cell lines transfected with a LHX4 expression vector; any of such cells can be used for the screening method of the present invention. The expression level of a LHX4 gene can be estimated by methods well known to one skilled in the art, for example, RT-PCR, Northern blot assay, Western blot assay, immunostaining and flow cytometry analysis. The phrase "reduce the expression level" as defined herein are preferably at least 10% reduction of expression level of LHX4 gene in comparison to the expression level in absence of the substance, more preferably at least 25%, 50% or 75% reduced level and most preferably at least 95% reduced level. The substance herein includes chemical compounds, double-strand nucleotides, and so on. The preparation of the double-strand nucleotides will be described bellow. In the method of screening, a substance that reduces the expression level of LHX4 gene can be selected as candidate substances to be used for the treatment or prevention of cancer.

[0167] Alternatively, the screening method of the present invention may include the following steps:

- (a) contacting a test substance with a cell into which a vector, including the transcriptional regulatory region of a LHX4 gene and a reporter gene that is expressed under the control of the transcriptional regulatory region, has been introduced;
- (b) measuring the expression or activity of the reporter gene; and
- (c) selecting the test substance that reduces the expression or activity of the reporter gene.

Suitable reporter genes and host cells are well known in the art. Illustrative reporter genes include, but are not limited to, 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 LHX4 gene. The transcriptional regulatory region of LHX4 gene herein is the region from transcription start site to at least 500bp upstream, preferably 1000bp, more preferably 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. The reporter construct required for the screening can be prepared by connecting reporter gene sequence to the transcriptional regulatory region of the gene. 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).

The vector containing such reporter construct is introduced into host cells and the expression or activity of the reporter gene is detected by methods well known in the art (e.g., using luminometer, absorption spectrometer, flow cytometer and so on).

"Reduces the expression or activity" as defined herein are preferably at least 10% reduction of the expression or activity of the reporter gene in comparison with in absence of the test substance, more preferably at least 25%, 50% or 75% reduction and most preferably at least 95% reduction.

[0168] Alternatively, in some embodiments, the present invention also provides a method for evaluating or estimating a therapeutic effect of a test substance on treating or preventing cancer or inhibiting cancer associated with over-expression of LHX4 gene, the method including steps of:

- (a) contacting a test substance with a cell into which a vector, including the transcriptional regulatory region of LHX4 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 the test substance reduces the expression level or activity of said reporter gene.

According to the present invention, the therapeutic effect of the test substance on inhibiting the cell growth or a candidate substance for treating or preventing C6orf16-associating disease such as 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 LHX4-associating disease.

[0169] According to another aspect, the present invention provides a method which 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 LHX4 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 of (b) with the therapeutic effect of the test substance.

In the present invention, the therapeutic effect may be correlated with the expression level or activity of said reporter gene. For example, when the test substance reduces the expression level or activity of said 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 said 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.

[0170] By screening for candidate substances that (i) bind to the LHX4 polypeptide; (ii) suppress/reduce the biological activity (e.g., the cell-proliferating activity, binding activity to a NFKBIL2 polypeptide, nuclear localization activity) of the LHX4 polypeptide; (iii) reduce the expression level of LHX4 gene, or (iv) inhibit or reduce the binding between LHX4 polypeptide and NFKBIL2 polypeptide, candidate substances that have the potential to treat or prevent cancers (e.g., lung cancer,) can be identified. The therapeutic potential of these candidate substances may be evaluated by second and/or further screening to identify therapeutic substance for cancers. For example, when a substance that binds to the LHX4 polypeptide inhibits the above-described activities of cancer, it may be concluded that such a substance has the LHX4-specific therapeutic effect.

[0171] III. Screening using the phosphorylation level of LHX4 as index

Furthermore, in the present invention, it was confirmed that the LHX4 proteins were phosphorylated (Fig. 9). Thus, a substance that inhibits the phosphorylation of LHX4 protein can be screened using such modification as an index. Therefore, the present invention also provides a method for screening a substance for inhibits the phosphorylation of LHX4 protein. Furthermore, the present invention also provides a method for screening a substance for treating or preventing cancer. The method is particularly suited for screening substances that may be used in treating and/or preventing cancer. More specifically, the method includes the steps of:

- (a) contacting a cell that expresses a polypeptide selected from the group consisting of:
 - (1) a polypeptide including the amino acid sequence of SEQ ID NO: 2;

(2) a polypeptide that includes the amino acid sequence of SEQ ID NO: 2 in which one or more amino acids are substituted, deleted, inserted, and/or added and that has a biological activity equivalent to a protein consisting of the amino acid sequence of SEQ ID NO: 2;

(3) a polypeptide that shares at least 90%, 93%, 95%, 96%, 97%, 98% or 99% sequence identity with a polypeptide including the amino acid sequence of SEQ ID NO: 2 wherein the polypeptide has a biological activity equivalent to a polypeptide of the amino acid sequence of SEQ ID NO: 2; and

(4) a polypeptide encoded by a polynucleotide that hybridizes under stringent conditions to a polynucleotide consisting of the nucleotide sequence of SEQ ID NO: 1, wherein the polypeptide has a biological activity equivalent to a polypeptide consisting of the amino acid sequence of SEQ ID NO: 2;

with a test substance;

(b) detecting the phosphorylation level of the polypeptide;

(c) comparing the phosphorylation level of the polypeptide with the phosphorylation level of the polypeptide detected in the absence of the test substance; and

(d) selecting the test substance that reduced the phosphorylation level of the polypeptide as an inhibitor of the phosphorylation of the polypeptide or a candidate substance for treating or preventing cancer.

Herein, any cell may be used so long as it expresses the LHX4 polypeptide or functional equivalents thereof. The cell used in the present screening may be a cell naturally expressing the LHX4 polypeptide including, for example, cells derived from and cell-lines established from lung cancer and testis. Cell-lines of lung cancer such as A427, A549, LC319, PC-3, PC-9, PC-14, NCI-H1373, NCI-H1781, NCI-H358, NCI-H226, EBC-1, NCI-H520, NCI-H1703, NCI-H2170, RERF-LC-AI, DMS114, DMS273, SBC-3, SBC-5, NCI-H196, and NCI-H446 can be employed.

[0172] Alternatively, the cell used in the screening may be a cell that naturally does not express the LHX4 polypeptide and which is transfected with an LHX4 polypeptide- or an LHX4 functional equivalent-expressing vector. Such recombinant cells can be obtained through known genetic engineering methods (e.g., Morrison DA., J. Bacteriology 1977, 132: 349-51; Clark-Curtiss & Curtiss, Methods in Enzymology (eds. Wu et al.) 1983, 101: 347-62) as mentioned above.

[0173] Any of the aforementioned test substances may be used for the present screening. However, it is preferred to select substances that can permeate into a cell. Alternatively, when the test substance is a polypeptide, the contact of a cell and the test substance in the present screening can be performed by transforming the cell with a vector that includes the nucleotide sequence coding for the test substance and expressing the test substance in the cell.

- [0174] In another embodiment, conditions suitable for phosphorylation of LHX4 polypeptide or functional equivalents thereof can be provided in vitro. This screening method includes the steps of:
- (a) contacting a test substance with the LHX4 polypeptide or fragment thereof;
 - (b) detecting the phosphorylation of the polypeptide of step (a); and
 - (c) selecting a test substance that suppresses the phosphorylation level of the polypeptide in comparison with the phosphorylation level detected in the absence of the test substance.

The skilled artisan can estimate phosphorylation level of the LHX4 polypeptide using conventional methods for detection of phosphorylation level of a polypeptide.

- [0175] Accordingly, in these embodiments, the present invention provides a method of screening a substance for inhibiting the phosphorylation of LHX4 or a candidate substance for preventing and/or treating cancer including the steps of:
- (a) contacting a polypeptide selected from the group consisting of:
 - (1) a polypeptide including the amino acid sequence of SEQ ID NO: 2;
 - (2) a polypeptide that includes the amino acid sequence of SEQ ID NO: 2 in which one or more amino acids are substituted, deleted, inserted, and/or added and that has a biological activity equivalent to a protein consisting of the amino acid sequence of SEQ ID NO: 2
 - (3) a polypeptide that shares at least 90%, 93%, 95%, 96%, 97%, 98% or 99% sequence identity with a polypeptide including the amino acid sequence of SEQ ID NO: 2 wherein the polypeptide has a biological activity equivalent to a polypeptide of the amino acid sequence of SEQ ID NO: 2; and
 - (4) a polypeptide encoded by a polynucleotide that hybridizes under stringent conditions to a polynucleotide consisting of the nucleotide sequence of SEQ ID NO: 1, wherein the polypeptide has a biological activity equivalent to a polypeptide consisting of the amino acid sequence of SEQ ID NO: 2; or a fragment thereof including a phosphorylation site
 - with a test substance under a condition that allows phosphorylation of the polypeptide;
 - (b) detecting the phosphorylation level of the polypeptide or the fragment thereof;
 - (c) comparing the phosphorylation level of the polypeptide with the phosphorylation level of the polypeptide detected in the absence of the test substance; and
 - (d) selecting the test substance that reduced the phosphorylation level of the polypeptide as a substance for inhibiting the phosphorylation of the polypeptide or a candidate substance for treating and/or preventing cancer.

In these embodiments, a condition that allows phosphorylation of LHX4 polypeptide can be provided by incubating the polypeptide with suitable kinase for phosphorylation

the LHX4 polypeptide and ATP. In some embodiments, a substance enhancing phosphorylation of the LHX4 polypeptide can be added to the reaction mixture of screening. When phosphorylation of the polypeptide is enhanced by the addition of the substance, the phosphorylation level can be determined with higher sensitivity.

The phosphorylation level of LHX4 polypeptide or functional equivalent thereof may be detected according to any method known in the art (e.g. see Examples).

[0176] Dominant negative polypeptides

As demonstrated herein, a fragment of LHX4 polypeptide that has the amino acid sequence of SEQ ID NO: 21 effectively suppresses growth of lung cancer cells (Figs. 8C and 8D). Accordingly, the present invention also provides polypeptides that has the amino acid sequence of SEQ ID NO: 21, which has the function to inhibit an activity of the LHX4 polypeptide.

The polypeptides of the present invention can be of any length, so long as the polypeptides retain the function to inhibit an activity of the LHX4 polypeptide. Specifically, the length of the amino acid sequence may range from 21 to 70 residues, for example, from 21 to 50, preferably from 21 to 30, more specifically from 21 to 25 amino acid residues.

[0177] Furthermore, the present invention relates to variants of the polypeptide having the amino acid sequence of SEQ ID NO: 21. In the present invention, the variants can be those which contain any mutations selected from addition, deletion, substitution and insertion of one, two or several amino acid residues and are functionally equivalent to the polypeptide having the amino acid sequence of SEQ ID NO: 21. The phrase "functionally equivalent to the polypeptide having the amino acid sequence of SEQ ID No: 21" refers to having the function to inhibit an activity of the LHX4 polypeptide. In general, the modifications of one, two or several amino acids in a polypeptide will not influence the function of the polypeptide, and in some cases will even enhance the desired function of the original polypeptide (See, "Genes and Polypeptides"). Thus, the polypeptide of the present invention encompass polypeptides that have an amino acid sequence in which one, two or several amino acids are substituted, deleted, inserted and/or added in the amino acid sequence of SEQ ID NO: 21 and have the function to inhibit an activity of the LHX4 polypeptide. In preferred embodiments, the modifications in the amino acid sequence of SEQ ID NO: 21 can be conservative amino acid substitutions (See, "Genes and Polypeptides"). However, the polypeptides of the present invention are not restricted thereto and may include non-conservative modifications, so long as the modified polypeptide retains the function to inhibit an activity of the LHX4 polypeptide. Furthermore, modified polypeptides should not exclude inhibitory polypeptides of polymorphic variants, interspecies homologues, and alleles of LHX4. To retain the function to inhibit the binding between the LHX4 polypeptide and

Ku protein, one can modify (insert, add, delete and/or substitute) a small number (for example, 1, 2 or several) or a small percentage of amino acids. Herein, the term "several" means 5 or fewer amino acids, for example, 4 or 3 or fewer. The percentage of amino acids to be modified is preferably 20% or less, more preferably 15% or less, and even more preferably 10% or less or 1 to 5%.

Alternatively, the number of amino acids that may be mutated is not particularly restricted, so long as the modified polypeptide retains the function to inhibit an activity of the LHX4 polypeptide. Likewise, the site of mutation is not particularly restricted, so long as the mutation does not result in the disruption of the function to inhibit an activity of the LHX4 polypeptide.

- [0178] As demonstrated in Examples, the amino acid sequence of SEQ ID NO: 21 corresponds to a binding domain to Ku protein of the LHX4 polypeptide (Fig. 8). Therefore, it is considered that the polypeptide having the amino acid sequence of SEQ ID NO: 21 competitively inhibits the binding of the LHX4 polypeptide to Ku protein and consequently, inhibits cell proliferation promoting activity of the LHX4 polypeptide. Accordingly, in some embodiments, the activities of the LHX4 polypeptide to be inhibited by the polypeptide of the present invention include cell proliferation promoting activity and binding activity to the Ku protein. Detection of cell proliferation promoting activity and binding activity to the Ku protein can be carried out by methods known in the art, for example, methods described above section "Screening for Anti-Lung Cancer Substances, (ii) Screening for a Substance that Suppresses the Biological Activity of LHX4: and (iii) Screening for a Substance that Inhibits the Binding between LHX4 and Ku protein:".

- [0179] The polypeptides of the present invention can be chemically synthesized. Methods used in the ordinary peptide chemistry can be used for the method of synthesizing polypeptides (See, "Screening for Anti-Lung Cancer Substances, I. Protein based screening methods"). Alternatively, the polypeptides of the present invention can be also prepared by known genetic engineering techniques (See, "Screening for Anti-Lung Cancer Substances, I. Protein based screening methods").

When genetic engineering techniques are used, the polypeptide of the present invention can be expressed as a fused protein with a peptide having a different amino acid sequence. A vector expressing a desired fusion protein can be obtained by linking a polynucleotide encoding the polypeptide of the present invention to a polynucleotide encoding a different peptide so that they are in the same reading frame, and then introducing the resulting nucleotide into an expression vector. The fusion protein is expressed by transforming an appropriate host with the resulting vector. Different peptides to be used in forming fusion proteins include the following peptides: FLAG (Hopp et al., (1988) BioTechnology 6, 1204-10), 6xHis consisting of six His (histidine)

residues, 10xHis, Influenza hemagglutinin (HA), Human c-myc fragment, VSV-GP fragment, p18 HIV fragment, T7-tag, HSV-tag, E-tag, SV40T antigen fragment, lck tag, alpha-tubulin fragment, B-tag, Protein C fragment, GST (glutathione-S-transferase), HA (Influenza hemagglutinin), Immunoglobulin constant region, beta-galactosidase, and MBP (maltose-binding protein).

- [0180] The polypeptide of the present invention can be obtained by treating the fusion protein thus produced with an appropriate protease, and then recovering the desired polypeptide. To purify the polypeptide, the fusion protein is captured in advance with affinity chromatography that binds with the fusion protein, and then the captured fusion protein can be treated with a protease. With the protease treatment, the desired polypeptide is separated from affinity chromatography, and the desired polypeptide with high purity is recovered.
- [0181] Furthermore, the polypeptides of the present invention may be modified by being linked to other substances. The other substances include organic compounds such as peptides, lipids, saccharides, and various naturally-occurring or synthetic polymers. The polypeptides of the present invention may be linked to any other substances so long as the polypeptides retain the function to inhibit an activity of the LHX4 polypeptide. Modifications can also confer additive functions on the polypeptides of the present invention. Examples of the additive functions include, but are not limited to, targetability, deliverability, permeability and stability.
- Examples of modifications in the present invention include the introduction of a cell-membrane permeable substance. Usually, the intracellular structure is cut off from the outside by the cell membrane. Therefore, it is difficult to efficiently introduce an extracellular substance into cells. Cell membrane permeability can be conferred on the polypeptides of the present invention by modifying the polypeptides with a cell-membrane permeable substance. As a result, by contacting the polypeptide of the present invention with a cell, the polypeptide can be delivered into the cell to act thereon.
- [0182] As used herein, the phrase "cell-membrane permeable substance" refers to a substance capable of penetrating the mammalian cell membrane to enter the cytoplasm. For example, a certain liposome fuses with the cell membrane to release the content into the cell. Meanwhile, a certain type of polypeptide penetrates the cytoplasmic membrane of mammalian cell to enter the inside of the cell. In one embodiment, the polypeptides of the present invention have the following general formula:

[R]-[D];

wherein,

[R] represents a cell-membrane permeable substance; [D] represents a polypeptide comprising the amino acid sequence of (a) or (b) below:

- (a) the amino acid sequence of SEQ ID NO: 21;
- (b) the amino acid sequence in which one, two or several amino acid is substituted, deleted, inserted and/or added in the amino acid sequence of SEQ ID NO: 21.

In the above-described general formula, [R] and [D] can be linked directly or indirectly through a linker. Peptides, compounds having multiple functional groups, or such can be used as a linker. Specifically, amino acid sequences containing -G- can be used as a linker. Alternatively, a cell-membrane permeable substance and the polypeptide can be bound to the surface of a minute particle. [R] can be linked to any positions of [D]. Specifically, [R] can be linked to the N terminal or C terminal of [D], or to a side chain of amino acids constituting [D]. Furthermore, more than one [R] molecule can be linked to one molecule of [D]. The [R] molecules can be introduced to different positions on the [D] molecule. Alternatively, [D] can be modified with a number of [R]s linked together.

- [0183] There have been reported a variety of naturally-occurring or artificially synthesized polypeptides having cell-membrane permeability (Joliot A. & Prochiantz A., Nat Cell Biol. 2004; 6: 189-96). All of these known cell-membrane permeable substances can be used for the polypeptides in the present invention. Examples of cell-membrane permeable substances include, for example:

poly-arginine; Matsushita et al., (2003) J. Neurosci.; 21, 6000-7.
 Tat / RKKRRQRRR (SEQ ID NO: 23) (Frankel et al., (1988) Cell 55, 1189-93., Green & Loewenstein (1988) Cell 55, 1179-88.);
 Penetratin / RQIKIWFQNRRMKWKK (SEQ ID NO: 24)(Derossi et al., (1994) J. Biol. Chem. 269, 10444-50.);
 Buforin II / TRSSRAGLQFPVGRVHRLRK (SEQ ID NO: 25)(Park et al., (2000) Proc. Natl Acad. Sci. USA 97, 8245-50.);
 Transportan / GWTLNSAGYLLGKINLKALAALAKKIL (SEQ ID NO: 26)(Pooga et al., (1998) FASEB J. 12, 67-77.);
 MAP (model amphipathic peptide) / KLALKLALKALKALKLA (SEQ ID NO: 27)(Oehlke et al., (1998) Biochim. Biophys. Acta. 1414, 127-39.);
 K-FGF / AAVALLPAVLLALLAP (SEQ ID NO: 28)(Lin et al., (1995) J. Biol. Chem. 270, 14255-8.);
 Ku70 / VPMLK (SEQ ID NO: 29)(Sawada et al., (2003) Nature Cell Biol. 5, 352-7.);
 Ku70 / PMLKE (SEQ ID NO: 30)(Sawada et al., (2003) Nature Cell Biol. 5, 352-7.);
 Prion / MANLGYWLLALFVTMWTDVGLCKKRKPK (SEQ ID NO: 31)(Lundberg et al., (2002) Biochem. Biophys. Res. Commun. 299, 85-90.);
 pVEC / LLIILRRRIRKQAHHSK (SEQ ID NO: 32)(Elmquist et al., (2001) Exp. Cell Res. 269, 237-44.);
 Pep-1 / KETWWETWWTEWSQPKKRKV (SEQ ID NO: 33)(Morris et al., (2001)

Nature Biotechnol. 19, 1173-6.);
SynB1 / RGGRLSYSRRRFSTSTGR (SEQ ID NO: 34)(Rousselle et al., (2000) Mol. Pharmacol. 57, 679-86.);
Pep-7 / SDLWEMMMVSLACQY (SEQ ID NO: 35)(Gao et al., (2002) Bioorg. Med. Chem. 10, 4057-65.); and
HN-1 / TSPLNIHNGQKL (SEQ ID NO: 36)(Hong & Clayman (2000) Cancer Res. 60, 6551-6.).

The poly-arginine, which is listed above as an example of cell-membrane permeable substances, may be constituted by any number of arginine residues. Specifically, for example, it may be constituted by consecutive 5-20 arginine residues. The preferable number of arginine residues is 11 (SEQ ID NO: 37).

[0184] Compositions and Methods for treating cancer using dominant negative polypeptides

The polypeptides of the present invention inhibit proliferation of cancer cells. Therefore, the present invention provides pharmaceutical compositions for treating and/or preventing cancer which contains as an active ingredient the polypeptide of the present invention; or a polynucleotide encoding the same. Alternatively, the present invention relates to methods for treating and/or preventing cancer including the step of administering the polypeptide of the present invention to a subject. Furthermore, the present invention relates to the use of the polypeptides of the present invention in manufacturing pharmaceutical compositions for treating and/or preventing cancer. Cancers to be treated or prevented by the present invention are not limited, so long as expression of LHX4 is up-regulated in the cancer cells. For example, the polypeptides of the present invention are useful for treating lung cancer.

[0185] Alternatively, the polypeptides of the present invention can be used to suppress growth of cancer cells. Therefore, the present invention provides compositions for suppressing cancer cell growth, which contain as an active ingredient the polypeptide of the present invention; or a polynucleotide encoding the same. Alternatively, the present invention relates to methods for suppressing cancer cell growth which include the step of administering the polypeptides of the present invention. Furthermore, the present invention relates to the use of polypeptides of the present invention in manufacturing pharmaceutical compositions for suppressing cancer cell growth.

When the polypeptides of the present invention are administered, as a prepared pharmaceutical, to human and other mammals such as mouse, rat, guinea pig, rabbit, cat, dog, sheep, pig, cattle, monkey, baboon and chimpanzee for treating and/or preventing cancer, the polypeptides of the present invention can be administered directly, or formulated into an appropriate dosage form using known methods for preparing pharmaceuticals. For example, if necessary, the pharmaceuticals can be orally administered as a sugar-coated tablet, capsule, elixir, and microcapsule, or alternatively parenterally

administered in the injection form that is a sterilized solution or suspension with water or any other pharmaceutically acceptable liquid. For example, the polypeptides of the present invention can be mixed with pharmacologically acceptable carriers or media, specifically sterilized water, physiological saline, plant oil, emulsifier, suspending agent, surfactant, stabilizer, corrigent, excipient, vehicle, preservative, and binder, in a unit dosage form necessary for producing a generally accepted pharmaceutical.

Depending on the amount of active ingredient in these formulations, a suitable dose within the specified range can be determined.

Examples of additives that can be mixed in tablets and capsules are binders such as gelatin, corn starch, tragacanth gum, and gum arabic; media such as crystalline cellulose; swelling agents such as corn starch, gelatin, and alginic acid; lubricants such as magnesium stearate; sweetening agents such as sucrose, lactose or saccharine; and corrigents such as peppermint, wintergreen oil and cherry. When the unit dosage form is capsule, liquid carriers such as oil can be further included in the above-described ingredients. Sterilized mixture for injection can be formulated using media such as distilled water for injection according to the realization of usual pharmaceuticals.

Physiological saline, glucose, and other isotonic solutions containing adjuvants such as D-sorbitol, D-mannose, D-mannitol, and sodium chloride can be used as an aqueous solution for injection. They can be used in combination with a suitable solubilizer, for example, alcohol, specifically ethanol and polyalcohols such as propylene glycol and polyethylene glycol, non-ionic surfactants such as Polysorbate 80TM and HCO-50.

Sesame oil or soybean oil can be used as an oleaginous liquid, and also used in combination with benzyl benzoate or benzyl alcohol as a solubilizer. Furthermore, they can be further formulated with buffers such as phosphate buffer and sodium acetate buffer; analgesics such as procaine hydrochloride; stabilizers such as benzyl alcohol and phenol; and antioxidants. Injections thus prepared can be loaded into appropriate ampoules.

- [0186] Methods well-known to those skilled in the art can be used for administering pharmaceutical compositions of the present invention to subjects, for example, by intraarterial, intravenous, or subcutaneous injection, and similarly, by intranasal, transtracheal, intramuscular, or oral administration. Doses and administration methods are varied depending on the body weight and age of patients as well as administration methods. However, those skilled in the art can routinely select them. DNA encoding the polypeptide of the present invention can be inserted into a vector for the gene therapy, and the vector can be administered for treatment. Although doses and administration methods are varied depending on the body weight, age, and symptoms of patients, those skilled in the art can appropriately select them. For example, a dose of the polypeptide of the present invention is, when orally administered to a normal adult

(body weight 60 kg), about 0.1 mg to about 100 mg/day, usually about 1.0 mg to about 50 mg/day, more usually about 1.0 mg to about 20 mg/day, although it is slightly varied depending on symptoms.

When the polypeptide of the present invention is parenterally administered to a normal adult (body weight 60 kg) in the injection form, it is convenient to intravenously inject a dose of about 0.01 mg to about 30 mg/day, usually about 0.1 mg to about 20 mg/day, more usually about 0.1 mg to about 10 mg/day, although it is slightly varied depending on patients, target organs, symptoms, and administration methods. Similarly, the polypeptide of the present invention can be administered to other animals in an amount converted from the dose for the body weight of 60 kg.

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

[0187] Example 1: General Methods

1. Lung cancer cell lines and tissue samples.

The human lung-cancer cell lines used in this study included five adenocarcinomas (ADCs; A549, LC319, NCI-H1373, PC14, and NCI-H1781), five squamous cell carcinomas (SCCs; LU61, NCI-H520, NCI-H1703, NCI-H2170, SK-MES-1), one large cell carcinoma (LCC; LX1), and six small cell lung cancers (SCLCs; DMS114, DMS273, SBC-3, SBC-5, NCI-H196, and NCI-H446). Human SAEC and BEAS-2B cells were used as controls (Table 1). Human small airway epithelial cells (SAEC) were grown in optimized medium purchased from Cambrex Bio Science, Inc. Primary lung cancer tissue samples had been obtained with informed consent as described previously (Kikuchi T, et al. Oncogene 2003;22:2192-205., Taniwaki M, et al. Int J Oncol 2006;29:567-75.). The other 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.

[Table 1]

List of lung cancer cell lines used for analyses

Cell line	Resource	Histology
SAEC	CAMBREX	airway epithelia
BEAS-2B	ATCC (American Type Culture Collection)	human bronchial epithelial cell
NCI-H1781	ATCC (American Type Culture Collection)	ADC
NCI-H1373	ATCC (American Type Culture Collection)	ADC
A549	ATCC (American Type Culture Collection)	ADC
LC319	Aichi cancer center	ADC
PC14	RIKEN BRC(BioResource Center)	ADC
SK-MES-1	ATCC (American Type Culture Collection)	SCC
NCI-H520	ATCC (American Type Culture Collection)	SCC
NCI-H1703	ATCC (American Type Culture Collection)	SCC
NCI-H2170	ATCC (American Type Culture Collection)	SCC
LU61	CIEA (Central Institute for Experimental Animals)	SCC
LX1	CIEA (Central Institute for Experimental Animals)	LCC
DMS114	ATCC (American Type Culture Collection) or Cancer Institute of Japanese foundation for cancer research	SCLC
DMS273	ECACC (European Collection of Animal Cell Cultures) or Cancer Institute of Japanese foundation for cancer research	SCLC
SBC-3	JCRB (Japanese Collection of Research Bioresources) or University of Tokushima	SCLC
SBC-5	JCRB (Japanese Collection of Research Bioresources) or University of Tokushima	SCLC
H196	Cancer Institute of Japanese foundation for cancer research	SCLC
H446	Cancer Institute of Japanese foundation for cancer research	SCLC

ADC adenocarcinoma , AS adenosquamous cell carcinoma ,

SCC squamous cell carcinoma , LCC large cell carcinoma,

SCLC small cell lung carcinoma

[0188] 2. Semiquantitative reverse transcription-PCR

Total RNA was extracted from cultured cells using the TRIzol reagent (Life Technologies, Inc.) according to the manufacturer's protocol. Extracted RNAs were treated with DNase I (Nippon Gene) and reversely transcribed using oligo(dT) primer and SuperScript II. Semiquantitative reverse transcription-PCR (RT-PCR) experiments were carried out with the following synthesized LHX4-specific primers or with beta-actin (ACTB)-specific primers as an internal control:

LHX4, 5'- GATCGAAAGTACGCCAATGTG-3' (SEQ ID NO: 3) and 5'-TGTGAGGCCTCTACCTTAGGAGTC-3' (SEQ ID NO: 4); ACTB, 5'-GAGGTGATAGCATTGCTTCG-3' (SEQ ID NO: 5) and 5'-CAAGTCAGTGTACAGGTAAGC-3' (SEQ ID NO: 6).

PCR reactions were optimized for the number of cycles to ensure product intensity within the logarithmic phase of amplification.

[0189] 3. Northern blot analysis

Human multiple-tissue blots (BD Biosciences Clontech) were hybridized with a ³²P-labeled PCR product of LHX4. The cDNA probe of LHX4 was prepared by RT-PCR using the following synthesized LHX4-specific primers: LHX4, 5'-CCAAAGAGAACGCCCTGAAG-3' (SEQ ID NO: 7) and 5'-CTGGGCTAGTTGGAAAGTCG-3' (SEQ ID NO: 8). Prehybridization, hybridization, and washing were done according to the supplier's recommendations. The blots were autoradiographed at room temperature for 30 hours with intensifying BAS screens (Bio-Rad).

[0190] 4. Anti-LHX4 Antibodies

Affinity-purified anti-LHX4 antibodies were produced using standard method after successive inoculation of soluble partial recombinant protein (Carboxyl-terminus of LHX4) into rabbits. It was confirmed that the antibody was specific to LHX4 on Western blots using lysates from cell lines that had been transfected with LHX4 expression vector and those from lung cancer cell lines, either of which expressed LHX4 endogenously or not, as well as by immunocytochemical staining of the cell lines.

[0191] 5. Immunofluorescence analysis

Cells were plated on glass coverslips (Becton Dickinson Labware), fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100 in PBS for 3 minutes at room temperature. Non-specific binding was blocked by CASBLOCK (ZYMED) for 10 minutes at room temperature. Cells were then incubated for 60 minutes at room temperature with a mouse monoclonal anti-Flag M2 antibody (sigma-aldrich) as a primary antibody diluted in PBS containing 3% BSA. Alternatively, Cells were then incubated for 60 minutes at room temperature with rabbit polyclonal anti-LHX4 antibody (generated to recombinant LHX4; please see above) as a primary antibody diluted in Antibody Diluent (DAKO). After being washed with PBS, the cells were stained by Alexa Fluor 488-conjugated secondary antibody (Molecular Probes) for 60 min at room temperature. After another wash with PBS, each specimen was mounted with Vectashield (Vector Laboratories, Inc.) containing 4', 6'-diamidino-2'-phenylindolendihydrochloride (DAPI) and visualized with Spectral Confocal Scanning Systems (TSC SP2 AOBS: Leica Microsystems).

[0192] 6. RNA interference assay

A vector-based RNA interference system, psiH1BX3.0 that was designed to synthesize small interfering RNAs (siRNA) has been established in mammalian cells (Suzuki C, et al., Cancer Res 2003;63:7038-41.). Ten micrograms of siRNA expression vector were transfected using 30 micro-L of LipofectAMINE 2000 (Invitrogen) into SCLC cell lines SBC-3 and SBC-5. The transfected cells were cultured for 7 days in the presence of appropriate concentrations of geneticin (G418); the number of colonies was counted by Giemsa staining; and viability of cells was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5 -diphenyltetrazolium bromide assay at 7 days after the treatment. 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 2 hours. Absorbance was then measured at 490 nm, and at 630 nm as a reference, with a Microplate Reader 550 (Bio-Rad). To confirm suppression of LHX4 mRNA expression, semiquantitative RT-PCR experiments were carried out. The target sequences of the synthetic oligonucleotides for RNA interference were as follows: control 1 (luciferase/LUC: *Photinus pyralis* luciferase gene), 5'-CGTACGCGGAATACTTCGA-3' (SEQ ID NO: 9); control 2 (scramble/SCR: chloroplast *Euglena gracilis* gene coding for 5S and 16S (rRNAs)), 5'-GCGCGCTTGTAGGATTG-3' (SEQ ID NO: 10); siRNA-LHX4-#A, 5'-GCAGTGTAGGCTATCCGA-3' (SEQ ID NO: 11); siRNA-LHX4-#B, 5'-CTAGCCCAGGCTTGGCT-3' (SEQ ID NO: 12); siRNA-Ku70, 5'-AAGCAATGAATAAAAGACT-3' (SEQ ID NO: 13); siRNA-Ku86, 5'- GCATAACTATGAGTGTAA-3' (SEQ ID NO: 14).

[0193] 7. 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). Protein samples were separated by SDS-polyacrylamide gels and electroblotted onto Hybond-ECL nitrocellulose membranes (GE Healthcare Bio-Sciences). Blots were incubated with a mouse monoclonal anti-myc antibody or rabbit polyclonal anti-LHX4 antibody, anti-Flag M2 antibody, anti-Ku70 antibody (santa cruz, sc-12729) and anti-Ku86 antibody (santa cruz, sc-56132). 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).

[0194] 8. Cell-growth assay

The entire coding sequence of LHX4 was cloned into the appropriate site of pcDNA3.1 myc/His plasmid vector (Invitrogen). COS-7 and, SBC-3 cells transfected either with plasmids expressing myc-tagged LHX4 or with mock plasmids were grown

for eight days in DMEM and RPMI respectively 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 2 hours. Absorbance was then measured at 450 nm as a reference, with a Microplate Reader 550 (BIO-RAD)

Alternatively, the entire coding sequence of LHX4 was cloned into the appropriate site of pCAGGSn3FC plasmid vector. COS-7 and HEK293 cells transfected either with plasmids expressing flag-tagged LHX4 or with mock plasmids were grown for eight days in DMEM and RPMI respectively 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 2 hours. Absorbance was then measured at 490 nm as a reference, with a Microplate Reader 550 (BIO-RAD)

[0195] 9. Immunoprecipitation and mass spectrometry

SBC-3 cells were plated onto 15-cm dishes (1 X 10⁶ cells/dish) and transfected with 20 micro-g of pCAGGSn3Fc-Mock (without insertion) or pCAGGSn3Fc-LHX4 using FuGENE6 reagent (Roche). After 48 h incubation, the cells were lysed with 0.1% NP-40 lysis buffer as described above. The cell lysates were precleaned with normal mouse IgG and rec-Protein G Sepharose 4B (Zymed) at 4 degrees C for 1 hour. Subsequently, the lysates were incubated with anti-Flag M2 agarose (Sigma-Aldrich) at 4 degrees C for 1 hour. After washing five times with lysis buffer, the protein samples were separated by SDS-PAGE (4-12% Bis-Tris gel) (Invitrogen). Proteins in polyacrylamide gel were silver stained with the SilverQuest Silver Staining Kit (Invitrogen). Bands that were specifically observed in the LHX4-transfected lane were excised, and the extracted proteins were subjected to PMF (peptide mass fingerprint) analysis using MALDI TOF-MS (Shimadzu Biotech).

[0196] 10. Flow Cytometry

Cells were collected in PBS, and fixed in cold 70% ethanol solution for 30 min. After treatment with 100 g /ml RNase (Sigma-Aldrich), the cells were stained with 50 g /ml propidium iodide (Sigma-Aldrich) in PBS. Flow cytometry was done on a Cell Lab QuantaTM SC (BECKMAN COULTER) and analyzed by CXP Analysis software ver.2.2 (BECKMAN COULTER). The cells selected from at least 15,000 cells were analyzed for DNA content.

[0197] 11. Cell cycle arrest and synchronization

SBC-5 cells were enriched in cell cycle G1/S phase by aphidicolin treatment. Synchronous cultures were obtained by releasing cultures from 3, 6, 9, 12, and 15 hours

after aphidicolin treatment (2 micro-g/ml).

[0198] 12. Dominant-Negative Peptide Assay

Twenty one-amino acid sequence derived from minimized Ku proteins-binding domain in LHX4 (codons 157-219; see Fig. 8A) was covalently linked at its N-terminus to a membrane transducing 11 poly-arginine sequence (11R) as described elsewhere (Hayama S, et al. Cancer Res 2006;66:10339-48., Hayama S, et al. Cancer Res 2007; 67:4113-22.). Three dominant-negative peptides were synthesized covering the codons 157-219 region: 11R-P1 157-177, RRRRRRRRRRR (SEQ ID NO: 37)-GGG-AKRPRTTITAKQLETLKNAYK (SEQ ID NO: 19); 11R-P2 178-198, RRRRRRRRRRR-GGG-NSPKPARHVREQLSSETGLDM (SEQ ID NO: 20); 11R-P3 199-219 RRRRRRRRRRR-GGG-RVVQVWFQNRRAKEKRLKKDA (SEQ ID NO: 21). Peptides were purified by preparative reverse-phase high-performance liquid chromatography to make >95% purity. Lung cancer DMS114 cells that expressed both LHX4 and Ku70/86 as well as normal human airway epithelia BEAS-2B cells that did not express LHX4 were incubated with the 11R peptides at the concentration of 5, 10, or 15 micro-M for 4 days. The viability of cells was evaluated by MTT assay at 4 days after the treatment.

[0199] Example 2: LHX4 expression in lung tumors and normal tissues

To search for novel target molecules for the development of therapeutic agents and/or diagnostic biomarkers for lung cancer, at first cDNA microarray analysis was performed to screen genes that showed 3-fold or higher level of expression in more than half of 120 lung cancer samples (Daigo Y, Nakamura Y. Gen Thorac Cardiovasc Surg 2008;56:43-53., Kikuchi T, et al. Oncogene 2003;22:2192-205., Kakiuchi S, et al. Mol Cancer Res 2003;1:485-99., Kakiuchi S, et al. Hum Mol Genet 2004;13:3029-43., Kikuchi T, et al. Int J Oncol 2006; 28:799-805., Taniwaki M, et al. Int J Oncol 2006;29:567-75.). Among 27,648 genes screened, the overexpression of LHX4 was identified in the great majority of small cell lung cancers (SCLCs) examined, and its transactivation was confirmed by semiquantitative RT-PCR experiments in 8 of 10 additional SCLC tissues and in 5 of 6 SCLC cell lines (Figs. 1A and 1B). On the other hand, the LHX4 was overexpressed in 3 of 10 non-small cell lung cancers (NSCLCs) (2 of 5 ADCs; 1 of 5 SCCs; Figs. 1E and 1F). It was also confirmed that overexpression of LHX4 protein (55 kDa) in lung cancer cell lines by Western blot analysis using anti-LHX4 polyclonal antibody (Fig. 1G). Northern-blot analysis showed that LHX4 was not expressed in normal tissues except testis (Fig. 1C). To determine the subcellular localization of endogenous LHX4 in lung cancer cells, immunofluorescence analysis was performed to examine the subcellular localization of exogenously expressed LHX4 protein in COS-7 cells. LHX4 was detected in nucleus (Fig. 1D). Further, the present inventors did immunocytochemical analysis using anti-

LHX4 polyclonal antibodies; LHX4 protein was localized in the nucleus of DMS114 cells which express LHX4, but was not detectable in BEAS-2B cells not expressing LHX4 (Fig. 1H). The present inventors subsequently examined expression of LHX4 protein in five normal tissues (heart, liver, lung, kidney, and testis), as well as lung cancers using anti-LHX4 polyclonal antibody, and found that it was hardly detectable in the former four tissues, whereas positive LHX4 staining was detected at nucleus of testis and lung cancer tissues (Fig. 7A). The expression levels of LHX4 protein in lung cancer were significantly higher than those in testis. The present inventors also evaluated LHX4 staining in lung cancers and adjacent normal lung tissues, and confirmed LHX4 protein to be positively stained in the majority of NSCLC tissues, but not in their corresponding normal lungs (Fig. 7B).

[0200] Example 3: Inhibition of growth of lung cancer cells by siRNA against LHX4

To assess whether LHX4 is essential for growth or survival of lung cancer cells, the plasmids to express siRNAs against LHX4 (si-LHX4-#A and -#B) as well as control plasmids (siRNAs for luciferase (LUC) and SCR) were constructed and transfected into SBC-3 and SBC-5 cells, which expressed LHX4. The LHX4-mRNA levels in cells transfected with si-LHX4-#A or -#B were significantly decreased in comparison with cells transfected with either control siRNAs (Fig. 2A). Significant decreases in the numbers of viable cells transfected with si-LHX4-#A or -#B were observed (Figs. 2B and 2C).

[0201] Example 4: Growth-promoting effect of LHX4

To elucidate a potential role of LHX4 in tumorigenesis, plasmids designed to express either LHX4 (pcDNA3.1/myc-His A vector) or mock vector were prepared. To determine the effects of LHX4 on growth of mammalian cells and human lung cancer cells, transient transfectants were generated using mammalian COS-7 cells or lung cancer SBC-3 cells which scarcely or weakly expressed endogenous LHX4. Further, the present inventors prepared plasmids designed to express either LHX4 (pCAGGSn3Fx vector) or mock vector, and generated transfectants using mammalian COS-7 cells or Human Embryonic Kidney 293 (HEK293) cells which scarcely expressed endogenous LHX4. All cells that transiently expressed exogenous LHX4 showed more rapid growth compared with the mock-transfected cells (Figs. 3A-D).

[0202] Example 5: Phosphorylation of LHX4

LHX4 protein was detected as double bands by Western blotting, indicating a possible modification of the LHX4 protein. Therefore, at first, extracts from COS-7 cells transfected with LHX4-expressing plasmids were incubated in the presence or absence of protein phosphatase, and analyzed the molecular size of LHX4 protein by Western blot analysis. The measured weight of the majority of exogenous LHX4 protein in the extracts treated with phosphatase was smaller than that in the untreated

cells. The data indicated that LHX4 was possibly phosphorylated in cells (Fig.9).

- [0203] Example 6: Identification of Ku70 and Ku86 protein as LHX4-interacting proteins.
Since the biological functions of LHX4 in cancer cells are totally unknown, proteins interacting with LHX4 were examined by immunoprecipitation and mass spectrometric analyses. Lysates of SBC-3 cells transfected with a pCAGGSn3Fc-LHX4 vector or a pCAGGSn3Fc-Mock (mock control) were extracted and immunoprecipitated with anti-Flag M2 monoclonal antibody. Protein complexes were silver-stained on SDS-PAGE gels. An about 85kDa protein, which was seen in immunoprecipitates of cell lysates transfected with the Flag-tagged LHX4 plasmid but not in those with mock control plasmid, was extracted and its peptide sequences were determined by mass-spectrometric analysis. This approach identified Ku86 protein as a candidate protein interacting with LHX4. To investigate the biological significance of their interaction, lysates of SBC-5 cells transfected with a pCAGGSn3Fc-LHX4 vector or a pCAGGSn3Fc-Mock were extracted and immunoprecipitated with anti-Flag M2 monoclonal antibody. Immunoblot of the precipitates using anti-Ku86 antibody indicated that LHX4-Flag was coprecipitated with endogenous Ku86 protein (Fig. 4A, left panels). Since Ku86 protein was known to form heterodimers with Ku70 protein, immunoblotting of the precipitates were performed using anti-Ku70 antibody, and identified that LHX4-Flag was also coprecipitated with endogenous Ku70 protein (Fig. 4A, right panels). It was further confirmed the co-localization of exogenous LHX4 protein and endogenous Ku70/86 proteins in nucleus of SBC-5 cells by immunocytochemical analysis (Fig. 4B).

- [0204] Example 7: The levels of Ku70/Ku86 and LHX4 proteins were elevated in S phase.
To analyze the functional role of LHX4-Ku70/86 interaction, LHX4-transfected SBC-5 cells were synchronized by aphidicolin in G1 phase and detected the levels of LHX4 and Ku86 after the release from G1 arrest. 6 hours after release of aphidicolin, the levels of LHX4 proteins started to increase, while Ku86 proteins were elevated at 3 hours (Fig. 5A). The mRNA levels of LHX4 and Ku86 did not change during the cell cycle (Fig. 5B). Flowcytometric analysis indicated that LHX4 and Ku86 proteins were increased around S phase (Fig. 5C). Furthermore, the Ku70 or Ku86 protein expression was knocked down by siRNA. As previously reported, reduction of Ku70 expression reduced the levels of Ku86 protein, while inhibition of Ku86 expression decreased Ku70 protein. Interestingly, LHX4 proteins were also decreased by siRNA for Ku70 or Ku86 (Fig. 6A left panels), whereas LHX4 transcript levels were not changed by these siRNA transfection (Fig. 6A right panels). On the other hand, introduction of Ku proteins into cells increased the level of LHX4 proteins (Fig. 6B). These results indicate that the interaction of LHX4 with Ku70/Ku86 proteins are needed for its protein stabilization.

[0205] Example 8: Growth Inhibition of Lung Cancer Cells by Dominant-Negative Peptides of LHX4.

To further investigate the biological importance of the interaction of these proteins, either of three partial constructs of LHX4 with Flag sequence at its N-terminus (L1 1-156, L2 157-219, and L3 220-390; Fig. 8A) was transfected into HEK293 cells. Immunoprecipitation with Flag M2 agarose indicated that only L2 157-219 construct which containing HOX domain was able to interact with endogenous Ku proteins (Fig. 8B). These data suggested that the 63-amino acid polypeptide (codons 157-219) in LHX4 should play an significant role in the interaction with Ku proteins.

To develop the bioactive cell-permeable peptides that can inhibit the functional interaction of LHX4 with Ku proteins, the present inventors synthesized three different kinds of 21-amino acid polypeptides covering the Ku-binding domain in L2 157-219 with a membrane-permeable 11 residues of arginine (11R) at its N-terminus (11R-P1 157-177, 11R-P2 178-198, and 11R-P3 199-219). To test the effect of these polyarginine-linked peptides on lung cancer cell growth/survival, the present inventors treated DMS114 with each of the three peptides. Addition of the 11R-P3 199-219 into the culture media resulted in significant decreases in cell viability, as measured by MTT assay (Fig. 8C). On the other hand, no effect on cell growth was observed when the cells were treated with the remaining two peptides (11R-P1 157-177 and 11R-P2 178-198). The 11R-P3 199-219 revealed no effect on cell viability of normal human epithelial cells BEAS-2B which LHX4 expression was hardly detectable (Fig. 8D). These data suggested that 11R-P3 199-219 peptides could inhibit a functional complex formation of LHX4 and Ku proteins and have no off-target toxic effect on normal human cells that do not express LHX4 protein.

[0206] Discussion

In the present invention, the treatment of lung cancer cells with specific siRNA to knockdown LHX4 expression resulted in suppression of cancer cell growth. In addition, evidence provided here establishes the significance of this pathway in carcinogenesis; for example, the expression of LHX4 resulted in significant promotion of the cellular growth in vitro. The results obtained here show that LHX4 is an important growth factor for lung cancer cells. Because LHX4 can be classified as a cancer antigen, selective inhibition of LHX4 activity by molecular targeted agents has a powerful biological activity against cancer with a minimal risk of adverse events.

LHX4 is a family member of LIM-homeodomain (LIM-HD) transcription factors. The LIM domain, known as a multifunctional protein-protein interaction domain, was first recognized in several other members of this class of transcription factors: LIN11, ISL1, and MEC3 (Hunter et al., 2005. Mol. Biol. Rep. 32, 67-77.). The large LIM protein superfamily also includes cytoskeletal proteins, signaling cascade transducers,

and transcriptional coactivators. In mammals there are at least twelve LIM-HD genes encoding developmental regulatory proteins featuring two LIM domains and a DNA-binding HD. The functions of LIMHD proteins are impacted by interacting proteins, such as the NLI/LDB/CLIM, MRG1, SLB, and RLIM proteins (Bach, I., 2000. Mech. Dev 91, 5-17.). Of the mammalian LIM-HD proteins, ISL1, ISL2, LHX2, LHX3, and LHX4 have been implicated in pituitary development. Herein, it was demonstrated that Ku70 and Ku86 proteins are interacting partner of LHX4 proteins. Ku is a complex of two protein subunits of 70 and 86 kDa, herein designated as Ku70 and Ku86, respectively. It was shown that Ku is a DNA-binding component of a DNA-dependent protein kinase (DNA-PK) that phosphorylates many nuclear proteins in vitro, e.g., p53, Artemis, XRCC4, DNA-PKcs, or Ku itself, and is involved in DSB repair and V(D)J recombination (S.P. Lees-Miller, Biochem. Cell Biol. 74 (1996) 503-512., S.E. Critchlow, et al., Curr. Biol. 7 (1997) 588-598., R. Leber, et al., J. Biol. Chem. 273 (1998) 1794- 1801., Y. Ma, et al., Cell 108 (2002) 781-794.). In addition to this main function, the Ku protein has other functions, some of which may be independent of DNA-PK activity (A. Nussenzweig, et al., Nature 382 (1996) 551-555., Y. Gu, K.J. et al., Immunity 7 (1997) 653- 665., Y. Gao, et al., Immunity 9 (1998) 367- 376.). Ku is also a multifunctional protein, which plays a key role in telomere maintenance, transcription regulation, and replication. The data disclosed herein show that interaction of LHX4 with Ku70/Ku86 plays an important role for its stabilization in S phase. The inhibition of the interaction of these molecules with dominant negative cell permeable peptide of LHX4 resulted in suppression of cancer cell growth, indicating that this interaction has a crucial role in the growth of cancer cells. Importantly, this permeable peptide has no toxic effect on normal human cells that do not express LHX4. Specific inhibition of the LHX4-Ku complex as well as LHX4 function is an effective approach to treat lung cancer.

- [0207] In summary, activation of LHX4 has a specific functional role for growth of cancer cells that provides new approaches for designing new anticancer drugs to specifically target the oncogenic activity of LHX4 for treatment of lung cancer patients.

Industrial Applicability

- [0208] As demonstrated herein, cell growth is suppressed by a double-stranded molecule that specifically targets the LHX4 gene. Thus, the double-stranded molecules are useful candidates for the development of an anti-cancer pharmaceutical. For example, agents that block the expression of LHX4 protein and/or prevent its activity find therapeutic utility as anti-cancer agents, particularly anti-cancer agents for the treatment of lung cancer.

The expression of human gene LHX4 is markedly elevated in lung cancer, as

compared to normal organs. Accordingly, the gene can be conveniently used as diagnostic marker of lung cancer and the protein encoded thereby find utility in diagnostic assays of lung cancer.

Furthermore, the methods described herein are also useful in diagnosis of lung cancer. Moreover, the present invention provides a candidate for development of therapeutic approaches for cancer including lung cancers.

Furthermore, LHX4 polypeptide is a useful target for the development of anti-cancer pharmaceuticals. For example, agents that bind LHX4 or block the expression of LHX4 or prevent its activity find therapeutic utility as anti-cancer agents, particularly anti-cancer agents for the treatment of lung cancer.

While the invention has been described in detail and with reference to specific embodiments thereof, it will be apparent to one skilled in the art that various changes and modifications can be made therein without departing from the spirit and scope of the invention, the metes and bounds of which are set by the appended claims.

Claims

- [Claim 1] A method for diagnosing lung cancer, said method comprising the steps of:
- (a) determining the expression level of the gene in a subject-derived biological sample by any one of the method selected from the group consisting of:
 - (i) detecting an mRNA of a LHX4 gene;
 - (ii) detecting a LHX4 protein;
 - (iii) detecting a biological activity of the LHX4 protein; and
 - (b) correlating an increase in the expression level determined in step (a) as compared to a normal control level of the gene to the presence of lung cancer.
- [Claim 2] The method of claim 1, wherein the expression level determined in step (a) is at least 10% greater than the normal control level.
- [Claim 3] The method of claim 1, wherein the expression level determined in step (a) is determined by detecting the hybridization of an oligonucleotide that hybridizes to the mRNA of the LHX4 or the binding of an antibody against the LHX4 protein.
- [Claim 4] The method of claim 1, wherein the subject-derived biological sample comprises a biopsy specimen, sputum, blood, pleural effusion or urine.
- [Claim 5] A kit for diagnosing lung cancer, which comprises a reagent selected from the group consisting of:
- (a) a reagent for detecting mRNA of a LHX4 gene;
 - (b) a reagent for detecting a LHX4 protein; and
 - (c) a reagent for detecting the biological activity of the LHX4 protein.
- [Claim 6] The kit of claim 5, wherein the reagent comprises an oligonucleotide that hybridizes to the mRNA of the LHX4 gene.
- [Claim 7] The kit of claim 5, wherein the reagent comprises an antibody against the LHX4 protein.
- [Claim 8] An isolated double-stranded molecule that, when introduced into a cell, inhibits expression of a LHX4 gene, a Ku70 gene or a Ku86 gene as well as cell proliferation, said molecule comprising a sense strand and an antisense strand complementary thereto, said strands hybridized to each other to form the double-stranded molecule.
- [Claim 9] The double-stranded molecule of claim 8, wherein the sense strand comprises the sequence corresponding to a target sequence selected from the group consisting of SEQ ID NOS: 11, 12, 13 and 14.

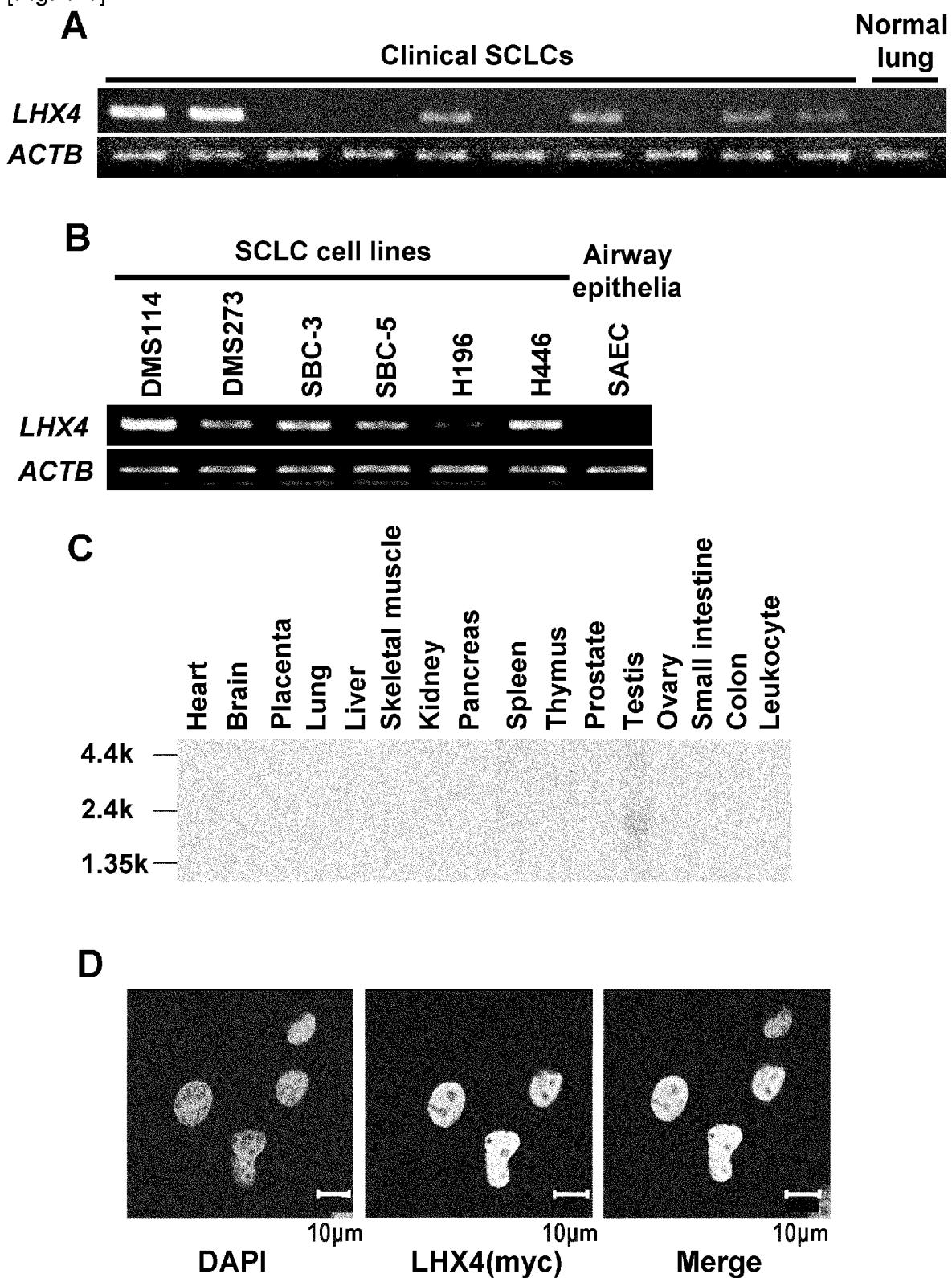
- [Claim 10] The double-stranded molecule of claim 8 or 9, wherein the sense strand hybridizes with the antisense strand at the target sequence to form the double-stranded molecule having between 19 and 25 nucleotide pairs in length.
- [Claim 11] The double-stranded molecule of any one of claims 8 to 10, which consists of a single polynucleotide comprising both the sense and antisense strands linked by an intervening single-strand.
- [Claim 12] The double-stranded molecule of claim 11, which has the general formula 5'-[A]-[B]-[A']-3' or 5'-[A']-[B]-[A]-3', wherein [A] is the sense strand comprising a sequence corresponding to a target sequence selected from the group consisting of SEQ ID NOs:11, 12, 13, and 14, [B] is the intervening single-strand consisting of 3 to 23 nucleotides, and [A'] is the antisense strand comprising a complementary sequence to the target sequence.
- [Claim 13] A vector encoding the double-stranded molecule of any one of claims 8 to 12.
- [Claim 14] Vectors comprising each of a combination of polynucleotide comprising a sense strand nucleic acid and an antisense strand nucleic acid, wherein said sense strand nucleic acid comprises nucleotide sequence of SEQ ID NOs: 11, 12, 13 or 14, and said antisense strand nucleic acid consists of a sequence complementary to the sense strand, wherein the transcripts of said sense strand and said antisense strand hybridize to each other to form a double-stranded molecule, and wherein said vectors, when introduced into a cell expressing the LHX4 gene, Ku70 gene or Ku86 gene, inhibit expression of said gene.
- [Claim 15] A method for treating and/or preventing cancer expressing a LHX4 gene, wherein the method comprises the step of administering at least one isolated double-stranded molecule against the LHX4 gene, a Ku70 gene or a Ku86 gene, or a vector encoding the double-stranded molecule.
- [Claim 16] The method of claim 15, wherein the double-stranded molecule is that of any one of claims 8 to 12.
- [Claim 17] The method of claim 15, wherein the vector is that of claim 13 or 14.
- [Claim 18] The method of any one of claims 15 to 18, wherein the cancer to be treated is lung cancer.
- [Claim 19] A composition for treating and/or preventing cancer expressing a LHX4 gene, wherein composition comprises at least one isolated double-stranded molecule against the LHX4 gene, a Ku70 gene or a

- Ku86gene, or a vector encoding the double-stranded molecule.
- [Claim 20] The composition of claim 19, wherein the double-stranded molecule is that of any one of claims 8 to 12.
- [Claim 21] The composition of claim 19, wherein the vector is that of claim 13 or 14.
- [Claim 22] The composition of any one of claim 19 to 21, wherein the cancer to be treated is lung cancer.
- [Claim 23] A method of screening for a candidate substance for treating and/or preventing lung cancer, or inhibiting lung cancer cell growth, said method comprising the steps of:
(a) contacting a test substance with a LHX4 polypeptide or functional equivalent thereof;
(b) detecting the binding activity between the polypeptide or the functional equivalent and the test substance; and
(c) selecting a substance that binds to the polypeptide or the functional equivalent.
- [Claim 24] A method of screening for a candidate substance for treating and/or preventing lung cancer, or inhibiting lung cancer cell growth, said method comprising the steps of:
(a) contacting a test substance with a LHX4 polypeptide or functional equivalent thereof ;
(b) detecting the biological activity of the polypeptide or the functional equivalent of step (a); and
(c) selecting the test substance that suppresses the biological activity of the polypeptide or the functional equivalent as compared to the biological activity detected in the absence of the test substance.
- [Claim 25] The method of claim 24, wherein the biological activity is selected from the group consisting of the facilitation of the cell proliferation or binding activity to a Ku protein.
- [Claim 26] A method of screening for a candidate substance for treating and/or preventing lung cancer or inhibiting lung cancer cell growth, said method comprising the steps of:
(a) contacting a test substance with a cell expressing a LHX4 gene;
(b) detecting an expression level of the LHX4 gene in the cell of step (a);
(c) selecting the test substance that reduces the expression level of LHX4 gene in comparison with the expression level detected in the absence of the test substance.

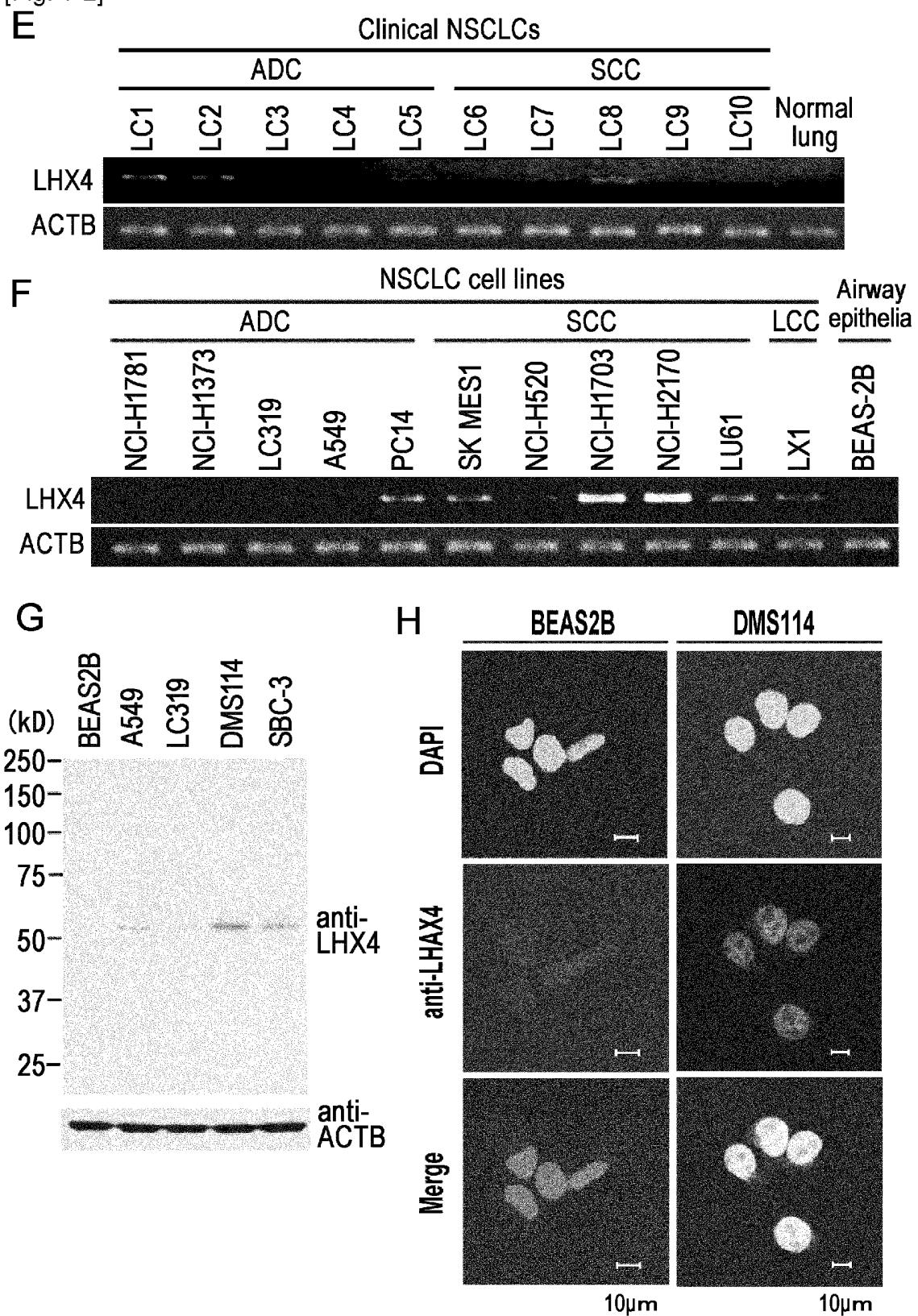
- [Claim 27] A method of screening for a candidate substance for treating and/or preventing lung cancer or inhibiting lung cancer cell growth, said method comprising the steps of:
(a) contacting a test substance with a cell into which a vector, comprising the transcriptional regulatory region of LHX4 gene and a reporter gene that is expressed under the control of the transcriptional regulatory region, has been introduced;
(b) measuring the expression or activity of said reporter gene; and
(c) selecting a 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 28] A method of screening for a candidate substance for inhibiting a binding between a LHX4 polypeptide and a Ku protein, or treating and/or preventing lung cancer, said method comprising steps of:
(a) contacting the LHX4 polypeptide or functional equivalent thereof with the Ku protein or functional equivalent thereof in presence of a test substance;
(b) detecting a binding between the polypeptides;
(c) comparing the binding level detected in the step (b) with the binding level detected in absence of the test substance; and
(d) selecting the test substance that reduces or inhibits the binding level.
- [Claim 29] A method of screening for a candidate substance for inhibiting the phosphorylation of a LHX4 polypeptide, or treating and/or preventing lung cancer, comprising the steps of:
(a) contacting the LHX4 polypeptide or functional equivalent thereof with a test substance under a condition that allows phosphorylation of the polypeptide;
(b) detecting the phosphorylation level of the polypeptide described in (a);
(c) comparing the phosphorylation level of the polypeptide with the phosphorylation level detected in the absence of the test substance; and
(d) selecting the test substance that reduced the phosphorylation level of the polypeptide as the candidate substance.
- [Claim 30] A polypeptide comprising the amino acid sequence of (a) or (b) below:
(a) the amino acid sequence of SEQ ID NO: 21;
(b) the amino acid sequence in which one, two or several amino acid is substituted, deleted, inserted and/or added in the amino acid sequence

- of SEQ ID NO: 21;
wherein the polypeptide inhibits a biological activity of the LHX4 polypeptide.
- [Claim 31] The polypeptide of claim 30, wherein the biological activity of the LHX4 polypeptide is a binding activity to the Ku protein.
- [Claim 32] The polypeptide of claim 30 or 31, which is modified with a cell-membrane permeable substance.
- [Claim 33] The polypeptide of claim 32, which has the following general formula:
[R]-[D];
wherein [R] represents the cell-membrane permeable substance; and
[D] represents a polypeptide comprising the amino acid sequence of (a) or (b) below:
(a) the amino acid sequence of SEQ ID NO: 21;
(b) the amino acid sequence in which one, two or several amino acid is substituted, deleted, inserted and/or added in the amino acid sequence of SEQ ID NO: 21,
wherein [R] and [D] are linked directly or indirectly through a linker.
A composition for treating and/or preventing cancer expressing a LHX4 gene, wherein the composition comprises the polypeptide of any one of claims 30 to 33 and a pharmaceutically acceptable carrier.
- [Claim 34] The composition of claim 34, wherein the cancer to be treated is lung cancer.
- [Claim 35] A method for treating and/or preventing cancer expressing a LHX4 gene, wherein the method comprises the step of administering the polypeptide of any one of claims 30 to 33 to a subject.
- [Claim 36] The method of claim 36, wherein the cancer to be treated is lung cancer.

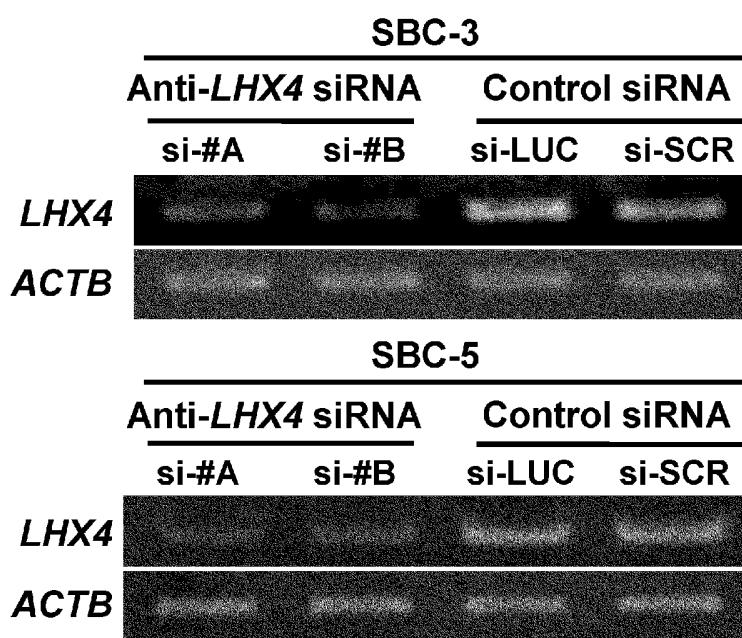
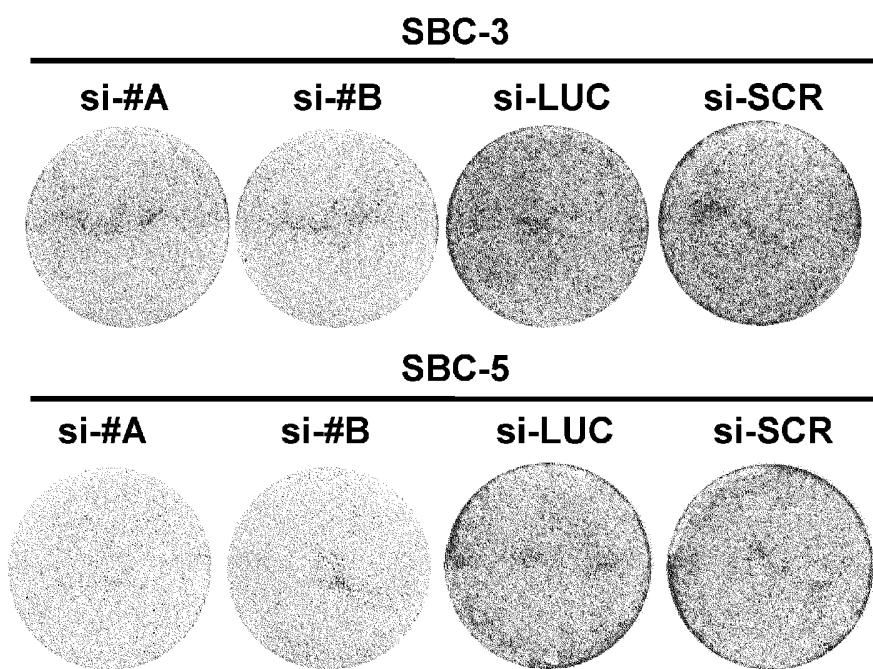
[Fig. 1-1]



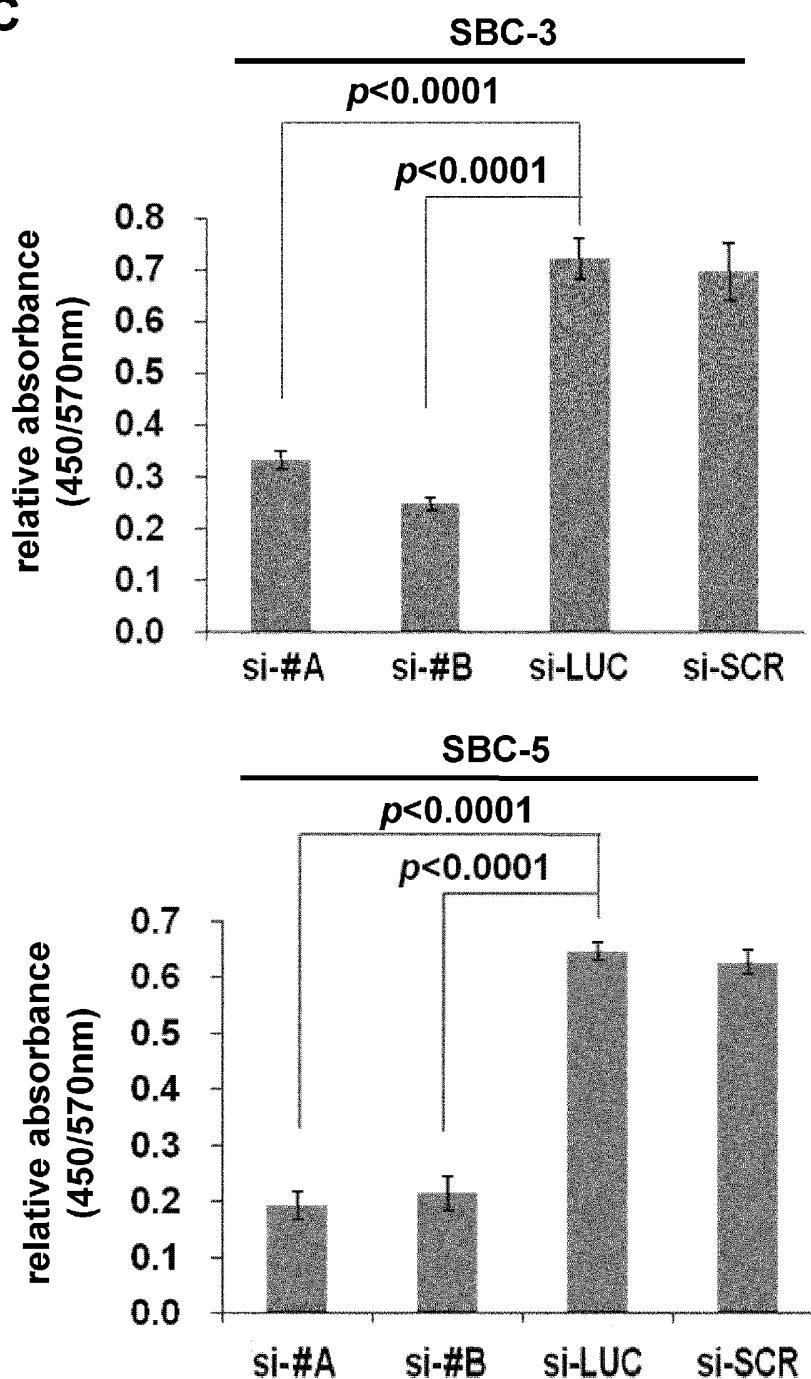
[Fig. 1-2]



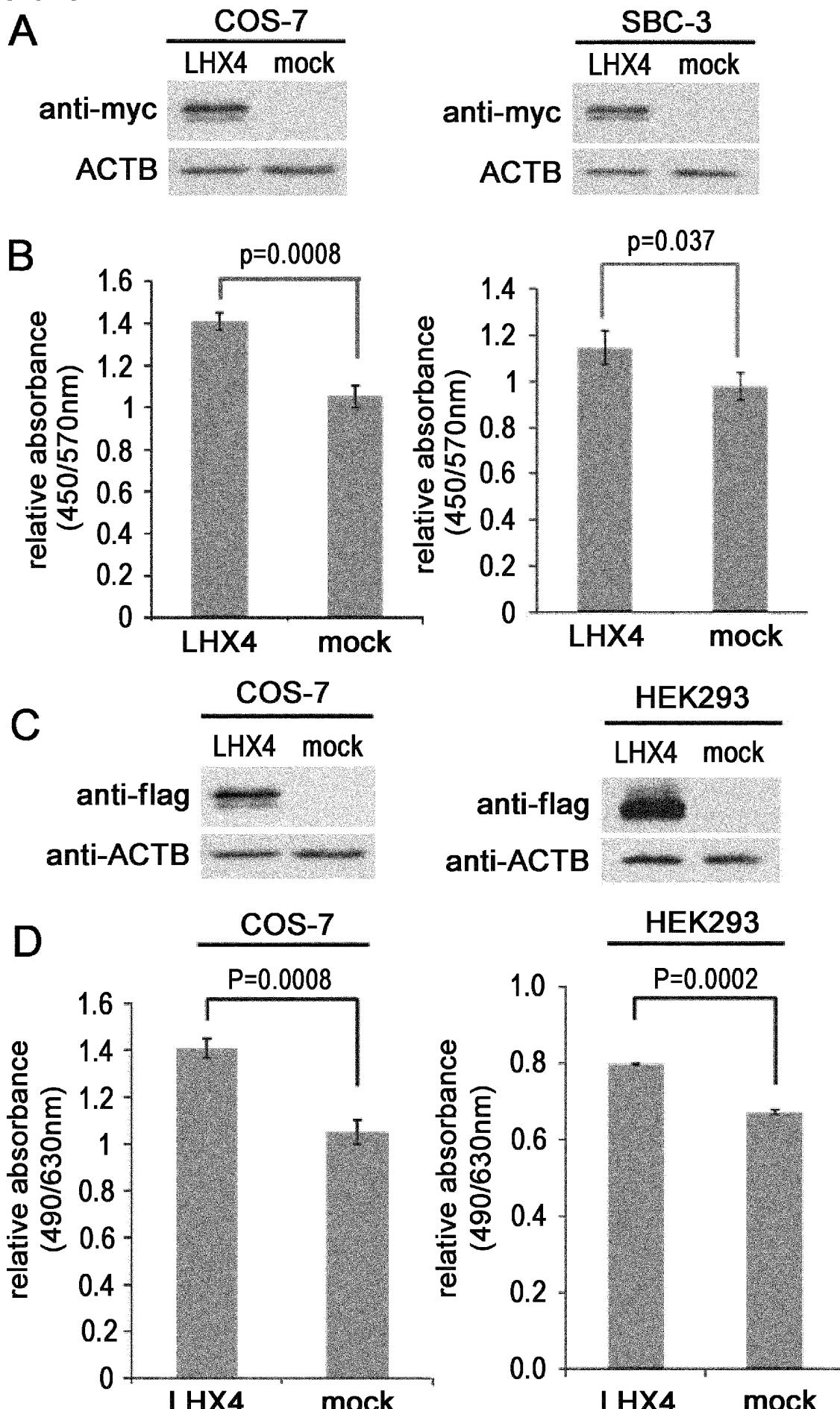
[Fig. 2-1]

A**B**

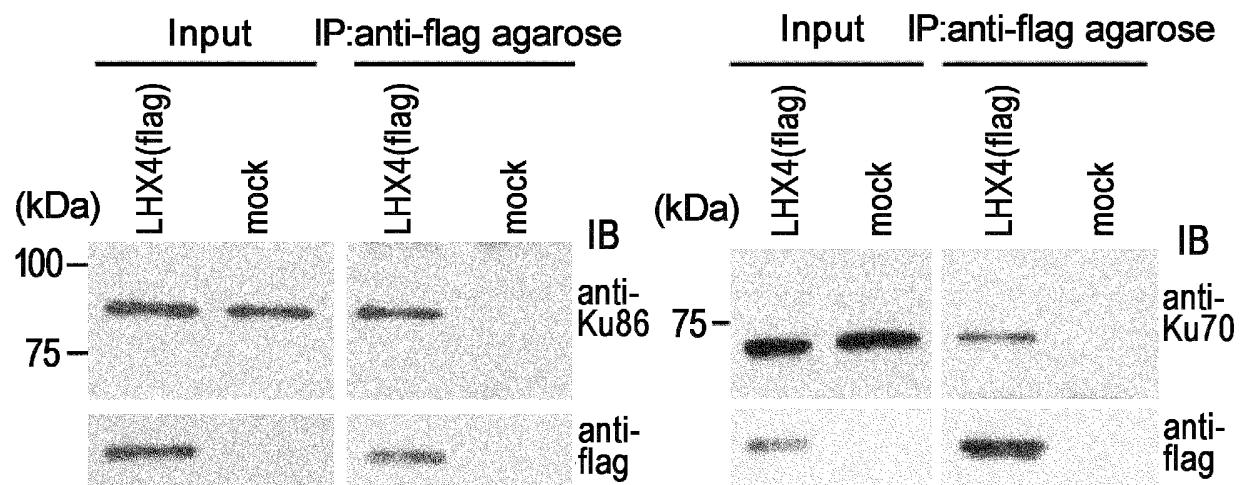
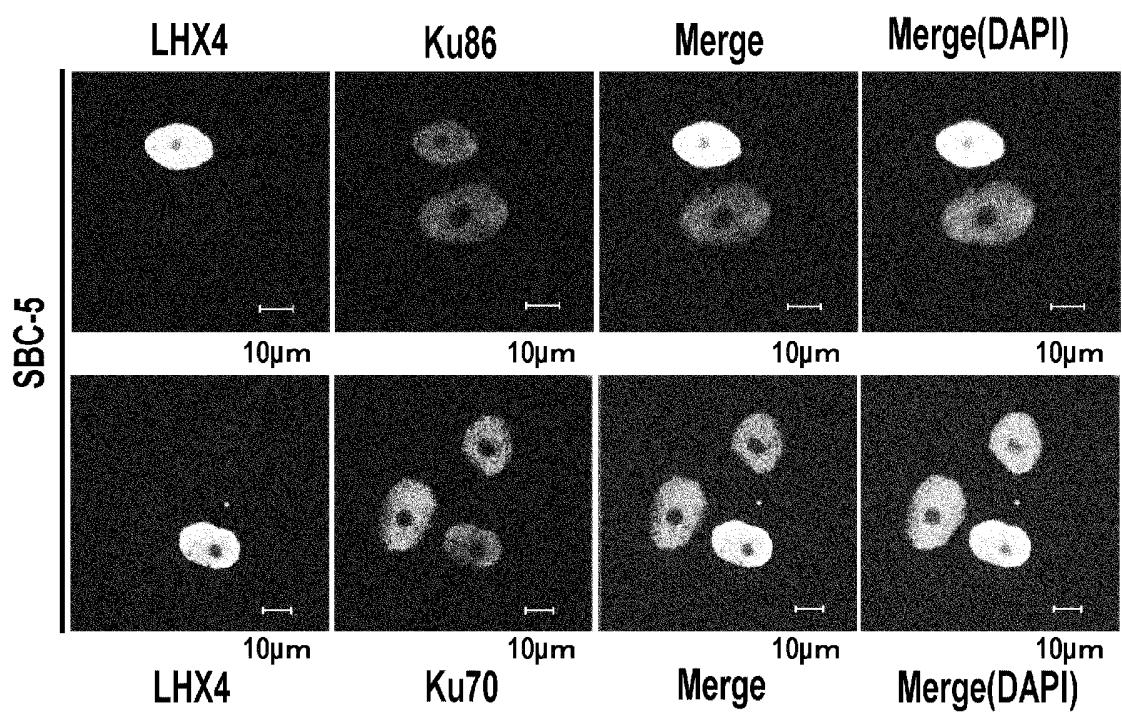
[Fig. 2-2]

C

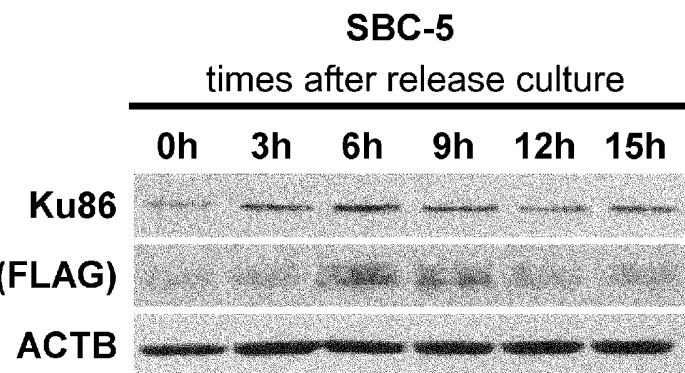
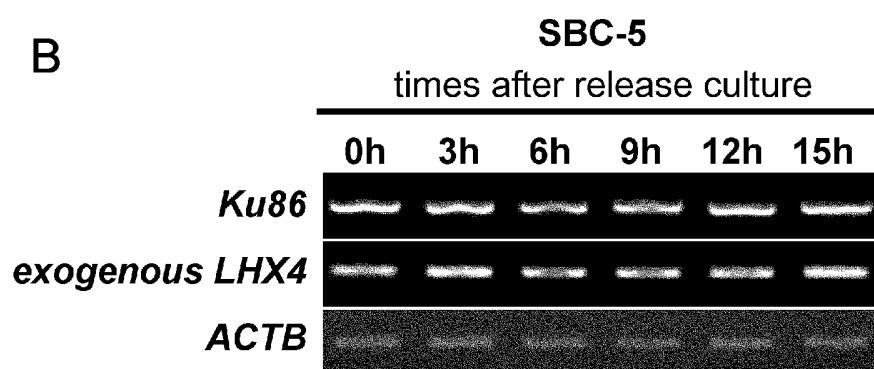
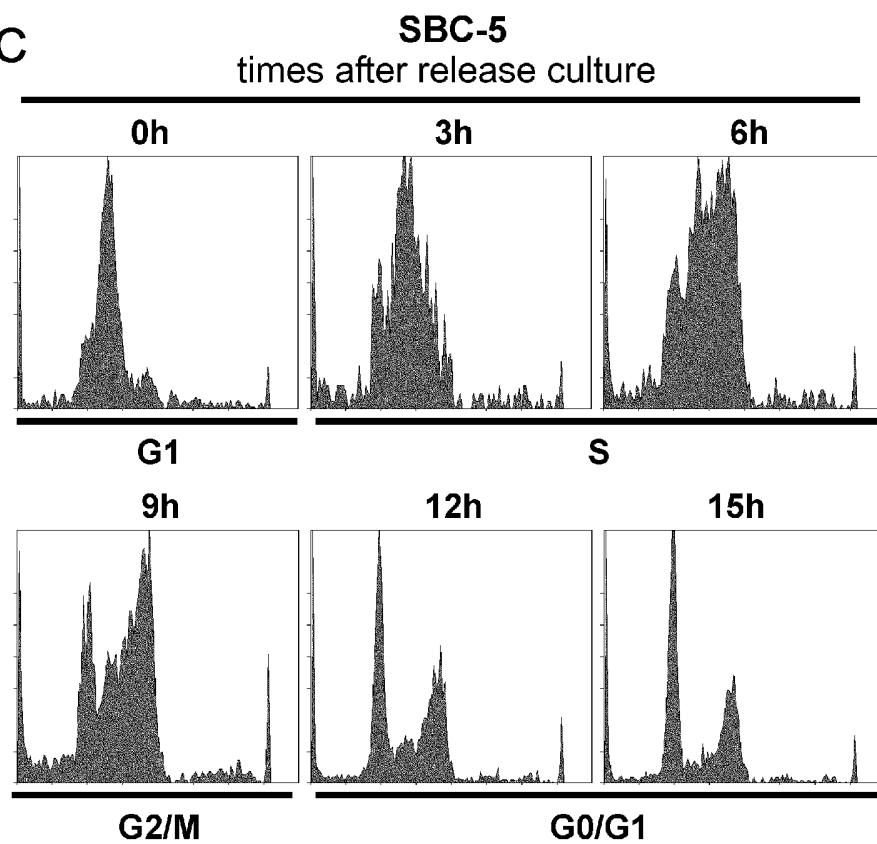
[Fig. 3]



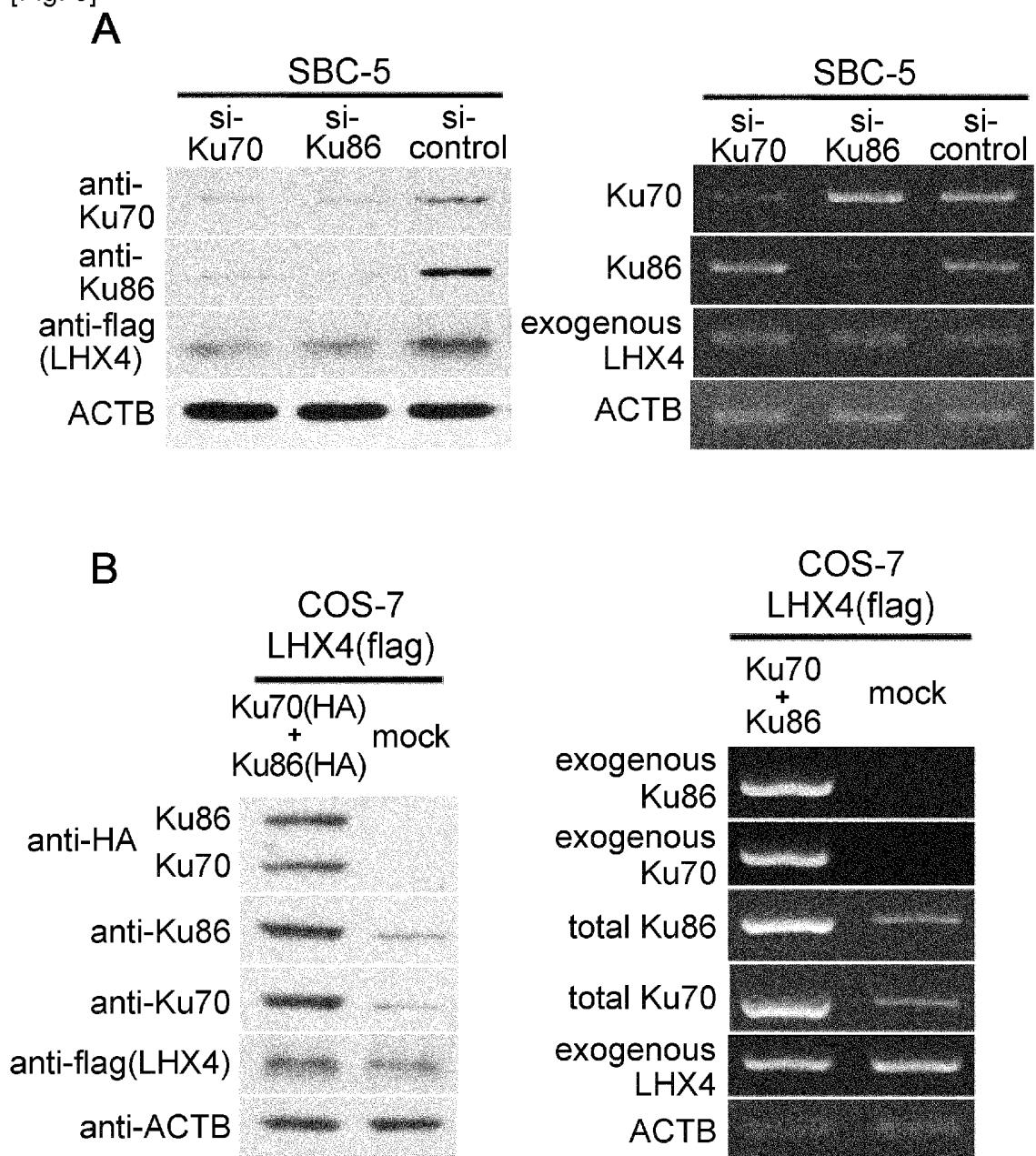
[Fig. 4]

A**B**

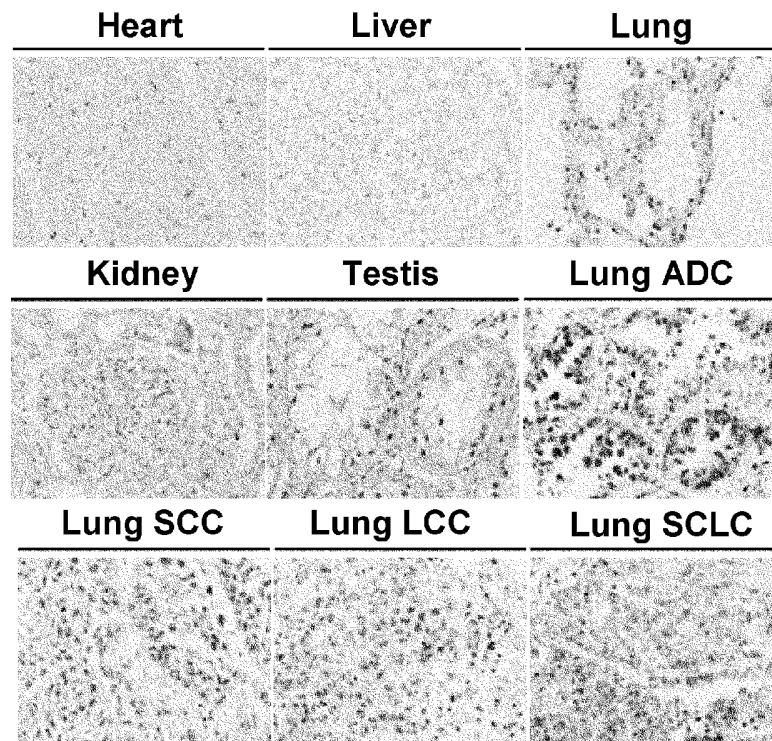
[Fig. 5]

A**B****C**

[Fig. 6]



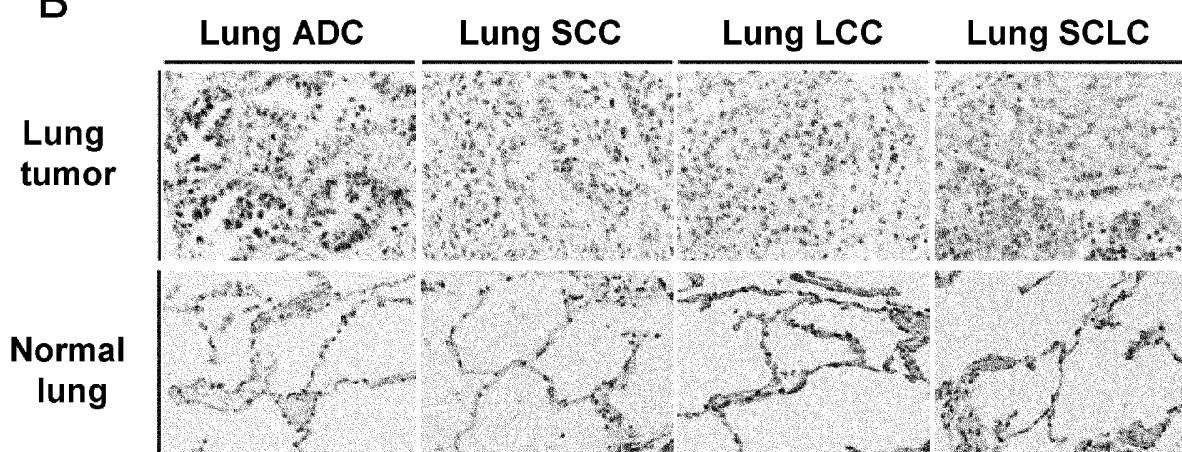
[Fig. 7]

A

Lung SCC

Lung LCC

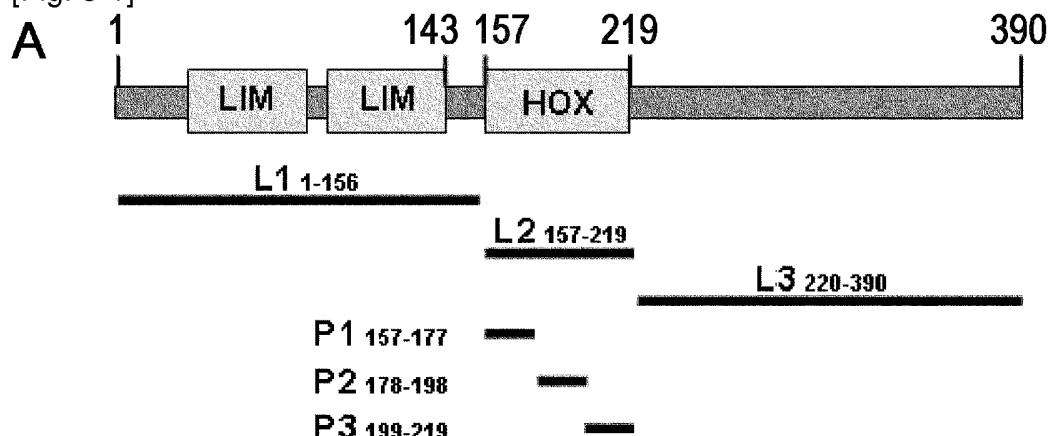
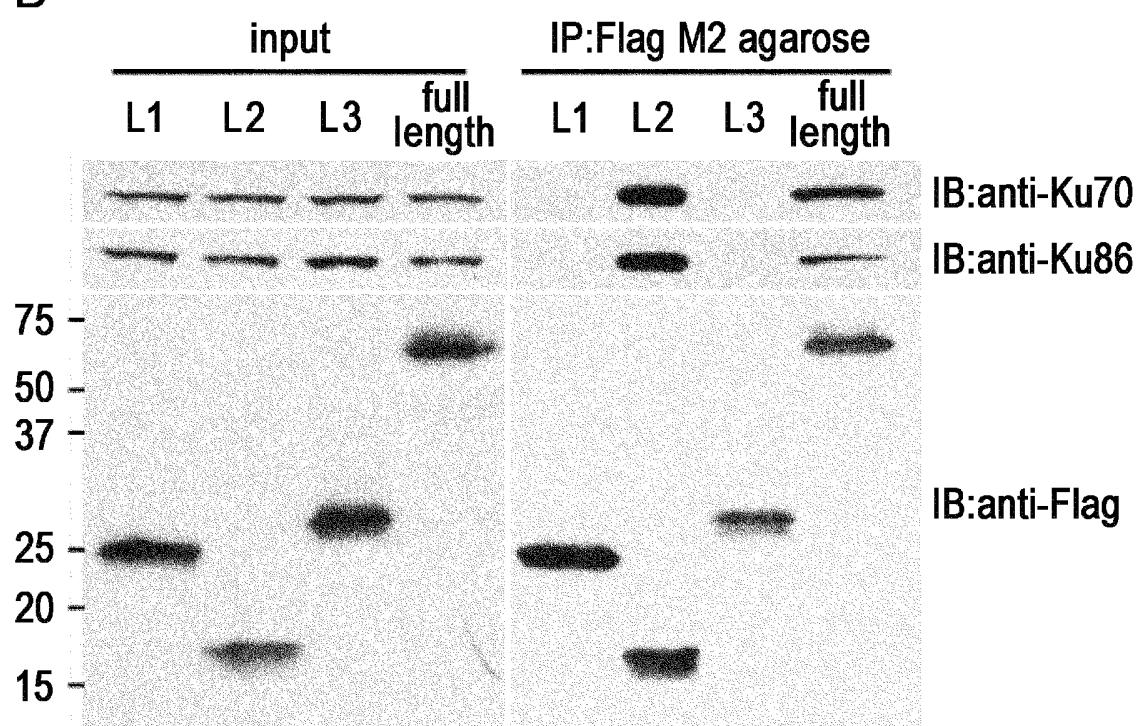
Lung SCLC

B

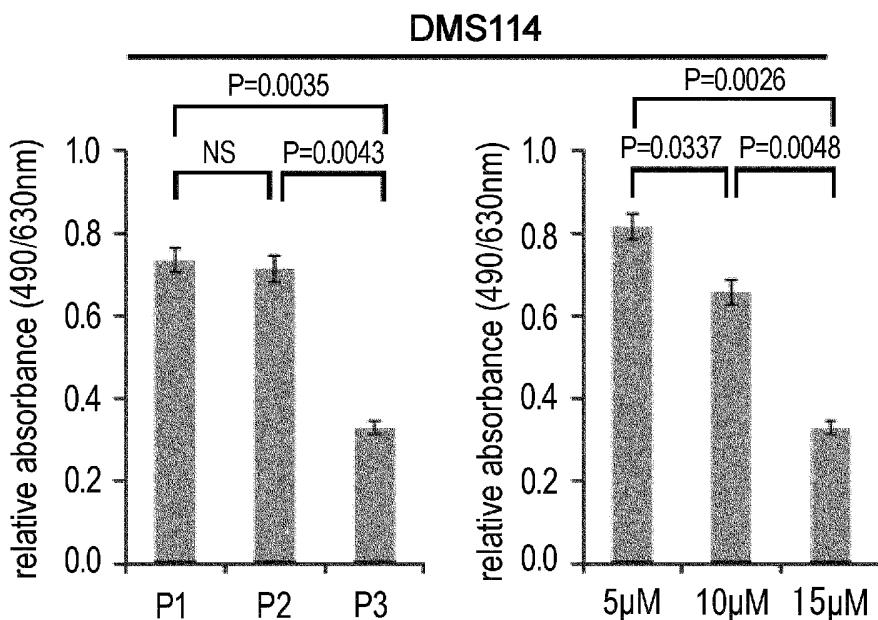
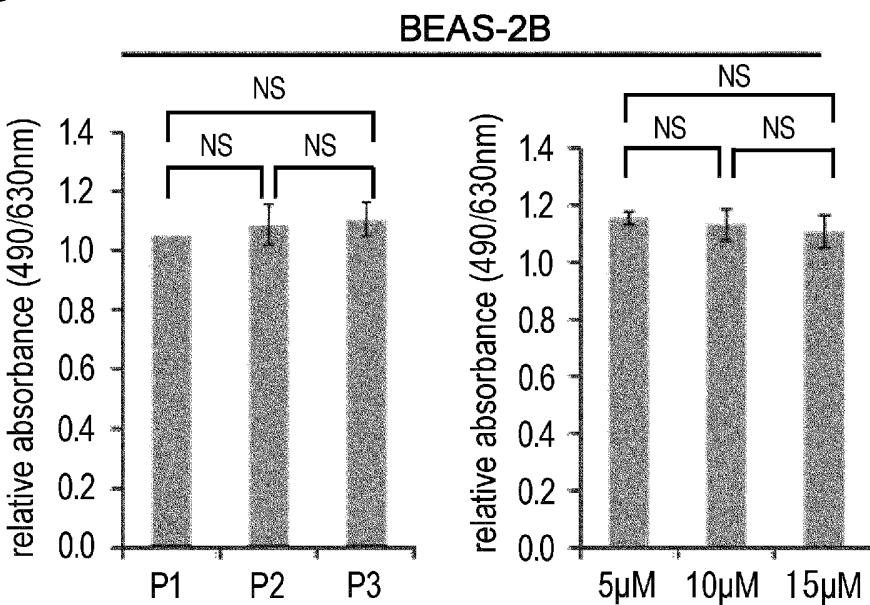
Lung tumor

Normal lung

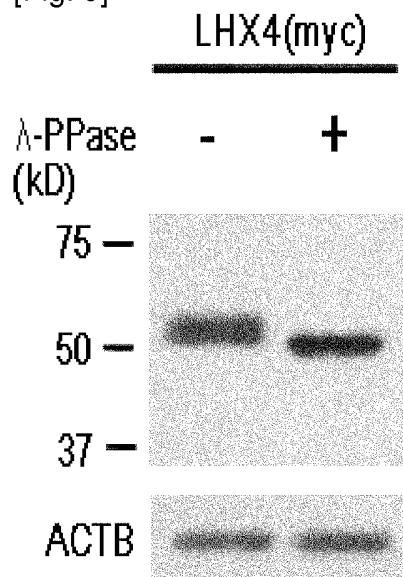
[Fig. 8-1]

**B**

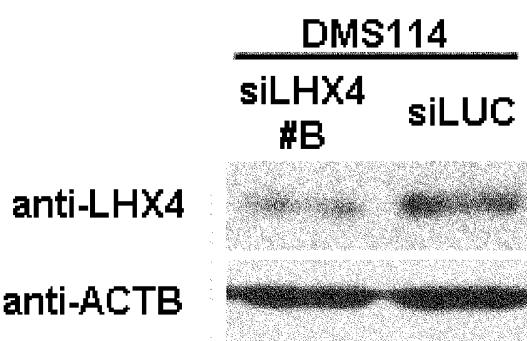
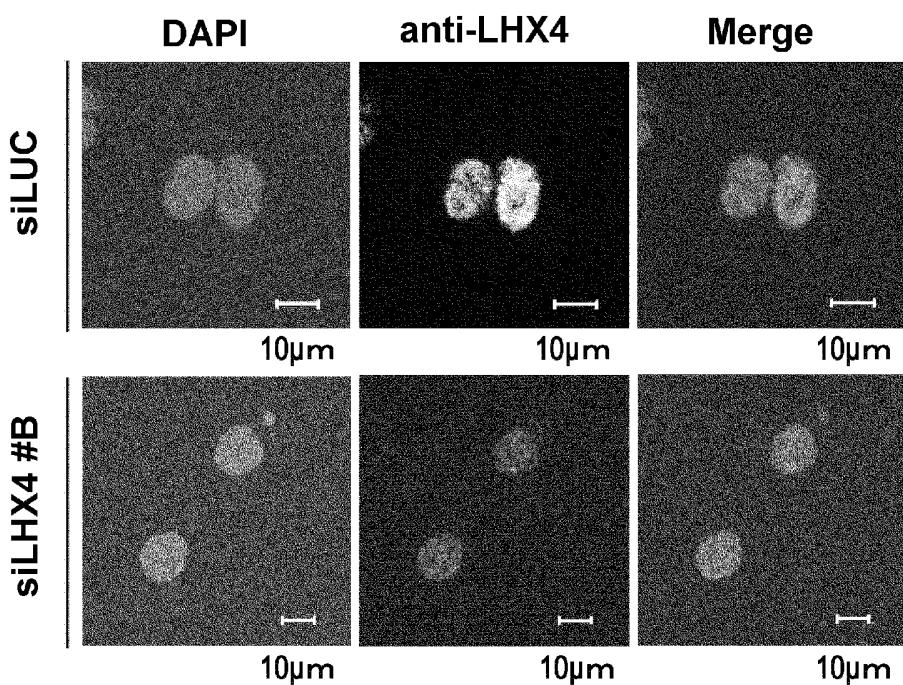
[Fig. 8-2]

C**D**

[Fig. 9]



[Fig. 10]

A**B**

INTERNATIONAL SEARCH REPORT

International application No. PCT/JP2011/004607
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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. C12N15/09, A61K31/7105, A61K38/16, A61P35/00, C07K14/47, C07K14/82, C12N15/113, G01N33/15

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)

CA/REGISTRY/BIOSIS/MEDLINE/WPIDS (STN), GenBank/EMBL/DDBJ/GeneSeq, UniProt/GeneSeq, JSTPlus (JDreamII)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
<u>X</u> <u>A</u>	ZHANG Q et al., Suppression of DNA-PK by RNAi has different quantitative effects on telomere dysfunction and mutagenesis in human lymphoblasts treated with gamma rays or HZE particles, Radiat. Res., 2005, Vol.164, No.4, Pt.2, pp.497-504	8-14 5-7, 19-35
<u>X</u> <u>A</u>	WINDHOFER F et al., Distinctive differences in DNA double-strand break repair between normal urothelial and urothelial carcinoma cells, Mutat. Res., 2008, Vol.638, No.1-2, pp.56-65	8-14 5-7, 19-35

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

30.09.2011

Date of mailing of the international search report

11.10.2011

Name and mailing address of the ISA/JP

Japan Patent Office

3-4-3, Kasumigaseki, Chiyoda-ku, Tokyo 100-8915, Japan

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2011/004607

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X A	KAWAMATA N et al., A novel chromosomal translocation t(1;14) (q25;q32) in pre-B acute lymphoblastic leukemia involves the LIM homeodomain protein gene, <i>Lhx4</i> , Oncogene, 2002, Vol.21, No.32, pp.4983-4991	<u>8-14, 29</u> 5-7, 19-28, 30-35
A	RAUCH T et al., MIRA-assisted microarray analysis, a new technology for the determination of DNA methylation patterns, identifies frequent methylation of homeodomain-containing genes in lung cancer cells, <i>Cancer Res.</i> , 2006, Vol.66, No.16, pp.7939-7947	5-14, 19-35
A	HARTMANN O et al., DNA methylation markers predict outcome in node-positive, estrogen receptor-positive breast cancer with adjuvant anthracycline-based chemotherapy, <i>Clin. Cancer Res.</i> , 2009, Vol.15, No.1, pp.315-323	5-14, 19-35
A	TAJIMA T et al., A novel missense mutation (P366T) of the LHX4 gene causes severe combined pituitary hormone deficiency with pituitary hypoplasia, ectopic posterior lobe and a poorly developed sella turcica, <i>Endocr. J.</i> , 2007, Vol.54, No.4, pp.637-641	5-14, 19-35
A	HOWARD PW et al., Identification of a conserved protein that interacts with specific LIM homeodomain transcription factors, <i>J. Biol. Chem.</i> , 2000, Vol.275, No.18, pp.13336-13342	5-14, 19-35
A	SAMUEL S et al., Homeobox gene expression in cancer: insights from developmental regulation and deregulation, <i>Eur. J. Cancer</i> , 2005, Vol.41, No.16, pp.2428-2437	5-14, 19-35
A	JP 2009-502116 A (ONCOTHERAPY SCI. INC.) 2009.01.29, & US 2009/0208514 A1 & EP 1907582 A1 & EP 2295602 A1 & EP 2298933 A1 & EP 2311985 A1 & EP 2311986 A1 & WO 2007/013671 A2	5-14, 19-35

INTERNATIONAL SEARCH REPORTInternational application No.
PCT/JP2011/004607

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JP 2009-502112 A (ONCOTHERAPY SCI. INC.) 2009.01.29, & EP 1907580 A1 & WO 2007/013575 A2	5-14, 19-35
A	WO 2002/40716 A2 (CEMINES, LLC) 2002.05.23, & US 2003/0092009 A1 & US 2007/0161023 A1 & EP 1337667 A1	5-14, 19-35
A	WO 2010/073218 A2 (KONINKLIJKE PHILIPS ELECTRONICS N.V.) 2010.07.01, (no family)	5-14, 19-35
P, A	WO 2010/123354 A2 (ERASMUS UNIVERSITY MEDICAL CENTER ROTTERDAM) 2010.10.28, (no family)	5-14, 19-35
P, A	HAH N et al., A rapid, extensive, and transient transcriptional response to estrogen signaling in breast cancer cells, Cell, 2011.05.13, Vol.145, No.4, pp.622-634, Epub. 2011.05.05	5-14, 19-35

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2011/004607

CLASSIFICATION OF SUBJECT MATTER

C12N15/09(2006.01)i, A61K31/7105(2006.01)i, A61K38/16(2006.01)i,
A61P35/00(2006.01)i, C07K14/47(2006.01)i, C07K14/82(2006.01)i,
C12N15/113(2010.01)i, G01N33/15(2006.01)i

INTERNATIONAL SEARCH REPORTInternational application No.
PCT/JP2011/004607**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.: 1–4, 15–18, 36, 37
because they relate to subject matter not required to be searched by this Authority, namely:
See extra sheet
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.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP2011/004607

(Box No.II)

The subject matter of claims 1-4 relates to a diagnostic methods practiced on the human or animal body, which does not require an intentional search by the International Searching Authority in accordance with PCT Article 17(2)(a)(i) and [Rule 39.1(iv)].

The subject matter of claims 15-18,36,37 relates to a method for treatment of the human body by therapy, which does not require an intentional search by the International Searching Authority in accordance with PCT Article 17(2)(a)(i) and [Rule 39.1(iv)].