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(54) **Title:** CANCER RELATED GENE RASGEF1A

(57) **Abstract:** The present invention provides methods for detecting and diagnosing cancer. According to an embodiment, the diagnostic method involves the determination of the expression level of the RASGEF1A gene which was discovered to discriminate cancer cells from normal cells. Furthermore, the present invention provides methods of screening for therapeutic agents useful in the treatment of cancer, methods for treating cancer, and methods for vaccinating a subject against cancer.

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DESCRIPTION

CANCER RELATED GENE RASGEF1A

This application claims the benefit of U.S. Provisional Application Serial No.60/704,054 filed July 28, 2005, the contents of which are hereby incorporated by reference in their entirety.

Technical Field

The present invention relates to methods for detecting and diagnosing cancer as well as methods for treating and preventing cancer.

Background Art

Intrahepatic cholangiocarcinoma (ICC) is the second most common type of primary hepatobiliary cancer. Its incidence has been increasing in Japan and Western countries, especially in the United Kingdom (Shaib YH *et al.*, J Hepatol 2004, 40:472-7; Taylor-Robinson SD *et al.*, Lancet 1997, 350:1142-3; Kato I *et al.*, Jpn J Clin Oncol 1990, 20:232-7). Hepatocellular carcinoma, the most common liver malignancy, has been intensively studied, and clinical advances have been achieved through development of various therapeutic strategies. However, the carcinogenetic processes involved in ICC are poorly understood, and prognosis of patients with ICC remains poor. In terms of treatment, surgical resection of the tumor has been almost the only way to overcome this disease (Okuda K *et al.*, J Gastroenterol Hepatol 2002, 17:1056-63), but complete removal of cancer cells from patients bearing advanced invasive ICC is barely possible. Furthermore, chemotherapy against ICC has been also unsatisfactory; for example, although 5-fluorouracil and Gemcitabine-based regimens have partial response rates of 20-30%, no survival benefits have been shown (Gores GJ, Hepatology 2003, 37:961-9). Thus, the development of novel therapeutic strategies for ICC is a matter of pressing concern.

The Ras family of GTPases plays a key role in integrating and transmitting signals from cell surface receptors to downstream effector pathways. Ras proteins exist predominantly in their inactive GDP-bound state in resting cells. They are inter-convertible, cycling between an active, GTP-bound form and an inactive, GDP-bound form (Hall A, Annu. Rev Cell Biol 1994, 10:31-54). Their activity is modulated by interaction with regulatory proteins that stimulate GDP/GTP exchange (GEF proteins) and GTP hydrolysis (GAP proteins). Upon activation by extracellular stimuli, guanine nucleotide exchange factors (GEFs) promote the release of GDP from Ras, permitting GTP to associate with Ras. This

GTP-bound Ras forms an active conformation and mediates signals to downstream effector proteins such as Raf, phosphoinositide 3-kinase, and RalGDS (Campbell SL *et al.*, Oncogene 1998, 17:1395-413; Quilliam LA *et al.*, Bioessays 1995, 17:395-404). Although a number of Ras family GTPases have been identified, the number of Ras GEFs whose activity have been clarified is limited; *e.g.* Sos, GRF and GRP for Ras, C3G, CalDAG1 and Epac for Rap1, and RalGDS family members for Ral (Quilliam LA *et al.*, Bioessays 1995, 17:395-404; Bos JL, EMBO J 1998, 17:6776-82; de Rooij J *et al.*, Nature 1998, 396:474-7; Ebinu JO *et al.*, Science 1998, 280:1082-6; Gotoh T *et al.*, J Biol Chem 1997, 272:18602-7; Kawasaki H *et al.*, Proc Natl Acad Sci USA 1998, 95:13278-83).

Gene-expression profiles generated by cDNA microarray analysis can provide considerably more detail about the nature of individual cancers than traditional histopathological methods. The promise of such information lies in its potential for improving clinical strategies for treating neoplastic diseases and developing novel drugs (Petricoin EF *et al.*, Nat Genet 2002, *32 Suppl*:474-9). To this aim, the present inventors have analyzed the expression profiles of tumors from various tissues by cDNA microarrays (Okabe H *et al.*, Cancer Res 2001, *61*:2129-37; Hasegawa S *et al.*, Cancer Res 2002, *62*:7012-7; Kaneta Y *et al.*, Jpn J Cancer Res 2002, *93*:849-56; Kaneta, Y. *et al.*, Int J Oncol 2003, *23*:681-91; Kitahara O *et al.*, Cancer Res 2001, *61*:3544-9; Lin Y *et al.* Oncogene 2002, *21*:4120-8; Nagayama S *et al.*, Cancer Res 2002, *62*:5859-66; Okutsu J *et al.*, Mol Cancer Ther 2002, *1*:1035-42; Kikuchi T *et al.*, Oncogene 2003, *22*:2192-205).

Studies designed to reveal mechanisms of carcinogenesis have already facilitated the identification of molecular targets for certain 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 post-translational farnesylation, have been shown to be effective in treating Ras-dependent tumors in animal models (Sun J *et al.*, Oncogene 1998, 16(11):1467-73). Similarly, clinical trials on humans using a combination of anti-tumor agents and the anti-HER2 monoclonal antibody, trastuzumab, with the aim of antagonizing the proto-oncogene receptor HER2/neu have achieved improved clinical response and overall survival of cancer patients (Molina MA *et al.*, Cancer Res 2001;61(12):4744-9.). Finally, 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 (O'Dwyer ME & Druker BJ, Curr Opin Oncol 2000;12(6):594-7.). Accordingly, it is apparent that gene products commonly up-regulated in cancer cells may serve as potential targets for developing novel anti-tumor agents.

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

In spite of significant progress in basic and clinical research concerning TAAs (Rosenberg *et al.*, Nature Med 1998, 4:321-7; Mukherji *et al.*, Proc Natl Acad Sci USA 1995, 92:8078-82; Hu *et al.*, Cancer Res 1996, 56:2479-83), only limited number of candidate TAAs for the treatment of adenocarcinomas, including colorectal cancer, are currently available. TAAs abundantly expressed in cancer cells yet whose 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 strategies for various types of cancers (Boon *et al.*, J Exp Med 1996, 183:725-9; van der Bruggen *et al.*, Science 1991, 254:1643-7; Brichard *et al.*, J Exp Med 1993, 178:489-95; Kawakami *et al.*, J Exp Med 1994, 180:347-52; Shichijo *et al.*, J Exp Med 1998, 187:277-88; Chen *et al.*, Proc Natl Acad Sci USA 1997, 94:1914-8; Harris, J Natl Cancer Inst 1996, 88:1442-55; Butterfield *et al.*, Cancer Res 1999, 59:3134-42; Vissers *et al.*, Cancer Res 1999, 59:5554-9; van der Burg *et al.*, J Immunol 1996, 156:3308-14; Tanaka *et al.*, Cancer Res 1997, 57:4465-8; Fujie *et al.*, Int J

Cancer 1999, 80:169-72; Kikuchi *et al.*, Int J Cancer 1999, 81:459-66; Oiso *et al.*, Int J Cancer 1999, 81:387-94).

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

Disclosure of the Invention

The present invention is based on the discovery of a specific expression pattern of the *RASGEF1A* gene in cancerous cells.

Through a previous analysis on genome-wide expression profiles of genes in 25 ICCs, a set of genes whose expression was commonly up-regulated was identified (Obama K *et al.*, Hepatology 2005 Jun, 41(6):1339-48.). Herein, among the genes, gene *RASGEF1A* (*RasGEF domain family, member 1A*) is focused. The expression of the *RASGEF1A* gene has been detected by the present inventors to be enhanced in bladder carcinomas (PCT/JP2006/302684) and suppressed in breast cancer (WO 2005/28678). Through the present invention, the *RASGEF1A* gene was further revealed to be frequently up-regulated in a wide range of human tumors including intrahepatic cholangiocarcinome (ICC), lung cancers, gastric cancer, colorectal cancers, prostate cancer, chronic myeloid leukemia, renal cancer, soft tissue sarcoma, pancreatic cancer, and seminoma. Furthermore, the protein encoded by the gene was discovered to enhance RAS activity. Moreover, since the suppression of the *RASGEF1A* gene by small interfering RNA (siRNA) resulted in growth inhibition and/or cell death of ICC

cells, this gene may serve as a novel therapeutic target for various types of human neoplasms.

The *RASGEF1A* gene identified herein as well as its transcription and translation products find diagnostic utility as a marker for cancer and as an oncogene target, the expression and/or activity of which may be altered to treat or alleviate a symptom of cancer. Similarly, by detecting the changes in the expression of the *RASGEF1A* gene due to a compound, various compounds can be identified as agents for treating or preventing cancer.

Accordingly, the present invention provides a method for diagnosing or determining a predisposition to cancer in a subject by determining the expression level of the *RASGEF1A* gene in a subject-derived biological sample, such as tissue sample. Increased expression level of the gene as compared to a normal control level indicates that the subject suffers from or is at risk of developing cancer. The normal control level can be determined using a normal cell obtained from a non-cancerous tissue.

In the present invention, preferred cancer is ICC.

In the context of the present invention, the phrase "control level" refers to the expression level of the *RASGEF1A* gene detected in a control sample and includes both normal control level and cancer control level. A control level can be a single expression pattern derived from a single reference population or from a plurality of expression patterns. For example, the control level can be a database of expression patterns from previously tested cells. A "normal control level" refers to a level of the *RASGEF1A* gene expression detected in a normal healthy individual or in a population of individuals known not to be suffering from cancer. A normal individual is one with no clinical symptom of cancer. On the other hand, a "cancer control level" refers to an expression level of the *RASGEF1A* gene found in an individual or population suffering from cancer.

An increase in the expression level of the *RASGEF1A* gene detected in a sample as compared to a normal control level indicates that the subject (from which the sample has been obtained) suffers from or is at risk of cancer.

Alternatively, expression levels of a panel of genes comprising the *RASGEF1A* gene in a sample can be compared to cancer control levels of the same panel of genes. A similarity between the expression levels of a sample and the cancer control levels indicates that the subject (from which the sample has been obtained) suffers from or is at risk of cancer.

Herein, gene expression levels are deemed to be "altered" when the gene expression increases or decreases by, for example, 10%, 25%, or 50% from, or at least 0.1 fold, at least 0.2 fold, at least 1 fold, at least 2 fold, at least 5 fold, or at least 10 fold or more compared to a

control level. The expression level of the *RASGEF1A* gene can be determined by detecting, *e.g.*, hybridization intensity of nucleic acid probes to gene transcripts in a sample.

In the context of the present invention, subject-derived tissue samples may be any tissues obtained from test subjects, *e.g.*, patients known to have or suspected of having cancer. For example, tissues may comprise epithelial cells. More particularly, tissues may be cancerous epithelial cells.

The present invention further provides methods for identifying compounds that inhibit or enhance the expression or activity of RASGEF1A, by contacting a test cell expressing RASGEF1A with test compounds and determining the expression level of the *RASGEF1A* gene or the activity of the gene product. The test cell may be an epithelial cell, such as cancerous epithelial cell. A decrease in the expression level of the gene or the activity of its gene product as compared to a control level in the absence of the test compound indicates that the test compound may be used to reduce symptoms of cancer.

The present invention also provides a kit that comprises at least one detection reagent which binds to the transcription or translation product of the *RASGEF1A* gene.

Therapeutic methods of the present invention include methods for treating or preventing cancer in a subject comprising the step of administering an antisense composition to the subject. In the context of the present invention, the antisense composition reduces the expressions of a specific target gene (*i.e.*, the *RASGEF1A* gene). For example, the antisense compositions may contain a nucleotide which is complementary to the *RASGEF1A* gene sequence. Alternatively, the present methods may include the step of administering siRNA composition to the subject. In the context of the present invention, the siRNA composition reduces the expression of the *RASGEF1A* gene. In yet another method, the treatment or prevention of cancer in a subject may be carried out by administering a ribozyme composition to the subject. In the context of the present invention, the nucleic acid-specific ribozyme composition reduce the expression of the *RASGEF1A* gene. Actually, the present inventors confirmed inhibitory effects of siRNAs for the *RASGEF1A* gene. For example, the inhibition of cell proliferation of cancer cells by the siRNAs are demonstrated in the Examples section, which supports the fact that the *RASGEF1A* gene serves as a preferable therapeutic target for cancer.

The present invention also includes vaccines and vaccination methods. For example, a method for treating or preventing cancer in a subject may involve administering to the subject a vaccine containing a polypeptide encoded by the *RASGEF1A* gene or an immunologically

active fragment of the polypeptide. In the context of the present invention, immunologically active fragments are polypeptides that are shorter in length than the full-length naturally-occurring protein yet which induce an immune response analogous to that induced by the full-length protein. For example, an immunologically active fragment should be at least 8 amino acid residues in length and capable of stimulating an immune cell such as a T cell or a B cell. Immune cell stimulation can be measured by detecting cell proliferation, elaboration of cytokines (*e.g.*, IL-2), or production of an antibody.

One advantage of the methods described herein is that the disease is identified prior to detection of overt clinical symptoms of cancers. Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

Brief Description of the Drawings

Fig. 1A shows the expression of *D4223* (*RASGEF1A*) gene in 13 ICC tissues, a mixture of non-cancerous biliary ductal cells, and five normal tissues. Semiquantitative RT-PCR was carried out using a set of *RASGEF1A* gene-specific primers. Expression of β-actin (*ACTB*) gene served as a quantitative control. Fig. 1B shows the multiple-tissue Northern blot analysis of the *D4223* (*RASGEF1A*) gene.

Fig. 2 shows the subcellular localization of RASGEF1A. Fig. 2A shows the result of fluorescent immunocytochemical staining of FLAG-tagged RASGEF1A using NIH3T3 cells transfected with a plasmid expressing FLAG-tagged RASGEF1A. FLAG-tagged protein was stained with anti-FLAG mouse antibody and subsequently visualized with goat anti-mouse secondary antibody conjugated with FITC (upper-right panel). Nuclei were counterstained with DAPI (upper-left panel). Merged image is shown in the lower left panel. In Fig. 2B, the expression of Flag-tagged protein in the cells was analyzed by Western blot analysis.

Fig. 3A shows the colony formation assay of COS7 cells transfected with plasmids expressing RASGEF1A. Cells transfected with pCAGGS-n3Fc-RASGEF1A, pCAGGS-n3Fc-RASGEF1A(-), or pCAGGS-n3Fc-Mock were stained with Giemsa solution. Fig. 3B shows the cell viability measured by MTT assay using a cell-counting kit. Fig. 3C shows the expression of *RASGEF1A* gene in the cells transfected with plasmids expressing siRNAs (si-B and si-E). Plasmids expressing EGFP (siEGFP) and mock plasmids (Mock) were used as controls. Semi-quantitative RT-PCR was carried out to examine the gene silencing effect of the siRNAs. Expression of *ACTB* gene served as a quantitative control. Fig. 3D shows the growth suppressive effect of siRNAs designated to suppress *RASGEF1A*. Viability of SSP25 cells in response to the siRNAs was measured using a cell-counting kit.

Fig. 4A shows the result of K-Ras (upper left panel), Ha-Ras (upper right panel) and N-RAS (lower panel) nucleotide dissociation assay. Relative amount of [^{3}H]-GDP interacted with Ras after the treatment with recombinant GST (open box), SOS (hatched box), or RASGEF1A (closed box) as compared to the result obtained without such treatment (shadowed box). Fig. 4B shows RAS-activating activity of RASGEF1A. Extracts from cells transfected with pCAGGS-n3Fc-RASGEF1A or those with mock plasmids were analyzed by GST-RafRBD pull-down assay. Expression of RASGEF1A in the cells was examined by immunoblot analyses with anti-FLAG antibody (left panel). Activated form of GTP-bound Ras was precipitated with recombinant Raf-1 protein, and analyzed by Western blot analysis using anti-pan-Ras antibody (right panels).

Fig.5A shows the result of wound-healing assay using COS7 cells transfected with RASGEF1A or mock vector. Representative images of migrating cells were captured 12 and 24 h after the scratch. Fig.5B shows the result of Matrigel invasion assays using the same cells. Average number of cells migrating through the Matrigel was determined by counting in five different fields. Similar results were obtained through repeated experiments.

DETAILED DESCRIPTION OF THE INVENTION

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

The terms "isolated" and "purified" used in relation with a substance (*e.g.*, polypeptide, antibody, polynucleotide, *etc.*) indicates that the substance is substantially free from at least one substance that may else be included in the natural source. Thus, an isolated or purified antibody refers to antibodies that is substantially free of cellular material such as carbohydrate, lipid, or other contaminating proteins from the cell or tissue source from which the protein (antibody) is derived, or substantially free of chemical precursors or other chemicals when chemically synthesized. The term "substantially free of cellular material" includes preparations of a polypeptide in which the polypeptide is separated from cellular components of the cells from which it is isolated or recombinantly produced. Thus, a polypeptide that is substantially free of cellular material includes preparations of polypeptide having less than about 30%, 20%, 10%, or 5% (by dry weight) of heterologous protein (also referred to herein as a "contaminating protein"). When the polypeptide is recombinantly produced, it is also preferably substantially free of culture medium, which includes preparations of polypeptide with culture medium less than about 20%, 10%, or 5%

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

An "isolated" or "purified" nucleic acid molecule, such as a cDNA molecule, can be substantially free of other cellular material, or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized. In a preferred embodiment, nucleic acid molecules encoding antibodies of the present invention are isolated or purified.

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

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

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

Nomenclature Commission.

The terms "polynucleotides", "nucleotides", "nucleic acids", and "nucleic acid molecules" are used interchangeably unless otherwise specifically indicated and are similarly to the amino acids referred to by their commonly accepted single-letter codes. Similar to the amino acids, they encompass both naturally-occuring and non-naturally occurring nucleic acid polymers.

The present invention is based in part on the discovery of elevated expression of the *RASGEF1A* gene in cells from patients of a wide range of cancers. The nucleotide sequence of human *RASGEF1A* gene is shown in SEQ ID NO: 1 and is also available as GenBank Accession No. AK095136. Herein, the *RASGEF1A* gene encompasses the human *RASGEF1A* gene as well as those of other animals including non-human primate, mouse, rat, dog, cat, horse, and cow but are not limited thereto, and includes allelic mutants and genes found in other animals as corresponding to the *RASGEF1A* gene.

The amino acid sequence encoded the human *RASGEF1A* gene is shown in SEQ ID NO: 2 and is also available as GenBank Accession No. BAC04491. In the present invention, the polypeptide encoded by the *RASGEF1A* gene is referred to as "RASGEF1A", and sometimes as "RASGEF1A polypeptide" or "RASGEF1A protein".

According to an aspect of the present invention, functional equivalents are also included in the RASGEF1A polypeptide. Herein, a "functional equivalent" of a protein is a polypeptide that has a biological activity equivalent to the protein. Namely, any polypeptide that retains the biological ability of the RASGEF1A protein may be used as such a functional equivalent in the present invention. Such functional equivalents include those wherein one or more amino acids are substituted, deleted, added, or inserted to the natural occurring amino acid sequence of the RASGEF1A protein. 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 the sequence of the respective proteins. In other embodiments, the polypeptide can be encoded by a polynucleotide that hybridizes under stringent conditions to the natural occurring nucleotide sequence of the *RASGEF1A* gene.

The phrase "stringent (hybridization) conditions" refers to conditions under which a nucleic acid molecule will hybridize to its target sequence, typically in a complex mixture of nucleic acids, but not detectably to other sequences. Stringent conditions are sequence-dependent and will be different in different circumstances. Longer sequences hybridize

specifically at higher temperatures. An extensive guide to the hybridization of nucleic acids is found in Tijssen, *Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Probes*, "Overview of principles of hybridization and the strategy of nucleic acid assays" (1993). Generally, stringent conditions are selected to be about 5-10°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 of background, preferably 10 times of 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.

Generally, it is known that modifications of one or more amino acid in a protein do not influence the function of the protein. One of skill in the art will recognize that individual additions, deletions, insertions, or substitutions to an amino acid sequence which alters a single amino acid or a small percentage of amino acids is a "conservative modification" wherein the alteration of a protein results in a protein with similar functions. Conservative substitution tables providing functionally similar amino acids are well known in the art. For example, the following eight groups each contain amino acids that are conservative substitutions for one another:

1) Alanine (A), Glycine (G);

2) Aspartic acid (d), Glutamic acid (E);

3) Aspargine (N), Glutamine (Q);

4) Arginine (R), Lysine (K);

5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V);

6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W);

7) Serine (S), Threonine (T); and

8) Cystein (C), Methionine (M) (see, *e.g.*, Creighton, Proteins 1984).

Such conservatively modified polypeptides are included in the present RASGEF1A protein. However, the present invention is not restricted thereto and the RASGEF1A

protein includes non-conservative modifications so long as they retain at least one biological activity of the RASGEF1A protein. Furthermore, the modified proteins do not exclude polymorphic variants, interspecies homologues, and those encoded by alleles of these proteins.

Moreover, the *RASGEF1A* gene of the present invention encompasses polynucleotides that encode such functional equivalents of the RASGEF1A protein.

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. In case of conflict, the present specification, including definitions, will control.

I. Diagnosing cancer:

I-1. Method for diagnosing cancer or a predisposition for developing cancer

The expression of the *RASGEF1A* gene was found to be specifically elevated in patients with cancer. Therefore, the gene identified herein as well as its transcription and translation products find diagnostic utility as a marker for cancer and by measuring the expression of the *RASGEF1A* gene in a cell sample, cancer can be diagnosed. Specifically, the present invention provides a method for diagnosing cancer or a predisposition for developing cancer in a subject by determining the expression level of the *RASGEF1A* gene in the subject.

Cancers that can be diagnosed by the present method include, but are not limited to, ICC, bladder carcinoma, lung cancer, gastric cancer, colorectal cancer, prostate cancer, chronic myeloid leukemia, renal cancer, soft tissue sarcoma, pancreatic cancer, and seminoma. The present method is particularly suited for diagnosing ICC.

According to another aspect of the present invention, the predisposition for developing at least any one of such cancers.

In the context of the present invention, the term "diagnosing" is intended to encompass predictions and likelihood analysis. The present method is intended to be used clinically in making decisions concerning treatment modalities, including therapeutic intervention, diagnostic criteria such as disease stages, and disease monitoring and surveillance for cancer.

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

It is preferred to collect a biological sample from the subject to be diagnosed to perform the diagnosis. Any biological material can be used as the biological sample for the determination so long as it comprises the objective transcription or translation product of the *RASGEF1A* gene. The biological samples include, but are not limited to, bodily tissues and fluids, such as blood, sputum, and urine. Preferably, the biological sample contains a cell population comprising an epithelial cell, more preferably a cancerous epithelial cell or an epithelial cell derived from tissue suspected to be cancerous. Further, if necessary, the cell may be purified from the obtained bodily tissues and fluids, and then used as the biological sample.

According to the present invention, the expression level of the *RASGEF1A* gene is determined in the subject-derived biological sample. The expression level can be determined at the transcription (nucleic acid) product level, using methods known in the art. For example, the mRNA of the *RASGEF1A* gene may be quantified using probes by hybridization methods (*e.g.*, Northern hybridization). The detection may be carried out on a chip or an array. The use of an array is preferable for detecting the expression level of a plurarity of genes (*e.g.*, various cancer specific genes) including the present *RASGEF1A* gene. Those skilled in the art can prepare such probes utilizing the sequence information of the *RASGEF1A* gene (SEQ ID NO: 1; GenBank Accession No. AK095136). For example, the cDNA of the *RASGEF1A* gene may be used as the probes. If necessary, the probe may be labeled with a suitable label, such as dyes and isotopes, and the expression level of the gene may be detected as the intensity of the hybridized labels.

Furthermore, the transcription product of the *RASGEF1A* gene may be quantified using primers by amplification-based detection methods (*e.g.*, RT-PCR). Such primers can also be prepared based on the available sequence information of the gene. For example, the primers (SEQ ID NOs: 5 and 6; or 7 and 8) used in the Example may be employed for the detection by RT-PCR, but the present invention is not restricted thereto.

Specifically, a probe or primer used for the present method hybridizes under stringent, moderately stringent, or low stringent conditions to the mRNA of the *RASGEF1A* gene. As used herein, the phrase "stringent (hybridization) conditions" refers to conditions under which a probe or primer will hybridize to its target sequence, but to no other sequences. Stringent conditions are sequence-dependent and will be different under different circumstances. Specific hybridization of longer sequences is observed at higher temperatures

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

Alternatively, the translation product may be detected for the diagnosis of the present invention. For example, the quantity of the RASGEF1A protein may be determined. A method for determining the quantity of the protein as the translation product includes immunoassay methods that use an antibody specifically recognizing the protein. The antibody may be monoclonal or polyclonal. Furthermore, any fragment or modification (*e.g.*, chimeric antibody, scFv, Fab, $F(ab')_2$, Fv, *etc.*) of the antibody may be used for the detection, so long as the fragment retains the binding ability to the RASGEF1A protein. Methods to prepare these kinds of antibodies for the detection of proteins are well known in the art, and any method may be employed in the present invention to prepare such antibodies and equivalents thereof.

As another method to detect the expression level of the *RASGEF1A* gene based on its translation product, the intensity of staining may be observed via immunohistochemical analysis using an antibody against the RASGEF1A protein. Namely, the observation of strong staining indicates increased presence of the protein and at the same time high expression level of the *RASGEF1A* gene.

Furthermore, the translation product may be detected based on its biological activity. Specifically, herein, the RASGEF1A protein was demonstrated to have a Ras nucleotide dissociation activity, RAS enhancing activity, and to be involved in the migration of cancer cells. Thus, such activities of the RASGEF1A protein may be used as an index of the RASGEF1A protein existing in the biological sample. For example, such activities may be detected following the method described in the Example.

Moreover, in addition to the expression level of the *RASGEF1A* gene, the

expression level of other cancer-associated genes, for example, genes known to be differentially expressed in ICC, may also be determined to improve the accuracy of the diagnosis.

The expression level of cancer marker gene including the *RASGEF1A* gene in a biological sample can be considered to be increased or decreased if it increases or decreases from the control level of the corresponding cancer marker gene by, for example, 10%, 25%, or 50%; or increases to more than 1.1 fold, more than 1.5 fold, more than 2.0 fold, more than 5.0 fold, more than 10.0 fold, or more; or decreases to at least 0.5 fold, at least 0.2 fold, at least 0.1 fold, or less.

The control level may be determined at the same time with the test biological sample by using a sample(s) previously collected and stored from a subject/subjects whose disease state (cancerous or non-cancerous) is/are known. Alternatively, the control level may be determined by a statistical method based on the results obtained by analyzing previously determined expression level(s) of the *RASGEF1A* gene in samples from subjects whose disease state are known. Furthermore, the control level can be a database of expression patterns from previously tested cells. Moreover, according to an aspect of the present invention, the expression level of the *RASGEF1A* gene in a biological sample may be compared to multiple control levels, which control levels are determined from multiple reference samples. It is preferred to use a control level determined from a reference sample derived from a tissue type similar to that of the patient-derived biological sample. Moreover, it is preferred, to use the standard value of the expression levels of the *RASGEF1A* gene in a population with a known disease state. The standard value may be obtained by any method known in the art. For example, a range of mean ± 2 S.D. or mean ± 3 S.D. may be used as standard value.

In the context of the present invention, a control level determined from a biological sample that is known not to be cancerous is called "normal control level". On the other hand, if the control level is determined from a cancerous biological sample, it will be called "cancerous control level".

When the expression level of the *RASGEF1A* gene is increased compared to the normal control level or is similar to the cancerous control level, the subject may be diagnosed to be suffering from or at a risk of developing cancer. Furthermore, in case where the expression levels of multiple cancer-related genes are compare, a similarity in the gene expression pattern between the sample and the reference which is cancerous indicates

that the subject is suffering from or at a risk of developing cancer.

Difference between the expression levels of a test biological sample and the control level can be normalized to the expression level of control nucleic acids, *e.g.* housekeeping genes. Genes whose expression levels are known not to differ depending on the cancerous or non-cancerous state of the cell. Exemplary control genes include, but are not limited to, β-actin, glyceraldehyde 3-phosphate dehydrogenase, and ribosomal protein P1.

I-2. Assessing efficacy of cancer treatment

The *RASGEF1A* gene differentially expressed between normal and cancerous cells also allow for the course of treatment of cancers to be monitored, and the above-described method for diagnosing cancer can be applied for assessing the efficacy of a treatment on cancer. Specifically, the efficacy of a treatment on cancer can be assessed by determining the expression level of the *RASGEF1A* gene in a cell(s) derived from a subject undergoing the treatment. If desired, test cell populations are obtained from the subject at various time points, before, during, and/or after the treatment. The expression level of the *RASGEF1A* gene can be, for example, determined following the method described above under the item of 'I-1. Method for diagnosing cancer or a predisposition for developing cancer'. In the context of the present invention, it is preferred that the control level to which the detected expression level is compared is determined from the *RASGEF1A* gene expression in a cell(s) not exposed to the treatment of interest.

If the expression level of the *RASGEF1A* gene is compared to a control level that is determined from a normal cell or a cell population containing no cancer cell, a similarity in the expression level indicates that the treatment of interest is efficacious and a difference in the expression level indicates less favorable clinical outcome or prognosis of that treatment. On the other hand, if the comparison is conducted against a control level that is determined from a cancer cell or a cell population containing a cancer cell(s), a difference in the expression level indicates efficacious treatment, while a similarity in the expression level indicates less favorable clinical outcome or prognosis.

Furthermore, the expression levels of the *RASGEF1A* gene before and after a treatment can be compared according to the present method to assess the efficacy of the treatment. Specifically, the expression level detected in a subject-derived biological sample after a treatment (*i.e.*, post-treatment level) is compared to the expression level detected in a biological sample obtained prior to treatment onset from the same subject (*i.e.*, pre-treatment level). A decrease in the post-treatment level compared to the pre-treatment level indicates

that the treatment of interest is efficacious while an increase in or similarity of the post-treatment level to the pre-treatment level indicates less favorable clinical outcome or prognosis.

As used herein, the term "efficacious" indicates that the treatment leads to a reduction in the expression of a pathologically up-regulated gene, an increase in the expression of a pathologically down-regulated gene or a decrease in size, prevalence, or metastatic potential of carcinoma in a subject. When a treatment of interest is applied prophylactically, "efficacious" means that the treatment retards or prevents the forming of tumor or retards, prevents, or alleviates at least one clinical symptom of cancer. Assessment of the state of tumor in a subject can be made using standard clinical protocols.

In addition, efficaciousness of a treatment can be determined in association with any known method for diagnosing cancer. Cancers can be diagnosed, for example, by identifying symptomatic anomalies, *e.g.,* weight loss, abdominal pain, back pain, anorexia, nausea, vomiting and generalized malaise, weakness, and jaundice.

I-3. Assessing prognosis of subject with cancer

The method for diagnosing cancer described above can also be used for assessing the prognosis of cancer in a subject. Thus, the present invention also provides a method for assessing the prognosis of a subject with cancer. The expression level of the *RASGEF1A* gene can be, for example, determined following the method described above under the item of 'I-1. Method for diagnosing cancer or a predisposition for developing cancer'. For example, the expression level of the *RASGEF1A* gene in biological samples derived from patients over a spectrum of disease stages can be used as control levels to be compared with the expression level of the gene detected for a subject. By comparing the expression level of the *RASGEF1A* gene in a subject and the control level(s) the prognosis of the subject can be assessed. Alternatively, by comparing over time the pattern of expression levels in a subject, the prognosis of the subject can be assessed.

For example, an increase in the expression level of *RASGEF1A* gene in a subject as compared to a normal control level indicates less favorable prognosis. Conversely, a similarity in the expression level as compared to normal control level indicates a more favorable prognosis for the subject.

II. Kits:

The present invention also provides reagents for detecting cancer, *i.e.*, reagents that can detect the transcription or translation product of the *RASGEF1A* gene. Specifically,

nucleic acids that specifically bind to or identify a transcription product of the *RASGEF1A* gene. For example, nucleic acid that specifically bind to or identify a transcription product of the *RASGEF1A* gene include such as oligonucleotides (*e.g.*, probes and primers) having a sequence that is complementary to a portion of the *RASGEF1A* gene transcription product. Alternatively, antibodies can be exemplified as reagents for detecting the translation product of the gene. The probes, primers, and antibodies described above under the item of 'I-1. Method for diagnosing cancer or a predisposition for developing cancer' can be mentioned as suitable examples of such reagents.

Furthermore, the translation product may be detected based on its biological activity. As shown in the Example, RASGEF1A protein was demonstrated to have a Ras nucleotide dissociation activity, RAS enhancing activity, and to be involved in the migration of cancer cells. Thus, materials necessary for Ras nucleotide dissociation assay, detecting Ras activity, or migration of cancer cells may be also used as reagents for detecting the expression level of the *RASGEF1A* gene according to the present invention.

These reagents may be used for the above-described diagnosis of cancer. The assay format for using the reagents may be Northern hybridization or sandwich ELISA, both of which are well-known in the art.

The detection reagents may be packaged together in the form of a kit. For example, the detection reagents may be packaged in separate containers. Furthermore, the detection reagents may be packaged with other reagents necessary for the detection. For example a kit may include a nucleic acid or antibody (either bound to a solid matrix or packaged separately with reagents for binding them to the matrix) as the detection reagent, a control reagent (positive and/or negative), and/or a detectable label. Instructions (*e.g.*, written, tape, VCR, CD-ROM, *etc.*) for carrying out the assay may also be included in the kit.

As an aspect of the present invention, the reagents for detecting cancer may be immobilized on a solid matrix, such as a porous strip, to form at least one site for detecting cancer. The measurement or detection region of the porous strip may include a plurality of sites, each containing a detection reagent (*e.g.*, nucleic acid). A test strip may also contain sites for negative and/or positive controls. Alternatively, control sites may be located on a separate strip from the test strip. Optionally, the different detection sites may contain different amounts of immobilized detection reagents (*e.g.*, nucleic acid), *i.e.*, a higher amount in the first detection site and lesser amounts in subsequent sites. Upon the addition of test biological sample, the number of sites displaying a detectable signal provides a quantitative

indication of the expression level of the *RASGEF1A* gene in the sample. The detection sites may be configured in any suitably detectable shape and are typically in the shape of a bar or dot spanning the width of a test strip.

III. Screening methods:

Using the *RASGEF1A* gene, polypeptides encoded by the gene or fragments thereof, or transcriptional regulatory region of the gene, it is possible to screen agents that alter the expression of the gene or the biological activity of a polypeptide encoded by the gene. Such agents can be used as pharmaceuticals for treating or preventing cancer. Thus, the present invention provides methods of screening for agents for treating or preventing cancer using the *RASGEF1A* gene, polypeptides encoded by the gene or fragments thereof, or transcriptional regulatory region of the gene.

An agent isolated by the screening method of the present invention is an agent that is expected to inhibit the expression of the *RASGEF1A* gene or the activity of the translation product of the gene, and thus, is a candidate for treating or preventing diseases attributed to, for example, cell proliferative diseases, such as cancer. The agents are expected to treat or prevent cancer selected from the group of ICC, bladder carcinoma, lung cancer, gastric cancer, colorectal cancer, prostate cancer, chronic myeloid leukemia, renal cancer, soft tissue sarcoma, pancreatic cancer, and seminoma. Namely, the agents screened through the present methods are deemed to have a clinical benefit and can be further tested for its ability to prevent cancer cell growth in animal models or test subjects.

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

Any test agent, for example, cell extracts, cell culture supernatant, products of fermenting microorganism, extracts from marine organism, plant extracts, purified or crude proteins, peptides, non-peptide compounds, synthetic micromolecular compounds (including nucleic acid constructs, such as antisense RNA, siRNA, libozymes, *etc.*) and natural compounds can be used in the screening methods of the present invention. The test agent of the present invention can be also obtained using any of the numerous approaches in combinatorial library methods known in the art, including (1) biological libraries, (2) spatially

addressable parallel solid phase or solution phase libraries, (3) synthetic library methods requiring deconvolution, (4) the "one-bead one-compound" library method and (5) synthetic library methods using affinity chromatography selection. The biological library methods using affinity chromatography selection is limited to peptide libraries, while the other four approaches are applicable to peptide, non-peptide oligomer or small molecule libraries of compounds (Lam, Anticancer Drug Des 1997, 12: 145-67). Examples of methods for the synthesis of molecular libraries can be found in the art (DeWitt *et al.*, Proc Natl Acad Sci USA 1993, 90: 6909-13; Erb *et al.*, Proc Natl Acad Sci USA 1994, 91: 11422-6; Zuckermann *et al.*, J Med Chem 37: 2678-85, 1994; Cho *et al.*, Science 1993, 261: 1303-5; Carell *et al.*, Angew Chem Int Ed Engl 1994, 33: 2059; Carell *et al.*, Angew Chem Int Ed Engl 1994, 33: 2061; Gallop *et al.*, J Med Chem 1994, 37: 1233-51). Libraries of compounds may be presented in solution (see Houghten, Bio/Techniques 1992, 13: 412-21) or on beads (Lam, Nature 1991, 354: 82-4), chips (Fodor, Nature 1993, 364: 555-6), bacteria (US Pat. No. 5,223,409), spores (US Pat. No. 5,571,698;5,403,484, and 5,223,409), plasmids (Cull *et al.*, Proc Natl Acad Sci USA 1992, 89: 1865-9) or phage (Scott and Smith, Science 1990, 249: 386-90; Devlin, Science 1990, 249: 404-6; Cwirla *et al.*, Proc Natl Acad Sci USA 1990, 87: 6378-82; Felici, J Mol Biol 1991, 222: 301-10; US Pat. Application 2002103360).

A compound in which a part of the structure of the compound screened by any of the present screening methods is converted by addition, deletion and/or replacement, is included in the agents obtained by the screening methods of the present invention.

Furthermore, when the screened test agent is a protein, for obtaining a DNA encoding the protein, either the whole amino acid sequence of the protein may be determined to deduce the nucleic acid sequence coding for the protein, or partial amino acid sequence of the obtained protein may be analyzed to prepare an oligo DNA as a probe based on the sequence, and screen cDNA libraries with the probe to obtain a DNA encoding the protein. The obtained DNA find use in preparing the test agent which is a candidate for treating or preventing cancer.

III-1. Protein based screening methods

According to the present invention, the expression of the *RASGEF1A* gene was suggested to be crucial for the growth and/or survival of cancer cells. Therefore, it was considered that agents which suppress the function of the polypeptide encoded by the gene inhibit the growth and/or survival of cancer cells, and find use in treating or preventing cancer. Thus, the present invention provides methods of screening an agent for treating or preventing

cancer, using the RASGEF1A polypeptide.

In addition to the RASGEF1A polypeptide, fragments of the polypeptide may be used for the present screening so long as retains at least one biological activity of natural occuring RASGEF1A polypeptide.

The polypeptide or fragments thereof may be further linked to other substances so long as the polypeptide and fragments retains at least one of its biological activity. Usable substances include: peptides, lipids, sugar and sugar chains, acetyl groups, natural and synthetic polymers, etc. These kinds of modifications may be performed to confer additional functions or to stabilize the polypeptide and fragments.

The polypeptide or fragments used for the present method may be obtained from nature as naturally occurring proteins via conventional purification methods or through chemical synthesis based on the selected amino acid sequence. For example, conventional peptide synthesis methods that can be adopted for the synthesis includes:

1) Peptide Synthesis, Interscience, New York, 1966;

2) The Proteins, Vol. 2, Academic Press, New York, 1976;

3) Peptide Synthesis (in Japanese), Maruzen Co., 1975;

4) Basics and Experiment of Peptide Synthesis (in Japanese), Maruzen Co., 1985;

5) Development of Pharmaceuticals (second volume) (in Japanese), Vol. 14 (peptide synthesis), Hirokawa, 1991;

6) WO99/67288; and

7) Barany G. & Merrifield R.B., Peptides Vol. 2, "Solid Phase Peptide Synthesis", Academic Press, New York, 1980, 100-118.

Alternatively, the protein may be obtained adopting any known genetic engineering methods for producing polypeptides (*e.g.*, Morrison J., J Bacteriology 1977, 132: 349-51; Clark-Curtiss & Curtiss, Methods in Enzymology (eds. Wu *et al.*) 1983, 101: 347-62). For example, first, a suitable vector comprising a polynucleotide encoding the objective protein in an expressible form (*e.g.*, downstream of a regulatory sequence comprising a promoter) is prepared, transformed into a suitable host cell, and then the host cell is cultured to produce the protein. More specifically, a gene encoding the RASGEF1A polypeptide is expressed in host (*e.g.*, animal) cells and such by inserting the gene into a vector for expressing foreign genes, such as pSV2neo, pcDNA I, pcDNA3.1, pCAGGS, or pCD8. A promoter may be used for the expression. Any commonly used promoters may be employed including, for example, the SV40 early promoter (Rigby in Williamson (ed.), Genetic Engineering, vol. 3. Academic

Press, London, 1982, 83-141), the EF-α promoter (Kim *et al.*, Gene 1990, 91:217-23), the CAG promoter (Niwa *et al.*, Gene 1991, 108:193), the RSV LTR promoter (Cullen, Methods in Enzymology 1987, 152:684-704), the SRα promoter (Takebe *et al.*, Mol Cell Biol 1988, 8:466), the CMV immediate early promoter (Seed *et al.*, Proc Natl Acad Sci USA 1987, 84:3365-9), the SV40 late promoter (Gheysen *et al.*, J Mol Appl Genet 1982, 1:385-94), the Adenovirus late promoter (Kaufman *et al.*, Mol Cell Biol 1989, 9:946), the HSV TK promoter, and such. The introduction of the vector into host cells to express the *RASGEF1A* gene can be performed according to any methods, for example, the electroporation method (Chu *et al.*, Nucleic Acids Res 1987, 15:1311-26), the calcium phosphate method (Chen *et al.*, Mol Cell Biol 1987, 7:2745-52), the DEAE dextran method (Lopata *et al.*, Nucleic Acids Res 1984, 12:5707-17; Sussman *et al.*, Mol Cell Biol 1985, 4:1642-3), the Lipofectin method (Derijard B, Cell 1994, 7:1025-37); Lamb *et al.*, Nature Genetics 1993, 5:22-30; Rabindran *et al.*, Science 1993, 259:230-4), and such.

The RASGEF1A protein may also be produced *in vitro* adopting an *in vitro* translation system.

The RASGEF1A polypeptide to be contacted with a test agent can be, for example, a purified polypeptide, a soluble protein, or a fusion protein fused with other polypeptides.

III-1-1. Identifying agents that bind to RASGEF1A polypeptide

An agent that binds to a protein is likely to alter the expression of the gene coding for the protein or the biological activity of the protein. Thus, as an aspect, the present invention provides a method of screening an agent for treating or preventing cancer, which comprises the steps of:

a) contacting a test agent with the RASGEF1A polypeptide or a fragment thereof;

b) detecting the binding between the polypeptide and the test agent; and

c) selecting the test agent that binds to the polypeptide.

The binding of a test agent to the RASGEF1A polypeptide may be, for example, detected by immunoprecipitation using an antibody against the polypeptide. Therefore, for the purpose for such detection, it is preferred that the RASGEF1A polypeptide or fragments thereof used for the screening contains an antibody recognition site. The antibody used for the screening may be one that recognizes an antigenic region (*e.g.*, epitope) of the present RASGEF1A polypeptide which preparation methods are well known in the art, and any method may be employed in the present invention to prepare such antibodies and equivalents thereof.

Alternatively, the RASGEF1A polypeptide or a fragment thereof may be expressed as a fusion protein comprising at its N- or C-terminus a recognition site (epitope) of a 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 1995, 13:85-90). Vectors which can express a fusion protein with, for example, β-galactosidase, maltose binding protein, glutathione S-transferase, green florescence protein (GFP), and such by the use of its multiple cloning sites are commercially available and can be used for the present invention. Furthermore, fusion proteins containing much smaller epitopes to be detected by immunoprecipitation with an antibody against the epitopes are also known in the art (Experimental Medicine 1995, 13:85-90). Such epitopes consisting of several to a dozen amino acids so as not to change the property of the RASGEF1A polypeptide or fragments thereof can also be used in the present invention. Examples include 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.

Glutathione S-transferase (GST) is also well-known as the counterpart of the fusion protein to be detected by immunoprecipitation. When GST is used as the protein to be fused with the RASGEF1A polypeptide or fragment thereof to form a fusion protein, the fusion protein can be detected either with an antibody against GST or a substance specifically binding to GST, *i.e.*, such as glutathione (*e.g.*, glutathione-Sepharose 4B).

In immunoprecipitation, an immune complex is formed by adding an antibody (recognizing the RASGEF1A polypeptide or a fragment thereof itself, or an epitope tagged to the polypeptide or fragment) to the reaction mixture of the RASGEF1A polypeptide and the test agent. If the test agent has the ability to bind the polypeptide, then the formed immune complex will consists of the RASGEF1A polypeptide, the test agent, and the antibody. On the contrary, if the test agent is devoid of such ability, then the formed immune complex only consists of the RASGEF1A polypeptide and the antibody. Therefore, the binding ability of a test agent to RASGEF1A polypeptide can be examined by, for example, measuring the size of the formed immune complex. Any method for detecting the size of a substance can be used, including chromatography, electrophoresis, and such. For example, when mouse IgG antibody is used for the detection, Protein A or Protein G sepharose can be used for quantitating the formed immune complex.

For more details on immunoprecipitation see, for example, Harlow *et al.*, Antibodies,

Cold Spring Harbor Laboratory publications, New York, 1988, 511-52.

Furthermore, the RASGEF1A polypeptide or a fragment thereof used for the screening of agents that bind to thereto may be bound to a carrier. Example of carriers that may be used for binding the polypeptides include insoluble polysaccharides, such as agarose, cellulose and dextran; and synthetic resins, such as polyacrylamide, polystyrene and silicon; preferably commercially 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 is also known in the art, and enables to readily isolate polypeptides and agents bound on the beads via magnetism.

The binding of a polypeptide to a carrier may be conducted according to routine methods, such as chemical bonding and physical adsorption. Alternatively, a polypeptide may be bound to a carrier via antibodies specifically recognizing the protein. Moreover, binding of a polypeptide to a carrier can also be conducted by means of interacting molecules, such as the combination of avidin and biotin.

Screening using such carrier-bound RASGEF1A polypeptide or fragments thereof include, for example, contacting a test agent to the carrier-bound polypeptide, incubating the mixture, washing the carrier, and detecting and/or measuring the agent bound to the carrier. The binding may be carried out in buffer, for example, but are not limited to, phosphate buffer and Tris buffer, as long as the buffer does not inhibit the binding.

A screening method wherein such carrier-bound RASGEF1A polypeptide or fragments thereof and a composition (*e.g.*, cell extracts, cell lysates, *etc.*) are used as the test agent, such method is generally called affinity chromatography. For example, the RASGEF1A polypeptide may be immobilized on a carrier of an affinity column, and a test agent, containing a substance capable of binding to the polypeptides, is applied to the column. After loading the test agent, the column is washed, and then the substance bound to the polypeptide is eluted with an appropriate buffer.

A biosensor using the surface plasmon resonance phenomenon may be used as a mean for detecting or quantifying the bound agent in the present invention. When such a biosensor is used, the interaction between the RASGEF1A polypeptide and a test agent can be observed real-time as a surface plasmon resonance signal, using only a minute amount of the polypeptide and without labeling (for example, BIAcore, Pharmacia). Therefore, it is possible to evaluate the binding between the polypeptide and test agent using a biosensor such as BIAcore.

Methods of screening for molecules that bind to a specific protein among synthetic chemical compounds, or molecules in natural substance banks or a random phage peptide display library by exposing the specific protein immobilized on a carrier to the molecules, and methods of high-throughput screening based on combinatorial chemistry techniques (Wrighton *et al.*, Science 1996, 273:458-64; Verdine, Nature 1996, 384:11-3) to isolate not only proteins but chemical compounds are also well-known to those skilled in the art. These methods can also be used for screening agents (including agonist and antagonist) that bind to the RASGEF1A protein or fragments thereof.

When the test agent is a protein, for example, West-Western blotting analysis (Skolnik *et al.*, Cell 1991, 65:83-90) can be used for the present method. Specifically, a protein binding to the RASGEF1A polypeptide can be obtained by preparing first a cDNA library is prepared from cells, tissues, organs, or cultured cells (*e.g.*, SSP25) expected to express at least one protein binding to the RASGEF1A polypeptide using a phage vector (*e.g.*, ZAP), expressing the proteins encoded by the vectors of the cDNA library on LB-agarose, fixing the expressed proteins on a filter, reacting the purified and labeled RASGEF1A polypeptide with the above filter, and detecting the plaques expressing proteins to which the RASGEF1A polypeptide has bound according to the label of the RASGEF1A polypeptide.

Labeling substances such as radioisotope (*e.g.*, ^{3}H, ^{14}C, ^{32}P, ^{33}P, ^{35}S, ^{125}I, ^{131}I), enzymes (*e.g.*, alkaline phosphatase, horseradish peroxidase, β-galactosidase, β-glucosidase), fluorescent substances (*e.g.*, fluorescein isothiosyanete (FITC), rhodamine) and biotin/avidin, may be used for the labeling of RASGEF1A polypeptide in the present method. When the protein is labeled with radioisotope, the detection or measurement can be carried out by liquid scintillation. Alternatively, when the protein is labeled with an enzyme, it 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.

Moreover, the RASGEF1A polypeptide bound to the protein can be detected or measured by utilizing an antibody that specifically binds to the RASGEF1A polypeptide, or a peptide or polypeptide (for example, GST) that is fused to the RASGEF1A polypeptide. 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 the RASGEF1A polypeptide 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 RASGEF1A polypeptide in the present screening may be detected or measured using protein G or protein A column.

Alternatively, in another embodiment of the screening method of the present invention, 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 *et al.*, Cell 1992, 68:597-612" and "Fields *et al.*, Trends Genet 1994, 10:286-92"). In two-hybrid system, RASGEF1A polypeptide or a fragment thereof is fused to the SRF-binding region or GAL4-binding region and expressed in yeast cells. A cDNA library is prepared from cells expected to express at least one protein binding to the RASGEF1A 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 RASGEF1A polypeptide is expressed in the yeast cells, the binding of the two activates a reporter gene, making positive clones detectable). A protein encoded by the cDNA can be prepared by introducing the cDNA isolated above to *E. coli* and expressing the protein.

As a reporter gene, for example, Ade2 gene, lacZ gene, CAT gene, luciferase gene and such can be used in addition to the HIS3 gene.

The agent isolated by this screening is a candidate for agonists or antagonists of the RASGEF1A polypeptide. The term "agonist" refers to molecules that activate the function of the polypeptide by binding thereto. On the other hand, the term "antagonist" refers to molecules that inhibit the function of the polypeptide by binding thereto. Moreover, an agent isolated by this screening as an antagonist is a candidate that inhibits the *in vivo* interaction of the RASGEF1A polypeptide with molecules (including nucleic acids (RNAs and DNAs) and proteins).

III-1-2. Identifying agents by detecting biological activity of the RASGEF1A polypeptide

According to the present invention, the expression of *RASGEF1A* gene was shown to by crucial for the growth and/or survival of cancer cells. Therefore, agents that suppress or inhibit the biological function of the translational product of the *RASGEF1A* gene is considered to serve as candidates for treating or preventing cancer. Thus, the present invention also provides a method for screening a compound for treating or preventing cancer

using the RASGEF1A polypeptide or fragments thereof comprising the steps as follows:

a) contacting a test agent with the RASGEF1A polypeptide or a fragment thereof; and

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

Any polypeptide can be used for the screening so long as it has one biological activity of the RASGEF1A polypeptide that can be used as an index in the present screening method. Since the RASGEF1A polypeptide has the activity of promoting cell proliferation of cancer cells, biological activities of the RASGEF1A polypeptide that can be used as an index for the screening include such cell-proliferating activity of the human RASGEF1A polypeptide. For example, a human RASGEF1A polypeptide can be used and polypeptides functionally equivalent thereto including fragments thereof can also be used. Such polypeptides may be expressed endogenously or exogenously by suitable cells.

When the biological activity to be detected in the present method is cell proliferation, it can be detected, for example, by preparing cells which express the RASGEF1A polypeptide or a fragment thereof, culturing the cells in the presence of a test agent, and determining the speed of cell proliferation, measuring the cell cycle and such, as well as by detecting wound-healing activity, conducting Matrigel invasion assay and measuring the colony forming activity as described in the Example (see, "Materials and Methods").

According to an aspect of the present invention, the screening further comprises after the above step (b) the step of :

c) selecting the test agent that suppresses the biological activity of the polypeptide as compared to the biological activity detected in the absence of the test agent.

The agent isolated by this screening is a candidate for an antagonist of the RASGEF1A polypeptide, and thus, is a candidate that inhibits the in vivo interaction of the polypeptide with molecules (including nucleic acids (RNAs and DNAs) and proteins).

As another aspect of the invention, agents serving as a candidate for agonist of the RASGEF1A polypeptide can be screened using the biological activity of the polypeptide as an index. According to such a method, agents that enhance the biological activity of the RASGEF1A polypeptide are selected. Specifically, the screening method further comprises after the above step (b) the step of :

c) selecting the test agent that enhances the biological activity of the polypeptide as compared to the biological activity detected in the absence of the test agent.

Herein, it was revealed that RASGEF1A polypeptide enhances the activity of RAS. Accordingly, an agent which decreases the RAS-enhancing activity, *i.e.*, one of the biological

activity of RASGEF1A polypeptide may be used for treating or preventing cancer. Therefore the present invention further provides a method for screening an agent for treating or preventing cancer. An embodiment of this screening method comprises the steps of:

(a) contacting the RASGEF1A polypeptide or a fragment thereof and RAS in the presence of a test agent;

(b) detecting the RAS activity; and

(c) selecting the test agent that decreases the RAS activity as compared to that detected in the absence of the test agent.

The RAS activity can be measured according to a method known in the art; for example, by GDP detection (see, *e.g.*, Hall BE et al., J Biol Chem 2001, 276 : 27629-37) or RAS activation assay. In the present invention, in order to estimate the RAS activation activity of RASGEF1A polypeptide under the presence or absence of a test agent, for example, a cell lysate of *RASGEF1A* gene expressing cell is incubated with GST-Ras fusion protein, GDP, and GTPγS in the presence or absence of the test compound. After the incubation, active or GTP-Ras is detected by Western blot analysis using anti-Ras antibody which detects the active or GTP-Ras at 21 kDa. A pull-down assay of active or GTP-Ras can be performed with glutathione disc. Commercially available RAS activation assay kit can also be used for the screening method of the present invention. For example, "StressXpress® RAS Activation Kit" (Stressgen Bioreagents Corp.) or "EZ-Detect TM Ras Activation Kit" (PIERCE Biotechnology) comprises all elements for the assay, *e.g.* anti RAS antibody, glutathione disc, GST fusion protein which contains the Ras binding domain (RBD) of Raf1, GDP, and GTPγS. Furthermore, Ras activity can be detected as described in the Example (see under the item of "8. Ras Nucleotide Dissociation Assay" and "9. Analysis of Ras activity" of "Materials and Methods").

III-2. Nucleotide based screening methods

III-2-1. Screening method using RASGEF1A gene

As discussed in detail above, by controlling the expression level of the *RASGEF1A* gene, one can control the onset and progression of cancer. Thus, agents that may be used in the treatment or prevention of cancers can be identified through screenings that use the expression levels of *RASGEF1A* gene as indices. In the context of the present invention, such screening may comprise, for example, the following steps:

a) contacting a test agent with a cell expressing the *RASGEF1A* gene;

b) detecting the expression level of the *RASGEF1A* gene; and

c) selecting the test agent that reduces the expression level of the *RASGEF1A* gene as compared to a level detected in the absence of the test agent.

An agent that inhibits the expression of the *RASGEF1A* gene or the activity of its gene product can be identified by contacting a cell expressing the *RASGEF1A* gene with a test agent and then determining the expression level of the *RASGEF1A* gene. Naturally, the identification may also be performed using a population of cells that express the gene in place of a single cell. A decreased expression level detected in the presence of an agent as compared to the expression level in the absence of the agent indicates the agent as being an inhibitor of the *RASGEF1A* gene, suggesting the possibility that the agent is useful for inhibiting cancer, thus a candidate agent to be used for the treatment or prevention of cancer.

The expression level of a gene can be estimated by methods well known to one skilled in the art. The expression level of the *RASGEF1A* gene can be, for example, determined following the method described above under the item of 'I-1. Method for diagnosing cancer or a predisposition for developing cancer'.

The cell or the cell population used for such identification may be any cell or any population of cells so long as it expresses the *RASGEF1A* gene. For example, the cell or population may be or contain an epithelial cell derived from a tissue. Alternatively, the cell or population may be or contain an immortalized cell derived from a carcinoma cell, including those derived from ICC, bladder carcinoma, lung cancer, gastric cancer, colorectal cancer, prostate cancer, chronic myeloid leukemia, renal cancer, soft tissue sarcoma, pancreatic cancer, and seminoma. Cells expressing the *RASGEF1A* gene include, for example, cell lines established from cancers (*e.g.*, SSP25). Furthermore, the cell or population may be or contain a cell which has been transfected with *RASGEF1A* gene.

The present method allows screening of various agents mentioned above and is particularly suited for screening functional nucleic acid molecules including antisense RNA, siRNA, and such.

III-2-2. Screening method using transcriptional regulatory region of RASGEF1A gene

According to another aspect, the present invention provides a method which comprises the following steps of:

a) contacting a test agent with a cell into which a vector, comprising the transcriptional regulatory region of the *RASGEF1A* gene and a reporter gene that is expressed under the control of the transcriptional regulatory region, has been introduced;

b) detecting the expression or activity of said reporter gene; and

c) selecting the test agent that reduces the expression or activity of said reporter gene as compared to a level detected in the absence of the test agent.

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 the *RASGEF1A* gene, which can be obtained as a nucleotide segment containing the transcriptional regulatory region from a genome library based on the nucleotide sequence information of the gene.

The transcriptional regulatory region may be, for example, the promoter sequence of the *RASGEF1A* gene.

When a cell(s) transfected with a reporter gene that is operably linked to the regulatory sequence (*e.g.* promoter sequence) of the *RASGEF1A* gene is used, an agent can be identified as inhibiting or enhancing the expression of the *RASGEF1A* gene through detecting the expression level of the reporter gene product.

As a reporter gene, for example, Ade2 gene, lacZ gene, CAT gene, luciferase gene, HIS3 gene, and such well-known in the art can be used. Methods for detection of the expression of these genes are well known in the art.

III-3. Selecting therapeutic agents that are appropriate for a particular individual

Differences in the genetic makeup of individuals can result in differences in their relative abilities to metabolize various drugs. An agent that is metabolized in a subject to act as an anti-tumor agent can manifest itself by inducing a change in a 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 *RASGEF1A* gene differentially expressed between cancerous and non-cancerous cells disclosed herein allow for a putative therapeutic or prophylactic inhibitor of cancer to be tested in a test cell population from a selected subject in order to determine if the agent is a suitable inhibitor of cancer in the subject.

To identify an inhibitor of cancer that is appropriate for a specific subject, a test cell population from the subject is exposed to a candidate therapeutic agent, and the expression of *RASGEF1A* gene is determined.

In the context of the method of the present invention, test cell populations contain cancer cells expressing the *RASGEF1A* gene. Preferably, the test cell is an epithelial cell.

Specifically, a test cell population may be incubated in the presence of a candidate therapeutic agent and the expression of the *RASGEF1A* gene in the test cell population may be

measured and compared to one or more reference profiles, *e.g.*, a cancerous reference expression profile or a non-cancerous reference expression profile.

A decrease in the expression of the *RASGEF1A* gene in a test cell population relative to a reference cell population containing cancer indicates that the agent has therapeutic potential.

IV. Pharmaceutical compositions for treating or preventing cancers:

The agents screened by any of the screening methods of the present invention, antisense nucleic acids and siRNAs of the *RASGEF1A* gene, and antibodies against the RASGEF1A polypeptide inhibit or suppress the expression of the *RASGEF1A* gene, or the biological activity of the RASGEF1A polypeptide and inhibit or disrupts cell cycle regulation and cell proliferation. Thus, the present invention provides compositions for treating or preventing cancer, which compositions include agents screened by any of the screening methods of the present invention, antisense nucleic acids and siRNAs of the *RASGEF1A* gene, or antibodies against the RASGEF1A polypeptide. The present compositions can be used for treating or preventing cancer such as ICC, bladder carcinoma, lung cancer, gastric cancer, colorectal cancer, prostate cancer, chronic myeloid leukemia, renal cancer, soft tissue sarcoma, pancreatic cancer, and seminoma.

The compositions may be used as pharmaceuticals for humans and other mammals, such as mice, rats, guinea-pigs, rabbits, cats, dogs, sheep, pigs, cattle, monkeys, baboons, and chimpanzees.

In the context of the present invention, suitable pharmaceutical formulations for the active ingredients of the present invention detailed below (including screened agents, antisense nucleic acids, siRNA, antibodies, *etc.*) include those suitable for oral, rectal, nasal, topical (including buccal and sub-lingual), vaginal or parenteral (including intramuscular, subcutaneous and intravenous) administration, or for administration by inhalation or insufflation. Preferably, administration is intravenous. The formulations are optionally packaged in discrete dosage units.

Pharmaceutical formulations suitable for oral administration include capsules, microcapsules, cachets and tablets, each containing a predetermined amount of active ingredient. Suitable formulations also include powders, elixirs, granules, solutions, suspensions and emulsions. The active ingredient is optionally administered as a bolus electuary or paste. Alternatively, according to needs, the pharmaceutical composition may be administered non-orally, in the form of injections of sterile solutions or suspensions with

water or any other pharmaceutically acceptable liquid. For example, the active ingredients of the present invention can be mixed with pharmaceutically acceptable carriers or media, specifically, sterilized water, physiological saline, plant-oils, emulsifiers, suspending agents, surfactants, stabilizers, flavoring agents, excipients, vehicles, preservatives, binders, and such, in a unit dose form required for generally accepted drug implementation. The amount of active ingredient contained in such a preparation makes a suitable dosage within the indicated range acquirable.

Examples of additives that can be admixed into tablets and capsules include, but are not limited to, binders, such as gelatin, corn starch, tragacanth gum and arabic gum; excipients, such as crystalline cellulose; swelling agents, such as corn starch, gelatin and alginic acid; lubricants, such as magnesium stearate; sweeteners, such as sucrose, lactose or saccharin; and flavoring agents, such as peppermint, Gaultheria adenothrix oil and cherry. A tablet may be made by compression or molding, optionally with one or more formulational 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. 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.

Furthermore, when the unit-dosage form is a capsule, a liquid carrier, such as oil, can be further included in addition to the above ingredients.

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.

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.

Moreover, sterile composites for injection can be formulated following normal drug implementations using vehicles, such as distilled water, suitable for injection. Physiological saline, glucose, and other isotonic liquids, including adjuvants, such as D-sorbitol, D-mannose, D-mannitol, and sodium chloride, can be used as aqueous solutions for injection. These can be used in conjunction with suitable solubilizers, such as alcohol, for example, ethanol; polyalcohols, such as propylene glycol and polyethylene glycol; and non-ionic surfactants, such as Polysorbate 80 (TM) and HCO-50.

Sesame oil or soy-bean oil can be used as an oleaginous liquid, which may be used in conjunction with benzyl benzoate or benzyl alcohol as a solubilizer, and may be formulated with a buffer, such as phosphate buffer and sodium acetate buffer; a pain-killer, such as procaine hydrochloride; a stabilizer, such as benzyl alcohol and phenol; and/or an anti-oxidant. A prepared injection may be filled into a suitable ampoule.

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.

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, dichiorotetrafluoroethane, 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.

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.

Other formulations include implantable devices and adhesive patches; which release a therapeutic agent.

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. It should be understood that in addition to the ingredients particularly mentioned above, the formulations of this invention may include other agents conventional in the art having regard to the type of formulation in question, for example, those suitable for oral administration may include flavoring agents.

Preferred unit dosage formulations are those containing an effective dose, as recited below under the item of 'Method for treating or preventing cancer', of each of the active ingredients of the present invention or an appropriate fraction thereof.

IV-1. Pharmaceutical compositions comprising screened agents

The present invention provides compositions for treating or preventing cancers comprising any of the agents selected by the above-described screening methods of the present invention.

An agent screened by the method of the present invention can be directly administered or can be formulated into a dosage form according to any conventional pharmaceutical preparation method detailed above.

IV-2. Pharmaceutical compositions comprising siRNA

An siRNA against the *RASGEF1A* gene (hereinafter, also referred to as 'RASGEF1A siRNA') can be used to reduce the expression level of the gene. Herein, the term "siRNA" refers to a double-stranded RNA molecule which prevents translation of a target mRNA. In the context of the present invention, the siRNA comprises a sense nucleic acid sequence and an anti-sense nucleic acid sequence against the up-regulated marker gene, *RASGEF1A*. The siRNA is constructed so that it both comprises a sense and complementary antisense sequences of the target gene, *i.e.*, a nucleotide comprising a hairpin structure.

An siRNA of the *RASGEF1A* gene hybridizes to target mRNA, *i.e.*, associates with the normally single-stranded mRNA transcript and thereby interfering with translation of the mRNA, which finally decreases or inhibits production (expression) of the polypeptide

encoded by the gene. Thus, an siRNA molecule of the invention can be defined by its ability to specifically hybridize to the mRNA of the *RASGEF1A* gene under stringent conditions.

In the context of the present invention, an siRNA is preferably less than 500, 200, 100, 50, or 25 nucleotides in length. More preferably an siRNA is 19-25 nucleotides in length. Exemplary target nucleic acid sequences of RASGEF1A siRNA includes the nucleotide sequences of SEQ ID NO: 9 or 13. The nucleotide "t" in the sequence should be replaced with "u" in RNA or derivatives thereof. Accordingly, for example, the present invention provides double-stranded RNA molecules comprising nucleotide sequence 5'-gagaauggcacagugaaga -3' (SEQ ID NO: 9) or 5'- caucuacuuccugcacaaa -3' (SEQ ID NO: 13). In order to enhance the inhibition activity of the siRNA, nucleotide "u" can be added to the 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 a single strand at the 3'end of the antisense strand of the siRNA.

A loop sequence consisting of an arbitrary nucleotide sequence can be located between the sense and antisense sequence in order to form the hairpin loop structure. Thus, the present invention also provides siRNA having the general formula 5'-[A]-[B]-[A']-3', wherein [A] is a ribonucleotide sequence corresponding to a sequence that specifically hybridizes to an mRNA or a cDNA of the *RASGEF1A* gene. In preferred embodiments, [A] is a ribonucleotide sequence corresponding to a sequence of the *RASGEF1A* gene; [B] is a ribonucleotide sequence consisting of 3 to 23 nucleotides; and [A'] is a ribonucleotide sequence consisting of the complementary sequence of [A]. The region [A] hybridizes to [A'], and then a loop consisting of region [B] is formed. The loop sequence may be preferably 3 to 23 nucleotide in length. The loop sequence, for example, can be selected from a group consisting of following sequences (http://www.ambion.com/techlib/tb/tb_506.html):

CCC, CCACC, or CCACACC: Jacque JM *et al.*, Nature 2002, 418: 435-8.

UUCG: Lee NS *et al.*, Nature Biotechnology 2002, 20:500-5; Fruscoloni P *et al.*, Proc Natl Acad Sci USA 2003, 100(4):1639-44.

UUCAAGAGA: Dykxhoorn DM *et al.*, Nature Reviews Molecular Cell Biology 2003, 4:457-67.

'UUCAAGAGA ("ttcaagaga" in DNA)' is a particularly suitable loop sequence. Furthermore, loop sequence consisting of 23 nucleotides also provides an active siRNA (Jacque J-M *et al.*, Nature 2002, 418:435-8).

Exemplary hairpin siRNA suitable for use in the context of the present invention

include, for RASGEF1A-siRNA,
5'-gagaauggcacagugaaga-[B]-ucuucacugugccauucuc-3' (for target sequence of SEQ ID NO: 9); and
5'-caucuacuuccugcacaaa-[B]-uuugugcaggaaguagaug-3' (for target sequence of SEQ ID NO: 13).

The nucleotide sequence of suitable siRNAs can be designed using an siRNA design computer program available from the Ambion website (http://www.ambion.com/techlib/misc/siRNA_finder.html). The computer program selects nucleotide sequences for siRNA synthesis based on the following protocol.

Selection of siRNA Target Sites:

1. Beginning with the AUG start codon of the object 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 Cev 1999, 13(24):3191-7 don't recommend against designing siRNA to the 5' and 3' untranslated regions (UTRs) and regions near the start codon (within 75 nucleotides) as these may be richer in regulatory protein binding sites. UTR-binding proteins and/or translation initiation complexes may interfere with binding of the siRNA endonuclease complex.
2. Compare the potential target sites to the 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 (Altschul SF *et al.*, Nucleic Acids Res 1997, 25:3389-402; J Mol Biol 1990, 215:403-10.), which can be found on the NCBI server at: www.ncbi.nlm.nih.gov/BLAST/.
3. Select qualifying target sequences for synthesis. At Ambion, preferably several target sequences can be selected along the length of the gene to evaluate.

Standard techniques for introducing siRNA into the cell may be used. For example, an siRNA of RASGEF1A can be directly introduced into the cells in a form that is capable of binding to the mRNA transcripts. In these embodiments, the siRNA molecules of the present invention 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 2003, 9:347-51).

Alternatively, a DNA encoding the siRNA may be carried in a vector (hereinafter, also referred to as 'siRNA vector'). Such vectors may be produced, for example, by cloning the

target *RASGEF1A* gene sequence into an expression vector having operatively-linked regulatory sequences (*e.g.*, a RNA polymerase III transcription unit from the small nuclear RNA (snRNA) U6 or the human H1 RNA promoter) flanking the sequence in a manner that allows for expression (by transcription of the DNA molecule) of both strands (Lee NS *et al.*, Nature Biotechnology 2002, 20: 500-5). For example, an RNA molecule that is antisense to mRNA of the *RASGEF1A* gene is transcribed by a first promoter (*e.g.*, a promoter sequence 3' of the cloned DNA) and an RNA molecule that is the sense strand for the mRNA of the *RASGEF1A* gene is transcribed by a second promoter (*e.g.*, a promoter sequence 5' of the cloned DNA). The sense and antisense strands hybridize *in vivo* to generate siRNA constructs for silencing the expression of the *RASGEF1A* gene. Alternatively, the two constructs can be utilized to create the sense and anti-sense strands of a single-stranded siRNA construct. In this case, a construct having secondary structure, *e.g.*, hairpin, is produced as a single transcript that comprises both the sense and complementary antisense sequences of the target gene.

Thus, the present pharmaceutical composition for treating or preventing cancer comprises either the siRNA or a vector expressing the siRNA *in vivo*.

For introducing the siRNA vector into the cell, transfection-enhancing agent can be used. FuGENE6 (Roche diagnostics), Lipofectamine 2000 (Invitrogen), Oligofectamine (Invitrogen), and Nucleofector (Wako pure Chemical) are useful as the transfection-enhancing agent. Therefore, the present pharmaceutical composition may further include such transfection-enhancing agents.

IV-3. Pharmaceutical compositions comprising antisense nucleic acids

Antisense nucleic acids corresponding to the nucleotide sequence of the *RASGEF1A* gene can be used to reduce the expression level of the gene, which is up-regulated in various cancerous cells, are useful for the treatment of cancer and thus are also encompassed by the present invention. An antisense nucleic acid acts by binding to the nucleotide sequence of the *RASGEF1A* gene, or mRNAs corresponding thereto, thereby inhibiting the transcription or translation of the gene, promoting the degradation of the mRNAs, and/or inhibiting the expression of the protein encoded by the gene. Thus, as a result, an antisence nucleic acid inhibits the RASGEF1A protein to function in the cancerous cell. Herein, the phrase "antisense nucleic acids" refers to nucleotides that specifically hybridize to a target sequence and includes not only nucleotides that are entirely complementary to the target sequence but also that comprise mismatches of one or more nucleotides. For example, the antisense nucleic

acids of the present invention include polynucleotides that have a homology of at least 70% or higher, preferably of at least 80% or higher, more preferably of at least 90% or higher, even more preferably of at least 95% or higher over a span of at least 15 continuous nucleotides of the *RASGEF1A* gene or the complementary sequence thereof. Algorithms known in the art can be used to determine such homology.

Antisense nucleic acids of the present invention act on cells producing proteins encoded by the *RASGEF1A* gene by binding to the DNA or mRNA of the gene, inhibiting their transcription or translation, promoting the degradation of the mRNA, and inhibiting the expression of the protein, finally inhibiting the protein to function.

Antisense nucleic acids of the present invention can be made into an external preparation, such as a liniment or a poultice, by admixing it with a suitable base material which is inactive against the nucleic acids.

Also, as needed, the antisense nucleic acids of the present invention can be formulated into tablets, powders, granules, capsules, liposome capsules, injections, solutions, nose-drops and freeze-drying agents by adding excipients, isotonic agents, solubilizers, stabilizers, preservatives, pain-killers, and such. An antisense-mounting medium can also be used to increase durability and membrane-permeability. Examples include, but are not limited to, liposomes, poly-L-lysine, lipids, cholesterol, lipofectin, or derivatives of these.These can be prepared by following known methods.

The antisense nucleic acids of the present invention inhibit the expression of the RASGEF1A protein and are useful for suppressing the biological activity of the protein. In addition, expression-inhibitors, comprising antisense nucleic acids of the present invention, are useful in that they can inhibit the biological activity of the RASGEF1A protein.

The antisense nucleic acids of present invention include modified oligonucleotides. For example, thioated oligonucleotides may be used to confer nuclease resistance to an oligonucleotide.

IV-4. Pharmaceutical compositions comprising antibodies

The function of a gene product of the *RASGEF1A* gene which is over-expressed in various cancers can be inhibited by administering a compound that binds to or otherwise inhibits the function of the gene products. An antibody against the RASGEF1A polypeptide can be mentioned as such a compound and can be used as the active ingredient of a pharmaceutical composition for treating or preventing cancer.

The present invention relates to the use of antibodies against a protein encoded by the

RASGEF1A gene, or fragments of the antibodies. As used herein, the term "antibody" refers to an immunoglobulin molecule having a specific structure, that interacts (*i.e.*, binds) only with the antigen that was used for synthesizing the antibody (*i.e.*, the gene product of an up-regulated marker) or with an antigen closely related thereto. Molecules comprising the antigen that was used for synthesizing the antibody and molecules comprising the epitope of the antigen recognized by the antibody can be mentioned as closely related antigens thereto.

Furthermore, an antibody used in the present pharmaceutical compositions may be a fragment of an antibody or a modified antibody, so long as it binds to the protein encoded by the *RASGEF1A* gene (*e.g.*, an immunologically active fragment of anti-RASGEF1A antibody). For instance, the antibody fragment may be Fab, $F(ab')_2$, Fv, or single chain Fv (scFv), in which Fv fragments from H and L chains are ligated by an appropriate linker (Huston JS *et al.*, Proc Natl Acad Sci USA 1988, 85:5879-83). Such antibody fragments 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 MS *et al.*, J Immunol 1994, 152:2968-76; Better M *et al.*, Methods Enzymol 1989, 178:476-96; Pluckthun A *et al.*, Methods Enzymol 1989, 178:497-515; Lamoyi E, Methods Enzymol 1986, 121:652-63; Rousseaux J *et al.*, Methods Enzymol 1986, 121:663-9; Bird RE *et al.*, Trends Biotechnol 1991, 9:132-7).

An antibody may be modified by conjugation with a variety of molecules, such as polyethylene glycol (PEG). The present invention includes such modified antibodies. The modified antibody can be obtained by chemically modifying an antibody. Such modification methods are conventional in the field.

Alternatively, the antibody used for the present invention may be a chimeric antibody having a variable region derived from a non-human antibody against the RASGEF1A polypeptide and a constant region derived from a human antibody, or a humanized antibody, comprising a complementarity determining region (CDR) derived from a non-human antibody, a frame work region (FR) and a constant region derived from a human antibody. Such antibodies can be prepared by using known technologies. Humanization can be performed by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody (see *e.g.*, Verhoeyen *et al.*, Science 1988, 239:1534-6). 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.

Complete 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 *et al.*, J Mol Biol 1992, 227:381). Similarly, human antibodies can be made by introducing 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; and 5,661,016.

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.

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, isoelectric 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).

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.

V. Methods for treating or preventing cancer:

Cancer therapies directed at specific molecular alterations that occur in cancer cells have been validated through clinical development and regulatory approval of anti-tumor pharmaceuticals such as trastuzumab (Herceptin) for the treatment of advanced cancers,

imatinib mesylate (Gleevec) for chronic myeloid leukemia, gefitinib (Iressa) for non-small cell lung cancer (NSCLC), and rituximab (anti-CD20 mAb) for B-cell lymphoma and mantle cell lymphoma (Ciardiello F *et al.*, Clin Cancer Res 2001, 7:2958-70, Review; Slamon DJ *et al.*, N Engl J Med 2001, 344:783-92; Rehwald U *et al.*, Blood 2003, 101:420-4; Fang G *et al.*, Blood 2000, 96:2246-53). These drugs are clinically effective and better tolerated than traditional anti-tumor agents because they target only transformed cells. Hence, such drugs not only improve survival and quality of life for cancer patients, but also validate the concept of molecularly targeted cancer therapy. Furthermore, targeted drugs can enhance the efficacy of standard chemotherapy when used in combination with it (Gianni L, Oncology 2002, 63 Suppl 1:47-56; Klejman A *et al.*, Oncogene 2002, 21:5868-76). Therefore, future cancer treatments will probably involve combining conventional drugs with target-specific agents aimed at different characteristics of tumor cells such as angiogenesis and invasiveness.

These modulatory methods can be performed *ex vivo* or *in vitro* (*e.g.*, by culturing the cell with the agent) or, alternatively, *in vivo* (*e.g.*, by administering the agent to a subject). The methods involve administering a protein or combination of proteins or a nucleic acid molecule or combination of nucleic acid molecules as therapy to counteract aberrant expression of the differentially expressed genes or aberrant activity of their gene products.

Diseases and disorders that are characterized by increased (relative to a subject not suffering from the disease or disorder) expression levels or biological activities of genes and gene products, respectively, may be treated with therapeutics that antagonize (*i.e.*, reduce or inhibit) activity of the over-expressed gene. Therapeutics that antagonize activity can be administered therapeutically or prophylactically.

Accordingly, therapeutics that may be utilized in the context of the present invention include, *e.g.*, (i) a polypeptide of the over-expressed *RASGEF1A* gene or analogs, derivatives, fragments or homologs thereof; (ii) antibodies to the over-expressed gene or gene products; (iii) nucleic acids encoding the over-expressed gene; (iv) antisense nucleic acids or nucleic acids that are "dysfunctional" (*i.e.*, due to a heterologous insertion within the nucleic acids of over-expressed gene); (v) small interfering RNA (siRNA); or (vi) modulators (*i.e.*, inhibitors, antagonists that alter the interaction between an over-expressed polypeptide and its binding partner). The dysfunctional antisense molecules are utilized to "knockout" endogenous function of a polypeptide by homologous recombination (see, *e.g.*, Capecchi, Science 1989, 244: 1288-92).

Increased levels can be readily detected by quantifying peptide and/or RNA, by

obtaining a patient tissue sample (*e.g.*, from biopsy tissue) and assaying it *in vitro* for RNA or peptide levels, structure and/or activity of the expressed peptides (or mRNAs of a gene whose expression is altered). Methods that are well-known within the art include, but are not limited to, immunoassays (*e.g.*, by Western blot analysis, immunoprecipitation followed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis, immunocytochemistry, *etc.*) and/or hybridization assays to detect expression of mRNAs (*e.g.*, Northern blot assays, dot blots, in situ hybridization, *etc.*).

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.

Therapeutic methods of the present invention may include the step of contacting a cell with an agent that modulates one or more of the activities of the RASGEF1A gene products. Examples of agent that modulates protein activity include, but are not limited to, nucleic acids, proteins, naturally occurring cognate ligands of such proteins, peptides, peptidomimetics, and other small molecule.

Thus, the present invention provides methods for treating or alleviating a symptom of cancer, or preventing cancer in a subject by decreasing the expression of the *RASGEF1A* gene or the activity of the gene product. The present method is particularly suited for treating or preventing ICC, bladder carcinoma, lung cancer, gastric cancer, colorectal cancer, prostate cancer, chronic myeloid leukemia, renal cancer, soft tissue sarcoma, pancreatic cancer, and seminoma.

Suitable therapeutics can be administered prophylactically or therapeutically to a subject suffering from or at risk of (or susceptible to) developing cancers. Such subjects can be identified by using standard clinical methods or by detecting an aberrant expression level ("up-regulation" or "over-expression") of the *RASGEF1A* gene or aberrant activity of the gene product.

According to an aspect of the present invention, an agent screened through the present method may be used for treating or preventing cancer. Methods well known to those skilled in the art may be used to administer the agents to patients, for example, as an intraarterial, intravenous, or percutaneous injection or as an intranasal, transbronchial, intramuscular, or oral administration. If said agent is encodable by a DNA, the DNA can be inserted into a vector for gene therapy and the vector administered to a patient to perform the therapy.

The dosage and methods for administration vary according to the body-weight, age,

sex, symptom, condition of the patient to be treated and the administration method; however, one skilled in the art can routinely select suitable dosage and administration method.

For example, although the dose of an agent that binds to the RASGEF1A polypeptide and regulates the activity of the polypeptide depends on the aforementioned various factors, the dose is generally 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 human (60 kg weight).

When administering the agent parenterally, in the form of an injection to a normal adult human (60 kg weight), although there are some differences according to the patient, target organ, symptoms and methods for 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. In the case of other animals, the appropriate dosage amount may be routinely calculated by converting to 60 kg of body-weight.

Similarly, a pharmaceutical composition of the present invention may be used for treating or preventing cancer. Methods well known to those skilled in the art may be used to administer the compositions to patients, for example, as an intraarterial, intravenous, or percutaneous injection or as an intranasal, transbronchial, intramuscular, or oral administration.

For each of the aforementioned conditions, the compositions, *e.g.*, polypeptides and organic compounds, can be administered orally or via injection at a dose ranging 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.

The dose employed will depend upon a number of factors, including the age, body weight 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. In any event, appropriate and optimum dosages may be routinely calculated by those skilled in the art, taking into consideration the above-mentioned factors.

In particular, an antisense nucleic acids against the *RASGEF1A* gene can be given to

the patient by direct application onto the ailing site or by injection into a blood vessel so that it will reach the site of ailment.

The dosage of the antisense nucleic acid derivatives of the present invention 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.

VI. Vaccinating against cancer:

The present invention relates to a method for treating or preventing cancers in a subject comprising the step of administering to said subject a vaccine comprising a polypeptide encoded by the *RASGEF1A* gene (*i.e.*, RAS polypeptide), an immunologically active fragment of said polypeptide, or a polynucleotide encoding such a polypeptide or fragment thereof. In the present invention, vaccines against cancers refer to substances that have the ability to induce anti-tumor immunity upon inoculation into animals.

Administration of such a vaccine induces anti-tumor immunity in the subject. According to the present invention, the RASGEF1A polypeptide and immunologically active fragments thereof were suggested to be HLA-A24 or HLA-A*0201 restricted epitope peptides that may induce potent and specific immune response against cancer cells expressing the *RASGEF1A* gene. Thus, the present invention also relates to a method for inducing anti-tumor immunity in a subject in need thereof by administering to said subject a vaccine comprising a polypeptide encoded by the *RASGEF1A* gene (*i.e.*, RAS polypeptide), an immunologically active fragment of said polypeptide, or a polynucleotide encoding such a polypeptide or fragment thereof.

These methods are particularly suited for treating or preventing ICC, bladder carcinoma, lung cancer, gastric cancer, colorectal cancer, prostate cancer, chronic myeloid leukemia, renal cancer, soft tissue sarcoma, pancreatic cancer, and seminoma.

In some cases, the RASGEF1A protein or immunologically active fragments thereof may be administered in a form bound to T cell receptors (TCRs) or presented by APCs, such as macrophage, dendritic cell (DC), B-cells, or PBMCs. Due to the strong antigen presenting ability of DC, the use of DC is most preferable among the APCs.

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.

Therefore, when a certain protein induces any one of these immune responses upon

inoculation into an animal, the protein is determined to have anti-tumor immunity inducing effect. Any protein fragment of the RASGEF1A polypeptide may be used as an immunologically active fragment of the present methods.

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.

For example, a method for detecting the induction of CTLs is well known. Specifically, a foreign substance that enters the living body is presented to T cells and B cells by the action of APCs. T cells that respond to the antigen presented by the APCs in an antigen specific manner differentiate into CTLs due to stimulation by the antigen, and then proliferate (this is referred to as activation of T cells). Therefore, CTL induction by a certain peptide can be evaluated by presenting the peptide to a T cell via an APC, and detecting the induction of CTLs. Furthermore, APCs have the effect of activating CD4+ T cells, CD8+ T cells, macrophages, eosinophils, and NK cells. Since CD4+ T cells and CD8+ 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.

A method for evaluating the inducing action of CTLs using DCs as the APC is well known in the art. DCs are representative APCs having the strongest CTL-inducing action among APCs. To determine whether a fragment of the RASGEF1A polypeptide is immunological active and can be used in the present method, a test polypeptide (*i.e.*, any fragment of the RASGEF1A polypeptide) is initially contacted with DCs, and then the DCs are 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 CTLs against tumors can be detected, for example, using the lysis of ^{51}Cr-labeled tumor cells as the indicator. Alternatively, the methods for evaluating the degree of tumor cell damage using ^{3}H-thymidine uptake activity or LDH (lactose dehydrogenase)-release as the indicator is also well known.

Apart from DCs, PBMCs may also be used as the APC. The induction of CTLs has been reported to be enhanced by culturing PBMCs in the presence of GM-CSF and IL-4. Similarly, CTLs have been shown to be induced by culturing PBMCs in the presence of keyhole limpet hemocyanin (KLH) and IL-7.

Test polypeptides confirmed to possess CTL-inducing activity by these methods are deemed to be polypeptides having DC activation effect and subsequent CTL-inducing activity. Therefore, polypeptides that induce CTLs against tumor cells are useful as vaccines against

tumors. Furthermore, APCs that have acquired the ability to induce CTLs against tumors through contact with the polypeptides are also useful as vaccines against tumors. Furthermore, CTLs that have acquired cytotoxicity due to presentation of the polypeptide antigens by APCs can be also used as vaccines against tumors. Such therapeutic methods for tumors, using anti-tumor immunity due to APCs and CTLs, are referred to as cellular immunotherapy.

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

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 of tumor cells is suppressed by these antibodies, the polypeptide is deemed to have the ability to induce anti-tumor immunity.

Anti-tumor immunity is induced by administering the vaccines of this invention, and the induction of anti-tumor immunity enables treatment and prevention of cancers. Therapy against cancer or prevention of the onset of cancer includes any of the following steps, such as inhibition of the growth of cancer cells, involution of cancer, and suppression of the occurrence of cancer. A decrease in mortality and morbidity of individuals having cancer, decrease in the levels of tumor markers in the blood, alleviation of detectable symptoms accompanying cancer, and such are also included in the therapy or prevention of cancer. 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 cell proliferative diseases 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.

The above-mentioned RASGEF1A polypeptide or fragments thereof having immunological activity or a vector encoding them may be combined with an adjuvant. An adjuvant refers to a compound that enhances the immune response against the RASGEF1A polypeptide when administered together (or successively) with the polypeptide or fragment having immunological activity. Exemplary adjuvants include, but are not limited to, cholera toxin, salmonella toxin, alum, and such, but are not limited thereto. Furthermore, the vaccines of this invention may be combined appropriately with a pharmaceutically acceptable carrier.

Examples of such carriers include 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 can be administered systemically or locally. Vaccine administration can be performed by single administration, or boosted by multiple administrations.

When using APCs or CTLs as the vaccines of this invention, tumors 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 RASGEF1A polypeptide or fragments thereof *ex vivo*, and following the induction of APCs or CTLs, the cells may be administered to the subject. APCs can be also induced by introducing a vector encoding the polypeptide into PBMCs *ex vivo*. APCs or CTLs 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, APCs and CTLs 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 tumors from other individuals.

Furthermore, pharmaceutical compositions for treating or preventing cell proliferative diseases, such as cancers, comprising a pharmaceutically effective amount of the RASGEF1A polypeptide or immunological active fragments thereof is provided. Such pharmaceutical compositions may be used for raising anti tumor immunity.

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

EXAMPLES

I. Materials and Methods

1. Cell lines and tissue specimens

Monkey kidney cell line COS7 and mouse fibroblast cell line NIH3T3 were obtained from the American Type Culture Collection (ATCC). Human cholangiocarcinoma cell line SSP25 was obtained from Japanese Collection of Research Bioresources (JCRB). All cells were cultured as monolayers in appropriate media, as follows: Dullbecco's Modified Eagle Medium (Sigma-Aldrich Corporation, St. Louis, MO) for COS7 and NIH3T3; and RPMI

1640 (Sigma-Aldrich Corporation, St. Louis, MO) for SSP25. Clinical tissues were obtained with informed consents from surgical specimens of patients who underwent hepatectomy.

2. RNA preparation and semiquantitative RT-PCR

The present inventors used laser microbeam microdissection technology to collect pure populations of cholangiocarcinoma cells as well as noncancerous bile duct epithelia from surgical specimens. Preparation of sections, laser microbeam microdissection, extraction of total RNA, T7-based amplification was performed as previously described (Nakamura T *et al.*, Oncogene 2004, 23:2385-400). A mixture of normal intrahepatic biliary epithelial cells in liver tissues from 10 patients without ICC was prepared as universal control. Total RNA was extracted from culture cells using TRIZOL reagent (Invitrogen) according to the manufacturer's protocol. Extracted RNA was reversely transcribed for single-stranded cDNAs using poly dT_{12-18} primer (Amersham Biosciences) with Superscript II reverse transcriptase (Life Technologies). Each single-stranded cDNA was diluted for subsequent PCR amplification. Standard RT-PCR was carried out in a 12 μl volume of PCR buffer (TAKARA), and amplified for 4 min at 94°C for denaturing, followed by 25 (for *ACTB*) or 30 (for *RASGEF1A*) cycles of 94°C for 20 s, 53°C (for *ACTB*) or 60°C (for *RASGEF1A*) for 30 s, 72°C for 30 s, in GeneAmp PCR system 9700 (Perkin-Elmer). Primer sequences were as follows; 5'-CAAGATGAGATTGGCATGG-3' (SEQ ID NO: 3) and 5'-TCTCCTTAGAGAGAAGTGG-3' (SEQ ID NO: 4) for *ACTB*; and 5'-TTTGCACTTTCTGAGGTTCTAGC-3' (SEQ ID NO: 5) and 5'-GTCCTGCATTATGGTACAGCTTC-3' (SEQ ID NO: 6) for *RASGEF1A*.

3. Northern-blot analysis

Human multiple-tissue northern blots (Clontech) were hybridized with ^{32}P-labeled *RASGEF1A* cDNA. Pre-hybridization, hybridization and washing were performed according to the supplier's recommendations. The blots were autoradiographed with intensifying screens at -80°C for 120 h.

4. Subcellular localization of RASGEF1A

The entire coding region of *RASGEF1A* (Genbank Accession No. AK095136 (SEQ ID NO: 1), encoding the amino acid sequence of SEQ ID NO: 2) was amplified by RT-PCR using a set of primers, 5'-ATTGAATTCCGGCCAGAATGTTCCTGGAGC-3' (SEQ ID NO: 7) and 5'-AATCTCGAGGGCTCTGTTCAGAAGGGTGGTC-3' (SEQ ID NO: 8), and cloned into an appropriated enzyme site of pCAGGS-n3Fc vector (pCAGGS-n3Fc--RASGEF1A).

NIH3T3 cells transiently transfected with plasmids expressing FLAG-tagged RASGEF1A were transferred onto chamber slides. Twenty-four hours after transfection, cultured cells were fixed with PBS containing 4% paraformaldehyde for 15 min, then rendered permeable with PBS containing 0.1% Triton X-100 for 2.5 min at room temperature. The cells were covered with 3% BSA in PBS for 24 h at 4°C to block non-specific hybridization and subsequently incubated with anti-FLAG antibody (SIGMA F-3165) as the first antibody. Antibodies were fluorescent stained with substrate-conjugated anti-mouse IgG secondary antibody (ICN/Cappel and Jackson Immuno Research). Nuclei were counter stained with 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI). Fluorescence image was obtained with TCS-SP2 spectral confocal scanning system (Leica).

5. Colony Formation assay

COS7 cells transfected with plasmids expressing *RASGEF1A* (pCAGGS-n3Fc-RASGEF1A). Cells expressing antisense *RASGEF1A* (pCAGGS-n3Fc-RASGEF1A(-)), or mock plasmids (pCAGGS-n3Fc-Mock) were incubated with an appropriate concentration of geneticin for 14 days. The cells were fixed with 100% methanol and stained by Giemsa solution. Cell viability was analyzed in triplicate using cell-counting kit (DOJINDO, kumamoto, Japan) according to the manufacturer's protocol.

6. Construction of siRNA expression vectors against *RASGEF1A*

Plasmids expressing siRNAs were prepared by cloning double-stranded oligonucleotides into the *Bbs*I site of psiH1BX3.0 vector as described previously (Shimokawa T *et al.*, Cancer Res 2003, 63:6116-20). The sequences of paired oligonucleotides were as follows:

5'-TCCCGAGAATGGCACAGTGAAGATTCAAGAGATCTTCACTGTGCCATTCTC-3', (SEQ ID NO: 10) and

5'-AAAAGAGAATGGCACAGTGAAGATCTCTTGAATCTTCACTGTGCCATTCTC-3' (SEQ ID NO: 11) for siRNA-B;

5'-TCCCCATCTACTTCCTGCACAAATTCAAGAGATTTGTGCAGGAAGTAGATG-3' (SEQ ID NO: 14), and

5'-AAAACATCTACTTCCTGCACAAATCTCTTGAATTTGTGCAGGAAGTAGATG-3' (SEQ ID NO: 15) for siRNA-E; and

5'-TCCCGAAGCAGCACGACTTCTTCTTCAAGAGAGAAGAAGTCGTGCTGCTTC-3' (SEQ ID NO: 18) and

5'-AAAAGAAGCAGCACGACTTCTTCTCTCTTGAAGAAGAAGTCGTGCTGCTTC-3'

(SEQ ID NO: 19) for siRNA-EGFP. After phosphorylation with T4-polynucleotide kinase, the paired oligonucleotides were boiled for 5 min, and were subsequently annealed to produce double-stranded oligonucleotides by slowly cooling down. Expression of *RASGEF1A* was examined by semi-quantitative RT-PCR 24 h after transfection. siRNA sequences used for the present invention are shown below.

Table 1

		SEQ ID NO	Sequence
RASGEF1 si#B position 569-587	Target	9	GAGAATGGCACAGTGAAGA
	Insert	10	TCCCGAGAATGGCACAGTGAAGATTCAAGAGATCTTCA CTGTGCCATTCTC
		11	AAAAGAGAATGGCACAGTGAAGATCTCTTGAATCTTCA CTGTGCCATTCTC
	Hairpin siRNA	12	GAGAATGGCACAGTGAAGATTCAAGAGATCTTCACTGT GCCATTCTC
RASGEF1 si#E position 1273-1291	Target	13	CATCTACTTCCTGCACAAA
	Insert	14	TCCCCATCTACTTCCTGCACAAATTCAAGAGATTTGTGC AGGAAGTAGATG
		15	AAAACATCTACTTCCTGCACAAATCTCTTGAATTTGTGC AGGAAGTAGATG
	Hairpin siRNA	16	CATCTACTTCCTGCACAAATTCAAGAGATTTGTGCAGGA AGTAGATG
EGFP control	Target	17	GAAGCAGCACGACTTCTTC
	Insert	18	TCCCGAAGCAGCACGACTTCTTCTTCAAGAGAGAAGAA GTCGTGCTGCTTC
		19	AAAAGAAGCAGCACGACTTCTTCTCTCTTGAAGAAGAA GTCGTGCTGCTTC
	Hairpin siRNA	20	GAAGCAGCACGACTTCTTCTTCAAGAGAGAAGAAGTCG TGCTGCTTC

7. Cell viability assay

SSP25 cells plated on 10-cm dish (1x 10^6 cells/dish) were transfected with plasmids expressing siRNA using either Nucleofector (AMAXA) or FuGENE6 reagent (Roche Diagnostics). Transfected cells were maintained in medium containing 10% fetal bovine serum supplemented with 0.9 mg/ml of geneticin for two weeks. Surviving cells were fixed with 100% methanol and stained with Giemsa solution. In another experiment, the viable cells were measured with cell-counting kit (DOJINDO, Kumamoto, Japan). To analyze the effect on RASGEF1A expression, total RNA was extracted 24 h after transfection from the cells and subjected to semi-quantitative RT-PCR analysis.

8. Ras Nucleotide Dissociation Assay

Entire coding region of K-Ras (amino acids 1-188), Ha-Ras (amino acids 1-188), N-RAS (amino acids 1-189), RASGEF1A, or the catalytic domain of Sos (amino acids 564-1049) was cloned into an appropriate cloning site (*Bam*HI and *Xho*I) of pGEX-6P bacterial

expression vector (Amersham Pharmacia Biotech). GST-fusion protein was expressed in BL21 bacterial strain of *Escherichia coli*, and the recombinant protein was purified from the whole cell lysate using glutathione-Sepharose 4B resin (Amersham Pharmacia Biotech) according to the manufacturer's protocol. Ras nucleotide dissociation assay was carried out as described elsewhere (Hall BE *et al.*, J Biol Chem 2001, 276:27629-37). GST-Ras fusion proteins were bound with [8-^{3}H]-labeled Guanosine 5'-diphosphate (GDP) by incubating 40 pmol of Ras in 100 ml of buffer containing 20 mM Tris (pH8.0), 50 mM NaCl, 1 mg/ml bovine serum albumin (BSA), 1 mM dithiothreitol, and 1 mM EDTA with 400 pmol of [8-^{3}H]-labeled GDP (12.4 Ci/mmol; Amersham Biosciences) for 15 min at 30°C on a thermoshaker. 20 ml of the mixture was further incubated with or without recombinant RASGEF1A in 20 ml of buffer containing 20 mM Tris (pH8.0), 50 mM NaCl, 1 mg/ml BSA, 1 mM dithiothreitol, 5 mM MgCl2, and 0.8 mM GTPgS for 15 min at 30°C. The catalytic domain of Sos served as positive control. The reaction was terminated by adding 40 ml of ice-cold stop solution (20 mM Tris (pH8.0), 50 mM NaCl, and 15 mM $MgCl_2$) and the mixture was spotted onto P81 phosphocellulose squares (Upstate; catalog #20-134). Washed twice with 3 ml of ice-cold stop solution, the filter was loaded on Aloka LSC-5100 liquid scintillation counter to measure the radioactivity of [^{3}H]-GDP that associated with Ras.

9. Analysis of Ras activity

Activation of Ras was measured by Raf-1 pull-down assay utilizing Ras Activation Assay Kit (Upstate, Catalog # 17-218 Lot # 26220) according to the supplier's protocol. Specifically, agarose beads-conjugated GST fusion protein containing the RBD of Raf-1 was prepared and incubated for 45 min at 4°C with lysates from COS7 cells transfected with plasmids expressing RASGEF1A or control plasmids (Mock). Proteins bound to Raf-1 were precipitated, separated on SDS–PAGE, and subsequently analyzed by Western blotting with anti-pan Ras antibody (Upstate).

10. Wound-healing and Matrigel invasion assays.

The entire coding region of RASGEF1A (GenBank accession: AK095136) was amplified by RT-PCR using a set of primers, 5'-ATTGAATTCCGGCCAGAATGTTCCTGGAGC-3' (SEQ ID NO: 7) and 5'-AATCTCGAGGGCTCTGTTCAGAAGGGTGGTC-3' (SEQ ID NO; 8), and cloned into an appropriate enzyme site of pCAGGS-n3Fc vector (pCAGGS-n3Fc-RASGEF1A). COS7 cells expressing RASGEF1A (COS7-RASGEF1A) and control cells (COS7-Mock) were

established by transfecting COS7 cells with pCAGGS-n3Fc-RASGEF1A or pCAGGS-Mock plasmids. The cells were maintained in culture media supplemented with 0.9 mg/ml of geneticin, and single colonies were selected two weeks after the transfection. Expression of RASGEF1A was confirmed by Western blotting with anti-FLAG antibody. Cell growth was analyzed in triplicate using cell-counting kit (DOJINDO, Kumamoto, Japan) according to the manufacturer's protocol. For wound-healing migration assay, cells were grown to confluence in six-well plates for 2 days, and a scrape in the form of a cross was made through the confluent monolayers with a plastic pipette tip. Several wounded areas were marked for orientation, observed and then photographed by phase-contact microscopy at indicated times after the scratch. The invasion of cells was examined using BD BioCoat™ Matrigel™ Invasion Chambers with 8-μm pores (BD Biosciences, San Jose, CA). The number of COS7-RASGEF1A and COS7-Mock cells that migrated through the chamber was counted in five independent visual fields under a microscope after 36 h of incubation. Statistical Analysis. Statistical significance was determined by Student's t test.

II. Results

1. Elevated expression of *RASGEF1A* in a wide range of human cancers

Using genome-wide cDNA microarray containing 27,648 genes, the present inventors analyzed expression profiles of 25 ICCs, and identified 52 genes whose expression was frequently up-regulated in tumors that passed the set cutoff-filter. In addition to the 52 genes, a gene with an inhouse accession number of D4223, corresponding to an EST of Hs. 125293 in the Unigene database (Build #183) in National Center for Biotechnology Information, was found to be significantly up-regulated more than two-fold in eleven of fourteen ICCs (78.6%) that passed the cutoff-filter, compared with normal intrahepatic bile duct epithelia. In the microarray data obtained by the present inventors, D4223 expression was also elevated more than two fold in eight out of nine bladder carcinomas (88.9%), 16 out of 26 lung cancers (61.5%), four out of five gastric cancers (80%), three out of five colorectal cancers (60%), one of three prostate cancers (33.3%), eight of 44 chronic myeloid leukemias (18.2%), all five renal cancers, all five soft tissue sarcomas (100%), all three pancreatic cancers (100%), and all three seminomas (100%) compared to the corresponding non-cancerous controls. Subsequent semi-quantitative RT-PCR analysis revealed enhanced D4223 expression in 9 of 13 ICCs that were subjected to the microarray analysis (Fig. 1A). To evaluate the expression levels of D4223 in human adult normal tissues, the present inventors performed multiple-tissue Northern blot analysis using D4223 cDNA as a probe and identified a transcript of

approximately 3.3 kb that was moderately expressed in the brain, spinal cord and weakly in the lymph node, adrenal gland but not in any of 19 other tissues examined (Fig. 1B). The gene was lately dubbed as *RASGEF1A*, because it contained a RAS-GEF domain. A homology search with the deduced RASGEF1A amino acid sequence showed 30% identity with PDZ-GEF2, and 29% with PDZ-GEF1.

2. Subcellular localization of RASGEF1A

To investigate the subcellular localization of RASGEF1A, the present inventors expressed FLAG-tagged RASGEF1A in NIH3T3 cells by transfection of plasmid (p3Xflag-RASGEF1A) (Fig. 2B), and carried out immunocytochemical staining. The immunocytochemical staining revealed that RASGEF1A protein localizes in the cytoplasm of transfected cells (Fig. 2A).

3. Association of RASGEF1A with ICC cells growth

Colony formation assay was carried out to examine the oncogenic activity of RASGEF1A in COS7 cells. As a result, significant increase in colony number was observed when the cells were transfected with plasmids expressing *RASGEF1A* (pCAGGS-n3Fc-RASGEF1A) compared to the transfection with control plasmids, pCAGGS-n3Fc-Mock, or pCAGGS-n3Fc-RASGEF1A(-) (Figs. 3A and 3B). To further evaluate its potential role in cell growth, plasmids psiH1BX-RASGEF1A-siB and psiH1BX-RASGEF1A-siE that express RASGEF1A specific siRNAs together with neomycin resistant gene were prepared. SSP25 ICC cells were transfected with either of the plasmids, or with control plasmids (psiH1BX-siEGFP, psiH1BX-Mock). Semiquantitative RT-PCR showed that *RASGEF1A* expression was suppressed by psiRNA-BX-RASGEF1A-siB and psiH1BX-RASGEF1A-siE transfections compared to the transfection of the control plasmids (Fig. 3C). Notably, marked growth retardation of transfected cells was inhibited with siRNA-B and siRNA-E compared to cells transfected with the control plasmids (Fig. 3D). These data suggest that expression of *RASGEF1A* is crucial for the growth and/or survival of ICC cells.

4. Ras nucleotide dissociation activity of RASGEF1A

Since RASEGF1A contained a putative RasGEF domain, its GDP dissociation activity has been examined. A reaction mixture either with or without recombinant RASGEF1A protein were subsequently added, and GDP-associated RAS was measured with scintillation counter. SOS, a known RASGEF protein, was used as positive control. SOS decreased the GDP-bound form K-RAS by approximately 40%. Similarly, RASGEF1A reduced GDP-bound K-RAS by approximately 50% (Fig. 4A, upper left panel). It also dissociated GDP

bound to H-RAS and N-RAS by approximately 40% and 80%, respectively (Fig. 4A upper right panel and lower panel). These data indicate that RASEGF1A shows GDP dissociation activity to all of the three examined RAS proteins.

5. Activation of RAS by RASGEF1A

Since RASGEF1A contained a putative RASGEF domain, RASGEF1A was further investigated whether it has the activity to stimulate RAS. This activity was measured using COS7 cells expressing exogenous RASGEF1A and control cells. Immunoblot analysis of extracts from cells transfected with pxFLAG-RASGEF1A plasmids with anti-FLAG antibody confirmed exogenous RASGEF1A expression in the transfected cells (Fig. 4B, upper left panel). Extracts from the cells or control cells were subjected to a pull-down assay of recombinant Raf-1, an effector of RAS. As a result, the amount of Ras that interacted with the RBD of Raf-1 measured by immunoblot analysis with anti-pan-Ras antibody increased in accordance with RASGEF1A expression (Fig. 4B, upper right panel), indicating enhanced activity of RAS by RASGEF1A.

6. Effect of RASGEF1A on cancer cell migration

RAS family proteins have been reported to play important roles in cell migration as well as cell growth. Therefore, the involvement of RASGEF1A in migration and invasion of cancer cells was examined. COS7-RASGEF1A cells expressing exogenous RASGEF1A and control cells (COS7-Mock) were established for wound healing assay. As shown in Fig 5A, COS7-RASGEF1A cells migrated rapidly, and filled in the wound significantly faster than COS7-Mock cells, implying the association of RASGEF1A in cellular migration. In addition, to investigate the effect of RASGEF1A on invasion, transwell assay using Matrigel was performed. Consistent with the result of the wound-healing assay, the number of COS7-RASGEF1A cells that went through the pores of chambers increased compared to those of the COS7-Mock cells (Fig 5B). Both cells had a similar proliferation rate, hence these data suggest the involvement of RASGEF1A in cellular migration.

III. Discussion on the Results

The preset inventors have here demonstrated elevated expression of RASGEF1A in human ICCs and its guanine nucleotide exchange activity to K-RAS, H-RAS and N-RAS, implying that its elevated expression plays some crucial role in the carcinogenesis of ICCs. So far approximately 20 members belonging to the Ras-GEF family that share the core catalytic domain possessing five structurally conserved regions have been reported (Boguski MS & McCornick F, Nature 1993, 366: 643-54 ; Rebhun JF *et al.*, J Biol Chem 2000, 275:

13406-10). Like other Ras-GEF members, RASGEF1A contained these five conserved regions. In addition, it had a REM (Ras exchange motif) or RasGEFN domain between codons 49 and 178, which is supposed to stabilize the large helical hairpin structure that pries open the GTP-binding pocket. However, since RASGEF1A lacks any additional conserved domain, such as the Dbl homology (DH) domain, the pleckstrin homology (PH) domain, the EF hands, the cysteine-rich C1 domain, and the PDZ domain, it is likely to exert different function from other members such as SOS, GRF1, RasGRP, or PDZ-GEF (Quilliam LA *et al.*, Progress in Nucleic Acid Research and Molecular Biology 2002, 71:391-444). In this invention, RASGEF1A was revealed to have a guanine-nucleotide exchange activity to K-RAS, H-RAS, and N-RAS. Members of other Ras-GEFs have different spectrums of substrates; for example, SOS1 modulates K-RAS, H-RAS, N-RAS, R-RAS2, R-RAS3, and Rac1, but not R-RAS; Ras-GRP2 stimulates K-RAS, H-RAS, N-RAS, R-RAS, R-RAS2, Rap1A, Rap2A, but not H-RAS (Mitin N *et al.*, Current Biology 2005, 15: R563-74.). Hence, RASGEF1A may also specifically activate substrates that have not been examined in the present study, and may be involved in the crosstalk of Ras-signaling pathways.

Recent studies indicated that Ras signaling is involved in not only cell proliferation and survival, but also cell motility and cell adhesion. Active Ras interacts and regulates multiple downstream effectors that stimulate diverse signaling pathways including Raf/MEK/ERK and phosphoinositide 3 kinase (PI3K)/Akt, RalGDS/Ral, and Tiam1/Rac1 pathways. Accumulating evidences suggest the involvement of Raf/MEK/ERK in Ras-mediated cell proliferation. The Raf-association assay demonstrated the increase of activated-RAS in the presence of RASGEF1A and thus, downstream Raf/MEK/ERK is supposed to be enhanced in cells expressing RASGEF1A. Consistently, suppression of RASGEF1A by introduction of specific siRNA decreased the growth of ICC cells, which is in line with the view that augmented RAF/MEK/ERK-signaling confers growth-promoting effect to cancer cells. In the colony formation assays using COS7 and NIH3T3 fibroblast cells to test the oncogenic activity of RASGEF1A, the number of colonies was unchanged between cells expressing exogenous RASGEF1A and controls. Therefore, it is likely that the introduction of RASGEF1A alone may not be sufficient to cause transformation of COS7 or NIH3T3 fibroblast cells. However, the oncogenic activity of RASGEF1A was suggested to be tissue- and/or cell-type dependent, and the siRNA experiments indicated that RASGEF1A is essential for maintaining cell growth or proliferation of ICCs. Therefore, inhibition of its guanine nucleotide exchange activity may be a promising therapeutic option for ICCs. In

addition to its growth promoting effect, Ras has been reported to play a role in cell motility by different mechanisms. Enhanced PI3 kinase activity resulted in increased invasion of breast cancer cells, which was mediated through beta 4 integrin in Rac1-dependent manner. PI3K also activates Rac GEFs, and induces actin reorganization and membrane ruffling, which are associated with cell motility. Additionally Ras enhances Tiam1, which results in activated form of Rac1 that is involved in migration and actin-cytoskeleton. In fibroblasts, activation of H-RAS suppresses activity of integrins, cell-surface receptors that play an important role in cell-extracellular matrix adhesion (Paul EH *et al.*, Mol Biol Cell 2002, 13:2256-65 ; Sechler JL *et al.*, J Cell Sci 2000, 113:1491-8). According to the present invention, it was discovered that RASGEF1A activates RAS proteins and that exogenous expression of RASGEF1A enhances cellular motility in Matrigel assay. Although further investigations is required to clarify the mechanisms of increased motility by RASGEF1A, it was suggested that RASGEF1A enhances cell motility through the activation of PI3K or Tiam1.

The present inventors further investigated the co-relation between K-Ras mutation status and RASGEF1A expression levels, but could not find any relation between them (data not shown). Since elevated RASGEF1A expression activates H-Ras and N-Ras in addition to K-Ras, RASGEF1A accumulation may have an additional role(s) in the carcinogenesis of ICC compared to mutation in K-Ras. Since suppression of RASGEF1A by the siRNAs lead to the inhibition of cancer cells, RASGEF1A may be a promising therapeutic target for ICCs.

Industrial Applicability

The gene-expression analysis of cancers described herein using the combination of laser-capture dissection and genome-wide cDNA microarray has identified specific genes as targets for cancer prevention and therapy. Based on the expression of a subset of these differentially expressed genes, the present invention provides molecular diagnostic markers for identifying and detecting cancers.

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

All patents, patent applications, and publications cited herein are incorporated by

reference in their entirety.

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

CLAIMS

1. A method for diagnosing cancer or a predisposition for developing cancer in a subject, comprising the step of determining the expression level of the *RASGEF1A* gene in a subject-derived biological sample, wherein an increase in said expression level as compared to a normal control level of said gene indicates that said subject suffers from or is at a risk of developing cancer.

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

3. The method of claim 1, wherein said expression level is determined by any of the methods selected from the group consisting of:
(a) detecting mRNA of the *RASGEF1A* gene;
(b) detecting a protein encoded by the *RASGEF1A* gene; and
(c) detecting a biological activity of the protein encoded by the *RASGEF1A* gene.

4. The method of claim 1, wherein said subject-derived biological sample comprises an epithelial cell.

5. The method of claim 1, wherein said subject-derived biological sample comprises a cancer cell.

6. The method of claim 1, wherein said subject-derived biological sample comprises a cancerous epithelial cell.

7. The method of claim 1, wherein said cancer is selected from the group consisting of ICC, bladder carcinoma, lung cancer, gastric cancer, colorectal cancer, prostate cancer, chronic myeloid leukemia, renal cancer, soft tissue sarcoma, pancreatic cancer, and seminoma.

8. A kit comprising a detection reagent which binds to the transcription or translation product of the *RASGEF1A* gene.

9. A method of screening an agent for treating or preventing cancer, which comprises the steps of:
a) contacting a test agent with the RASGEF1A polypeptide or a fragment thereof;
b) detecting the binding between the polypeptide and the test agent; and
c) selecting the test agent that binds to the polypeptide.

10. A method of screening an agent for treating or preventing cancer, wherein said method comprises the steps of:
a) contacting a test agent with the RASGEF1A polypeptide or a fragment thereof;

b) detecting the biological activity of the polypeptide; and

c) selecting the test agent that suppresses the biological activity of the polypeptide as compared to that detected in the absence of the test agent.

11. A method of screening an agent for treating or preventing cancer, which comprises the steps of:

(a) contacting the RASGEF1A polypeptide or a fragment thereof with RAS in the presence of a test agent;

(b) detecting the RAS activity; and

(c) selecting the test agent that decreases the RAS activity as compared to that detected in the absence of the test agent.

12. A method of screening an agent for treating or preventing cancer, which comprises the steps of:

a) contacting a test agent with a cell expressing the *RASGEF1A* gene;

b) detecting the expression level of the *RASGEF1A* gene; and

c) selecting the test agent that reduces the expression level of said gene as compared to that detected in the absence of the test agent.

13. The method of claim 12, wherein said cell is derived from ICC, bladder carcinoma, lung cancer, gastric cancer, colorectal cancer, prostate cancer, chronic myeloid leukemia, renal cancer, soft tissue sarcoma, pancreatic cancer, or seminoma.

14. A method of screening an agent for treating or preventing cancer, wherein said method comprises the steps of:

a) contacting a test agent with a cell introduced with a vector that comprises the transcriptional regulatory region of the *RASGEF1A* gene and a reporter gene expressed under the control of the transcriptional regulatory region;

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

c) selecting the test agent that reduces the expression or activity of said reporter gene as compared to that detected in the absence of the test agent.

15. The method of any one of claims 9 to 14, wherein the cancer is selected from the group consisting of ICC, bladder carcinoma, lung cancer, gastric cancer, colorectal cancer, prostate cancer, chronic myeloid leukemia, renal cancer, soft tissue sarcoma, pancreatic cancer, and seminoma.

16. A composition for treating or preventing cancer, which comprises as an active ingredient a pharmaceutically effective amount of an agent selected by any of the

methods of claims 9 to15, and a pharmaceutically acceptable carrier.

17. A composition for treating or preventing cancer, which comprises a pharmaceutically effective amount of an antisense polynucleotide or siRNA against a polynucleotide encoded by the *RASGEF1A* gene.

18. The composition of claim 17, wherein said siRNA comprises the sense strand of the *RASGEF1A* gene comprising the nucleotide sequence of SEQ ID NOs: 9 or 13.

19. The composition of claim 18, wherein said siRNA has the general formula 5'-[A]-[B]-[A']-3', wherein [A] is a ribonucleotide sequence corresponding to a sequence of SEQ ID NOs: 9 or 13, [B] is a ribonucleotide loop sequence consisting of 3 to 23 nucleotides, and [A'] is a ribonucleotide sequence complementary to [A].

20. A composition for treating or preventing cancer, which comprises a pharmaceutically effective amount of an antibody or fragment thereof that binds to the RASGEF1A polypeptide.

21. The composition of any one of claims 16 to 20, wherein the cancer is selected from the group consisting of ICC, bladder carcinoma, lung cancer, gastric cancer, colorectal cancer, prostate cancer, chronic myeloid leukemia, renal cancer, soft tissue sarcoma, pancreatic cancer, and seminoma.

22. A method for treating or preventing cancer in a subject, which comprises the step of administering an agent obtained by any of the methods according to claims 9 to 15.

23. A method for treating or preventing cancer in a subject, which comprises the step of administering to said subject the composition of any one of claims 16 to 20.

24. A method for treating or preventing cancer in a subject, which comprises the step of administering to the subject a pharmaceutically effective amount of an antibody or immunologically active fragment thereof, that binds to the RASGEF1A polypeptide.

25. A method for treating or preventing cancer in a subject, which comprises the step of administering to said subject a vaccine, which comprises (a) the RASGEF1A polypeptide, (b) an immunologically active fragment of said polypeptide, or (c) a polynucleotide coding for the polypeptide or the fragment.

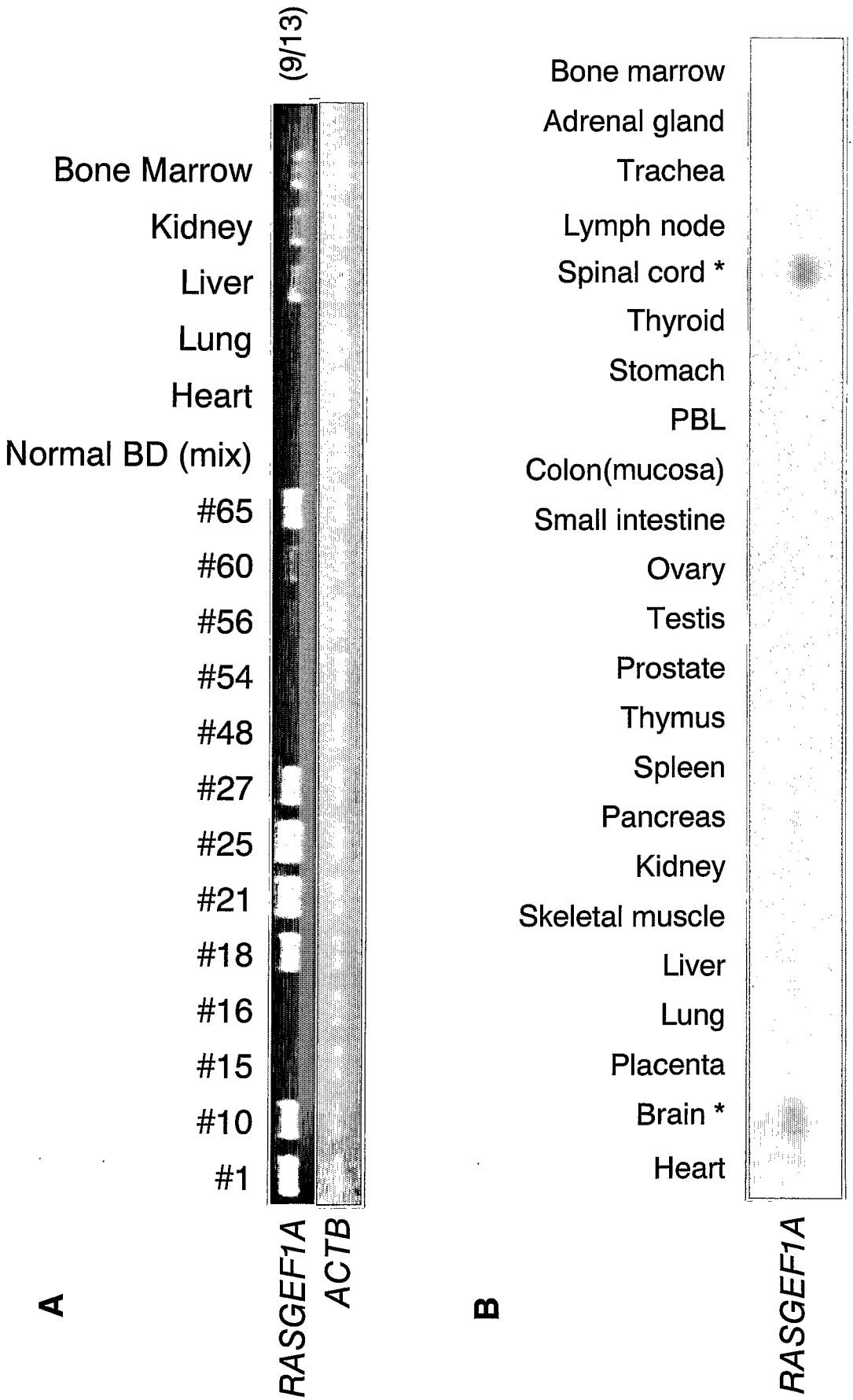
26. A method for inducing an anti-tumor immunity, which comprises the step of contacting an APC with the RASGEF1A polypeptide or a fragment thereof, a polynucleotide encoding the polypeptide or the fragment, or a vector comprising the polynucleotide.

27. The method for inducing an anti-tumor immunity of claim 26, wherein the method

further comprises the step of administering the APC to a subject.

28. The method of any one of claims 22 to 27, wherein the cancer is selected from the group consisting of ICC, bladder carcinoma, lung cancer, gastric cancer, colorectal cancer, prostate cancer, chronic myeloid leukemia, renal cancer, soft tissue sarcoma, pancreatic cancer, and seminoma.

Fig. 1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Spinal cord *
Thyroid
Stomach
PBL
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Ovary
Testis
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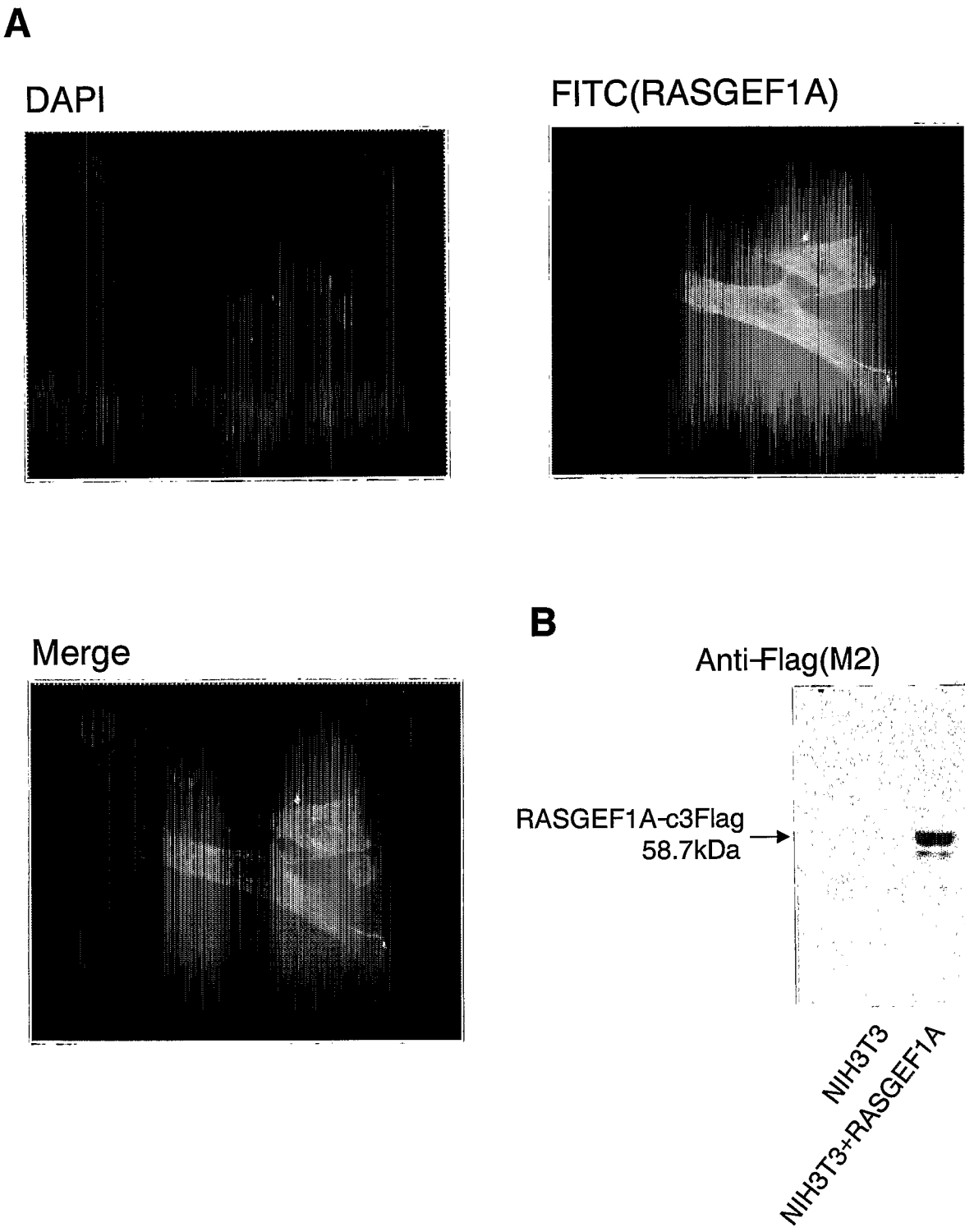
Fig. 2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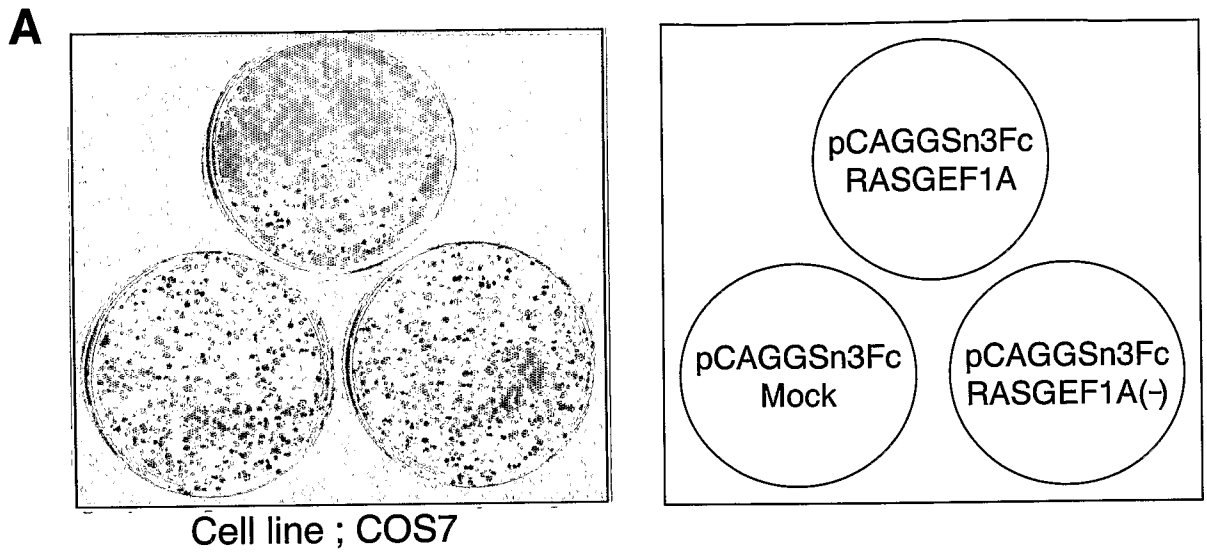
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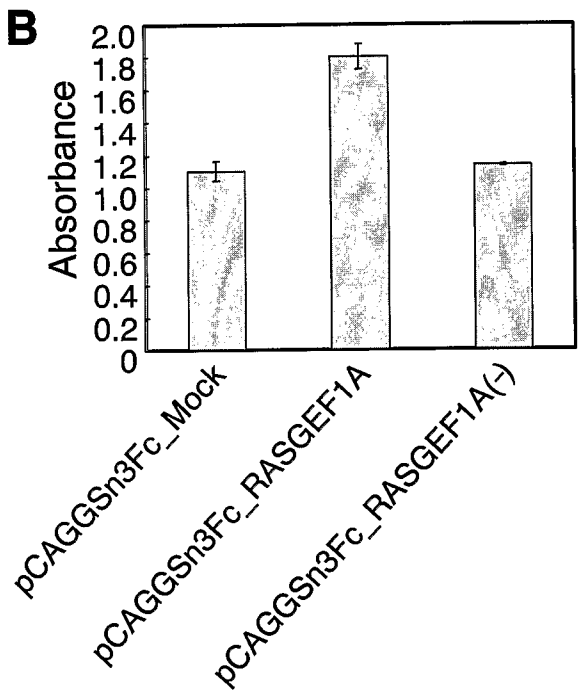
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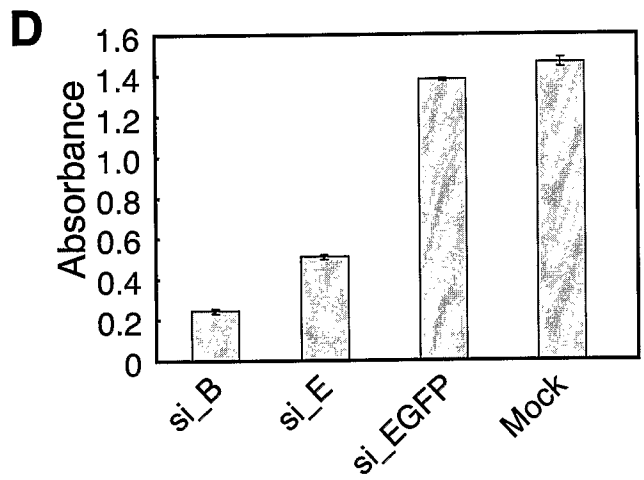
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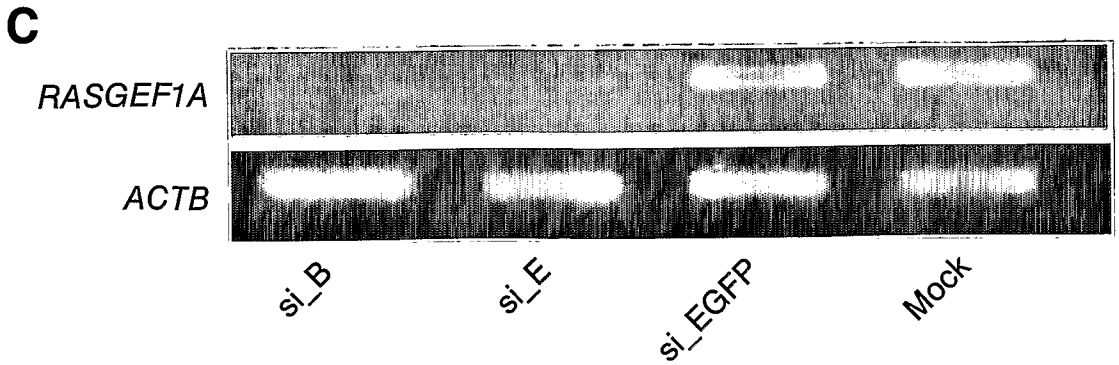
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Fig. 4

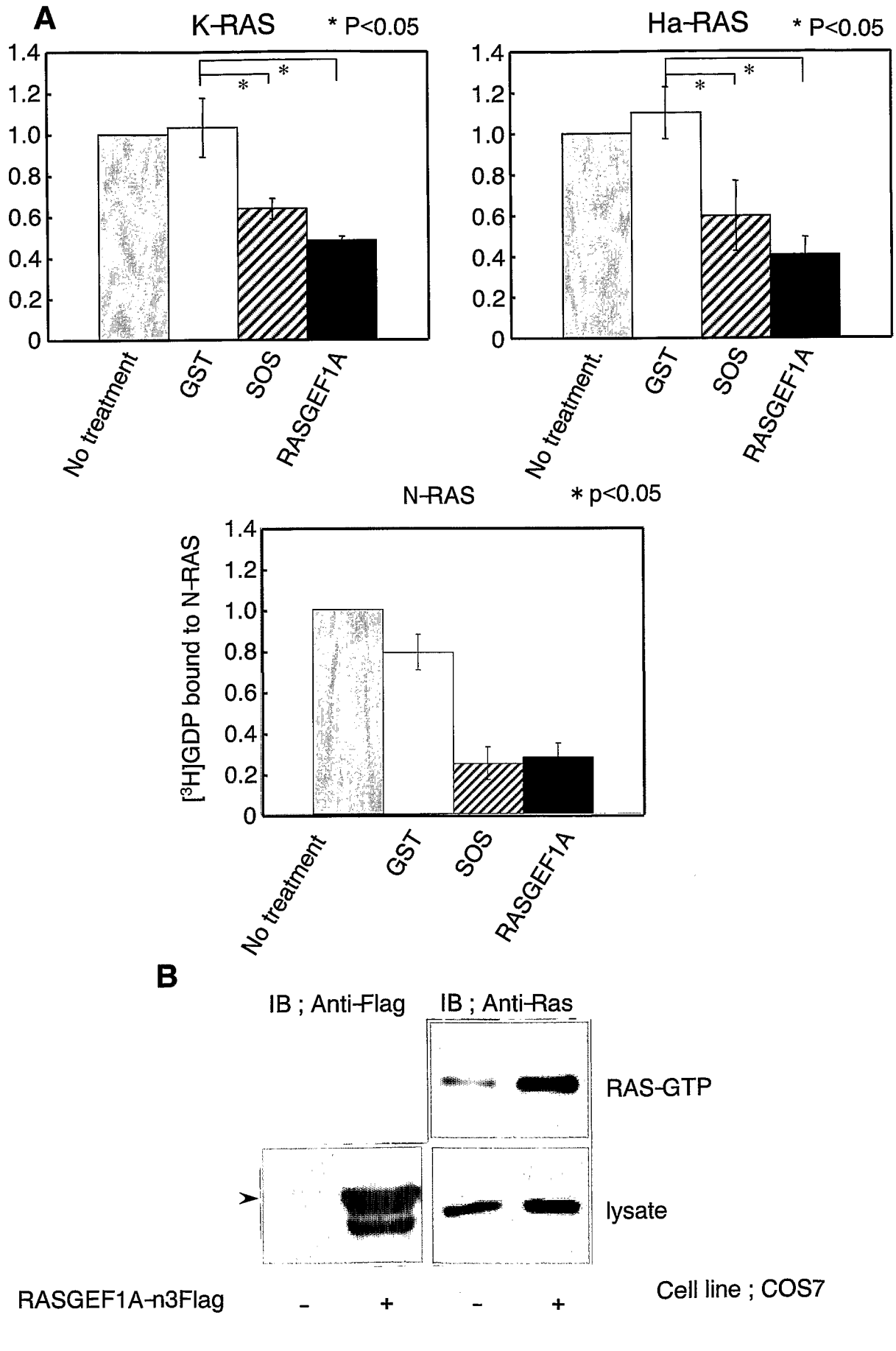
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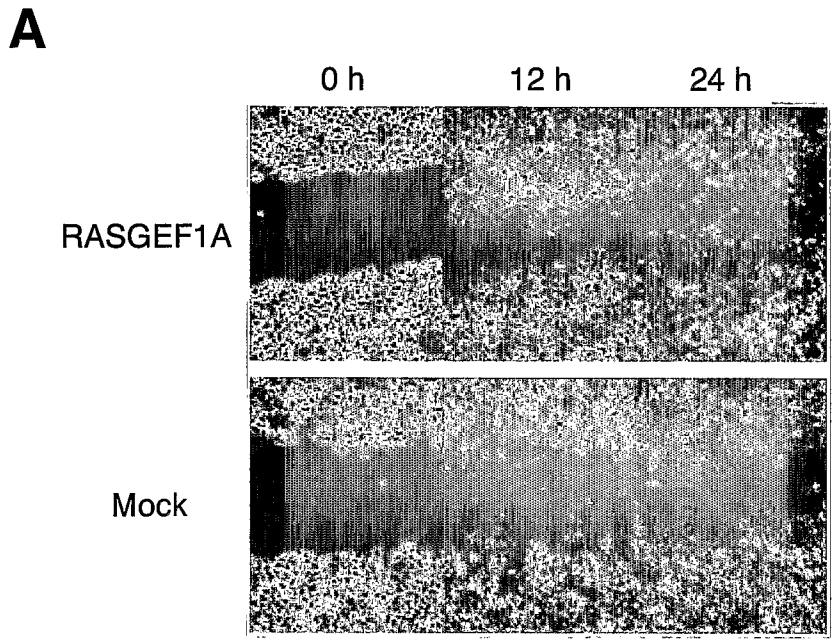
Fig. 5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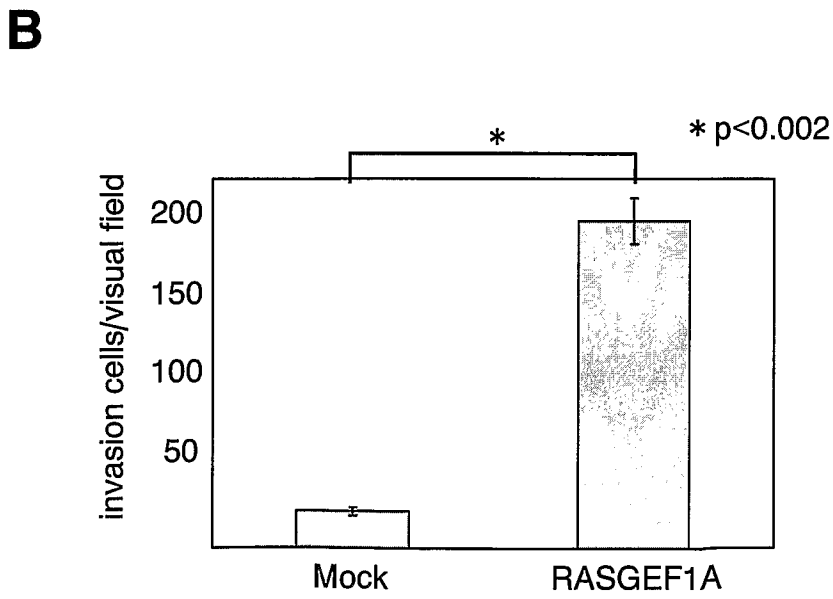
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Gly Val Cys Cys Asp Pro Leu Val Leu Ala Gln Gln Leu Thr His Ile
220 225 230

gag ctg gac agg gtc agc agc att tac cct gag gac ttg atg cag atc 832
Glu Leu Asp Arg Val Ser Ser Ile Tyr Pro Glu Asp Leu Met Gln Ile
235 240 245

gtc agc cac atg gac tcc ttg gac aac cac agg tgc cga ggg gac ctg 880
Val Ser His Met Asp Ser Leu Asp Asn His Arg Cys Arg Gly Asp Leu
250 255 260 265

acc aag acc tac agc ctg gag gcc tat gac aac tgg ttc aac tgc ctg 928
Thr Lys Thr Tyr Ser Leu Glu Ala Tyr Asp Asn Trp Phe Asn Cys Leu
270 275 280

agc atg ctg gtg gcc act gag gtg tgc cgg gtg gtg aag aag aaa cac 976
Ser Met Leu Val Ala Thr Glu Val Cys Arg Val Val Lys Lys Lys His
285 290 295

cgg acc cgc atg ttg gag ttc ttc att gat gtg gcc cgg gag tgc ttc 1024
Arg Thr Arg Met Leu Glu Phe Phe Ile Asp Val Ala Arg Glu Cys Phe
300 305 310

aac atc ggg aac ttc aac tcc atg atg gcc atc atc tct ggc atg aac 1072
Asn Ile Gly Asn Phe Asn Ser Met Met Ala Ile Ile Ser Gly Met Asn
315 320 325

ctc agt cct gtg gca agg ctg aag aaa act tgg tcc aag gtc aag aca 1120
Leu Ser Pro Val Ala Arg Leu Lys Lys Thr Trp Ser Lys Val Lys Thr
330 335 340 345

gcc aag ttt gat gtc ttg gag cat cac atg gac ccg tcc agc aac ttc 1168
Ala Lys Phe Asp Val Leu Glu His His Met Asp Pro Ser Ser Asn Phe
350 355 360

tgc aac tac cgt aca gcc ctg cag ggg gcc acg cag agg tcc cag atg 1216
Cys Asn Tyr Arg Thr Ala Leu Gln Gly Ala Thr Gln Arg Ser Gln Met
365 370 375

gcc aac agc agc cgt gaa aag atc gtc atc cct gtg ttc aac ctc ttc 1264
Ala Asn Ser Ser Arg Glu Lys Ile Val Ile Pro Val Phe Asn Leu Phe
380 385 390

gtt aag gac atc tac ttc ctg cac aaa atc cat acc aac cac ctg ccc 1312

Val Lys Asp Ile Tyr Phe Leu His Lys Ile His Thr Asn His Leu Pro
395 400 405

aac ggg cac att aac ttt aag aaa ttc tgg gag atc tcc aga cag atc 1360
Asn Gly His Ile Asn Phe Lys Lys Phe Trp Glu Ile Ser Arg Gln Ile
410 415 420 425

cat gag ttc atg aca tgg aca cag gta gag tgt cct ttc gag aag gac 1408
His Glu Phe Met Thr Trp Thr Gln Val Glu Cys Pro Phe Glu Lys Asp
430 435 440

aag aag att cag agt tac ctg ctc acg gcg ccc atc tac agc gag gaa 1456
Lys Lys Ile Gln Ser Tyr Leu Leu Thr Ala Pro Ile Tyr Ser Glu Glu
445 450 455

gct ctc ttc gtc gcc tcc ttt gaa agt gag ggt ccc gag aac cac atg 1504
Ala Leu Phe Val Ala Ser Phe Glu Ser Glu Gly Pro Glu Asn His Met
460 465 470

gaa aaa gac agc tgg aag acc ctc agg acc acc ctt ctg aac aga gcc 1552
Glu Lys Asp Ser Trp Lys Thr Leu Arg Thr Thr Leu Leu Asn Arg Ala
475 480 485

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Gly Glu Arg Gly Gly Gly Ala Gly Gly Gly Ser Gly Asp Leu Ile Phe
35 40 45

Gln Asp Gly His Leu Ile Ser Gly Ser Leu Glu Ala Leu Met Glu His
50 55 60

Leu Val Pro Thr Val Asp Tyr Tyr Pro Asp Arg Thr Tyr Ile Phe Thr
65 70 75 80

Phe Leu Leu Ser Ser Arg Val Phe Met Pro Pro His Asp Leu Leu Ala

85 90 95

Arg Val Gly Gln Ile Cys Val Glu Gln Lys Gln Gln Leu Glu Ala Gly
100 105 110

Pro Glu Lys Ala Lys Leu Lys Ser Phe Ser Ala Lys Ile Val Gln Leu
115 120 125

Leu Lys Glu Trp Thr Glu Ala Phe Pro Tyr Asp Phe Gln Asp Glu Lys
130 135 140

Ala Met Ala Glu Leu Lys Ala Ile Thr His Arg Val Thr Gln Cys Asp
145 150 155 160

Glu Glu Asn Gly Thr Val Lys Lys Ala Ile Ala Gln Met Thr Gln Ser
165 170 175

Leu Leu Leu Ser Leu Ala Ala Arg Ser Gln Leu Gln Glu Leu Arg Glu
180 185 190

Lys Leu Arg Pro Pro Ala Val Asp Lys Gly Pro Ile Leu Lys Thr Lys
195 200 205

Pro Pro Ala Ala Gln Lys Asp Ile Leu Gly Val Cys Cys Asp Pro Leu
210 215 220

Val Leu Ala Gln Gln Leu Thr His Ile Glu Leu Asp Arg Val Ser Ser
225 230 235 240

Ile Tyr Pro Glu Asp Leu Met Gln Ile Val Ser His Met Asp Ser Leu
245 250 255

Asp Asn His Arg Cys Arg Gly Asp Leu Thr Lys Thr Tyr Ser Leu Glu
260 265 270

Ala Tyr Asp Asn Trp Phe Asn Cys Leu Ser Met Leu Val Ala Thr Glu
275 280 285

Val Cys Arg Val Val Lys Lys Lys His Arg Thr Arg Met Leu Glu Phe
290 295 300

Phe Ile Asp Val Ala Arg Glu Cys Phe Asn Ile Gly Asn Phe Asn Ser
305 310 315 320

Met Met Ala Ile Ile Ser Gly Met Asn Leu Ser Pro Val Ala Arg Leu
325 330 335

Lys Lys Thr Trp Ser Lys Val Lys Thr Ala Lys Phe Asp Val Leu Glu
340 345 350

His His Met Asp Pro Ser Ser Asn Phe Cys Asn Tyr Arg Thr Ala Leu
355 360 365

Gln Gly Ala Thr Gln Arg Ser Gln Met Ala Asn Ser Ser Arg Glu Lys
370 375 380

Ile Val Ile Pro Val Phe Asn Leu Phe Val Lys Asp Ile Tyr Phe Leu
385 390 395 400

His Lys Ile His Thr Asn His Leu Pro Asn Gly His Ile Asn Phe Lys
405 410 415

Lys Phe Trp Glu Ile Ser Arg Gln Ile His Glu Phe Met Thr Trp Thr
420 425 430

Gln Val Glu Cys Pro Phe Glu Lys Asp Lys Lys Ile Gln Ser Tyr Leu
435 440 445

Leu Thr Ala Pro Ile Tyr Ser Glu Glu Ala Leu Phe Val Ala Ser Phe
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