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VERFAHREN ZUR DIAGNOSE VON NICHT-KLEINZELLIGEM LUNGENKREBS

PROCÉDÉ SERVANT À DIAGNOSTIQUER UN CANCER DES POUMONS NON À PETITES CELLULES

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**10177195.4 / 2 343 384**(73) Proprietor: **Oncotherapy Science, Inc.****Kawasaki-shi  
Kanagawa 213-0012 (JP)**

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(72) Inventors:

- **NAKAMURA, Yusuke  
Tokyo 1088639 (JP)**
- **DAIGO, Yataro  
Tokyo 1088639 (JP)**
- **NAKATSURU, Shuichi  
Kawasaki-shi,  
Kanagawa 2130012 (JP)**

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**Description**

**[0001]** This application claims the benefit of U.S. Provisional Application Serial No.60/555,789 filed March 23, 2004

5 FIELD OF THE INVENTION

**[0002]** The present invention relates to the field of biological science, more specifically to the field of cancer therapy and diagnosis. In particular, disclosed herein methods of diagnosing non-small cell lung cancers using genes, KIF11, GHSR1b, NTSR1, and FOXM1, that show elevated expression in such cancerous cells.

10 BACKGROUND OF THE INVENTION

**[0003]** Lung cancer is one of the most commonly fatal human tumors. Many genetic alterations associated with the development and progression of lung cancer have been reported. Although genetic changes can aid prognostic efforts and predictions of metastatic risk or response to certain treatments, information about a single or a limited number of molecular markers generally fails to provide satisfactory results for clinical diagnosis of non-small cell lung cancer (NSCLC) (Mitsudomi et al., Clin Cancer Res 6: 4055-63 (2000); Niklinski et al., Lung Cancer. 34 Suppl 2: S53-8 (2001); Watine, BMJ 320: 379-80 (2000)). NSCLC is by far the most common form, accounting for nearly 80% of lung tumors (Society, A.C. Cancer Facts and Figures 2001 (2001)). The overall 10-year survival rate remains as low as 10% despite recent advances in multi-modality therapy, because the majority of NSCLCs are not diagnosed until advanced stages (Fry, W.A. et al., Cancer 86: 1867-76 (1999)). Although chemotherapy regimens based on platinum are considered the reference standards for treatment of NSCLC, those drugs are able to extend survival of patients with advanced NSCLC only about six weeks (Non-small Cell Lung Cancer Collaborative Group, BMJ. 311: 899-909 (1995)). Numerous targeted therapies are being investigated for this disease, including tyrosine kinase inhibitors, but so far promising results have been achieved in only a limited number of patients and some recipients suffer severe adverse reactions (Kris M.N.R., Herbst R.S. Proc. Am. Soc. Clin. Oncol. 21: 292a(A1166) (2002)).

**[0004]** Many genetic alterations associated with development and progression of lung cancer have been reported, but the precise molecular mechanisms remain unclear (Sozzi, G. Eur. J. Cancer 37: 63-73 (2001)). Over the last decade newly developed cytotoxic agents including paclitaxel, docetaxel, gemcitabine, and vinorelbine have emerged to offer multiple therapeutic choices for patients with advanced NSCLC; however, each of the new regimens can provide only modest survival benefits compared with cisplatin-based therapies (Schiller, J.H. et al., N. Engl. J. Med. 346: 92-98 (2002); Kelly, K. et al., J. Clin. Oncol. 19: 3210-3218 (2001)). Hence, new therapeutic strategies, such as development of molecular-targeted agents, are eagerly awaited by clinicians.

**[0005]** Systematic analysis of expression levels of thousands of genes on cDNA microarrays is an effective approach to identifying unknown molecules involved in pathways of carcinogenesis (Kikuchi, T. et al., Oncogene 22: 2192-2205 (2003); Kakiuchi, S. et al., Mol. Cancer Res. 1: 485-499 (2003); Zembutsu, H. et al., Int. J. Oncol. 23: 29-39 (2003); Suzuki, C. et al., Cancer Res. 63: 7038-7041 (2003)) and can reveal candidate targets for development of novel anti-cancer drugs and tumor markers. To isolate novel molecular targets for diagnosis, treatment and prevention of NSCLC, pure populations of tumor cells were prepared from 37 cancer tissues by laser-capture microdissection and genome-wide expression profiles of NSCLC cells were analyzed on a cDNA microarray containing 23,040 genes (Kikuchi, T. et al., Oncogene 22: 2192-2205 (2003)). In the course of those experiments, KOC1 (GenBank Accession No.NM\_006547) and neuromedin U (NMU; GenBank Accession No. NM\_006681) were identified as genes that were frequently over-expressed in lung tumors and indispensable for growth of NSCLC cells.

**[0006]** Cell-to-cell communication is a prerequisite for development and maintenance of multicellular organisms. Several intercellular information-exchange systems such as chemical synapses, gap junctions, and plasmodesmata in plant cells have long been observed, but a new transporting system involving a highly sensitive nanotubular structure, tunneling nanotubes (TNTs) between the cells, was only recently reported in mammalian cells (Rustom, A. et al., Science 303, 1007-1010 (2004)). Such a structure would facilitate the selective transfer of membrane vesicles and organelles; therefore TNTs in mammalian somatic cells might contribute to a cell-to-cell transporting system(s) by carrying transcription factors or ribonucleoproteins (RNPs), as in plants (Nakajima, K. et al., Nature 413, 307-311 (2001); Lucas, W.J. et al., Nat. Rev. Mol. Cell Biol. 2, 849-857 (2001)). Some investigators have documented interactions between some RNA-binding proteins and motor proteins like kinesin and dynein within mammalian somatic cells, as well as intercellular mRNA transport in mammalian germ cells (Brendza, R.P. et al., Science 289, 2120-2122 (2000); Chennathukuzhi, V. et al., Proc. Natl. Acad. Sci. USA 100, 15566-15571 (2003); Villace, P. et al., Nucleic Acids Res. 32, 2411-2420 (2004).; Morales, C.R. et al. Dev. Biol.246, 480-494 (2002).). However, no report has emerged describing an intercellular mRNA transporting system in mammalian somatic cells involving a complex of RNA-binding proteins and motor proteins.

**[0007]** The phenomenon of mRNA localization has been reported in oocytes and developing embryos of *Drosophila* and *Xenopus* and in somatic cells such as fibroblasts and neurons (King, M.L. et al., Bioessays 21: 546-557 (1999);

Mowry, K.L., Cote, C.A. *FASEB J.* 13: 435-445 (1999); Lasko, P. *J. Cell Biol.* 150: F51-56 (2000); Steward, O. *Neuron* 18: 9-12 (1997)). Beta actin (ACTB) mRNA is localized at the leading lamellae of chicken embryo fibroblasts (CEFs) (Lawrence, J.B., Singer, R.H. *Cell* 45: 407-415 (1986)) and at the growth cone of developing neurons (Bassell, G.J. et al., *J. Neurosci.* 18: 251-265 (1998)). The localization of ACTB mRNA is dependent on the zipcode, a cis-acting element located in the 3' UTR of the mRNA (Kislauskis, E.H. et al., *J. Cell Biol.* 123: 165-172 (1993)). The trans-acting factor, zipcode binding protein 1 (ZBP1), was affinity purified with the zipcode of ACTB mRNA (Ross, A.F. et al., *Mol. Cell Biol.* 17: 2158-2165 (1997)). After the identification of ZBP1, additional homologues were identified in a wide range of organisms including *Xenopus*, *Drosophila*, human, and mouse (Mueller-Pillasch, F. et al., *Oncogene* 14: 2729-2733 (1997); Deshler, J.O. et al., *Science* 276: 1128-1131 (1997); Doyle, G.A. et al., *Nucleic Acids Res.* 26: 5036-5044 (1998)). ZBP1 family members are expressed in germ embryonic fibroblasts and in several types of cancer (Mueller-Pillasch, F. et al., *Oncogene* 14: 2729-2733 (1997); Mueller, F. et al., *Br. J. Cancer* 88: 699-701 (2003)). ZBP1-like proteins contain two RNA-recognition motifs (RRMs) at the NH2-terminal part of the protein and four hnRNP K homology (KH) domains at the COOH-terminal end.

**[0008]** KOC1 (alias IGF-II mRNA-binding protein 3: IMP-3) is one of the IMPs (IMP-1, IMP-2, and IMP-3), which belong to the ZBP1 family members and exhibit multiple attachments to IGF-II leader 3 mRNA and the reciprocally imprinted H19 RNA (Mueller-Pillasch, F. et al., *Oncogene* 14: 2729-2733 (1997)). Although KOC1 was initially reported to be over-expressed in pancreatic cancer (Mueller-Pillasch, F. et al., *Oncogene* 14: 2729-2733 (1997); Mueller, F. et al., *Br. J. Cancer* 88: 699-701 (2003)), its precise function in cancer cells or even in normal mammalian somatic cells remains unclear.

**[0009]** KOC1 is orthologous to the *Xenopus* Vg1 RNA-binding protein (Vg1RBP/Vera), which mediates the localization of Vg1 mRNA to the vegetal pole of the oocyte during oocyte maturation, and IMP-1 is orthologous to the ZBP1. IMP is mainly located at the cytoplasm and its cellular distribution ranges from a distinct concentration in perinuclear regions and lamellipodia to a completely delocalized pattern. H19 RNA co-localized with IMP, and removal of the high-affinity attachment site led to delocalization of the truncated RNA (Runge, S. et al., *J. Biol. Chem.* 275: 29562-29569 (2000)), suggesting that EMPs are involved in cytoplasmic trafficking of RNA. IMP-1 was able to associate with microtubules (Nielsen, F.C. et al., *J. Cell Sci.* 115: 2087-2097 (2002); Havin, L. et al., *Genes Dev.* 12: 1593-1598 (1998)), and is likely to involve a motor protein such as kinesin, myosin, and dyenin. On the other hand, Oskar mRNA localization to the posterior pole requires Kinesin I (Palacios, I.M., St. Johnston D. *Development* 129: 5473-5485 (2002); Brendza, R.P. et al., *Science* 289: 2120-2102 (2000)).

**[0010]** KIF11 (alias EG5) is a member of kinesin family, and plays a role in establishing and/or determining the stability of specific microtubule arrays that form during cell division. This role may encompass the ability of KIF 11 to influence the distribution of other protein components associated with cell division (Whitehead, C.M., Rattner, J.B. *J. Cell Sci.* 111: 2551-2561 (1998); Mayer, T.U. et al., *Science* 286: 971-974 (1999)).

**[0011]** NMU is a neuropeptide that was first isolated from porcine spinal cord. It has potent activity on smooth muscles (Minamino, N. et al., *Biochem. Biophys. Res. Commun.* 130: 1078-1085 (1985); Domin, J. et al., *Biochem. Biophys. Res. Commun.* 140: 1127-1134 (1986); Conlon, J.M. et al., *J. Neurochem.* 51: 988-991 (1988); Minamino, N. et al., *Biochem. Biophys. Res. Commun.* 156: 355-360 (1988); Domin, J. et al., *J. Biol. Chem.* 264: 20881-20885 (1989); O'Harte, F. et al., *Peptides* 12: 809-812 (1991); Kage, R. et al., *Regul. Pept.* 33: 191-198 (1991); Austin, C. et al., *J. Mol. Endocrinol.* 12: 257-263 (1994); Fujii, R. et al., *J. Biol. Chem.* 275: 21068-21074 (2000)), and in mammalian species NMU is distributed predominantly in the gastrointestinal tract and central nervous system (Howard, A.D. et al., *Nature* 406: 70-74 (2000); Funes, S. et al., *Peptides* 23: 1607-1615 (2002)). Peripheral activities of NMU include stimulation of smooth muscle, elevation of blood pressure, alternation of ion transport in the gut, and regulation of feeding (Minamino, N. et al., *Biochem. Biophys. Res. Commun.* 130: 1078-1085 (1985)); however, the role of NMU during carcinogenesis has not been addressed. Neuropeptides function peripherally as paracrine and autocrine factors to regulate diverse physiologic processes and act as neurotransmitters or neuromodulators in the nervous system. In general, receptors that mediate signaling by binding neuropeptides are members of the superfamily of G protein-coupled receptors (GPCRs) having seven transmembrane-spanning domains. Two known receptors for NMU, NMU1R and NMU2R, show a high degree of homology to other neuropeptide receptors such as GHSR and NTSR1, for which the corresponding known ligands are Ghrelin (GHRL) and neurotensin (NTS), respectively. NMU1R (FM3/GPR66) and NMU2R (FM4) have seven predicted alpha-helical transmembrane domains containing highly conserved motifs, as do other members of the rhodopsin GPCR family (Fujii, R. et al., *J. Biol. Chem.* 275: 21068-21074 (2000); Howard, A.D. et al., *Nature* 406: 70-74 (2000); Funes, S. et al., *Peptides* 23: 1607-1615 (2002)).

**[0012]** A C-terminal asparaginamide structure and the C-terminal hepatapeptide core of NMU protein are essential for its contractile activity in smooth-muscle cells (Westfall, T.D. et al., *J. Pharmacol. Exp. Ther.* 301: 987-992 (2002); Austin, C. *J. Mol. Endocrinol.* 14: 157-169 (1995)). Recent studies have contributed evidence that NMU acts at the hypothalamic level to inhibit food intake; therefore this protein might be a physiological regulator of feeding and body weight (Howard, A.D. et al., *Nature* 406: 70-74 (2000); Maggi, C.A. et al., *Br. J. Pharmacol.* 99: 186-188 (1990); Wren, A.M. et al., *Endocrinology* 143: 227-234 (2002); Ivanov, T.R. et al., *Endocrinology* 143: 3813-3821 (2002)). However,

so far no reports have suggested involvement of NMU over-expression in carcinogenesis.

**[0013]** Studies designed to reveal mechanisms of carcinogenesis have already facilitated identification of molecular targets for anti-tumor agents. For example, inhibitors of farnesyltransferase (FTIs) which were originally developed to inhibit the growth-signaling pathway related to Ras, whose activation depends on posttranslational farnesylation, has been effective in treating Ras-dependent tumors in animal models (He et al., Cell 99:335-45 (1999)). Clinical trials on human using a combination of anti-cancer drugs and anti-HER2 monoclonal antibody, trastuzumab, have been conducted to antagonize the proto-oncogene receptor HER2/neu; and have been achieving improved clinical response and overall survival of breast-cancer patients (Lin et al., Cancer Res. 61:6345-9 (2001)). A tyrosine kinase inhibitor, STI-571, which selectively inactivates bcr-abl fusion proteins, has been developed to treat chronic myelogenous leukemias wherein constitutive activation of bcr-abl tyrosine kinase plays a crucial role in the transformation of leukocytes. Agents of these kinds are designed to suppress oncogenic activity of specific gene products (Fujita et al., Cancer Res. 61:7722-6 (2001)). Therefore, gene products commonly up-regulated in cancerous cells may serve as potential targets for developing novel anti-cancer agents.

**[0014]** It has been demonstrated that CD8+ cytotoxic T lymphocytes (CTLs) recognize epitope peptides derived from tumor-associated antigens (TAAs) presented on MHC Class I molecule, and lyse tumor cells. Since the discovery of MAGE family as the first example of TAAs, many other TAAs have been discovered using immunological approaches (Boon, Int. J. Cancer 54: 177-80 (1993); Boon and van der Bruggen, J. Exp. Med. 183: 725-9 (1996); van der Bruggen et al., Science 254: 1643-7 (1991); Brichard et al., J. Exp. Med. 178: 489-95 (1993); Kawakami et al., J. Exp. Med. 180: 347-52 (1994)). Some of the discovered TAAs are now in the stage of clinical development as targets of immunotherapy. TAAs discovered so far include MAGE (van der Bruggen et al., Science 254: 1643-7 (1991)), gp100 (Kawakami et al., J. Exp. Med. 180: 347-52 (1994)), SART (Shichijo et al., J. Exp. Med. 187: 277-88 (1998)), and NY-ESO-1 (Chen et al., Proc. Natl. Acad. Sci. USA 94: 1914-8 (1997)). On the other hand, gene products which had been demonstrated to be specifically over-expressed in tumor cells, have been shown to be recognized as targets inducing cellular immune responses. Such gene products include p53 (Umano et al., Brit. J. Cancer 84: 1052-7 (2001)), HER2/neu (Tanaka et al., Brit. J. Cancer 84: 94-9 (2001)), CEA (Nukaya et al., Int. J. Cancer 80: 92-7 (1999)), and so on.

**[0015]** In spite of significant progress in basic and clinical research concerning TAAs (Rosenbeg et al., Nature Med 4: 321-7 (1998); Mukherji et al., Proc. Natl. Acad. Sci. USA 92: 8078-82 (1995); Hu et al., Cancer Res. 56: 2479-83 (1996)), only limited number of candidate TAAs for the treatment of cancer are available. TAAs abundantly expressed in cancer cells, and at the same time which expression is restricted to cancer cells would be promising candidates as immunotherapeutic targets. Further, identification of new TAAs inducing potent and specific antitumor immune responses is expected to encourage clinical use of peptide vaccination strategy in various types of cancer (Boon and van der Bruggen, J. Exp. Med. 183: 725-9 (1996); van der Bruggen et al., Science 254: 1643-7 (1991); Brichard et al., J. Exp. Med. 178: 489-95 (1993); Kawakami et al., J. Exp. Med. 180: 347-52 (1994); Shichijo et al., J. Exp. Med. 187: 277-88 (1998); Chen et al., Proc. Natl. Acad. Sci. USA 94: 1914-8 (1997); Harris, J. Natl. Cancer Inst. 88: 1442-5 (1996); Butterfield et al., Cancer Res. 59: 3134-42 (1999); Vissers et al., Cancer Res. 59: 5554-9 (1999); van der Burg et al., J Immunol 156: 3308-14 (1996); Tanaka et al., Cancer Res. 57: 4465-8 (1997); Fujie et al., Int. J. Cancer 80: 169-72 (1999); Kikuchi et al., Int. J. Cancer 81: 459-66 (1999); Oiso et al., Int. J. Cancer 81: 387-94 (1999)).

**[0016]** It has been repeatedly reported that peptide-stimulated peripheral blood mononuclear cells (PBMCs) from certain healthy donors produce significant levels of IFN- $\gamma$  in response to the peptide, but rarely exert cytotoxicity against tumor cells in an HLA-A24 or -A0201 restricted manner in  $^{51}\text{Cr}$ -release assays (Kawano et al., Cancer Res. 60: 3550-8 (2000); Nishizaka et al., Cancer Res. 60: 4830-7 (2000); Tamura et al., Jpn. J. Cancer Res. 92: 762-7 (2001)). However, both of HLA-A24 and HLA-A0201 are one of the popular HLA alleles in Japanese, as well as Caucasian (Date et al., Tissue Antigens 47: 93-101 (1996); Kondo et al., J. Immunol. 155: 4307-12 (1995); Kubo et al., J. Immunol. 152: 3913-24 (1994); Imanishi et al., Proceeding of the eleventh International Histocompatibility Workshop and Conference Oxford University Press, Oxford, 1065 (1992); Williams et al., Tissue Antigen 49: 129 (1997)). Thus, antigenic peptides of cancers presented by these HLAs may be especially useful for the treatment of cancers among Japanese and Caucasian. Further, it is known that the induction of low-affinity CTL *in vitro* usually results from the use of peptide at a high concentration, generating a high level of specific peptide/MHC complexes on antigen presenting cells (APCs), which will effectively activate these CTL (Alexander-Miller et al., Proc. Natl. Acad. Sci. USA 93: 4102-7 (1996)).

**[0017]** U.S. Patent No. 6,544,766 describes methods of producing kinesins from, for example, bacterial cells. U.S. Patent No. 6,472,521 describes an oligonucleotide that corresponds to a particular fragment of a nucleic acid sequence which encodes human eg5. WO 03/030832 describes antisense nucleotides that target human kinesin genes for treating diseases involving aberrant cell proliferation. WO 03/099224 describes antisense compounds for modulating the expression of kinesin-like 1. Kaiser, A. et al., J. Biol. Chem. (1999) 274(27):18925-18931, identifies kinesin-related protein HsEg5 as a differentially expressed all-*trans*-retinoic acid (ATRA)-responsive gene in pancreatic carcinoma cells. In Weil, D. et al., BioTechniques (2002) 33:1244-1248, it is reported that a small interfering RNA (siRNA) targeting the mRNA of the kinesin Eg5 induces a rapid mitotic, which provides an assay for optimization of siRNA transfection. In Sharp, D.J et al., J. Cell Biol. (1999) 144(1):125-138, it is reported that KLP61F, the essential mitotic bipolar kinesin,

functions in a sliding filament mechanism to hold spindle poles apart during metaphase and anaphase A and to drive spindle elongation during anaphase B. Blangy A. et al., Cell (1995) 83:1159-1169, report that microinjection of antibodies against human Eg5 (HsEg5) blocks centrosome migration and causes HeLa cells to arrest in mitosis with monoastal microtubule arrays. Houlston, E. et al, Devel. Biol. (1994) 164(1):147-159, examined the changing abundance and distribution of the kinesin-related protein Eg5 during oogenesis and early development in *Xenopus laevis*. U.S. Patent No. 6,331,396 describes methods employing an array with gene probes for use in identifying and characterizing proteins that mimic or inhibit the activity of all interferons. Methods and compositions for the construction of custom cDNA microarrays are described in U.S. Patent No. 6,706,867. Uses of nucleic acids encoding the kinesin KSP and their gene products to identify modulators of cell proliferation are described in WO 01/31335. Yarrow et al., Combinatorial Chemistry & High Throughput Screening 2003 6:279-286 describes cells screening methods to identify compounds that result in mitotic arrest. Processes to determine the cancerous status of a test cell, based on determining the expression of a gene that is differently expressed in comparison to non-cancerous cells, are disclosed in WO 01/94629.

**[0018]** Although advances have been made in the development of molecular-targeting drugs for cancer therapy, the ranges of tumor types that respond as well as the effectiveness of the treatments are still very limited. Hence, it is urgent to develop new anti-cancer agents that target molecules highly specific to malignant cells and are likely to cause minimal or no adverse reactions. To achieve the goal molecules whose physiological mechanisms are well defined need to be identified. A powerful strategy toward these ends would combine screening of up-regulated genes in cancer cells on the basis of genetic information obtained on cDNA microarrays with high-throughput screening of their effect on cell growth, by inducing loss-of-function phenotypes with RNAi systems (Kikuchi, T. et al., Oncogene 22: 2192-2205 (2003)).

## SUMMARY OF THE INVENTION

**[0019]** The invention described herein provides a method of diagnosing non-small cell lung cancer (NSCLC) or a predisposition to developing non-small cell lung cancer in a subject, comprising determining the expression level of a non-small cell lung cancer-associated gene in a biological sample derived from the subject, wherein an increase of said expression level compared to a normal control level of said gene indicates that said subject suffers from or is at risk of developing NSCLC, wherein said NSCLC-associated gene is KIF11.

**[0020]** The invention provides an in vitro method of identifying a compound for treating or preventing NSCLC, comprising the steps of:

- (1) contacting a test cell expressing said NSCLC-associated gene with a test compound;
- (2) detecting the expression level of said NSCLC-associated gene; and
- (3) determining the compound that suppresses said expression level compared to a normal control level of said gene wherein said NSCLC-associated gene is KIF11 and said compound is for use in treating or preventing NSCLC.

**[0021]** The invention provides an in vitro method of screening for a compound for treating or preventing NSCLC, said method comprising the steps of:

- (1) contacting a test compound with a polypeptide of KIF11;
- (2) detecting the binding activity between the polypeptide and the test compound; and
- (3) selecting a compound that binds to the polypeptide wherein said compound is for use in treating or preventing NSCLC.

**[0022]** The invention provides an in vitro method of screening for a compound for treating or preventing NSCLC, said method comprising the steps of:

- (a) contacting a test compound with a polypeptide encoded by a polynucleotide of KIF11;
- (b) detecting the biological activity of the polypeptide of step (a); and
- (c) selecting a compound that suppresses the biological activity of the polypeptide of KIF11 in comparison with the biological activity detected in the absence of the test compound

wherein said compound is for use in treating or preventing NSCLC.

**[0023]** The invention provides an in vitro method of screening for a compound for treating or preventing NSCLC, said method comprising the steps of:

- (1) contacting a test compound with a cell expressing KIF11; and

(2) selecting a compound that reduces the expression level of KIF11

wherein said compound is for use in treating or preventing NSCLC.

**[0024]** The invention provides an in vitro method of screening for a compound for treating or preventing NSCLC, said method comprising the steps of:

- (1) contacting a test compound with a cell into which a vector comprising the transcriptional regulatory region of KIF11 and a reporter gene that is expressed under the control of the transcriptional regulatory region has been introduced;
- (2) measuring the activity of said reporter gene; and
- (3) selecting a compound that reduces the expression level of said reporter gene, as compared to a control

wherein said compound is for use in treating or preventing NSCLC.

**[0025]** The invention provides in vitro method of screening for a compound for treating or preventing NSCLC, said method comprising the steps of:

- (1) contacting a KIF11 polypeptide or functional equivalent thereof with KOC1 polypeptide or functional equivalent thereof in the presence of a test compound;
- (2) detecting the binding between the polypeptides; and
- (3) selecting the test compound that inhibits the binding between the polypeptides.

**[0026]** The invention provides in vitro method of measuring RNA transporting activity of a polypeptide, said method comprising the steps of:

(a) contacting a polypeptide selected from the group consisting of:

- i. a polypeptide comprising the amino acid sequence of SEQ ID NO: 2 (*KIF11*);
- ii. a polypeptide comprising the amino acid sequence of SEQ ID NO: 2 wherein one or more amino acids are substituted, deleted, or inserted, and said polypeptide has a biological activity equivalent to the polypeptide consisting of the amino acid sequence of SEQ ID NO: 2;
- iii. a polypeptide that comprises the amino acid sequence having at least about 80% homology to SEQ ID NO: 2; and
- iv. 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 an RNA to be transported and under the condition capable of RNA transporter formation;

(b) detecting the level of the transported RNA; and

(c) measuring the RNA transporting activity by correlating the level of the transported RNA of step (b) with the RNA transporting activity.

**[0027]** The invention provides in vitro method of identifying an agent that modulates RNA transporting activity, said method comprising the steps of:

(a) contacting the agent with a polypeptide selected from the group consisting of:

- i. a polypeptide comprising the amino acid sequence of SEQ ID NO: 2 (*KIF11*);
- ii. a polypeptide comprising the amino acid sequence of SEQ ID NO: 2 wherein one or more amino acids are substituted, deleted, or inserted, and said polypeptide has a biological activity equivalent to the polypeptide consisting of the amino acid sequence of SEQ ID NO: 2;
- iii. a polypeptide that comprises the amino acid sequence having at least about 80% homology to SEQ ID NO: 2; and
- iv. 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 an RNA to be transported and under the condition capable of RNA transporter formation;

(b) detecting the level of the transported RNA; and

(c) comparing the level of the transported RNA to a control level in the absence of the agent wherein an increase or decrease in the level of the transported RNA compared to control level indicates that the test compound modulates RNA transporting activity.

**[0028]** The invention provides a kit for detecting for an activity of a test compound to regulate RNA transporting activity, said kit comprising an isolated cell expressing the components of a to d, and culture medium supporting the cell growth.

(a) a polypeptide selected from the group consisting of:

- i. a polypeptide comprising the amino acid sequence of SEQ ID NO: 2 (*KIF11*);
- ii. a polypeptide comprising the amino acid sequence of SEQ ID NO: 2 wherein one or more amino acids are substituted, deleted, or inserted, and said polypeptide has a biological activity equivalent to the polypeptide consisting of the amino acid sequence of SEQ ID NO: 2;
- iii. a polypeptide that comprises the amino acid sequence having at least about 80% homology to SEQ ID NO: 2; and
- iv. 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;

(b) a polypeptide selected from the group consisting of:

- i. a polypeptide comprising the amino acid sequence of SEQ ID NO: 105 (*KOC1*);
- ii. a polypeptide comprising the amino acid sequence of SEQ ID NO: 105 wherein one or more amino acids are substituted, deleted, or inserted, and said polypeptide has a biological activity equivalent to the polypeptide consisting of the amino acid sequence of SEQ ID NO: 105;
- iii. a polypeptide that comprises the amino acid sequence having at least about 80% homology to SEQ ID NO: 105; and
- iv. a polypeptide encoded by a polynucleotide that hybridizes under stringent conditions to a polynucleotide consisting of the nucleotide sequence of SEQ ID NO: 104, wherein the polypeptide has a biological activity equivalent to a polypeptide consisting of the amino acid sequence of SEQ ID NO: 105; and

(c) an RNA to be transported; and  
(d) DCTN1.

**[0029]** The invention provides the use of

- (i) an antisense composition, said composition comprising a nucleotide sequence complementary to a coding sequence of *KIF11*;
- (ii) an siRNA composition comprising an siRNA, wherein said composition reduces the expression of *KIF11*; or
- (iii) an antibody or fragment thereof that binds to a polypeptide encoded by *KIF11*; for the preparation of a pharmaceutical composition of treating or preventing NSCLC in a subject.

**[0030]** The invention provides the use of a polypeptide encoded by *KIF11* or an immunologically active fragment of said polypeptide, or a polynucleotide encoding the polypeptide for the preparation of a vaccine for treating or preventing NSCLC in a subject.

**[0031]** The invention provides a double-stranded molecule comprising a sense strand and an antisense strand, wherein the sense strand comprises a ribonucleotide sequence corresponding to a *KIF11* target sequence selected from the group consisting of SEQ ID NOs: 32, 33 and 34, and wherein the antisense strand comprises a ribonucleotide sequence which is complementary to said sense strand, wherein said sense strand and said antisense strand hybridize to each other to form said double-stranded molecule, and wherein said double-stranded molecule, when introduced into a cell expressing a *KIF11* gene, inhibits the expression of said gene, further wherein the double-stranded molecule is an oligonucleotide of between about 19 and about 25 nucleotides in length.

**[0032]** The invention provides a pharmaceutical composition comprising an siRNA against *KIF11* gene, wherein the siRNA comprises a sense strand comprising the nucleotide sequence of SEQ ID NO: 32, 33 and 34 as the target sequence and having about 19 to about 25 nucleotides in length.

**[0033]** The invention provides a method of predicting an NSCLC prognosis, wherein the method comprises the steps of:

(a) detecting expressing level of *KIF11* in a specimen collected from a subject whose NSCLC prognosis is to be



predicted, and

(b) indicating a poor prognosis when an elevation of the expressing level of KIF11 is detected.

**[0034]** Disclosed is a method of diagnosing or determining a predisposition to non-small cell lung cancer (NSCLC) in a subject by determining an expression level of a non-small cell lung cancer-associated gene that is selected from the group of KIF11, GHSR1b, NTSR1, and FOXM1 in a patient derived biological sample. An increase of the expression level of any of the genes compared to a normal control level of the genes indicates that the subject suffers from or is at risk of developing NSCLC.

**[0035]** Disclosed are also methods of providing a prognosis of a patient diagnosed with NSCLC. In particular, the methods involve detecting expression of KOC1, KIF11, or KOC1 in combination with expression of KIF11.

**[0036]** A "normal control level" indicates an expression level of any of the genes detected in a normal, healthy individual or in a population of individuals known not to be suffering from NSCLC. A control level is a single expression pattern derived from a single reference population or from a plurality of expression patterns. In contrast to a "normal control level", the "control level" is an expression level of the gene detected in an individual or a population of individuals whose background of the disease state is known (i.e., cancerous or non-cancerous). Thus, the control level may be determined base on the expression level of the gene in a normal, healthy individual, in a population of individuals known not to be suffering from NSCLC, a patient of NSCLC or a population of the patients. The control level corresponding to the expression level of the gene in a patient of non-small cell lung cancer or a population of the patients is referred to as "NSCLC control level". Furthermore, the control level can be a database of expression patterns from previously tested cells.

**[0037]** An increase in the expression level of any one of the genes of KIF11, GHSR1b, NTSR1, and FOXM1 detected in a test biological sample compared to a normal control level indicates that the subject (from which the sample was obtained) suffers from NSCLC. Alternatively, the expression level of any one or all of the genes in a biological sample may be compared to an NSCLC control level of the same gene(s).

**[0038]** Gene expression is increased or decreased 10%, 25%, 50% or more compared to the control level. Alternatively, gene expression is increased or decreased 1, 2, 5 or more fold compared to the control level. Expression is determined by detecting hybridization, e.g., on a chip or an array, of an NSCLC gene probe to a gene transcript of a patient-derived biological sample. The patient-derived biological sample may be any sample derived from a subject, e.g., a patient known to or suspected of having NSCLC. For example, the biological sample may be tissue containing sputum, blood, serum, plasma or lung cell.

**[0039]** Disclosed is a non-small cell lung cancer reference expression profile comprising a pattern of gene expression levels of two or more genes selected from the group of KIF11, GHSR1b, NTSR1, and FOXM1.

**[0040]** Also disclosed is a kit comprising two or more detection reagents which detects the expression of one or more of genes selected from the group of KIF11, GHSR1b, NTSR1, and FOXM1 (e.g., via detecting mRNA and polypeptide). Also provided is an array of polynucleotides that binds to one or more of the genes selected from the group of KIF11, GHSR1b, NTSR1, and FOXM1. The kits disclosed may also comprise reagents used to detect the expression of KIF11 and KOC1 to be used for the prognosis of NSCLC.

**[0041]** Also disclosed are kits for the detection of compounds that regulate RNA transporting activity. The kits may comprise a cell expressing a KIF11 polypeptide, or functional equivalent, a KOC1 polypeptide, or functional equivalent, and RNA to be transported, and DCTN1. The kits of the invention may also be used to screen for compounds for treating or preventing NSCLC. The kits may comprise a KOC1 polypeptide, or functional equivalent, and an RNA that is bound by the KOC1 polypeptide or functional equivalent.

**[0042]** Disclosed herein are methods of identifying compounds that inhibit the expression level of an NSCLC-associated gene (KIF11, GHSR1b, NTSR1 or FOXM1) by contacting a test cell expressing an NSCLC-associated gene with a test compound and determining the expression level of the NSCLC-associated gene. The test cell may be an NSCLC cell. A decrease of the expression level compared to a normal control level of the gene indicates that the test compound is an inhibitor of the expression or function of the NSCLC-associated gene. Therefore, if a compound suppresses the expression level of KIF11, GHSR1b, NTSR1 or FOXM1 compared to a control level, the compound is expected to reduce a symptom of NSCLC.

**[0043]** Alternatively, disclosed herein is a method of screening for a compound for treating or preventing NSCLC. The method includes contacting a polypeptide selected from the group of KIF11, GHSR1b, NTSR1, and FOXM1 with a test compound, and selecting the test compound that binds to or suppresses the biological activity of the polypeptide. The disclosure further provides a method of screening for a compound for treating or preventing NSCLC, which includes the steps of contacting a test compound with a cell that expresses KIF11, GHSR1b, NTSR1 or FOXM1 protein or introduced with a vector comprising the transcriptional regulatory region of KIF11, GHSR1b, NTSR1 or FOXM1 gene upstream of a reporter gene, and then selecting the test compound that reduces the Expression level of the KIF11, GHSR1b, NTSR1 or FOXM1 protein or protein encoded by the reporter gene. According to these screening methods, the test compound that suppresses the biological activity or the expression level compared to a control level is expected to reduce a symptom

of NSCLC. Furthermore, the present disclosure provides a method of screening for a compound for tre2 or preventing NSCLC wherein the binding between KIF11 and KOC1, or GHSR1b or NTSR1 and NMU is detected. Compounds that inhibit the binding between KIF11 and KOC 1, or GHSR1b or NTSR1 and NMU are expected to reduce a symptom of NSCLC.

**[0044]** We detected a novel intra-cellular and inter-cellular RNA-transporting system in lung carcinomas, involving transactivation of KOC1 and KIF11. A complex of these two molecules in lung tumors was able to bind mRNAs encoding proteins known to function in intercellular adhesion, cancer-cell progression, and oncogenesis, and transport them to neighboring cells through ultrafine intercellular structures. In particular, evidence provided here shows that KOC1 binds to KIF11 at the RRM domain in the N-terminal region of KOC1. In addition, evidence provided here shows inhibition of their binding by dominant-negative KOC1 mutants effectively suppressed growth of NSCLC cells *in vitro*. For example, KOC1 fragments (or nucleic acids encoding them) comprising the RRM domains of KOC1 can be used as dominant negative fragments to suppress cell proliferation and thus treat cancer. Alternatively, the KOC1 fragment may comprise the ribonucleoprotein K-homologous (KH) domain.

**[0045]** The disclosure also provides methods of identifying polypeptides and other compounds that modulate RNA transport activity. For example, a polypeptide can be tested for RNA transporting activity by contacting the polypeptide with a KIF11 polypeptide or a functional equivalent thereof with an RNA that can be transported by KIF11 under conditions suitable for transportation of RNA. Alternatively, agents that modulate RNA transporting activity can be tested by contacting a test agent with a KIF11 polypeptide or a functional equivalent thereof with an RNA that can be transported by KIF11 under conditions suitable for transportation of RNA. Test agents useful for treating NSCLC by testing the agents for the ability to inhibit binding between a KOC1 polypeptide, or a functional equivalent and an RNA that is bound by KOC1 or the complex of KOC1 and KIF11.

**[0046]** Immunohistochemical analysis of lung-cancer tissue microarrays demonstrated that transactivation of KOC1 and KIF11 was significantly associated with poor prognosis of lung-cancer patients.

**[0047]** Methods for treating or preventing NSCLC and compositions to be used for such methods are also provided. Therapeutic methods include a method of treating or preventing NSCLC in a subject by administering to the subject a composition of an antisense, short interfering RNA (siRNA) or a ribozyme that reduce the expression of KIF11, GHSR1b, NTSR1 or FOXM1 gene, or a composition comprising an antibody or fragment thereof that binds and suppresses the function of a polypeptide encoded by the gene. The compositions herein disclosed may also comprise a dominant negative KOC1 mutant (or nucleic acids encoding it) comprising a KOC1 fragment that contains one or more RRM domains and/or KH domains of KOC1.

**[0048]** Disclosed are also vaccines and vaccination methods. For example, a method of treating or preventing NSCLC in a subject is carried out by administering to the subject a vaccine containing a polypeptide encoded by KIF11, GHSR1b, NTSR1, or FOXM1 gene, or an immunologically active fragment of the polypeptide. An immunologically active fragment is a polypeptide that is shorter in length than the full-length naturally-occurring protein and which induces an immune response upon introduction into the body. For example, an immunologically active fragment includes a polypeptide of at least 8 residues in length that stimulates an immune cell such as a T cell or a B cell *in vivo*. Immune cell stimulation can be measured by detecting cell proliferation, elaboration of cytokines (e.g., IL-2) or production of antibody.

**[0049]** Other therapeutic methods include those wherein a compound selected by the screening method disclosed is administered.

**[0050]** Also disclosed are double-stranded molecules that comprise a sense strand and an antisense strand. The sense strand comprises a ribonucleotide sequence corresponding to a target sequence comprised within the mRNA of a KIF11, GHSR1b, NTSR1 or FOXM1 gene, and the antisense strand is a complementary sequence to the sense strand. Such double-stranded molecules disclosed herein, can be used as siRNAs against KIF11, GHSR1b, NTSR1 or FOXM1 gene. Furthermore the disclosure relates to vectors encoding the double-stranded molecules of the present disclosure.

**[0051]** The disclosure also provides a composition for treating and/or preventing NSCLC using any of the antisense polynucleotides or siRNAs against KIF11, GHSR1b, NTSR1 or FOXM1 gene, or an antibody that binds to a polypeptide encoded by KIF11, GHSR1b, NTSR1 or FOXM1 gene. Other compositions include those that contain a compound selected by the screening method of the present disclosure as an active ingredient.

**[0052]** It is to be understood that both the foregoing summary of the invention and the following detailed description are of a preferred embodiment, and not restrictive of the invention or other alternate embodiments.

#### BRIEF DESCRIPTION OF THE DRAWINGS

**[0053]** **Figure. 1** shows photographs confirming the relationship between KOC1 and KIF11.

(a) depicts the result of co-immunoprecipitation of KOC1 and KIF11 confirming the interaction between KOC 1 and KIF11. A549 cells were transiently co-transfected with Flag-tagged KIF11 and myc-tagged KOC1, immunoprecipitated with anti-Flag M2 agarose, and subsequently immunoblotted with anti-myc antibody In contrast, using the

same combination of vectors and cells, the cells were immunoprecipitated with anti-myc agarose and immunoblotted with anti-Flag M2 antibody. A band corresponding to the immunoblotted protein was found only when both constructs were co-transfected.

(b) depicts the result of immunocytochemical staining showing the co-localization of KOC1 and KIF11. COS-7 cells were transiently transfected with FLAG-tagged KIF11 and myc-tagged KOC1, and their co-localization was detected mainly in the cytoplasm using FITC-labeled anti-FLAG antibody and rhomamine-labeled anti-myc antibody.

(c) depicts the result of reciprocal co-immunoprecipitation of endogenous KOC 1 and KIF11 from extracts of lung-cancer cell lines A549 and LC319. (*upper panel*) Western-blot analysis of both cell extracts immunoprecipitated with anti-KOC1 antibodies, with KIF11 protein detected in the immunoprecipitate. (*lower panel*) Western-blot of extracts immunoprecipitated with anti-KIF11 antibodies, with KOC1 protein detected in the immunoprecipitate.

**[0054] Figure. 2** shows photographs confirming co-activation of *KOC1* and *KIF11* in lung tumors and normal tissues.

(a) depicts the result of QRT-PCR examining expression of *KOC1* and *KIF11* in clinical samples of NSCLC and corresponding normal lung tissues. Y-axis indicates the relative expression rate of the two genes (*KOC1* or *KIF11/ACTB*).

(b) depicts the result of QRT-PCR examining expression of *KOC1* and *KIF11* among 20 lung-cancer cell lines.

(c) depicts the result of Northern-blot analysis detecting expression of *KOC1* and *KIF11* in normal human tissues.

**[0055] Figure. 3** shows photographs confirming the relationship between KOC 1 and KIF11.

(a) shows schematic drawing of five KOC1 deletion mutants lacking either or both of the terminal regions, with N- and C-terminals tagged with FLAG and HA respectively. KH, ribonucleoprotein K-homologous domain.

(b) depicts the result of immunoprecipitation experiments for identification of the region of KOC1 that binds to KIF11. The KOC1DEL4 and KOC1DEL5 constructs, which lacked two RNA-recognition motifs, (RRM) did not retain any appreciable ability to interact with endogenous KIF11.

**[0056] Figure. 4** shows photographs confirming the relationship between KOC1 and KOC1-associated mRNAs.

(a) depicts the result of Western blotting with immunoprecipitated KOC1 deletion mutants and DIG-labeled RAB35 full length mRNA for identification of the mRNA-binding region in KOC1.

(b) depicts the result of Northwestern with immunoprecipitated KOC1 deletion mutants and DIG-labeled RAB35 full length mRNA for identification of the mRNA-binding region in KOC1. The KOC1DEL3 and KOC1DEL5, did not bind to any of these mRNAs, and the KOC1DEL4, which is a construct with the four KH domains only, showed similar binding affinities for mRNAs to the KOC1DEL2, a construct without C-terminal two KH domains.

(c) depicts the result of IP-RT-PCR for confirmation of IP-microarray and the ability of various KOC1 deletion mutants transfected into A549 cells to bind directly to representative eight endogenous mRNAs (*CCT2*, *SBP2*, *SLC25A3*, *RAB35*, *PSMB7*, *GL*, *PKP4*, and *WINS1*) among 55 candidate genes (see Table2).

**[0057] Figure. 5** shows photographs showing movement of KOC1-KIF11-mRNA ribonucleoprotein complexes in living cultured mammalian cells.

(a) are photographs showing transport of the KOC1-KIF11 protein complex. Small particles that expressed fluorescent cyan (ECFP) KOC1 and yellow (EYFP) KIF11 proteins were co-localized, and transferred together between connected COS-7 cells through ultrafine intercellular structures (arrows).

(b) are photographs showing transport of KOC1-RAB35 mRNA RNP complex from one COS-7 cell that contains a high level of KOC1-RNP complex (cell A) to another cell with a lower level of the complex (stained simply with CellTracker (blue); cell B). Small particles of KOC1 (green)-RAB35 mRNA (red) complex as well as KOC1 particles (green) were transferred from cell A to cell B through ultrafine intercellular structures (arrows).

**[0058] Figure. 6** shows photographs showing localization of KOC1-KIF11-mRNA ribonucleoprotein complexes.

(a) depicts the result of immunoprecipitation of cell extracts from A549 and LC319 confirming of direct interaction between endogenous KIF11 and DCTN1 (upper and lower panels).

**[0059] Figure. 7** shows photographs showing translation of KOC1-associated mRNAs transported into the recipient cells.

(a) are photographs showing translation of mRNA transported into the recipient cells monitored by *in situ* hybridization. (b) are photographs showing protein synthesis based on transported mRNA in receiving cell. Constructs with full length RAB35 mRNA fused in frame to a myc tag sequence (upper panel). Co-localization of myc-tagged RAB35 proteins in the cytoplasm of CellTracker-stained receiving cells (blue) using immunocytochemistry (lower panels). (c) are photographs showing protein synthesis based on transported mRNA in receiving cell. Constructs with full length RAB35 mRNA fused in frame to a EGFP protein sequence. (d) are photographs showing protein synthesis based on transported mRNA in receiving cell. Expression of EGFP-fused RAB35 proteins in CellTracker-positive receiving cells (blue) using time-lapse video microscopy. EGFP and related-DIC image were shown. (e) are photographs showing that no significant difference in the protein level of RAB35-EGFP fused-protein was found between COS-7 cells that were co-transfected with RAB35-EGFP and HA-tagged-KOC1 vectors, and those with RAB35-EGFP and mock plasmid vectors. This indicates that KOC1 is not likely to interfere with translation of RAB35-EGFP mRNA.

**[0060] Figure. 8** shows the effect of KIF 11 siRNAs on cells.

(a) depicts the inhibition on the growth of NSCLC cells by siRNAs against KIF 11. The expression of KIF 1 in response to specific siRNAs (si-KIF#1, #2, and #3) or control siRNAs (EGFP, LUC, SC) in A549 cells, was analyzed by semiquantitative RT-PCR. (b) depicts the viability of A549 cells in response to si-KIF#1, #2, #3, EGFP, LUC, or SC, evaluated by triplicate MTT assays.

**[0061] Figure. 9** shows the effect of KOC1 dominant-negative on cells.

(a) depicts the results of immunoprecipitation confirming interaction of KOC 1 deletion-mutant KOC1DEL3 with endogenous KIF11 in LC319 cells. (b) depicts the results of immunoprecipitation confirming reduction of the complex formation between endogenous KOC1 and KIF11 in LC319 cells over-expressing the RRM domains. (c) depicts the viability of LC319 cells in response to dose-dependent dominant-negative effect of KOC1DEL3 evaluated by triplicate MTT assays. X-axis indicates dosage of KOC1DEL3 plasmid-DNA ( $\mu$ g) transfected into LC319 cells in individual assays. (d) depicts the results of immunoprecipitation detecting reduction of the complex formation between endogenous KOC 1 and KIF11 in A549 cells that were transfected with the KOC1DEL2 construct. (e) depicts the results of immunoprecipitation detecting interaction of the KOC1DEL2 with endogenous KIF 11 in A549 cells. (f) depicts the viability of A549 cells in response to dose-dependent dominant-negative effect of KOC1DEL2 evaluated by triplicate MTT assays. X-axis indicates dosage of KOC1DEL2 plasmid-DNA ( $\mu$ g) transfected into A549 cells in individual assays.

**[0062] Figure. 10** shows the effect of RAB35 siRNAs on cells.

(a) depicts the result of semi-quantitative RT-PCR analyzing mRNA knock-down effect in response to si-RAB35 or control siRNAs in A549 cells. (b) show results of MTT assays of A549 cells transfected with specific siRNA or control plasmids (EGFP, Scramble, or Luciferase). Error bars represent the standard deviation of triplicate assays.

**[0063] Figure.11** shows association of KOC1 and KIF11 over-expression with worse outcomes in NSCLC.

(a) depicts the results of immunohistochemical evaluation of representative samples from surgically-resected SCC tissues, using anti-KOC1 (left) and anti-KIF11 (right) polyclonal antibodies on tissue microarrays (X200). (b) depicts the results of Kaplan-Meier analysis of tumor-specific survival times according to KOC1 expression (left panel) and KIF11 expression (right panel) on tissue microarrays.

**[0064] Figure. 12** is schematic model for the mechanism of intracellular and cell-to-cell mRNA transport by KOC1-KIF11-DCTN1 complexes on microtubules. The KOC1 ribonucleoprotein complex, including KIF11 motor-protein and DCTN1, transports KOC1-associated mRNAs through the structure of microtubules within or between mammalian somatic cells. This model implies that proliferating cancer-cells may communicate actively by engaging this molecular complex in a system that transports mRNAs critical for cancer growth or progression from one cell to another Fig. 8

shows the relationship between NMU and GHSR1b/NTSR1.

**[0065]** **Figure. 13 (a)** shows the result of semiquantitative RT-PCR analysis depicting the expression of NMU, candidate receptors, and their known ligands detected in NSCLC cell lines.

(b) shows GHSR1b Expression in normal human tissues.

(c) depicts the result of immunocytochemical staining using FITC-labeled anti-FLAG antibody showing the co-localization of NMU and GHSR1b/NTSR1 on the cell surface of COS-7 cells that were transiently transfected with FLAG-tagged GHSR1b or NTS1.

(d) depicts the interaction of NMU with GHSR1b/NTSR1. COS-7 cells were transiently transfected with the same vectors, and binding of rhodamine-labeled NMU-25 to the cell surface was detected by flow cytometry. As negative controls for these assays, three ligand/cell combinations were prepared: 1) non-transfected COS-7 cells; 2) NMU-25-rhodamine vs non-transfected COS-7 cells; and 3) COS-7 cells transfected only with GHSR1b or NTSR1.

(e) depicts the results of receptor-ligand binding assay using the LC319 and PC-14 cells treated with NMU-25.

(f) depicts cAMP production of NMU-treated NSCLC cells.

**[0066]** **Figure. 14** shows the effect of siRNAs on cells.

(a) depicts the inhibition on the growth of NSCLC cells by siRNAs against GHSR1b and NTSR1. Expression of GHSR or NTSR1 in response to specific siRNAs (si-GHSR or si-NTSR1) or control siRNAs (EGFP, LUC, SCR) in A549 and LC319 cells were analyzed by semiquantitative RT-PCR.

(b) depicts the result of triplicate MTT assays evaluating viability of A549 or LC319 cells in response to si-GHSR, NTSR1, EGFP, LUC, or SCR.

**[0067]** **Figure. 15** shows validation of candidate downstream genes of NMU.

(a) depicts time-dependent reduction of NMU transcript by si-NMU.

(b) depicts the result of semiquantitative RT-PCR experiments of mRNAs from LC319 cells treated with si-NMU, with gene-specific primers confirming time-dependent reduction of candidate downstream target gene expression.

(c) depicts the result of semiquantitative RT-PCR using mRNAs from LC319 cells incubated with NMU-25 or BSA (control) (100  $\mu$ M) detecting induction of FOXM1 as the candidate downstream target gene of NMU.

**[0068]** **Figure. 16** shows the effect of FOXM1 siRNAs on cells.

(a) depicts inhibition of growth of NSCLC cells by siRNAs against FOXM1. Expression of FOXM1 in response to specific siRNAs (si-FOXM1) or control siRNAs (EGFP, LUC, SCR) in A549 cells, analyzed by semiquantitative RT-PCR (*upper panel*). Viability of A549 cells, evaluated by triplicate MTT assays, in response to si-FOXM1, EGFP, LUC, or SCR (*lower panel*).

(b) depicts inhibition of growth of NSCLC cells by siRNAs against FOXM1. Expression of FOXM1 in response to specific siRNAs (si-FOXM1) or control siRNAs (EGFP, LUC, SCR) in LC319 cells, analyzed by semiquantitative RT-PCR (*upper panel*). Viability of A549 cells, evaluated by triplicate MTT assays, in response to si-FOXM1, EGFP, LUC, or SCR (*lower panel*).

**[0069]** **Figure. 17** is a schematic model for promotion of cancer cell growth and invasion through the NMU-receptor interaction in the autocrine NMU-GHSR1b oncogenic signaling pathway. Binding of NMU to GHSR1 b and/or NTSR1 leads to the activation of adenylate cyclase, accumulation of intracellular cAMP and following activation of cAMP-dependent protein kinase (PKA). The release of catalytic subunits of PKA (C) from the regulatory subunits (R) is resulting in the activation of downstream FOXM1 gene and/or related target genes.

#### DETAILED DESCRIPTION OF THE METHODS DISCLOSED

**[0070]** The words "a", "an" and "the" as used herein mean "at least one" unless otherwise specifically indicated. The terms "protein" and "polypeptide" are used interchangeably. Furthermore, the terms "gene", "polynucleotide", and "nucleic acids" are used interchangeably unless otherwise specifically indicated.

**[0071]** To investigate the mechanisms of lung carcinogenesis and identify genes that might be useful as diagnostic markers or targets for development of new molecular therapies, genes specifically up-regulated in non-small cell lung cancers (NSCLC) were searched by means of cDNA microarray. Through the analysis, a couple of candidate therapeutic target genes were identified. Two genes, KH domain containing protein over-expressed in cancer (KOC1) and neuro-medin U (NMU) were abundantly expressed in clinical NSCLC samples as well as NSCLC cell lines examined. However,

their expression was hardly detectable in corresponding non-cancerous lung tissue. The growth of NSCLC cells that over-expressed endogenous NMU was significantly inhibited by anti-NMU antibody. Furthermore, the treatment of NSCLC cells with siRNA against KOC1 and/or NMU suppressed the expression of the gene and resulted in growth inhibition of the NSCLC cells. Furthermore, KOC1 was identified to bind to kinesin family member 11 (KIF11) of the cancer cells, whereas NMU bound to the neuropeptide G protein-coupled receptors (GPCRs), growth hormone secretagogue receptor 1b (GHSR1b) and neurotensin receptor 1 (NTSR1). NMU ligand-receptor system was identified to activate Homo sapiens forkhead box M1 (FOXO1). Interestingly, GHSR1b, NTS1, FOXO1, and KIF11 were all specifically over-expressed in NSCLC cells.

**[0072]** RNA binding protein KOC 1 and microtubules motor protein KIF 11 is required for the localization of some kinds of mRNA needed in embryogenesis and carcinogenesis (Fig. 12). As previously reported by the present inventors, treatment of NSCLC cells with specific siRNA to reduce expression of KOC1 resulted in growth suppression. In this study, KIF11 was demonstrated to associate with KOC1 in NSCLC cells and to be the target for the growth-promoting effect of KOC1 in lung tumors. The present inventors revealed that KOC1 not only co-localized with KIF1 1 in human normal tissues, NSCLCs, and cell lines, but also directly interacted with KIF11 in NSCLC cells *in vitro*, and that the treatment of NSCLC cells with siRNAs for KIF11 reduced its expression and led to growth suppression. The results show that KOC1-KIF11 signaling affects growth of NSCLC cells. As shown below, dominant negative fragments of KOC1 (e.g., those containing the RRM domains) can be used to inhibit proliferation of cancer cells. By expression analysis, increased expression of KOC1 and KIF 11 was detected in the majority of NSCLC samples, but not in normal lung tissues. Since most of the clinical NSCLC samples used for the present analysis were at an early and operable stage, KOC1 and KIF11 can be conveniently used as a biomarker for diagnosing early-stage lung cancer, in combination with fiberoptic transbronchial biopsy (TBB) of sputum cytology.

**[0073]** Therefore, KOC1 and KIF11 are essential for an oncogenic pathway in NSCLCs. The data reported here provide evidence for designing new anti-cancer drugs, specific for lung cancer, which target the KOC1-KIF11 pathway. They also show that siRNAs can be used to treat chemotherapy-resistant, advanced lung cancers.

**[0074]** A significant increase in the sub-G1 fraction of NSCLC cells transfected with siRNA-NMU suggested that blocking the autocrine NMU-signaling pathway could induce apoptosis. The present inventors also found other evidence supporting the significance of this pathway in carcinogenesis; e.g., addition of NMU into the medium promoted the growth of COS-7 cells in a dose-dependent manner, and addition of anti-NMU antibody into the culture medium inhibited this NMU-enhanced cell growth, possibly by neutralizing NMU activity. Moreover, the growth of NSCLC cells that endogenously over-expressed NMU was significantly inhibited by anti-NMU antibody. The expression of NMU also resulted in significant promotion of COS-7 cell invasion in *in vitro* assays. These results show that NMU is an important growth factor for NSCLC and is associated with cancer cell invasion, functioning in an autocrine manner, and that screening molecules targeting the NMU-receptor growth-promoting pathway is a useful therapeutic approach for treating NSCLCs. By immunohistochemical analysis, increased expression of NMU protein was detected in the majority of NSCLC (SCC, ADC, LCC, and BAC) and SCLC samples, but not in normal lung tissues. Since NMU is a secreted protein and most of the clinical NSCLC samples used for the present analysis were at an early and operable stage, NMU can be conveniently used as a biomarker for diagnosis of early-stage lung cancer, in combination with fiberoptic transbronchial biopsy (TBB), sputum cytology, or blood tests.

**[0075]** Two receptors, NMU1R (FM3/GPR66) and NMU2R (FM4) are known to interact with NMU. The results presented here, however, indicated that these two known receptors were not the targets for the autocrine NMU-signaling pathway in NSCLCs; instead, GHSR1b and NTSR1 proved to be the targets for the growth-promoting effect of NMU in lung tumors. The present inventors revealed that NMU-25 bound to these receptors on the cell surface, and that treatment of NSCLC cells with siRNAs for GHSR1b or NTSR1 reduced expression of the receptors and led to apoptosis. The results show that NMU affects growth of NSCLC cells by acting through GHSR1b and/or NTSR1 (Fig. 14). GHSR is a known receptor of Ghrelin (GHRL), a recently identified 28-amino-acid peptide capable of stimulating release of pituitary growth hormone and appetite in humans (Lambert, P.D. et al., Proc. Natl. Acad. Sci. 98: 4652-4657 (2001); Petersenn, S. et al., Endocrinology 142: 2649-2659 (2001); Kim K. et al., Clin. Endocrinol. 54: 705-860 (2001); Kojima, M. et al., Nature 402: 656-660 (1999)). Of the two transcripts known to be receptors for GHRL, GHSR1a and GHSR1b, over-expression of only GHSR1b was detected in NSCLC tissues and cell lines. Since GHRL was not expressed in the NSCLCs examined, GHSR1b was suspected to have a growth-promoting function in lung tumors through binding to NMU, but not to GHRL.

**[0076]** NTSR1 is one of three receptors of neurotensin (NTS), a brain and gastrointestinal peptide that fulfills many central and peripheral functions (Heasley, L.E. Oncogene 20: 1563-1569 (2001)). NTS modulates transmission of dopamine and secretion of pituitary hormones, and exerts hypothermic and analgesic effects in the brain while it functions as a peripheral hormone in the digestive tract and cardiovascular system. Others have reported that NTS is produced and secreted in several human cancers, including small-cell lung cancers (SCLC) (Heasley, L.E. Oncogene 20: 1563-1569 (2001)). The expression of NTS was detected in four of the 15 NSCLC cell lines that were examined as disclosed herein (Fig. 13a), but the expression pattern of NTS was not necessarily concordant with that of NMU or

NTSR1. Therefore NTS may, along with NMU, contribute to the growth of NSCLC through NTSR1 or other receptor(s) in a small subset of NSCLCs. In the present experiments the majority of the cancer cell lines and clinical NSCLCs that expressed NMU also expressed GHSR1b and/or NTSR1, indicating that these ligand-receptor interactions were involved in a pathway that is central to the growth-promoting activity of NMU in NSCLC.

**[0077]** NMU signaling pathway affects the growth promotion of lung-cancer cells by transactivating a set of downstream genes including *FOXM1*. *FOXM1* was known to be over-expressed in several types of human cancers (Teh, M.T. et al., Cancer Res. 62, 4773-4780.; van den Boom, J. et al., (2003). Am. J. Pathol. 163, 1033-1043.; Kalinichenko, V.V. et al., (2004). Genes. Dev. 18, 830-850). The "forkhead" gene family, originally identified in *Drosophila*, comprises transcription factors with a conserved 100-amino acid DNA-binding motif, and has been shown to play important roles in regulating the expression of genes involved in cell growth, proliferation, differentiation, longevity, and transformation. Cotransfection assays in the human hepatoma HepG2 cell line demonstrated that *FOXM1* protein stimulated expression of both the cyclin B1 (*CCNB1*) and cyclin D1 (*CCND1*) (Wang, X. et al., (2002). Proc. Nat. Acad Sci. 99, 16881-16886.), suggesting that these cyclin genes are direct *FOXM1* transcription targets and that *FOXM1* controls the transcription network of genes that are essential for cell division and exit from mitosis. It should be noted that we observed activation of *CCNB1* in the majority of a series of NSCLC and its good concordance of the expression to *FOXM1* (data not shown). The promotion of cell growth in NSCLC cells by NMU might reflect transactivation of *FOXM1*, which would affect the function of those molecular pathways in consequence. Therefore, NMU, two newly revealed receptors for this molecule, GHSR1b and NTSR1, and their downstream gene *FOXM1* are involved in an autocrine growth-promoting pathway in NSCLCs. The data reported here provide the basis for designing new anti-cancer drugs, specific for lung cancer, that target the NMU-GHSR1b/NTSR1-*FOXM1* pathway.

**[0078]** They also show that siRNAs that interfere with this pathway can be used to treat chemotherapy-resistant, advanced lung cancers.

**[0079]** These data show that KOC1-KIF11 signaling pathway is frequently up-regulated in lung carcinogenesis, and that NMU an important autocrine growth factor for NSCLC, acting through GHSR1b and NTSR1 receptor molecules. Thus, selective suppression of components of these complexes can suppress the development and/or progression of lung carcinogenesis and targeting these pathways are conveniently used in therapeutic and diagnostic strategies for the treatment of lung-cancer patients.

#### *Diagnosing non-small cell lung cancer (NSCLC)*

**[0080]** By measuring the expression level of KIF11, GHSR1b, NTSR1 or *FOXM1* gene in a biological derived from a subject, the occurrence of NSCLC or a predisposition to develop NSCLC in the subject can be determined. The disclosure involves determining (e.g., measuring) the expression level of at least one, and up to all of KIF11, GHSR1b, NTSR1, and *FOXM1* gene in the biological sample.

**[0081]** According to the disclosure, a gene transcript of NSCLC-associated gene, KIF11, GHSR1b, NTSR1 or *FOXM1*, is detected for determining the expression level of the gene. The expression level of a gene can be detected by detecting the expression products of the gene, including both transcriptional and translational products, such as mRNA and proteins. Based on the sequence information provided by the GenBank™ database entries for the known sequences, KIF11 (NM\_004523), GHSR1b (NM\_004122), NTSR1 (NM\_002531), and *FOXM1* (No.NM\_202003) genes can be detected and measured using techniques well known to one of ordinary skill in the art. The nucleotide sequences of the KIF11, GHSR1b, NTSR1, and *FOXM1* genes are described as SEQ ID NOs: 1, 3, 5, and 106, respectively, and the amino acid sequences of the proteins encoded by the genes are described as SEQ ID NOs: 2, 4, 6, and 107.

**[0082]** For example, sequences within the sequence database entries corresponding to KIF11, GHSR1b, NTSR1 or *FOXM1* gene can be used to construct probes for detecting their mRNAs by, e.g., Northern blot hybridization analysis. The hybridization of the probe to a gene transcript in a subject biological sample can be also carried out on a DNA array. The use of an array is preferred for detecting the expression level of a plurality of the NSC genes (KIF11, GHSR1b, NTSR1, and *FOXM1*). As another example, the sequences can be used to construct primers for specifically amplifying KIF11, GHSR1b, NTSR1 or *FOXM1* gene in, e.g., amplification-based detection methods such as reverse-transcription based polymerase chain reaction (RT-PCR). Furthermore, the expression level of KIF11, GHSR1b, NTSR1 or *FOXM1* gene can be analyzed based on the quantity of the expressed proteins encoded by the gene. A method for determining the quantity of the expressed protein includes immunoassay methods. Alternatively, the expression level of KIF11, GHSR1b, NTSR1 or *FOXM1* gene can also be determined based on the biological activity of the expressed protein encoded by the gene. For example, a protein encoded by KIF11 gene is known to bind to KOC1, and thus the expression level of the gene can be detected by measuring the binding ability to KOC1 due to the expressed protein. Furthermore, KIF11 protein is known to have a cell proliferating activity. Therefore, the expression level of KIF11 gene can be determined using such cell proliferating activity as an index. On the other hand GHSR1 b and NTSR1 proteins are known to bind to NMU, and also have a cell proliferating activity. Thus, similarly to KIF11, the expression levels of GHSR1b and NTSR1 genes can be detected by measuring their binding ability to NMU or cell proliferating activity due to the expressed protein.

**[0083]** Any biological materials may be used as the biological sample for determining the expression level so long as any of the KIF11, GHSR1b, NTSR1, and FOXM1 genes can be detected in the sample and includes test cell populations (*i.e.*, subject derived tissue sample). Preferably, the biological sample comprises a lung cell (a cell obtained from the lung). Gene Expression may also be measured in blood, serum or other bodily fluids such as sputum. Furthermore, the

**[0084]** The subject diagnosed for NSCLC according to the method is preferably a mammal and includes human, non-human primate, mouse, rat, dog, cat, horse and cow.

**[0085]** The expression level of one or more of KIF11, GHSR1b, NTSR1 or FOXM1 gene in the biological sample is compared to the expression level(s) of the same genes in a reference sample. The reference sample includes one or more cells with known parameters, *i.e.*, cancerous or non-cancerous. The reference sample should be derived from a tissue type similar to that of the test sample. Alternatively, the control expression level may be determined based on a database of molecular information derived from cells for which the assayed parameter or condition is known.

**[0086]** Whether or not a pattern of the gene expression levels in a biological sample indicates the presence of NSCLC depends upon the composition of the reference cell population. For example, when the reference cell population is composed of non-cancerous cells, a similar gene expression level in the test biological sample to that of the reference indicates that the test biological sample is non-cancerous. On the other hand, when the reference cell population is composed of cancerous cells, a similar gene expression profile in the biological sample to that of the reference indicates that the test biological sample includes cancerous cells.

**[0087]** The test biological sample may be compared to multiple reference samples. Each of the multiple reference samples may differ in the known parameter. Thus, a test sample may be compared to a reference sample known to contain, *e.g.*, NSCLC cells, and at the same time to a second reference sample known to contain, *e.g.*, non-NSCLC cells (normal cells).

**[0088]** According to the disclosure, the expression of one or more of the NSCLC-associated genes,

**[0089]** KIF11, GHSR1b, NTSR1, and FOXM1, is determined in the biological sample and compared to the normal control level of the same gene. The phrase "normal control level" refers to an expression profile of KIF11, GHSR1b, NTSR1 or FOXM1 gene typically found in a biological sample derived from a population not suffering from NSCLC. The expression level of KIF11, GHSR1b, NTSR1 or FOXM1 gene in the biological samples from a controls and test subjects may be determined at the same time or the normal control level may be determined by a statistical method based on the results obtained by analyzing the expression level of the gene in samples previously collected from a control group. An increase of the expression level of KIF11, GHSR1b, NTSR1 or FOXM1 gene in the biological sample derived from a patient derived tissue sample indicates that the subject is suffering from or is at risk of developing NSCLC.

**[0090]** An expression level of KIF11, GHSR1b, NTSR1 or FOXM1 gene in a test biological sample can be considered altered when the expression level differs from that of the reference by more than 1.0, 1.5, 2.0, 5.0, 10.0 or more fold. Alternatively, an expression level of KIF11, GHSR1 b, NTSR1 or FOXM1 gene in a test biological sample can be considered altered, when the expression level is increased or decreased to that of the reference at least 50%, 60%, 80%, 90% or more.

**[0091]** The difference in gene expression between the test sample and a reference sample may be normalized to a control, *e.g.*, housekeeping gene. For example, a control polynucleotide includes those whose Expression levels are known not to differ between the cancerous and non-cancerous cells. The expression levels of the control polynucleotide in the test and reference samples can be used to normalize the expression levels detected for KIF11, GHSR1b, NTSR1 or FOXM1 gene. The control genes to be used in the present disclosure include  $\beta$ -action, glyceraldehyde 3-phosphate dehydrogenase and ribosomal protein P1.

**[0092]** The differentially expressed KIF11, GHSR1b, NTSR1 or FOXM1 gene identified herein also allow for monitoring the course of treatment of NSCLC. In this method, a test biological sample is provided from a subject undergoing treatment for NSCLC. If desired, multiple test biological samples are obtained from the subject at various time points before, during or after the treatment. The expression of one or more of KIF11, GHSR1b, NTSR1 or FOXM1 gene in the sample is then determined and compared to a reference sample with a known state of NSCLC that has not been exposed to the treatment.

**[0093]** If the reference sample contains no NSCLC cells, a similarity in the expression level of KIF11, GHSR1b, NTSR1 or FOXM1 gene in the test biological sample and the reference sample indicates the efficaciousness of the treatment. However, a difference in the expression level of KIF11, GHSR1b, NTSR1 or FOXM1 gene in the test and the reference samples indicates a less favorable clinical outcome or prognosis. In particular, increased expression of KOC1, KIF11, or KOC1 in combination with increased Expression of KIF11 is significantly associated with poor prognosis.

**[0094]** The term "efficacious" refers that the treatment leads to a reduction in the expression of a pathologically up-regulated gene (including the present indicator genes, KIF11, GHSR1b, NTSR1, and FOXM1), or a decrease in size, prevalence or metastatic potential of NSCLC in a subject. When a treatment is applied prophylactically, "efficacious" means that the treatment retards or prevents occurrence of NSCLC or alleviates a clinical symptom of NSCLC. The assessment of NSCLC can be made using standard clinical protocols. Furthermore, the efficaciousness of a treatment



is determined in association with any known method for diagnosing or treating NSCLC. For example, NSCLC is diagnosed histopathologically or by identifying symptomatic anomalies such as chronic cough, hoarseness, coughing up blood, weight loss, loss of appetite, shortness of breath, wheezing, repeated bouts of bronchitis or pneumonia and chest pain.

**[0095]** Moreover, the present method for diagnosing NSCLC may also be applied for assessing the prognosis of a patient with the cancer by comparing the expression level of KIF11, KOC 1, GHSR1b, NTSR1, FOXM1 gene, or a combination thereof (e.g., KOC1 and KIF11) in the patient-derived biological sample. Alternatively, the expression level of the gene(s) in the biological sample may be measured over a spectrum of disease stages to assess the prognosis of the patient

**[0096]** An increase in the expression level of KIF11, KOC1, GHSR1b, NTSR1 or FOXM1 gene compared to a normal control level indicates less favorable prognosis. A similarity in the Expression level of KIF11, KOC1, GHSR1b, NTSR1 or FOXM1 gene compared to a normal control level indicates a more favorable prognosis of the patient. Preferably, the prognosis of a subject can be assessed by comparing the expression profile of KIF11, KOC1, GHSR1 b, NTSR1 or FOXM1 gene. In some embodiments, expression levels of KIF11 and KOC1 are determined.

#### *Expression profile*

**[0097]** The disclosure also provides an NSCLC reference expression profile comprising a pattern of gene expression levels of two or more of KIF11, KOC1, GHSR1b, NTSR1 and FOXM1 genes. The expression profile serves as a control for the diagnosis of NSCLC or predisposition for developing the disease, monitoring the course of treatment and assessing prognosis of a subject with the disease.

#### *Kits disclosed herein*

**[0098]** Disclosed herein is a kit comprising two or more detection reagents, e.g., a nucleic acid that specifically binds to or identifies one or more of KIF11, KOC1, GHSR1b, NTSR1 and FOXM1 genes. Such nucleic acids specifically binding to or identifying one or more of KIF11, KOC1, GHSR1b, NTSR1 and FOXM1 genes are exemplified by oligo-nucleotide sequences that are complementary to a portion of KIF11, KOC1, GHSR1b, NTSR1 or FOXM1 polynucleotides or antibodies which bind to polypeptides encoded by the KIF11, KOC1, GHSR1b, NTSR1 or FOXM1 gene. The reagents are packaged together in the form of a kit. The reagents, such as a nucleic acid or antibody (either bound to a solid matrix or packaged separately with reagents for binding them to the matrix), a control reagent (positive and/or negative) and/or a means of detection of the nucleic acid or antibody are preferably packaged in separate containers. Instructions (e.g., written, tape, VCR, CD-ROM, etc.) for carrying out the assay may be included in the kit. The assay format of the kit may be Northern hybridization or sandwich ELISA known in the art.

**[0099]** For example, a detection reagent is 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 detection sites, each detection site containing a detection reagent. A test strip may also contain sites for negative and/or positive controls. Alternatively, control site(s) is located on a separate strip from the test strip. Optionally, the different detection sites may contain different amounts of immobilized reagents, i.e., a higher amount in the first detection site and lesser amounts in subsequent sites. Upon the addition of a test biological sample, the number of sites displaying a detectable signal provides a quantitative indication of the amount of KIF11, GHSR1b, NTSR1 or FOXM1 gene, or polypeptides encoded by the gene 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 teststrip.

**[0100]** Alternatively, the kit contains a nucleic acid substrate array comprising two or more of the KIF11, GHSR1b, NTSR1, and FOXM1 gene sequences. The expression of 2 or 3 of the genes represented by KIF11, GHSR1b, NTSR1, and FOXM1 genes are identified by virtue of the level of binding to an array test strip or chip. The substrate array can be on, e.g., a solid substrate, e.g., a "chip" as described in U.S. Patent No. 5,744,305.

**[0101]** In some embodiments, the kits can be used for predicting an NSCLC prognosis. The kits in these embodiments, can comprise a reagent for detecting mRNA encoding the amino acid sequence of KIF11 or KOC1, a reagent for detecting the proteins or reagents for detecting the biological activity of the KIF11 or KOC1 protein.

**[0102]** Also disclosed herein are ; kits for the detection of a compound that regulates RNA transporting activity. The kits may comprise a cell expressing a KIF11 polypeptide, or functional equivalent, a KOC1 polypeptide, or functional equivalent, and RNA to be transported, and DCTN1.

**[0103]** The kits disclosed may also be used to screen for compounds for treating or preventing NSCLC. The kits may comprise a KOC1 polypeptide, or functional equivalent, and an RNA that is bound by the KOC polypeptide or functional equivalent. In the present disclosure any RNA transportable with RNA transporter activity of KOC1-KIF11 complex can be used as the RNA to be transported. Prefer RNA can be selected from transcripts of genes shown in table 2, or fragment thereof. An RNA to be transported may also be labeled for detecting RNA transporter activity. Furthermore, in the present disclosure, KOC1 and KIF11 polypeptide or functional equivalent thereof is expressed as fusion protein with

signal generating protein for observation by microscopy or cell imaging systems. For example, ECFP, EYFP, and EGFP may be used for signal generating protein.

#### *Array and pluralities*

**[0104]** The disclosure also includes a nucleic acid substrate array comprising one or more of the KIF11, GHSR1b, NTSR1, and FOXM1 genes. The nucleic acids on the array specifically correspond to one or more polynucleotide sequences represented by KIF11, GHSR1b, NTS1, and FOXM1 genes. The expression level of 2, 3 or 4 of the KIF11, GHSR1b, NTSR1, and FOXM1 genes is identified by detecting the binding of nucleic acid to the array.

**[0105]** The disclosure also includes an isolated plurality (*i.e.*, a mixture of two or more nucleic acids) of nucleic acids. The nucleic acids are in a liquid phase or a solid phase, *e.g.*, immobilized on a solid support such as a nitrocellulose membrane. The plurality includes one or more of the polynucleotides represented by KIF11, GHSR1b, NTS1, and FOXM1 genes. According to a further embodiment disclosed herein the plurality includes 2, 3, or 4 of the polynucleotides represented by KIF11, GHSR1b, NTS1, and FOXM1 genes.

#### *Chips*

**[0106]** The DNA chip is a device that is convenient to compare the expression levels of a number of genes at the same time. DNA chip-based expression profiling can be carried out, for example, by the method as disclosed in "Microarray Biochip Technology" (Mark Schena, Eaton Publishing, 2000), etc.

**[0107]** A DNA chip comprises immobilized high-density probes to detect a number of genes. Thus, the expression levels of many genes can be estimated at the same time by a single-round analysis. Namely, the expression profile of a specimen can be determined with a DNA chip. The DNA chip-based method of the present disclosure, comprises the following steps of:

- (1) synthesizing aRNAs or cDNAs corresponding to the marker genes;
- (2) hybridizing the aRNAs or cDNAs with probes for marker genes; and
- (3) detecting the aRNA or cDNA hybridizing with the probes and quantifying the amount of mRNA thereof.

**[0108]** The term "aRNA" refers to RNA transcribed from a template cDNA with RNA polymerase. An aRNA transcription kit for DNA chip-based expression profiling is commercially available. With such a kit, aRNA can be synthesized from T7 promoter-attached cDNA as a template using T7 RNA polymerase. On the other hand, by PCR using random primer, cDNA can be amplified using as a template a cDNA synthesized from mRNA.

**[0109]** Alternatively, the DNA chip comprises probes, which have been spotted thereon, to detect the marker genes of the disclosure (KIF11, GHSR1b, NTSR1 or FOXM1 gene). There is no limitation on the number of marker genes spotted on the DNA chip, and 1, 2, 3 or all of the genes, KIF11, GHSR1b, NTSR1, and FOXM1, may be used. Any other genes as well as the marker genes can be spotted on the DNA chip. For example, a probe for a gene whose expression level is hardly altered may be spotted on the DNA chip. Such a gene can be used to normalize assay results when the assay results are intended to be compared between multiple chips or between different assays.

**[0110]** A probe is designed for each marker gene selected, and spotted on a DNA chip. Such a probe may be, for example, an oligonucleotide comprising 5-50 nucleotide residues. A method for synthesizing such oligonucleotides on a DNA chip is known to those skilled in the art. Longer DNAs can be synthesized by PCR or chemically. A method for spotting long DNA, which is synthesized by PCR or the like, onto a glass slide is also known to those skilled in the art. A DNA chip that is obtained by the method as described above can be used for diagnosing NSCLC according to the present disclosure.

**[0111]** The prepared DNA chip is contacted with aRNA, followed by the detection of hybridization between the probe and aRNA. The aRNA can be previously labeled with a fluorescent dye. A fluorescent dye such as Cy3 (red) and Cy5 (green) can be used to label an aRNA. aRNAs from a subject and a control are labeled with different fluorescent dyes, respectively. The difference in the expression level between the two can be estimated based on a difference in the signal intensity. The signal of fluorescent dye on the DNA chip can be detected by a scanner and analyzed using a special program. For example, the Suite from Affymetrix is a software package for DNA chip analysis.

#### *Identifying compounds that inhibit NSCLC-associated gene expression*

**[0112]** A compound that inhibits the expression or activity of a target NSCLC-associated gene (KIF11, GHSR1b, NTSR1 or FOXM1 gene) is identified by contacting a test cell expressing the NSCLC-associated gene with a test

compound and determining the expression level or activity of the NSCLC-associated gene. A decrease in expression compared to the normal control level indicates that the compound is an inhibitor of the NSCLC-associated gene. Such compounds identified according to the method are useful for inhibiting NSCLC.

**[0113]** The test cell may be a population of cells and includes any cells as long as the cell expresses the target NSCLC-associated gene(s). For example, the test cell may be an immortalized cell line derived from an NSCLC cell. Alternatively, the test cell may be a cell transfected with any of the KIF11, GHSR1b, NTSR1, and FOXM1 genes, or which has been transfected with the regulatory sequence (e.g., promoter) of any of the genes that is operably linked to a reporter gene.

#### *Screening compounds*

**[0114]** Using KIF11, GHSR1b, NTSR1 or FOXM1 gene, proteins encoded by the gene or transcriptional regulatory region of the gene, compounds can be screened that alter the expression of the gene or biological activity of a polypeptide encoded by the gene. Such compounds are expected to serve as pharmaceuticals for treating or preventing NSCLC.

**[0115]** Therefore, the present disclosure provides a method of screening for a compound for treating or preventing NSCLC using the polypeptide herein disclosed. An embodiment of this screening method comprises the steps of: (a) contacting a test compound with a polypeptide encoded by KIF11, GHSR1b, NTSR1 or FOXM1 gene; (b) detecting the binding activity between the polypeptide herein disclosed and the test compound; and (c) selecting the compound that binds to the polypeptide.

**[0116]** As explained in more detail below, KOC1 and KIF11 form a complex that has RNA transporting activity. Thus, the present disclosure provides methods of identifying polypeptides and other compounds that modulate RNA transport activity. For example, a polypeptide can be tested for RNA transporting activity by contacting a KIF11 polypeptide (SEQ ID NO: 2) or a functional equivalent thereof with an RNA that can be transported by KIF11 under conditions suitable for transportation of RNA. The level of RNA transported can be measured using well known techniques, such as by RNA immunoprecipitation, as described in detail below.

**[0117]** A functional equivalent of a KIF 11 polypeptide is a polypeptide that has a biological activity equivalent to a polypeptide consisting of the amino acid sequence of SEQ ID NO: 2 and, for example, comprising the amino acid sequence of SEQ ID NO: 2 (*KIF11*), wherein one or more amino acids (usually less than five) are substituted, deleted, or inserted. Alternatively, the polypeptide may be one that comprises an amino acid sequence having at least about 80% homology (also referred to as sequence identity) to SEQ ID NO: 2. In other embodiments, the polypeptide can be encoded by a polynucleotide that hybridizes under stringent conditions (as defined below) to a polynucleotide consisting of the nucleotide sequence of SEQ ID NO: 1.

**[0118]** In some embodiments, the KIF11 polypeptide or functional equivalent is contacted with a KOC1 polypeptide or functional equivalent thereof. A functional equivalent of a KOC1 polypeptide is a polypeptide that has a biological activity equivalent to a polypeptide consisting of the amino acid sequence of SEQ ID NO: 105 and, for example, comprising the amino acid sequence of SEQ ID NO: 105, wherein one or more amino acids (usually less than five) are substituted, deleted, or inserted. Alternatively, the polypeptide may be one that comprises an amino acid sequence having at least about 80% homology (also referred to as sequence identity) to SEQ ID NO: 105. In other embodiments, the polypeptide can be encoded by a polynucleotide that hybridizes under stringent conditions (as defined below) to a polynucleotide consisting of the nucleotide sequence of SEQ ID NO: 104. In some embodiments, a functional equivalent comprises at least one RRM or KH domain.

**[0119]** The disclosure also provides methods of identifying agents that modulate RNA transporting activity. In these methods, an agent suspected of modulating RNA transporting activity with a KIF11 polypeptide or functional equivalent. The level of transported RNA is detected and compared to the level in a control in the absence of the agent.

**[0120]** The polypeptide to be used for the 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 compound can be, for example, a purified polypeptide, a soluble protein in a form bound to a carrier or a fusion protein fused with other polypeptides.

**[0121]** As a method of screening for proteins that bind to KIF11, GHSR1b, NTSR1 or FOXM1 polypeptide, many methods well known by a person skilled in the art can be used. Such a screening can be conducted by, for example, immunoprecipitation method using methods well known in the art. The proteins disclosed herein can be recombinantly produced using standard procedures. For example, a gene encoding any of the KIF11, GHSR1b, NTSR1, and FOXM1 polypeptides is expressed in animal cells by inserting the gene into an expression vector for foreign genes, such as pSV2neo, pcDNA I, pcDNA3.1, pCAGGS and pCD8. The promoter to be used for the expression may be any promoter that can be used commonly and include, for example, the SV40 early promoter (Rigby in Williamson (ed.), Genetic Engineering, vol. 3. Academic Press, London, 83-141 (1982)), the EF- $\alpha$  promoter (Kim et al., Gene 91: 217-23 (1990)), the CAG promoter (Niwa et al., Gene 108: 193-200 (1991)), the RSV LTR promoter (Cullen, Methods in Enzymology 152: 684-704 (1987)) the SR $\alpha$  promoter (Takebe et al., Mol Cell Biol 8: 466 (1988)), the CMV immediate early promoter (Seed and Aruffo, Proc. Natl Acad Sci USA 84: 3365-9 (1987)), the SV40 late promoter (Gheysen and Fiers, J Mol Appl Genet 1: 385-94 (1982)), the Adenovirus late promoter (Kaufman et al., Mol Cell Biol 9: 946 (1989)), the HSV TK promoter

and so on. The introduction of the gene into animal cells to express a foreign gene can be performed according to any methods, for example, the electroporation method (Chu et al., Nucleic Acids Res 15: 1311-26 (1987)), the calcium phosphate method (Chen and Okayama, Mol Cell Biol 7: 2745-52 (1987)), the DEAE dextran method (Lopata et al., Nucleic Acids Res 12: 5707-17 (1984); Sussman and Milman, Mol Cell Biol 4: 1642-3 (1985)), the Lipofectin method (Derijard, B Cell 7: 1025-37 (1994); Lamb et al., Nature Genetics 5: 22-30 (1993); Rabindran et al., Science 259: 230-4 (1993)), and so on. The NSC polypeptide can also be expressed as a fusion protein comprising a recognition site (epitope) of a monoclonal antibody by introducing the epitope of the monoclonal antibody, whose specificity has been revealed, to the N- or C- terminus of the polypeptide. A commercially available epitope-antibody system can be used (Experimental Medicine 13: 85-90 (1995)). Vectors which can express a fusion protein with, for example,  $\beta$ -galactosidase, maltose binding protein, glutathione S-transferase, green fluorescence protein (GFP), and so on, by the use of its multiple cloning sites are commercially available.

**[0122]** A fusion protein prepared by introducing only small epitopes consisting of several to a dozen amino acids so as not to change the property of the original polypeptide by the fusion is also reported. 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 monoclonal antibodies recognizing them can be used as the epitope-antibody system for screening proteins binding to the KIF11, GHSR1b, NTSR1 or FOXM1 polypeptide (Experimental Medicine 13: 85-90 (1995)).

**[0123]** 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 KIF11, GHSR1b, NTSR1 or FOXM1 polypeptide, a polypeptide comprising the binding ability with the polypeptide, and an antibody. Immunoprecipitation can be also conducted using antibodies against the KIF11, GHSR1b, NTSR1 or FOXM1 polypeptide, in addition to the use of antibodies against the above epitopes, which antibodies can be prepared according to conventional methods and may be in any form, such as monoclonal or polyclonal antibodies, and includes antiserum obtained by immunizing an animal such as a rabbit with the polypeptide, all classes of polyclonal and monoclonal antibodies, as well as recombinant antibodies (e.g., humanized antibodies).

**[0124]** Specifically, antibodies against KIF11, GHSR1b, NTSR1 or FOXM1 polypeptide can be prepared using techniques well known in the art. For example, KIF11, GHSR1b, NTSR1 or FOXM1 polypeptide used as an antigen to obtain an antibody may be derived from any animal species, but preferably is derived from a mammal such as a human, mouse, or rat, more preferably from a human. The polypeptide used as the antigen can be recombinantly produced or isolated from natural sources. According to the present disclosure, the polypeptide to be used as an immunization antigen may be a complete protein or a partial peptide of the KIF11, GHSR1b, NTSR1 or FOXM1 polypeptide. A partial peptide may comprise, for example, the amino (N)-terminal or carboxy (C)-terminal fragment of the KIF11, GHSR1b, NTSR1 or FOXM1 polypeptide.

**[0125]** Any mammalian animal may be immunized with the antigen, but preferably the compatibility with parental cells used for cell fusion is taken into account. In general, animals of Rodentia, Lagomorpha or Primates are used. Animals of Rodentia include, for example, mouse, rat and hamster. Animals of Lagomorpha include, for example, rabbit. Animals of Primates include, for example, a monkey of Catarrhini (old world monkey) such as *Macaca fascicularis*, rhesus monkey, sacred baboon and chimpanzees.

**[0126]** Methods for immunizing animals with antigens are known in the art. Intraperitoneal injection or subcutaneous injection of antigens is a standard method for immunization of mammals. More specifically, antigens may be diluted and suspended in an appropriate amount of phosphate buffered saline (PBS), physiological saline, etc. If desired, the antigen suspension may be mixed with an appropriate amount of a standard adjuvant, such as Freund's complete adjuvant, made into emulsion, and then administered to mammalian animals. Preferably, it is followed by several administrations of the antigen mixed with an appropriately amount of Freund's incomplete adjuvant every 4 to 21 days. An appropriate carriers may also be used for immunization. After immunization as above, the serum is examined by a standard method for an increase in the amount of desired antibodies.

**[0127]** Polyclonal antibodies against KIF11, GHSR1 b, NTSR1 or FOXM1 polypeptide may be prepared by collecting blood from the immunized mammal examined for the increase of desired antibodies in the serum, and by separating serum from the blood by any conventional method. Polyclonal antibodies include serum containing the polyclonal antibodies, as well as the fraction containing the polyclonal antibodies may be isolated from the serum. Immunoglobulin G or M can be prepared from a fraction which recognizes only the KIF11, GHSR1b, NTSR1 or FOXM1 polypeptide using, for example, an affinity column coupled with the polypeptide, and further purifying this fraction using protein A or protein G column.

**[0128]** To prepare monoclonal antibodies, immune cells are collected from the mammal immunized with the antigen and checked for the increased level of desired antibodies in the serum as described above, and are subjected to cell fusion. The immune cells used for cell fusion are preferably obtained from spleen. Other preferred parental cells to be fused with the above immunocyte include, for example, myeloma cells of mammals, and more preferably myeloma cells having an acquired property for the selection of fused cells by drugs.

**[0129]** The above immunocyte and myeloma cells can be fused according to known methods, for example, the method of Milstein *et al.*, (Galfre and Milstein, *Methods Enzymol* 73: 3-46 (1981)).

**[0130]** Resulting hybridomas obtained by the cell fusion may be selected by cultivating them in a standard selection medium, such as HAT medium (hypoxanthine, aminopterin, and thymidine containing medium). The cell culture is typically continued in the HAT medium for several days to several weeks, the time being sufficient to allow all the other cells, with the exception of the desired hybridoma (non-fused cells), to die. Then, the standard limiting dilution is performed to screen and clone a hybridoma cell producing the desired antibody.

**[0131]** In addition to the above method, in which a non-human animal is immunized with an antigen for preparing hybridoma, human lymphocytes such as those infected by EB virus may be immunized with KIF11, GHSR1b, NTSR1 or FOXM1 polypeptide, cells expressing the polypeptide, or their lysates *in vitro*. Then, the immunized lymphocytes are fused with human-derived myeloma cells that are capable of indefinitely dividing, such as U266, to yield a hybridoma producing a desired human antibody that is able to bind to the KIF11, GHSR1b, NTSR1 or FOXM1 polypeptide can be obtained (Unexamined Published Japanese Patent Application No. (JP-A) Sho 63-17688).

**[0132]** The obtained hybridomas are subsequently transplanted into the abdominal cavity of a mouse and the ascites are extracted. The obtained monoclonal antibodies can be purified by, for example, ammonium sulfate precipitation, a protein A or protein G column, DEAE ion exchange chromatography, or an affinity column to which any of the target proteins disclosed herein (KIF11, GHSR1b, NTSR1, and FOXM1 polypeptide) is coupled. The antibody can be used not only in the present screening method, but also for purification and detection of KIF11, GHSR1b, NTSR1 or FOXM1 polypeptide, and serve also as candidates for agonists and antagonists of the polypeptide. In addition, this antibody can be applied to the antibody treatment for diseases related to the KIF11, GHSR1b, NTSR1 or FOXM1 polypeptide including NSCLC as described *infra*.

**[0133]** Monoclonal antibodies thus obtained can be also recombinantly prepared using genetic engineering techniques (see, for example, Borrebaeck and Larrick, *Therapeutic Monoclonal Antibodies*, published in the United Kingdom by MacMillan Publishers LTD (1990)). For example, a DNA encoding an antibody may be cloned from an immune cell, such as a hybridoma or an immunized lymphocyte producing the antibody, inserted into an appropriate vector, and introduced into host cells to prepare a recombinant antibody. Such recombinant antibody can also be used for the present screening.

**[0134]** Furthermore, an antibody used in the screening and so on may be a fragment of an antibody or modified antibody, so long as it binds to one or more of KIF11, GHSR1b, NTSR1, and FOXM1 polypeptides. For instance, the antibody fragment may be Fab, F(ab')<sub>2</sub>, Fv, or single chain Fv (scFv), in which Fv fragments from H and L chains are ligated by an appropriate linker (Huston *et al.*, *Proc Natl Acad Sci USA* 85: 5879-83 (1988)). More specifically, an antibody fragment may be generated by treating an antibody with an enzyme, such as papain or pepsin. Alternatively, a gene encoding the antibody fragment may be constructed, inserted into an Expression vector, and expressed in an appropriate host cell (see, for example, Co *et al.*, *J Immunol* 152: 2968-76 (1994); Better and Horwitz, *Methods Enzymol* 178: 476-96 (1989); Pluckthun and Skerra, *Methods Enzymol* 178: 497-515 (1989); Lamoyi, *Methods Enzymol* 121: 652-63 (1986); Rousseaux *et al.*, *Methods Enzymol* 121: 663-9 (1986); Bird and Walker, *Trends Biotechnol* 9: 132-7 (1991)).

**[0135]** An antibody may be modified by conjugation with a variety of molecules, such as polyethylene glycol (PEG). Modified antibodies can be obtained through chemically modification of an antibody. These modification methods are conventional in the field.

**[0136]** Alternatively, an antibody may be obtained as a chimeric antibody, between a variable region derived from nonhuman antibody and the constant region derived from human antibody, or as a humanized antibody, comprising the complementarity determining region (CDR) derived from nonhuman antibody, the frame work region (FR) derived from human antibody, and the constant region. Such antibodies can be prepared using known technology.

**[0137]** Humanization can be performed by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody (see *e.g.*, Verhoeven *et al.*, *Science* 239:1534-1536 (1988)). Accordingly, such humanized antibodies are chimeric antibodies, wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species.

**[0138]** Fully human antibodies comprising human variable regions in addition to human framework and constant regions can also be used. Such antibodies can be produced using various techniques known in the art. For example *in vitro* methods involve use of recombinant libraries of human antibody fragments displayed on bacteriophage (*e.g.*, Hoogenboom & Winter, *J. Mol. Biol.* 227:381 (1991)). Similarly, human antibodies can be made by introducing of human immunoglobulin loci into transgenic animals, *e.g.*, mice in which the endogenous immunoglobulin genes have been partially or completely inactivated. This approach is described, *e.g.*, in U.S. Patent Nos. 6,150,584; 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,661,016.

**[0139]** Antibodies obtained as above may be purified to homogeneity. For example, the separation and purification of the antibody can be performed according to separation and purification methods used for general proteins. For example, the antibody may be separated and isolated by the appropriately selected and combined use of column chromatographies, such as affinity chromatography, filter, ultrafiltration, salting-out, dialysis, SDS polyacrylamide gel electrophoresis, isoe-

lectric focusing, and others (Antibodies: A Laboratory Manual. Ed Harlow and David Lane, Cold Spring Harbor Laboratory (1988)), but are not limited thereto. A protein A column and protein G column can be used as the affinity column. Exemplary protein A columns to be used include, for example, Hyper D, POROS, and Sepharose F.F. (Pharmacia).

**[0140]** Exemplary chromatography, with the exception of affinity includes, for example, ion-exchange chromatography, hydrophobic chromatography, gel filtration, reverse-phase chromatography, adsorption chromatography, and the like (Strategies for Protein Purification and Characterization: A Laboratory Course Manual. Ed Daniel R. Marshak et al., Cold Spring Harbor Laboratory Press (1996)). The chromatographic procedures can be carried out by liquid-phase chromatography, such as HPLC and FPLC.

**[0141]** An immune complex can be precipitated, for example with Protein A sepharose or Protein G sepharose when the antibody is a mouse IgG antibody. If the KIF11, GHSR1b, NTSR1 or FOXM1 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 KIF11, GHSR1b, NTSR1 or FOXM1 polypeptide, using a substance specifically binding to these epitopes, such as glutathione-Sepharose 4B.

**[0142]** 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)).

**[0143]** 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 KIF11, GHSR1b, NTSR1 or FOXM1 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, <sup>35</sup>S-methionine or <sup>35</sup>S-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 the protein has been revealed.

**[0144]** As a method for screening proteins binding to any of KIF11, GHSR1b, NTSR1, and FOXM1 polypeptides using the polypeptide, for example, West-Western blotting analysis (Skolnik et al., Cell 65: 83-90 (1991)) can be used. Specifically, a protein binding to KIF11, GHSR1b, NTSR1 or FOXM1 polypeptide can be obtained by preparing a cDNA library from cells, tissues, organs (for example, tissues such as lung cells) or cultured cells (particularly those derived from NSCLC cells) expected to express a protein binding to the KIF11, GHSR1b, NTSR1 or FOXM1 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 KIF11, GHSR1b, NTSR1 or FOXM1 polypeptide with the above filter, and detecting the plaques expressing proteins bound to the KIF11, GHSR1b, NTSR1 or FOXM1 polypeptide according to the label. The KIF11, GHSR1b, NTSR1 or FOXM1 polypeptide may be labeled by utilizing the binding between biotin and avidin, or by utilizing an antibody that specifically binds to the KIF11, GHSR1b, NTSR1 or FOXM1 polypeptide, or a peptide or polypeptide (for example, GST) that is fused to the KIF11, GHSR1b, NTSR1 or FOXM1 polypeptide. Methods using radioisotope or fluorescence and such may be also used.

**[0145]** Alternatively, in another embodiment of the screening method of the present disclosure, 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)").

**[0146]** In the two-hybrid system, KIF11, GHSR1b, NTSR1 or FOXM1 polypeptide is fused to the SRF-binding region or GAL4-binding region and expressed in yeast cells. A DNA library is prepared from cells expected to express a protein binding to the KIF11, GHSR1b, NTSR1 or FOXM1 polypeptide, such that the library, when expressed, is fused to the VP16 or GAL4 transcriptional activation region. The cDNA library is then introduced into the above yeast cells and the cDNA derived from the library is isolated from the positive clones detected (when a protein binding to the polypeptide disclosed herein is expressed in yeast cells, the binding of the two activates a reporter gene, making positive clones detectable). A protein encoded by the DNA can be prepared by introducing the cDNA isolated above to *E. coli* and expressing the protein.

**[0147]** 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.

**[0148]** A compound binding to KIF11, GHSR1b, NTSR1 or FOXM1 polypeptide can also be screened using affinity chromatography. For example, KIF11, GHSR1b, NTSR1 or FOXM1 polypeptide may be immobilized on a carrier of an affinity column, and a test compound, containing a protein capable of binding to KIF11, GHSR1b, NTSR1 or FOXM1 polypeptide, is applied to the column. A test compound herein may be, for example, cell extracts, cell lysates, etc. After loading the test compound, the column is washed, and compounds bound to KIF11, GHSR1b, NTSR1 or FOXM1 polypeptide can be prepared.

**[0149]** When the test compound 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.

**[0150]** A biosensor using the surface plasmon resonance phenomenon may be used as a mean for detecting or

quantifying the bound compound as disclosed herein. When such a biosensor is used, the interaction between KIF11, GHSR1b, NTSR1 or FOXM1 polypeptide and a test compound 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 KIF11, GHSR1b, NTSR1 or FOXM1 polypeptide and a test compound using a biosensor such as BIAcore.

**[0151]** The methods of screening for molecules that bind when an immobilized KIF11, GHSR1b, NTSR1 or FOXM1 polypeptide is exposed to synthetic chemical compounds, or natural substance banks or a random phage peptide display library, and the methods of screening using high-throughput based on combinatorial chemistry techniques (Wrighton et al., Science 273: 458-64 (1996); Verdine, Nature 384: 11-13 (1996); Hogan, Nature 384: 17-9 (1996)) to isolate not only proteins but chemical compounds that bind to KIF11, GHSR1b, NTSR1 or FOXM1 protein (including agonist and antagonist) are well known to one skilled in the art.

**[0152]** Alternatively, the present disclosure provides a method of screening for a compound for treating or preventing NSCLC using KIF11, GHSR1b, NTSR1 or FOXM1 polypeptide comprising the steps as follows:

- (a) contacting a test compound with KIF11, GHSR1b, NTSR1 or FOXM1 polypeptide;
- (b) detecting the biological activity of the KIF11, GHSR1b, NTSR1 or FOXM1 polypeptide of step (a); and
- (c) selecting a compound that suppresses the biological activity of the KIF11, GHSR1b, NTSR1 or FOXM1 polypeptide in comparison with the biological activity detected in the absence of the test compound.

**[0153]** Since proteins encoded by any of the genes of KIF11, GHSR1b, NTSR1, and FOXM1 have the activity of promoting cell proliferation of NSCLC cells, a compound which inhibits this activity of one of these proteins can be screened using this activity as an index.

**[0154]** Any polypeptides can be used for screening so long as they comprise the biological activity of KIF11, GHSR1b, NTSR1 or FOXM1 proteins. Such biological activity includes cell-proliferating activity and binding ability to other proteins of the proteins encoded by KIF11, GHSR1b, NTSR1 or FOXM1 gene. For example, a human protein encoded by KIF11, GHSR1b, NTSR1 or FOXM1 gene can be used and polypeptides functionally equivalent to these proteins can also be used. Such polypeptides may be expressed endogenously or exogenously by cells.

**[0155]** The compound isolated by this screening is a candidate for antagonists of the KIF11, GHSR1b, NTSR1 or FOXM1 polypeptide. The term "antagonist" refers to molecules that inhibit the function of KIF11, GHSR1b, NTSR1 or FOXM1 polypeptide by binding thereto. Moreover, a compound isolated by this screening is a candidate for compounds which inhibit the *in vivo* interaction of KIF11, GHSR1b, NTSR1 or FOXM1 polypeptide with molecules (including DNAs and proteins).

**[0156]** 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 KIF11, GHSR1b, NTSR1 or FOXM1 polypeptide, culturing the cells in the presence of a test compound, and determining the speed of cell proliferation, measuring the cell cycle and such, as well as by measuring the colony forming activity.

**[0157]** As discussed in detail above, by controlling the expression levels of KIF11, GHSR1 b, NTSR1 or FOXM1 gene, one can control the onset and progression of NSCLC. Thus, compounds that may be used in the treatment or prevention of NSCLC, can be identified through screenings that use the expression levels of one or more of KIF11, GHSR1b, NTSR1, and FOXM1 genes as indices. In the context of the present disclosure, such screening may comprise, for example, the following steps:

- (a) contacting a test compound with a cell expressing one or more of KIF11, GHSR1b, NTSR1, and FOXM1 genes; and
- (b) selecting a compound that reduces the expression level of one or more of the genes in comparison with the expression level detected in the absence of the test compound.

**[0158]** Cells expressing at least one of KIF11, GHSR1b, NTSR1, and FOXM1 genes include, for example, cell lines established from NSCLC cells; such cells can be used for the above screening disclosed herein (e.g., A549, NCI-H226, NCI-H522, LC319). The expression level can be estimated by methods well known to one skilled in the art. In the method of screening, a compound that reduces the expression level of at least one of the genes can be selected as candidate agents to be used for the treatment or prevention of NSCLC.

**[0159]** Alternatively, the screening method of the disclosure may comprise the following steps:

- (a) contacting a test compound with a cell into which a vector comprising the transcriptional regulatory region of one or more of the marker genes and a reporter gene that is expressed under the control of the transcriptional regulatory region has been introduced, wherein the marker genes are selected from the group of KIF11, GHSR1b, NTSR1, and FOXM1;

- (b) measuring the activity of said reporter gene; and
- (c) selecting a compound that reduces the expression level of said reporter gene as compared to a control.

**[0160]** Suitable reporter genes and host cells are well known in the art. The reporter construct required for the screening can be prepared using the transcriptional regulatory region of a marker gene. When the transcriptional regulatory region of a marker gene has been known to those skilled in the art, a reporter construct can be prepared using the previous sequence information. When the transcriptional regulatory region of a marker gene remains unidentified, a nucleotide segment containing the transcriptional regulatory region can be isolated from a genome library based on the nucleotide sequence information of the marker gene (e.g., based the 5' upstream sequence information).

**[0161]** In a further embodiment of the method of screening for a compound for treating or preventing NSCLC disclosed herein, the method utilizes the binding ability of KIF11 to KOC1, or GHSR1 b or NTSR1 to NMU.

**[0162]** As described above, the present inventors revealed that KOC1 not only co-localized with KIF11 in human normal tissues, NSCLCs, and cell lines, but also directly interacted with KIF11 in NSCLC cells *in vitro*, and that the treatment of NSCLC cells with siRNAs for KIF11 reduced its expression and led to growth suppression. The results suggest that KOC1 -KIF11 signaling affects growth of NSCLC cells. Thus, it is expected that the inhibition of the binding between KOC1 and KIF11 leads to the suppression of cell proliferation, and compounds inhibiting the binding serve as pharmaceuticals for treating or preventing NSCLCs. This screening method includes the steps of: (a) contacting a KIF11 polypeptide or functional equivalent thereof with KOC1, or a functional equivalent thereof, in the presence of a test compound; (b) detecting the binding between the polypeptide and KOC1; and (c) selecting the test compound that inhibits the binding between the polypeptide and KOC1.

**[0163]** Furthermore, as described above, the present inventors revealed GHSR1b and NTSR1 as the likely targets for the growth-promoting effect of NMU in lung tumors. The present inventors revealed that NMU-25 bound to these receptors on the cell surface, and that treatment of NSCLC cells with siRNAs for GHSR1 or NTSR1 reduced expression of the receptors and led to apoptosis. The results suggest that NMU affects growth of NSCLC cells by acting through GHSR1b and/or NTSR1 (Fig. 14). Thus, it is expected that the inhibition of binding between GHSR1b or NTSR1 and NMU leads to the suppression of cell proliferation, and compounds inhibiting the binding serve as pharmaceuticals for treating or preventing NSCLCs. This screening method includes the steps of: (a) contacting a GHSR1 b or NTSR1 polypeptide or functional equivalent thereof with NMU in the presence of a test compound; (b) detecting binding between the polypeptide and NMU; and (c) selecting the test compound that inhibits binding between the polypeptide and NMU.

**[0164]** KOC1 and KIF11 polypeptides, or GHSR1b or NTSR1 and NMU polypeptides to be used for the screening may be a recombinant polypeptide or a protein derived from the nature, or may also be a partial peptide thereof so long as it retains the binding ability to each other. Such partial peptides retaining the binding ability are herein referred to as "functional equivalents". The KOC1 and KIF11 polypeptides, or GHSR1b or NTSR1 and NMU polypeptides to be used in the screening can be, for example, a purified polypeptide, a soluble protein, a form bound to a carrier or a fusion protein fused with other polypeptides.

**[0165]** As a method of screening for compounds that inhibit binding between KOC1 and KIF11, or GHSR1b or NTSR1 and NMU, many methods well known by one skilled in the art can be used. Such a screening can be carried out as an *in vitro* assay system, for example, in a cellular system. More specifically, first, either KOC1 or KIF11, or GHSR1b or NTSR1, or NMU is bound to a support, and the other protein is added together with a test compound thereto. Next, the mixture is incubated, washed and the other protein bound to the support is detected and/or measured.

**[0166]** Examples of supports that may be used for binding proteins include insoluble polysaccharides, such as agarose, cellulose and dextran; and synthetic resins, such as polyacrylamide, polystyrene and silicon; preferably commercial available beads and plates (e.g., multi-well plates, biosensor chip, etc.) prepared from the above materials may be used. When using beads, they may be filled into a column. Alternatively, the use of magnetic beads of also known in the art, and enables to readily isolate proteins bound on the beads via magnetism.

**[0167]** The binding of a protein to a support may be conducted according to routine methods, such as chemical bonding and physical adsorption. Alternatively, a protein may be bound to a support via antibodies specifically recognizing the protein. Moreover, binding of a protein to a support can be also conducted by means of avidin and biotin.

**[0168]** The binding between proteins is carried out in buffer, for example, but are not limited to, phosphate buffer and Tris buffer, as long as the buffer does not inhibit binding between the proteins.

**[0169]** In the present disclosure, a biosensor using the surface plasmon resonance phenomenon may be used as a mean for detecting or quantifying the bound protein. When such a biosensor is used, the interaction between the proteins 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 binding between the KOC1 and KIF11, or GHSR1b or NTSR1 and NMU using a biosensor such as BIAcore.

**[0170]** Alternatively, either KOC1 or KIF11, or GHSR1b or NTSR1, or NMU may be labeled, and the label of the bound protein may be used to detect or measure the bound protein. Specifically, after pre-labeling one of the proteins, the labeled protein is contacted with the other protein in the presence of a test compound, and then bound proteins are



detected or measured according to the label after washing.

**[0171]** Labeling substances such as radioisotope (e.g.,  $^3\text{H}$ ,  $^{14}\text{C}$ ,  $^{32}\text{P}$ ,  $^{33}\text{P}$ ,  $^{35}\text{S}$ ,  $^{125}\text{I}$ ,  $^{131}\text{I}$ ), enzymes (e.g., alkaline phosphatase, horseradish peroxidase,  $\beta$ -galactosidase,  $\beta$ -glucosidase), fluorescent substances (e.g., fluorescein isothiocyanate (FITC), rhodamine) and biotin/avidin, may be used for the labeling of a protein in the present method. When the protein is labeled with radioisotope, the detection or measurement can be carried out by liquid scintillation. Alternatively, proteins labeled with enzymes can be detected or measured by adding a substrate of the enzyme to detect the enzymatic change of the substrate, such as generation of color, with absorptiometer. Further, in case where a fluorescent substance is used as the label, the bound protein may be detected or measured using fluorophotometer.

**[0172]** Furthermore, binding of KOC1 and KIF11, or GHSR1b or NTSR1 and NMU can be also detected or measured using antibodies to the KOC1 and KIF11, or GHSR1b or NTSR1 and NMU. For example, after contacting the KOC1 polypeptide immobilized on a support with a test compound and KIF11, the mixture is incubated and washed, and detection or measurement can be conducted using an antibody against KIF11. Alternatively, KIF11 may be immobilized on a support, and an antibody against KOC1 may be used as the antibody. When the combination of GHSR1b or NTSR1 and NMU is used, GHSR1b or NTSR1 polypeptide may be immobilized on a support with a test compound and NMU, the mixture is incubated and washed, and detection or measurement can be conducted using an antibody against NMU. Alternatively NMU may be immobilized on a support, and an antibody against GHSR1 b or NTSR1 may be used as the antibody.

**[0173]** In case of using an antibody in the present screening, the antibody is preferably labeled with one of the labeling substances mentioned above, and detected or measured based on the labeling substance. Alternatively, the antibody against KOC1 or KIF11, or GHSR1b or NTSR1, or NMU may be used as a primary antibody to be detected with a secondary antibody that is labeled with a labeling substance. Furthermore, the antibody bound to the protein in the screening of the present disclosure may be detected or measured using protein G or protein A column.

**[0174]** Alternatively, in another embodiment of the screening method herein disclosed, 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)").

**[0175]** In the two-hybrid system, for example, KOC1 polypeptide is fused to the SRF-binding region or GAL4-binding region and expressed in yeast cells. KIF11 polypeptide that binds to KOC1 polypeptide is fused to the VP16 or GAL4 transcriptional activation region and also expressed in the yeast cells in the existence of a test compound. Alternatively, KIF11 polypeptide may be fused to the SRF-binding region or GAL4-binding region, and KOC1 polypeptide to the VP16 or GAL4 transcriptional activation region. When the combination of GHSR1b or NTSR1 and NMU is used in the two-hybrid system, for example, GHSR1b or NTSR1 polypeptide is fused to the SRF-binding region or GAL4-binding region and expressed in yeast cells. NMU polypeptide that binds to GHSR1b or NTSR1 polypeptide is fused to the VP16 or GAL4 transcriptional activation region and also expressed in the yeast cells in the existence of a test compound. Alternatively, NMU polypeptide may be fused to the SRF-binding region or GAL4-binding region, and GHSR1b or NTSR1 polypeptide to the VP16 or GAL4 transcriptional activation region. When the test compound does not inhibit the binding between KOC1 and KIF11, or GHSR1b or NTSR1 and NMU, the binding of the two activates a reporter gene, making positive clones detectable.

**[0176]** As a reporter gene, for example, Ade2 gene, lacZ gene, CAT gene, luciferase gene and such can be used besides HIS3 gene.

**[0177]** Moreover, when the combination of GHSR1b or NTSR1 and NMU is used in the screening method, since GHSR1b and NTSR1 are polypeptides naturally expressed on the cell surface, in a preferable embodiment of the present screening method, the polypeptides are expressed on the surface of a living cell. When the polypeptides are expressed on the surface of a living cell, the binding between the polypeptide and NMU can be detected by methods detecting the autocrine and paracrine signaling leading to stimulation of tumor cell growth (Heasley, Oncogene 20: 1563-1569 (2001)). For example, the binding between GHSR1 or NTSR1 polypeptide and NMU can be detected by:

(1) detecting the concentration of calcium or cAMP in the cell (e.g. FLIPR assay, Biochem. Biophys. Res. Commun. 276: 435-438, 2000; Nature 406: 70-74, 2000; J. Biol. Chem. 275:21068-21074, 2000);

(2) detecting the activation of the polypeptide;

(3) detecting the interaction between the polypeptide and G-protein (e.g. FLIPR assay, Biochem. Biophys. Res. Commun. 276: 435-438, 2000; Nature 406: 70-74, 2000; J. Biol. Chem. 275:21068-21074, 2000, or binding assay with  $^{125}\text{I}$  labeled peptide);

(4) detecting the activation of phospholipase C or its down stream pathway (Oncogene 20:1563-1569,2001);

(5) detecting the activation of kinases of the protein kinase cascade, such as Raf, MEK, ERKs, and protein kinase D (PKD) (Oncogene 20:1563-1569, 2001);

(6) detecting the activation of a member of Src/TecBmx-family of tyrosine kinases (Oncogene 20:1563-1569, 2001);

(7) detecting the activation of a member of the Ras and Rho family, regulation of a member of the JNK members of MAP families, or the reorganization of the actin cytoskeleton (Oncogene 20:1563-1569, 2001);

(8) detecting the activation of any signal complex mediated by the polypeptide activation;

(9) detecting the change in subcellular localization of the polypeptide including the ligand-induced internalization/endocytosis of the polypeptide (J. Cell Sci., 113: 2963-2975, 2000; J. Histochem. Cytochem. 48:1553-1563, 2000; Endocrinology October 23, 2003. as doi: 10.1210/en. 2003-0974);

(10) detecting the activation of any transcription factor downstream of the polypeptides or the activation of their downstream gene; and

(11) detecting cell proliferation, transformation, or any other oncogenic phenotype of the cell.

**[0178]** Any test compound, for example, cell extracts, cell culture supernatant, products of fermenting microorganism, extracts from marine organism, plant extract, purified or crude proteins, peptides, non-peptide compounds, synthetic micromolecular compounds and natural compounds can be used in the screening methods of the present disclosure. The test compound herein disclosed 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 compounds (Lam, Anticancer Drug Des. 12: 145 (1997)). Examples of methods for the synthesis of molecular libraries can be found in the art (DeWitt et al., Proc. Natl. Acad. Sci. USA 90: 6909 (1993); Erb et al., Proc. Natl. Acad. Sci. USA 91: 11422 (1994); Zuckermann et al., J. Med. Chem. 37: 2678 (1994); Cho et al., Science 261: 1303 (1993); Carell et al., Angew. Chem. Int. Ed Engl. 33: 2059 (1994); Carell et al., Angew. Chem. Int. Ed Engl. 33: 2061 (1994); Gallop et al., J. Med. Chem. 37: 1233 (1994)). Libraries of compounds may be presented in solution (see Houghten, Bio/Techniques 13: 412 (1992)) or on beads (Lam, Nature 354: 82 (1991)), chips (Fodor, Nature 364: 555 (1993)), 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 89: 1865 (1992)) or phage (Scott and Smith, Science 249: 386 (1990); Delvin, Science 249: 404 (1990); Cwirla et al., Proc. Natl. Acad. Sci. USA 87: 6378 (1990); Felici, J. Mol. Biol. 222: 301 (1991); US Pat. Application 2002103360). The test compound exposed to a cell or protein according to the screening methods disclosed herein may be a single compound or a combination of compounds. When a combination of compounds are used in the screening methods disclosed herein, the compounds may be contacted sequentially or simultaneously.

**[0179]** A compound isolated by the screening methods disclosed herein is a candidate for drugs which inhibit the activity of KIF11, GHSR1b, NTSR1 or FOXM1 polypeptide, for treating or preventing diseases attributed to, for example, cell proliferative diseases, such as NSCLC. A compound in which a part of the structure of the compound obtained by the present screening methods disclosed herein is converted by addition, deletion and/or replacement, is included in the compounds obtained by the screening methods disclosed herein. A compound effective in suppressing the expression of over-expressed genes, i.e., KIF11, GHSR1b, NTSR1 or FOXM1 gene, is deemed to have a clinical benefit and can be further tested for its ability to prevent cancer cell growth in animal models or test subjects.

*Selecting a therapeutic agent for treating and/or preventing NSCLC that is appropriate for a particular individual*

**[0180]** Differences in the genetic makeup of individuals can result in differences in their relative abilities to metabolize various drugs. A compound that is metabolized in a subject to act as an anti-NSCLC agent can manifest itself by inducing a change in gene expression pattern in the subject's cells from that characteristic of a cancerous state to a gene expression pattern characteristic of a non-cancerous state. Accordingly, the differentially expressed KIF11, GHSR1b, NTSR1, and FOXM1 genes disclosed herein allow for selection of a putative therapeutic or prophylactic inhibitor of NSCLC specifically adequate for a subject by testing candidate compounds in a test cell (or test cell population) derived from the selected subject.

**[0181]** To identify an anti-NSCLC agent, that is appropriate for a specific subject, a test cell or test cell population derived from the subject is exposed to a therapeutic agent and the expression of one or more of the KIF11, GHSR1b,

NTSR1, and FOXM1 genes is determined.

**[0182]** The test cell is or the test cell population contains an NSCLC cell expressing an NSCLC-associated gene. Preferably, the test cell is or the test cell population contains a lung cell. For example, the test cell or test cell population is incubated in the presence of a candidate agent and the pattern of gene expression of the test cell or cell population is measured and compared to one or more reference profiles, e.g., an NSCLC reference expression profile or a non-NSCLC reference expression profile.

**[0183]** A decrease in the expression of one or more of KIF11, GHSR1b, NTSR1, and FOXM1 in a test cell or test cell population relative to a reference cell population containing NSCLC is indicative that the agent is therapeutic.

**[0184]** The test agent can be any compound or composition. For example, the test agent is an immunomodulatory agent.

#### *Methods for treating or preventing NSCLC*

**[0185]** The present disclosure provides a method for treating, alleviating or preventing NSCLC in a subject. Therapeutic compounds are administered prophylactically or therapeutically to subjects suffering from or at risk of (or susceptible to) developing NSCLC. Such subjects are identified using standard clinical methods or by detecting an aberrant level of expression or activity of KIF11, GHSR1b, NTSR1 or FOXM1 gene or polypeptide. Prophylactic administration occurs prior to the manifestation of overt clinical symptoms of disease, such that a disease or disorder is prevented or alternatively delayed in its progression.

**[0186]** The method includes decreasing the expression or function, or both, of one or more gene products of genes whose expression is aberrantly increased ("over-expressed gene"; KIF11, GHSR1 b, NTSR1 or FOXM1 gene) in an NSCLC cell relative to normal cells of the same tissue type from which the NSCLC cells are derived. The expression may be inhibited by any method known in the art. For example, a subject may be treated with an effective amount of a compound that decreases the amount of one or more of the KIF11, GHSR1b, NTSR1 or FOXM1 gene in the subject. Administration of the compound can be systemic or local. Such therapeutic compounds include compounds that decrease the expression level of such gene that endogenously exists in the NSCLC cells (*i.e.*, compounds that down-regulate the expression of the over-expressed gene(s), KIF11, GHSR1b and/or NTSR1 genes). The administration of such therapeutic compounds counter the effects of aberrantly-over expressed gene(s) in the subjects NSCLC cells and are expected to improve the clinical condition of the subject. Such compounds can be obtained by the screening method of the present disclosure described above.

**[0187]** The compounds that modulate the activity of a protein encoded by KIF11, GHSR1b, NTSR1 or FOXM1 gene that can be used for treating or preventing NSCLC of the present disclosure include besides proteins, naturally-occurring cognate ligand of these proteins, peptides, peptidomimetics and other small molecules.

**[0188]** Alternatively, the expression of these over-expressed gene(s) (KIF11, GHSR1b, NTSR1 and/or FOXM1) can be inhibited by administering to the subject a nucleic acid that inhibits or antagonizes the expression of the over-expressed gene(s). Antisense oligonucleotides, siRNAs or ribozymes which disrupt the expression of the over-expressed gene(s) can be used for inhibiting the expression of the over-expressed gene(s).

**[0189]** As noted above, antisense-oligonucleotides corresponding to any of the nucleotide sequence of KIF11, GHSR1b, NTSR1 or FOXM1 gene can be used to reduce the expression level of the gene. Antisense-oligonucleotides corresponding to KIF11, GHSR1b, NTSR1, and FOXM1 genes that are up-regulated in NSCLC are useful for the treatment or prevention of NSCLC. Specifically, the antisense-oligonucleotides against the genes may act by binding to any of the corresponding polypeptides encoded by these genes, or mRNAs corresponding thereto, thereby inhibiting the transcription or translation of the genes, promoting the degradation of the mRNAs, and/or inhibiting the expression of proteins encoded by the KIF11, GHSR1b, NTSR1, and FOXM1 nucleotides, and finally inhibiting the function of the proteins. The term "antisense-oligonucleotides" as used herein encompasses both nucleotides that are entirely complementary to the target sequence and those having a mismatch of one or more nucleotides, so long as the antisense-oligonucleotides can specifically hybridize to the target sequence. For example, the antisense-oligonucleotides disclosed herein include polynucleotides that have a homology (also referred to as sequence identity) of at least 70% or higher, preferably at 80% or higher, more preferably 90% or higher, even more preferably 95% or higher over a span of at least 15 continuous nucleotides up to the full length sequence of any of the nucleotide sequences of KIF11, GHSR1b, NTSR1 or FOXM1 gene. Algorithms known in the art can be used to determine the homology. Furthermore, derivatives or modified products of the antisense-oligonucleotides can also be used as antisense-oligonucleotides disclosed herein. Examples of such modified products include lower alkyl phosphonate modifications such as methyl-phosphonate-type or ethyl-phosphonate-type, phosphorothioate modifications and phosphoroamidate modifications. siRNA molecules of the disclosure can also be defined by their ability to hybridize specifically to mRNA or cDNA from the genes disclosed here. For the purposes of this disclosure the terms "hybridize" or "hybridize specifically" are used to refer the ability of two nucleic acid molecules to hybridize under "stringent hybridization conditions." 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

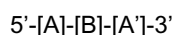
specifically at higher temperatures. An expensive guide to the hybridization of nucleic acids is found in Tijssen, Techniques in Biochemistry and Molecular Biology-Hybridization with Nucleic Probes, "Overview of principles of hybridization and the strategy of nucleic acid assays" (1993). Generally, stringent conditions are selected to be about 5-10°C lower than the thermal melting point ( $T_m$ ) for the specific sequence at a defined ionic strength pH. The  $T_m$  is the temperature (under defined ionic strength, pH, and nucleic concentration) at which 50% of the probes complementary to the target hybridize to the target sequence at equilibrium (as the target sequences are present in excess, at  $T_m$ , 50% of the probes are occupied at equilibrium). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. For selective or specific hybridization, a positive signal is at least two times background, preferably 10 times background hybridization. Exemplary stringent hybridization conditions can be as following: 50% formamide, 5x SSC, and 1 % SDS, incubating at 42°C, or, 5x SSC, 1% SDS, incubating at 65°C, with wash in 0.2x SSC, and 0.1% SDS at 50°C. The antisense-oligonucleotides and derivatives thereof act on cells producing the proteins encoded by KIF11, GHSR1b, NTSR1 or FOXM1 gene by binding to the DNA or mRNA encoding the protein, inhibiting transcription or translation thereof, promoting the degradation of the mRNAs and inhibiting the expression of the protein, thereby resulting in the inhibition of the protein function.

**[0190]** An antisense-oligonucleotides and derivatives thereof can be made into an external preparation, such as a liniment or a poultice, by mixing with a suitable base material which is inactive against the derivative.

**[0191]** The antisense-oligonucleotides disclosed herein inhibit the expression of at least one protein encoded by any one of KIF11, GHSR1b, NTSR1, and FOXM1 genes, and thus are useful for suppressing the biological activity of the protein.

**[0192]** The polynucleotides that inhibit one or more gene products of over-expressed genes also include small interfering RNAs (siRNA) comprising a combination of a sense strand nucleic acid and an antisense strand nucleic acid of the nucleotide sequence encoding an over-expressed protein encoded by KIF11, GHSR1b, NTSR1 or FOXM1 gene. 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 can be used in the treatment or prevention disclosed herein, including those in which DNA is a template from which RNA is transcribed. The siRNA is constructed such that a single transcript has both the sense and complementary antisense sequences from the target gene, e.g., a hairpin.

**[0193]** The method is used to suppress gene expression of a cell with up-regulated Expression of KIF11, GHSR1b, NTSR1 or FOXM1 gene. Binding of the siRNA to KIF11, GHSR1b, NTSR1 or FOXM1 gene transcript in the target cell results in a reduction of KIF11, GHSR1b, NTSR1 or FOXM1 protein production by the cell. The length of the oligonucleotide is at least about 10 nucleotides and may be as long as the naturally occurring transcript. Preferably, the oligonucleotide is about 19 to about 25 nucleotides in length. Most preferably, the oligonucleotide is less than about 75, about 50 or about 25 nucleotides in length. Preferable siRNA of the present disclosure include the polynucleotides having the nucleotide sequence of SEQ ID NO: 32, 33, 34, 35, 36, 37, or 108 as the target sequence, which all proved to be effective for suppressing cell viability of NSCLC cell lines. Specifically, a preferable siRNA used in the present disclosure has the general formula:



wherein [A] is a ribonucleotide sequence corresponding to a target sequence of KIF11, GHSR1b, NTSR1 or FOXM1; [B] is a ribonucleotide sequence consisting of about 3 to about 23 nucleotides; and [A'] is a ribonucleotide sequence complementary to [A]. Herein, the phrase a "target sequence of KIF11, GHSR1b, NTSR1 or FOXM1 gene" refers to a sequence that, when introduced into NSCLC cell lines, is effective for suppressing cell viability. Preferred target sequence of KIF11, GHSR1b, NTSR1 or FOXM1 gene includes nucleotide sequences comprising: SEQ ID NOs: 32, 33, 34, 35, 36, 37, and 108. The complementary sequence [A'] and [A] hybridize to each other to form a double strand, and the whole siRNA molecule with the general formula 5'-[A]-[B]-[A']-3' forms a hairpin loop structure. As used herein, the term "complementary" refers to a Watson-Crick or Hoogsteen base pairing between nucleotide units of a polynucleotide, and hybridization or binding of nucleotide units indicates physical or chemical interaction between the units under appropriate conditions to form a stable duplex (double-stranded configuration) containing few or no mismatches. In a preferred embodiment, such duplexes contain no more than 1 mismatch for every 10 base pairs. Particularly preferred duplexes are fully complementary and contain no mismatch. The siRNA against the mRNA of KIF11, GHSR1b, NTSR1 or FOXM1 gene to be used in the present disclosure contains a target sequence shorter than the whole mRNA of KIF11, GHSR1b, NTSR1 or FOXM1 gene, and has a sequence of 500, 200, or 75 nucleotides as the whole length.

**[0194]** Also disclosed herein is a vector containing one or more of the nucleic acids described herein, and a cell containing the vectors. The isolated nucleic acids of the present disclosure are useful for siRNA against KIF11, GHSR1b, NTSR1 or FOXM1 or DNA encoding the siRNA. When the nucleic acids are used for siRNA or coding DNA thereof, the sense strand is preferably longer than about 19 nucleotides, and more preferably longer than about 21 nucleotides.

**[0195]** Furthermore, the nucleotide sequence of siRNAs may be designed using a siRNA design computer program available from the Ambion website ([http://www.ambion.com/techlib/misc/siRNA\\_finder.html](http://www.ambion.com/techlib/misc/siRNA_finder.html)). The nucleotide sequences

for the siRNA are selected by the computer program based on the following protocol:

#### Selection of siRNA Target Sites:

1. Beginning with the AUG start codon of the transcript, scan downstream for AA dinucleotide sequences. Record the occurrence of each AA and the 3' adjacent 19 nucleotides as potential siRNA target sites. Tuschl, et al. *Genes Dev* 13(24): 3191-7 (1999), not recommend against designing siRNA against 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 thus the complex of endonuclease and siRNAs that were designed against these regions may interfere with the binding of UTR-binding proteins and/or translation initiation complexes.

2. Compare the potential target sites to the human genome database and eliminate from consideration any target sequences with significant homology to other coding sequences. The homology search can be performed using BLAST, which can be found on the NCBI server at: [www.ncbi.nlm.nih.gov/BLAST/](http://www.ncbi.nlm.nih.gov/BLAST/)

3. Select qualifying target sequences for synthesis. On the website of Ambion, several preferable target sequences can be selected along the length of the gene for evaluation.

**[0196]** The siRNAs inhibit the expression of over-expressed KIF11, GHSR1b, NTSR1 or FOXM1 protein and is thereby useful for suppressing the biological activity of the protein. Therefore, a composition comprising the siRNA is useful in treating or preventing non-small cell lung cancer.

**[0197]** The nucleic acids that inhibit one or more gene products of over-expressed genes KIF11, GHSR1b, NTSR1, and FOXM1 also include ribozymes against the gene(s).

**[0198]** The ribozymes inhibit the expression of over-expressed KIF11, GHSR1b, NTSR1 or FOXM1 protein and is thereby useful for suppressing the biological activity of the protein. Therefore, a composition comprising the ribozyme is useful in treating or preventing NSCLC.

**[0199]** Generally, ribozymes are classified into large ribozymes and small ribozymes. A large ribozyme is known as an enzyme that cleaves the phosphate ester bond of nucleic acids. After the reaction with the large ribozyme, the reacted site consists of a 5'-phosphate and 3'-hydroxyl group. The large ribozyme is further classified into (1) group I intron RNA catalyzing transesterification at the 5'-splice site by guanosine; (2) group II intron RNA catalyzing self-splicing through a two step reaction via lariat structure; and (3) RNA component of the ribonuclease P that cleaves the tRNA precursor at the 5' site through hydrolysis. On the other hand, small ribozymes have a smaller size (about 40 bp) compared to the large ribozymes and cleave RNAs to generate a 5'-hydroxyl group and a 2'-3' cyclic phosphate. Hammerhead type ribozymes (Koizumi et al., *FEBS Lett.* 228: 225 (1988)) and hairpin type ribozymes (Buzayan, *Nature* 323: 349 (1986); Kiluchi and Sasaki, *Nucleic Acids Res.* 19: 6751 (1992)) are included in the small ribozymes. Methods for designing and constructing ribozymes are known in the art (see Koizumi et al., *FEBS Lett.* 228: 225 (1988); Koizumi et al., *Nucleic Acids Res.* 17: 7059 (1989); Kikuchi and Sasaki, *Nucleic Acids Res.* 19: 6751 (1992)) and ribozymes inhibiting the expression of an over-expressed NSC protein can be constructed based on the sequence information of the nucleotide sequence encoding KIF11, GHSR1b, NTSR1 or FOXM1 protein according to conventional methods for producing ribozymes.

**[0200]** The ribozymes inhibit the expression of over-expressed KIF11, GHSR1b, NTSR1 or FOXM1 protein and is thereby useful for suppressing the biological activity of the protein. Therefore, a composition comprising the ribozyme is useful in treating or preventing NSCLC.

**[0201]** Alternatively, the function of one or more gene products of the over-expressed genes is inhibited by administering a compound that binds to or otherwise inhibits the function of the gene products. For example, the compound is an antibody which binds to the over-expressed gene product or gene products.

**[0202]** The present disclosure refers to the use of antibodies, particularly antibodies against a protein encoded by any of the up-regulated genes KIF11, GHSR1b, NTSR1 or FOXM1, or a fragment of the antibody. As used herein, the term "antibody" refers to an immunoglobulin molecule having a specific structure that interacts (binds) specifically with a molecule comprising the antigen used for synthesizing the antibody (*i.e.*, the up-regulated gene product) or with an antigen closely related to it. An antibody that binds to the over-expressed KIF11, GHSR1 b, NTSR1 or FOXM1 nucleotide may be in any form, such as monoclonal or polyclonal antibodies, and includes antiserum obtained by immunizing an animal such as a rabbit with the polypeptide, all classes of polyclonal and monoclonal antibodies, human antibodies and humanized antibodies produced by genetic recombination. Furthermore, the antibody used in the method of treating or preventing NSCLC, disclosed herein may be a fragment of an antibody or a modified antibody, so long as it binds to one or more of the proteins encoded by the marker genes (KIF11, GHSR1b, NTSR1 or FOXM1 gene). The antibodies and antibody fragments used in the present method of treating or preventing NSCLC may be modified, and include chemically modified and chimeric antibodies. Such antibodies and antibody fragments can be obtained according to the

above-mentioned methods, *supra*.

**[0203]** When the obtained antibody is to be administered to the human body (antibody treatment), a human antibody or a humanized antibody is preferable for reducing immunogenicity.

**[0204]** For example, transgenic animals having a repertoire of human antibody genes may be immunized with an antigen such as KIF11, GHSR1b, NTSR1 or FOXM1 polypeptide, cells expressing the polypeptide, or their lysates. Antibody producing cells are then collected from the animals and fused with myeloma cells to obtain hybridoma, from which human antibodies against the polypeptide can be prepared (see WO92-03918, W093-2227, WO94-02602, WO94-25585, WO96-33735, and WO96-34096).

**[0205]** Alternatively, an immune cell, such as an immunized lymphocyte, producing antibodies may be immortalized by an oncogene and used for preparing monoclonal antibodies.

**[0206]** The present disclosure provides a method for treating or preventing NSCLC, using an antibody against an over-expressed KIF11, GHSR1b, NTSR1 or FOXM1 polypeptide. According to the method, a pharmaceutically effective amount of an antibody against KIF11, GHSR1b, NTSR1 or FOXM1 polypeptide is administered. An antibody against an over-expressed KIF11, GHSR1b, NTSR1 or FOXM1 polypeptide is administered at a dosage sufficient to reduce the activity of KIF11, GHSR1b, NTSR1 or FOXM1 protein. Alternatively, an antibody binding to a cell surface marker specific for tumor cells can be used as a tool for drug delivery. Thus, for example, an antibody against an over-expressed KIF11, GHSR1b, NTSR1 or FOXM1 polypeptide conjugated with a cytotoxic agent may be administered at a dosage sufficient to injure tumor cells.

**[0207]** In addition, dominant negative mutants of the proteins disclosed here can be used to treat or prevent NSCLC. For example, fragments of KOC1 that specifically bind KIF11 can be used. As used here "dominant negative fragment of KOC1" is a mutated form of KOC1 that is capable of complexing with either of KIF11 and RNA to be transported such that the RNA transporter activity of the complex is diminished. Thus, a dominant negative fragment is one that is not functionally equivalent to the full length KOC1 polypeptide. Preferred dominant negative fragments are those that comprise at least one RRM domain of KOC1. Alternatively, in another embodiment, the dominant negative fragments have two RRM domains and zero to three of KH domains. For example KOC1DEL2 (2xRRM+2xKH) and KOC1DEL3 (2xRRM without KH) are preferable fragment for dominant negative effect. The amino acid sequences of KOC1DEL2 and KOC1DEL3 consist of positions 1 to 406 and 1-197 of SEQ ID NO:105, respectively. The fragments are typically less than about 300 amino acids, typically less than about 200 amino acids.

**[0208]** The present disclosure also relates to a method of treating or preventing NSCLC in a subject comprising administering to said subject a vaccine comprising a polypeptide encoded by a nucleic acid selected from the group consisting of KIF11, GHSR1b, NTSR1, and FOXM1 genes or an immunologically active fragment of said polypeptide, or a polynucleotide encoding the polypeptide or the fragment thereof. Administration of the polypeptide induces an anti-tumor immunity in a subject. Thus, the present disclosure further provides a method for inducing anti tumor immunity. The polypeptide or the immunologically active fragments thereof are useful as vaccines against NSCLC. In some cases the proteins or fragments thereof may be administered in a form bound to the T cell receptor (TCR) or presented on an antigen presenting cell (APC), such as macrophage, dendritic cell (DC) or B-cells. Due to the strong antigen presenting ability of DC, the use of DC is most preferable among the APCs.

**[0209]** In the present disclosure, the phrase "vaccine against NSCLC" refers to a substance that has the function to induce anti-tumor immunity or immunity to suppress NSCLC upon inoculation into animals. In general, anti-tumor immunity includes immune responses such as follows:

- induction of cytotoxic lymphocytes against tumors,
- induction of antibodies that recognize tumors, and
- induction of anti-tumor cytokine production.

**[0210]** Therefore, when a certain protein induces any one of these immune responses upon inoculation into an animal, the protein is decided to have anti-tumor immunity inducing effect. The induction of the anti-tumor immunity by a protein can be detected by observing *in vivo* or *in vitro* the response of the immune system in the host against the protein.

**[0211]** For example, a method for detecting the induction of cytotoxic T lymphocytes is well known. A foreign substance that enters the living body is presented to T cells and B cells by the action of antigen presenting cells (APCs). T cells that respond to the antigen presented by APC in antigen specific manner differentiate into cytotoxic T cells (or cytotoxic T lymphocytes; CTLs) due to stimulation by the antigen, and then proliferate (this is referred to as activation of T cells).

**[0212]** Therefore, CTL induction by a certain peptide can be evaluated by presenting the peptide to T cell by APC, and detecting the induction of CTL. Furthermore, APC has the effect of activating CD4+ T cells, CD8+ T cells, macrophages, eosinophils and NK cells. Since CD4+ T cells are also important in anti-tumor immunity, the anti-tumor immunity inducing action of the peptide can be evaluated using the activation effect of these cells as indicators.

**[0213]** A method for evaluating the inducing action of CTL using dendritic cells (DCs) as APC is well known in the art. DC is a representative APC having the strongest CTL inducing action among APCs. In this method, the test polypeptide is initially contacted with DC and then this DC is contacted with T cells. Detection of T cells having cytotoxic effects against the cells of interest after the contact with DC shows that the test polypeptide has an activity of inducing the cytotoxic T cells. Activity of CTL against tumors can be detected, for example, using the lysis of <sup>51</sup>Cr-labeled tumor cells as the indicator. Alternatively, the method of evaluating the degree of tumor cell damage using <sup>3</sup>H-thymidine uptake activity or LDH (lactose dehydrogenase)-release as the indicator is also well known.

**[0214]** Apart from DC, peripheral blood mononuclear cells (PBMCs) may also be used as the APC. The induction of CTL is reported to be enhanced by culturing PBMC in the presence of GM-CSF and IL-4. Similarly, CTL is shown to be induced by culturing PBMC in the presence of keyhole limpet hemocyanin (KLH) and IL-7.

**[0215]** The test polypeptides confirmed to possess CTL inducing activity by these methods are polypeptides having DC activation effect and subsequent CTL inducing activity. Therefore, polypeptides that induce CTL against tumor cells are useful as vaccines against NSCLC. Furthermore, APC that acquired the ability to induce CTL against NSCLC by contacting with the polypeptides are useful as vaccines against NSCLC. Furthermore, CTL that acquired cytotoxicity due to presentation of the polypeptide antigens by APC can be also used as vaccines against NSCLC. Such therapeutic methods for NSCLC using anti-tumor immunity due to APC and CTL are referred to as cellular immunotherapy.

**[0216]** Generally, when using a polypeptide for cellular immunotherapy, efficiency of the CTL-induction is known to increase by combining a plurality of polypeptides having different structures and contacting them with DC. Therefore, when stimulating DC with protein fragments, it is advantageous to use a mixture of multiple types of fragments.

**[0217]** Alternatively, the induction of anti-tumor immunity by a polypeptide can be confirmed by observing the induction of antibody production against tumors. For example, when antibodies against a polypeptide are induced in a laboratory animal immunized with the polypeptide, and when growth, proliferation or metastasis of tumor cells is suppressed by those antibodies, the polypeptide can be determined to have an ability to induce anti-tumor immunity.

**[0218]** Anti-tumor immunity is induced by administering the vaccine disclosed herein and the induction of anti-tumor immunity enables treatment and prevention of NSCLC. Therapy against or prevention of the onset of NSCLC includes any of the steps, such as inhibition of the growth of NSCLC cells, involution of NSCLC cells and suppression of occurrence of NSCLC cells. Decrease in mortality of individuals having NSCLC, decrease of marker genes (in addition to KIF11, GHSR1b and/or NTSR1 genes) in the blood, alleviation of detectable symptoms accompanying NSCLC and such are also included in the therapy or prevention of NSCLC. Such therapeutic and preventive effects are preferably statistically significant. For example, in observation, at a significance level of 5% or less, wherein the therapeutic or preventive effect of a vaccine against NSCLC is compared to a control without vaccine administration. For example, Student's t-test, the Mann-Whitney U-test or ANOVA may be used for statistical analysis.

**[0219]** The above-mentioned protein having immunological activity, or a polynucleotide or vector encoding the protein may be combined with an adjuvant. An adjuvant refers to a compound that enhances the immune response against the protein when administered together (or successively) with the protein having immunological activity. Examples of adjuvants include cholera toxin, salmonella toxin, alum and such, but are not limited thereto. Furthermore, the vaccine disclosed herein may be combined appropriately with a pharmaceutically acceptable carrier. Examples of such carriers are sterilized water, physiological saline, phosphate buffer, culture fluid and such. Furthermore, the vaccine may contain as necessary, stabilizers, suspensions, preservatives, surfactants and such. The vaccine is administered systemically or locally. Vaccine administration may be performed by single administration or boosted by multiple administrations.

**[0220]** When using APC or CTL as the vaccine disclosed herein NSCLC can be treated or prevented, for example, by the *ex vivo* method. More specifically, PBMCs of the subject receiving treatment or prevention are collected, the cells are contacted with the polypeptide *ex vivo*, and following the induction of APC or CTL, the cells may be administered to the subject. APC can be also induced by introducing a vector encoding the polypeptide into PBMCs *ex vivo*. APC or CTL induced *in vitro* can be cloned prior to administration. By cloning and growing cells having high activity of damaging target cells, cellular immunotherapy can be performed more effectively. Furthermore, APC and CTL isolated in this manner may be used for cellular immunotherapy not only against individuals from whom the cells are derived, but also against similar types of diseases in other individuals.

**[0221]** Moreover, the present disclosure provides a method for treating or preventing NSCLC in a subject, wherein a compound obtained according to any of the above-described screening methods is administered to the subject. Any compound that are obtained according to any of the screening methods disclosed herein can be administered to the subject so long as it : decreases the expression or function, or both, of one or more gene products of KIF11, GHSR1b, NTSR1, and FOXM1 genes.

*siRNA and vectors encoding them*

**[0222]** Transfection of vectors expressing siRNA for KIF11, GHSR1b, NTSR1 or FOXM1 leads to growth inhibition of NSCLC cells. Thus, it is another aspect of the present disclosure to provide a double-stranded molecule comprising a

sense-strand and antisense-strand which molecule functions as an siRNA for KIF11, GHSR1b, NTSR1 or FOXM1, and a vector encoding the double-stranded molecule.

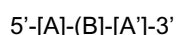
**[0223]** The double-stranded molecule herein disclosed comprises a sense strand and an antisense strand, wherein the sense strand comprises a ribonucleotide sequence corresponding to a KIF11, GHSR1b, NTSR1 or FOXM1 target sequence, and wherein the antisense strand comprises a ribonucleotide sequence which is complementary to said sense strand, wherein said sense strand and said antisense strand hybridize to each other to form said double-stranded molecule, and wherein said double-stranded molecule, when introduced into a cell expressing a KIF11, GHSR1b, NTSR1 or FOXM1 gene, inhibits expression of said gene.

**[0224]** The double-stranded molecule herein disclosed may be a polynucleotide derived from its original environment (i.e., when it is a naturally occurring molecule, the natural environment), physically or chemically altered from its natural state, or chemically synthesized. According to the present disclosure, such double-stranded molecules include those composed of DNA, RNA, and derivatives thereof. A DNA is suitably composed of bases such as A, T, C and G, and T is replaced by U in an RNA.

**[0225]** As described above, the term "complementary" refers to a Watson-Crick or Hoogsteen base pairing between nucleotide units of a polynucleotide, and hybridization or binding of nucleotide units indicates physical or chemical interaction between the units under appropriate conditions to form a stable duplex (double-stranded configuration) containing few or no mismatches. In a preferred embodiment, such duplexes contain no more than 1 mismatch for every 10 base pairs. Particularly preferred duplexes are fully complementary and contain no mismatch.

**[0226]** The double-stranded molecule herein disclosed contains a ribonucleotide sequence corresponding to a KIF11, GHSR1b, NTSR1 or FOXM1 target sequence shorter than the whole mRNA of KIF11, GHSR1b, NTSR1 or FOXM1 gene. Herein, the phrase a "target sequence of KIF11, GHSR1b, NTSR1 or FOXM1 gene" refers to a sequence that, when introduced into NSCLC cell lines, is effective for suppressing cell viability. Specifically, the target sequence comprises at least about 10, or suitably about 19 to about 25 contiguous nucleotides from the nucleotide sequences selected from the group of SEQ ID NOs: 1, 3, 5, and 106. That is, the sense strand of the present double-stranded molecule consists of at least about 10 nucleotides, suitably is longer than 19 nucleotides, and more preferably longer than 21 nucleotides. Preferred target sequences include the sequences of SEQ ID NOs: 32,33, 34, 35, 36, 37, and 108. The present double-stranded molecule including the sense strand and the antisense strand is an oligonucleotide shorter than about 100, preferably 75, more preferably 50 and most preferably 25 nucleotides in length. A suitable double-stranded molecule of the present disclosure is an oligonucleotide of a length of about 19 to about 25 nucleotides. Furthermore, in order to enhance the inhibition activity of the siRNA, nucleotide "u" can be added to 3' end of the antisense strand of the target sequence. The number of "u"s to be added is at least 2, generally 2 to 10, preferably 2 to 5. The added "u"s form single strand at the 3' end of the antisense strand of the siRNA- In these embodiments, the siRNA molecules of the disclosure are typically modified as described above for antisense molecules. Other modifications are also possible, for example, cholesterol-conjugated siRNAs have shown improved pharmacological properties (Song et al. Nature Med 9:347-351 (2003):).

**[0227]** Furthermore, the double-stranded molecule of the present disclosure may be a single ribonucleotide transcript comprising the sense strand and the antisense strand linked via a single-stranded ribonucleotide sequence. Namely, the present double-stranded molecule may have the general formula:



wherein [A] is a ribonucleotide sequence corresponding to a target sequence of KIF11, GHSR1b, NTSR1 or FOXM1;

[B] is a ribonucleotide sequence (loop sequence) consisting of 3 to 23 nucleotides; and

[A'] is a ribonucleotide sequence complementary to [A]. The complementary sequence [A'] and [A] hybridize to each other to form a double strand, and the whole siRNA molecule with the general formula 5'-[A]-(B)-[A']-3' forms a hairpin loop structure.

**[0228]** The region [A] hybridizes to [A'], and then a loop consisting of region [B] is formed. The loop sequence can be selected from those describe in [http://www.ambion.com/techlib/tb/tb\\_506.html](http://www.ambion.com/techlib/tb/tb_506.html), or those described in Jacque, J.-M. et al., Nature 418:435-438 (2002). Additional examples of the loop sequence that can be included in the present double-stranded molecules include:

CCC, CCACC or CCACACC: Jacque, J. M. et al., Nature, Vol. 418: 435-438 (2002);

UUCG: Lee, N.S. et al., Nature Biotechnology 20:500-505 (2002); Fruscoloni, P. et.al., Proc. Natl. Acad Sci. USA 100(4): 1639-1644 (2003); and



UUCAAGAGA: Dykxhoorn, D. M. et al., Nature Reviews Molecular- Cell Biology 4:457-467 (2002).

**[0229]** Preferable siRNAs having hairpin loop structure disclosed herein are shown below. In the following structure, the loop sequence can be selected from the group consisting of: CCC, UUCG, CCACC, CCACACC, and UUCAAGAGA. Among these sequences, the most preferable loop sequence is UUCAAGAGA (corresponding to "ttcaagaga" in a DNA):

guuaguguac gaacuggag-[B]-cuccaguuc guacacuaac (for the target sequence of SEQ ID NO:32);  
gugucucugu uggagaucu-[B]-agaucucca acagagacac (for the target sequence of SEQ ID NO:33);  
gaaggcaguu gaccaacac-[B]-guguugguc aacugccuuc (for the target sequence of SEQ ID NO:34);  
ccucuaccug uccagcaug-[B]-caugcugga cagguagagg (for the target sequence of SEQ ID NO:35);  
guucaucagc gccaucugg-[B]-ccagauggc gcugaugaac (for the target sequence of SEQ ID NO:36);  
ggucgucuaa caggucaac-[B]-guugaccug uaugacgacc (for the target sequence of SEQ ID NO:37);  
and  
gcagcagaaa cgaccgaau-[B]-auucggucg uuucugcugc (for the target sequence of SEQ ID NO:108).

**[0230]** The present disclosure further provides a vector encoding the double-stranded molecule herein disclosed. The vector encodes a transcript having a secondary structure and which comprises the sense strand and the antisense strand, and suitably comprises a single-stranded ribonucleotide sequence linking said sense strand and said antisense strand. The vector preferably comprises a regulatory sequence adjacent to the region encoding the present double-stranded molecule that directs the expression of the molecule in an adequate cell. For example, the double-stranded molecules disclosed herein are intracellularly transcribed by cloning their coding sequence 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.

**[0231]** Alternatively, the present vectors are produced, for example, by cloning the target sequence into an expression vector so the objective sequence is operatively-linked to a regulatory sequence of the vector in a manner to allow expression thereof (transcription of the DNA molecule) (Lee, N.S. et al., Nature Biotechnology 20: 500-505 (2002)). For example, the transcription of an RNA molecule having an antisense sequence to the target sequence is driven by a first promoter (e.g., a promoter sequence linked to the 3'-end of the cloned DNA) and that having the sense strand to the target sequence by a second promoter (e.g., a promoter sequence linked to the 5'-end of the cloned DNA). The expressed sense and antisense strands hybridize to each other *in vivo* to generate a siRNA construct to silence a gene that comprises the target sequence. Furthermore, two constructs (vectors) may be utilized to respectively produce the sense and anti-sense strands of a siRNA construct.

**[0232]** For introducing the vectors into a cell, transfection-enhancing agent can be used. FuGENE (Rochediagnos-tics), Lipofectamine 2000 (Invitrogen), Oligofectamine (Invitrogen), and Nucleofector (Wako pure Chemical) are useful as the transfection-enhancing agent.

#### *Pharmaceutical compositions for treating or preventing NSCLC*

**[0233]** The present disclosure provides compositions for treating or preventing NSCLC comprising a compound selected by the present method of screening for a compound that alters the expression or activity of an NSCLC-associated gene.

**[0234]** When administering a compound isolated by the screening method herein disclosed as a pharmaceutical for humans and other mammals, such as mice, rats, guinea-pig, rabbits, cats, dogs, sheep, pigs, cattle, monkeys, baboons or chimpanzees for treating a cell proliferative disease (e.g., non-small cell lung cancer), the isolated compound can be directly administered or can be formulated into a dosage form using conventional pharmaceutical preparation methods. Such pharmaceutical formulations of the present compositions include those suitable for oral, rectal, nasal, topical (including buccal and sub-lingual), vaginal or parenteral (including intramuscular, sub-cutaneous and intravenous) administration, or for administration by inhalation or insufflation. The formulations are optionally packaged in discrete dosage units.

**[0235]** Pharmaceutical formulations suitable for oral administration include capsules, cachets or tablets, each containing a predetermined amount of the active ingredient. Formulations also include powders, granules, solutions, suspensions or emulsions. The active ingredient is optionally administered as a bolus electuary or paste. Tablets and capsules for oral administration may contain conventional excipients such as binding agents, fillers, lubricants, disintegrant or wetting agents. A tablet may be made by compression or molding, optionally with one or more formulation ingredients. Compressed tablets may be prepared by compressing in a suitable machine the active ingredients in a free-flowing form such as powder or granules, optionally mixed with a binder, lubricant, inert diluent, lubricating, surface active or dispersing agent. Molded tablets may be made via molding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent. The tablets may be coated according to methods well known in the art. Oral fluid preparations may be in the form of, for example, aqueous or oily suspensions, solutions, emulsions, syrups

or elixirs, or may be presented as a dry product for reconstitution with water or other suitable vehicle prior to use. Such liquid preparations may contain conventional additives such as suspending agents, emulsifying agents, non-aqueous vehicles (which may include edible oils) or preservatives. The tablets may optionally be formulated so as to provide slow or controlled release of the active ingredient *in vivo*. A package of tablets may contain one tablet to be taken on each of the month. The formulation or dose of medicament in these preparations makes a suitable dosage within the indicated range acquirable.

**[0236]** Formulations for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example, saline, water-for-injection, immediately prior to use. Alternatively, the formulations may be presented for continuous infusion. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

**[0237]** Formulations for rectal administration include suppositories with standard carriers such as cocoa butter or polyethylene glycol. Formulations for topical administration in the mouth, for example, buccally or sublingually, include lozenges, which contain the active ingredient in a flavored base such as sucrose and acacia or tragacanth, and pastilles comprising the active ingredient in a base such as gelatin, glycerin, sucrose or acacia. For intra-nasal administration of an active ingredient, a liquid spray or dispersible powder or in the form of drops may be used. Drops may be formulated with an aqueous or non-aqueous base also comprising one or more dispersing agents, solubilizing agents or suspending agents.

**[0238]** For administration by inhalation the compositions are conveniently delivered from an insufflator, nebulizer, pressurized packs or other convenient means of delivering an aerosol spray. Pressurized packs may comprise a suitable propellant such as dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount

**[0239]** Alternatively, for administration by inhalation or insufflation, the compositions may take the form of a dry powder composition, for example, a powder mix of an active ingredient and a suitable powder base such as lactose or starch. The powder composition may be presented in unit dosage form in, for example, capsules, cartridges, gelatin or blister packs from which the powder may be administered with the aid of an inhalator or insufflators.

**[0240]** Other formulations include implantable devices and adhesive patches; which release a therapeutic agent.

**[0241]** When desired, the above-described formulations, adapted to give sustained release of the active ingredient, may be employed. The pharmaceutical compositions may also contain other active ingredients such as antimicrobial agents, immunosuppressants or preservatives.

**[0242]** It should be understood that in addition to the ingredients particularly mentioned above, the formulations of this disclosure 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.

**[0243]** Preferred unit dosage formulations are those containing an effective dose, as recited below, of the active ingredient or an appropriate fraction thereof.

**[0244]** For each of the aforementioned conditions, the compositions, e.g., polypeptides and organic compounds are administered orally or via injection at a dose of from about 0.1 to about 250 mg/kg per day. The dose range for adult humans is generally from about 5 mg to about 17.5 g/day, preferably about 5 mg to about 10 g/day, and most preferably about 100 mg to about 3 g/day. Tablets or other unit dosage forms of presentation provided in discrete units may conveniently contain an amount which is effective at such dosage or as a multiple of the same, for instance, units containing about 5 mg to about 500 mg, usually from about 100 mg to about 500 mg.

**[0245]** The dose employed will depend upon a number of factors, including the age and sex of the subject, the precise disorder being treated, and its severity. Also the route of administration may vary depending upon the condition and its severity.

**[0246]** The present disclosure further provides a composition for treating or preventing NSCLC comprising active ingredient that inhibits the expression of any one of the gene selected from the group of KIF11, GHSR1b, NTSR1, and FOXM1 genes. Such active ingredient can be an antisense-oligonucleotide, siRNA or ribozyme against the gene, or derivatives, such as expression vector, of the antisense-oligonucleotide, siRNA or ribozyme. The active ingredient may be made into an external preparation, such as liniment or a poultice, by mixing with a suitable base material which is inactive against the derivatives.

**[0247]** Also, as needed, the active ingredient can be formulated into tablets, powders, granules, capsules, liposome capsules, injections, solutions, nose-drops and freeze-drying agents by adding excipients, isotonic agents, solubilizers, preservatives, pain-killers and such. These can be prepared according to conventional methods for preparing nucleic acid containing pharmaceuticals.

**[0248]** Preferably, the antisense-oligonucleotide derivative, siRNA derivative or ribozyme derivative is given to the patient by direct application to the ailing site or by injection into a blood vessel so that it will reach the site of ailment. A mounting medium can also be used in the composition to increase durability and membrane-permeability. Examples of mounting mediums include liposome, poly-L-lysine, lipid, cholesterol, lipofectin and derivatives thereof.

**[0249]** The dosage of such compositions can be adjusted suitably according to the patient's condition and used in desired amounts. For example, a dose range of 0.1 to 100 mg/kg, preferably 0.1 to 50 mg/kg can be administered.

**[0250]** Another embodiment disclosed herein is a composition for treating or preventing NSCLC comprising an antibody against a polypeptide encoded by any one of the genes selected from the group of KIF11, GHSR1 b, NTSR1, and FOXM1 genes or fragments of the antibody that bind to the polypeptide.

**[0251]** Although there are some differences according to the symptoms, the dose of an antibody or fragments thereof for treating or preventing NSCLC is about 0.1 mg to about 100 mg per day, preferably about 1.0 mg to about 50 mg per day and more preferably about 1.0 mg to about 20 mg per day, when administered orally to a normal adult (weight 60 kg).

**[0252]** When administering parenterally, in the form of an injection to a normal adult (weight 60 kg), although there are some differences according to the condition of the patient, symptoms of the disease and method of administration, it is convenient to intravenously inject a dose of about 0.01 mg to about 30 mg per day, preferably about 0.1 to about 20 mg per day and more preferably about 0.1 to about 10 mg per day. Also, in the case of other animals too, it is possible to administer an amount converted to 60 kg of body-weight.

**[0253]** The following examples are presented to illustrate the present invention and to assist one of ordinary skill in making and using the same. The examples are not intended in any way to otherwise limit the scope of the invention.

**[0254]** 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 this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below.

## BEST MODE FOR CARRING OUT THE METHODS DISCLOSED HEREIN

### Materials and methods

#### (1) Patients and tissue samples

**[0255]** Primary NSCLC samples, of which 22 were classified as adenocarcinomas (ADCs), 14 as squamous-cell carcinomas (SCCs), and one as adenosquamous carcinoma, had been obtained earlier with informed consent from 37 patients (Kikuchi, T. et al., *Oncogene* 22, 2192-2205 (2003)). Fifteen additional primary NSCLCs, including seven ADCs and eight SCCs, were obtained along with adjacent normal lung tissue samples from patients undergoing surgery at our institutes.

#### (2) Cell lines

**[0256]** The 30 human NSCLC and four SCLC cell lines used in this study were as follows: adenocarcinomas (ADCs) A427, A549, NCI-H23, NCI-H522, LC 174, LC 176, LC319, PC3, PC9, PC14, PG14-PE6, NCI-H1373, NCI-H1435, NCI-H1793, SK-LU-1, NCI-H358, NCI-H1650 and SW1573; adenosquamous carcinomas (ASCs) NCI-H226, NCI-H596 and NCI-H647; squamous-cell carcinomas (SCCs) RERF-LC-AI, SW-900, SK-MES-1, Ebb-1, LU61, NCI-H520, NCI-H1703, and NCI-H2170; large-cell carcinoma (LCC) LX1; and SCLCs DMS114, DMS273, SBC-3, and SBC-5. Human small airway epithelial cells, SAEC were grown in optimized medium (SAGM) purchased from Cambrex Bio Science Inc. A human bronchial epithelial cell line, BEAS2B cells were also served.

**[0257]** Thirty-four human NSCLC or SCLC cancer cell lines and two normal bronchial epithelium cell lines were grown in monolayers in appropriate medium supplemented with 5 or 10% fetal bovine serum (see Table 1).

Table 1

Cell line name	Medium	Provider
adenocarcinoma (ADC)		
A427	EMEM (10%FBS)	ATCC(HTB-53)
A549	RPMI- 1640 (10%FBS)	ATCC(CCL-185)
NCI- H23	RPMI- 1640 (10%FBS)	ATCC(CRL-5800)

(continued)						
Cell line name			Medium		Provider	
BAC	NCI-	H522	RPMI-	1640	(10%FBS)	ATCC(CRL-5810)
	LC174		RPMI-	1640	(10%FBS)	Aichi Cancer Center
	LC176		RPMI-	1640	(10%FBS)	Aichi Cancer Center
	LC319		RPMI-	1640	(10%FBS)	Aichi Cancer Center
	PC-	3	DMEM		(10%FBS)	Tokushima Univeraity
	PC-	9	DMEM		(10%FBS)	Tokushima Univeraity
	PC14		RPMI-	1640	(10%FBS)	Tokushima Univeraity
	PC14-	PE6	RPMI-	1640	(10%FBS)	Tokushima Univeraity
	NCI-	H1373	RPMI-	1640	(10%FBS)	ATCC(CRL-5866)
	NCI-	H1435	F12+DMEM(5%FBS)+EGF(+)		SNU Bank	
	NCI-	H1793	F12+DMEM (5%FBS) +Glu		SNU Bank	
	SK-	LU- 1	DMEM		(10%FBS)	SNU Bank
	NCI-	H358	RPMI-	1640	(10%FBS)	SNU Bank
	NCI-	H1650	RPMI-	1640	(10%FBS)	ATCC(CRL-5883)
	SW1573		Leibovitz's L-15(10%FBS)		ATCC(CRL-2170)	
adenosquamous carcinoma (ASCs)						
NCI-	H226	RPMI-	1640	(10%FBS)	ATCC(CRL-5826)	
NCI-	H647	RPMI-	1640	(10%FBS)	ATCC(CRL-5834)	
NCI-	H596	RPMI-	1640	(10%FBS)	SNU Bank	
squamous cell carcinoma (SCC)						
RERF-	LC-	AI	DMEM	(10%FBS)	Tokushima Univeraity	
SW-		900	Leibovitz's L-15(10%FBS)		SNU Bank	
SK-	MES-	1	DMEM	(10%FBS)	SNU Bank	
EBC-		1	DMEM	(10%FBS)	Tokushima Univeraity	
	LU61		DMEM(10%FBS)		Central Institute for Experimental Animals	
NCI-		H520	RPMI-	1640	(10%FBS)	ATCC(HTB-182)
NCI-		H1703	RPMI-	1640	(10%FBS)	ATCC(CRL-5889)
NCI-		H2170	RPMI-	1640	(10%FBS)	ATCC8(CRL-5928)
large-cell carcinoma (LCC)						
LX1			DMEM	(10%FBS)	Central Institute for Experimental Animals	
small-cell lung carcinoma (SCLCS)						
DMS114			RPMI-	1640	(10%FBS)	ATCC(CRL-2066)
DMS273			RPMI-	1640	(10%FBS)	Japanese foundation for cancer research
SBC-		3	RPMI-	1640	(10%FBS)	Tokushima Univeraity
SBC-		5	EMEM	(10%FBS)	Tokushima Univeraity	
small airway epithelial cells						
SAEC			SAGM		Cambrex Bio Science Inc.	
human bronchial cell line						
BEAS2B			RPMI-	1640	(10%FBS)	ATCC(CRL-9609)

## (3) Semiquantitative RT-PCR analysis

**[0258]** Total RNA was extracted from cultured cells and clinical tissues using Trizol reagent (Life Technologies, Inc.) according to the manufacturer's protocol. Extracted RNAs and normal human tissue poly(A) RNAs were treated with DNase I (Nippon Gene) and reverse-transcribed using oligo(dT) primer and SuperScript II reverse transcriptase (Invit-

rogen). Semiquantitative RT-PCR experiments were carried out with the following synthesized gene-specific primers or with beta-actin (ACTB)-specific primers as an internal control:

KOC1, 5'-TAAATGGCTTCAGGAGACTTCAG-3' (SEQ.ID.NO.7) and  
5'-GGTTTTAAATGCAGCTCCTATGTG-3' (SEQ.ID.NO.8);  
KIF11, 5'-CTGAACAGTGGGTATCTTCCTTA-3' (SEQ.ID.NO.9) and  
5'-GATGGCTCTTGACTTAGAGGTTTC-3' (SEQ.ID.NO.10);  
NMU, 5'-TGAAGAGATTCAGAGTGGACGA-3' (SEQ.ID.NO.11) and  
5'-ACTGAGAACATTGACAACACAGG-3' (SEQ.ID.NO.12);  
NMU1R, 5'-AAGAGGGACAGGGACAAGTAGT-3' (SEQ.ID.NO.13) and  
5'-ATGCCACTGTTACTGCTTCAG-3' (SEQ.ID.NO.14);  
NMU2R, 5'-GGCTCTTACAACCTCATGTACCCA-3' (SEQ.ID.NO.15) and  
5'-TGATACAGAGACATGAAGTGAGCA-3' (SEQ.ID.NO.16);  
GHSR1a, 5'-TGGTGTTCCTTCATCCT-3' (SEQ.ID.NO.17) and  
5'-GAATCCCAGAAGTCTGAACA-3' (SEQ.ID.NO.18);  
GHSR1b, 5'-ACGGTCCTCTACAGTCTCA-3' (SEQ.ID.NO.19) and  
5'-CACAGGGAGAGGATAGGA-3' (SEQ.ID.NO.20);  
NTSR1, 5'-AGTGGGCTCAGAGTCTAGCAAAT-3' (SEQ.ID.NO.21) and  
5'-TATTGAGAGATACACGGGGTTTG-3' (SEQ.ID.NO.22);  
GHRL, 5'-TGAGCCCTGAACACCAGAGAG-3' (SEQ.ID.NO.23) and  
5'-AAAGCCAGATGAGCGCTTCTA-3' (SEQ.ID.NO.24);  
NTS, 5'-TCTTCAGCATGATGTGTGTGT-3' (SEQ.ID.NO.25) and  
5'-TGAGAGATTCATGAGGAAGCTTG-3' (SEQ.ID.NO.26);  
ACTB, 5'-GAGGTGATAGCATTGCTTTTCG-3' (SEQ.ID.NO.27) and  
5'-CAAGTCAGTGACAGGTAAGC-3' (SEQ.ID.NO.28).

**[0259]** PCR reactions were optimized for the number of cycles to ensure product intensity within the logarithmic phase of amplification.

#### Quantitative real-time RT-PCR (QRT-PCR) analysis and northern-blot analyses

**[0260]** Expression levels of the *KOC1* and *KIF11* genes were measured by QRT-PCR using the ABI Prism 7700 sequence detection system (Applied Biosystems). Total RNA was extracted from cultured cells and clinical tissues using Trizol reagent (Life Technologies, Inc.) according to the manufacturer's protocol. Extracted RNAs and normal human tissue poly(A) RNAs were treated with DNase I (Nippon Gene) and were reverse-transcribed using oligo (dT) primer and SuperScript II reverse transcriptase (Invitrogen). The TaqMan Pre-Developed Assay Human ACTB (Applied Biosystems; #4333762F) was used for *ACTB* gene as a quantitative control. A primer pair and a TaqMan probe for each gene were designed by using Primer Express software as follows:

*KOC1*, 5'-ACGAACCTCATTTGCTCACTCCTT-3' (sense) (SEQ.ID.NO.98),  
5'-ACCCACACCCAACACAATTGT-3' (antisense) (SEQ.ID.NO.99),  
5'-ACAGCAAAGCCC-3' (TaqMan-MGB probe) (SEQ.ID.NO.100);  
*KIF11*, 5'-TTCACCCTGACAGAGTTCACAAA-3' (sense) (SEQ.ID.NO.101)  
5'-GGGTGGTCTCCATAATAGCAA-3' (antisense) (SEQ.ID.NO.102),  
5'-AGCCCACTTTAGAGTATAC-3' (TaqMan-MGB probe) (SEQ.ID.NO.103).

**[0261]** PCR for each gene and the *ACTB* gene was performed in a single tube in duplicate. Results were expressed as the average of these two independent tests.

#### (4) Northern-blot analysis

**[0262]** Human multiple-tissue blots (BD Biosciences Clontech) were hybridized with <sup>32</sup>P-labeled PCR products of *KOC1*, *KIF11* and *GHSR1*. cDNA probes of *KOC1*, *KIF11* and *GHSR1* were prepared by RT-PCR using primers similarly

as above. Prehybridization, hybridization, and washing were performed according to the supplier's recommendations. The blots were autoradiographed with intensifying BAS screens (BIO-RAD) at room temperature (RT) for 30 to 168 hours.

#### Generation of anti-KOC1 and -KIF11 antibodies

**[0263]** Plasmids expressing KOC1 (full-length) and KIF11 (partial amino acid sequence corresponding to codons 361-1056), each containing His-tagged epitope at the N-terminal, were prepared using pET28 vector (Novagen). Recombinant proteins were expressed in *Escherichia coli* BL21 codon-plus strain (Stratagene), purified using TALON resin (BD Biosciences Clontech) according to the supplier's protocol, and inoculated into rabbits. The immune sera were purified on affinity columns according to standard methodology. Affinity-purified anti-KOC1 and anti-KIF11 antibodies were used for western-blot analysis, immunoprecipitation, and immunostaining. We confirmed by western-blot analysis that anti-KOC1 antibody are specific to KOC1 and do not cross-react with other homologous proteins, IMP-1 and IMP-2 using lysates of NCI-H520 cells, which expressed neither of endogenous IMP-1, -2, and -3, but had been transfected with HA-tagged IMP-1, -2, and -3 expression vector.

#### Construction of KOC1 deletion mutants and immunoprecipitation assays for identification of the KOC1-KIF11 binding region

**[0264]** KOC1 and several of its domains (Fig. 3a) were cloned into appropriate sites of N-terminal FLAG-tagged and C-terminal HA-tagged pCAGGS vector. COS-7 cells transfected only with an KOC1 deletion mutant, were immunoprecipitated with anti-HA agarose (SIGMA). Endogenous KIF11 bands were detected with affinity-purified anti-KIF11 antibody by western blotting.

Table3 Primer sequence for construction of deletion mutant by RT-PCR

	F	SEQ ID NO.	R	SEQ ID NO.
full length	5'- ATGAACAACTGTATATCGG- 3'	69	5'-CTTCCGTCTTGACTGAGG-3'	70
KOC1 DEL1	5'- ATGAACAACTGTATATCGG- 3'	71	5'-ATGAGCTTCAAGTTTCACC- 3'	72
KOC1 DEL2	5'- ATGAACAACTGTATATCGG- 3'	73	5'-CTCCGTTTCTGATTGCTC-3'	74
KOC1 DEL3	5'- ATGAACAACTGTATATCGG- 3'	75	5'- AGGCAAATCACATGGTTTCTG- 3'	76
KOC1 DEL4	5'-TTGCCTCTGCGCCTGCTG- 3'	77	5'-CTTCCGTCTTGACTGAGG-3'	78
KOC1 DEL5	5'-TTGCCTCTGCGCCTGCTG- 3'	79	5'-CTCCGTTTCTGATTGCTC-3'	80

#### RNA-immunoprecipitation and cDNA microarray screening for identification of KOC1-associated mRNAs

**[0265]** We adopted the RNA immunoprecipitation protocol of Niranjana Kumari *et al.* (Niranjana Kumari, S. *et al.* Methods 26, 182-190 (2002)) to analyze RNA-protein interactions involving KOC1 *in vivo*. Immunoprecipitated RNAs were isolated from five lung-cancer cell lines (A549, LC319, PC14, RERF-LC-AI, and SK-MES-1). A 2.5-μg aliquot of T7-based amplified RNAs (aRNAs) from each immunoprecipitated RNA (IP-RNA) and from the total RNA (control) were reversely transcribed in the presence of Cy5-dCTP and Cy3-dCTP respectively as described previously (Kikuchi, T. *et al.* Oncogene 22, 2192-2205 (2003)), for hybridization to a cDNA microarray representing 32,256 genes (IP-microarray analysis). To confirm the binding to KOC1 of the mRNAs identified by IP-microarray analysis, we carried out RT-PCR experiments using gene-specific primers and RNAs from NSCLC cell extracts immunoprecipitated with anti-KOC1 antibody (IP-RT PCR). To confirm the region of KOC1 required for binding to the KOC1-associated mRNAs, we also carried out north-western blot analysis as below and IP-RT-PCR of KOC1-associated mRNAs from these immunoprecipitated extracts

transfected with various KOC1 deletion mutants.

Table4 Primer sequence for IP-RT-PCR

	F	SEQ ID NO.	R	SEQ ID NO.
CCT2	5'-TTATCCTGAACAGCTCT TTGGTG-3'	81	5'-AAGCGAAGGTCAGCTAAATA TCC-3'	82
SBP2	5'-CTTTCTGAGCACACTAC GGATCT-3'	83	5'-AAGCCCTCTTACTTACAGGG AAA-3'	84
SLC25A3	5'-GGTTCCCCTGGATTAG TGAA-3'	85	5'-CAACAGTAAATCTGAAACTC TTGCC-3'	86
RAB35	5'-GACAAAGGTAGCAAGA GGATTC-3'	87	5'-CTGGTGTTAAACTCGGTTCT TC-3'	88
PSMB7	5'-CTAGTGAGTGAGGCTAT TGCAGC-3'	89	5'-GTCTCTTCTAGCACCTCAAT CTCC-3'	90
GL	5'-ATCTGACTTTCTGTCCA CTGCAT-3'	91	5'-TAATTCAGCATAAGCCAAAG CC-3'	92
PKP4	5'-ACACAGTATGGACTGAA ATCGAC-3'	93	5'-CACCTCAATCTGAACAAGGT TAG-3'	94
WINS1	5'-GGCCTCTCAAAGTCTGG TAGATT-3'	95	5'-ATATTCCCACTTCAGAGACG ACA-3'	96

#### Northwestern blot analysis

[0266] Immunoprecipitated extracts from cells transfected with the KOC1 deletion mutants ( $\mu$ M) were boiled in 2x SDS-sample buffer, electrophoresed through 10-20% gradient polyacrylamide gels (BIO-RAD) and transferred to a polyvinylidene difluoride membrane (Hybond-P). The membrane was then blocked for 1 hour at room temperature in blocking buffer (10 mM Tris-HCl (pH 7.8), 150 mM NaCl, 1 mg/ml yeast tRNA), and washed twice with 50 ml of 10 mM Tris-HCl (pH 7.8) for 5 min and incubated with DIG-labeled RNA probe in 5 ml of NWB buffer (10 mM Tris-HCl (pH 7.8), 1 mM EDTA, 50 mM NaCl, 0.02% Ficoll, 0.02% polyvinyl pyrrolidone, 0.02% BSA) for 2 hours at room temperature. The membrane was washed four times with NWB buffer and the RNA probe bound to the proteins was then detected using DIG nucleic acid detection kit (Roche) according to the supplier's protocol.

#### Living-cell imaging of KOC1 and KIF11 proteins and KOC1-associated RAB35 mRNA

[0267] Plasmids expressing ECFP-fused KOC1 (ECFP-KOC1) protein were prepared using pECFP-N1 vectors (BD Biosciences Clontech). Plasmids expressing EYFP-fused KIF11 (EYFP-KIF11) protein were also prepared, using pEYFP-N1 vectors (BD Biosciences Clontech). Time-lapse images of COS-7 cells transfected with plasmids expressing ECFP-KOC1 or EYFP-KIF11 proteins were captured for 5-15 hours by the Live Cell Imaging System (Power IX81, OLYMPUS) and a confocal microscope (TCS SP2-AOBS, Leica Microsystems; FV1000 FLUOVIEW, OLYMPUS).

[0268] *In vitro* transcription of linearized plasmids carrying the full-length cDNA sequence of an KOC1-associated gene, *RAB35*, was performed using DAVIS Lab's protocol (<http://www.ed.ac.uk/~ilan>). To generate fluorescent riboprobes for *in vivo* co-localization with KOC1, the plasmids were transcribed using the mCAP RNA capping kit (Stratagene) in the presence of Alexa Fluor 546-labeled UTP (Molecular Probes). We constructed plasmids expressing EGFP-fused

KOC1 (EGFP-KOC1) protein were prepared using pEGFP-N1 vectors (BD Biosciences Clontech). For live-cell imaging of co-localized EGFP-KOC1 and Alexa Fluor 546-labeled *RAB35* mRNA, COS-7 cells that had been transfected initially with pEGFP-KOC1 were additionally transfected 36 hours later with Alexa Fluor 546-labeled *RAB35* mRNA (3 µg per 3.5-cm culture dish) in the presence of RNase Inhibitor (TAKARA). The plasmid-DNA and RNA samples were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocols. The cells were washed twice with PBS, and fresh medium was added 90 min after transfection with the labeled mRNA. The cells were allowed to recover in the incubator (37°C, 5% CO<sub>2</sub>) for 30 min before live-cell imaging for 3-6 hours with a confocal microscope (FV1000 FLUOVIEW, OLYMPUS). To investigate the specific transport of mRNAs by KOC1-RNP complex from one cell to another cell, we prepared two different COS-7-derived cells; the COS-7 cells transfected with pEGFP-KOC1 and Alexa Fluor 546-labeled *RAB35* mRNA and the other, parental COS-7 cells simply labeled with CellTracker (Molecular Probes) according to the supplier's protocols. These two cell populations were mixed and co-cultured for 12 hours before live-cell imaging with confocal microscope for 6 hours.

**[0269]** To investigate the translation of the mRNA transported by KOC1-RNP complex in the recipient cells, we prepared two types of COS-7-derived cell; one type was COS-7 cells co-transfected with pCAGGS-FLAG tagged-KOC1 and -*KIF11*. After 24 hours culture, plasmid containing EGFP-fused *RAB35* full length mRNA were re-transfected into these cells. The other type was COS-7 cells simply labeled with CellTracker (blue). These two cell-types were mixed and co-cultured for 24 hours before live-cell imaging with video microscope for 12 hours. Synthesis of EGFP-tagged *RAB35* mRNAs and correspondind proteins in the CellTracker-stained recipient cells (blue) as well as on the ultrafine structure between the two cells was examined by *in situ* hybridization and and time-lapse video microscopy.

#### Fluorescent *In situ* hybridization

**[0270]** We carried out *in situ* hybridization with DIG-labeled probes complementary to *RAB35* or EGFP mRNA at 60°C for 16 hours. The DIG label was detected using NBT-BCIP, an alkaline phosphatase color substrate. Cells were washed, mounted and visualized on light microscope. Fixed cells were hybridized with a mixture of DIG-labeled complementary to *RAB35* mRNA for 16 hours in 50% formamide/ 2X SSC at 42°C. Cells were washed, mounted and visualized on confocal microscope.

#### (5) RNA interference assay

**[0271]** To prepare plasmid vector expressing short interfering RNA (siRNA), we amplified the genomic fragment of *H1RNA* gene containing its promoter region by PCR using a set of primers, 5'-TGGTAGCCAAGTGCAGGTTATA-3' (SEQ ID No: 44), and 5'- CCAAAGGGTTTCTGCAGTTTCA-3' (SEQ ID No: 45) and human placental DNA as a template. The product was purified and cloned into pCR2.0 plasmid vector using a TA cloning kit according to the supplier's protocol (Invitrogen). The *Bam*HI and *Xho*I fragment containing *H1RNA* was into pcDNA3.1 (+) between nucleotides 1257 and 56, and the fragment was amplified by PCR using

5'-TGCGGATCCAGAGCAGATTGTACTGAGAGT-3' (SEQ ID No: 46) and  
5'- CTCTATCTCGAGTGAGGCGGAAAGAACCA-3' (SEQ ID No: 47),

**[0272]** The ligated DNA became the template for PCR amplification with primers,

5'- TTTAAGCTTGAAGACCATTTTTGGAAAAAAAAAAAAAAAAAAAAAAC-3' (SEQ ID No: 48) and  
5'-TTTAAGCTTGAAGACATGGGAAAGAGTGGTCTCA-3' (SEQ ID No: 49).

**[0273]** The product was digested with *Hind*III and subsequently self-ligated to produce psiH1BX3.0 vector plasmid having a nucleotide sequence shown in SEQ ID NO: 50.

**[0274]** The DNA fragment encoding siRNA was inserted into the GAP at nucleotide 489-492 as indicated (-) in the following plasmid sequence (SEQ ID NO: 50).



GACGGATCGGGAGATCTCCCGATCCCCTATGGTGCACTCTCAGTACAATCTGCTCTGGATCCA  
CTAGTAACGGCCGCCAGTGTGCTGGAATTTCGGCTTGGTAGCCAAGTGCAGGTTATAGGGAGC  
TGAAGGGAAGGGGGTACAGTAGGTGGCATCGTTCCTTTCTGACTGCCCCCCCCCGCATGC  
CGTCCCGCGATATTGAGCTCCGAACCTCTCGCCCTGCCGCCGCCGGTGCTCCGTCGCCGCCG  
CGCCGCCATGGAATTCGAACGCTGACGTCATCAACCCGCTCCAAGGAATCGCGGGCCCACT  
GTCCTAGGCGGGAACACCCAGCGCGCGTGCGCCCTGGCAGGAAGATGGCTGTGAGGGAC  
AGGGGAGTGGCGCCCTGCAATATTTGCATGTCGCTATGTGTTCTGGGAAATCACCATAAACGT  
GAAATGTCTTTGGATTGGAATCTTATAAGTTCTGTATGAGACCACTCTTTCCC—TTTTTGG  
GAAAAAAAAAAAAAAAAAAAAACGAAACCGGGCCGGCGCGGTGGTTCACGCCTATAAT  
CCCAGCACTTTGGGAGGCCGAGGCGGGCGGATCACAAGGTCAGGAGGTCGAGACCATCCA  
GGCTAACACGGTGAAACCCCCCCCCATCTCTACTAAAAAAAAAAAAATACAAAAAATTAGCCA  
TTAGCCGGGCGTGGTGGCGGGCGCCTATAATCCAGCTACTTGGGAGGCTGAAGCAGAATG  
GCGTGAACCCGGGAGGCGGACGTTGCAGTGAGCCGAGATCGCGCCGACTGCATTCCAGCCT  
GGGCGACAGAGCGAGTCTCAAAAAAAAAAACCGAGTGGAATGTGAAAAGCTCCGTGAAACT  
GCAGAAACCCAAGCCGAATTCTGCAGATATCCATCACACTGGCGGCCGCTCGAGTGAGGCG  
GAAAGAACCAGCTGGGGCTCTAGGGGGTATCCCCACGCGCCCTGTAGCGGCGCATTAAGCG  
CGGCGGGTGTGGTGGTTACGCGCAGCGTGACCGCTACACTTGCCAGCGCCCTAGCGCCCGC  
TCCTTTTCGCTTTCTTCCCTTCCTTTCTCGCCACGTTCCGCCGCTTTCCCCGTCAAGCTCTAAAT  
CGGGGGCTCCCTTTAGGGTTCCGATTTAGTGCTTTACGGCACCTCGACCCCAAAAACTTGA

TTAGGGTGATGGTTCACGTAGTGGGCCATCGCCCTGATAGACGGTTTTTCGCCCTTTGACGTT  
 GGAGTCCACGTTCTTTAATAGTGGACTCTTGTTCCAACTGGAACAACACTCAACCCTATCT  
 5 CGGTCTATTCTTTTGATTATAAGGGATTTTGCCGATTTCCGGCTATTGGTTAAAAAATGAGCT  
 GATTTAACAAAAATTTAACGCGAATTAATTCTGTGGAATGTGTGTCAGTTAGGGTGTGGAAA  
 GTCCCCAGGCTCCCCAGCAGGCAGAAGTATGCAAAGCATGCATCTCAATTAGTCAGCAACCA  
 GGTGTGGAAAGTCCCCAGGCTCCCCAGCAGGCAGAAGTATGCAAAGCATGCATCTCAATTA  
 10 GTCAGCAACCATAGTCCCGCCCCCTAACTCCGCCCATCCCGCCCCCTAACTCCGCCCAGTTCCG  
 CCCATTCTCCGCCCATGGCTGACTAATTTTTTTTATTATGTCAGAGGCCGAGGCCGCCTCTG  
 CCTCTGAGCTATTCCAGAAGTAGTGAGGAGGCTTTTTTGGAGGCCTAGGCTTTTGCAAAAAG  
 CTCCCGGGAGCTTGTATATCCATTTTCGGATCTGATCAAGAGACAGGATGAGGATCGTTTCGC  
 ATGATTGAACAAGATGGATTGCACGCAGGTTCTCCGGCCGCTTGGGTGGAGAGGCTATTCCG  
 CTATGACTGGGCACAACAGACAATCGGCTGCTCTGATGCCGCCGTGTTCGGCTGTGACGCG  
 15 AGGGGCGCCCGGTTCTTTTTGTCAAGACCGACCTGTCCGGTGCCCTGAATGAACTGCAGGA  
 CGAGGCAGCGCGGCTATCGTGGCTGGCCACGACGGGCGTTTCTTGCGCAGCTGTGCTCGAC  
 GTTGTCACTGAAGCGGGAAGGGACTGGCTGCTATTGGGCGAAGTGCCGGGGCAGGATCTCC  
 TGTCACTCACCTTGCTCCTGCCGAGAAAGTATCCATCATGGCTGATGCAATGCGGCGGCTGC  
 ATACGCTTGATCCGGCTACCTGCCCATTCGACCACCAAGCGAAACATCGCATCGAGCGAGCA  
 20 CGTACTCGGATGGAAGCCGGTCTTGTCTGATCAGGATGATCTGGACGAAGAGCATCAGGGGCT  
 CGCGCCAGCCGAAGTGTTCGCCAGGCTCAAGGCGCGCATGCCCGACGGCGAGGATCTCGTC  
 GTGACCCATGGCGATGCCTGCTTGCCGAATATCATGGTGGAATAATGGCCGCTTTTCTGGATT  
 ATCGACTGTGGCCGGCTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGA  
 TATTGCTGAAGAGCTTGGCGGCGAATGGGCTGACCGCTTCTCGTGCTTTACGGTATCGCCG  
 25 CTCCCGATTGCGAGCGCATCGCCTTCTATCGCCTTCTTGACGAGTTCTTCTGAGCGGGACTCT  
 GGGGTTGCAAATGACCGACCAAGCGACGCCCAACCTGCCATCACGAGATTCGATTCCACC  
 GCCGCCTTCTATGAAAGGTTGGGCTTCGGAATCGTTTTCCGGGACGCCGGCTGGATGATCCT  
 CCAGCGCGGGGATCTCATGCTGGAGTTCTTCGCCACCCCAACTTGTTTATTGCAGCTTATAA  
 TGGTTACAAATAAAGCAATAGCATCACAAATTCACAAATAAAGCATTTTTTTCTACTGCATTC  
 30 TAGTTGTGGTTTGTCCAACTCATCAATGTATCTTATCATGTCTGTATACCGTCGACCTCTAGC  
 TAGAGCTTGGCGTAATCATGGTCATAGCTGTTTCTGTGTGAAATTGTTATCCGCTCACAATTC  
 CACACAACATACGAGCCGGAAGCATAAAGTGTAAGCCTGGGGTGCCTAATGAGTGAGCTA  
 ACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTCCAGTCGGGAAACCTGTCTGCCAGC  
 TGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCCTATTGGGCGCTCTTCCGCT  
 35 TCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCCGGCTGCGGCGAGCGGTATCAGCTCACTC  
 AAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCA  
 AAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCTTGGCTGGCGTTTTTCCATAGGC  
 TCCGCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGAC  
 AGGACTATAAAGATAACAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCTGTTCCGA  
 40 CCCTGCCGCTTACCGGATACCTGTCCGCCTTCTCCCTTCGGGAAGCGTGCGCTTCTCATA  
 GCTCACGCTGTAGGTATCTCAGTTCCGGTGTAGGTGCTTCGCTCCAAGCTGGGCTGTGTGCAC  
 GAACCCCCCGTTACGCCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCC  
 GGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAAACAGGATTAGCAGAGCGAGG  
 TATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGCCCTAACTACGGCTACACTAGAAGAAC  
 45 AGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTT  
 GATCCGGCAAACAAACCACCGCTGGTAGCGGTTTTTTTTGTTTGCAAGCAGCAGATTACGCGC  
 AGAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGA  
 CGAAAACTCACGTAAAGGGATTTTGGTCAAGATTATCAAAAAGGATCTTCACCTAGATCCT  
 TTTAAATTAATAAAGTATTAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGT  
 50 TACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCTGTTTATCCATAGTTG  
 CCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCT  
 GCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTATCAGCAATAAACCAGCCAGC  
 CGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATT  
 GTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATT

GCTACAGGCATCGTGGTGTACGCTCGTCGTTTGGTATGGCTTCATTAGCTCCGGTCCCAA  
 CGATCAAGGCGAGTTACATGATCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCC  
 TCCGATCGTTGTCAGAAGTAAGTTGGCCGAGTGTATCACTCATGGTTATGGCAGCACTGCA  
 TAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAA  
 GTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAA  
 TACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAA  
 AACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAAC  
 TGATCTTCAGCATCTTTTACTTTTACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAA  
 TGCCGCAAAAAGGGAATAAGGGCGACACGGAATGTTGAATACTCATACTCTTCCTTTTTC  
 AATATTATTGAAGCATTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTAG  
 AAAAATAAACAAATAGGGGTTCCGCGCACATTCCCCGAAAAGTGCCACCTGACGTC

**[0275]** Using 30µl of Lipofectamine 2000 (Invitrogen), 10µg of siRNA-expression vector were transfected into NSCLC cell lines, A549 and LC319, both endogenously over-expressing KOC1, KIF11, NMU, GHSR1b, NTSR1, RAB35, and FOXM1. More than 90% of the transfected cells expressed the synthetic siRNAs, and endogenous expression of target genes (KIF11, GHSR1b, NTSR1, RAB35, or FOXM1) in these cells was effectively suppressed. The transfected cells were cultured for five days in the presence of appropriate concentrations of geneticin (G418), and then, cell numbers and viability were measured by Giemsa staining and triplicate MTT assays. The target sequences of the synthetic oligonucleotides for RNAi were as follows: control 1 (EGFP: enhanced green fluorescent protein (EGFP) gene, a mutant of *Aequorea victoria* EGFP), 5'-GAAGCAGCAGCACTTCTTC-3' (SEQ.ID.NO.29); control 2 (Luciferase: *Photinus pyralis* luciferase gene), 5'-CGTACGCGGAATACTTCGA-3' (SEQ.ID.NO.30); control 3 (Scramble: chloroplast *Euglena gracilis* gene coding for 5S and 16S rRNAs), 5'-GCGCGCTTTGTAGGATTGCG-3' (SEQ.ID.NO.31); siRNA-KIF11-1 (#1), 5'-GTTAGTGTACGAAGTGGAG-3' (SEQ.ID.NO.32); siRNA-KIF11-2 (#2), 5'-GTGTCTCTGTTGGAGATCT-3' (SEQ.ID.NO.33); siRNA-KIF11-3 (#3), 5'-GAAGGCAGTTGACCAACAC-3' (SEQ.ID.NO.34); siRNA-GHSR-1 (si-GHSR-1), 5'-CCTCTACCTGTCCAGCATG-3' (SEQ.ID.NO.35); siRNA-NTSR1-1 (si-NTSR1-1), 5'-GTTTCATCAGCGCCATCTGG-3' (SEQ.ID.NO.36); siRNA-NTSR1-2 (si-NTSR1-2), 5'-GGTCGTCATACAGGTCAAC-3' (SEQ.ID.NO.37); siRNA-RAB35 (si-RAB35), 5'-GAGATGTTCAACTGCATCA-3' (SEQ.ID.NO.114); siRNA-FOXM1 (si-FOXM1), 5'-GCAGCA-GAAACGACCGAAT-3' (SEQ.ID.NO.108).

**[0276]** The oligonucleotides used for these siRNAs are shown below. Each constructs were prepared by cloning the following double-stranded oligonucleotide into the BbsI site in the psiH1BX3.0 vector. The corresponding nucleotide position relative to the KIF11, GHSR1b, NTSR1, RAB35 and FOXM1 nucleic acid sequence of SEQ ID NOs:1, 3, 5, 112, and 106 are listed for each oligonucleotide sequence. Each oligonucleotide is a combination of a sense nucleotide sequence and an antisense nucleotide sequence of the target sequence of KIF11, GHSR1b, NTSR1, RAB35 and FOXM1. The nucleotide sequences of the hairpin loop structure of each siRNAs are also shown below. (endonuclease recognition sites are eliminated from each hairpin loop structure sequence).

KIF11	si1 288-306 (for the target sequence of gttagtgtac gaactggag/ SEQ ID NO:32) (insert F) Tccc gttagtgtacgaactggag ttcaagaga ctccagttcgtactaac/SEQ ID NO:51 (insert R) Aaaa gttagtgtacgaactggag tctcttgaa ctccagttcgtactaac/SEQ ID NO:52 (hairpin) gttagtgtacgaactggag ttcaagaga ctccagttcgtactaac/SEQ ID NO:53
KIF11	si2 612-630 (for the target sequence of gtgtctctgt tggagatct/ SEQ ID NO:33) (insert F) Tccc gtgtctctgt tggagatct ttcaagaga agatctccaacagagacac/SEQ ID NO:54 (insert R) Aaaa gtgtctctgt tggagatct tctcttgaa agatctccaacagagacac/SEQ ID NO:55 (hairpin) gtgtctctgt tggagatct ttcaagaga agatctccaacagagacac/SEQ ID NO:56
KIF11	si3 1700-1718 (for the target sequence of gaaggcagtt gaccaacac/ SEQ ID NO:34) (insert F) Tccc gaaggcagtt gaccaacac ttcaagaga gtgttggtcaactgccttc/SEQ ID NO:57 (insert R) Aaaa gaaggcagtt gaccaacac tctcttgaa gtgttggtcaactgccttc/SEQ ID NO:58 (hairpin) gaaggcagtt gaccaacac ttcaagaga gtgttggtcaactgccttc/SEQ ID NO:59
GHSR1b	si1 237-255 (for the target sequence of cctctacctg tccagcatg/ SEQ ID NO:35) (insert F) Tccc cctctacctg tccagcatg ttcaagaga catgctggacaggtagagg/SEQ ID NO:60 (insert R) Aaaa cctctacctg tccagcatg tctcttgaa catgctggacaggtagagg/SEQ ID NO:61

(continued)

(hairpin) cctctacctg tccagcatg ttcaagaga catgctggacaggttagagg/SEQ ID NO:62

5	NTSR1	si1 933-951 (for the target sequence of gttcatcagc gccatctgg/ SEQ ID NO:36) (insert F) Tccc gttcatcagc gccatctgg ttcaagaga ccagatggcgctgatgaac/SEQ ID NO:63 (insert R) Aaaa gttcatcagc gccatctgg tctcttgaa ccagatggcgctgatgaac/SEQ ID NO:64 (hairpin) gttcatcagc gccatctgg ttcaagaga ccagatggcgctgatgaac/SEQ ID NO:65
10	NTSR1	si2 1074-1092 (for the target sequence of ggctgcata caggtaac/ SEQ ID NO:37) (insert F) Tccc ggctgcata caggtaac ttcaagaga gttgacctgtatgacgacc/SEQ ID NO:66 (insert R) Aaaa ggctgcata caggtaac tctcttgaa gttgacctgtatgacgacc/SEQ ID NO:67 (hairpin) ggctgcata caggtaac ttcaagaga gttgacctgtatgacgacc/SEQ ID NO:68
15	RAB35	si 620-638 (for the target sequence of gagatgttca actgcatca/ SEQ ID NO:114) (insert F) Tccc gagatgttca actgcatca ttcaagaga tgatgcagt tgaacatctc/SEQ ID NO:115 (insert R) Aaaa gagatgttca actgcatca tctcttgaa tgatgcagt tgaacatctc /SEQ ID NO:116 (hairpin) gagatgttca actgcatca ttcaagaga tgatgcagt tgaacatctc /SEQ ID NO:117
20	FOXO1	si 1240-1258 (for the target sequence of gcagcagaaaegaccgaatt SEQ ID NO: 108) (insert F) Tccc gcagcagaaa cgaccgaat ttcaagaga attcggtcg ttctgtctgc/SEQ ID NO:109 (insert R) Aaaa gcagcagaaa cgaccgaat tctcttgaa attcggtcg ttctgtctgc/SEQ ID NO:110 (hairpin) gcagcagaaa cgaccgaat ttcaagaga attcggtcg ttctgtctgc /SEQ ID NO:111.
25		

**[0277]** To validate RNAi system of the present disclosure, individual control siRNAs (EGFP, Luciferase, and Scramble) were initially confirmed using semiquantitative RT-PCR to decrease the expression of the corresponding target genes that had been transiently transfected into COS-7 cells. Down-regulation of KIF11, GHSR1b, NTSR1, RAB35 and FOXO1 expression by their respective siRNAs (si-KIF11-1, si-KIF11-2, si-KIF11-3, si-GHSR-1, si-NTSR1-1, si-NTSR1-2, si-RAB35 and si-F/FOXO1), but not by controls, was confirmed with semiquantitative RT-PCR in the cell lines used for this assay.

#### Dominant-negative assays

**[0278]** We performed dominant-negative assays using the KOC1 deletion mutants to investigate the functional role of the KOC1-KIF11 complex in growth or survival of lung-cancer cells. The KOC1DEL3 and KOC1DEL2 construct (Fig. 3 a; 10  $\mu$ g), mock plasmid (10  $\mu$ g), or plasmid mixtures of both constructs in the final dose of 10- $\mu$ g DNA (KOC1DEL3 or KOC1DEL2 vs mock ( $\mu$ g), 7.5 : 2.5; 5 : 5; or 2.5 : 7.5, individually) were transfected into LC319 cells. The transfected cells were cultured for 7 days in the presence of G418 and their viability was measured by triplicate MTT assays.

#### (6) Co-immunoprecipitation and MALDI-TOF mass spectrometry

**[0279]** Human lung cancer cell line LC319 cells were transfected with bilateral-tagged pCAGGS-n3FH (NH<sub>2</sub>-terminal FLAG, COOH-terminal HA)-KOC1 expression vector or empty vector (mock transfection). Cells were extracted in IP-buffer (0.5% NP-40, 50 mM Tris-HCl, 150 mM NaCl, and protease inhibitor) for 30 min on ice. Extracts were centrifuged at 14,000 rpm for 15 min, and supernatants were subjected to immunoprecipitation using anti-Flag M2 agarose (Sigma-Aldrich) and anti-HA beads (Sigma-Aldrich) for 1-2 hours. The beads were washed six times with IP-buffer, and protein was eluted by boiling the beads in Laemmli sample buffer after removing the final wash fraction. The eluted protein was resolved by SDS-PAGE and stained with silver staining. A 125 kDa-band was extracted by gel extraction, and used for mass spectrometric sequencing using MALDI-TOF mass spectrometry. This analysis identified KIF11 as the 125 kDa product

**[0280]** To confirm the interaction between KOC1 and KIF11, A549 cells were transiently co-transfected with Flag-tagged KIF 11 and myc-tagged KOC1 and the cells were immunoprecipitated with anti-Flag M2 agarose. Subsequently, the cells were immunoblotted with anti-myc antibody (9E10; Santa Cruz). Next, using the same combination of vectors and cells, the cells were immunoprecipitated with anti-myc agarose (SIGMA) and immunoblotted with anti-Flag M2 antibody (Sigma-Aldrich).

**[0281]** To further confirm this interaction, A549 cells were transiently co-transfected with Flag-tagged KIF11 and myc-

tagged KOC1, and co-localization of FITC-labeled KIF11 and rhodamine-labeled KOC1 mainly in the cytoplasm was detected by immunocytochemical staining using FITC-labeled anti-FLAG antibody and rhodamine-labeled anti-myc antibody, as described below.

#### (7) Immunocytochemistry

**[0282]** A549 cells grown on coverslips were cultured for 24 hours after passage, and were co-transfected with Flag-tagged KIF 11 and myc-tagged KOC1. After 24-hours incubation, the cells were fixed with acetone/methanol (1:1) for 5 min on ice, blocked in CAS BLOCK (ZYMED) for 7 min at RT, and then incubated with rabbit anti-Flag polyclonal antibody (SIGMA) for 1 hour at RT. The fixed cells were washed 3 times with PBS, reacted with anti-rabbit IgG-FITC for 1 hour at RT. Then the cells were blocked again, and incubated with anti-myc antibody (9E10; Santa Cruz) for 1 hour at RT. Finally anti-mouse IgG-rhodamin was applied to the cells for 1 hour at RT. The cells were viewed on a Leica TCS SP2-AOBS confocal microscope.

#### Immunohistochemistry and tissue-microarray analysis

**[0283]** Tumor-tissue microarrays using formalin-fixed NSCLCs were constructed as published elsewhere (Kononen, J. et al., Nat. Med. 4, 844-847 (1998); Sauter, G. et al., Nat. Rev. Drug Discov. 2, 962-972 (2003)). KOC1 and KIF11 positivity were assessed semi-quantitatively as absent or positive according to staining intensity, by three independent investigators with no prior knowledge of clinical follow-up data.

#### (8) Ligand-receptor binding assay

**[0284]** To identify direct binding of NMU-25 to its candidate receptors, GHSR1a, GHSR1b and NTSR1, the following experiments were performed. The entire coding region of each receptor gene was amplified by RT-PCR using primers

GHSR1a (5'-GGAATTCATGTGGAACGCGACGCCAGCGAA-3' (SEQ.ID.NO.38) and 5'-CGCGGATCCGCGTGTATTAATACTAGATTCTGTCCAGGCC-3'(SEQ.ID.NO.39)),  
GHSR1b (5'-GGAATTCATGTGGAACGCGACGCCAGCGAA-3' (SEQ.ID.NO.40) and 5'-CGCGGATCCGCGGAGAGAAGGGAGAAGGCACAGGGA-3' (SEQ.ID.NO.41)), and  
NTSR1 (5'-GGAATTCATGCGCCTCAACAGCTCCGCGCCGGGAA-3' (SEQ.ID.NO.42) and 5'-CGCGGATCCGCGGTACAGCGTCTCGCGGGTGGCATTGCT-3' (SEQ.ID.NO.43)).

**[0285]** The products were digested with *Eco*R1 and *Bam*H1 and cloned into appropriate sites of p3XFLAG-CMV10 vector (Sigma-Aldrich Co.). COS-7 cells were transfected with GHSR1b or NTSR1 expression plasmids using FuGENE6, as described above. Transfected COS-7 cells were cultured with 0.5  $\mu$ M rhodamine-labeled NMU-25 peptide (NMU-25-rhodamine: Phoenix Pharmaceuticals. Inc.) for 12 hours, washed five times in PBS(-), and fixed in 4% paraformaldehyde solution for 60 min at room temperature. Then the cells were incubated with antibodies to FLAG-tagged GHSR1a, GHSR1b, or NTSR1 proteins (Sigma-Aldrich Co.), stained with a goat anti-mouse secondary antibody conjugated to FITC (Cappel) and viewed under laser-confocal microscopy (TCS SP2 AOBS: Leica Microsystems). In addition, three different negative controls were prepared for this assay: 1) non-transfected COS-7 cells without addition of NMU-25-rhodamine; 2) non-transfected COS-7 cells treated with NMU-25-rhodamine; and 3) COS-7 cells transfected with GHSR1a, GHSR1b, or NTSR1 without NMU-25-rhodamine. COS-7 cells transfected with a known NMU receptor (NMU1R) served as a positive control for the assay.

**[0286]** To confirm the binding of NMU-25 to the candidate receptors, flow-cytometric analysis was performed using the same series of COS-7 cells. Specifically, COS-7 cells were plated at a density of 1 X 10<sup>5</sup> cells/100-mm dish and transfected with either GHSR1b, NTSR1, or NMU1R expression vectors; 24 hours after transfection, cells were incubated with 0.5  $\mu$ M NMU-25-rhodamine for 12 hours, washed, trypsinized, collected in PBS, and washed once more in PBS. The population of cells binding to rhodamine-labeled NMU-25 was determined by flow cytometry.

**[0287]** To further confirm binding of NMU-25 to the endogenous candidate receptors on the NSCLC cells, we performed receptor-ligand binding assay using the LC319 and PC-14 cells. Briefly, these cells trypsinized were seeded onto 96-well black-wall, clear-bottom microtiter plates 24 hours prior to the assay. The medium was removed and the cells were incubated with Cy5-NMU-25 with a 10-fold excess of unlabeled competitor. The plate was incubated in the dark for 24 hours at 37°C and was scanned on the 8200 Cellular Detection System (Applied Biosystems). 8200 Analysis Software creates a digitized gray scale image, quantitates the amount of fluorescence bound on the surface of each cell, and enumerates the fluorescent cells.

**Measurement of cAMP levels**

**[0288]** Trypsinized LC319 cells were seeded onto 96-well microtiter plate ( $5.0 \times 10^4$  cells) and cultured in 10% FCS (+) RPMI-1640 medium for 24 hours, and then medium was changed to serum free RPMI-1640 medium / 1 mM IBMX (isobutylmethylxanthine) for 20 min prior to assay. Cells were incubated with NMU-25 peptides for 20 min prior to measuring the cAMP level using the cAMP EIA System (Amersham Biosystems).

**Intracellular  $\text{Ca}^{2+}$  mobilization assay**

**[0289]** Trypsinized LC319 cells were seeded onto poly-D-lysine coated 384-well black-wall, clear-bottom microtiter plate ( $1.0 \times 10^4$  cells/ml) 24 hours prior to assay. Cells were loaded for 1 hour with 1 mM Fluo-4-AM fluorescent indicator dye in assay buffer (Hank's balanced salts solution, 20 mM HEPES, 2.5 mM probenecid), washed three times with assay buffer, and then returned to the incubator for 10 min before assay on a fluorometric imaging plate reader (FLIPR, Molecular Devices). Maximum change in fluorescence over base line was used to determine the response of the cells to the NMU-25 peptides stimulation.

**Identification of downstream genes of NMU by cDNA microarray**

**[0290]** LC319 cells were transfected with either siRNA against NMU (si-NMU) or Luciferase (control siRNA). mRNAs were extracted 12, 24, and 36 hours after transfection, labelled with Cy5 or Cy3 dye and subjected to co-hybridization onto cDNA microarray slides containing 32,256 genes as described (Kakiuchi, S., et al., (2004). Hum. Mol. Genet. 13, 3029-3043., Ochi, K. et al., (2004). Int. J. Oncol. 24, 647-655.). After normalization of the data, genes with signals higher than the cut-off value were analyzed further. Genes whose intensity were significantly decreased in accordance with the time-dependent reduction of NMU expression were initially selected using SOM cluster analysis. Validation of candidate downstream genes of NMU was performed using semiquantitative RT-PCR experiments of the same mRNAs from LC319 cells used for microarray hybridization, with gene-specific primers listed below.

FLJ42024 (5'-AAAAAGGGGATGCCTAGAACTC-3'(SEQ.ID.NO.118) and  
5'-CTTTCAGCACGTCAAGGACAT-3' (SEQ.ID.NO.119)),  
GCDH (5'-ACACCTACGAAGGTACACATGAC-3' (SEQ.ID.NO.120) and  
(5'-GCTATTTTCAGGGTAAATGGAGTC-3' (SEQ.ID.NO.121)),  
CDK5RAP1 (5'-CAGAGATGGAGGATGTCAATAAC-3' (SEQ.ID.NO.122) and  
(5'-CATAGCAGCTTTAAAGAGACACG-3'(SEQ.ID.NO.123)),  
LOC134145 (5'-CCACCATAACAGTGGAGTGGG-3' (SEQ.ID.NO.124)  
(5'-CAGTTACAGGTGTATGACTGGGAG-3'(SEQ.ID.NO.125)),  
NUP188 (5'-CTGAATACAACCTTCCTGTTTGCC-3'(SEQ.ID.NO.126) and  
(5'-GACCACAGAATTACCAAACTGC-3'(SEQ.ID.NO.127)).

**[0291]** Expression of the candidate genes was additionally detected by semiquantitative RT-PCR using mRNAs isolated at 72 and 96 hours from LC319 cells treated with 1  $\mu\text{M}$  NMU-25 or BSA at the time point of 0 and 48 hours.

**Results****(1) Identification of KIF11 as a protein interacting with KOC1**

**[0292]** LC319 cells transfected with pCAGGS- n3FH-KOC1 vector were extracted and immunoprecipitated with anti-Flag M2 monoclonal antibody, and subsequently immunoprecipitated with anti-HA monoclonal antibody. The protein complex including KOC1 was stained with silver staining on SDS-PAGE gel. A 125 kDa band that was absent in mock transfection was extracted and determined to be KIF11 (NMU\_004523; SEQ.ID.NO.1) by Mass spectrometric sequencing.

**(2) Confirmation of interaction between KOC1 and KIF11**

**[0293]** The A549 cells co-transfected with Flag-tagged KIF11 and myc-tagged KOC1, the cells transfected with either KIF11 or KOC1, and the non-transfected cells were immunoprecipitated with anti-Flag M2 agarose and subsequently immunoblotted with anti-myc antibody. In contrast, the same series of A549 cells were immunoprecipitated with anti-myc agarose and immunoblotted with anti-Flag M2 antibody. A single band was found only when both constructs were co-transfected (Fig. 1a). Immunocytochemistry showed that FLAG-tagged FITC-labeled KIF 11 co-localized in cytoplasm

of A549 with myc-tagged rhomamine-labeled KOC1 (Fig. 1b).

[0294] Next we confirmed by western blot analysis that anti-KOC1 antibody are specific to KOC1 and do not cross-react with other homologous proteins, IMP-1 and IMP-2 using H520 cell lysate, which had been confirmed to be not expressed endogenous IMP-1, -2, and -3(KOC1), but had been transfected with HA-tagged IMP-1, -2, and -3(KOC1) expression vector. Lysates of LC319 cells transfected with pCAGGS- FLAG-tagged-KOC1 vector or mock vector (control) were extracted and immunoprecipitated with anti-FLAG M2 monoclonal antibody. The protein complex including KOC1 was stained with SilverQuest (Invitrogen) on an SDS-PAGE gel. A 125-kDa band was detected specifically in immunoprecipitates from lysates of cells transfected with KOC1 expressing plasmids, but not in control lysates (mock plasmids). Subsequent MALDI-TOF mass spectrometric analysis identified this 125-kDa protein as KIF 11, a member of the kinesin family. We confirmed direct interaction between endogenous KOC1 and KIF11 by immunoprecipitation experiments with extracts from A549 and LC319, using affinity-purified anti-KOC1 and anti-KIF11 polyclonal antibodies (Fig. 1c).

### (3) KIF11 expression in NSCLC

[0295] Validation of KIF11 expression was performed in primary NSCLCs (clinical samples) and lung cancer cell lines. Increased KIF11 expression was confirmed in 12 of 16 NSCLC cases (5 of 8 ADCs and in 7 of 8 SCCs. In addition, up-regulation of KIF1 were observed in 14 of the 15 NSCLC cell lines.

[0296] We subsequently re-examined primary NSCLC tissues and lung-cancer cell lines, and found increased *KIF11* expression in 18 NSCLC clinical samples as well as in all of the 20 NSCLC or SCLC cell lines examined by quantitative RT-PCR (Fig. 2a,b). The mRNA levels of the *KOC1* and *KIF11* genes relative to *ACTB* genes were significantly correlated ( $r = 0.702$ ,  $P = 0.0029$  by the Spearman rank correlation). These two genes were coactivated in almost lung cancer cell lines ( $r = 0.458$ ,  $P = 0.0359$  by the Spearman rank correlation).

### (4) KIF11 expression in normal human tissues

[0297] Northern blotting with KIF11 cDNA as a probe identified 4.5- and 5.5-kb transcripts as very weak bands, only seen in placenta, testis, and bone marrow, among the 23 normal human tissues examined. The reported cDNA sequence of *KIF11* was considered to correspond to the larger transcript. To investigate the transcript corresponding to the smaller band, we reversely transcribed mRNAs isolated from tissues of the testis and NSCLC cell lines. We amplified the entire sequence of *KIF11* cDNA by PCR using four primer sets, but found no alternatively-spliced transcript in these samples. Therefore, the smaller band may reflect cross-hybridization to the transcript of some related gene(s). The expression pattern of KIF11 in normal human tissues was significantly correlated with that of KOC1 (Fig. 2c).

### Identification of the KIF11-binding region in KOC1

[0298] To determine the specific domain of KOC1 required for interaction with KIF11, we transfected into COS-7 cells one of five deletion-constructs of KOC1 with NH<sub>2</sub> (N)-terminal FLAG- or COOH (C)-terminal HA-tagged sequences (KOC1DEL1-5; Fig. 3a). Immunoprecipitation with monoclonal anti-HA indicated that the KOC1DEL4 and KOC1DEL5 constructs, which both lacked two RNA-recognition motifs (RRMs), were unable to interact with endogenous KIF11, while all KOC1 constructs possessing the two RRMs retained binding affinity for KIF11 (Fig. 3b).

### Isolation of mRNAs associated with the KOC1-KIF11 complex using RNA-immunoprecipitation and cDNA microarray

[0299] KOC1 protein is known to exhibit multiple attachments to IGF2 leader-3 mRNA, possibly through its two functional RRMs and four K-homologous (KH) domains (Nielsen, J. et al., Mol. Cell Biol. 19, 1262-1270 (1999)). However, we did not detect expression of IGF2 mRNA in any of the lung-cancer cell lines or clinical NSCLC tissue samples we examined. Therefore, to elucidate the function of KOC1 in lung carcinogenesis, we searched for mRNA(s) that would interact with KOC1 and might thereby play important roles in growth and/or progression of lung cancer. First we immunoprecipitated mRNAs using anti-KOC1 antibody and five NSCLC cell lines (A549, LC319, PC14, RERF-LC-AI, and SK-MES-1). Then, Cy-5-labeled immunoprecipitated RNAs (IP-mRNA) and Cy-3-labeled total RNAs isolated from each matching cell line, were co-hybridized on human cDNA microarrays (IP-microarray). Among 32,256 genes screened, we identified a total of 55 that were enriched in IP-mRNA compared with total RNA in at least four of the five NSCLC cell lines tested (see Table2), and confirmed enrichment of all those candidates by RT-PCR using the IP-mRNAs as templates (IP-RT-PCR). To examine the specificity of RNA-immunoprecipitation, we performed RT-PCR experiments with beta-actin (ACTB) mRNA using IP-mRNA as template; no ACTB was precipitated by anti-KOC1 antibody. As background controls of RNA-immunoprecipitation, we precipitated mRNAs using normal rabbit IgG and five NSCLC cell lines, and confirmed that none of eight KOC1 associated mRNAs tested (CCT2, SBP2, SLC25A3, RAB35, PSMB7, GL,

PKP4, and WINS1) was precipitated by normal rabbit IgG. We also confirmed elevated expression of many of the candidate genes in NSCLC samples by RT-PCR (data not shown). To examine whether the KOC1-KIF11 complex formation requires the co-presence of these KOC1-associated mRNAs, we performed immunoprecipitation experiments using cell lysates which were treated or untreated *in vitro* with 30 units of RNase T1 (SIGMA), and found no difference in the interaction of the two proteins in the presence or absence of mRNAs, suggesting that the KOC1-KIF11 complex formation is unlikely to require these specific mRNAs.

**[0300]** By pursuing that strategy we have been able to show that *KOC1* and *KIF11* not only are co-over-expressed in the great majority of clinical NSCLC samples and cell lines, but also that a complex formed by the products of these genes is indispensable for growth and progression of NSCLC cells, by contributing to an intra- and inter-cellular mRNA-transporting system. Intracellular mRNA transport by RNA-binding proteins has been reported in oocytes and developing embryos of *Drosophila* and *Xenopus*, and in somatic cells such as fibroblasts and neurons (King, M.L. et al., *Bioessays* 21, 546-557 (1999); Mowry, K.L. & Cote, C.A. *Faseb. J.* 13, 435-445 (1999); Lasko, P., *J. Cell Biol.* 150, F51-56 (2000); Steward, O. *Neuron* 18, 9-12 (1997)) beta-actin mRNA is transported to the leading lamellae of chicken-embryo fibroblasts (CEFs) and to the growth cones of developing neurons (Lawrence, J.B. & Singer, R.H. *Cell* 45, 407-415 (1986); Bassell, G.J. et al., *J. Neurosci.* 18, 251-265 (1998)). The localization of *ACTB* mRNA depends on the "zipcode", a cis-acting element in the 3' UTR of the mRNA (Kislauskis, E.H. et al., *J. Cell Biol.* 123, 165-172 (1993)). The respective trans-acting factor, zipcode-binding protein 1 (ZBP1), was identified by affinity purification with the zipcode of *ACTB* mRNA; (Ross, A.F. et al., *Mol. Cell Biol.* 17, 2158-2165 (1997)) homologues of ZBP1 have since been identified in a wide range of organisms including *Xenopus*, *Drosophila*, mouse, and human (Mueller-Pillasch, F. et al., *Oncogene* 14, 2729-2733 (1997); Deshler, J.O. et al., *Science* 276, 1128-1131 (1997); Doyle, G.A. et al., *Nucleic Acids Res.* 26, 5036-5044 (1998)). ZBP1-like proteins contain two RRM domains in the N-terminal region and four hnRNP KH (ribonucleoprotein K-homology) domains at the C-terminal end. KOC1, one of the *IGF2* mRNA-binding proteins, is considered to be a member of the ZBP1 family; it exhibits multiple attachments to *IGF2* leader-3 mRNA (Nielsen, J. et al., *Mol. Cell Biol.* 19, 1262-1270 (1999)) and is over-expressed in several types of cancers (Mueller-Pillasch, F. et al., *Oncogene* 14, 2729-2733 (1997); Zhang, J.Y. et al., *Clin. Immunol.* 100, 149-156 (2001); Mueller, F. et al., *Br. J. Cancer* 88, 699-701 (2003); Wang, T. et al., *Br. J. Cancer* 88, 887-894 (2003)). However, since we failed to detect expression of *IGF2* leader-3 mRNA in most of the NSCLC cell lines or clinical samples we examined, we suspected that KOC1 could mediate growth of lung-cancer cells through interaction with, and transport of, other mRNA(s). When we undertook RNA-immunoprecipitation experiments coupled with cDNA microarrays (IP-microarray), we identified dozens of candidate mRNAs that were likely to be associated with KOC1 in NSCLC cells (see Table2). That list included genes encoding proteins with functions of cell-adhesion (PKP4, L1CAM1), cancer-cell progression and invasion (IGFBP2), and binding of small GTPs (RAB35), (Papagerakis, S. et al., *Hum. Pathol.* 34, 565-572 (2003); Fogel, M. et al., *Cancer Lett.* 189, 237-247 (2003); Wang, H. et al., *Cancer Res.* 63, 4315-4321 (2003); Zhao, H. et al., *Biochem. Biophys. Res. Commun.* 293, 1060-1065 (2002)) and many of them were expressed at high levels in clinical NSCLC samples (data not shown). Activation of a system that transports mRNAs whose products are associated with growth or movement of cells is very interesting, and further investigations along this line could lead to a better understanding of pulmonary carcinogenesis.

Table2

RANK <sup>(1)</sup>	GENE	ACCESSION	A549	LC319	PC14	RERF	SKMES1	SUM*
1	LOC283050	AA843724	6.0	7.7	5.4	6.4	9.2	34.7
2	KIAA0169	R49113	6.2	8.9	4.9	5.7	6.8	32.5
3	CCT2	AF026166	4.1	8.0	5.4	5.7	9.1	32.3
4	LOH11CR2A	NM_014622	9.1	5.6	5.8	3.9	4.5	28.8
5	SNTB2	AA625860	6.0	5.0	6.8	4.0	5.0	26.9
6	CFLAR	U97074	5.3	5.7	3.8	4.3	5.9	25.0
7	SBP2	AF380995	5.0	6.1	3.9	2.9	6.1	24.0
8	LOC56267	AA420728	4.8	5.4	5.4	2.5	5.0	23.1
9	SLC25A3	NM_002635	3.5	5.8	2.9	4.1	5.3	21.7
10	IFIT1	M24594	4.4	4.2	3.8	3.6	5.2	21.2
11	OSTalpha	H79642	2.3	5.9	5.6	3.8	2.8	20.4
12	FILIP1	XM_029179	10.3	2.3	2.1	2.7	2.5	19.9



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(continued)

	RANK <sup>(1)</sup>	GENE	ACCESSION	A549	LC319	PC14	RERF	SKMES1	SUM*
5	13	ZNF415	AY283600	3.5	5.2	3.3	4.0	3.8	19.8
	14	RAB35	BX344673; NM_006861	3.0	4.1	4.2	4.0	4.6	19.8
	15	APG-1	AW966019	0.0	6.2	6.2	2.5	4.4	19.4
	16	INPP4B	AA759168	3.4	3.2	4.4	3.6	4.5	19.1
10	17	na	AI160370	3.6	4.1	2.9	4.6	3.1	18.3
	18	N33	NM_006765	4.5	0.0	4.0	4.7	5.0	18.1
	19	RPS3A	BX343424	1.6	4.5	3.2	3.1	4.3	16.8
15	20	PSMB7	BM837906	4.0	4.2	2.2	3.1	2.9	16.5
	21	GIT2	NM_057169	3.4	4.2	2.3	2.9	3.4	16.1
	22	GL	AJ420489	4.4	3.2	3.2	2.3	2.9	16.0
	23	SOS2	XM_043720	2.1	3.5	2.5	2.3	4.7	15.1
20	24	L1CAM	M77640	3.4	2.9	2.5	2.6	3.6	14.9
	25	BRUNOL4	BM671360	2.8	3.8	1.7	2.5	4.1	14.9
	26	RRAGA	U41654	2.9	4.3	2.4	2.4	2.8	14.8
25	27	IGFBP2	BC004312	3.9	3.7	2.0	2.5	2.6	14.8
	28	SRPK1	BC038292	3.3	2.5	2.8	2.8	3.4	14.8
	29	FLJ12649	R41135	1.2	2.8	2.6	3.4	4.5	14.4
	30	AGL	NM_000028	4.0	2.8	2.6	2.5	2.4	14.3
30	31	FLJ23468	BX355581	3.0	3.4	3.0	2.1	2.4	13.9
	32	MGC4730	BM665147	2.6	2.8	2.6	2.3	3.1	13.4
	33	GAB2	NM_012296	3.7	3.4	1.3	2.8	2.2	13.4
35	34	USP15	AF106069	2.0	3.0	2.4	2.6	3.2	13.1
	35	KIAA0657	AB014557	0.0	4.7	2.2	3.0	3.1	13.1
	36	C6orf134	AI146643	3.2	0.0	2.5	2.7	4.5	12.8
	37	MSCP	AK093931	2.5	3.0	4.2	3.0	0.0	12.7
40	38	ACAA2	D16294	2.1	2.0	3.0	2.6	3.1	12.7
	39	PKP4	AI681111	3.2	2.5	2.9	1.7	2.3	12.6
	40	RGS5	BX537427	2.0	3.0	1.3	3.4	2.8	12.5
45	41	CYFIP1	BC005097	2.2	2.1	2.6	1.3	4.0	12.2
	42	PLAGL2	AK026951	1.2	2.7	2.3	2.4	3.3	11.9
	43	EHD4	AW779971	2.7	2.3	2.3	1.9	2.6	11.9
	44	KIAA1666	XM_300791	2.1	2.9	2.4	2.3	2.2	11.9
50	45	RAP80	BX537376	2.4	2.1	3.0	1.8	2.5	11.7
	46	LOC118812	BG537484	0.0	2.3	2.3	3.2	3.7	11.5
	47	UTX	AF000993	1.0	2.2	2.8	3.2	2.2	11.4
55	48	PCBP3	AK094301	2.4	2.9	1.3	2.3	2.5	11.4
	49	AP3S2	BC002785	2.4	2.3	1.2	2.8	2.3	11.0
	50	WINS1	AA741459	1.4	3.0	2.2	2.1	2.2	10.9

(continued)

RANK <sup>(1)</sup>	GENE	ACCESSION	A549	LC319	PC14	RERF	SKMES1	SUM*
51	na <sup>(2)</sup>	AF504647	0.8	2.0	2.1	2.1	3.6	10.7
52	LOC203859	AL832374	2.0	2.6	2.5	3.4	0.0	10.5
53	HNMT	NM_006895	2.3	2.0	1.9	2.1	2.2	10.5
54	LOC282965	XM_210833	1.1	2.8	2.0	2.2	2.0	10.1
55	PDK2	AK055119	1.0	2.4	2.2	2.4	2.0	10.0
N/C <sup>(3)</sup>	ACTB	BC053988	0.0	0.0	0.0	0.0	0.0	0.0
(1) Probe sets are ranked by the sum(*) of the fold change value (IP-mRNA / input RNA) of all five cell lines.								
(2) na : not annotated								
(3) N/C: negative control								

#### Identification of the mRNA-binding region in KOC1

**[0301]** To determine the region of KOC1 that is required for binding to KOC1-associated mRNAs, we performed northwestern blot analysis using immunoprecipitated recombinant proteins of KOC1 deletion-mutants expressed in A549 cells (Fig. 4a) and DIG-labeled *RAB35* mRNA, which is one of the KOC1 associated mRNAs. The KOC1DEL3, which lacked four KH domains, and KOC1DEL5, which lacked N-terminal two RRM and C-terminal two KH domains, did not bind to the *RAB35* mRNA. On the other hand, the KOC1DEL4, which is a construct with only the four KH domains and the KOC1DEL2, a construct without C-terminal two KH domains showed very weak binding affinities for mRNAs compared to the full-length KOC1 construct (Fig. 4b), suggesting the importance of two RRM as well as of C-terminal two KH domains for binding to KOC1-associated mRNAs.

**[0302]** We further expressed five of the KOC1 deletion-mutants in A549 cells and performed immunoprecipitation experiments twice with the cell lysates, first with monoclonal anti-HA and then with monoclonal anti-FLAG M2 antibody. Using IP-mRNA, we examined the ability of each deleted-protein to bind to eight endogenous mRNAs (CCT2, SBP2, SLC25A3, *RAB35*, PSMB7, GL, PKP4, and WINS1) selected from the above list (see Table2). The results were completely concordant to that of northwestern blot analysis, independently confirming that both C-terminal two KH domains and two RRM in the N-terminal are indispensable for effective binding of KOC1 to mRNAs (Fig. 4c).

#### Microtubule dependent intra- and inter-cellular transport of an KOC1-KIF11 ribonucleoprotein complex and KOC1-associated mRNAs

**[0303]** To further investigate the functional roles of KOC1 and KIF11, we prepared plasmids designed to express ECFP-KOC1 (cyan) and EYFP-KIF11 (yellow). We then transfected the two plasmids together into COS-7 cells, and examined their localization using immunofluorescence video-microscopy and real-time confocal microscopy. Cells expressing both KOC1 and KIF11 protruded into the processes, and then connected with adjacent cells (data not shown). A more detailed observation of living cells found that the KOC1 had formed a complex with KIF11 (KOC1-KIF11 RNP complex; green particle) that was transported from one cell to another through an ultrafine structure connecting the two cells (Fig. 5a). Movement of the KOC1-KIF11 complex appeared to be unidirectional from one cell to another.

**[0304]** Furthermore, to examine whether KOC1-KIF11 complex could specifically transport KOC1-associated-mRNAs from one cell having a high level of KOC1-RNP complex to another having a lower level of the complex, we mixed and co-cultured two different cell populations; one is COS-7 cells that had been transfected with pEGFP-KOC1 (green) as well as Alexa Fluor 546-labeled full-length *RAB35* mRNA (red), and the other is parental COS-7 cells simply labeled with CellTracker (blue). We observed that not only KOC1 particles (green), but also RNP particles of KOC1-*RAB35* mRNA (yellow) were transferred through the ultrafine structure from the former cells to the latter ones (Fig. 5b). Using *in situ* hybridization on A549 cells in which both *KOC1* and *KIF11* were over-expressed, we further confirmed that the endogenous *RAB35* mRNA (green) localized on the ultrafine intercellular structures as well as in the cytoplasm (data not shown).

**[0305]** We also investigated the endogenous location of KOC1 and KIF11 particles on the ultrafine structure of microtubules bridging individual A549 cells by an immunocytochemical study, using affinity-purified anti-KOC1- or anti-KIF11 for primary antibody and Alexa594 -labeled anti-rabbit IgG for secondary antibody (Molecular Probe) and anti-alpha-tubulin-FITC monoclonal antibody. A549 cells treated with 10  $\mu$ M of the microtubule disrupting agent nocodazole (SIGMA) for four hours showed collapse and aggregation of endogenous KOC1 and KIF11, along with the depolymerization of microtubules in the cytoplasm. Moreover, no particle was found on the residual structure between the cells.

The result suggested the possibility of a microtubule-dependent transporting mechanism involving the KOC1-KIF11 complex. To further clarify the detailed mechanism by which the KOC1-KIF11 complex transports mRNAs in NSCLC cells, we have searched for other component(s) that might be interacting with KIF11. Immunoprecipitation with anti-KIF11 polyclonal antibody using a lysate of LC319 cells identified a 150-kDa protein, which was later determined to be a dynactin 1 (DCTN1; p150, glued homolog, Drosophila) by MALDI-TOF mass-spectrometric analysis. DCTN1 is the largest subunit of DCTN, which binds to the cytoplasmic motor-protein dynein and activates vesicle transport along microtubules (Holzbaur, E.L. & Tokito, M.K. Genomics 31, 398-399 (1996); Tokito, M.K. et al., Mol. Biol. Cell 7, 1167-1180 (1996)), or binds to KIF11 to probably participate in centrosome separation (Blangy, A. et al., J. Biol. Chem. 272, 19418-19424 (1997)). We observed endogenous co-localization of KOC1/KIF11 and DCTN1 on the ultrafine structure between the individual A549 cells by immunocytochemistry, using the combination of affinity-purified anti-KOC1- or anti-KIF11- polyclonal antibodies for primary antibody and Alexa488-labeled anti-rabbit IgG for secondary antibody, and the combination of anti-DCTN1 monoclonal antibody (BD transduction Laboratories, #610473) for primary antibody and anti-Alexa594-labeled anti-rabbit IgG for secondary antibody. And we confirmed direct interaction between endogenous KIF11 and DCTN1 by immunoprecipitation experiments with extracts from A549 and LC319 cells, using anti-KIF11 polyclonal antibody and anti-DCTN1 monoclonal antibody (BD transduction Laboratories, #610473) (Fig. 6a).

**[0306]** To further demonstrate the KIF11-dependent intercellular transport of mRNA, we examined the effect of monastrol, the cell-permeable inhibitor that specifically inhibits the KIF11. Previous reports indicated that monastrol partially inhibits KIF11 ATPase activity through binding directly to the motor domain (DeBonis, S. et al., Biochemistry 42, 338-349 (2003); Kononen, J. et al., Nat. Med 4, 844-847 (1998)). Treatment of A549 cells with 150  $\mu$ M monastrol (SIGMA) for 24 hours induced the accumulation of endogenous KIF11 and exogenous EYFP-KIF11 at the center of monoaster along microtubules and the cell cycle arrest in mitosis with monopolar spindles, which is called "monoastal spindle". Treatment of A549 cells with 150  $\mu$ M of monastrol for 24 hours induced cell cycle arrest for mitotic cells with monopolar spindles that is called "monoastal spindle" and also caused accumulation of endogenous KIF11 at the center of monoaster along microtubules. On the other hand, most non-mitotic cells lost protrusion into the processes and then lost connection to adjacent cells within 2-hour of the monastrol treatment. Further quantitative analysis by counting the number of inter-cellular ultrafine structures (n = 252 structures) with time-lapse video-microscopy demonstrated that more than a half of the cell-to-cell connections in non-mitotic cells tested disappeared by the one-hour monastrol treatment. However, six hours after the release of the cells from the monastrol exposure, the intercellular bridge formation was re-constituted and cells at normal mitosis process was observed, indicating that KIF11 was indispensable for the formation of ultrafine intercellular structures (data not shown).

**[0307]** Some cells lost protrusion into the processes and then did not connected with adjacent cells. A more detailed observation of living cells found that no KOC1-KIF11 RNP complex (green particle) was transported from one cell to another through an ultrafine structure connecting the two cells, which subsequently disappeared during observation.

**[0308]** In this study we demonstrated endogenous interaction of KOC1, KIF11 and DCTN1 in human lung cancers, and revealed a possible role of those complexes in transport of mRNAs from one cell to another. DCTN1, the largest subunit of DCTN, binds to the cytoplasmic motor protein dynein and activates vesicle transport along microtubules (Holzbaur, E.L. & Tokito, M.K. Genomics 31, 398-399 (1996)). Dynein-DCTN interaction is probably a key component of the mechanism of axonal transport of vesicles and organelles (Holzbaur, E.L. & Tokito, M.K. Genomics 31, 398-399 (1996); Tokito, M.K. et al., Mol. Biol. Cell 7, 1167-1180 (1996)). The binding of DCTN to dynein is reportedly critical for neuronal function, since antibodies that specifically disrupt this binding block vesicle motility along microtubules. *In vitro* interaction of DCTN1 and KIF11, and their co-localization during mitosis have been observed (Blangy, A. et al., J. Biol. Chem. 272, 19418-19424 (1997)), but no report has shown an intercellular transporting system involving this complex. Since in our experiments KIF11, a member of the kinesin family, was over-expressed in NSCLCs along with KOC1, we suggest that direct interaction of KOC1, KIF11, and DCTN1 could play a significant role in establishing specific alignment of microtubules between lung-cancer cells.

#### **Protein synthesis by transported KOC1-associated mRNAs in the receiving cells**

**[0309]** To elucidate whether the mRNA transport by KOC1-KIF11 RNP complex is physiologically relevant (the recipient cell can synthesize the protein by translating the mRNAs transported), we constructed an expression vector of full length *RAB35* mRNA, one of the binding targets of the KOC1/KIF11 complex, fused in frame to myc tagged and an EGFP protein sequences. We then investigated whether this chimeric mRNA could be transportable from one cell to another and subsequently translated into the protein production in the recipient cell. FLAG-tagged KOC1 and KIF11 expressing-COS7 cells were transfected with constructs with these *RAB35* mRNA-expressing construct (cell A). Parental mRNA-recipient COS-7 cells were simply stained with CellTracker (blue; cell B). These two cell populations were mixed together and co-cultured for 24 hours. We first confirmed the intercellular transportation of *RAB35*-EGFP mRNAs between cells A and B by *in situ* hybridization using antisense EGFP as a probe; after co-culture of the cells for 24 hours, weak-staining of *RAB35*-EGFP mRNAs were detected in the CellTracker-stained cell B as well as on the ultrafine structure between

the two cell types (Fig. 7a). We then examined a presence of the EGFP-fused RAB35 proteins in the CellTracker-stained B-type cells were found using immunocytochemistry and time-lapse video microscopy, respectively (Fig. 7b and 7c). During these observations using time-lapse video microscopy, no visible EGFP-protein particle was transported from the type-A to type-B cells, but the EGFP protein gradually appeared in the apparatus of cytoplasm, which seemed to be endoplasmic reticulum (ER) of in the type-B cells (Figure 7d). These results have indicated that KOC1 and KIF11 should functionally associate with a subset of mRNAs, which encode proteins possibly inducing cell proliferation and/or adhesion, and that the presence of KOC1 and KIF11 is indispensable to the cell-to-cell transportation. Although previous reports suggested that high KOC 1 levels might interfere with translation of bound mRNAs such as *IGF2* leader-3, our experiment of co-transfecting KOC1 and full-length RAB35-EGFP mRNA constructs together into COS-7 cells detected no decrease of RAB35-EGFP-fused protein levels (Fig. 7e).

**[0310]** Our experiments also revealed formation of protruding processes connecting adjacent cells, and showed pre-dominant co-distribution of transfected RAB35 mRNAs and KOC1 protein on ultra-fine intercellular structures in two lung-cancer cell lines (A549 and LC319) that expressed high levels of endogenous KOC1 and KIF11. On the other hand, we did not find specific localization of transfected RAB35 mRNAs in NCI-H520 cells, which express KIF11 but not KOC1. That observation supported the importance of co-activation of KOC 1 and KIF11 for communication among cancer cells. Among the known cell-to-cell communication systems in human cancers, formation of functional gap-junctions between malignant glioma cells and vascular endothelial cells appears to influence angiogenesis in the tumors (Zhang, W. et al., Cancer Res. 59, 1994-2003 (1999); Zhang, W. et al., J. Neurosurg. 98, 846-853 (2003)). However, to our knowledge ours is the first report to describe inter-cellular transport of mRNA by means of ribonucleoprotein particles combined with motor proteins in mammalian somatic cells and to assess its biological significance for formation of an inter-cellular network critical for growth and survival of cancer cells.

#### (5) Inhibition of growth of NSCLC cells by siRNA against KIF11

**[0311]** Transfection of either siRNA plasmids for KIF11 into A549 (Fig. 8a) or LC319 (data not shown) cells suppressed mRNA expression of the KIF11 in comparison to cells containing any of the three control siRNAs and mock transfection. In accordance with the reduced mRNA expression, A549 and LC319 cells showed significant decreases in cell viability and colony numbers measured by MTT (Fig. 8b) and colony-formation assays (data not shown). We also investigated the effect by siRNA against KIF11 on intercellular transport using time-lapse videomicroscopy. A similar phenomenon to monastrol treatment was observed; some cells reduced protrusion into the processes and the disappearance of the ultrafine structure connecting the two cells.

**[0312]** To investigate the functional significance of KOC1-KIF11 interaction for growth or survival of lung-cancer cells, a deletion fragment of KOC1 containing the two RRM domains, which was able to interact with KIF11 (KOC1DEL3; Fig. 3a, b) was examined for a dominant-negative function of suppressing direct interaction between endogenous KOC1 and KIF11. We transfected KOC1DEL3 and mock plasmid (control) into LC319 cells and detected interaction of KOC1DEL3 with endogenous KIF11. We further verified that overexpression of the RRM domains reduce complex formation between KOC1 and KIF11 by immunoprecipitation (Fig. 9a,b). Expectedly, transfection of that fragment resulted in significant dose-dependent decreases in cell viability as measured by MTT assay ( $P < 0.001$ , KOC1DEL3 vs mock; Fig. 9c). We also confirmed that transfection of construct containing only KH-domains control have no effect on proliferation.

**[0313]** Furthermore, to investigate the functional significance of KOC1-KIF11 interaction for growth or survival of lung-cancer cells, a deletion fragment of KOC1, which lacked the C-terminal two KH-domains indispensable for mRNA binding but was able to interact with KIF11 (KOC1DEL2; Fig. 3a, b), was examined for a dominant-negative function of suppressing direct interaction between endogenous KOC1 and KIF11. We transfected KOC1DEL2 and mock plasmid (control) into A549 cells and detected interaction of KOC1DEL2 with endogenous KIF11 (Fig. 9d). We further verified by immunoprecipitation that over-expression of the KOC1DEL2 reduced complex formation between endogenous KOC1 and KIF11 (Fig. 9e). Expectedly, transfection of the dominant-negative fragment resulted in significant dose-dependent decreases in cell viability as measured by MTT assay ( $P = 0.0006$ , KOC1DEL2 vs mock; Fig. 9f).

**[0314]** We also examined some biological role(s) of these KIF11-transporting mRNAs in controlling the cell growth or survival of lung-cancer cells, we constructed plasmid to express siRNA against RAB35 (si-RAB35), which was identified as the KOC1-RNP complex-associated mRNAs. Transfection of the plasmids (si-RAB35) into A549 cells significantly suppressed expression of endogenous RAB35 in comparison with the controls, and resulted in significant decreases in cell viability and colony numbers measured by MTT and colony-formation assays (Fig. 10a,b).

#### Association of KOC1 and KIF11 over-expression with poor prognosis of NSCLC patients

**[0315]** We performed immunohistochemical analysis with anti-KOC1 and anti-KIF11 polyclonal antibodies using tissue microarrays consisting of 265 NSCLC tissues (Fig. 11a). Of the 265 cases, KOC1 staining was positive for 172 (64.9%); 129 cases were positive for KIF11 (48.7%). The expression pattern of KOC1 was significantly concordant with KIF11

expression in these tumors ( $X^2=60.8$ ,  $P<0.0001$ ). We then asked whether KOC1 and/or KIF11 over-expression could be associated with clinical outcome. We found that expression of KOC1 in NSCLCs was significantly associated with pT factor status ( $X^2= 23.1$ ,  $P < 0.0001$ ) and with tumor-specific 5-year survival ( $P = 0.0115$  by the Log-rank test) (Fig. 11b, *upper panel*). Expression of KIF11 in NSCLCs was significantly associated with pT factor ( $X^2 = 15.0$ ,  $P < 0.0001$ ), pN factor ( $X^2= 4.4$ ,  $P = 0.0356$ ), and 5 year-survival ( $P = 0.0008$  by the Log-rank test) (Fig. 11b, *lower panel*). By univariate analysis pT, pN, gender, and KOC1/KIF11 expression were each significantly related to a poor tumor-specific survival among NSCLC patients. Furthermore, KOC1 and KIF11 were determined to be independent prognostic factors by multivariate analysis using a Cox proportional-hazard model ( $P = 0.0499$  and  $P = 0.0259$ , respectively).

#### (6) Screening of candidate receptors for NMU in NSCLC

**[0316]** Two known NMU receptors, NMU1R (FM3/GPR66) and NMU2R (FM4) play important roles in energy homeostasis (Fujii, R. et al., J. Biol. Chem. 275: 21068-21074 (2000); Howard, A.D. et al., Nature 406: 70-74 (2000); Funes, S. et al., Peptides 23: 1607-1615 (2002)). NMU1R is present in many peripheral human tissues (Fujii, R. et al., J. Biol. Chem. 275: 21068-21074 (2000); Howard, A.D. et al., Nature 406: 70-74 (2000); Funes, S. et al., Peptides 23:1607-1615 (2002)), but NMU2R is located only in brain. To investigate whether NMU1R and NMU2R genes were expressed in NSCLCs, expression of these NMU receptors were analyzed in normal human brain and lung, in NSCLC cell lines, and in clinical tissues by semiquantitative RT-PCR experiments. Neither NMU1R nor NMU2R expression was detected in any of the cell lines or clinical samples examined, although NMU1R was expressed in lung and NMU2R in brain (data now shown), suggesting that NMU could be mediating growth of lung-cancer cells through interaction with other receptor(s).

**[0317]** Since NMU2R and NMU1R were originally isolated as homologues of known neuropeptide GPCRs, unidentified NMU receptor(s) were speculated to be members of the same family that would show some degree of homology to NMU1R/NMU2R. Hence, candidate NMU receptors were searched using the BLAST program. The results and their high expression levels in NSCLCs in the expression profile data of the present inventors indicated GHSR1b (NM\_004122; SEQ ID NOs: 3 and 4) and NTSR1 (NM\_002531; SEQ ID NOs: 5 and 6) to be good candidates. GHSR1b has two transcripts, types 1a and 1b. The full-length human type 1a cDNA encodes a predicted polypeptide of 366 amino acids with seven transmembrane domains, a typical feature of G protein-coupled receptors. A single intron divides its open reading frame into two exons encoding transmembrane domains 1-5 and 6-7, thus placing the GHSR1a into the intron-containing class of GPCRs. Type 1b is a non-spliced mRNA variant transcribed from a single exon that encodes a polypeptide of 289 amino acids with five transmembrane domains. The semiquantitative RT-PCR analysis using specific primers for each variant indicated that GHSR1a was not expressed in NSCLCs. On the other hand, GHSR1b and NTSR1 were expressed at a relatively high level in some NSCLC cell lines, but not at all in normal lung (Fig. 13a). The GHSR1b product has 46% homology to NMU1R, and NTSR1 encodes 418 amino acids with 47% homology to NMU1R.

#### (7) Identification of candidate receptors for NMU in NSCLC

**[0318]** To confirm direct interaction between NMU and GHSR1b/NTSR1, COS-7 cells were transiently transfected with plasmids designed to express FLAG-tagged GHSR1b or NTSR1, and cultured in the presence of rhodamine-labeled NMU-25. Then the localization of FLAG-tagged GHSR1b/NTSR1 and NMU-25-rhodamine in the cells were examined using anti-FLAG antibodies conjugated to FITC, and found that NMU-25 and either of both receptors were located together on the cell membrane (Fig. 13c). Co-localization of NMU-25 with these receptors was not observed in control assays involving either of the following ligand/cell combinations: 1) NMU-25-rhodamine incubated with COS-7 cells that were not transfected with either of the receptor plasmids; 2) non-transfected COS-7 cells incubated without NMU-25-rhodamine; and 3) COS-7 cells transfected with either of the receptor plasmids, but incubated without NMU-25-rhodamine. The result was confirmed by flow cytometry, which revealed binding of rhodamine-labeled NMU-25 to the surface of COS-7 cells that expressed either of the two receptors (Fig. 13d) and binding of rhodamine-labeled NMU-25 to the surface of COS-7 cells in a dose dependent manner.

#### (8) GHSR1b expression in normal human tissues

**[0319]** As the expression of GHSR1b in normal human tissues was not precisely reported at the time, the distribution of GHSR1b was determined using human multiple tissue Northern-blot. Northern blotting with GHSR1b cDNA as a probe identified a 0.9-kb transcript as a very weak signal band in comparison with a 1.1-kb transcript GHSR1a, seen in the heart, liver, skeletal muscle, pancreas, and stomach, among the 23 normal human tissues examined (Fig. 13b).

**[0320]** To further confirm binding of NMU-25 to the endogenous GHSR1b and NTSR1 on the NSCLC cells, we performed receptor-ligand binding assay using the LC319 and PC-14 cells treated with NMU-25. We detected binding of Cy5-labeled NMU-25 to the surface of these two cell lines that expressed both of the two receptors, but scarcely

expressed NMU1R/NMU2R (Figure 13e).

[0321] Biologically active ligands for GPCRs have been reported to bind specifically to their cognate receptors and cause an increase in second-messengers such as intracellular- $\text{Ca}^{2+}$  and cAMP levels. We therefore determined the ability of NMU to induce these second-messengers in LC319 cells through its interaction with GHSR1b/NTSR1. cAMP production, but not  $\text{Ca}^{2+}$  flux in LC319 cells, which express both GHSR1b and NTSR1 was observed in a NMU-25 dose dependent manner, when the cells were cultured in the presence of NMU-25 at final concentrations of 3-100  $\mu\text{M}$  in the culture media. The results demonstrate that NSCLC cells express functional GHSR1b/NTSR1 (Figure 13f left panel). This effect was confirmed to be NMU-25 specific by adding other reported ligands for GHSR1b/NTSR1, GHRL or NTS (Figure 13f right panel). In addition, GHRL and NTS caused the mobilization response of intracellular calcium in LC319 cells (data not shown), suggesting a variety of function for the poorly understood for GHSR1b and/or NTSR1.

#### (9) Inhibition of growth of NSCLC cells by siRNA against GHSR/NTSR1

[0322] Furthermore, the biological significance of the NMU-receptor interaction in pulmonary carcinogenesis was examined using plasmids designed to express siRNA against GHSR or NTSR1 (si-GHSR-1, si-NTSR1-1, and si-NTSR1-2). Transfection of either of these plasmids into A549 or LC319 cells suppressed expression of the endogenous receptor in comparison to cells containing any of the three control siRNAs (Fig. 14a). In accordance with the reduced expression of the receptors, A549 and LC319 cells showed significant decreases in cell viability (Fig. 14b) and numbers of colonies (data not shown). These results strongly supported the possibility that NMU, by interaction with GHSR1b and NTSR1, might play a very significant role in development/progression of NSCLC.

#### Identification of downstream genes of NMU

[0323] To further elucidate the NMU-signaling pathway and identify downstream genes regulated by NMU, siRNA against *NMU* (si-*NMU*) or LUC (control siRNA) were transfected into LC319 cells which had overexpressed *NMU* and down-regulations in gene expression were monitored using a cDNA microarray that contained 32,256 genes. Among hundreds of genes detected by this method, we performed Self-organizing map (SOM) clustering analysis to further select candidate genes. SOM clustering is data mining and visualization method originally developed by Kohonen (Kohonen, T. (1990). The self-organizing map. IEEE 78, 1464-1480.) and applied to the analysis of gene expression data from microarrays. The clustering method is similar to k-means clustering (Kaeck, S. M., et al., (2002). Cell 111, 837-851.) but differs in that genes are divided into groups based on expression patterns, and relationships between groups are illustrated by two-dimensional maps. The genes passing our variation filter were grouped by a 5 x 4 SOM.

[0324] We initially selected 70 genes using SOM cluster analysis, whose intensity were significantly decreased in accordance with the reduction of *NMU* expression (Figure 15a). Semiquantitative RT-PCR analysis confirmed reduction of candidate transcripts in a time-dependent manner in LC319 cells transfected with si-*NMU*, but not with control siRNA for LUC (Figure 15b). These transcripts were also confirmed to be up-regulated greater than 2-fold in LC319 cells expressing exogenous NMU, compared with that of normal lung tissues. Overexpression of these genes in accordance with *NMU* expression were evaluated as well in lung-cancer tissues and cell lines (data not shown). We finally identified 6 candidate NMU target genes, which satisfied the above selection criteria; *FOXM1*, *FLJ42024*, *GCDH*, *CDK5RAP1*, *LOC134145*, and *NUP188* (Figure 15b).

[0325] *FOXM1* mRNA levels were significantly elevated in lung cancers compared with normal lung tissues and its expression showed good concordance with NMU and two receptors for NMU, GHSR1b and NTSR1, whereas the function of *FOXM1* in lung carcinogenesis remains unclear. Therefore, we chose *FOXM1* for further analysis. To determine specific induction of the *FOXM1* by the NMU ligand-receptor signaling, LC319 cells expressing GHSR1b and NTSR1 were cultured in the presence of NMU-25 or BSA (control) at final concentrations of 100  $\mu\text{M}$  in the culture media. NMU-25-treated cells showed higher expression of *FOXM1* compared to the control cells (Figure 15c). Furthermore, *FOXM1* was also confirmed to be up-regulated in LC319 cells expressing exogenous NMU, compared with that of control cells transfected with mock vector (data not shown).

[0326] We then examined the biological significance of the *FOXM1* activation by NMU signaling for growth or survival of lung-cancer cells, using plasmids designed to express siRNA against *FOXM1* (si-*FOXM1*). Transfection of si-*FOXM1* into A549 or LC319 cells suppressed expression of the endogenous *FOXM1* in comparison to cells containing any of the three control siRNAs (Figure 16a and 16b). In accordance with the reduced expression of the *FOXM1*, A549 and LC319 cells showed significant decreases in cell viability and numbers of colonies (Figure 16a and b). These results strongly demonstrated that NMU, by the interaction with GHSR1b/NTSR1 and subsequent activation of its downstream targets, such as *FOXM1*, could significantly affect the growth of lung-cancer cells.

[0327] Microarray data of LC319 cells treated with siRNA for *NMU* presented herein proved that NMU signaling pathway could affect the growth promotion of lung-cancer cells by transactivating a set of downstream genes involving transcripts whose protein products can function as a transcription factor and are capable of controlling cell growth or

participating in signal transduction. We provided evidence that the FOXM1 transcription factor is a downstream target of NMU signaling by additional biological assays. FOXM1 was known to be over-expressed in several types of human cancers (Teh, M.T. et al., Cancer Res. 62, 4773-4780.; van den Boom, J. et al., (2003). Am. J. Pathol. 163, 1033-1043.; Kalinichenko, VV et al., (2004). Genes. Dev. 18, 830-850). The "forkhead" gene family, originally identified in *Drosophila*, comprises transcription factors with a conserved 100-amino acid DNA-binding motif, and has been shown to play important roles in regulating the expression of genes involved in cell growth, proliferation, differentiation, longevity, and transformation. Cotransfection assays in the human hepatoma HepG2 cell line demonstrated that FOXM1 protein stimulated expression of both the cyclin B1 (CCNB1) and cyclin D1 (CCND1) (Wang, X. et al., (2002). Proc. Nat. Acad Sci. 99, 16881-16886.), suggesting that these cyclin genes are direct FOXM1 transcription targets and that FOXM1 controls the transcription network of genes that are essential for cell division and exit from mitosis. It should be noted that we observed activation of *CCNB1* in the majority of a series of NSCLC we examined and its good concordance of the expression to *FOXM1* (data not shown). On the other hand, it was also demonstrated that p27 (Kip1) and p19 (Arf) (CDKN2A) interact with FOXM1 and inhibit FOXM1 transcriptional activity (Kalinichenko, V.V. et al., (2004). Genes. Dev. 18, 830-850). The promotion of cell growth in NSCLC cells by NMU might reflect transactivation of FOXM1, which would affect the function of those molecular pathways in consequence.

**[0328]** By immunohistochemical analysis on tissue microarray, we detected increased expression of NMU protein in the majority of NSCLC (SCC, ADC, LCC, and BAC) and SCLC samples, but not in normal lung tissues. Since NMU is a secreted protein and most of the clinical NSCLC samples used for our analysis were at an early and operable stage, NMU might serve as a biomarker for diagnosis of early-stage lung cancer, in combination with fiberoptic transbronchial biopsy (TBB) or blood tests.

**[0329]** In summary, we have shown that NMU and two newly revealed receptors for this molecule, GHSR1b and NTSR1, are likely to play an essential role for an autocrine growth-promoting pathway in NSCLCs by modulating transcription of down stream target genes. The data reported here strongly imply the possibility of designing new anti-cancer drugs, specific for lung cancer, that target the NMU-GHSR1b/NTSR1 pathway. They also suggest a potential for siRNAs themselves to interfere with this pathway, as a novel approach to treatment of chemotherapy-resistant, advanced lung cancers.

#### Industrial Applicability

**[0330]** The expression of human genes KIF11, GHSR1b, NTSR1 and FOXM1 are markedly elevated in non-small cell lung cancer (NSCLC) as compared to normal lung tissues. Accordingly, these genes can be conveniently used as diagnostic markers of NSCLC and the proteins encoded thereby may be used in diagnostic assays of NSCLC.

**[0331]** The present inventors have also shown that the expression of KIF11, GHSR1b, NTSR1 or FOXM1 promotes cell growth whereas cell growth is suppressed by small interfering RNAs corresponding to KIF11, GHSR1b, NTSR1 or FOXM1 gene. These findings show that each of KIF11, KOC1, GHSR1b, NTSR1 and FOXM1 proteins stimulate oncogenic activity. Thus, each of these oncoproteins is a useful target for the development of anti-cancer pharmaceuticals. For example, agents that block the expression of KIF11, KOC1, GHSR1b, NTSR1 or FOXM1, or prevent its activity may find therapeutic utility as anti-cancer agents, particularly anti-cancer agents for the treatment of NSCLC. Examples of such agents include antisense oligonucleotides, small interfering RNAs, and ribozymes against the KIF11, KOC1, GHSR1b, NTSR1 or FOXM1 gene, and antibodies that recognize KIF11, KOC1, GHSR1b, NTSR1 or FOXM1 polypeptide.

**[0332]** 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

#### SEQUENCE LISTING

**[0333]**

<110> ONCOTHERAPY SCIENCE, INC.  
THE UNIVERSITY OF TOKYO

<120> METHOD FOR DIAGNOSING NON-SMALL CELL LUNG CANCER

<130> ONC-A0401P

<150> US 60/555,789

<151> 2004-03-23

<160> 127

<170> PatentIn version 3.3

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	gaa gtt att atg ggc tat aat tgc act atc ttt gcg tat ggc caa act																461
10	Glu	Val	Ile	Met	Gly	Tyr	Asn	Cys	Thr	Ile	Phe	Ala	Tyr	Gly	Gln	Thr	
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15	ggc act gga aaa act ttt aca atg gaa ggt gaa agg tca cct aat gaa																509
20	Gly	Thr	Gly	Lys	Thr	Phe	Thr	Met	Glu	Gly	Glu	Arg	Ser	Pro	Asn	Glu	
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25	gag tat acc tgg gaa gag gat ccc ttg gct ggt ata att cca cgt acc																557
	Glu	Tyr	Thr	Trp	Glu	Glu	Asp	Pro	Leu	Ala	Gly	Ile	Ile	Pro	Arg	Thr	
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	Leu	His	Gln	Ile	Phe	Glu	Lys	Leu	Thr	Asp	Asn	Gly	Thr	Glu	Phe	Ser	
	140				145					150				155			
40	gtc aaa gtg tct ctg ttg gag atc tat aat gaa gag ctt ttt gat ctt																653
45	Val	Lys	Val	Ser	Leu	Leu	Glu	Ile	Tyr	Asn	Glu	Glu	Leu	Phe	Asp	Leu	
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	Leu	Asn	Pro	Ser	Ser	Asp	Val	Ser	Glu	Arg	Leu	Gln	Met	Phe	Asp	Asp	
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 Val His Asn Lys Asp Glu Val Tyr Gln Ile Leu Glu Lys Gly Ala Ala  
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20      aaa agg aca act gca gct act ctg atg aat gca tac tct agt cgt tcc      845  
 Lys Arg Thr Thr Ala Ala Thr Leu Met Asn Ala Tyr Ser Ser Arg Ser  
          220                              225                              230                              235

25      cac tca gtt ttc tct gtt aca ata cat atg aaa gaa act acg att gat      893  
 30      His Ser Val Phe Ser Val Thr Ile His Met Lys Glu Thr Thr Ile Asp  
                                  240                              245                              250

35      gga gaa gag ctt gtt aaa atc gga aag ttg aac ttg gtt gat ctt gca      941  
 Gly Glu Glu Leu Val Lys Ile Gly Lys Leu Asn Leu Val Asp Leu Ala  
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45      gga agt gaa aac att ggc cgt tct gga gct gtt gat aag aga gct cgg      989  
 Gly Ser Glu Asn Ile Gly Arg Ser Gly Ala Val Asp Lys Arg Ala Arg  
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50      gaa gct gga aat ata aat caa tcc ctg ttg act ttg gga agg gtc att      1037  
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	Thr Ala Leu Val Glu Arg Thr Pro His Val Pro Tyr Arg Glu Ser Lys	
	300                                      305                                      310                                      315	
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	Leu Thr Arg Ile Leu Gln Asp Ser Leu Gly Gly Arg Thr Arg Thr Ser	
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	Ile Ile Ala Thr Ile Ser Pro Ala Ser Leu Asn Leu Glu Glu Thr Leu	
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	Glu Glu Ile Glu Arg Leu Lys Arg Asp Leu Ala Ala Ala Arg Glu Lys	
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	Leu Thr Val Gln Glu Glu Gln Ile Val Glu Leu Ile Glu Lys Ile Gly	
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	Ala Val Glu Glu Glu Leu Asn Arg Val Thr Glu Leu Phe Met Asp Asn	
	430 435 440	
25	aaa aat gaa ctt gac cag tgt aaa tct gac ctg caa aat aaa aca caa	1517
30	Lys Asn Glu Leu Asp Gln Cys Lys Ser Asp Leu Gln Asn Lys Thr Gln	
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45	gtt aaa gaa gaa tat atc aca tca gct ttg gaa agt act gag gag aaa	1613
	Val Lys Glu Glu Tyr Ile Thr Ser Ala Leu Glu Ser Thr Glu Glu Lys	
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Leu His Asp Ala Ala Ser Lys Leu Leu Asn Thr Val Glu Glu Thr Thr  
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 Ser Leu Thr Ser Ile Pro Glu Asn Val Ser Thr His Val Ser Gln Ile  
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5            ttt aat atg ata cta aaa gaa caa tca tta gca gca gaa agt aaa act            1997  
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           605                            610                            615

10           gta cta cag gaa ttg att aat gta ctc aag act gat ctt cta agt tca            2045  
           Val Leu Gln Glu Leu Ile Asn Val Leu Lys Thr Asp Leu Leu Ser Ser  
 15           620                            625                            630                            635

20           ctg gaa atg att tta tcc cca act gtg gtg tct ata ctg aaa atc aat            2093  
           Leu Glu Met Ile Leu Ser Pro Thr Val Val Ser Ile Leu Lys Ile Asn  
    640                            645                            650

25           agt caa cta aag cat att ttc aag act tca ttg aca gtg gcc gat aag            2141  
           Ser Gln Leu Lys His Ile Phe Lys Thr Ser Leu Thr Val Ala Asp Lys  
 30     655                            660                            665

35           ata gaa gat caa aaa aag gaa cta gat ggc ttt ctc agt ata ctg tgt            2189  
           Ile Glu Asp Gln Lys Lys Glu Leu Asp Gly Phe Leu Ser Ile Leu Cys  
 40     670                            675                            680

45           aac aat cta cat gaa cta caa gaa aat acc att tgt tcc ttg gtt gag            2237  
           Asn Asn Leu His Glu Leu Gln Glu Asn Thr Ile Cys Ser Leu Val Glu  
    685                            690                            695

50           tca caa aag caa tgt gga aac cta act gaa gac ctg aag aca ata aag            2285

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Ser Gln Lys Gln Cys Gly Asn Leu Thr Glu Asp Leu Lys Thr Ile Lys  
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 30 750 755 760  
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 35 Lys Met Thr Phe His Ser Gln Lys Phe Cys Ala Asp Ser Asp Gly Phe  
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 780 785 790 795  
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 55 800 805 810



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25	tca gac atc act gag aaa tca gat gga cgt aag gca gct cat gag aaa	2765
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35	cag cat aac att ttt ctt gat cag atg act att gat gaa gat aaa ttg	2813
	Gln His Asn Ile Phe Leu Asp Gln Met Thr Ile Asp Glu Asp Lys Leu	
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	Ile Ala Gln Asn Leu Glu Leu Asn Glu Thr Ile Lys Ile Gly Leu Thr	
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Lys Leu Asn Cys Phe Leu Glu Gln Asp Leu Lys Leu Asp Ile Pro Thr

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Gly Thr Thr Pro Gln Arg Lys Ser Tyr Leu Tyr Pro Ser Thr Leu Val

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cct gag ctg tta atg atg cta aac tgt tca gaa aac aac aaa gaa gag 3053

Pro Glu Leu Leu Met Met Leu Asn Cys Ser Glu Asn Asn Lys Glu Glu

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975

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1010

1015

5      gga aaa   gac aaa   gaa aac   aga   ggc att   aac aca   ctg   gag agg   tct   3239  
      Gly Lys   Asp Lys   Glu Asn   Arg   Gly Ile   Asn Thr   Leu   Glu Arg   Ser  
          1020                      1025                      1030

10      aaa gtg   gaa gaa   act aca   gag   cac ttg   gtt aca   aag   agc aga   tta   3284  
      Lys Val   Glu Glu   Thr Thr   Glu   His Leu   Val Thr   Lys   Ser Arg   Leu  
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20      cct ctg   cga gcc   cag atc   aac   ctt taa   ttcacttggg   ggttggcaat   3331  
      Pro Leu   Arg Ala   Gln Ile   Asn   Leu  
          1050                      1055

25      tttattttta   aagaaaactt   aaaaataaaa   cctgaaaccc   cagaacttga   gccttgtgta   3391

30      tagattttta   aagaatatat   atatcagccg   ggcgcggtgg   ctcatgcctg   taatcccagc   3451

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Arg Lys Ala Ser Ala His Ser Ile Val Glu Cys Asp Pro Val Arg Lys

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Glu Val Ser Val Arg Thr Gly Gly Leu Ala Asp Lys Ser Ser Arg Lys

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Thr Tyr Thr Phe Asp Met Val Phe Gly Ala Ser Thr Lys Gln Ile Asp

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Val Tyr Arg Ser Val Val Cys Pro Ile Leu Asp Glu Val Ile Met Gly

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Tyr Asn Cys Thr Ile Phe Ala Tyr Gly Gln Thr Gly Thr Gly Lys Thr

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Phe Thr Met Glu Gly Glu Arg Ser Pro Asn Glu Glu Tyr Thr Trp Glu

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Glu Lys Leu Thr Asp Asn Gly Thr Glu Phe Ser Val Lys Val Ser Leu

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Leu Glu Ile Tyr Asn Glu Glu Leu Phe Asp Leu Leu Asn Pro Ser Ser

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Asp Val Ser Glu Arg Leu Gln Met Phe Asp Asp Pro Arg Asn Lys Arg

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Gly Val Ile Ile Lys Gly Leu Glu Glu Ile Thr Val His Asn Lys Asp

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Glu Val Tyr Gln Ile Leu Glu Lys Gly Ala Ala Lys Arg Thr Thr Ala

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Lys Ile Gly Lys Leu Asn Leu Val Asp Leu Ala Gly Ser Glu Asn Ile  
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Gly Arg Ser Gly Ala Val Asp Lys Arg Ala Arg Glu Ala Gly Asn Ile  
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Gln Asp Ser Leu Gly Gly Arg Thr Arg Thr Ser Ile Ile Ala Thr Ile



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Glu Gln Ile Val Glu Leu Ile Glu Lys Ile Gly Ala Val Glu Glu Glu

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Gln Cys Lys Ser Asp Leu Gln Asn Lys Thr Gln Glu Leu Glu Thr Thr

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Ser Lys Leu Leu Asn Thr Val Glu Glu Thr Thr Lys Asp Val Ser Gly

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Ser Pro Thr Val Val Ser Ile Leu Lys Ile Asn Ser Gln Leu Lys His

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Lys Glu Leu Asp Gly Phe Leu Ser Ile Leu Cys Asn Asn Leu His Glu

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680

685

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Leu Gln Glu Asn Thr Ile Cys Ser Leu Val Glu Ser Gln Lys Gln Cys

690

695

700

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Gly Asn Leu Thr Glu Asp Leu Lys Thr Ile Lys Gln Thr His Ser Gln

705

710

715

720

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Glu Leu Cys Lys Leu Met Asn Leu Trp Thr Glu Arg Phe Cys Ala Leu

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735

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Glu Glu Lys Cys Glu Asn Ile Gln Lys Pro Leu Ser Ser Val Gln Glu

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	820	825	830
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55	Trp Val Ser Ser Leu Asn Glu Arg Glu Gln Glu Leu His Asn Leu Leu		
	835	840	845

	Glu	Val	Val	Ser	Gln	Cys	Cys	Glu	Ala	Ser	Ser	Ser	Asp	Ile	Thr	Glu
5		850					855					860				
10	Lys	Ser	Asp	Gly	Arg	Lys	Ala	Ala	His	Glu	Lys	Gln	His	Asn	Ile	Phe
	865					870					875				880	
15																
20	Leu	Asp	Gln	Met	Thr	Ile	Asp	Glu	Asp	Lys	Leu	Ile	Ala	Gln	Asn	Leu
						885				890				895		
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30	Glu	Leu	Asn	Glu	Thr	Ile	Lys	Ile	Gly	Leu	Thr	Lys	Leu	Asn	Cys	Phe
			900						905					910		
35																
40	Leu	Glu	Gln	Asp	Leu	Lys	Leu	Asp	Ile	Pro	Thr	Gly	Thr	Thr	Pro	Gln
		915							920					925		
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50	Arg	Lys	Ser	Tyr	Leu	Tyr	Pro	Ser	Thr	Leu	Val	Arg	Thr	Glu	Pro	Arg
		930						935					940			
55																
	Glu	His	Leu	Leu	Asp	Gln	Leu	Lys	Arg	Lys	Gln	Pro	Glu	Leu	Leu	Met

945 950 955 960

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Met Leu Asn Cys Ser Glu Asn Asn Lys Glu Glu Thr Ile Pro Asp Val

10

965 970 975

15

Asp Val Glu Glu Ala Val Leu Gly Gln Tyr Thr Glu Glu Pro Leu Ser

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980 985 990

25

Gln Glu Pro Ser Val Asp Ala Gly Val Asp Cys Ser Ser Ile Gly Gly

30

995 1000 1005

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Val Pro Phe Phe Gln His Lys Lys Ser His Gly Lys Asp Lys Glu

1010 1015 1020

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Asn Arg Gly Ile Asn Thr Leu Glu Arg Ser Lys Val Glu Glu Thr

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gcc gac ctg gac tgg gat gct tcc ccc ggc aac gac tcg ctg ggc gac 96

Ala Asp Leu Asp Trp Asp Ala Ser Pro Gly Asn Asp Ser Leu Gly Asp

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gag ctg ctg cag ctc ttc ccc gcg ccg ctg ctg gcg ggc gtc aca gcc 144

Glu Leu Leu Gln Leu Phe Pro Ala Pro Leu Leu Ala Gly Val Thr Ala



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	Thr Cys Val Ala Leu Phe Val Val Gly Ile Ala Gly Asn Leu Leu Thr			
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	atg ctg gtg gtg tcg cgc ttc cgc gag ctg cgc acc acc acc aac ctc			240
	Met Leu Val Val Ser Arg Phe Arg Glu Leu Arg Thr Thr Thr Asn Leu			
20	65	70	75	80
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	tac ctg tcc agc atg gcc ttc tcc gat ctg ctc atc ttc ctc tgc atg			288
	Tyr Leu Ser Ser Met Ala Phe Ser Asp Leu Leu Ile Phe Leu Cys Met			
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	ccc ctg gac ctc gtt cgc ctc tgg cag tac cgg ccc tgg aac ttc ggc			336
	Pro Leu Asp Leu Val Arg Leu Trp Gln Tyr Arg Pro Trp Asn Phe Gly			
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	Ala Thr Val Leu Thr Ile Thr Ala Leu Ser Val Glu Arg Tyr Phe Ala			
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	Trp Asp Thr Asn Glu Cys Arg Pro Thr Glu Phe Ala Val Arg Ser Gly	
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Glu Leu Leu Gln Leu Phe Pro Ala Pro Leu Leu Ala Gly Val Thr Ala

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Thr Cys Val Ala Leu Phe Val Val Gly Ile Ala Gly Asn Leu Leu Thr

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Met Leu Val Val Ser Arg Phe Arg Glu Leu Arg Thr Thr Thr Asn Leu

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70

75

80

35

Tyr Leu Ser Ser Met Ala Phe Ser Asp Leu Leu Ile Phe Leu Cys Met

85

90

95

40

45

Pro Leu Asp Leu Val Arg Leu Trp Gln Tyr Arg Pro Trp Asn Phe Gly

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Lys Leu Val Ile Phe Val Ile Trp Ala Val Ala Phe Cys Ser Ala Gly  
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Pro Ile Phe Val Leu Val Gly Val Glu His Glu Asn Gly Thr Asp Pro  
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Trp Asp Thr Asn Glu Cys Arg Pro Thr Glu Phe Ala Val Arg Ser Gly  
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Val Phe Cys Leu Thr Val Leu Tyr Ser Leu Ile Gly Arg Lys Leu Trp

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Arg Arg Arg Arg Gly Asp Ala Val Val Gly Ala Ser Leu Arg Asp Gln

245 250 255

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Leu Leu Ala Pro Gly Phe Gly Asn Ala Ser Gly Asn Ala Ser Glu Arg  
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	Leu Val Cys Thr Pro Thr Ile His Thr Ala Thr Val Lys Val Val Ile	
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Gln Val Asn Thr Phe Met Ser Phe Ile Phe Pro Met Val Val Ile Ser .

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Val Arg Val Leu Arg Ala Val Val Ile Ala Phe Val Val Cys Trp Leu

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Pro Gly Phe Gly Asn Ala Ser Gly Asn Ala Ser Glu Arg Val Leu Ala

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35

Ala Pro Ser Ser Glu Leu Asp Val Asn Thr Asp Ile Tyr Ser Lys Val

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Lys Thr Leu Met Ser Arg Ser Arg Thr Lys Lys Phe Ile Ser Ala Ile

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Val Arg Arg Leu Met Phe Cys Tyr Ile Ser Asp Glu Gln Trp Thr Pro

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Phe Leu Tyr Asp Phe Tyr His Tyr Phe Tyr Met Val Thr Asn Ala Leu

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Phe Tyr Val Ser Ser Thr Ile Asn Pro Ile Leu Tyr Asn Leu Val Ser

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# EP 1 737 979 B9

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15	att gag gac cac ttt ccc tac ttt aag cac att gcc aag cca ggc tgg			1108
	Ile Glu Asp His Phe Pro Tyr Phe Lys His Ile Ala Lys Pro Gly Trp			
20		270	275	280
25	aag aac tcc atc cgc cac aac ctt tcc ctg cac gac atg ttt gtc cgg			1156
	Lys Asn Ser Ile Arg His Asn Leu Ser Leu His Asp Met Phe Val Arg			
30		285	290	295
35	gag acg tct gcc aat ggc aag gtc tcc ttc tgg acc att cac ccc agt			1204
	Glu Thr Ser Ala Asn Gly Lys Val Ser Phe Trp Thr Ile His Pro Ser			
40		300	305	310
45	gcc aac cgc tac ttg aca ttg gac cag gtg ttt aag cag cag aaa cga			1252
	Ala Asn Arg Tyr Leu Thr Leu Asp Gln Val Phe Lys Gln Gln Lys Arg			
50		315	320	325
55	ccg aat cca gag ctc cgc cgg aac atg acc atc aaa acc gaa ctc ccc			1300
	Pro Asn Pro Glu Leu Arg Arg Asn Met Thr Ile Lys Thr Glu Leu Pro			
	330	335	340	345

ctg ggc gca cgg cgg aag atg aag cca ctg cta cca cgg gtc agc tca 1348  
 Leu Gly Ala Arg Arg Lys Met Lys Pro Leu Leu Pro Arg Val Ser Ser  
 5 350 355 360

tac ctg gta cct atc cag ttc ccg gtg aac cag tca ctg gtg ttg cag 1396  
 Tyr Leu Val Pro Ile Gln Phe Pro Val Asn Gln Ser Leu Val Leu Gln  
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ccc tcg gtg aag gtg cca ttg ccc ctg gcg gct tcc ctc atg agc tca 1444  
 Pro Ser Val Lys Val Pro Leu Pro Leu Ala Ala Ser Leu Met Ser Ser  
 15 380 385 390

gag ctt gcc cgc cat agc aag cga gtc cgc att gcc ccc aag gtg ctg 1492  
 Glu Leu Ala Arg His Ser Lys Arg Val Arg Ile Ala Pro Lys Val Leu  
 20 395 400 405

cta gct gag gag ggg ata gct cct ctt tct tct gca gga cca ggg aaa 1540  
 Leu Ala Glu Glu Gly Ile Ala Pro Leu Ser Ser Ala Gly Pro Gly Lys  
 25 410 415 420 425

gag gag aaa ctc ctg ttt gga gaa ggg ttt tct cct ttg ctt cca gtt 1588  
 Glu Glu Lys Leu Leu Phe Gly Glu Gly Phe Ser Pro Leu Leu Pro Val  
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cag act atc aag gag gaa gaa atc cag cct ggg gag gaa atg cca cac 1636  
 Gln Thr Ile Lys Glu Glu Glu Ile Gln Pro Gly Glu Glu Met Pro His  
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	445	450	455	
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	Ser Pro Ala Pro Ser Phe Lys Glu Glu Ser Ser His Ser Trp Glu Asp			
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	Ser Ser Gln Ser Pro Thr Pro Arg Pro Lys Lys Ser Tyr Ser Gly Leu			
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	agg tcc cca acc cgg tgt gtc tcg gaa atg ctt gtg att caa cac agg	1828		
35	Arg Ser Pro Thr Arg Cys Val Ser Glu Met Leu Val Ile Gln His Arg			
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	gag agg agg gag agg agc cgg tct cgg agg aaa cag cat cta ctg cct	1876		
	Glu Arg Arg Glu Arg Ser Arg Ser Arg Arg Lys Gln His Leu Leu Pro			
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	ccc tgt gtg gat gag ccg gag ctg ctc ttc tca gag ggg ccc agt act	1924		
	Pro Cys Val Asp Glu Pro Glu Leu Leu Phe Ser Glu Gly Pro Ser Thr			
	540	545	550	

55



tcc cgc tgg gcc gca gag ctc ccg ttc cca gca gac tcc tct gac cct 1972  
 5 Ser Arg Trp Ala Ala Glu Leu Pro Phe Pro Ala Asp Ser Ser Asp Pro  
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gcc tcc cag ctc agc tac tcc cag gaa gtg gga gga cct ttt aag aca 2020  
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 15 570 575 580 585

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ctc ccc aga acc cct gaa tcc tgg agg ctc acg ccc cca gcc aaa gta 2116  
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ggg gga ctg gat ttc agc cca gta caa acc tcc cag ggt gcc tct gac 2164  
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ccc ttg cct gac ccc ctg ggg ctg atg gat ctc agc acc act ccc ttg 2212  
 45 Pro Leu Pro Asp Pro Leu Gly Leu Met Asp Leu Ser Thr Thr Pro Leu  
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caa agt gct ccc ccc ctt gaa tca ccg caa agg ctc ctc agt tca gaa 2260  
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	650	655	660	665	
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	ata gac gtc ccc aag cca ggc tcc ccg gag cca cag gtt tct ggc ctt	2356			
	Ile Asp Val Pro Lys Pro Gly Ser Pro Glu Pro Gln Val Ser Gly Leu				
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	gca gcc aat cgt tct ctg aca gaa ggc ctg gtc ctg gac aca atg aat	2404			
	Ala Ala Asn Arg Ser Leu Thr Glu Gly Leu Val Leu Asp Thr Met Asn				
	700	705	710		
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	gac agc ctc agc aag atc ctg ctg gac atc agc ttt cct ggc ctg gac	2452			
	Asp Ser Leu Ser Lys Ile Leu Leu Asp Ile Ser Phe Pro Gly Leu Asp				
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	gag gac cca ctg ggc cct gac aac atc aac tgg tcc cag ttt att cct	2500			
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	Glu Leu Gln				
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<212> PRT  
<213> Homo sapiens

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<400> 107

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Pro Leu Pro Val Gln Asn Ala Pro Ser Glu Thr Ser Glu Glu Glu Pro

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20 25 30

40

Lys Arg Ser Pro Ala Gln Gln Glu Ser Asn Gln Ala Glu Ala Ser Lys

45

35 40 45

50

55

Glu Val Ala Glu Ser Asn Ser Cys Lys Phe Pro Ala Gly Ile Lys Ile

50

55

60

5

Ile Asn His Pro Thr Met Pro Asn Thr Gln Val Val Ala Ile Pro Asn

65

70

75

80

10

15

Asn Ala Asn Ile His Ser Ile Ile Thr Ala Leu Thr Ala Lys Gly Lys

85

90

95

20

25

Glu Ser Gly Ser Ser Gly Pro Asn Lys Phe Ile Leu Ile Ser Cys Gly

100

105

110

30

35

Gly Ala Pro Thr Gln Pro Pro Gly Leu Arg Pro Gln Thr Gln Thr Ser

115

120

125

40

Tyr Asp Ala Lys Arg Thr Glu Val Thr Leu Glu Thr Leu Gly Pro Lys

130

135

140

45

50

Pro Ala Ala Arg Asp Val Asn Leu Pro Arg Pro Pro Gly Ala Leu Cys

145

150

155

160

55

Glu Gln Lys Arg Glu Thr Cys Ala Asp Gly Glu Ala Ala Gly Cys Thr

5

165

170

175

10

Ile Asn Asn Ser Leu Ser Asn Ile Gln Trp Leu Arg Lys Met Ser Ser

15

180

185

190

20

Asp Gly Leu Gly Ser Arg Ser Ile Lys Gln Glu Met Glu Glu Lys Glu

195

200

205

25

Asn Cys His Leu Glu Gln Arg Gln Val Lys Val Glu Glu Pro Ser Arg

30

210

215

220

35

Pro Ser Ala Ser Trp Gln Asn Ser Val Ser Glu Arg Pro Pro Tyr Ser

40

225

230

235

240

45

Tyr Met Ala Met Ile Gln Phe Ala Ile Asn Ser Thr Glu Arg Lys Arg

50

245

250

255

55

Met Thr Leu Lys Asp Ile Tyr Thr Trp Ile Glu Asp His Phe Pro Tyr

260

265

270

5

Phe Lys His Ile Ala Lys Pro Gly Trp Lys Asn Ser Ile Arg His Asn

275

280

285

10

15

Leu Ser Leu His Asp Met Phe Val Arg Glu Thr Ser Ala Asn Gly Lys

290

295

300

20

25

Val Ser Phe Trp Thr Ile His Pro Ser Ala Asn Arg Tyr Leu Thr Leu

305

310

315

320

30

35

Asp Gln Val Phe Lys Gln Gln Lys Arg Pro Asn Pro Glu Leu Arg Arg

325

330

335

40

Asn Met Thr Ile Lys Thr Glu Leu Pro Leu Gly Ala Arg Arg Lys Met

340

345

350

45

50

Lys Pro Leu Leu Pro Arg Val Ser Ser Tyr Leu Val Pro Ile Gln Phe

355

360

365

55

Pro Val Asn Gln Ser Leu Val Leu Gln Pro Ser Val Lys Val Pro Leu

5                    370                    375                    380

Pro Leu Ala Ala Ser Leu Met Ser Ser Glu Leu Ala Arg His Ser Lys

10                    385                    390                    395                    400

Arg Val Arg Ile Ala Pro Lys Val Leu Leu Ala Glu Glu Gly Ile Ala

15                    405                    410                    415

Pro Leu Ser Ser Ala Gly Pro Gly Lys Glu Glu Lys Leu Leu Phe Gly

20                    420                    425                    430

Glu Gly Phe Ser Pro Leu Leu Pro Val Gln Thr Ile Lys Glu Glu Glu

25                    435                    440                    445

Ile Gln Pro Gly Glu Glu Met Pro His Leu Ala Arg Pro Ile Lys Val

30                    450                    455                    460

35

40

45

50

55



Glu Ser Pro Pro Leu Glu Glu Trp Pro Ser Pro Ala Pro Ser Phe Lys

465 470 475 480

Glu Glu Ser Ser His Ser Trp Glu Asp Ser Ser Gln Ser Pro Thr Pro

485 490 495

Arg Pro Lys Lys Ser Tyr Ser Gly Leu Arg Ser Pro Thr Arg Cys Val

500 505 510

Ser Glu Met Leu Val Ile Gln His Arg Glu Arg Arg Glu Arg Ser Arg

515 520 525

Ser Arg Arg Lys Gln His Leu Leu Pro Pro Cys Val Asp Glu Pro Glu

530 535 540

Leu Leu Phe Ser Glu Gly Pro Ser Thr Ser Arg Trp Ala Ala Glu Leu

545 550 555 560

Pro Phe Pro Ala Asp Ser Ser Asp Pro Ala Ser Gln Leu Ser Tyr Ser

565 570 575

Gln Glu Val Gly Gly Pro Phe Lys Thr Pro Ile Lys Glu Thr Leu Pro

5

580

585

590

10

Ile Ser Ser Thr Pro Ser Lys Ser Val Leu Pro Arg Thr Pro Glu Ser

595

600

605

15

20

Trp Arg Leu Thr Pro Pro Ala Lys Val Gly Gly Leu Asp Phe Ser Pro

610

615

620

25

30

Val Gln Thr Ser Gln Gly Ala Ser Asp Pro Leu Pro Asp Pro Leu Gly

625

630

635

640

35

Leu Met Asp Leu Ser Thr Thr Pro Leu Gln Ser Ala Pro Pro Leu Glu

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645

650

655

45

Ser Pro Gln Arg Leu Leu Ser Ser Glu Pro Leu Asp Leu Ile Ser Val

660

665

670

50

55

Pro Phe Gly Asn Ser Ser Pro Ser Asp Ile Asp Val Pro Lys Pro Gly

5

675

680

685

10

Ser Pro Glu Pro Gln Val Ser Gly Leu Ala Ala Asn Arg Ser Leu Thr

690

695

700

15

20

Glu Gly Leu Val Leu Asp Thr Met Asn Asp Ser Leu Ser Lys Ile Leu

705

710

715

720

25

Leu Asp Ile Ser Phe Pro Gly Leu Asp Glu Asp Pro Leu Gly Pro Asp

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Asn Ile Asn Trp Ser Gln Phe Ile Pro Glu Leu Gln

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740

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<211> 19

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<212> DNA

<213> Artificial

<220>

<223> A target sequence for siRNA.

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<400> 108

gcagcagaaa cgaccgaat 19

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<210> 109

<211> 51

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<400> 109

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<212> DNA

10 <213> Artificial

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<400> 110

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<211> 47

20 <212> DNA

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<400> 111

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<212> DNA

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<400> 112

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 cccgcgcgca ccgccccgc ccgcc atg gcc cgg gac tac gac cac ctc ttc 172  
 Met Ala Arg Asp Tyr Asp His Leu Phe  
 15 1 5  
 aag ctg ctc atc atc ggc gac agc ggt gtg ggc aag agc agt tta ctg 220  
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 Lys Leu Leu Ile Ile Gly Asp Ser Gly Val Gly Lys Ser Ser Leu Leu  
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 Lys Leu Gln Ile Trp Asp Thr Ala Gly Gln Glu Arg Phe Arg Thr Ile  
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Thr Ser Thr Tyr Tyr Arg Gly Thr His Gly Val Ile Val Val Tyr Asp

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Val Thr Ser Ala Glu Ser Phe Val Asn Val Lys Arg Trp Leu His Glu

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Ile Asn Gln Asn Cys Asp Asp Val Cys Arg Ile Leu Val Gly Asn Lys

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155

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<211> 201  
<212> PRT

EP 1 737 979 B9

<213> Homo sapiens

<400> 113

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50 55 60  
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45 Ala Gly Gln Glu Arg Phe Arg Thr Ile Thr Ser Thr Tyr Tyr Arg Gly  
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						Val	Thr
						Ser	Ala
						Glu	Ser
						Phe	
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						His	Glu
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						Gln	Asn
						Cys	Asp
						Asp	
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						Asp	Pro
						Glu	Arg
						Lys	
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						Phe	Ala
						Gly	Gln
						Met	Gly
						Ile	
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						Asn	Val
						Glu	Glu
						Met	
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						Arg	Ala
						Lys	Lys
						Asp	Asn
						Leu	
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&lt;210&gt; 114

&lt;211&gt; 19

&lt;212&gt; DNA

&lt;213&gt; Artificial

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&lt;220&gt;

&lt;223&gt; A target sequence for siRNA.

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&lt;400&gt; 114

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&lt;210&gt; 115

&lt;211&gt; 51

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50

## Claims

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1. A method of diagnosing non-small cell lung cancer (NSCLC) or a predisposition to developing non-small cell lung cancer in a subject, comprising determining the expression level of a non-small cell lung cancer-associated gene in a biological sample derived from the subject, wherein an increase of said expression level compared to a normal

control level of said gene indicates that said subject suffers from or is at risk of developing NSCLC, wherein said NSCLC-associated gene is KIF11.

2. The method of claim 1, wherein said increase is at least 10% greater than said normal control level.

3. The method of claim 1 or 2, wherein said method further comprises determining the expression level of a plurality of NSCLC-associated genes.

4. The method of any one of claims 1 to 3, wherein said expression level is determined by any one method selected from the group consisting of:

- (1) detecting mRNA of the NSCLC-associated gene;
- (2) detecting protein encoded by the NSCLC-associated gene; and
- (3) detecting the biological activity of protein encoded by the NSCLC-associated gene.

5. The method of any one of claims 1 to 3, wherein said expression level is determined by detecting hybridization of an NSCLC-associated gene probe to a gene transcript of said patient-derived biological sample.

6. The method of claim 5, wherein said hybridization step is carried out on a DNA array.

7. The method of any one of claims 1 to 6, wherein said biological sample comprises sputum or blood.

8. An in vitro method of identifying a compound for treating or preventing NSCLC, comprising the steps of:

- (1) contacting a test cell expressing said NSCLC-associated gene with a test compound;
- (2) detecting the expression level of said NSCLC-associated gene; and
- (3) determining the compound that suppresses said expression level compared to a normal control level of said gene

wherein said NSCLC-associated gene is KIF11 and said compound is for use in treating or preventing NSCLC.

9. The method of claim 8, wherein said test cell is NSCLC cell.

10. An in vitro method of screening for a compound for treating or preventing NSCLC, said method comprising the steps of:

- (1) contacting a test compound with a polypeptide of KIF11;
- (2) detecting the binding activity between the polypeptide and the test compound; and
- (3) selecting a compound that binds to the polypeptide

wherein said compound is for use in treating or preventing NSCLC.

11. An in vitro method of screening for a compound for treating or preventing NSCLC, said method comprising the steps of:

- (a) contacting a test compound with a polypeptide encoded by a polynucleotide of KIF11;
- (b) detecting the biological activity of the polypeptide of step (a); and
- (c) selecting a compound that suppresses the biological activity of the polypeptide of KIF11 in comparison with the biological activity detected in the absence of the test compound

wherein said compound is for use in treating or preventing NSCLC.

12. The method of claim 11, wherein said biological activity is cell proliferative activity.

13. An in vitro method of screening for a compound for treating or preventing NSCLC, said method comprising the steps of:

- (1) contacting a test compound with a cell expressing KIF11; and
- (2) selecting a compound that reduces the expression level of KIF11

wherein said compound is for use in treating or preventing NSCLC.

14. The method of claim 13, wherein said cell is NSCLC cell.

15. An in vitro method of screening for a compound for treating or preventing NSCLC, said method comprising the steps of:

- (1) contacting a test compound with a cell into which a vector comprising the transcriptional regulatory region of KIF11 and a reporter gene that is expressed under the control of the transcriptional regulatory region has been introduced;
- (2) measuring the activity of said reporter gene; and
- (3) selecting a compound that reduces the expression level of said reporter gene, as compared to a control

wherein said compound is for use in treating or preventing NSCLC.

16. An in vitro method of screening for a compound for treating or preventing NSCLC, said method comprising the steps of:

- (1) contacting a KIF11 polypeptide or functional equivalent thereof with KOC1 polypeptide or functional equivalent thereof in the presence of a test compound;
- (2) detecting the binding between the polypeptides; and
- (3) selecting the test compound that inhibits the binding between the polypeptides.

17. The method of claim 16, wherein the functional equivalent of KIF11 polypeptide comprises an amino acid sequence of KOC1 binding domain.

18. The method of claim 16, wherein the functional equivalent of KOC1 polypeptide comprises an amino acid sequence of KIF11 binding domain.

19. An in vitro method of measuring RNA transporting activity of a polypeptide, said method comprising the steps of:

(a) contacting a polypeptide selected from the group consisting of:

- i. a polypeptide comprising the amino acid sequence of SEQ ID NO: 2 (*KIF11*);
- ii. a polypeptide comprising the amino acid sequence of SEQ ID NO: 2 wherein one or more amino acids are substituted, deleted, or inserted, and said polypeptide has a biological activity equivalent to the polypeptide consisting of the amino acid sequence of SEQ ID NO: 2;
- iii. a polypeptide that comprises the amino acid sequence having at least about 80% homology to SEQ ID NO: 2; and
- iv. 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 an RNA to be transported and under the condition capable of RNA transporter formation;

(b) detecting the level of the transported RNA; and

(c) measuring the RNA transporting activity by correlating the level of the transported RNA of step (b) with the RNA transporting activity.

20. The method of claim 19, wherein the condition capable of RNA transporter formation is provided in the existence of KOC1 polypeptide or functional equivalent thereof.

21. The method of claim 20, wherein the functional equivalent of KOC1 polypeptide is a polypeptide selected from the group consisting of:

- (i) a polypeptide comprising the amino acid sequence of SEQ ID NO: 105 (*KOC1*);
- (ii) a polypeptide comprising the amino acid sequence of SEQ ID NO: 105 wherein one or more amino acids are substituted, deleted, or inserted, and said polypeptide has a biological activity equivalent to the polypeptide consisting of the amino acid sequence of SEQ ID NO: 105;
- (iii) a polypeptide that comprises the amino acid sequence having at least about 80% homology to SEQ ID NO: 105; and
- (iv) a polypeptide encoded by a polynucleotide that hybridizes under stringent conditions to a polynucleotide consisting of the nucleotide sequence of SEQ ID NO: 104, wherein the polypeptide has a biological activity



equivalent to a polypeptide consisting of the amino acid sequence of SEQ ID NO: 105.

22. An in vitro method of identifying an agent that modulates RNA transporting activity, said method comprising the steps of:

(a) contacting the agent with a polypeptide selected from the group consisting of:

- i. a polypeptide comprising the amino acid sequence of SEQ ID NO: 2 (*KIF11*);
- ii. a polypeptide comprising the amino acid sequence of SEQ ID NO: 2 wherein one or more amino acids are substituted, deleted, or inserted, and said polypeptide has a biological activity equivalent to the polypeptide consisting of the amino acid sequence of SEQ ID NO: 2;
- iii. a polypeptide that comprises the amino acid sequence having at least about 80% homology to SEQ ID NO: 2; and
- iv. 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 an RNA to be transported and under the condition capable of RNA transporter formation;

(b) detecting the level of the transported RNA; and

(c) comparing the level of the transported RNA to a control level in the absence of the agent wherein an increase or decrease in the level of the transported RNA compared to control level indicates that the test compound modulates RNA transporting activity.

23. A kit for detecting for an activity of a test compound to regulate RNA transporting activity, said kit comprising an isolated cell expressing the components of a to d, and culture medium supporting the cell growth:

(a) a polypeptide selected from the group consisting of:

- i. a polypeptide comprising the amino acid sequence of SEQ ID NO: 2 (*KIF11*);
- ii. a polypeptide comprising the amino acid sequence of SEQ ID NO: 2 wherein one or more amino acids are substituted, deleted, or inserted, and said polypeptide has a biological activity equivalent to the polypeptide consisting of the amino acid sequence of SEQ ID NO: 2;
- iii. a polypeptide that comprises the amino acid sequence having at least about 80% homology to SEQ ID NO: 2; and
- iv. 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;

(b) a polypeptide selected from the group consisting of:

- i. a polypeptide comprising the amino acid sequence of SEQ ID NO: 105 (*KOC1*);
- ii. a polypeptide comprising the amino acid sequence of SEQ ID NO: 105 wherein one or more amino acids are substituted, deleted, or inserted, and said polypeptide has a biological activity equivalent to the polypeptide consisting of the amino acid sequence of SEQ ID NO: 105;
- iii. a polypeptide that comprises the amino acid sequence having at least about 80% homology to SEQ ID NO: 105; and
- iv. a polypeptide encoded by a polynucleotide that hybridizes under stringent conditions to a polynucleotide consisting of the nucleotide sequence of SEQ ID NO: 104, wherein the polypeptide has a biological activity equivalent to a polypeptide consisting of the amino acid sequence of SEQ ID NO: 105; and

(c) an RNA to be transported; and

(d) DCTN1.

24. Use of

- (i) an antisense composition, said composition comprising a nucleotide sequence complementary to a coding sequence of *KIF11*;
- (ii) an siRNA composition comprising an siRNA, wherein said composition reduces the expression of *KIF11*; or

(iii) an antibody or fragment thereof that binds to a polypeptide encoded by KIF11;  
for the preparation of a pharmaceutical composition of treating or preventing NSCLC in a subject.

25. The use of claim 24, wherein the siRNA is a sense strand comprising the nucleotide sequence selected from the group of SEQ ID NOs: 32, 33 and 34 as the target sequence.

26. The use of claim 25, wherein the siRNA has the general formula 5'-[A]-[B]-[A']-3' wherein [A] is a ribonucleotide sequence corresponding to a sequence selected from the group consisting of SEQ ID NOs: 32, 33 and 34; [B] is a ribonucleotide sequence consisting of 3 to 23 nucleotides; and [A'] is a ribonucleotide sequence complementary to [A].

27. Use of a polypeptide encoded by KIF11 or an immunologically active fragment of said polypeptide, or a polynucleotide encoding the polypeptide for the preparation of a vaccine for treating or preventing NSCLC in a subject.

28. A double-stranded molecule comprising a sense strand and an antisense strand, wherein the sense strand comprises a ribonucleotide sequence corresponding to a KIF11 target sequence selected from the group consisting of SEQ ID NOs: 32, 33 and 34, and wherein the antisense strand comprises a ribonucleotide sequence which is complementary to said sense strand, wherein said sense strand and said antisense strand hybridize to each other to form said double-stranded molecule, and wherein said double-stranded molecule, when introduced into a cell expressing a KIF11 gene, inhibits the expression of said gene, further wherein the double-stranded molecule is an oligonucleotide of between about 19 and about 25 nucleotides in length.

29. The double-stranded molecule of claim 28, wherein said double-stranded molecule is a single ribonucleotide transcript comprising the sense strand and the antisense strand linked via a single-stranded ribonucleotide sequence.

30. A vector encoding the double-stranded molecule of any one of claims 28 to 29.

31. The vector of claim 30, wherein the vector encodes a transcript having a secondary structure and comprises the sense strand and the antisense strand.

32. The vector of claim 31, wherein the transcript further comprises a single-stranded ribonucleotide sequence linking said sense strand and said antisense strand.

33. The vector of claim 32, wherein said polynucleotide has the general formula 5'-[A]-[B]-[A']-3' wherein [A] is a nucleotide sequence selected from the group consisting of SEQ ID NOs: 32, 33 and 34; [B] is a nucleotide sequence consisting of 3 to 23 nucleotides; and [A'] is a nucleotide sequence complementary to [A].

34. A pharmaceutical composition comprising an siRNA against KIF11 gene, wherein the siRNA comprises a sense strand comprising the nucleotide sequence of SEQ ID NO: 32, 33 and 34 as the target sequence and having about 19 to about 25 nucleotides in length.

35. A method of predicting an NSCLC prognosis, wherein the method comprises the steps of:

- (a) detecting expressing level of KIF11 in a specimen collected from a subject whose NSCLC prognosis is to be predicted, and
- (b) indicating a poor prognosis when an elevation of the expressing level of KIF11 is detected.

36. The method of claim 35, wherein the expression level is detected by any one of the method selected from the group consisting of:

- (a) detecting the mRNA encoding the amino acid sequence of SEQ ID NO: 2 (*KIF11*),
- (b) detecting the protein comprising the amino acid sequence of SEQ ID NO: 2 (*KIF11*), and
- (c) detecting the biological activity of the protein comprising the amino acid sequence of SEQ ID NO: 2 (*KIF11*).

## Patentansprüche

1. Verfahren zur Diagnose von nicht-kleinzelligem Lungenkrebs (NSCLC) oder einer Veranlagung zur Entwicklung

von nicht-kleinzelligem Lungenkrebs in einem Individuum, umfassend das Bestimmen des Expressionsspiegels eines Gens, das mit nicht-kleinzelligem Lungenkrebs assoziiert ist, in einer biologischen Probe, die von dem Individuum stammt, wobei eine Erhöhung des Expressionsspiegels verglichen mit einem normalen Kontrollspiegel des Gens anzeigt, dass das Individuum an NSCLC leidet oder gefährdet ist, NSCLC zu entwickeln, wobei das NSCLC-assoziierte Gen KIF11 ist.

2. Verfahren nach Anspruch 1, wobei die Erhöhung mindestens 10% höher ist als der normale Kontrollspiegel.
3. Verfahren nach Anspruch 1 oder 2, wobei das Verfahren zudem die Bestimmung des Expressionsspiegels einer Vielzahl von NSCLC-assoziierten Genen umfasst.
4. Verfahren nach einem der Ansprüche 1 bis 3, wobei der Expressionsspiegel bestimmt wird durch ein Verfahren, das ausgewählt ist aus der Gruppe bestehend aus:

- (1) Nachweis der mRNA des NSCLC-assoziierten Gens;
- (2) Nachweis des Proteins, das durch das NSCLC-assoziierte Gen codiert wird;
- und
- (3) Nachweis der biologischen Aktivität des Proteins, das durch das NSCLC-assoziierte Gen codiert wird.

5. Verfahren nach einem der Ansprüche 1 bis 3, wobei der Expressionsspiegel bestimmt wird durch den Nachweis der Hybridisierung einer NSCLC-assoziierten Gensonde an ein Transkript eines Gens der biologischen Probe, die von dem Patienten stammt.

6. Verfahren nach Anspruch 5, wobei der Hybridisierungsschritt auf einem DNA-Array ausgeführt wird.

7. Verfahren nach einem der Ansprüche 1 bis 6, wobei die biologische Probe Sputum oder Blut umfasst.

8. In-vitro-Verfahren zur Identifizierung einer Verbindung zur Behandlung oder Vorbeugung von NSCLC, die Schritte umfassend:

- (1) Inkontaktbringen einer Testzelle, die das NSCLC-assoziierte Gen exprimiert, mit einer Testverbindung;
- (2) Nachweis des Expressionsspiegels des NSCLC-assoziierten Gens; und
- (3) Bestimmen der Verbindung, die den Expressionsspiegel unterdrückt verglichen mit einem normalen Kontrollspiegel des Gens,

wobei das NSCLC-assoziierte Gen KIF11 ist und die Verbindung für die Verwendung bei der Behandlung oder Vorbeugung von NSCLC ist.

9. Verfahren nach Anspruch 8, wobei die Testzelle eine NSCLC-Zelle ist.

10. In-vitro-Verfahren zum Durchsuchen nach einer Verbindung zur Behandlung oder Vorbeugung von NSCLC, wobei das Verfahren die Schritte umfasst:

- (1) Inkontaktbringen einer Testverbindung mit einem Polypeptid von KIF11;
- (2) Nachweis der Bindungsaktivität zwischen dem Polypeptid und der Testverbindung; und
- (3) Auswählen einer Verbindung, die an das Polypeptid bindet,

wobei die Verbindung für die Verwendung bei der Behandlung oder Vorbeugung von NSCLC ist.

11. In-vitro-Verfahren zum Durchsuchen nach einer Verbindung zur Behandlung oder Vorbeugung von NSCLC, wobei das Verfahren die Schritte umfasst:

- (a) Inkontaktbringen einer Testverbindung mit einem Polypeptid, das durch ein Polynucleotid von KIF11 codiert ist;
- (b) Nachweis der biologischen Aktivität des Polypeptids aus Schritt (a); und
- (c) Auswählen einer Verbindung, die die biologische Aktivität des Polypeptids von KIF11 unterdrückt im Vergleich zur biologischen Aktivität, die in der Abwesenheit der Testverbindung nachgewiesen wurde,

wobei die Verbindung für die Verwendung bei der Behandlung oder Vorbeugung von NSCLC ist.

12. Verfahren nach Anspruch 11, wobei die biologische Aktivität Zellproliferationsaktivität ist.

13. In-vitro-Verfahren zum Durchsuchen nach einer Verbindung zur Behandlung oder Vorbeugung von NSCLC, wobei das Verfahren die Schritte umfasst:

- (1) Inkontaktbringen einer Testverbindung mit einer Zelle, die KIF11 exprimiert; und
- (2) Auswählen einer Verbindung, die den Expressionsspiegel von KIF11 reduziert, wobei die Verbindung für die Verwendung bei der Behandlung oder Vorbeugung von NSCLC ist.

14. Verfahren nach Anspruch 13, wobei die Zelle eine NSCLC-Zelle ist.

15. In-vitro-Verfahren zum Durchsuchen nach einer Verbindung zur Behandlung oder Vorbeugung von NSCLC, wobei das Verfahren die Schritte umfasst:

- (1) Inkontaktbringen einer Testverbindung mit einer Zelle, in die ein Vektor eingeführt wurde, der die Transkriptionsregulationsregion von KIF11 und ein Reportergen umfasst, das unter der Kontrolle der Transkriptionsregulationsregion exprimiert wird;
- (2) Messen der Aktivität des Reportergens; und
- (3) Auswählen einer Verbindung, die den Expressionsspiegel des Reportergens reduziert, verglichen mit einer Kontrolle,

wobei die Verbindung für die Verwendung bei der Behandlung oder Vorbeugung von NSCLC ist.

16. In-vitro-Verfahren zum Durchsuchen nach einer Verbindung zur Behandlung oder Vorbeugung von NSCLC, wobei das Verfahren die Schritte umfasst:

- (1) Inkontaktbringen eines KIF11-Polypeptids oder eines funktionellen Äquivalents davon mit KOC1-Polypeptid oder einem funktionellen Äquivalent davon in der Gegenwart einer Testverbindung;
- (2) Nachweis der Bindung zwischen den Polypeptiden; und
- (3) Auswählen der Testverbindung, die die Bindung zwischen den Polypeptiden inhibiert.

17. Verfahren nach Anspruch 16, wobei das funktionelle Äquivalent des KIF11-Polypeptids eine Aminosäuresequenz der KOC1-Bindungsdomäne umfasst.

18. Verfahren nach Anspruch 16, wobei das funktionelle Äquivalent des KOC1-Polypeptids eine Aminosäuresequenz der KIF11-Bindungsdomäne umfasst.

19. In-vitro-Verfahren zum Messen der RNA-Transportaktivität eines Polypeptids, wobei das Verfahren die Schritte umfasst:

(a) Inkontaktbringen eines Polypeptids, das ausgewählt ist aus der Gruppe bestehend aus:

- i. einem Polypeptid, das die Aminosäuresequenz von SEQ ID NO:2 (*KIF11*) umfasst;
- ii. einem Polypeptid, das die Aminosäuresequenz von SEQ ID NO:2 umfasst, wobei eine oder mehrere Aminosäuren substituiert, deletiert oder inseriert sind, und das Polypeptid eine biologische Aktivität hat, die äquivalent ist zu der des Polypeptids, das aus der Aminosäuresequenz von SEQ ID NO:2 besteht;
- iii. einem Polypeptid, das die Aminosäuresequenz umfasst, die mindestens etwa 80% Homologie zu SEQ ID NO:2 hat; und
- iv. einem Polypeptid, das codiert wird durch ein Polynucleotid, das unter stringenten Bedingungen an ein Polynucleotid hybridisiert, das aus der Nucleotidsequenz von SEQ ID NO:1 besteht, wobei das Polypeptid eine biologische Aktivität hat, die äquivalent ist zu der eines Polypeptids, das aus der Aminosäuresequenz von SEQ ID NO:2 besteht;

mit einer zu transportierenden RNA und unter der Bedingung der Fähigkeit zur Bildung eines RNA-Transporters;

(b) Nachweis des Spiegels der transportierten RNA; und

(c) Messen der RNA-Transportaktivität durch Korrelieren des Spiegels der transportierten RNA aus Schritt (b)

mit der RNA-Transportaktivität.

20. Verfahren nach Anspruch 19, wobei die Bedingung der Fähigkeit zur Bildung eines RNA-Transporters bereitgestellt wird durch die Existenz des KOC1-Polypeptids oder eines funktionellen Äquivalents davon.

21. Verfahren nach Anspruch 20, wobei das funktionelle Äquivalent des KOC1-Polypeptids ein Polypeptid ist, das ausgewählt ist aus der Gruppe bestehend aus:

- (i) einem Polypeptid, das die Aminosäuresequenz von SEQ ID NO:105 (*KOC1*) umfasst;
- (ii) einem Polypeptid, das die Aminosäuresequenz von SEQ ID NO:105 umfasst, wobei eine oder mehrere Aminosäuren substituiert, deletiert oder inseriert sind, und das Polypeptid eine biologische Aktivität hat, die äquivalent ist zu der des Polypeptids, das aus der Aminosäuresequenz von SEQ ID NO: 105 besteht;
- (iii) einem Polypeptid, das die Aminosäuresequenz umfasst, die mindestens etwa 80% Homologie zu SEQ ID NO:105 hat; und
- (iv) einem Polypeptid, das codiert wird durch ein Polynucleotid, das unter stringenten Bedingungen an ein Polynucleotid hybridisiert, das aus der Nucleotidsequenz von SEQ ID NO:104 besteht, wobei das Polypeptid eine biologische Aktivität hat, die äquivalent ist zu der eines Polypeptids, das aus der Aminosäuresequenz von SEQ ID NO:105 besteht.

22. In-vitro-Verfahren zur Identifizierung eines Wirkstoffs, der die RNA-Transportaktivität moduliert, wobei das Verfahren die Schritte umfasst:

(a) Inkontaktbringen des Wirkstoffs mit einem Polypeptid, das ausgewählt ist aus der Gruppe bestehend aus:

- i. einem Polypeptid, das die Aminosäuresequenz von SEQ ID NO:2 (*KIF11*) umfasst;
- ii. einem Polypeptid, das die Aminosäuresequenz von SEQ ID NO:2 umfasst, wobei eine oder mehrere Aminosäuren substituiert, deletiert oder inseriert sind, und das Polypeptid eine biologische Aktivität hat, die äquivalent ist zu der des Polypeptids, das aus der Aminosäuresequenz von SEQ ID NO:2 besteht;
- iii. einem Polypeptid, das die Aminosäuresequenz umfasst, die mindestens etwa 80% Homologie zu SEQ ID NO:2 hat; und
- iv. einem Polypeptid, das codiert wird durch ein Polynucleotid, das unter stringenten Bedingungen an ein Polynucleotid hybridisiert, das aus der Nucleotidsequenz von SEQ ID NO:1 besteht, wobei das Polypeptid eine biologische Aktivität hat, die äquivalent ist zu der eines Polypeptids, das aus der Aminosäuresequenz von SEQ ID NO:2 besteht;

mit einer zu transportierenden RNA und unter der Bedingung der Fähigkeit zur Bildung eines RNA-Transporters;

(b) Nachweis des Spiegels der transportierten RNA; und

(c) Vergleichen des Spiegels der transportierten RNA mit einem Kontrollspiegel in der Abwesenheit des Wirkstoffs, wobei eine Erhöhung oder Verringerung des Spiegels der transportierten RNA verglichen mit dem Kontrollspiegel anzeigt, dass die Testverbindung die RNA-Transportaktivität moduliert.

23. Kit zum Nachweis einer Aktivität einer Testverbindung zur Regulation der RNA-Transportaktivität, wobei der Kit eine isolierte Zelle umfasst, die die Komponenten a bis d exprimiert, und Kulturmedium, das das Zellwachstum unterstützt:

(a) ein Polypeptid, ausgewählt aus der Gruppe bestehend aus:

- i. einem Polypeptid, das die Aminosäuresequenz von SEQ ID NO:2 (*KIF11*) umfasst;
- ii. einem Polypeptid, das die Aminosäuresequenz von SEQ ID NO:2 umfasst, wobei eine oder mehrere Aminosäuren substituiert, deletiert oder inseriert sind, und das Polypeptid eine biologische Aktivität hat, die äquivalent ist zu der des Polypeptids, das aus der Aminosäuresequenz von SEQ ID NO:2 besteht;
- iii. einem Polypeptid, das die Aminosäuresequenz umfasst, die mindestens etwa 80% Homologie zu SEQ ID NO:2 hat; und
- iv. einem Polypeptid, das codiert wird durch ein Polynucleotid, das unter stringenten Bedingungen an ein Polynucleotid hybridisiert, das aus der Nucleotidsequenz von SEQ ID NO:1 besteht, wobei das Polypeptid eine biologische Aktivität hat, die äquivalent ist zu der eines Polypeptids, das aus der Aminosäuresequenz von SEQ ID NO:2 besteht;

(b) ein Polypeptid, ausgewählt aus der Gruppe bestehend aus:

- (i) einem Polypeptid, das die Aminosäuresequenz von SEQ ID NO:105 (*KOC1*) umfasst;
- (ii) einem Polypeptid, das die Aminosäuresequenz von SEQ ID NO:105 umfasst, wobei eine oder mehrere Aminosäuren substituiert, deletiert oder inseriert sind, und das Polypeptid eine biologische Aktivität hat, die äquivalent ist zu der des Polypeptids, das aus der Aminosäuresequenz von SEQ ID NO:105 besteht;
- (iii) einem Polypeptid, das die Aminosäuresequenz umfasst, die mindestens etwa 80% Homologie zu SEQ ID NO:105 hat; und
- (iv) einem Polypeptid, das codiert wird durch ein Polynucleotid, das, unter stringenten Bedingungen an ein Polynucleotid hybridisiert, das aus der Nucleotidsequenz von SEQ ID NO:104 besteht, wobei das Polypeptid eine biologische Aktivität hat, die äquivalent ist zu der eines Polypeptids, das aus der Aminosäuresequenz von SEQ ID NO:105 besteht; und

(c) eine zu transportierende RNA; und

(d) *DCTN1*.

#### 24. Verwendung von

- (i) einer Antisense-Zusammensetzung, wobei die Zusammensetzung eine Nucleotidsequenz umfasst, die komplementär ist zu einer codierenden Sequenz von *KIF11*;
- (ii) einer siRNA-Zusammensetzung umfassend eine siRNA, wobei die Zusammensetzung die Expression von *KIF11* reduziert; oder
- (iii) einem Antikörper oder Fragment davon, der an ein durch *KIF11* codiertes Polypeptid bindet;

für die Herstellung eines Arzneimittels zur Behandlung oder Vorbeugung von NSCLC in einem Individuum.

**25.** Verwendung nach Anspruch 24, wobei die siRNA ein Sensestrang ist, der die Nucleotidsequenz umfasst, die ausgewählt ist aus der Gruppe bestehend aus SEQ ID NOs: 32, 33 und 34 als die Zielsequenz.

**26.** Verwendung nach Anspruch 25, wobei die siRNA die allgemeine Formel 5'-[A]-[B]-[A']-3' hat, wobei [A] eine Ribonucleotidsequenz ist, die einer Sequenz entspricht, die ausgewählt ist aus der Gruppe bestehend aus SEQ ID NOs: 32, 33 und 34; [B] eine Ribonucleotidsequenz ist, die aus 3 bis 23 Nucleotiden besteht; und [A'] eine Ribonucleotidsequenz ist, die komplementär zu [A] ist.

**27.** Verwendung eines durch *KIF11* codierten Polypeptids oder eines immunologisch aktiven Fragments von diesem Polypeptid, oder eines Polynucleotids, das das Polypeptid codiert, für die Herstellung eines Impfstoffs zur Behandlung oder Vorbeugung von NSCLC in einem Individuum.

**28.** Doppelsträngiges Molekül umfassend einen Sensestrang und einen Antisensestrang, wobei der Sensestrang eine Ribonucleotidsequenz umfasst, die einer *KIF11*-Zielsequenz entspricht, die ausgewählt ist aus der Gruppe bestehend aus SEQ ID NOs: 32, 33 und 34, und wobei der Antisensestrang eine Ribonucleotidsequenz umfasst, die komplementär ist zu dem Sensestrang, wobei der Sensestrang und Antisensestrang miteinander hybridisieren unter Bildung des doppelsträngigen Moleküls und wobei das doppelsträngige Molekül, wenn es in eine Zelle eingeführt wird, die ein *KIF11*-Gen exprimiert, die Expression dieses Gens inhibiert, wobei zudem das doppelsträngige Molekül ein Oligonucleotid mit einer Länge zwischen etwa 19 und etwa 25 Nucleotiden ist.

**29.** Doppelsträngiges Molekül nach Anspruch 28, wobei das doppelsträngige Molekül ein einzelnes Ribonucleotidtranskript ist, das den Sensestrang und den Antisensestrang verbunden über eine einzelsträngige Ribonucleotidsequenz umfasst.

**30.** Vektor, der das doppelsträngige Molekül nach einem der Ansprüche 28 bis 29 codiert.

**31.** Vektor nach Anspruch 30, wobei der Vektor ein Transkript codiert, das eine Sekundärstruktur hat und den Sensestrang und den Antisensestrang umfasst.

**32.** Vektor nach Anspruch 31, wobei das Transkript zudem eine einzelsträngige Ribonucleotidsequenz umfasst, die den Sensestrang und den Antisensestrang verbindet.

33. Vektor nach Anspruch 32, wobei das Polynucleotid die allgemeine Formel 5'-[A]-[B]-[A']-3' hat, wobei [A] eine Nucleotidsequenz ist, die ausgewählt ist aus der Gruppe bestehend aus SEQ ID NOs: 32, 33 und 34; [B] eine Nucleotidsequenz ist, die aus 3 bis 23 Nucleotiden besteht; und [A'] eine Nucleotidsequenz ist, die komplementär zu [A] ist.

34. Arzneimittel umfassend eine siRNA gegen das KIF11-Gen, wobei die siRNA einen Sensestrang umfasst, der die Nucleotidsequenz von SEQ ID NO:32, 33 und 34 als die Zielsequenz umfasst und eine Länge von etwa 19 bis etwa 25 Nucleotiden hat.

35. Verfahren zur Vorhersage einer NSCLC-Prognose, wobei das Verfahren die Schritte umfasst:

(a) Nachweis des Expressionsspiegels von KIF11 in einer Probe, die gewonnen wurde von einem Individuum, dessen NSCLC-Prognose vorherzusagen ist; und

(b) Anzeigen einer schlechten Prognose, wenn eine Anhebung des Expressionsspiegels von KIF11 nachgewiesen wird.

36. Verfahren nach Anspruch 35, wobei der Expressionsspiegel ermittelt wird durch ein Verfahren ausgewählt aus der Gruppe bestehend aus:

(a) Nachweis der mRNA, die die Aminosäuresequenz von SEQ ID NO:2 (*KIF11*) codiert;

(b) Nachweis des Proteins, das die Aminosäuresequenz von SEQ ID NO:2 (*KIF11*) umfasst; und

(c) Nachweis der biologischen Aktivität des Proteins, das die Aminosäuresequenz von SEQ ID NO:2 (*KIF11*) umfasst.

## Revendications

1. Procédé pour diagnostiquer le cancer du poumon non à petites cellules (CPNPC) ou une prédisposition au développement du cancer du poumon non à petites cellules chez un sujet, consistant à déterminer le taux d'expression d'un gène associé au cancer du poumon non à petites cellules dans un échantillon biologique dérivé du sujet, dans lequel une augmentation dudit taux d'expression par comparaison à un taux étalon normal dudit gène indique que ledit sujet souffre ou est exposé à un risque de développer un CPNPC, où ledit gène associé au CPNPC est KIF11.

2. Procédé selon la revendication 1, dans lequel ladite augmentation est au moins 10 % plus élevée que ledit taux étalon normal.

3. Procédé selon la revendication 1 ou 2, dans lequel ledit procédé consiste en outre à déterminer le taux d'expression d'une pluralité de gènes associés au CPNPC.

4. Procédé selon l'une quelconque des revendications 1 à 3, dans lequel ledit taux d'expression est déterminé par l'un quelconque des procédés choisis dans le groupe consistant en :

(1) détecter l'ARNm du gène associé au CPNPC;

(2) détecter une protéine codée par le gène associé au CPNPC ; et

(3) détecter l'activité biologique d'une protéine codée par le gène associé au CPNPC.

5. Procédé selon l'une quelconque des revendications 1 à 3, dans lequel ledit taux d'expression est déterminé en détectant l'hybridation d'une sonde d'un gène associé au CPNPC à un transcrit de gène dudit échantillon biologique dérivé du patient.

6. Procédé selon la revendication 5, dans lequel ladite étape d'hybridation est réalisée sur une puce à ADN.

7. Procédé selon l'une quelconque des revendications 1 à 6, dans lequel ledit échantillon biologique comprend des expectorations ou du sang.

8. Procédé *in vitro* pour identifier un composé destiné au traitement ou à la prévention du CPNPC, comprenant les étapes consistant à :

- (1) mettre en contact une cellule d'essai exprimant ledit gène associé au CPNPC avec un composé d'essai ;
- (2) détecter le taux d'expression dudit gène associé au CPNPC ; et
- (3) déterminer le composé qui supprime ledit taux d'expression par comparaison à un taux étalon normal dudit gène

où ledit gène associé au CPNPC est KIF11 et ledit composé est destiné à être utilisé dans le traitement ou la prévention du CPNPC.

9. Procédé selon la revendication 8, dans lequel ladite cellule d'essai est une cellule du CPNPC.

10. Procédé *in vitro* pour dépister un composé destiné au traitement ou à la prévention du CPNPC, ledit procédé comprenant les étapes consistant à :

- (1) mettre en contact un composé d'essai avec un polypeptide de KIF11 ;
- (2) détecter l'activité de liaison entre le polypeptide et le composé d'essai ; et
- (3) sélectionner un composé qui se lie au polypeptide où ledit composé est destiné à être utilisé dans le traitement ou la prévention du CPNPC.

11. Procédé *in vitro* pour dépister un composé destiné au traitement ou à la prévention du CPNPC, ledit procédé comprenant les étapes consistant à :

- (a) mettre en contact un composé d'essai avec un polypeptide codé par un polynucléotide de KIF11 ;
- (b) détecter l'activité biologique du polypeptide de l'étape (a) ; et
- (c) sélectionner un composé qui supprime l'activité biologique du polypeptide de KIF11 par comparaison à l'activité biologique détectée en l'absence du composé d'essai

où ledit composé est destiné au traitement ou à la prévention du CPNPC.

12. Procédé selon la revendication 11, dans lequel ladite activité biologique est une activité de prolifération cellulaire.

13. Procédé *in vitro* pour dépister un composé destiné au traitement ou à la prévention du CPNPC, ledit procédé comprenant les étapes consistant à :

- (1) mettre en contact un composé d'essai avec une cellule exprimant KIF11 ; et
- (2) sélectionner un composé qui réduit le taux d'expression de KIF11 où ledit composé est destiné à être utilisé dans le traitement ou la prévention du CPNPC.

14. Procédé selon la revendication 13, dans lequel ladite cellule est une cellule du CPNPC.

15. Procédé *in vitro* pour dépister un composé destiné au traitement ou à la prévention du CPNPC, ledit procédé comprenant les étapes consistant à :

- (1) mettre en contact un composé d'essai avec une cellule dans laquelle a été introduit un vecteur comprenant la région régulatrice de la transcription de KIF11 et un gène rapporteur qui est exprimé sous le contrôle de la région régulatrice de la transcription ;
- (2) mesurer l'activité dudit gène rapporteur ; et
- (3) sélectionner un composé qui réduit le taux d'expression dudit gène rapporteur, par comparaison à un contrôle

où ledit composé est destiné à être utilisé dans le traitement ou la prévention du CPNPC.

16. Procédé *in vitro* pour dépister un composé destiné au traitement ou à la prévention du CPNPC, ledit procédé comprenant les étapes consistant à :

- (1) mettre en contact un polypeptide KIF11 1 ou un équivalent fonctionnel de celui-ci avec un polypeptide KOC1 ou un équivalent fonctionnel de celui-ci en présence d'un composé d'essai ;
- (2) détecter la liaison entre les polypeptides ; et
- (3) sélectionner le composé d'essai qui inhibe la liaison entre les polypeptides.



17. Procédé selon la revendication 16, dans lequel l'équivalent fonctionnel du polypeptide KIF11 comprend une séquence d'acides aminés du domaine de liaison de KOC1.

18. Procédé selon la revendication 16, dans lequel l'équivalent fonctionnel du polypeptide KOC1 comprend une séquence d'acides aminés du domaine de liaison de KIF11.

19. Procédé *in vitro* pour mesurer une activité de transport d'ARN d'un polypeptide, ledit procédé comprenant les étapes consistant à :

(a) mettre en contact un polypeptide choisi dans le groupe consistant en :

- i. un polypeptide comprenant la séquence d'acides aminés de SEQ ID NO: 2 (*KIF11*) ;
- ii. un polypeptide comprenant la séquence d'acides aminés de SEQ ID NO: 2, où un ou plusieurs acides aminés sont substitués, supprimés, ou insérés, et ledit polypeptide possède une activité biologique équivalente au polypeptide consistant en la séquence d'acides aminés de SEQ ID NO: 2 ;
- iii. un polypeptide qui comprend la séquence d'acides aminés ayant au moins environ 80 % d'homologie avec SEQ ID NO: 2 ; et
- iv. un polypeptide codé par un polynucléotide qui s'hybride dans des conditions stringentes à un polynucléotide consistant en la séquence de nucléotides de SEQ ID NO: 1, où le polypeptide possède une activité biologique équivalente à un polypeptide consistant en la séquence d'acides aminés de SEQ ID NO: 2 ;

avec un ARN devant être transporté et dans la condition capable de former un transporteur d'ARN ;

(b) détecter le taux de l'ARN transporté ; et

(c) mesurer l'activité de transport d'ARN en corrélant le taux de l'ARN transporté de l'étape (b) avec l'activité de transport d'ARN.

20. Procédé selon la revendication 19, dans lequel la condition capable de former un transporteur d'ARN est obtenue par l'existence d'un polypeptide KOC1 ou d'un équivalent fonctionnel de celui-ci.

21. Procédé selon la revendication 20, dans lequel l'équivalent fonctionnel d'un polypeptide KOC1 est un polypeptide choisi dans le groupe consistant en :

- (i) un polypeptide comprenant la séquence d'acides aminés de SEQ ID NO: 105 (*KOC1*) ;
- (ii) un polypeptide comprenant la séquence d'acides aminés de SEQ ID NO: 105, où un ou plusieurs acides aminés sont substitués, supprimés, ou insérés, et ledit polypeptide possède une activité biologique équivalente au polypeptide consistant en la séquence d'acides aminés de SEQ ID NO: 105 ;
- (iii) un polypeptide qui comprend la séquence d'acides aminés ayant au moins environ 80 % d'homologie avec SEQ ID NO: 105 ; et
- (iv) un polypeptide codé par un polynucléotide qui s'hybride dans des conditions stringentes à un polynucléotide consistant en la séquence de nucléotides de SEQ ID NO: 104, où le polypeptide possède une activité biologique équivalente à un polypeptide consistant en la séquence d'acides aminés de SEQ ID NO: 105.

22. Procédé *in vitro* pour identifier un agent qui module une activité de transport de l'ARN, ledit procédé comprenant les étapes consistant à :

(a) mettre en contact l'agent avec un polypeptide choisi dans le groupe consistant en :

- i. un polypeptide comprenant la séquence d'acides aminés de SEQ ID NO: 2 (*KIF11*) ;
- ii. un polypeptide comprenant la séquence d'acides aminés de SEQ ID NO: 2, où un ou plusieurs acides aminés sont substitués, supprimés, ou insérés, et ledit polypeptide possède une activité biologique équivalente au polypeptide consistant en la séquence d'acides aminés de SEQ ID NO: 2 ;
- iii. un polypeptide qui comprend la séquence d'acides aminés ayant au moins environ 80 % d'homologie avec SEQ ID NO: 2 ; et
- iv. un polypeptide codé par un polynucléotide qui s'hybride dans des conditions stringentes à un polynucléotide consistant en la séquence de nucléotides de SEQ ID NO: 1, où le polypeptide possède une activité biologique équivalente à un polypeptide consistant en la séquence d'acides aminés de SEQ ID NO: 2 ;

avec un ARN devant être transporté et dans la condition capable de former un transporteur d'ARN ;

(b) détecter le taux de l'ARN transporté ; et

(c) comparer le taux de l'ARN transporté à un taux étalon en l'absence de l'agent, où une augmentation ou une réduction du taux de l'ARN transporté par comparaison à un taux étalon indique que le composé d'essai module l'activité de transport de l'ARN.

23. Kit servant à détecter l'activité de régulation d'une activité de transport de l'ARN d'un composé d'essai, ledit kit comprenant une cellule isolée exprimant les composants de a à d, et un milieu de culture pour soutenir la croissance cellulaire :

(a) un polypeptide choisi dans le groupe consistant en :

- i. un polypeptide comprenant la séquence d'acides aminés de SEQ ID NO: 2 (*KIF11*) ;
- ii. un polypeptide comprenant la séquence d'acides aminés de SEQ ID NO: 2, où un ou plusieurs acides aminés sont substitués, supprimés, ou insérés, et ledit polypeptide possède une activité biologique équivalente au polypeptide consistant en la séquence d'acides aminés de SEQ ID NO: 2 ;
- iii. un polypeptide qui comprend la séquence d'acides aminés ayant au moins environ 80 % d'homologie avec SEQ ID NO: 2 ; et
- iv. un polypeptide codé par un polynucléotide qui s'hybride dans des conditions stringentes à un polynucléotide consistant en la séquence de nucléotides de SEQ ID NO: 1, où le polypeptide possède une activité biologique équivalente à un polypeptide consistant en la séquence d'acides aminés de SEQ ID NO: 2 ;

(b) un polypeptide choisi dans le groupe consistant en :

- i. un polypeptide comprenant la séquence d'acides aminés de SEQ ID NO: 105 (*KOC1*) ;
- ii. un polypeptide comprenant la séquence d'acides aminés de SEQ ID NO: 105, où un ou plusieurs acides aminés sont substitués, supprimés, ou insérés, et ledit polypeptide possède une activité biologique équivalente au polypeptide consistant en la séquence d'acides aminés de SEQ ID NO: 105 ;
- iii. un polypeptide qui comprend la séquence d'acides aminés ayant au moins environ 80 % d'homologie avec SEQ ID NO: 105 ; et
- iv. un polypeptide codé par un polynucléotide qui s'hybride dans des conditions stringentes à un polynucléotide consistant en la séquence de nucléotides de SEQ ID NO: 104, où le polypeptide possède une activité biologique équivalente à un polypeptide consistant en la séquence d'acides aminés de SEQ ID NO: 105 ; et

(c) un ARN devant être transporté ; et

(d) DCTN1.

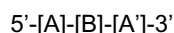
24. Utilisation de

- (i) une composition antisens, ladite composition comprenant une séquence de nucléotides complémentaire à une séquence codante de *KIF11* ;
- (ii) une composition d'ARNsi comprenant un ARNsi, où ladite composition réduit l'expression de *KIF11* ; ou
- (iii) un anticorps ou un fragment de celui-ci qui se lie à un polypeptide codé par *KIF11* ;

pour la préparation d'une composition pharmaceutique destinée au traitement ou à la prévention du CPNPC chez un sujet.

25. Utilisation selon la revendication 24, dans laquelle l'ARNsi est un brin sens comprenant la séquence de nucléotides choisie dans le groupe consistant en SEQ ID NO: 32, 33 et 34 en tant que séquence cible.

26. Utilisation selon la revendication 25, dans laquelle l'ARNsi possède la formule générale :



où [A] est une séquence de ribonucléotides correspondant à une séquence choisie dans le groupe consistant en SEQ ID NO: 32, 33 et 34 ; [B] est une séquence de ribonucléotides consistant en 3 à 23 nucléotides ; et [A'] est une séquence de ribonucléotides complémentaire à [A].

27. Utilisation d'un polypeptide codé par KIF11 ou d'un fragment immunologiquement actif dudit polypeptide, ou d'un polynucléotide codant pour le polypeptide, pour la préparation d'un vaccin destiné au traitement ou à la prévention du CPNPC chez un sujet.

28. Molécule double brin comprenant un brin sens et un brin antisens, où le brin sens comprend une séquence de ribonucléotides correspondant à une séquence cible KIF11 choisie dans le groupe consistant en SEQ ID NO: 32, 33 et 34, et où le brin antisens comprend une séquence de ribonucléotides qui est complémentaire audit brin sens, où ledit brin sens et ledit brin antisens s'hybrident entre eux pour former ladite molécule double brin, et où ladite molécule double brin, lorsqu'elle est introduite dans une cellule exprimant un gène KIF11, inhibe l'expression dudit gène, où en outre la molécule double brin est un oligonucléotide ayant une longueur comprise entre environ 19 et environ 25 nucléotides.

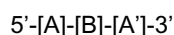
29. Molécule double brin selon la revendication 28, où ladite molécule double brin est un transcrit de ribonucléotides unique comprenant le brin sens et le brin antisens liés par une séquence de ribonucléotides simple brin.

30. Vecteur codant pour la molécule double brin selon l'une quelconque des revendications 28 à 29.

31. Vecteur selon la revendication 30, où le vecteur code pour un transcrit ayant une structure secondaire et comprend le brin sens et le brin antisens.

32. Vecteur selon la revendication 31, où le transcrit comprend en outre une séquence de ribonucléotides simple brin liant ledit brin sens et ledit brin antisens.

33. Vecteur selon la revendication 32, dans lequel ledit polynucléotide possède la formule générale



où [A] est une séquence de nucléotides choisie dans le groupe consistant en SEQ ID NO: 32, 33 et 34 ; [B] est une séquence de nucléotides consistant en 3 à 23 nucléotides ; et [A'] est une séquence de nucléotides complémentaire à [A].

34. Composition pharmaceutique comprenant un ARNsi dirigé contre un gène KIF11, où l'ARNsi comprend un brin sens comprenant la séquence de nucléotides de SEQ ID NO: 32, 33 et 34 en tant que séquence cible et ayant une longueur comprise entre environ 19 et environ 25 nucléotides.

35. Procédé pour prédire un pronostic de CPNPC, où le procédé comprend les étapes consistant à :

(a) détecter un taux d'expression de KIF11 dans un spécimen collecté chez un sujet chez qui le pronostic de CPNPC doit être prédit, et

(b) indiquer un mauvais pronostic lorsqu'une élévation du taux d'expression de KIF11 est détectée.

36. Procédé selon la revendication 35, dans lequel le taux d'expression est détecté par l'un quelconque des procédés choisis dans le groupe consistant en :

(a) détecter l'ARNm codant pour la séquence d'acides aminés de SEQ ID NO: 2 (KIF11),

(b) détecter la protéine comprenant la séquence d'acides aminés de SEQ ID NO: 2 (KIF11), et

(c) détecter l'activité biologique de la protéine comprenant la séquence d'acides aminés de SEQ ID NO: 2 (KIF11).



Fig. 1

**C**

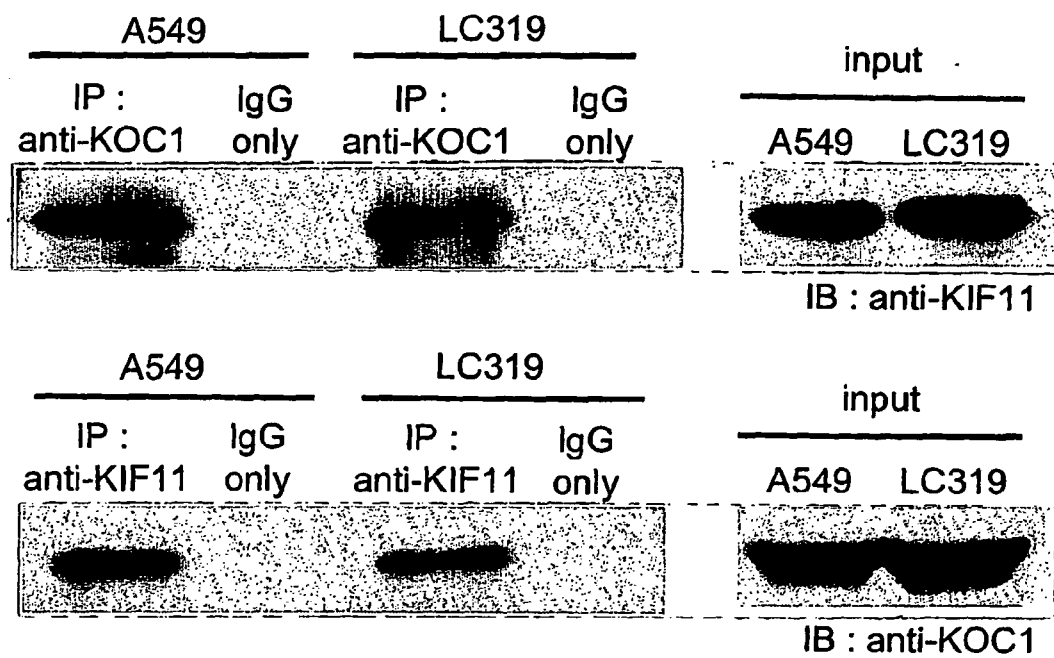


Fig. 2

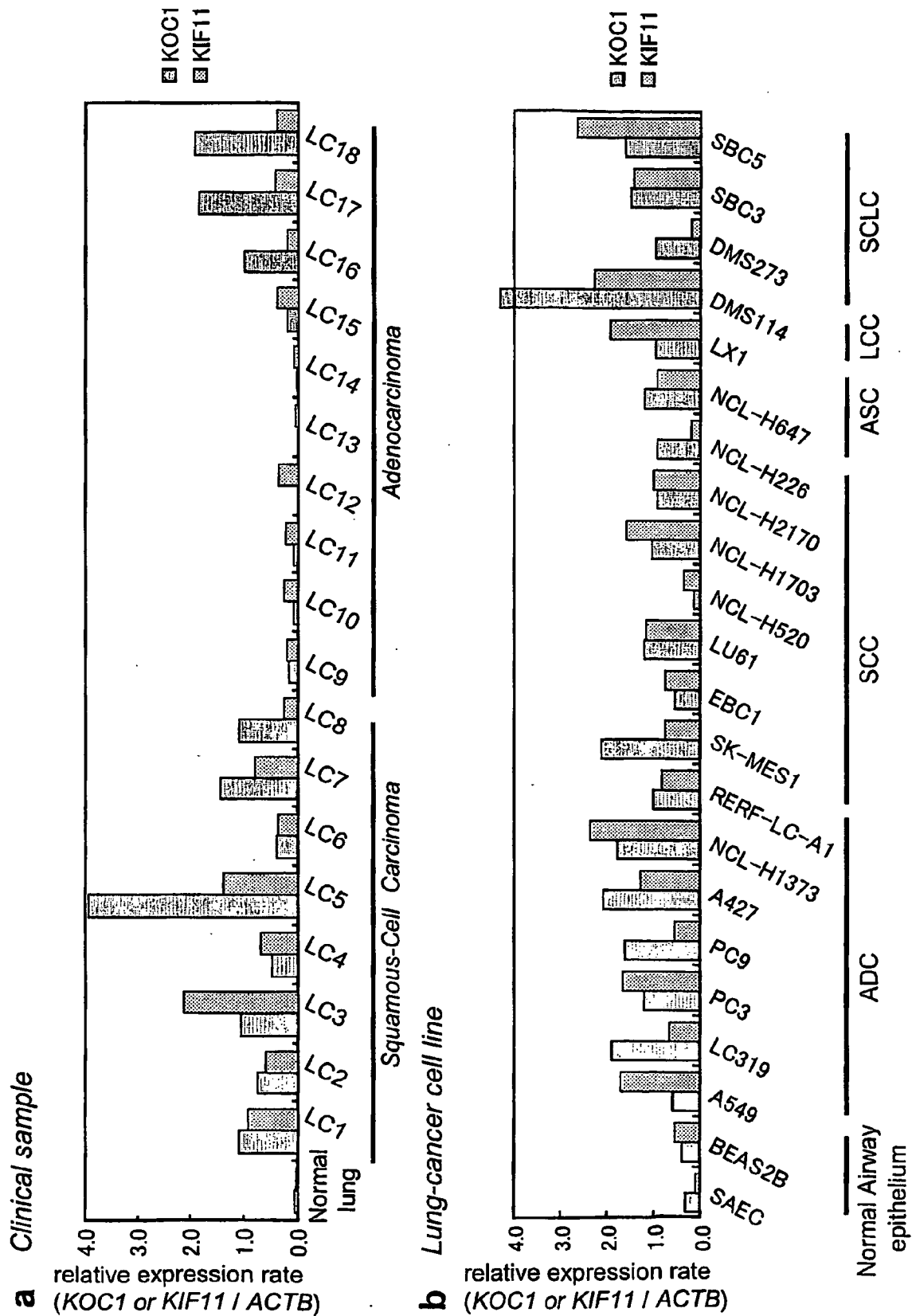
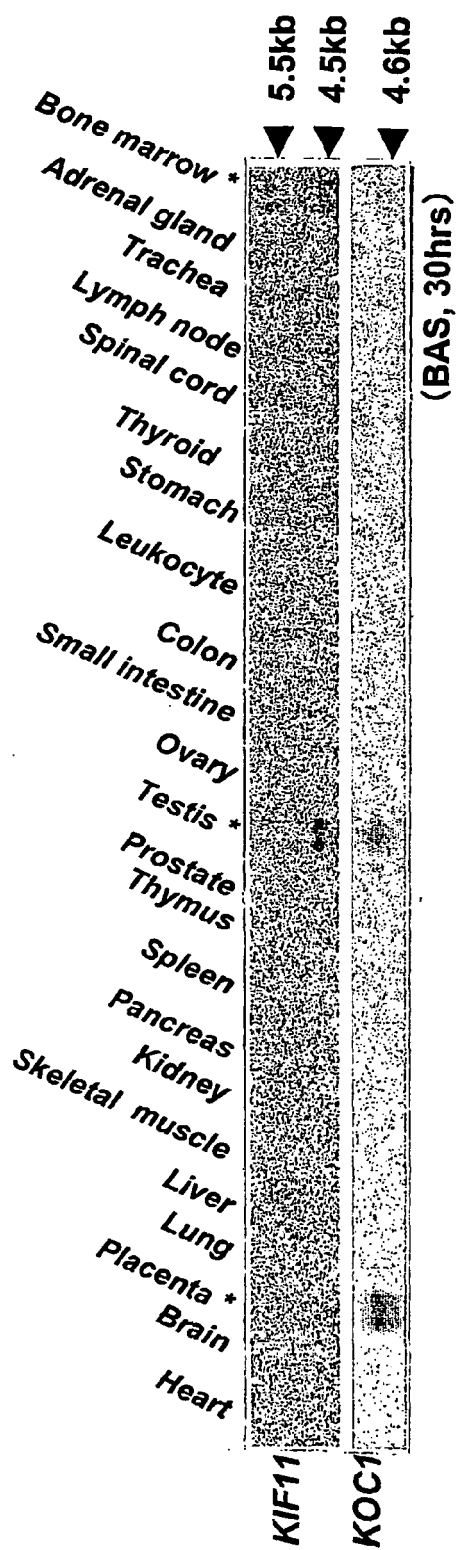


Fig. 2



c

Fig. 3

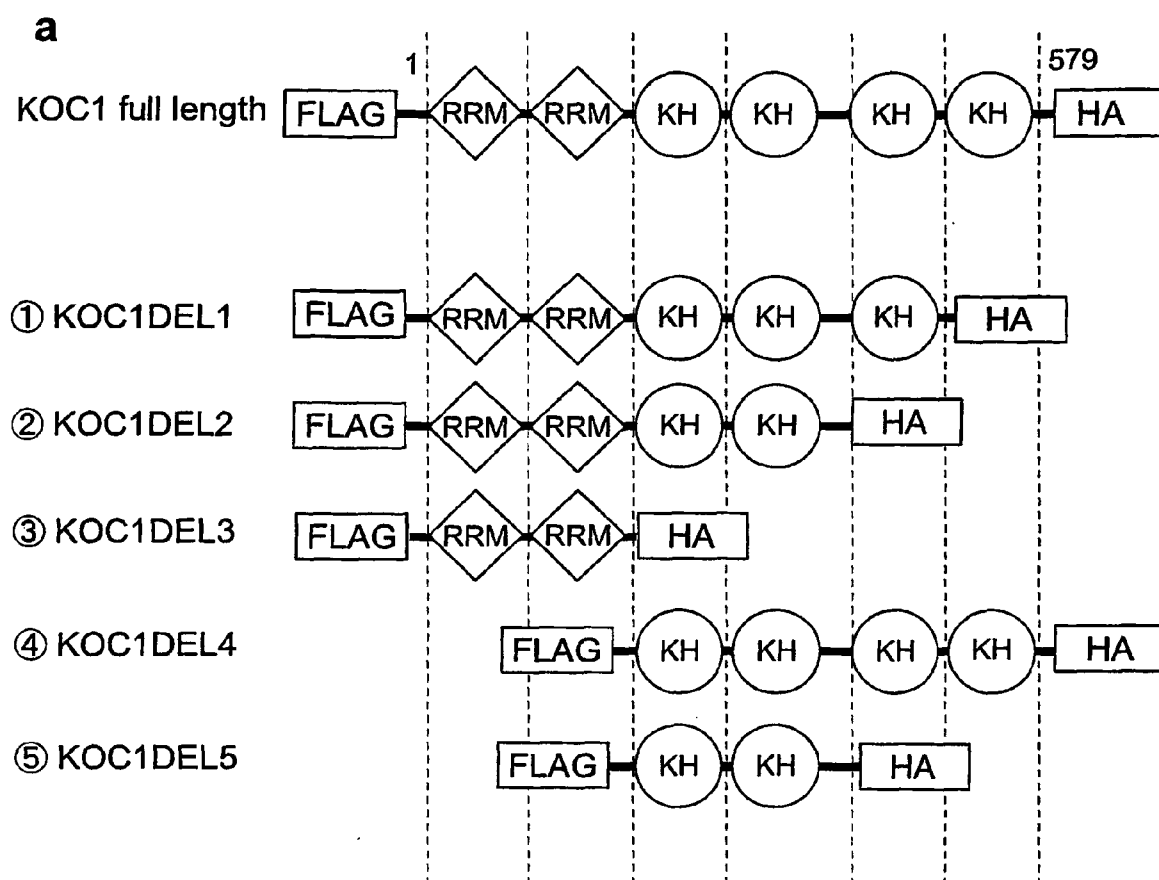
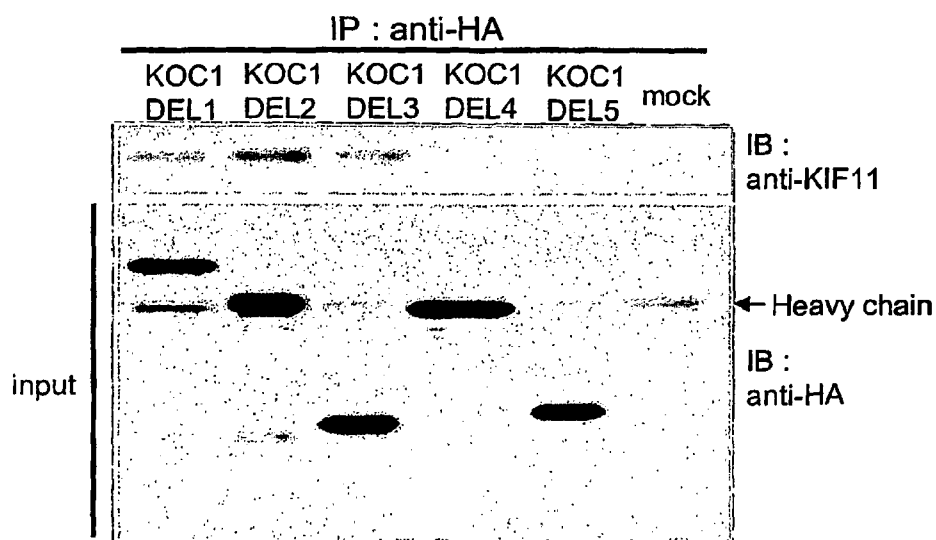
**b**



Fig. 4

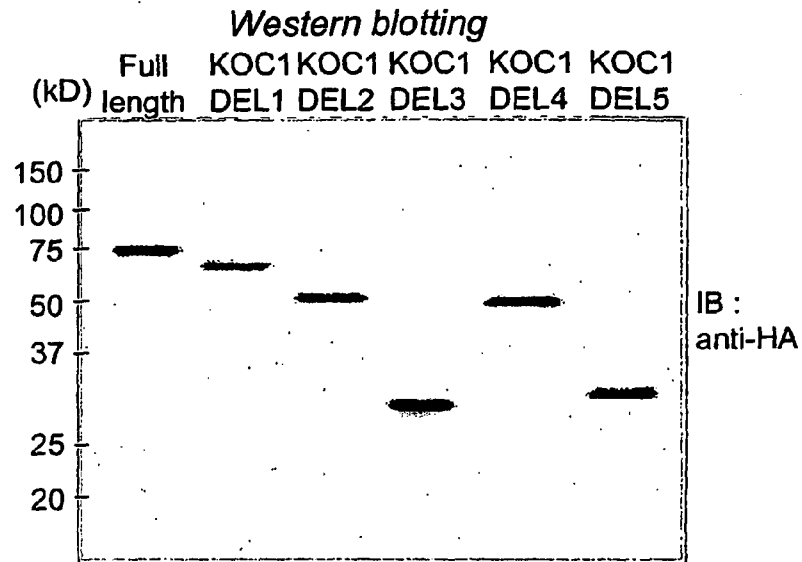
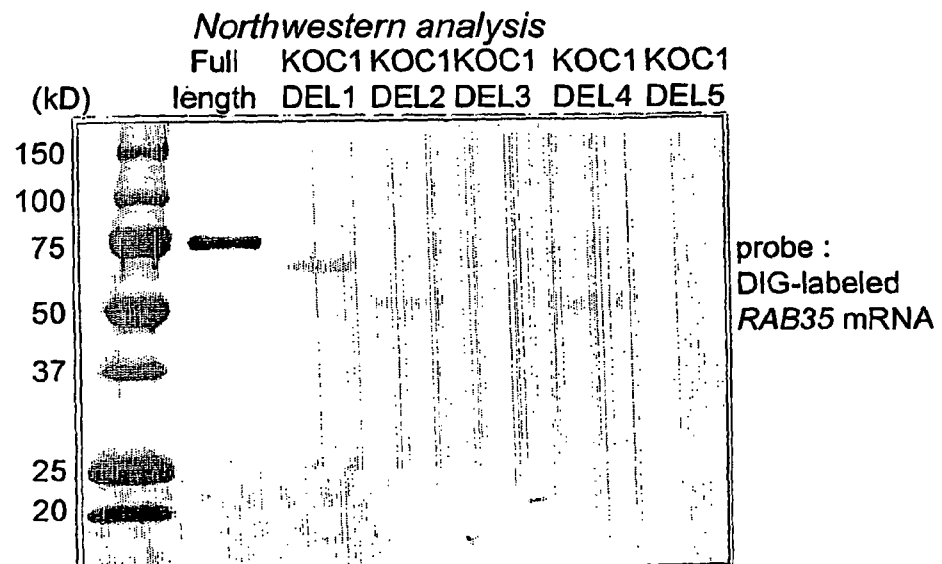
**a****b**

Fig. 4

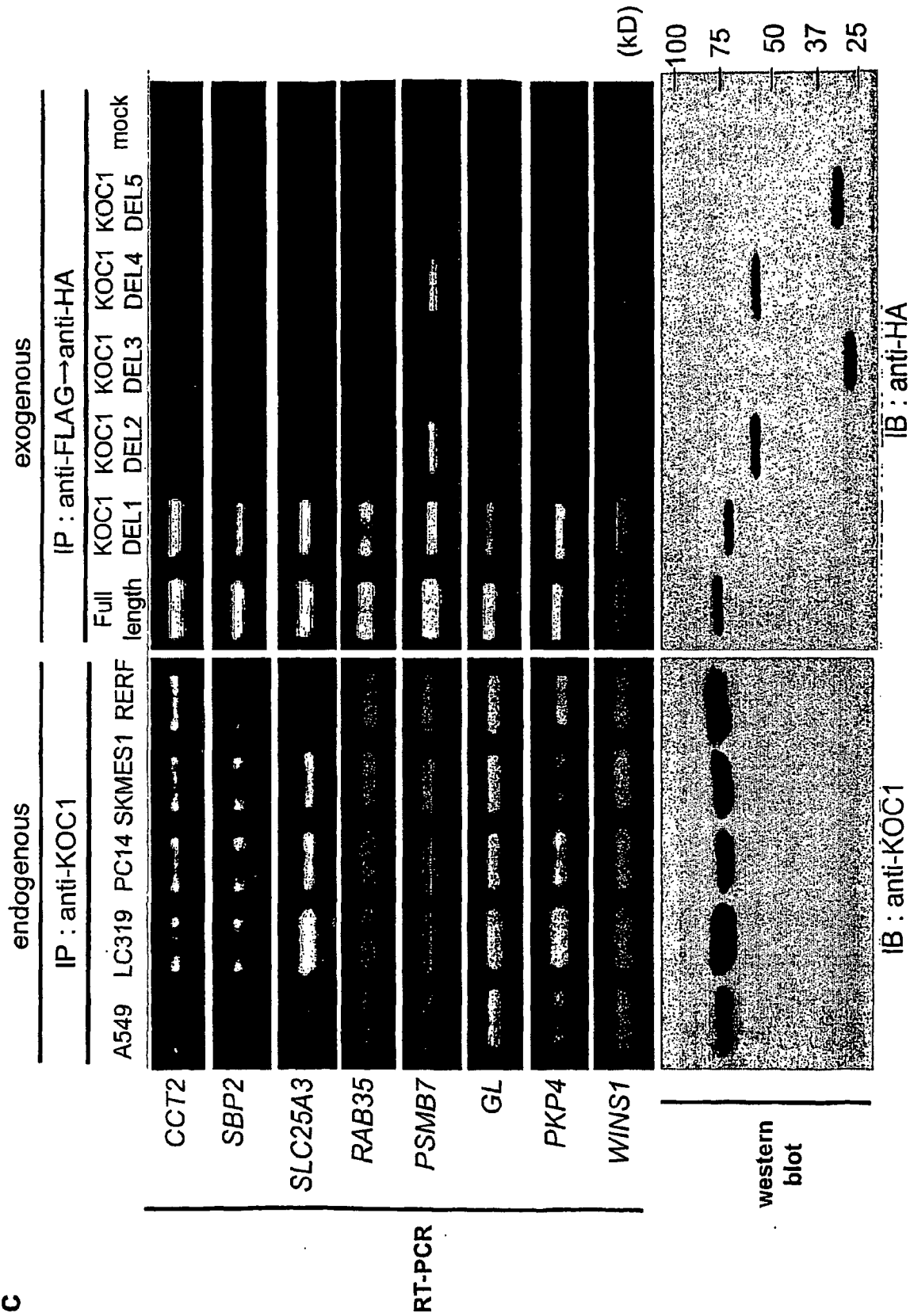


Fig. 5

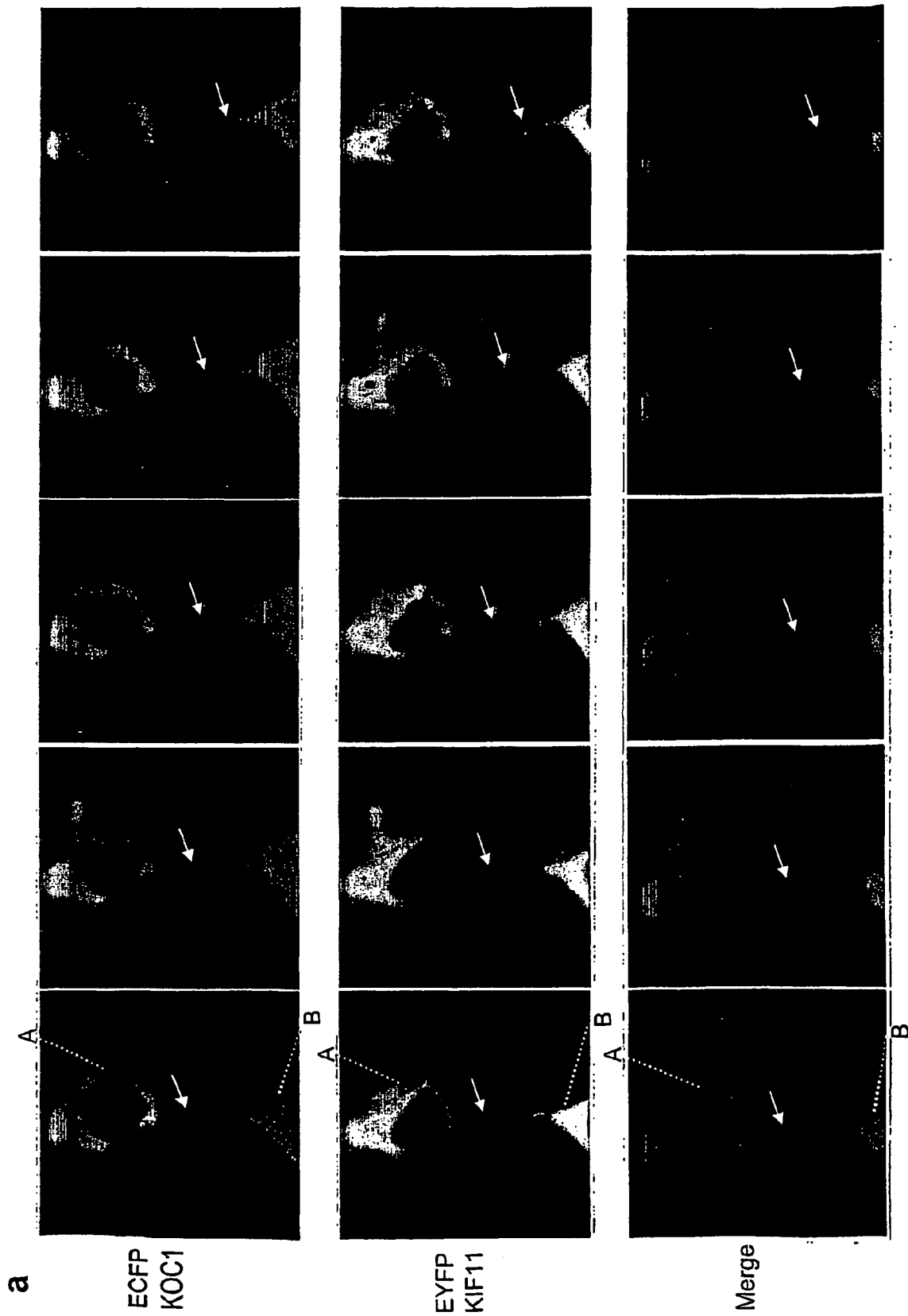


Fig. 5

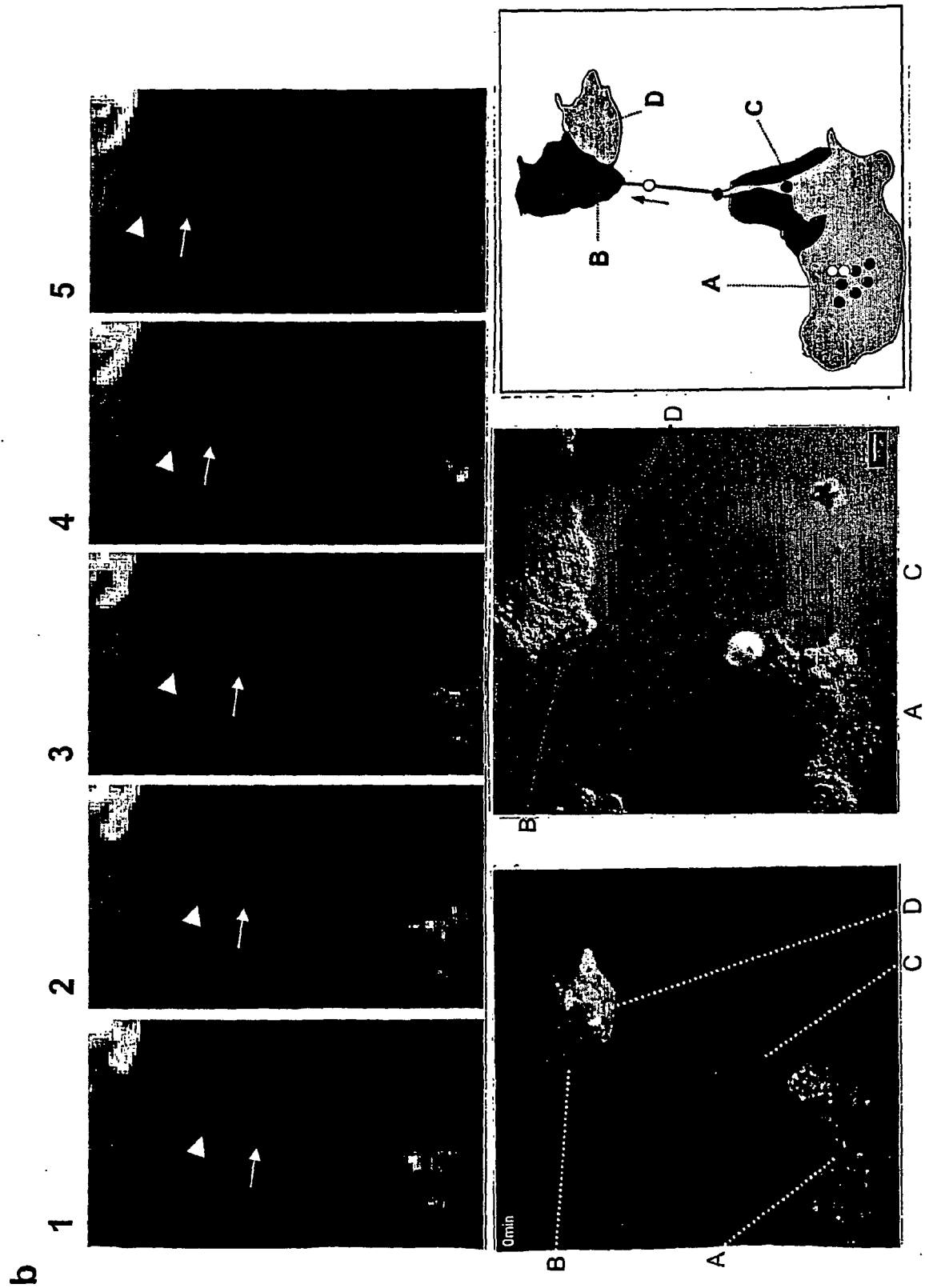


Fig. 6

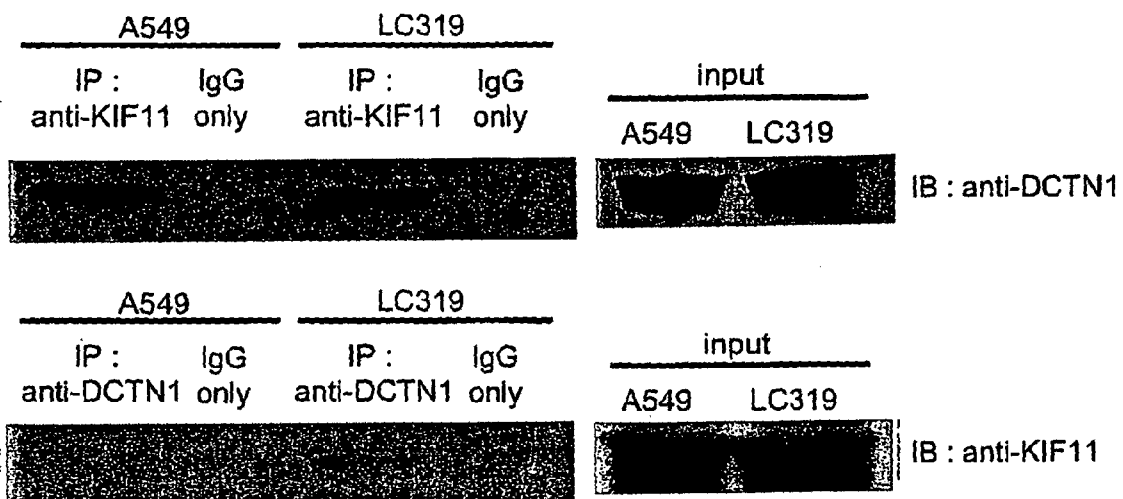
**a**

Fig. 7

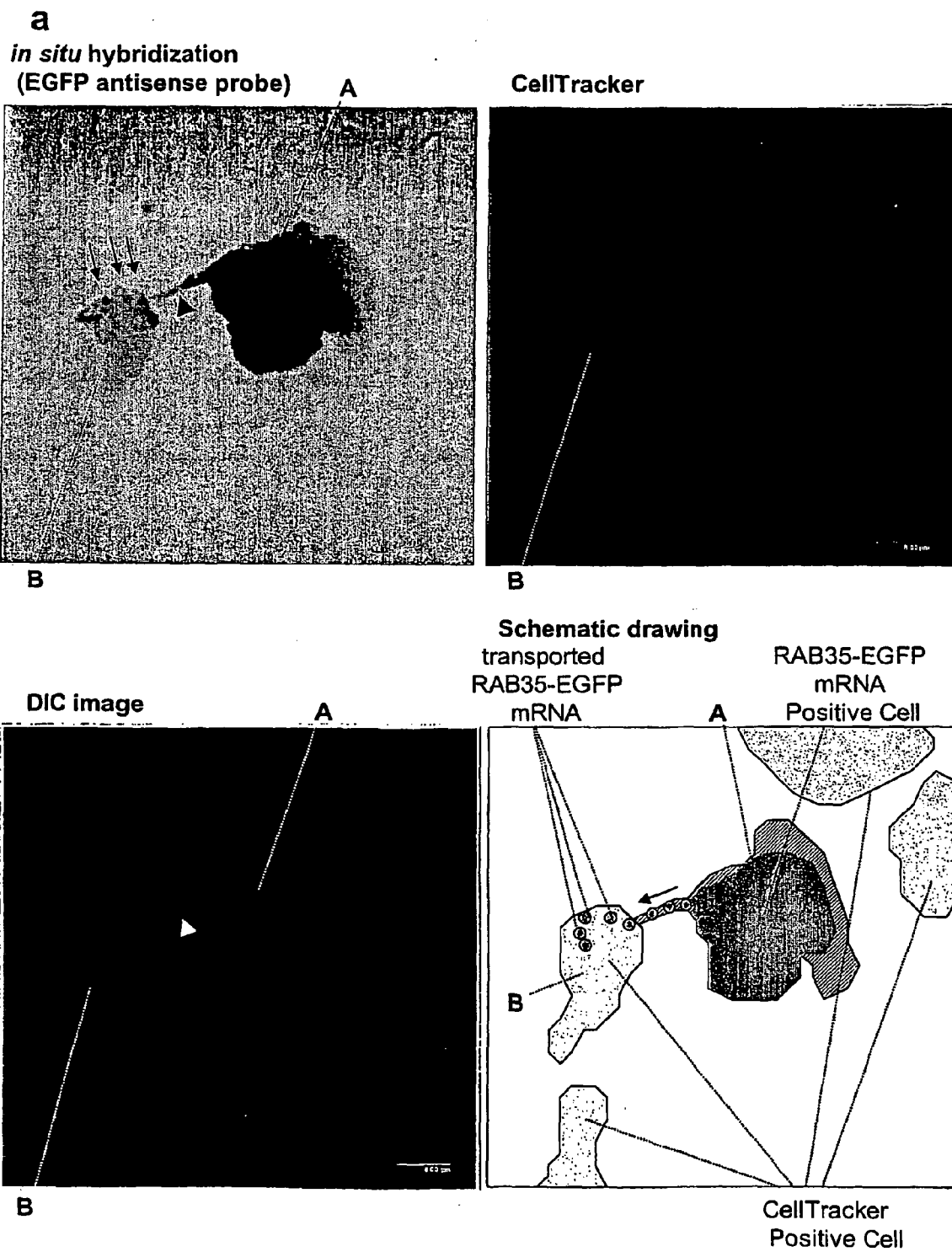


Fig. 7

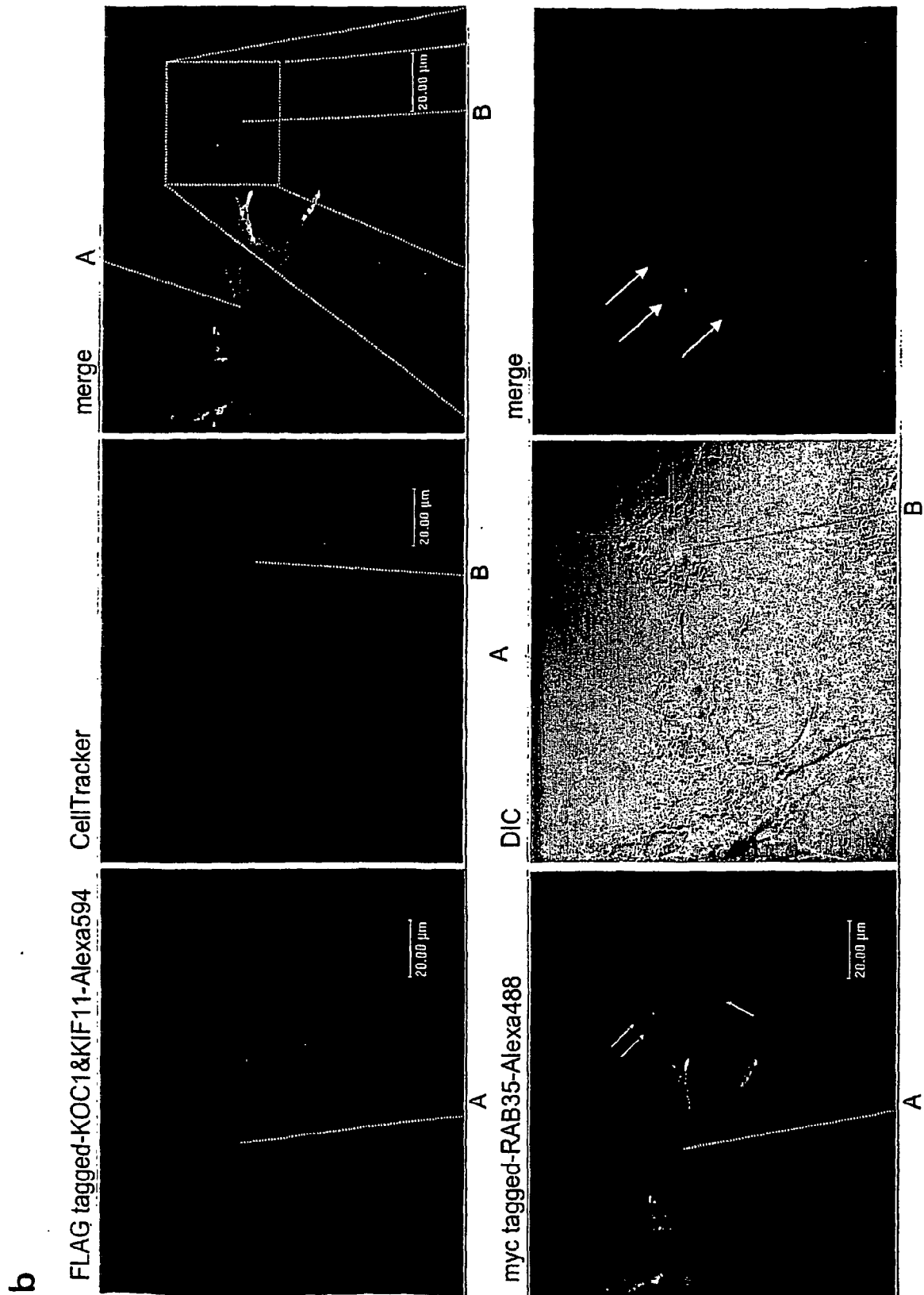


Fig. 7

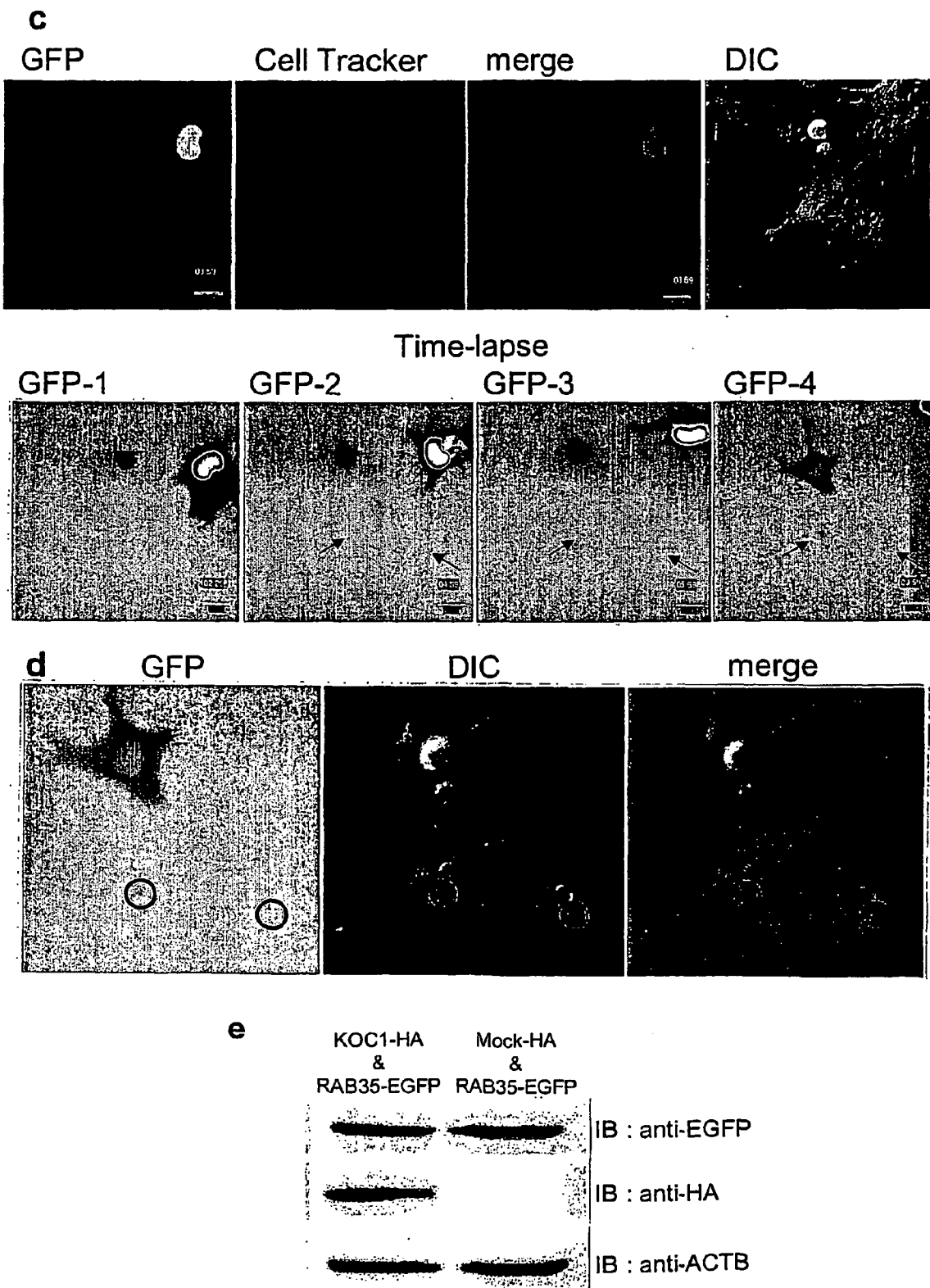




Fig. 8

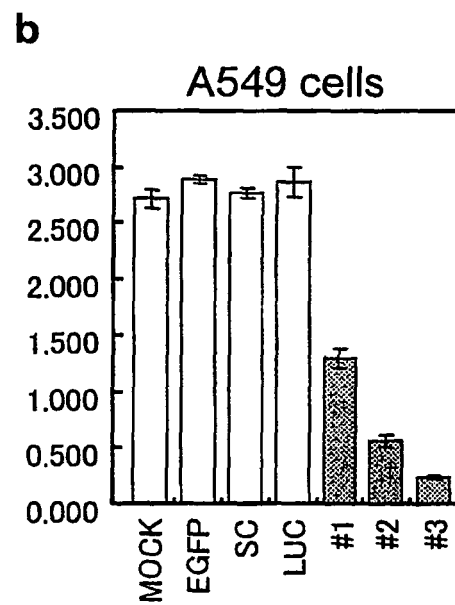
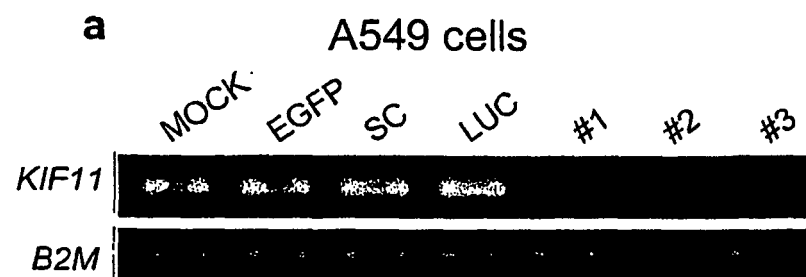


Fig. 9

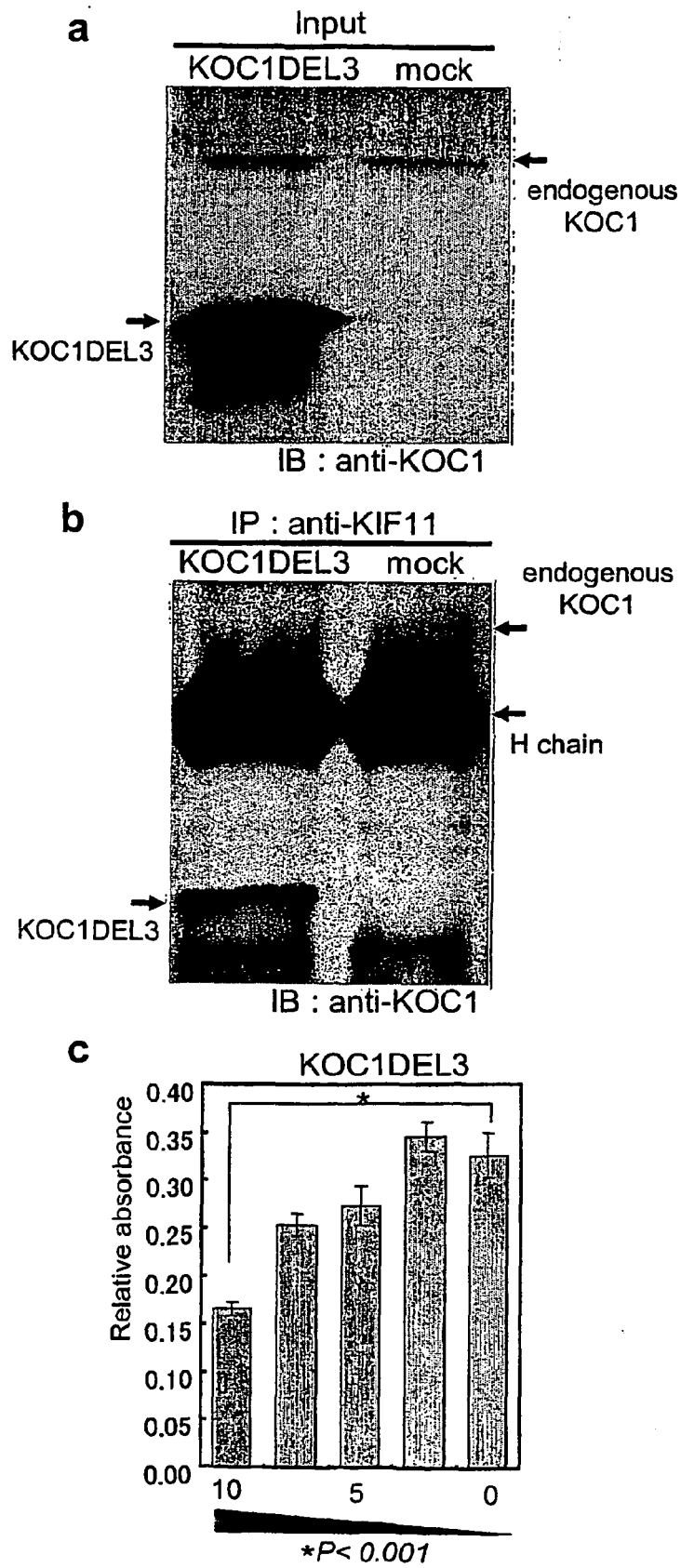


Fig. 9

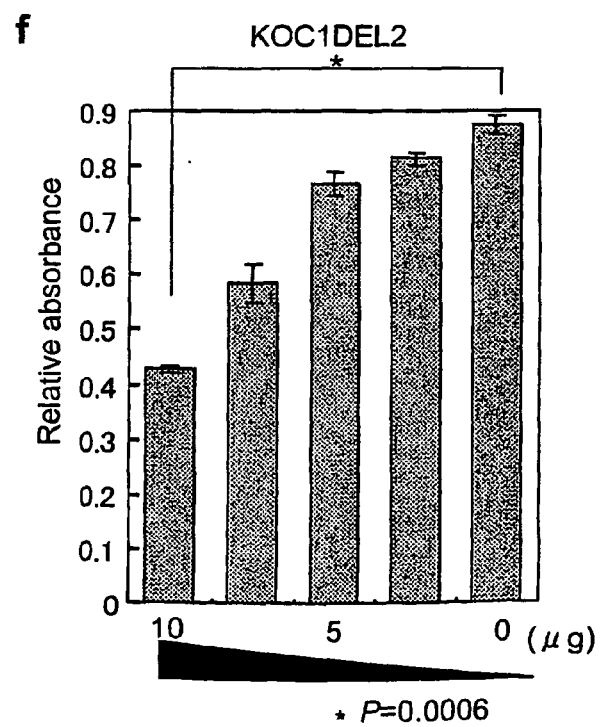
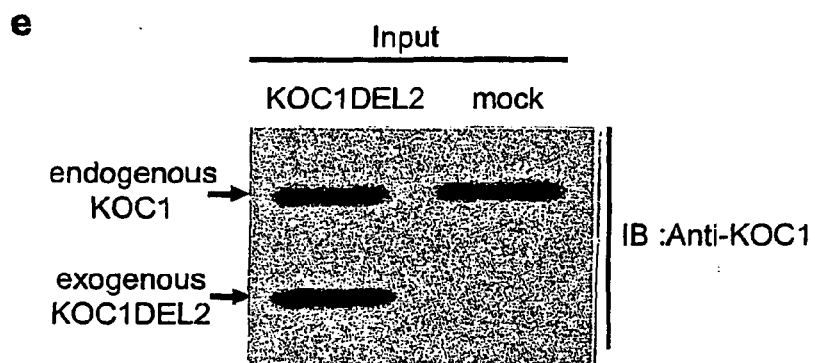
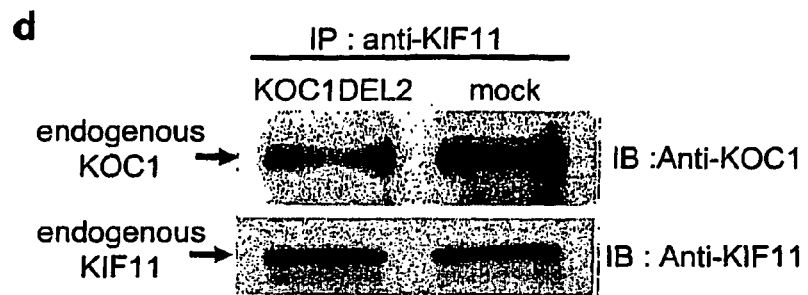


Fig. 10

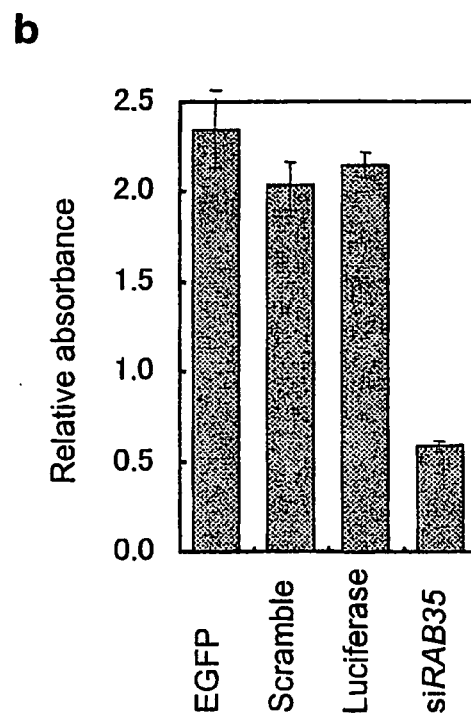
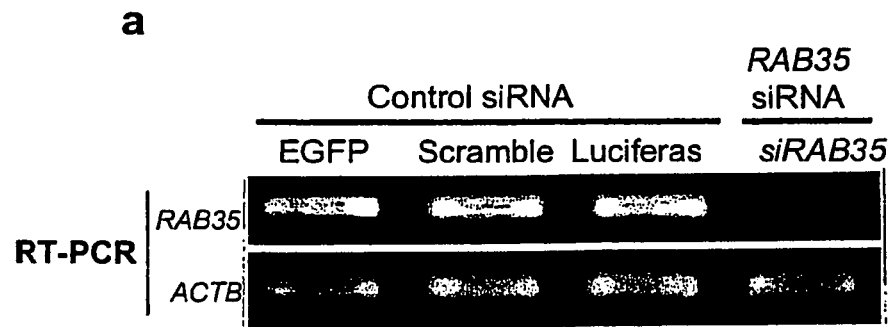


Fig. 11

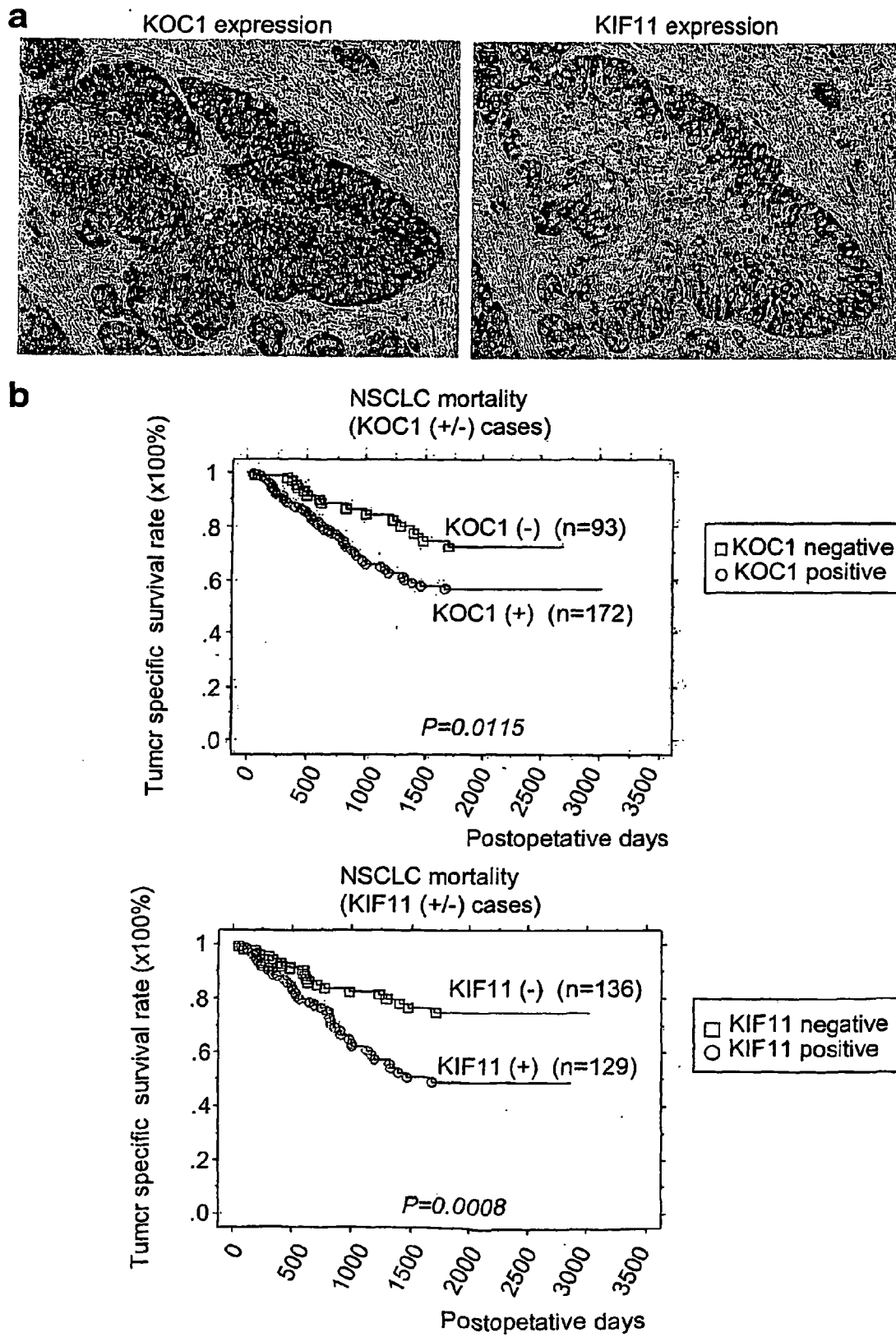


Fig. 12

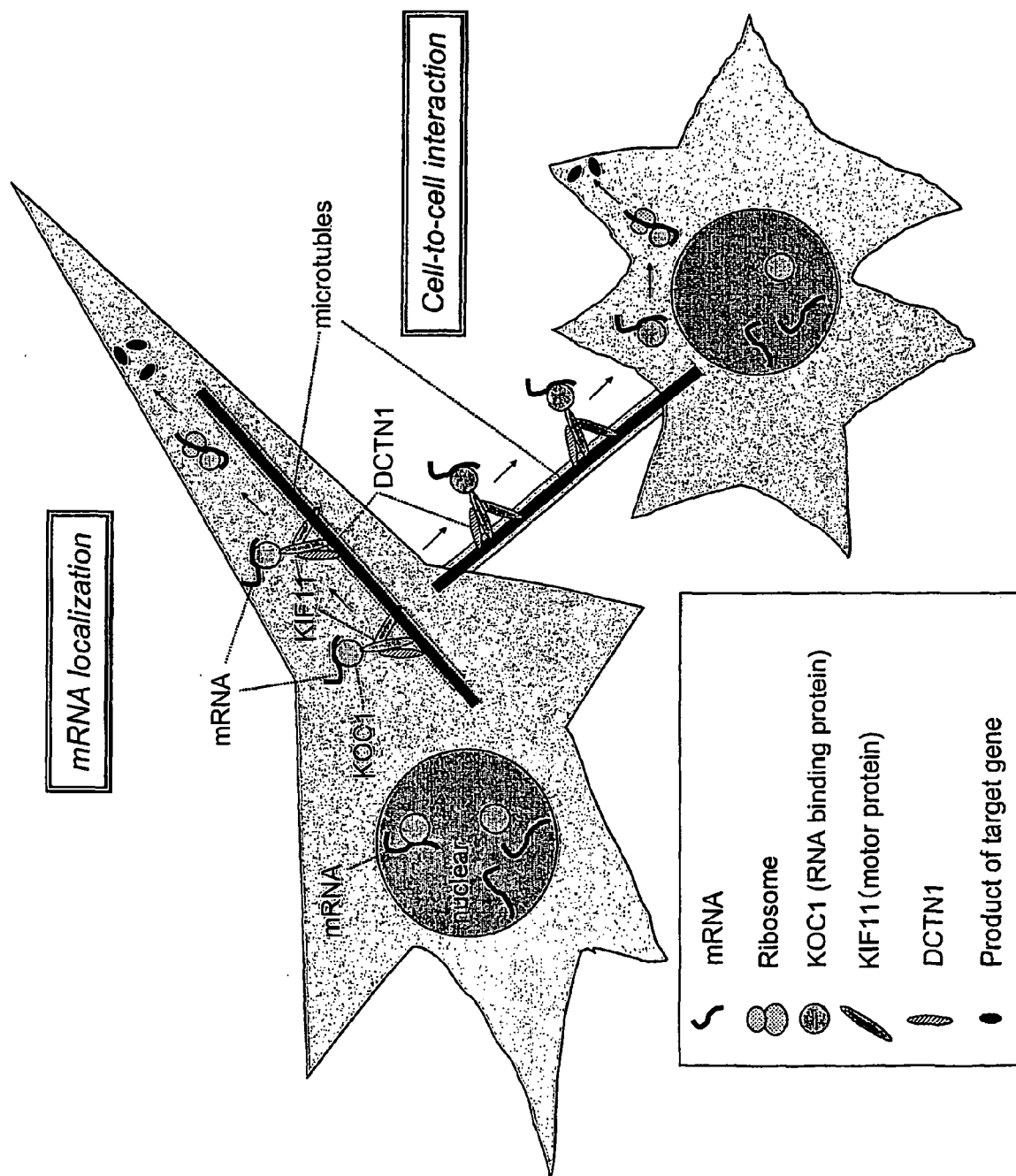


Fig. 13

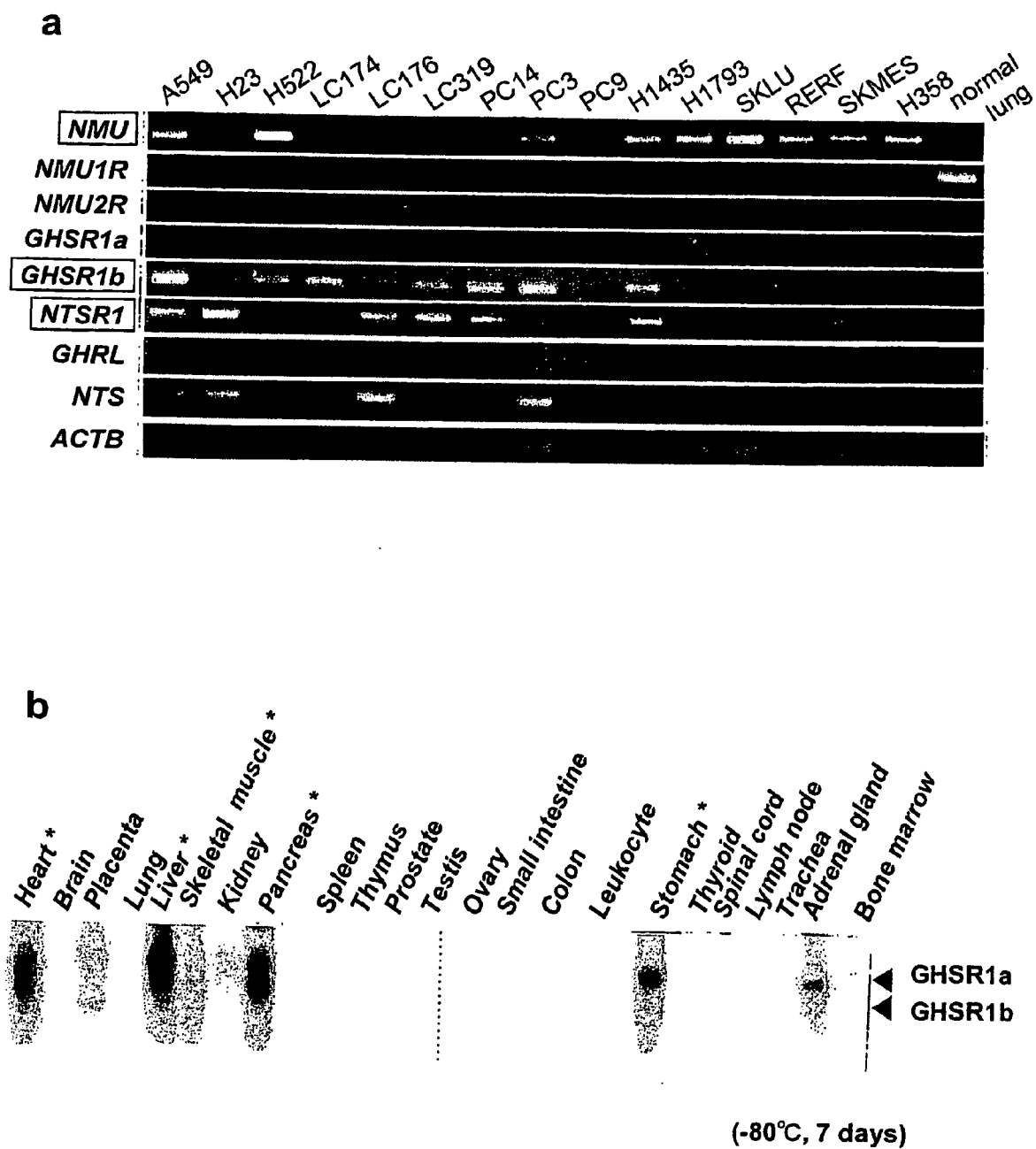
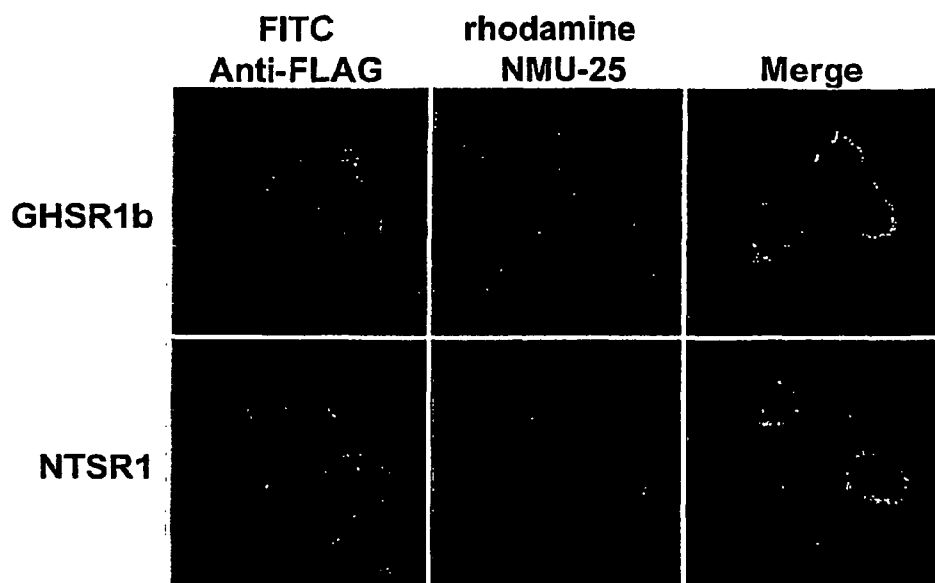
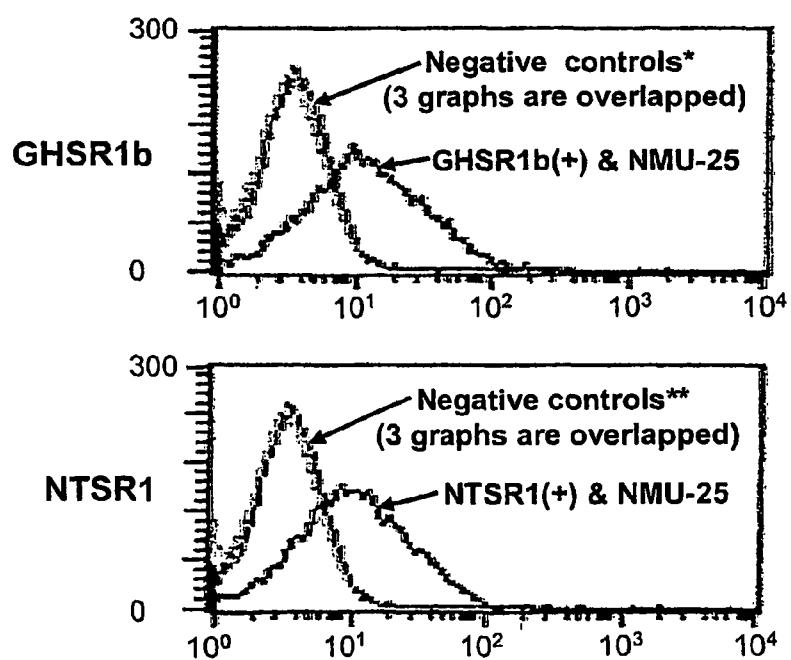


Fig. 13

c



d



\*Three control assays used for this study:

1. COS-7 cells (no receptor) only
2. No receptor & NMU-25
3. GHSR1b(+)-COS-7 cells only

\*\*Three control assays used for this study:

1. COS-7 cells (no receptor) only
2. No receptor & NMU-25
3. NTSR1(+)-COS-7 cells only



Fig. 13

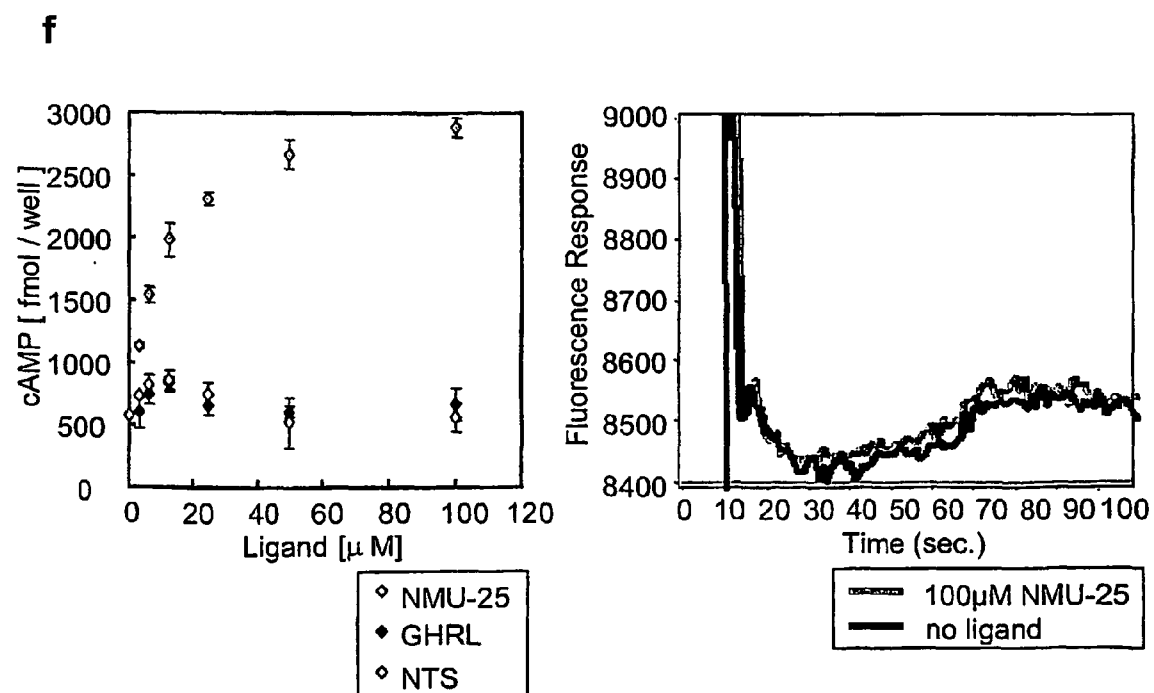
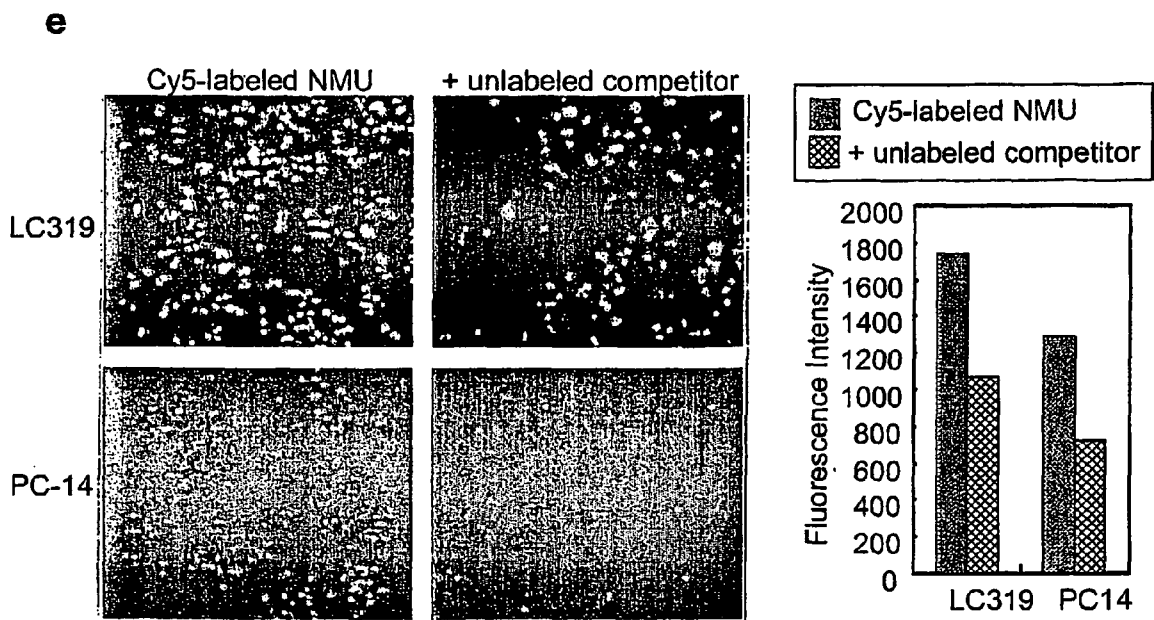


Fig. 14

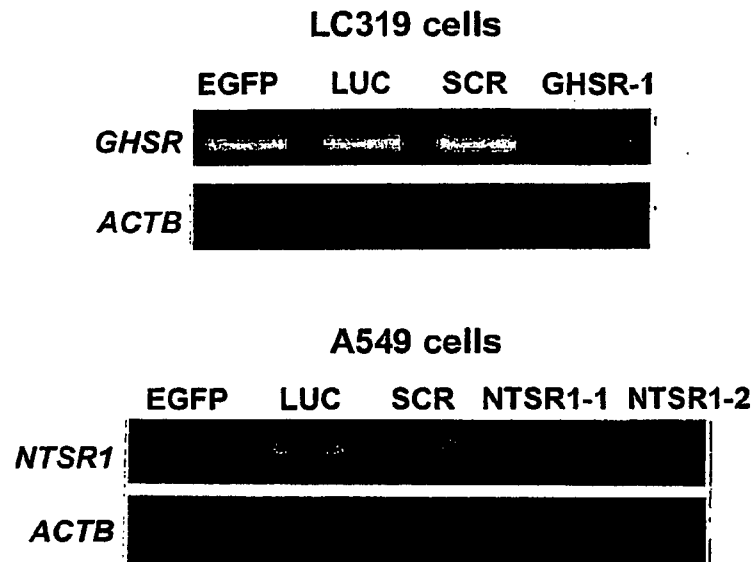
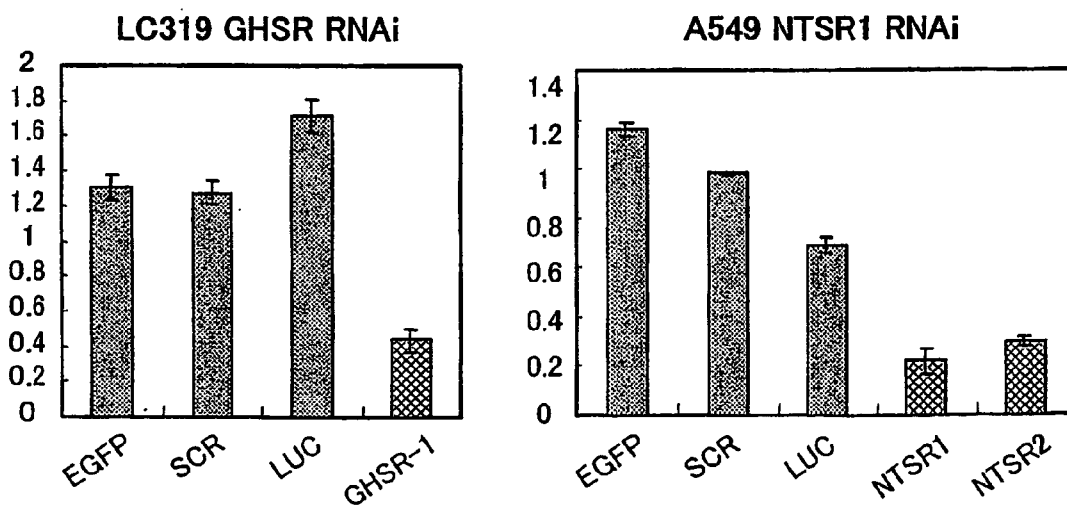
**a****b****MTT assay**

Fig. 15

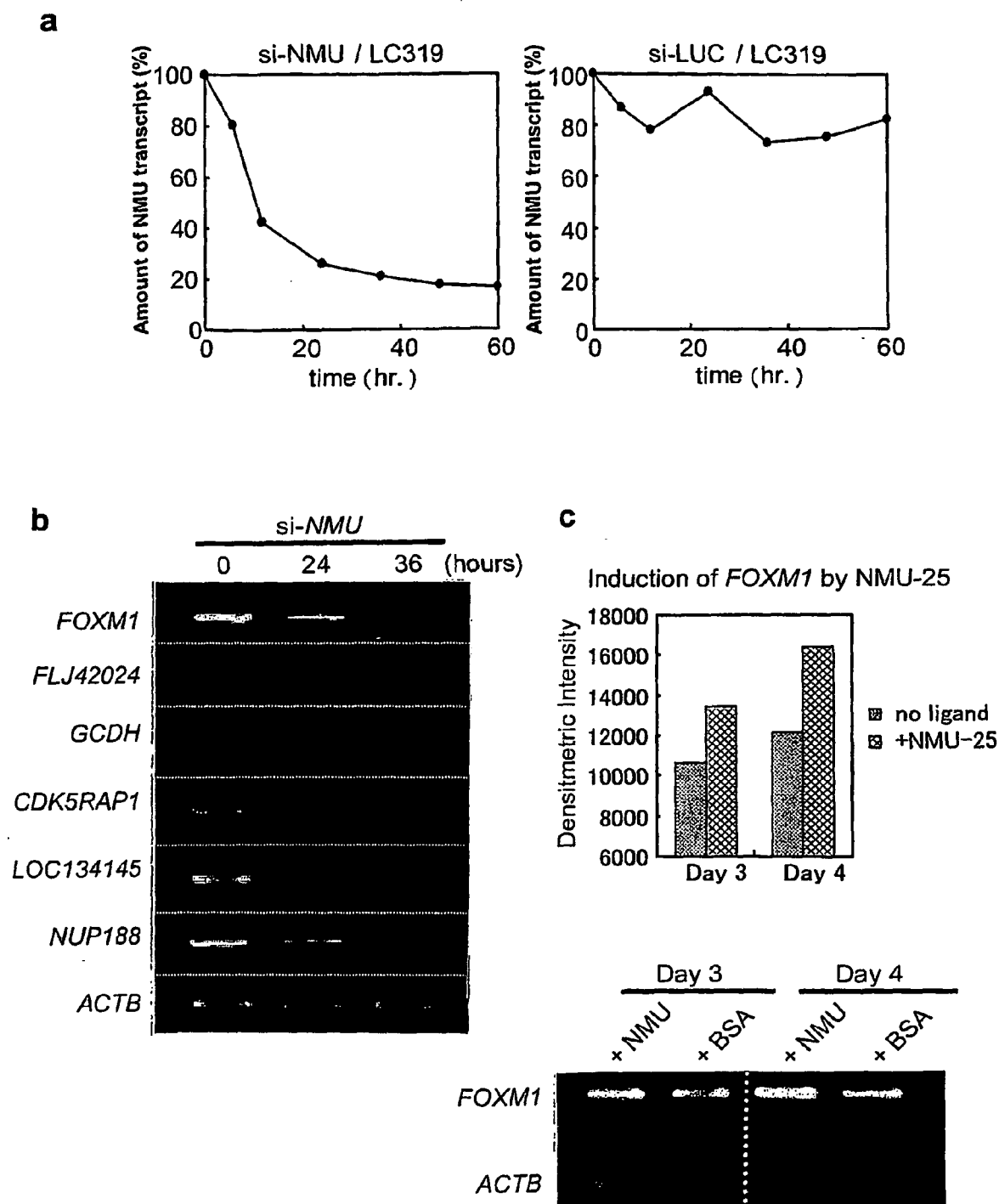
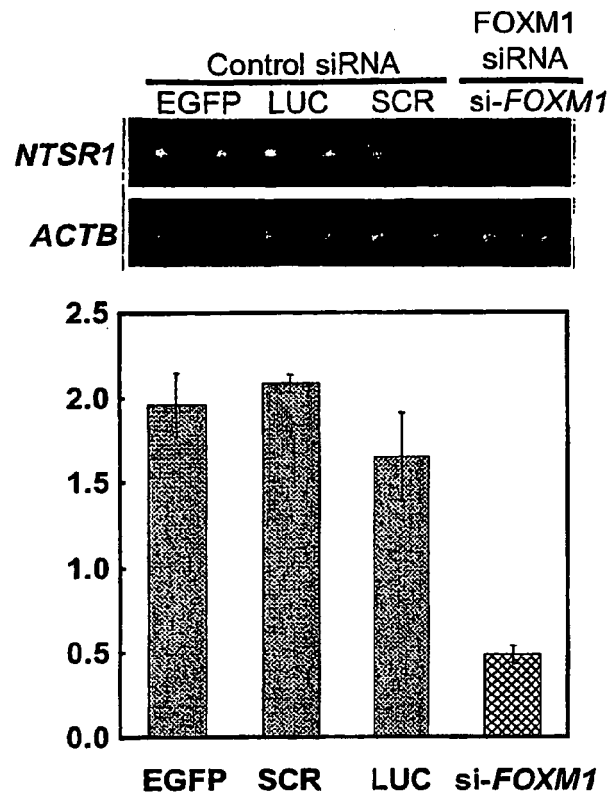


Fig. 16

a



b

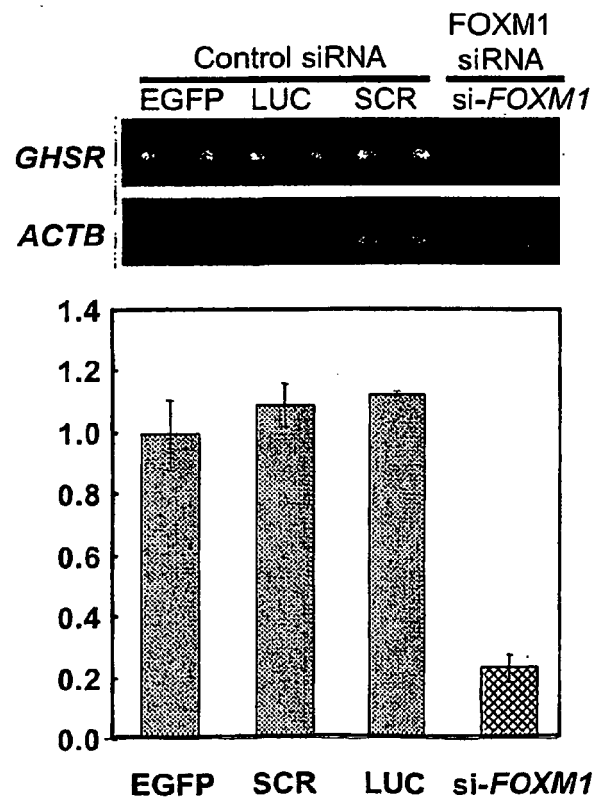
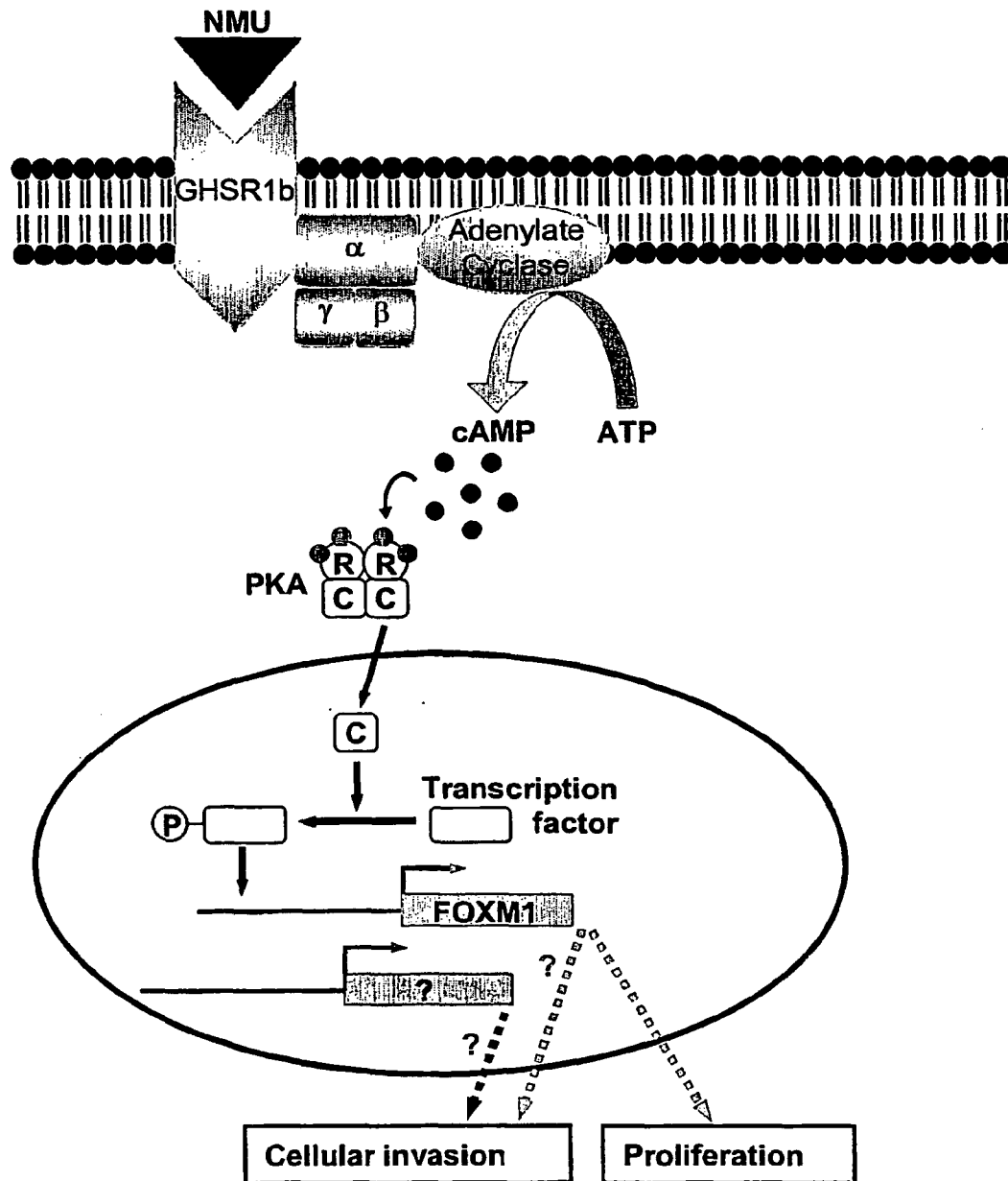


Fig. 17



## REFERENCES CITED IN THE DESCRIPTION

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