DESCRIPTION

GENES AND POLYPEPTIDES RELATING TO HUMAN COLON CANCERS

The present application is related to USSN 60/386,985, filed June 6, 2002, USSN 60/415,209, filed September 30, 2002, and USSN 60/451,013, filed February 28, 2003 which is incorporated herein by reference.

Technical Field

The present invention relates to the field of biological science, more specifically to the field of cancer research. In particular, the present invention relates to novel genes, *RNF43*, *CXADRL1*, and *GCUD1*, involved in the proliferation mechanism of cells, as well as polypeptides encoded by the genes. The genes and polypeptides of the present invention can be used, for example, in the diagnosis of cell proliferative disease, and as target molecules for developing drugs against the disease.

Background Art

Gastric cancers and colorectal cancers are leading causes of cancer death worldwide. In spite of recent progress in diagnostic and therapeutic strategies, prognosis of patients with advanced cancers remains very poor. Although molecular studies have revealed the involvement of alterations in tumor suppressor genes and/or oncogenes in carcinogenesis, the precise mechanisms still remain to be elucidated.

cDNA microarray technologies have enabled to obtain comprehensive profiles of gene expression in normal and malignant cells, and compare the gene expression in malignant and corresponding normal cells (Okabe et al., Cancer Res 61:2129-37 (2001); Kitahara et al., Cancer Res 61: 3544-9 (2001); Lin et al., Oncogene 21:4120-8 (2002); Hasegawa et al., Cancer Res 62:7012-7 (2002)). This approach enables to disclose the complex nature of cancer cells, and helps to understand the mechanism of carcinogenesis. Identification of genes that are deregulated in tumors can lead to more precise and accurate diagnosis of individual cancers, and to develop novel therapeutic targets (Bienz and Clevers, Cell 103:311-20 (2000)). To disclose mechanisms underlying tumors from a genome-wide point of view, and discover target molecules for diagnosis and development of novel therapeutic drugs, the present inventors have been analyzing the expression profiles of tumor cells using a cDNA microarray of 23040 genes (Okabe et al., Cancer Res 61:2129-37 (2001); Kitahara et al., Cancer Res 61:3544-9 (2001); Lin et al., Oncogene 21:4120-8 (2002); Hasegawa et al., Cancer Res 62:7012-7 (2002)).

Studies designed to reveal mechanisms of carcinogenesis have already facilitated

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

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

In spite of significant progress in basic and clinical research concerning TAAs (Rosenbeg et al., Nature Med 4: 321-7 (1998); Mukherji et al., Proc Natl Acad Sci USA 92: 8078-82 (1995); Hu et al., Cancer Res 56: 2479-83 (1996)), only limited number of candidate TAAs for the treatment of adenocarcinomas, including colorectal cancer, are available. TAAs abundantly expressed in cancer cells, and at the same time which expression is restricted to cancer cells would be promising candidates as

immunotherapeutic targets. Further, identification of new TAAs inducing potent and specific antitumor immune responses is expected to encourage clinical use of peptide vaccination strategy in various types of cancer (Boon and can der Bruggen, J Exp Med 183: 725-9 (1996); van der Bruggen et al., Science 254:1643-7 (1991); Brichard et al., J Exp Med 178:489-95 (1993); Kawakami et al.,J Exp Med 180: 347-52 (1994); Shichijo et al., J Exp Med 187: 277-88 (1998); Chen et al., Proc Natl Acad Sci USA 94: 1914-8 (1997); Harris, J Natl Cancer Inst 88:1442-5 (1996); Butterfield et al., Cancer Res 59: 3134-42 (1999); Vissers et al., Cancer Res 59: 5554-9 (1999); van der Burg et al., J Immunol 156:3308-14 (1996); Tanaka et al., Cancer Res 57: 4465-8 (1997); Fujie et al., Int J Cancer 80: 169-72 (1999); Kikuchi et al., Int J Cancer 81: 459-66 (1999); Oiso et al., Int J Cancer 81: 387-94 (1999)).

Other researchers have also speculated on the potential of various nucleotide sequences and their corresponding peptide sequences as diagnostic and therapeutic targets for a plethora of cancers and other diseases, such as autoimmune disease, nerve system disorders and arthritis (WO 01/66689 (HYSEQ, INC), 2001; Kawakami et al., DATABASE UNIPROT Accession No. Q9NXD0, 2000). The researchers in WO 01/66689, for example, have merely asserted that the disclosed sequences (derived from various public databases) are capable of modulating the overall activity of the target gene products and exhibit various biological activities. Kawakami et al. is merely a database submission that discloses a polypeptide sequence. Neither WO 01/66689 nor Kawakami et al. have provided any factual evidence that the disclosed sequences exhibited the asserted activities nor how the disclosed sequences could be used in any practical industrial applications. Neither WO 01/66689 nor Kawakami et al. have shown any correlation between the up/down-regulation of the disclosed sequences and the proliferation / inhibition of colorectal cancer cells. Hence, none of these researchers have shown that the suppression and/or expression of these sequences would result in the inhibition of tumor cell proliferation, particularly colorectal cancer cell proliferation.

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

Summary of the Invention

An object of the present invention is to provide novel proteins involved in the proliferation mechanism of gastric or colorectal cancer cells and the genes encoding the proteins, as well as methods for producing and using the same in the diagnosis and treatment of gastric cancer or colorectal cancer.

To disclose the mechanism of gastric and colorectal carcinogenesis and identify novel diagnostic markers and/or drug targets for the treatment of these tumors, the present

inventors analyzed the expression profiles of genes in gastric and colorectal carcinogenesis using a genome-wide cDNA microarray containing 23040 genes. From the pharmacological point of view, suppressing oncogenic signals is easier in practice than activating tumor-suppressive effects. Thus, the present inventors searched for genes that are up-regulated during gastric and colorectal carcinogenesis.

Among the transcripts that were commonly up-regulated in gastric cancers, novel human genes *CXADRL1* (coxsackie and adenovirus receptor like 1) and *GCUD1* (up-regulated in gastric cancer) were identified on chromosome band 3q13 and 7p14, respectively. Gene transfer of *CXADRL1* or *GCUD1* promoted proliferation of cells. Furthermore, reduction of *CXADRL1* or *GCUD1* expression by transfection of their specific antisense S-oligonucleotides or small interfering RNAs inhibited the growth of gastric cancer cells. Many anticancer drugs, such as inhibitors of DNA and/or RNA synthesis, metabolic suppressors, and DNA intercalators, are not only toxic to cancer cells but also for normally growing cells. However, agents suppressing the expression of CXADRL1 may not adversely affect other organs due to the fact that normal expression of the gene is restricted to the testis and ovary, and thus may be of great importance for treating cancer.

Further, among the transcripts that were commonly up-regulated in colorectal cancers, gene *RNF43* (Ring finger protein 43) assigned at chromosomal band 17pter-p13.1 was identified. In addition, yeast two-hybrid screening assay revealed that RNF43 protein associated with NOTCH2 or STRIN.

NOTCH2 is a large transmembrane receptor protein that is a component of an evolutionarily conserved intercellular signaling mechanism. NOTCH2 is a protein member of the Notch signaling pathway and is reported to be involved in glomerulogenesis in the kidney and development of heart and eye vasculature (McCright et al., Development 128: 491-502 (2001)). Three Delta/Serrate/Lag-2 (DSL) proteins, Delta1, Jaggaed1, and Jaggaed2, are reported as functional ligands for NOTCH2 (Shimizu et al., Mol Cell Biol 20: 6913-22 (2000)). The signal induced by ligand binding in the Notch signaling pathway is transmitted intracellulaly by a process involving proteolysis of the receptor and nuclear translocation of the intracellular domain of the NOTCH protein (see reviews Artavanis-Tsakonas et al., Annu Rev Cell Biol 7: 427-52 (1999); Weinmaster, Curr Opin Genet Dev 10: 363-9 (2000)). Furthermore, reduction of *RNF43* expression by transfection of specific antisense S-oligonucleotides or small interfering RNAs corresponding to *RNF43* inhibited the growth of colorectal cancer cells. As already described above many anticancer drugs, are not only toxic to cancer cells but also for normally growing cells. However, agents suppressing the expression of RNF43 may also

not adversely affect other organs due to the fact that normal expression of the gene is restricted to fetus, more specifically fetal lung and fetal kidney, and thus may be of great importance for treating cancer.

Thus, the embodiments of the present disclosure provide isolated novel genes, *CXADRL1, GCUD1*, and *RNF43*, which are candidates as diagnostic markers for cancer as well as promising potential targets for developing new strategies for diagnosis and effective anti-cancer agents. Further, the embodiments of the present disclosure provide polypeptides encoded by these genes, as well as the production and the use of the same. More specifically, the embodiments of the present disclosure provide the following:

The present application provides novel human polypeptides, CXADRL1, GCUD1, and RNF43, or a functional equivalent thereof, that promotes cell proliferation and is up-regulated in cell proliferative diseases, such as gastric and colorectal cancers.

In a preferred embodiment, the CXADRL1 polypeptide includes a putative 431 amino acid protein with about 37% identity to CXADR (coxsackie and adenovirus receptor). CXADRL1 is encoded by the open reading frame of SEQ ID NO: 1 and contains two immunoglobin domains at codons 29-124 and 158-232, as well as a transmembrane domain at codons 246-268. The CXADRL1 polypeptide preferably includes the amino acid sequence set forth in SEQ ID NO: 2. The present application also provides an isolated protein encoded from at least a portion of the *CXADRL1* polynucleotide sequence, or polynucleotide sequences at least 30%, and more preferably at least 40% complementary to the sequence set forth in SEQ ID NO: 1.

On the other hand, in a preferred embodiment, the GCUD1 polypeptide includes a putative 414 amino acid protein encoded by the open reading frame of SEQ ID NO: 3. The GCUD1 polypeptide preferably includes the amino acid sequence set forth in SEQ ID NO: 4. The present application also provides an isolated protein encoded from at least a portion of the *GCUD1* polynucleotide sequence, or polynucleotide sequences at least 15%, and more preferably at least 25% complementary to the sequence set forth in SEQ ID NO: 3.

Furthermore, in a preferred embodiment, the RNF43 polypeptide includes a putative 783 amino acid protein encoded by the open reading frame of SEQ ID NO: 5. The RNF43 polypeptide preferably includes the amino acid sequence set forth in SEQ ID NO: 6 and contains a Ring finger motif at codons 272-312. The RNF43 polypeptide showed 38% homology to RING finger protein homolog DKFZp566H073.1 (GenBank Accession Number: T08729). The present application also provides an isolated protein encoded from at least a portion of the *RNF43* polynucleotide sequence, or polynucleotide sequences at least 30%, and more preferably at least 40% complementary to the sequence

set forth in SEQ ID NO: 5.

The present disclosure further provides novel human genes, *CXADRL1* and *GCUD1*, whose expressions are markedly elevated in a great majority of gastric cancers as compared to corresponding non-cancerous mucosae. In addition to gastric cancers, CXADRL1 and GCUD1 were also highly expressed in colorectal cancer and liver cancer. The isolated *CXADRL1* gene includes a polynucleotide sequence as described in SEQ ID NO: 1. In particular, the *CXADRL1* cDNA includes 3423 nucleotides that contain an open reading frame of 1296 nucleotides (SEQ ID NO: 1). The embodiments of the present disclosure further encompass polynucleotides which hybridize to and which are at least 30%, and more preferably at least 40% complementary to the polynucleotide sequence set forth in SEQ ID NO: 1, to the extent that they encode a CXADRL1 protein or a functional equivalent thereof. Examples of such polynucleotides are degenerates and allelic mutants of SEQ ID NO: 1. On the other hand, the isolated *GCUD1* gene includes a polynucleotide sequence as described in SEQ ID NO: 3. In particular, the *GCUD1* cDNA includes 4987 nucleotides that contain an open reading frame of 1245 nucleotides (SEQ ID NO: 3). The embodiments of the present disclosure further encompass polynucleotides which hybridize to and which are at least 15%, and more preferably at least 25% complementary to the polynucleotide sequence set forth in SEQ ID NO: 3, to the extent that they encode a GCUD1 protein or a functional equivalent thereof. Examples of such polynucleotides are degenerates and allelic mutants of SEQ ID NO: 3.

Furthermore, the embodiments of the present disclosure provide a novel human gene *RNF43*, whose expression is markedly elevated in a great majority of colorectal cancers as compared to corresponding non-cancerous mucosae. In addition to colorectal cancersl RNF43 was also highly expressed in lung cancer, gastric cancer, and liver cancer. The isolated *RNF43* gene includes a polynucleotide sequence as described in SEQ ID NO: 5. In particular, the *RNF43* cDNA includes 5345 nucleotides that contain an open reading frame of 2352 nucleotides (SEQ ID NO: 5). The embodiments of the present disclosure further encompass polynucleotides which hybridize to and which are at least 30%, and more preferably at least 40% complementary to the polynucleotide sequence set forth in SEQ ID NO: 5, to the extent that they encode a RNF43 protein or a functional equivalent thereof. Examples of such polynucleotides are degenerates and allelic mutants of SEQ ID NO: 5.

As used herein, an isolated gene is a polynucleotide the structure of which is not identical to that of any naturally occurring polynucleotide or to that of any fragment of a naturally occurring genomic polynucleotide spanning more than three separate genes. The term therefore includes, for example, (a) a DNA which has the sequence of part of a naturally occurring genomic DNA molecule in the genome of the organism in which it

naturally occurs; (b) a polynucleotide incorporated into a vector or into the genomic DNA of a prokaryote or eukaryote in a manner such that the resulting molecule is not identical to any naturally occurring vector or genomic DNA; (c) a separate molecule such as a cDNA, a genomic fragment, a fragment produced by polymerase chain reaction (PCR), or a restriction fragment; and (d) a recombinant nucleotide sequence that is part of a hybrid gene, i.e., a gene encoding a fusion polypeptide.

Accordingly, the embodiments of the disclosure provide an isolated polynucleotide that encodes a polypeptide described herein or a fragment thereof. Preferably, the isolated polypeptide includes a nucleotide sequence that is at least 60% identical to the nucleotide sequence shown in SEQ ID NO: 1, 3, or 5. More preferably, the isolated nucleic acid molecule is at least 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more, identical to the nucleotide sequence shown in SEQ ID NO: 1, 3, or 5. In the case of an isolated polynucleotide which is longer than or equivalent in length to the reference sequence, e.g., SEQ ID NO: 1, 3, or 5, the comparison is made with the full length of the reference sequence. Where the isolated polynucleotide is shorter than the reference sequence, e.g., shorter than SEQ ID NO: 1, 3, or 5, the comparison is made to segment of the reference sequence of the same length (excluding any loop required by the homology calculation).

The embodiments of the present disclosure also provide a method of producing a protein by disclosure transfecting or transforming a host cell with a polynucleotide sequence encoding the CXADRL1, GCUD1, or RNF43 protein, and expressing the polynucleotide sequence. In addition, the embodiments of the present disclosure provide vectors comprising a nucleotide sequence encoding the CXADRL1, GCUD1, or RNF43 protein, and host cells harboring a polynucleotide encoding the CXADRL1, GCUD1, or RNF43 protein. Such vectors and host cells may be used for producing the CXADRL1, GCUD1, or RNF43 protein.

An antibody that recognizes the CXADRL1, GCUD1, or RNF43 protein is also provided by the present application. In part, an antisense polynucleotide (e.g., antisense DNA), ribozyme, and siRNA (small interfering RNA) of the *CXADRL1*, *GCUD1*, or *RNF43* gene is also provided.

The embodiments of the present disclosure further provide a method for diagnosis of cell proliferative diseases that includes determining an expression level of the gene in biological sample of specimen, comparing the expression level of *CXADRL1*, *GCUD1*, or *RNF43* gene with that in normal sample, and defining a high expression level of the *CXADRL1*, *GCUD1*, or *RNF43* gene in the sample as having a cell proliferative disease such as cancer. The disease diagnosed by the expression level of *CXADRL1* or *GCUD1* is suitably a gastric, colorectal, and liver cancer; and that detected by the expression level of *RNF43* is

colorectal, lung, gastric, and liver cancer.

Further, a method of screening for a compound for treating a cell proliferative disease is provided. The method includes contacting the CXADRL1, GCUD1, or RNF43 polypeptide with test compounds, and selecting test compounds that bind to the CXADRL1, GCUD1, or RNF43 polypeptide.

The embodiments of the present disclosure further provide a method of screening for a compound for treating a cell proliferative disease, wherein the method includes contacting the CXADRL1, GCUD1, or RNF43 polypeptide with a test compound, and selecting the test compound that suppresses the expression level or biological activity of the CXADRL1, GCUD1, or RNF43 polypeptide.

Alternatively, the embodiments of the present disclosure provide a method of screening for a compound for treating a cell proliferative disease, wherein the method includes contacting CXADRL1 and AIP1 in the presence of a test compound, and selecting the test compound that inhibits the binding of CXADRL1 and AIP1.

Furthermore, the embodiments of the present disclosure provide a method of screening for a compound for treating a cell proliferative disease, wherein the method includes contacting RNF43 and NOTCH2 or STRIN in the presence of a test compound, and selecting the test compound that inhibits the binding of RNF43 and NOTCH2 or STRIN.

The embodiments of the present disclosure also provide a pharmaceutical composition for treating cell proliferative disease, such as cancer. The pharmaceutical composition may be, for example, an anti-cancer agent. The pharmaceutical composition can be described as at least a portion of the antisense S-oligonucleotides or siRNA of the *CXADRL1*, *GCUD1*, or *RNF43* polynucleotide sequence shown and described in SEQ ID NO: 1, 3, or 5, respectively. A suitable antisense S-oligonucleotide has the nucleotide sequence selected from the group of SEQ ID NO: 23, 25, 27, 29, or 31. The antisense S-oligonucleotide of *CXADRL1* including those having the nucleotide sequence of SEQ ID NO: 23 or 25 may be suitably used to treat gastric cancer; the antisense S-oligonucleotide of *GCUD1* including those having the nucleotide sequence of SEQ ID NO: 27 or 29 suitably to treat gastric, colorectal, or liver cancer; and the antisense S-oligonucleotide of *RNF43* including those having the nucleotide sequence of SEQ ID NO: 31 suitably for colorectal, lung, gastric, or liver cancer. A suitable siRNA may consist of a set of nucleotides with the nucleotide sequences selected from the group of SEQ ID NOs: 40 and 41, 42 and 43, or 62 and 63. The siRNA of *CXADRL1* consisting of a set of nucleotides with the nucleotide sequence of SEQ ID NOs: 40 and 41, or 42 and 43 may be suitably used to treat gastric, colorectal, or liver cancer; and the siRNA of *RNF43* consisting of a set of nucleotides with the nucleotide sequence of SEQ ID NOs: 62 and 63 suitably for colorectal, lung, gastric, or

liver cancer. The pharmaceutical compositions may be also those comprising the compounds selected by the present methods of screening for compounds for treating cell proliferative diseases.

The course of action of the pharmaceutical composition is desirably to inhibit growth of the cancerous cells. The pharmaceutical composition may be applied to mammals including humans and domesticated mammals.

The present disclosure further provides methods for treating a cell proliferative disease using the pharmaceutical composition provided by the present disclosure.

In addition, the present disclosure provides method for treating or preventing cancer, which method comprises the step of administering the CXADRL1, GCUD1, or RNF43 polypeptide. It is expected that anti tumor immunity be induced by the administration of the CXADRL1, GCUD1, or RNF43 polypeptide. Thus, the present disclosure also provides method for inducing anti tumor immunity, which method comprises the step of administering the CXADRL1, GCUD1, or RNF43 polypeptide, as well as pharmaceutical composition for treating or preventing cancer comprising the CXADRL1, GCUD1, or RNF43 polypeptide.

In one aspect, there is provided a small interfering RNA against the polynucleotide encoding the polypeptide consisting of the amino acid sequence of SEQ ID NO: 6, wherein the sense strand of said small interfering RNA is the nucleotide sequence of SEQ ID NO: 112 as the target sequence.

In another aspect, there is provided an in vitro method for diagnosing colorectal cancer, said method comprising the steps of:

(a) detecting the expression level of the gene encoding the amino acid sequence of SEQ ID NO:6 in a biological sample of specimen; and

(b) relating an elevation of the expression level to colorectal cancer.

In another aspect, there is provided a method of screening for a compound for treating colorectal cancer, said method comprising the steps of:

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

(1) a polypeptide consisting of the amino acid sequence of SEQ ID NO: 6;

(2) a polypeptide that consists of the amino acid sequence of SEQ ID NO: 6 in which one or more amino acids are substituted, deleted, inserted, and/or added and that has a cell-proliferating activity equivalent to a protein consisting of the amino acid sequence of SEQ ID NO: 6; and

(3) a polypeptide encoded by a polynucleotide that hybridizes under stringent conditions to a polynucleotide consisting of the nucleotide sequence of SEQ ID NO: 5, wherein the polypeptide has a cell-proliferating activity equivalent to a polypeptide consisting of the amino acid sequence of SEQ ID NO: 6;

(b) detecting the binding activity between the polypeptide and the test compound; and

(c) selecting a compound that binds to the polypeptide.

In another aspect, there is provided a method of screening for a compound for treating colorectal cancer, said method comprising the steps of:

(a) contacting a candidate compound with a cell expressing one or more polynucleotides consisting of the nucleotide sequence of SEQ ID NO: 5; and

(b) selecting a compound that reduces the expression level of one or more polynucleotides consisting of the nucleotide sequence of SEQ ID NO: 5 in comparison with the expression level detected in the absence of the test compound.

In another aspect, there is provided a method of screening for a compound for treating a colorectal cancer, said method comprising the steps of:

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

(1) a polypeptide consisting of the amino acid sequence of SEQ ID NO: 6;

(2) a polypeptide that consists of the amino acid sequence of SEQ ID NO: 6 in which one or more amino acids are substituted, deleted, inserted, and/or added and that has a cell-proliferating activity equivalent to a protein consisting of the amino acid sequence of SEQ ID NO: 6; and

(3) a polypeptide encoded by a polynucleotide that hybridizes under stringent conditions to a polynucleotide consisting of the nucleotide sequence of SEQ ID NO: 5, wherein the polypeptide has a cell-proliferating activity equivalent to a polypeptide consisting of the amino acid sequence of SEQ ID NO: 6;

(b) detecting the cell-proliferating activity of the polypeptide of step (a); and
(c) selecting a compound that suppresses the cell-proliferating activity of the polypeptide in comparison with the cell- proliferating activity detected in the absence of the test compound.

In another aspect, there is provided a method of screening for a compound for treating a cell proliferative disease, said method comprising the steps of:

(a) contacting a polypeptide selected from the group consisting of:
(1) a polypeptide consisting of the amino acid sequence of SEQ ID NO: 6;
(2) a polypeptide that consists of the amino acid sequence of SEQ ID NO: 6 in which one or more amino acids are substituted, deleted, inserted, and/or added and that has a biological activity equivalent to a protein consisting of the amino acid sequence of SEQ ID NO: 6;
(3) a polypeptide encoded by a polynucleotide that hybridizes under stringent conditions to a polynucleotide consisting of the nucleotide sequence of SEQ ID NO: 5, wherein the polypeptide has a biological cell-proliferating activity equivalent to a polypeptide consisting of the amino acid sequence of SEQ ID NO: 6;
with NOTCH2 or STRIN in the existence of a test compound;

(b) detecting the binding between the polypeptide and NOTCH2 or STRIN; and
(c) selecting the test compound that inhibits the binding between the polypeptide and NOTCH2 or STRIN.

In another aspect, there is provided a method of screening for a compound for treating colorectal cancer, said method comprising the steps of:

a) contacting a candidate compound with a cell into which a vector comprising the transcriptional regulatory region of a marker gene and a reporter gene that is expressed under the control of the transcriptional regulatory region has been introduced, wherein the marker gene consists of the nucleotide sequence of SEQ ID:NO 5,
b) measuring the activity of said reporter gene; and
c) selecting a compound that reduces the expression level of said reporter gene as compared to a control.

In another aspect, there is provided a composition for treating colorectal cancer, said composition comprising a pharmaceutically effective amount of small interfering RNA against a polynucleotide encoding a polypeptide that consists of the amino acid sequence of SEQ ID NO: 6 as an active ingredient, and a pharmaceutically acceptable carrier, wherein the sense strand of said small interfering RNA is the nucleotide sequence of SEQ ID NO: 112 as the target sequence.

In another aspect, there is provided use of an antisense polynucleotide or small interfering RNA against a polynucleotide encoding a polypeptide selected from the group consisting of:

(1) a polypeptide consisting of the amino acid sequence of SEQ ID NO: 6;

(2) a polypeptide that consists of the amino acid sequence of SEQ ID NO: 6 in which one or more amino acids are substituted, deleted, inserted, and/or added and that has a cell-proliferating activity equivalent to a protein consisting of the amino acid sequence of SEQ ID NO: 6; and

(3) a polypeptide encoded by a polynucleotide that hybridizes under stringent conditions to a polynucleotide consisting of the nucleotide sequence of SEQ ID NO: 5, wherein the polypeptide has a cell-proliferating activity equivalent to a polypeptide consisting of the amino acid sequence of SEQ ID NO: 6

for the preparation of a pharmaceutical composition for treating colorectal cancer.

In another aspect, there is provided use of an antibody against a polypeptide selected from the group consisting of:

(a) a polypeptide that consists of the amino acid sequence of SEQ ID NO: 6;

(b) a polypeptide that consists of the amino acid sequence of SEQ ID NO: 6 in which one or more amino acids are substituted, deleted, inserted, and/or added and that has a cell-proliferating activity equivalent to a protein consisting of the amino acid sequence of SEQ ID NO: 6; and

(c) a polypeptide encoded by a polynucleotide that hybridizes under stringent conditions to a polynucleotide consisting of the nucleotide sequence of SEQ ID NO: 5, wherein the polypeptide has a cell-proliferating activity equivalent to a polypeptide consisting of the amino acid sequence of SEQ ID NO: 6.

for the preparation of a pharmaceutical composition for treating colorectal cancer.

In another aspect, there is provided use of a polypeptide selected from the group consisting of (a)-(c), or a polynucleotide encoding the polypeptide:

(a) a polypeptide consisting of the amino acid sequence of SEQ ID NO: 6 or fragment thereof;

(b) a polypeptide that consists of the amino acid sequence of SEQ ID NO: 6 in which one or more amino acids are substituted, deleted , inserted, and/or added and that has a cell-proliferating activity equivalent to a protein consisting of the amino acid sequence of SEQ ID NO: 6, or fragment thereof;

(c) a polypeptide encoded by a polynucleotide that hybridizes under stringent conditions to a polynucleotide consisting of the nucleotide sequence of SEQ ID NO: 5, wherein the polypeptide has a cell-proliferating activity equivalent to a polypeptide consisting of the amino acid sequence of SEQ ID NO: 6, or fragment thereof

for the preparation of a pharmaceutical composition for treating or preventing colorectal cancer.

In another aspect, there is provided use of a polypeptide consisting of the amino acid sequence of SEQ ID NO: 80, 97, or 108, or a polynucleotide encoding said polypeptide or vector comprising said polynucleotide for the preparation of a pharmaceutical composition for inducing an anti tumor immunity.

In another aspect, there is provided use of a polypeptide consisting of the amino acid sequence SEQ ID NO: 80, 97, or 108, or a polynucleotide encoding said polypeptide or vector comprising said polynucleotide for the preparation of the antigen presenting cells.

In another aspect, there is provided a pharmaceutical composition for treating or preventing colorectal cancer, said composition comprising a pharmaceutically effective amount of a polypeptide consisting of the amino acid sequence selected from the group of SEQ ID NO: 80, 97, and 108.

In another aspect, there is provided a substantially pure polypeptide consisting of the amino acid sequence of SEQ ID NO: 80, 97 or 108.

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

Brief Description of the Drawings

Fig. 1a to 1d depict the expression of *A5928* (*CXADRL1*) and *C8121(GCUD1)* in gastric cancers. Fig. 1a depicts the relative expression ratios (cancer/non-cancer) of *A5928* in primary 14 gastric cancers examined by cDNA microarray. Its expression was up-regulated (Cy3:Cy5 intensity ratio, >2.0) in 14 of the 14 gastric cancers that passed through the cutoff filter (both Cy3 and Cy5 signals greater than 25,000). Fig. 1b depicts the relative expression ratios (cancer/non-cancer) of *C8121* in primary 12 gastric cancers examined by cDNA microarray. Its expression was up-regulated (Cy3:Cy5 intensity ratio, >2.0) in 10 of the 12 gastric cancers that passed through the cutoff filter. Fig 1c depicts the expression of *CXADRL1* analyzed by semi-quantitative RT-PCR using 10 gastric cancer cases. Fig. 1d depicts the expression of *GCUD1* analyzed by semi-quantitative RT-PCT using 9 gastric cancer cases. Expression of *GAPDH* served as an internal control for both the expression analyses of *CXADRL1* and *GCUD1*.

Fig. 2a and 2b depict the expression of *CXADRL1* in various human tissues and the predicted protein structure and protein motifs of *CXADRL1*. Fig. 2a is a photograph depicting expression of *CXADRL1* in various human tissues analyzed by multiple-tissue

northern blot analysis. Fig. 2b depicts the predicted protein structure of CXADRL1. The *CXADRL1* cDNA consists of 3,423 nucleotides with an ORF of 1,296 nucleotides and is composed of 7 exons.

Fig. 3a to 3c depict the growth-promoting effect of CXADRL1. Fig. 3a is a photograph depicting the result of colony formation assays of NIH3T3 cells transfected with *CXADRL1*. Fig. 3b depicts the expression of exogeneous *CXADRL1* in NIH3T3-CXADRL1 cells analyzed by semi-quantitative RT-PCR. Expression of *GAPDH* served as an internal control. #2, #5, #6, and #7 all indicate NIH3T3 cells transfected with CXADRL1. Fig. 3c depicts the number of NIH3T3 cells. Growth of NIH3T3-CXADRL1 cells was statistically higher than that of mock (NIH3T3-LacZ) cells in culture media containing 10% FBS ($P<0.05$).

Fig. 4 depicts the growth-inhibitory effect of antisense S-oligonucleotides designated to suppress *CXADRL1* in MKN-1 cells. CXADRL1-AS4 and CXADRL1-AS5 were demonstrated to suppress the growth of MKN-1 cells.

Fig. 5A to 5C depict the growth suppressive effect of CXADRL1-siRNA on St-4 cells. Fig. 5A presents photographs depicting the expression of *CXADRL1* and GAPDH (control) in St-4 cells transfected with mock or CXADRL1-siRNA#7. Fig. 5B depicts photographs depicting the result of Giemsa's staining of viable cells treated with control-siRNA or CXADRL1-siRNA#7. Fig. 5C depicts the result of MTT assay on cells transfected with control plasmid or plasmids expressing CXADRL1-siRNA7.

Fig.6 depicts a photograph demonstrating the result of immunoblot analysis of cells expressing exogeneous Flag-tagged CXADRL1 protein with anti-CXADRL1 antisera or ant-Flag antibody.

Fig. 7 depicts the interaction between CXADRL1 and AIP1 examined by yeast two-hybrid system. Fig. 7 is a photograph depicting the interaction of CXADRL1 with AIP1 examined by the two-hybrid system.

Fig. 8 depicts the peptide specific cytotoxicity of CTL line raised by CXADRL1-207 stimulation. The CTL line showed high cytotoxic activity on target cells (T2) pulsed with CXADRL1-207, whereas no significant cytotoxic activity was detected on the same target cells (T2) pulsed without peptides.

Fig. 9 depicts the cytotoxic activity of CXADRL1-207 CTL Clone on SNU475, MKN74, and SNU-C4. CXADRL1-207 CTL Clone showed high cytotoxic activity on SNU475 that expresses both CXADRL1 and HLA-A*0201. On the other hand, CXADRL1-207 CTL Clone showed no significant cytotoxic activity on MKN74, which expresses CXADRL1 but not HLA-A*0201. Furthermore, this CTL Clone did not show significant cytotoxic activity on SNU-C4, which expresses HLA-A*0201 but not

CXADRL1.

Fig. 10 depicts the result of the cold target inhibition assay. CXADRL1-207 CTL Clone specifically recognizes CXADRL1-207 in an HLA-A*0201 restricted manner. SNU475 labeled with $Na_2{}^{51}Cr\ O_4$ was prepared as a hot target, while CXADRL1-207 peptide-pulsed T2 (Peptide +) was used as a cold target (Inhibitors). E/T ratio was fixed to 20. The cytotoxic activity on SNU475 was inhibited by the addition of T2 pulsed with the identical peptide, while almost no inhibition by the addition of T2 without peptide pulse.

Fig. 11 depicts the result of the blocking assay showing the effect of antibodies raised against HLA-Class I, HLA-Class II, CD4, and CD8 on the cytotoxic activity of CXADRL1-207 CTL Clone. CXADRL1-207 CTL Clone showed cytotoxic activity in HLA-Class I and CD8 restricted manner. To examine the characteristics of CTL clone raised with CXADRL1 peptide, antibodies against HLA-Class I, HLA-Class II, CD4, and CD8 were tested for their ability to inhibit the cytotoxic activity. The horizontal axis reveals % inhibition of the cytotoxicity. The cytotoxicity of CTL clone on SNU475 targets was significantly reduced when anti class I and CD8 antibodies were used. This result indicates that the CTL clone recognizes the CXADRL1 derived peptide in a HLA-Class I and CD8 dependant manner.

Fig. 12 is a photograph depicting the result of Northern blot analysis of *GCUD1* in various human tissues. The transcript of *GCUD1* is approximately 3.5-kb by size.

Fig. 13 shows a photograph depicting the subcellular localization of GCUD1 observed by immunocytochemistry of cells transfected with pcDNA3.1myc/His-GCUD1. cMyc-tagged GCUD1 protein expressed from the plasmid localized in the cytoplasm.

Fig. 14 is a photograph showing the growth-promoting effect of GCUD1on NIH3T3 cells examined by colony formation assays.

Fig. 15 depicts the growth-inhibitory effect of antisense S-oligonucleotides designated to suppress *GCUD1* on MKN-28 cells. GCUD1-AS5 and GCUD1-AS8 were revealed to suppress the growth of MKN-28 cells.

Fig. 16 depicts a photograph showing the purification of recombinant GCUD1 protein.

Fig. 17 depicts a photograph demonstrating the result of immunoblot analysis of cells expressing exogenous Flag-tagged GCUD1 protein with anti-GCUD1 antisera or ant-Flag antibody.

Fig. 18A and 18B depict the peptide specific cytotoxicity of CTL line raised by GCUD1-196 (A) or GCUD1-272 (B) stimulation. The CTL line showed high cytotoxic activity on target cells (T2) pulsed with GCUD1-196 or GCUD1-272, whereas no

significant cytotoxic activity

was detected on the same target cells (T2) pulsed without peptides.

Fig. 19 depicts the cytotoxic activity of GCUD1-196 CTL Clone on SNU475 and MKN45. GCUD1-196 CTL Clone showed high cytotoxic activity on SNU475 that expresses both GCUD1 and HLA-A*0201. On the other hand, GCUD1-196 CTL Clone showed no significant cytotoxic activity on MKN45, which expresses GCUD1 but not HLA-A*0201.

Fig. 20 depicts the result of the cold target inhibition assay. GCUD1-196 CTL Clone specifically recognizes GCUD1-196 in an HLA-A*0201 restricted manner. SNU475 labeled with $Na_2{}^{51}Cr\ O_4$ was prepared as a hot target, while GCUD1-196 peptide-pulsed T2 (Peptide +) was used as a cold target (Inhibitors). E/T ratio was fixed to 20. The cytotoxic activity on SNU475 was inhibited by the addition of T2 pulsed with the identical peptide, while almost no inhibition by the addition of T2 without peptide pulse.

Fig. 21 depicts the result of the blocking assay showing the effect of antibodies raised against HLA-Class I, HLA-Class II, CD4, and CD8 on the cytotoxic activity of GCUD1-196 CTL Clone. GCUD1-196 CTL Clone showed cytotoxic activity in HLA-Class I and CD8 restricted manner. To examine the characteristics of CTL clone raised with GCUD1 peptide, antibodies against HLA-Class I, HLA-Class II, CD4, and CD8 were tested for their ability to inhibit the cytotoxic activity. The horizontal axis reveals % inhibition of the cytotxicity. The cytotoxicity of CTL clone on SNU475 targets was significantly reduced when anti class I and CD8 antibodies were used. This result indicates that the CTL clone recognizes the GCUD1 derived peptide in a HLA Class I and CD8 dependent manner.

Fig. 22a and 22b depict the expression of *FLJ20315* in colon cancer. Fig. 22a depicts the relative expression ratios (cancer/non-cancer) of *FLJ20315* in 11 primary colon cancer cases examined by cDNA microarray. Its expression was up-regulated (Cy3:Cy5 intensity ratio, >2.0) in 10 of the 11 colon cancer cases that passed through the cut-off filter (both Cy3 and Cy5 signals greater than 25,000). Fig. 22b depicts the expression of *FLJ20315* analyzed by semi-quantitative RT-PCR using additional 18 colon cancer cases (T, tumor tissue; N, normal tissue). Expression of *GAPDH* served as an internal control.

Fig. 23a depicts a photograph showing the result of fetal-tissue northern blot analysis of *RNF43* in various human fetal tissues. Fig. 23b depicts the predicted protein structure of RNF43.

Fig. 24a and 24b show photographs depicting the subcellular localization of myc-tagged RNF43 protein. Fig. 24a is a photograph depicting the result of Western-blot analysis of myc-tagged RNF43 protein using extracts from COS7 cells transfected with

either pcDNA3.1-myc/His-RNF43 or control plasmids (mock). Fig. 24b presents photographs of the transfected cells that were stained with mouse anti-myc antibody and visualized by FITC-conjugated secondary antibody. Nuclei were counter-stained with DAPI.

Fig. 25a to 25c depicts the effect of *RNF43* on cell growth. Fig. 25a is a photograph depicting the result of colony formation assay of *RNF43* in NIH3T3 cells. Fig. 25b presents photographs depicting the expression of *RNF43* in mock (COS7-pcDNA) and COS7-RNF43 cells that was established by the transfection of COS7 cells with pcDNA-RNF43. Fig. 25c depicts the result of comparison on cell growth between COS7-RNF43 cells stably expressing exogenous *RNF43* and mock cells.

Fig. 26a and 26b depict the growth-inhibitory effect of antisense S-oligonucleotides designed to suppress *RNF43*. Fig. 26a presents photographs depicting the expression of RNF43 in LoVo cells treated for 12 h with either control (RNF43-S1) or antisense S-oligonucleotides (RNF43-AS1) analyzed by semi-quantitative RT-PCR. Fig. 26b depicts the cell viability of LoVo cells after treatment with the control or antisense S-oligonucleotides measured by MTT assay. The MTT assay was carried out in triplicate.

Fig. 27A to 27C depict the growth suppressive effect of RNF43-siRNAs. Fig. 27A presents photographs depicting the effect of RNF43-siRNAs on the expression of RNF43. Fig. 27B presents photographs depicting the result of Giemsa's staining of viable cells after the treatment with control- siRNA or RNF43-siRNAs. Fig. 27C depicts the result of MTT assay on cells transfected with control plasmid or plasmids expressing RNF43-siRNAs. *, a significant difference ($p < 0.05$) as determined by a Fisher's protected least significant difference test.

Fig. 28A and 28B depict the expression of tagged RNF43 protein. Fig. 28A is a photograph depicting the result of Western-blot analysis of Flag-tagged RNF43 protein secreted in the culture media of COS7 cells transfected with pFLAG-5CMV-RNF43 (lane 2) or mock vector (lane 1). Fig. 28B is a photograph depicting the result of Western-blot analysis of Myc-tagged RNF43 protein secreted in the culture media of COS7 cells transfected with pcDNA3.1-Myc/His-RNF43 (lane 2) or mock vector (lane 1).

Fig. 29A and 29B depict the growth promoting effect of conditioned media containing the Myc-tagged or Flag-tagged RNF43 protein. Fig. 29A presents photographs depicting the morphology of NIH3T3 cells cultured in control media (1) or in conditioned media of COS7 cells transfected with mock vector (2), pcDNA3.1-Myc/His-RNF43 (3), or pFLAG-5CMV-RNF43 (4). Fig. 29B depicts the number of NIH3T3 cells cultured in the indicated media described in Fig. 29A. Data are

shown as means of triplicate experiments for each group; *bars*, ±SE. *, significant difference when compared with control, mock($p<0.05$).

Fig. 30A to 30C depict the preparation of N-terminal (N1) and C-terminal (C1) recombinant protein of RNF43. Fig. 30A depicts the schematic structure of the recombinant protein RNF43-N1 and -C1. Fig. 30B is a photograph depicting the expression of Nus™-tagged RNF43-N1 protein in *E. coli* with (lane2) or without (lane 1) 0.2 mM of IPTG. Fig. 30C is a photograph depicting the expression of Nus™-tagged RNF43-C1 protein in *E. coli* with (lane2) or without (lane 1) 1mM of IPTG.

Fig. 31A and 31B depict the interaction between RNF43 and NOTCH2 examined by yeast two-hybrid system. Fig. 31A depicts the predicted structure and the interacting region of NOTCH2. (a) shows the predicted full length structure of NOTCH2 protein, and (b) shows the predicted responsible region for the interaction (ECD, Extracellular domain; TM, transmembrane domain; ICD, Intracellular domain). Fig. 31B is a photograph depicting the interaction of RNF43 with NOTCH2 examined by the two-hybrid system.

Fig. 32A and 32B depict the interaction between RNF43 and STRIN examined by the yeast two-hybrid system. Fig. 32A depicts the predicted structure and the interacting region of STRIN. (a) shows the predicted full length structure of STRIN protein, and (b) shows the predicted responsible region for the interaction (RING, RING domain). Fig. 32B is a photograph depicting the interaction of RNF43 with STRIN examined by the two-hybrid system.

Fig. 33 depicts the peptide specific cytotoxicity of CTL line raised by RNF43-721 stimulation. The CTL line showed high cytotoxic activity on target cells (TISI) pulsed with RNF43-721 (quadrilateral line), whereas no significant cytotoxic activity was detected on the same target cells (TISI) pulsed without peptides (triangular line). CTL line was demonstrated to have a peptide specific cytotoxicity.

Fig. 34 depicts the peptide specific cytotoxicity of CTL clones raised by RNF43-721 stimulation. The cytotoxic activity of 13 RNF43-721 CTL clones on peptide-pulsed targets (TISI) was tested as described in the Materials and Methods. The established RNF43-721 CTL clones had very potent cytotoxic activity on target cells (TISI) pulsed with the peptides without showing any significant cytotoxic activity on the same target cells (TISI) that were not pulsed with any peptides.

Fig. 35 depicts the cytotoxic activity of RNF43-721 CTL Clone 45 on HT29, WiDR and HCT116. RNF43-721 CTL Clone recognizes and lyses tumor cells that endogenously express RNF43 in an HLA restricted fashion. HT29, WiDR and HCT116 all endogenously express RNF43, and RNF43-721 CTL Clone 45 served as an effector cell.

TISI was used as the target that does not express RNF43. RNF43-721 CTL Clone 45 showed high cytotoxic activity on HT29 (filled triangular line) and WiDR (diamond line) that express both RNF43 and HLA-A24. On the other hand, RNF43-721 CTL Clone 45 showed no significant cytotoxic activity on HCT116 (empty triangular line), which expresses RNF43 but not HLA-A24, and TISI (empty quadrilateral line), which expresses HLA-A24 but not RNF43. Moreover, RNF43-721 CTL Clone 45 showed no cytotoxic activity on irrelevant peptide pulsed TISI (filled quadrilateral dotted line) and SNU-C4 (filled circle line) which expresses RNF43 but little HLA-A24.

Fig. 36 depicts the result of the cold target inhibition assay. RNF43-721 CTL Clone specifically recognizes RNF 43-721 in an HLA-A24 restricted manner. HT29 labeled with $Na_2{}^{51}Cr\ O_4$ was prepared as a hot target, while RNF43-721 peptide-pulsed TISI (Peptide +) was used as a cold target (Inhibitors). E/T ratio was fixed to 20. The cytotoxic activity on HT29 was inhibited by the addition of TISI pulsed with the identical peptide (filled quadrilateral line), while almost no inhibition occurred by the addition of TISI without peptide pulsing (empty quadrilateral line).

Fig. 37 depicts the result of the blocking assay showing the effect of antibodies raised against HLA-Class I, HLA-Class II, CD3, CD4, and CD8 on the cytotoxic activity of RNF43-721 CTL Clone. RNF43-721 CTL Clone showed cytotoxic activity in HLA-Class I, CD3 and CD8 restricted manner. To examine the characteristics of CTL clone raised with RNF43 peptide, antibodies against HLA-Class I, HLA-Class II, CD3, CD4, and CD8 were tested for their ability to inhibit the cytotoxic activity. The horizontal axis reveals % inhibition of the cytotoxicity. The cytotoxicity of CTL clone on WiDR targets was significantly reduced when anti class I, CD3, and CD8 antibodies were used. This result indicates that the CTL clone recognizes the RNF43 derived peptide in a HLA Class I, CD3 and CD8 dependent manner.

Fig. 38A and 38B depict the peptide specific cytotoxicity of the CTL lines raised with RNF43-11-9 (A) or RNF43-11-10 (B). These CTL lines showed high cytotoxic activity on target cells (T2) pulsed with RNF43-11-9 or RNF43-11-10, whereas no significant cytotoxic activity was observed on the same target cells (T2) pulsed without peptides.

Fig. 39A and 39B depict the peptide specific cytotoxicity of CTL clones raised by RNF43-11-9 (A) or RNF43-11-10 (B) stimulation. Cytotoxic activity of 4 RNF43-11-9 CTL clones or 7 RNF43-11-10 clones on peptide-pulsed targets (T2) was tested as described in the Materials and Methods. The established RNF43-11-9 and RNF43-11-10 CTL clones had very potent cytotoxic activities on target cells (T2) pulsed with the peptides without showing any significant cytotoxic activity on the same target cells (T2)

that were not pulsed with any peptides.

Fig. 40A and 40B depict the cytotoxic activity of RNF43-5 CTL Clone 90 and RNF43-17 CTL Clone 25 on HT29 and DLD-1. RNF43-5 CTL Clone 90 and RNF43-17 CTL Clone 25 recognize and lyses tumor cells that endogenously express RNF43 in an HLA restricted fashion. HT29 and DLD-1 all endogenously express RNF43, and RNF43-5 CTL Clone 90 and RNF43-17 CTL Clone 25 served as an effector cell. T2 was used as the target that does not express RNF43. RNF43-5 CTL Clone 90 and RNF43-17 CTL Clone 25 showed high cytotoxic activity on DLD-1 that express both RNF43 and HLA-A*0201. On the other hand, RNF43-5 CTL Clone 90 and RNF43-17 CTL Clone 25 showed no significant cytotoxic activity on HT29, which expresses RNF43 but not HLA-A*0201.

Fig. 41 depicts the result of cold target inhibition assay. RNF43-11-9 CTL Clone specifically recognizes RNF 43-11-9 in a HLA-A2 restricted manner. HCT116 labeled with $Na_2{}^{51}Cr\ O_4$ was prepared as a hot target, while RNF43-11-9 peptide-pulsed T2 (Peptide +) was used as a cold target (Inhibitors). E/T ratio was fixed to 20. The cytotoxic activity on HCT116 was inhibited by the addition of T2 pulsed with the identical peptide, while almost no inhibition observed by the addition of TISI without peptide pulse.

Detailed Description of the Invention

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

The present application identifies novel human genes *CXADRL1* and *GCUD1* whose expression is markedly elevated in gastric cancer compared to corresponding non-cancerous tissues. The *CXADRL1* cDNA consists of 3423 nucleotides that contain an open reading frame of 1296 nucleotides as set forth in SEQ ID NO: 1. The open reading frame encodes a putative 431-amino acid protein. CXADRL1 associates with AIP1. AIP1(atripin-1-interacting protein 1) is a protein that associates with atropin-1, a gene responsible fora hereditary disease, dentatorubral- pallidoluysian atrophy. AIP1 encodes a deduced 1455-amino acid protein conatining guanylate kinase-like domain, two WW domains and five PDZ domains. The mouse homolog of AIP1 was shown to interact with activin type IIA. However, the function of AIP1 remains to be resolved. The predicted amino acid sequence showed an identity of about 37% to CXADR (coxsackie and adenovirus receptor). Therefore this protein was dubbed CXADRL1 (coxsackie and adenovirus receptor like 1). On the other hand, the *GCUD1* cDNA consists of 4987 nucleotides that contain an open reading frame of 1245 nucleotides as set forth in SEQ ID NO: 3. The open reading frame encodes a putative 414-amino acid

protein. Since the expression of the protein was up-regulated in gastric cancer, the protein was dubbed GCUD1 (up-regulated in gastric cancer).

Furthermore, the present invention encompasses novel human gene *RNF43* whose expression is markedly elevated in colorectal cancer compared to corresponding non-cancerous tissue. The *RNF43* cDNA consists of 5345 nucleotides that contain an open reading frame of 2352 nucleotides as set forth in SEQ ID NO: 5. The open reading frame encodes a putative 783-amino acid protein. RNF43 associates with NOTCH2 and STRIN. NOTCH2 is reported as a large transmembrane receptor protein that is a component of an evolutionarily conserved intercellular signaling mechanism. NOTCH2 is a protein member of the Notch signaling pathway and is reported to be involved in glomerulogenesis in the kidney and development of heart and eye vasculature. Furthermore, three Delta/Serrate/Lag-2 (DSL) proteins, Delta1, Jaggaed1, and Jaggaed2, are reported as functional ligands for NOTCH2. STRIN encodes a putative protein that shares 79% identity with mouse Trif. The function of STRIN or Trif remains to be clarified.

Consistently, exogenous expression of *CXADRL1*, *GCUD1*, or *RNF43* into cells conferred increased cell growth, while suppression of its expression with antisense S-oligonucleotides or small interfering RNA (siRNA) resulted in significant growth-inhibition of cancerous cells. These findings suggest that CXADRL1, GCUD1, and RNF43 render oncogenic activities to cancer cells, and that inhibition of the activity of these proteins could be a promising strategy for the treatment of cancer.

The present invention encompasses novel human gene *CXADRL1*, including a polynucleotide sequence as described in SEQ ID NO: 1, as well as degenerates and mutants thereof, to the extent that they encode a CXADRL1 protein, including the amino acid sequence set forth in SEQ ID NO: 2 or its functional equivalent. Examples of polypeptides functionally equivalent to CXADRL1 include, for example, homologous proteins of other organisms corresponding to the human CXADRL1 protein, as well as mutants of human CXADRL1 proteins.

The present invention also encompasses novel human gene *GCUD1*, including a polynucleotide sequence as described in SEQ ID NO: 3, as well as degenerates and mutants thereof, to the extent that they encode a GCUD1 protein, including the amino acid sequence set forth in SEQ ID NO: 4 or its functional equivalent. Examples of polypeptides functionally equivalent to GCUD1 include, for example, homologous proteins of other organisms corresponding to the human GCUD1 protein, as well as mutants of human GCUD1 proteins.

Furthermore, the present invention encompasses novel human gene *RNF43*,

including a polynucleotide sequence as described in SEQ ID NO: 5, as well as degenerates and mutants thereof, to the extent that they encode a RNF43 protein, including the amino acid sequence set forth in SEQ ID NO: 6 or its functional equivalent. Examples of polypeptides functionally equivalent to RNF43 include, for example, homologous proteins of other organisms corresponding to the human RNF43 protein, as well as mutants of human RNF43 proteins.

In the present invention, the term "functionally equivalent" means that the subject polypeptide has the activity to promote cell proliferation like CXADRL1, GCUD1, or RNF43 protein and to confer oncogenic activity to cancer cells. Whether the subject polypeptide has a cell proliferation activity or not can be judged by introducing the DNA encoding the subject polypeptide into a cell expressing the respective polypeptide, and detecting promotion of proliferation of the cells or increase in colony forming activity. Such cells include, for example, NIH3T3 cells for CXADRL1 and GCUD1; and NIH3T3 cells, SW480 cells, and COS7 cells for RNF43. Alternatively, whether the subject polypeptide is functionally equivalent to CXADRL1 may be judged by detecting its binding ability to AIP1. Furthermore, whether the subject polypeptide is functionally equivalent to RNF43 may be judged by detecting its binding ability to NOTCH2 or STRIN.

Methods for preparing polypeptides functionally equivalent to a given protein are well known by a person skilled in the art and include known methods of introducing mutations into the protein. For example, one skilled in the art can prepare polypeptides functionally equivalent to the human CXADRL1, GCUD1, or RNF43 protein by introducing an appropriate mutation in the amino acid sequence of either of these proteins by site-directed mutagenesis (Hashimoto-Gotoh et al., Gene 152:271-5 (1995); Zoller and Smith, Methods Enzymol 100: 468-500 (1983); Kramer et al., Nucleic Acids Res. 12:9441-9456 (1984); Kramer and Fritz, Methods Enzymol 154: 350-67 (1987); Kunkel, Proc Natl Acad Sci USA 82: 488-92 (1985); Kunkel, Methods Enzymol 85: 2763-6 (1988)). Amino acid mutations can occur in nature, too. The polypeptide of the present invention includes those proteins having the amino acid sequences of the human CXADRL1, GCUD1, or RNF43 protein in which one or more amino acids are mutated, provided the resulting mutated polypeptides are functionally equivalent to the human CXADRL1, GCUD1, or RNF43 protein. The number of amino acids to be mutated in such a mutant is generally 10 amino acids or less, preferably 6 amino acids or less, and more preferably 3 amino acids or less.

Mutated or modified proteins, proteins having amino acid sequences modified by substituting, deleting, inserting, and/or adding one or more amino acid residues of a certain

amino acid sequence, have been known to retain the original biological activity (Mark et al., Proc Natl Acad Sci USA 81: 5662-6 (1984); Zoller and Smith, Nucleic Acids Res 10:6487-500 (1982); Dalbadie-McFarland et al., Proc Natl Acad Sci USA 79: 6409-13 (1982)).

The amino acid residue to be mutated is preferably mutated into a different amino acid in which the properties of the amino acid side-chain are conserved (a process known as conservative amino acid substitution). Examples of properties of amino acid side chains are hydrophobic amino acids (A, I, L, M, F, P, W, Y, V), hydrophilic amino acids (R, D, N, C, E, Q, G, H, K, S, T), and side chains having the following functional groups or characteristics in common: an aliphatic side-chain (G, A, V, L, I, P); a hydroxyl group containing side-chain (S, T, Y); a sulfur atom containing side-chain (C, M); a carboxylic acid and amide containing side-chain (D, N, E, Q); a base containing side-chain (R, K, H); and an aromatic containing side-chain (H, F, Y, W). Note, the parenthetic letters indicate the one-letter codes of amino acids.

An example of a polypeptide to which one or more amino acids residues are added to the amino acid sequence of human CXADRL1, GCUD1, or RNF43 protein is a fusion protein containing the human CXADRL1, GCUD1, or RNF43 protein. Fusion proteins are, fusions of the human CXADRL1, GCUD1, or RNF43 protein and other peptides or proteins, and are included in the present invention. Fusion proteins can be made by techniques well known to a person skilled in the art, such as by linking the DNA encoding the human CXADRL1, GCUD1, or RNF43 protein of the invention with DNA encoding other peptides or proteins, so that the frames match, inserting the fusion DNA into an expression vector and expressing it in a host. There is no restriction as to the peptides or proteins fused to the protein of the present invention.

Known peptides that can be used as peptides that are fused to the protein of the present invention include, for example, FLAG (Hopp et al., Biotechnology 6: 1204-10 (1988)), 6xHis containing six His (histidine) residues, 10xHis, Influenza agglutinin (HA), human c-myc fragment, VSP-GP fragment, p18HIV fragment, T7-tag, HSV-tag, E-tag, SV40T antigen fragment, lck tag, α-tubulin fragment, B-tag, Protein C fragment, and the like. Examples of proteins that may be fused to a protein of the invention include GST (glutathione-S-transferase), Influenza agglutinin (HA), immunoglobulin constant region, β-galactosidase, MBP (maltose-binding protein), and such.

Fusion proteins can be prepared by fusing commercially available DNA, encoding the fusion peptides or proteins discussed above, with the DNA encoding the polypeptide of the present invention and expressing the fused DNA prepared.

An alternative method known in the art to isolate functionally equivalent

polypeptides is, for example, the method using a hybridization technique (Sambrook et al., Molecular Cloning 2nd ed. 9.47-9.58, Cold Spring Harbor Lab. Press (1989)). One skilled in the art can readily isolate a DNA having high homology with a whole or part of the DNA sequence encoding the human CXADRL1, GCUD1, or RNF43 protein (i.e., SEQ ID NO: 1, 3, or 5), and isolate functionally equivalent polypeptides to the human CXADRL1, GCUD1, or RNF43 protein from the isolated DNA. The polypeptides of the present invention include those that are encoded by DNA that hybridize with a whole or part of the DNA sequence encoding the human CXADRL1, GCUD1, or RNF43 protein and are functionally equivalent to the human CXADRL1, GCUD1, or RNF43 protein. These polypeptides include mammal homologues corresponding to the protein derived from human (for example, a polypeptide encoded by a monkey, rat, rabbit and bovine gene). In isolating a cDNA highly homologous to the DNA encoding the human CXADRL1 protein from animals, it is particularly preferable to use tissues from testis or ovary. Alternatively, in isolating a cDNA highly homologous to the DNA encoding the human GCUD1 from animals, it is particularly preferable to use tissues from testis, ovary, or brain. Further, in isolating a cDNA highly homologous to the DNA encoding the human RNF43 protein from animals, it is particularly preferable to use tissue from fetal lung or fetal kidney.

The condition of hybridization for isolating a DNA encoding a polypeptide functionally equivalent to the human CXADRL1, GCUD1, or RNF43 protein can be routinely selected by a person skilled in the art. For example, hybridization may be performed by conducting prehybridization at 68°C for 30 min or longer using "Rapid-hyb buffer" (Amersham LIFE SCIENCE), adding a labeled probe, and warming at 68°C for 1 hour or longer. The following washing step can be conducted, for example, in a low stringent condition. A low stringent condition is, for example, 42°C, 2X SSC, 0.1% SDS, or preferably 50°C, 2X SSC, 0.1% SDS. More preferably, high stringent conditions are used. A high stringent condition is, for example, washing 3 times in 2X SSC, 0.01% SDS at room temperature for 20 min, then washing 3 times in 1x SSC, 0.1% SDS at 37°C for 20 min, and washing twice in 1x SSC, 0.1% SDS at 50°C for 20 min. However, several factors, such as temperature and salt concentration, can influence the stringency of hybridization and one skilled in the art can suitably select the factors to achieve the requisite stringency.

In place of hybridization, a gene amplification method, for example, the polymerase chain reaction (PCR) method, can be utilized to isolate a DNA encoding a polypeptide functionally equivalent to the human CXADRL1, GCUD1, or RNF43 protein, using a primer synthesized based on the sequence information of the protein encoding

DNA (SEQ ID NO: 1, 3, or 5).

Polypeptides that are functionally equivalent to the human CXADRL1, GCUD1, or RNF43 protein encoded by the DNA isolated through the above hybridization techniques or gene amplification techniques, normally have a high homology to the amino acid sequence of the human CXADRL1, GCUD1, or RNF43 protein. "High homology" typically refers to a homology of 40% or higher, preferably 60% or higher, more preferably 80% or higher, even more preferably 95% or higher. The homology of a polypeptide can be determined by following the algorithm in "Wilbur and Lipman, Proc Natl Acad Sci USA 80: 726-30 (1983)".

A polypeptide of the present invention may have variations in amino acid sequence, molecular weight, isoelectric point, the presence or absence of sugar chains, or form, depending on the cell or host used to produce it or the purification method utilized. Nevertheless, so long as it has a function equivalent to that of the human CXADRL1, GCUD1, or RNF43 protein of the present invention, it is within the scope of the present invention.

The polypeptides of the present invention can be prepared as recombinant proteins or natural proteins, by methods well known to those skilled in the art. A recombinant protein can be prepared by inserting a DNA, which encodes the polypeptide of the present invention (for example, the DNA comprising the nucleotide sequence of SEQ ID NO: 1, 3, or 5), into an appropriate expression vector, introducing the vector into an appropriate host cell, obtaining the extract, and purifying the polypeptide by subjecting the extract to chromatography, for example, ion exchange chromatography, reverse phase chromatography, gel filtration, or affinity chromatography utilizing a column to which antibodies against the protein of the present invention is fixed, or by combining more than one of aforementioned columns.

Also when the polypeptide of the present invention is expressed within host cells (for example, animal cells and *E. coli*) as a fusion protein with glutathione-S-transferase protein or as a recombinant protein supplemented with multiple histidines, the expressed recombinant protein can be purified using a glutathione column or nickel column. Alternatively, when the polypeptide of the present invention is expressed as a protein tagged with c-myc, multiple histidines, or FLAG, it can be detected and purified using antibodies to c-myc, His, or FLAG, respectively.

After purifying the fusion protein, it is also possible to exclude regions other than the objective polypeptide by cutting with thrombin or factor-Xa as required.

A natural protein can be isolated by methods known to a person skilled in the art, for example, by contacting the affinity column, in which antibodies binding to the

CXADRL1, GCUD1, or RNF43 protein described below are bound, with the extract of tissues or cells expressing the polypeptide of the present invention. The antibodies can be polyclonal antibodies or monoclonal antibodies.

The present invention also encompasses partial peptides of the polypeptide of the present invention. The partial peptide has an amino acid sequence specific to the polypeptide of the present invention and consists of at least 7 amino acids, preferably 8 amino acids or more, and more preferably 9 amino acids or more. The partial peptide can be used, for example, for preparing antibodies against the polypeptide of the present invention, screening for a compound that binds to the polypeptide of the present invention, and screening for accelerators or inhibitors of the polypeptide of the present invention.

A partial peptide of the invention can be produced by genetic engineering, by known methods of peptide synthesis, or by digesting the polypeptide of the invention with an appropriate peptidase. For peptide synthesis, for example, solid phase synthesis or liquid phase synthesis may be used.

Furthermore, the present invention provides polynucleotides encoding the polypeptide of the present invention. The polynucleotides of the present invention can be used for the *in vivo* or *in vitro* production of the polypeptide of the present invention as described above, or can be applied to gene therapy for diseases attributed to genetic abnormality in the gene encoding the protein of the present invention. Any form of the polynucleotide of the present invention can be used so long as it encodes the polypeptide of the present invention, including mRNA, RNA, cDNA, genomic DNA, chemically synthesized polynucleotides. The polynucleotide of the present invention include a DNA comprising a given nucleotide sequences as well as its degenerate sequences, so long as the resulting DNA encodes a polypeptide of the present invention.

The polynucleotide of the present invention can be prepared by methods known to a person skilled in the art. For example, the polynucleotide of the present invention can be prepared by: preparing a cDNA library from cells which express the polypeptide of the present invention, and conducting hybridization using a partial sequence of the DNA of the present invention (for example, SEQ ID NO: 1, 3, or 5) as a probe. A cDNA library can be prepared, for example, by the method described in Sambrook et al., Molecular Cloning, Cold Spring Harbor Laboratory Press (1989); alternatively, commercially available cDNA libraries may be used. A cDNA library can be also prepared by: extracting RNAs from cells expressing the polypeptide of the present invention, synthesizing oligo DNAs based on the sequence of the DNA of the present invention (for example, SEQ ID NO: 1, 3, or 5), conducting PCR using the oligo DNAs as primers, and amplifying cDNAs encoding the protein of the present invention.

In addition, by sequencing the nucleotides of the obtained cDNA, the translation region encoded by the cDNA can be routinely determined, and the amino acid sequence of the polypeptide of the present invention can be easily obtained. Moreover, by screening the genomic DNA library using the obtained cDNA or parts thereof as a probe, the genomic DNA can be isolated.

More specifically, mRNAs may first be prepared from a cell, tissue, or organ (e.g., testis or ovary for *CXADRL1*; testis, ovary, or brain for *GCUD1*; and fetal lung, or fetal kidney for *RNF43*) in which the object polypeptide of the invention is expressed. Known methods can be used to isolate mRNAs; for instance, total RNA may be prepared by guanidine ultracentrifugation (Chirgwin et al., Biochemistry 18:5294-9 (1979)) or AGPC method (Chomczynski and Sacchi, Anal Biochem 162:156-9 (1987)). In addition, mRNA may be purified from total RNA using mRNA Purification Kit (Pharmacia) and such or, alternatively, mRNA may be directly purified by QuickPrep mRNA Purification Kit (Pharmacia).

The obtained mRNA is used to synthesize cDNA using reverse transcriptase. cDNA may be synthesized using a commercially available kit, such as the AMV Reverse Transcriptase First-strand cDNA Synthesis Kit (Seikagaku Kogyo). Alternatively, cDNA may be synthesized and amplified following the 5'-RACE method (Frohman et al., Proc Natl Acad Sci USA 85: 8998-9002 (1988); Belyavsky et al., Nucleic Acids Res 17: 2919-32 (1989)), which uses a primer and such, described herein, the 5'-Ampli FINDER RACE Kit (Clontech), and polymerase chain reaction (PCR).

A desired DNA fragment is prepared from the PCR products and ligated with a vector DNA. The recombinant vectors are used to transform *E. coli* and such, and a desired recombinant vector is prepared from a selected colony. The nucleotide sequence of the desired DNA can be verified by conventional methods, such as dideoxynucleotide chain termination.

The nucleotide sequence of a polynucleotide of the invention may be designed to be expressed more efficiently by taking into account the frequency of codon usage in the host to be used for expression (Grantham et al., Nucleic Acids Res 9: 43-74 (1981)). The sequence of the polynucleotide of the present invention may be altered by a commercially available kit or a conventional method. For instance, the sequence may be altered by digestion with restriction enzymes, insertion of a synthetic oligonucleotide or an appropriate polynucleotide fragment, addition of a linker, or insertion of the initiation codon (ATG) and/or the stop codon (TAA, TGA, or TAG).

Specifically, the polynucleotide of the present invention encompasses the DNA comprising the nucleotide sequence of SEQ ID NO: 1, 3, or 5.

Furthermore, the present invention provides a polynucleotide that hybridizes under stringent conditions with a polynucleotide having a nucleotide sequence of SEQ ID NO: 1, 3, or 5, and encodes a polypeptide functionally equivalent to the CXADRL1, GCUD1, or RNF43 protein of the invention described above. One skilled in the art may appropriately choose stringent conditions. For example, low stringent condition can be used. More preferably, high stringent condition can be used. These conditions are the same as that described above. The hybridizing DNA above is preferably a cDNA or a chromosomal DNA.

The present invention also provides a vector into which a polynucleotide of the present invention is inserted. A vector of the present invention is useful to keep a polynucleotide, especially a DNA, of the present invention in host cell, to express the polypeptide of the present invention, or to administer the polynucleotide of the present invention for gene therapy.

When *E. coli* is a host cell and the vector is amplified and produced in a large amount in *E. coli* (e.g., JM109, DH5a, HB101, or XL1Blue), the vector should have "ori" to be amplified in *E. coli* and a marker gene for selecting transformed *E. coli* (e.g., a drug-resistance gene selected by a drug such as ampicillin, tetracycline, kanamycin, chloramphenicol or the like). For example, M13-series vectors, pUC-series vectors, pBR322, pBluescript, pCR-Script, etc. can be used. In addition, pGEM-T, pDIRECT, and pT7 can also be used for subcloning and extracting cDNA as well as the vectors described above. When a vector is used to produce the protein of the present invention, an expression vector is especially useful. For example, an expression vector to be expressed in *E. coli* should have the above characteristics to be amplified in *E. coli*. When *E. coli*, such as JM109, DH5a, HB101, or XL1 Blue, are used as a host cell, the vector should have a promoter, for example, lacZ promoter (Ward et al., Nature 341: 544-6 (1989); FASEB J 6: 2422-7 (1992)), araB promoter (Better et al., Science 240: 1041-3 (1988)), or T7 promoter or the like, that can efficiently express the desired gene in *E. coli*. In that respect, pGEX-5X-1 (Pharmacia), "QIAexpress system" (Qiagen), pEGFP and pET (in this case, the host is preferably BL21 which expresses T7 RNA polymerase), for example, can be used instead of the above vectors. Additionally, the vector may also contain a signal sequence for polypeptide secretion. An exemplary signal sequence that directs the polypeptide to be secreted to the periplasm of the *E. coli* is the pelB signal sequence (Lei et al., J Bacteriol 169: 4379 (1987)). Means for introducing of the vectors into the target host cells include, for example, the calcium chloride method, and the electroporation method.

In addition to *E. coli*, for example, expression vectors derived from mammals (for

example, pcDNA3 (Invitrogen) and pEGF-BOS (Nucleic Acids Res 18(17): 5322 (1990)), pEF, pCDM8), expression vectors derived from insect cells (for example, "Bac-to-BAC baculovirus expression system" (GIBCO BRL), pBacPAK8), expression vectors derived from plants (e.g., pMH1, pMH2), expression vectors derived from animal viruses (e.g., pHSV, pMV, pAdexLcw), expression vectors derived from retroviruses (e.g., pZIpneo), expression vector derived from yeast (e.g., "Pichia Expression Kit" (Invitrogen), pNV11, SP-Q01), and expression vectors derived from *Bacillus subtilis* (e.g., pPL608, pKTH50) can be used for producing the polypeptide of the present invention.

In order to express the vector in animal cells, such as CHO, COS, or NIH3T3 cells, the vector should have a promoter necessary for expression in such cells, for example, the SV40 promoter (Mulligan et al., Nature 277: 108 (1979)), the MMLV-LTR promoter, the EF1a promoter (Mizushima et al., Nucleic Acids Res 18: 5322 (1990)), the CMV promoter, and the like, and preferably a marker gene for selecting transformants (for example, a drug resistance gene selected by a drug (e.g., neomycin, G418)). Examples of known vectors with these characteristics include, for example, pMAM, pDR2, pBK-RSV, pBK-CMV, pOPRSV, and pOP13.

In addition, methods may be used to express a gene stably and, at the same time, to amplify the copy number of the gene in cells. For example, a vector comprising the complementary DHFR gene (e.g., pCHO I) may be introduced into CHO cells in which the nucleic acid synthesizing pathway is deleted, and then amplified by methotrexate (MTX). Furthermore, in case of transient expression of a gene, the method wherein a vector comprising a replication origin of SV40 (pcD, etc.) is transformed into COS cells comprising the SV40 T antigen expressing gene on the chromosome can be used.

A polypeptide of the present invention obtained as above may be isolated from inside or outside (such as medium) of host cells, and purified as a substantially pure homogeneous polypeptide. The term "substantially pure" as used herein in reference to a given polypeptide means that the polypeptide is substantially free from other biological macromolecules. The substantially pure polypeptide is at least 75% (e.g., at least 80, 85, 95, or 99%) pure by dry weight. Purity can be measured by any appropriate standard method, for example by column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis. The method for polypeptide isolation and purification is not limited to any specific method; in fact, any standard method may be used.

For instance, column chromatography, filter, ultrafiltration, salt precipitation, solvent precipitation, solvent extraction, distillation, immunoprecipitation, SDS-polyacrylamide gel electrophoresis, isoelectric point electrophoresis, dialysis, and recrystallization may be appropriately selected and combined to isolate and purify the

polypeptide.

Examples of chromatography include, for example, affinity chromatography, ion-exchange chromatography, hydrophobic chromatography, gel filtration, reverse phase chromatography, adsorption chromatography, and such (Strategies for Protein Purification and Characterization: A Laboratory Course Manual. Ed. Daniel R. Marshak et al., Cold Spring Harbor Laboratory Press (1996)). These chromatographies may be performed by liquid chromatography, such as HPLC and FPLC. Thus, the present invention provides for highly purified polypeptides prepared by the above methods.

A polypeptide of the present invention may be optionally modified or partially deleted by treating it with an appropriate protein modification enzyme before or after purification. Useful protein modification enzymes include, but are not limited to, trypsin, chymotrypsin, lysylendopeptidase, protein kinase, glucosidase, and so on.

The present invention provides an antibody that binds to the polypeptide of the invention. The antibody of the invention can be used in any form, such as monoclonal or polyclonal antibodies, and includes antiserum obtained by immunizing an animal such as a rabbit with the polypeptide of the invention, all classes of polyclonal and monoclonal antibodies, human antibodies, and humanized antibodies produced by genetic recombination.

A polypeptide of the invention used as an antigen to obtain an antibody may be derived from any animal species, but preferably is derived from a mammal such as a human, mouse, or rat, more preferably from a human. A human-derived polypeptide may be obtained from the nucleotide or amino acid sequences disclosed herein.
According to the present invention, the polypeptide to be used as an immunization antigen may be a complete protein or a partial peptide of the protein. A partial peptide may comprise, for example, the amino (N)-terminal or carboxy (C)-terminal fragment of a polypeptide of the present invention. More specifically, a polypeptide of CXADRL1 encompassing the codons from 235 to 276, from 493 to 537, or from 70 to 111 can be used as partial peptides for producing antibodies against CXADRL1 of the present invention.

Alternatively, for the production of antibodies against the polypeptide of the present invention, peptides comprising any one of following amino acid sequences may be used.

- RNF43 ; SEQ ID No: 80, 97, or 108
- CXADRL1 ; SEQ ID No: 124
- GCUD1 ; SEQ ID No: 164

Herein, an antibody is defined as a protein that reacts with either the full length or a fragment of a polypeptide of the present invention.

A gene encoding a polypeptide of the invention or its fragment may be inserted into

a known expression vector, which is then used to transform a host cell as described herein. The desired polypeptide or its fragment may be recovered from the outside or inside of host cells by any standard method, and may subsequently be used as an antigen. Alternatively, whole cells expressing the polypeptide or their lysates, or a chemically synthesized polypeptide may be used as the antigen.

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

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

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

To prepare monoclonal antibodies, immune cells are collected from the mammal immunized with the antigen and checked for the increased level of desired antibodies in the serum as described above, and are subjected to cell fusion. The immune cells used for cell fusion are preferably obtained from spleen. Other preferred parental cells to be fused with the above immunocyte include, for example, myeloma cells of mammalians, and

more preferably myeloma cells having an acquired property for the selection of fused cells by drugs.

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

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

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

The obtained hybridomas are subsequently transplanted into the abdominal cavity of a mouse and the ascites are extracted. The obtained monoclonal antibodies can be purified by, for example, ammonium sulfate precipitation, a protein A or protein G column, DEAE ion exchange chromatography, or an affinity column to which the polypeptide of the present invention is coupled. The antibody of the present invention can be used not only for purification and detection of the polypeptide of the present invention, but also as a candidate for agonists and antagonists of the polypeptide of the present invention. In addition, this antibody can be applied to the antibody treatment for diseases related to the polypeptide of the present invention. 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.

For example, transgenic animals having a repertory of human antibody genes may be immunized with an antigen selected from a polypeptide, polypeptide expressing cells, or their lysates. Antibody producing cells are then collected from the animals and fused with myeloma cells to obtain hybridoma, from which human antibodies against the polypeptide can be prepared (see WO92-03918, WO93-2227, WO94-02602, WO94-25585, WO96-33735, and WO96-34096).

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

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

Furthermore, an antibody of the present invention may be a fragment of an antibody or modified antibody, so long as it binds to one or more of the polypeptides of the invention. 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 et al., Proc Natl Acad Sci USA 85: 5879-83 (1988)). More specifically, an antibody fragment may be generated by treating an antibody with an enzyme, such as papain or pepsin. Alternatively, a gene encoding the antibody fragment may be constructed, inserted into an expression vector, and expressed in an appropriate host cell (see, for example, Co et al., J Immunol 152: 2968-76 (1994); Better and Horwitz, Methods Enzymol 178: 476-96 (1989); Pluckthun and Skerra, Methods Enzymol 178: 497-515 (1989); Lamoyi, Methods Enzymol 121: 652-63 (1986); Rousseaux et al., Methods Enzymol 121: 663-9 (1986); Bird and Walker, Trends Biotechnol 9: 132-7 (1991)).

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

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

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.

For example, measurement of absorbance, enzyme-linked immunosorbent assay (ELISA), enzyme immunoassay (EIA), radioimmunoassay (RIA), and/or immunofluorescence may be used to measure the antigen binding activity of the antibody of the invention. In ELISA, the antibody of the present invention is immobilized on a plate, a polypeptide of the invention is applied to the plate, and then a sample containing a desired antibody, such as culture supernatant of antibody producing cells or purified antibodies, is applied. Then, a secondary antibody that recognizes the primary antibody and is labeled with an enzyme, such as alkaline phosphatase, is applied, and the plate is incubated. Next, after washing, an enzyme substrate, such as *p*-nitrophenyl phosphate, is added to the plate, and the absorbance is measured to evaluate the antigen binding activity of the sample. A fragment of the polypeptide, such as a C-terminal or N-terminal fragment, may be used as the antigen to evaluate the binding activity of the antibody. BIAcore (Pharmacia) may be used to evaluate the activity of the antibody according to the present invention.

The above methods allow for the detection or measurement of the polypeptide of the invention, by exposing the antibody of the invention to a sample assumed to contain the polypeptide of the invention, and detecting or measuring the immune complex formed by the antibody and the polypeptide.

Because the method of detection or measurement of the polypeptide according to the invention can specifically detect or measure a polypeptide, the method may be useful in a variety of experiments in which the polypeptide is used.

The present invention also provides a polynucleotide which hybridizes with the polynucleotide encoding human CXADRL1, GCUD1, or RNF43 protein (SEQ ID NO: 1, 3, or 5) or the complementary strand thereof, and which comprises at least 15 nucleotides.

The polynucleotide of the present invention is preferably a polynucleotide which specifically hybridizes with the DNA encoding the polypeptide of the present invention. The term "specifically hybridize" as used herein, means that cross-hybridization does not occur significantly with DNA encoding other proteins, under the usual hybridizing conditions, preferably under stringent hybridizing conditions. Such polynucleotides include, probes, primers, nucleotides and nucleotide derivatives (for example, antisense oligonucleotides and ribozymes), which specifically hybridize with DNA encoding the polypeptide of the invention or its complementary strand. Moreover, such polynucleotide can be utilized for the preparation of DNA chip.

The present invention includes an antisense oligonucleotide that hybridizes with any site within the nucleotide sequence of SEQ ID NO: 1, 3, or 5. This antisense oligonucleotide is preferably against at least 15 continuous nucleotides of the nucleotide sequence of SEQ ID NO: 1, 3, or 5. The above-mentioned antisense oligonucleotide, which contains an initiation codon in the above-mentioned at least 15 continuous nucleotides, is even more preferred. More specifically, such antisense oligonucleotides include those comprising the nucleotide sequence of SEQ ID NO: 23 or 25 for suppressing the expression of CXADRL1; SEQ ID NO: 27, or 29 for GCUD1; and SEQ ID NO: 31 for RNF43.

Derivatives or modified products of antisense oligonucleotides can be used as antisense oligonucleotides. Examples of such modified products include lower alkyl phosphonate modifications such as methyl-phosphonate-type or ethyl-phosphonate-type, phosphorothioate modifications and phosphoroamidate modifications.

The term "antisense oligonucleotides" as used herein means, not only those in which the nucleotides corresponding to those constituting a specified region of a DNA or mRNA are entirely complementary, but also those having a mismatch of one or more nucleotides, as long as the DNA or mRNA and the antisense oligonucleotide can specifically hybridize with the nucleotide sequence of SEQ ID NO: 1, 3, or 5.

Such polynucleotides are contained as those having, in the "at least 15 continuous nucleotide sequence region", a homology of at least 70% or higher, preferably at 80% or higher, more preferably 90% or higher, even more preferably 95% or higher. The algorithm stated herein can be used to determine the homology. Such polynucleotides are useful as probes for the isolation or detection of DNA encoding the polypeptide of the invention as stated in a later example or as a primer used for amplifications.

The antisense oligonucleotide derivatives of the present invention act upon cells producing the polypeptide of the invention by binding to the DNA or mRNA encoding the polypeptide, inhibiting its transcription or translation, promoting the degradation of the

mRNA, and inhibiting the expression of the polypeptide of the invention, thereby resulting in the inhibition of the polypeptide's function.

An antisense oligonucleotide derivative of the present invention can be made into an external preparation, such as a liniment or a poultice, by mixing with a suitable base material which is inactive against the derivatives.

Also, as needed, the derivatives 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. These can be prepared by following usual methods.

The antisense oligonucleotide derivative is given to the patient by directly applying onto the ailing site or by injecting into a blood vessel so that it will reach the site of ailment. An antisense-mounting medium can also be used to increase durability and membrane-permeability. Examples are, liposome, poly-L-lysine, lipid, cholesterol, lipofectin or derivatives of these.

The dosage of the antisense oligonucleotide derivative 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.

The present invention also includes small interfering RNAs (siRNA) comprising a combination of a sense strand nucleic acid and an antisense strand nucleic acid of the nucleotide sequence of SEQ ID NO: 1, 3, or 5. More specifically, such siRNA for suppressing the expression of RNF43 include those whose sense strand comprises the nucleotide sequence of SEQ ID NO: 40, 41, 42, or 43. Alternatively, siRNA for suppressing the expression of CXADRL1 include those whose sense strand comprises the nucleotide sequence of SEQ ID NO: 62, or 63.

The term "siRNA" refers to a double stranded RNA molecule which prevents translation of a target mRNA. Standard techniques are used for introducing siRNA into cells, including those wherein DNA is used as the template to transcribe RNA. The siRNA comprises a sense nucleic acid sequence and an anti-sense nucleic acid sequence of the polynucleotide encoding human CXADRL1, GCUD1, or RNF43 protein (SEQ ID NO: 1, 3, or 5). The siRNA is constructed such that a single transcript (double stranded RNA) has both the sense and complementary antisense sequences from the target gene, *e.g.,* a hairpin.

The method is used to alter gene expression of a cell, i.e., up-regulate the expression of CXADRL1, GCUD1, or RNF43, *e.g.,* as a result of malignant transformation of the cells. Binding of the siRNA to CXADRL1, GCUD1, or RNF43 transcript in the

target cell results in a reduction of protein production by the cell. The length of the oligonucleotide is at least 10 nucleotides and may be as long as the naturally occurring the transcript. Preferably, the oligonucleotide is19-25 nucleotides in length. Most preferably, the oligonucleotide is less than 75, 50, 25 nucleotides in length. Examples of CXADRL1, GCUD1, or RNF43 siRNA oligonucleotides which inhibit the expression in mammalian cells include oligonucleotides containing any of SEQ ID NO: 112-114. These sequences are target sequence of the following siRNA sequences respectively.

SEQ ID NO: 112, SEQ ID NOs: 40, 41 (RNF43);

SEQ ID NO: 113, SEQ ID NOs: 42, 43 (RNF43); and

SEQ ID NO: 114, SEQ ID NOs: 62, 63 (CXADRL1).

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

Selection of siRNA Target Sites:

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

The antisense oligonucleotide or siRNA of the invention inhibit the expression of the polypeptide of the invention and is thereby useful for suppressing the biological activity of the polypeptide of the invention. Also, expression-inhibitors, comprising the antisense oligonucleotide or siRNA of the invention, are useful in the point that they can inhibit the biological activity of the polypeptide of the invention. Therefore, a composition comprising the antisense oligonucleotide or siRNA of the present invention is useful in treating a cell proliferative disease such as cancer.

Moreover, the present invention provides a method for diagnosing a cell

proliferative disease using the expression level of the polypeptides of the present invention as a diagnostic marker.

This diagnosing method comprises the steps of: (a) detecting the expression level of the *CXADRL1*, *GCUD1*, or *RNF43* gene of the present invention; and (b) relating an elevation of the expression level to the cell proliferative disease, such as cancer.

The expression levels of the the *CXADRL1*, *GCUD1*, or *RNF43* gene in a particular specimen can be estimated by quantifying mRNA corresponding to or protein encoded by the *CXADRL1*, *GCUD1*, or *RNF43* gene. Quantification methods for mRNA are known to those skilled in the art. For example, the levels of mRNAs corresponding to the *CXADRL1*, *GCUD1*, or *RNF43* gene can be estimated by Northern blotting or RT-PCR. Since the full-length nucleotide sequences of the *CXADRL1*, *GCUD1*, or *RNF43* genes are shown in SEQ ID NO: 1, 3, or 5, anyone skilled in the art can design the nucleotide sequences for probes or primers to quantify the *CXADRL1*, *GCUD1*, or *RNF43* gene.

Also the expression level of the *CXADRL1*, *GCUD1*, or *RNF43* gene can be analyzed based on the ctivity or quantity of protein encoded by the gene. A method for determining the quantity of the CXADRL1, GCUD1, or RNF43 protein is shown in bellow. For example, immunoassay method is useful for the determination of the proteins in biological materials. Any biological materials can be used for the determination of the protein or it's activity. For example, blood sample is analyzed for estimation of the protein encoded by a serum marker. On the other hand, a suitable method can be selected for the determination of the activity of a protein encoded by the *CXADRL1*, *GCUD1*, or *RNF43* gene according to the activity of each protein to be analyzed.

Expression levels of the *CXADRL1*, *GCUD1*, or *RNF43* gene in a specimen (test sample) are estimated and compared with those in a normal sample. When such a comparison shows that the expression level of the target gene is higher than those in the normal sample, the subject is judged to be affected with a cell proliferative disease. The expression level of *CXADRL1*, *GCUD1*, or *RNF43* gene in the specimens from the normal sample and subject may be determined at the same time. Alternatively, normal ranges of the expression levels can be determined by a statistical method based on the results obtained by analyzing the expression level of the gene in specimens previously collected from a control group. A result obtained by comparing the sample of a subject is compared with the normal range; when the result does not fall within the normal range, the subject is judged to be affected with the cell proliferative disease. In the present invention, the cell proliferative disease to be diagnosed is preferably cancer. More preferably, when the expression level of the *CXADRL1*, or *GCUD1* gene is estimated and compared with those in a normal sample, the cell proliferative disease to be diagnosed is

gastric, colorectal, or liver cancer; and when the *RNF43* gene is estimated for its expression level, then the disease to be diagnosed is colorectal, lung, gastric, or liver cancer.

In the present invention, a diagnostic agent for diagnosing cell proliferative disease, such as cancer including gastric, colorectal, lung, and liver cancers, is also provided. The diagnostic agent of the present invention comprises a compound that binds to a polynucleotide or a polypeptide of the present invention. Preferably, an oligonucleotide that hybridizes to the polynucleotide of the present invention, or an antibody that binds to the polypeptide of the present invention may be used as such a compound.

Moreover, the present invention provides a method of screening for a compound for treating a cell proliferative disease using the polypeptide of the present invention. An embodiment of this screening method comprises the steps of: (a) contacting a test compound with a polypeptide of the present invention, (b) detecting the binding activity between the polypeptide of the present invention and the test compound, and (c) selecting a compound that binds to the polypeptide of the present invention.

The polypeptide of the present invention to be used for screening may be a recombinant polypeptide or a protein derived from the nature, or a partial peptide thereof. Any test compound, 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 and natural compounds, can be used. The polypeptide of the present invention to be contacted with a test compound can be, for example, a purified polypeptide, a soluble protein, a form bound to a carrier, or a fusion protein fused with other polypeptides.

As a method of screening for proteins, for example, that bind to the polypeptide of the present invention using the polypeptide of the present invention, many methods well known by a person skilled in the art can be used. Such a screening can be conducted by, for example, immunoprecipitation method, specifically, in the following manner. The gene encoding the polypeptide of the present invention is expressed in animal cells and so on by inserting the gene to an expression vector for foreign genes, such as pSV2neo, pcDNA I, and pCD8. The promoter to be used for the expression may be any promoter that can be used commonly and include, for example, the SV40 early promoter (Rigby in Williamson (ed.), Genetic Engineering, vol. 3. Academic Press, London, 83-141 (1982)), the EF-1a promoter (Kim et al., Gene 91: 217-23 (1990)), the CAG promoter (Niwa et al., Gene 108: 193-200 (1991)), the RSV LTR promoter (Cullen, Methods in Enzymology 152: 684-704 (1987)) the SRa promoter (Takebe et al., Mol Cell Biol 8: 466 (1988)), the CMV immediate early promoter (Seed and Aruffo, Proc Natl Acad Sci USA 84: 3365-9 (1987)),

the SV40 late promoter (Gheysen and Fiers, J Mol Appl Genet 1: 385-94 (1982)), the Adenovirus late promoter (Kaufman et al., Mol Cell Biol 9: 946 (1989)), the HSV TK promoter, and so on. The introduction of the gene into animal cells to express a foreign gene can be performed according to any methods, for example, the electroporation method (Chu et al., Nucleic Acids Res 15: 1311-26 (1987)), the calcium phosphate method (Chen and Okayama, Mol Cell Biol 7: 2745-52 (1987)), the DEAE dextran method (Lopata et al., Nucleic Acids Res 12: 5707-17 (1984); Sussman and Milman, Mol Cell Biol 4: 1642-3 (1985)), the Lipofectin method (Derijard, B Cell 7: 1025-37 (1994); Lamb et al., Nature Genetics 5: 22-30 (1993): Rabindran et al., Science 259: 230-4 (1993)), and so on. The polypeptide of the present invention can be expressed as a fusion protein comprising a recognition site (epitope) of a monoclonal antibody by introducing the epitope of the monoclonal antibody, whose specificity has been revealed, to the N- or C- terminus of the polypeptide of the present invention. A commercially available epitope-antibody system can be used (Experimental Medicine 13: 85-90 (1995)). Vectors which can express a fusion protein with, for example, β-galactosidase, maltose binding protein, glutathione S-transferase, green florescence protein (GFP) and so on by the use of its multiple cloning sites are commercially available.

A fusion protein prepared by introducing only small epitopes consisting of several to a dozen amino acids so as not to change the property of the polypeptide of the present invention by the fusion is also reported. Epitopes, such as polyhistidine (His-tag), influenza aggregate HA, human c-myc, FLAG, Vesicular stomatitis virus glycoprotein (VSV-GP), T7 gene 10 protein (T7-tag), human simple herpes virus glycoprotein (HSV-tag), E-tag (an epitope on monoclonal phage), and such, and monoclonal antibodies recognizing them can be used as the epitope-antibody system for screening proteins binding to the polypeptide of the present invention (Experimental Medicine 13: 85-90 (1995)).

In immunoprecipitation, an immune complex is formed by adding these antibodies to cell lysate prepared using an appropriate detergent. The immune complex consists of the polypeptide of the present invention, a polypeptide comprising the binding ability with the polypeptide, and an antibody. Immunoprecipitation can be also conducted using antibodies against the polypeptide of the present invention, besides using antibodies against the above epitopes, which antibodies can be prepared as described above.

An immune complex can be precipitated, for example by Protein A sepharose or Protein G sepharose when the antibody is a mouse IgG antibody. If the polypeptide of the present invention is prepared as a fusion protein with an epitope, such as GST, an immune complex can be formed in the same manner as in the use of the antibody against the

polypeptide of the present invention, using a substance specifically binding to these epitopes, such as glutathione-Sepharose 4B.

Immunoprecipitation can be performed by following or according to, for example, the methods in the literature (Harlow and Lane, Antibodies, 511-52, Cold Spring Harbor Laboratory publications, New York (1988)).

SDS-PAGE is commonly used for analysis of immunoprecipitated proteins and the bound protein can be analyzed by the molecular weight of the protein using gels with an appropriate concentration. Since the protein bound to the polypeptide of the present invention is difficult to detect by a common staining method, such as Coomassie staining or silver staining, the detection sensitivity for the protein can be improved by culturing cells in culture medium containing radioactive isotope, ^{35}S-methionine or ^{35}S-cystein, labeling proteins in the cells, and detecting the proteins. The target protein can be purified directly from the SDS-polyacrylamide gel and its sequence can be determined, when the molecular weight of a protein has been revealed.

As a method for screening proteins binding to the polypeptide of the present invention using the polypeptide, for example, West-Western blotting analysis (Skolnik et al., Cell 65: 83-90 (1991)) can be used. Specifically, a protein binding to the polypeptide of the present invention can be obtained by preparing a cDNA library from cells, tissues, organs (for example, tissues such as testis and ovary for screening proteins binding to CXADRL1; testis, ovary, and brain for screening proteins binding to GCUD1; and fetal lung, and fetal kidney for those binding to RNF43), or cultured cells expected to express a protein binding to the polypeptide of the present invention using a phage vector (e.g., ZAP), expressing the protein on LB-agarose, fixing the protein expressed on a filter, reacting the purified and labeled polypeptide of the present invention with the above filter, and detecting the plaques expressing proteins bound to the polypeptide of the present invention according to the label. The polypeptide of the invention may be labeled by utilizing the binding between biotin and avidin, or by utilizing an antibody that specifically binds to the polypeptide of the present invention, or a peptide or polypeptide (for example, GST) that is fused to the polypeptide of the present invention. Methods using radioisotope or fluorescence and such may be also used.

Alternatively, in another embodiment of the screening method of the present invention, a two-hybrid system utilizing cells may be used ("MATCHMAKER Two-Hybrid system", "Mammalian MATCHMAKER Two-Hybrid Assay Kit", "MATCHMAKER one-Hybrid system" (Clontech); "HybriZAP Two-Hybrid Vector System" (Stratagene); the references "Dalton and Treisman, Cell 68: 597-612 (1992)", "Fields and Sternglanz, Trends Genet 10: 286-92 (1994)").

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

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

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

When the test compound is a protein, the amino acid sequence of the obtained protein is analyzed, an oligo DNA is synthesized based on the sequence, and cDNA libraries are screened using the oligo DNA as a probe to obtain a DNA encoding the protein.

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

The methods of screening for molecules that bind when the immobilized polypeptide of the present invention is exposed to synthetic chemical compounds, or natural substance banks, or a random phage peptide display library, and the methods of screening using high-throughput based on combinatorial chemistry techniques (Wrighton et al., Science 273: 458-64 (1996); Verdine, Nature 384: 11-13 (1996); Hogan, Nature 384: 17-9 (1996)) to isolate not only proteins but chemical compounds that bind to the protein

of the present invention (including agonist and antagonist) are well known to one skilled in the art.

Alternatively, the screening method of the present invention may comprise the following steps:

a) contacting a candidate compound with a cell into which a vector comprising the transcriptional regulatory region of one or more marker genes and a reporter gene that is expressed under the control of the transcriptional regulatory region has been introduced, wherein the one or more marker genes are selected from the group consisting of CXADRL1, GCUD1, and RNF43,

b) measuring the activity of said reporter gene; and

c) selecting a compound that reduces the expression level of said reporter gene as compared to a control.

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

A compound isolated by the screening is a candidate for drugs which promote or inhibit the activity of the polypeptide of the present invention, for treating or preventing diseases attributed to, for example, cell proliferative diseases, such as cancer. A compound in which a part of the structure of the compound obtained by the present screening method having the activity of binding to the polypeptide of the present invention is converted by addition, deletion and/or replacement, is included in the compounds obtained by the screening method of the present invention.

In a further embodiment, the present invention provides methods for screening candidate agents which are potential targets in the treatment of cell proliferative disease. As discussed in detail above, by controlling the expression levels of the CXADRL1, GCUD1, or RNF43, one can control the onset and progression of either gastric cancer, or colorectal, lung, gastric, or liver cancer. Thus, candidate agents, which are potential targets in the treatment of cell proliferative disease, can be identified through screenings that use the expression levels and activities of CXADRL1, GCUD1, or RNF43 as indices. In the context of the present invention, such screening may comprise, for example, the following steps:

a) contacting a candidate compound with a cell expressing the CXADRL1, GCUD1, or RNF43; and

b) selecting a compound that reduces the expression level of CXADRL1, GCUD1, or RNF43 in comparison with the expression level detected in the absence of the test compound.

Cells expressing at least one of the CXADRL1, GCUD1, or RNF43 include, for example, cell lines established from gastric, colorectal, lung, or liver cancers; such cells can be used for the above screening of the present invention. The expression level can be estimated by methods well known to one skilled in the art. In the method of screening, a compound that reduces the expression level of at least one of CXADRL1, GCUD1, or RNF43 can be selected as candidate agents.

In another embodiment of the method for screening a compound for treating a cell proliferative disease of the present invention, the method utilizes biological activity of the polypeptide of the present invention as an index. Since the CXADRL1, GCUD1, and RNF43 proteins of the present invention have the activity of promoting cell proliferation, a compound which promotes or inhibits this activity of one of these proteins of the present invention can be screened using this activity as an index. This screening method includes the steps of: (a) contacting a test compound with the polypeptide of the present invention; (b) detecting the biological activity of the polypeptide of step (a); and (c) selecting a compound that suppresses the biological activity of the polypeptide in comparison with the biological activity detected in the absence of the test compound.

Any polypeptides can be used for screening so long as they comprise the biological activity of the CXADRL1, GCUD1, or RNF43 protein. Such biological activity include cell-proliferating activity of the human CXADRL1, GCUD1, or RNF43 protein, the activity of RNF43 to bind to NOTCH2 or STRIN. For example, a human CXADRL1, GCUD1, or RNF43 protein can be used and polypeptides functionally equivalent to these proteins can also be used. Such polypeptides may be expressed endogenously or exogenously by cells.

Any test compounds, for example, cell extracts, cell culture supernatant, products of fermenting microorganism, extracts of marine organism, plant extracts, purified or crude proteins, peptides, non-peptide compounds, synthetic micromolecular compounds, natural compounds, can be used.

The compound isolated by this screening is a candidate for agonists or antagonists of the polypeptide of the present invention. The term "agonist" refers to molecules that activate the function of the polypeptide of the present invention by binding thereto. Likewise, the term "antagonist" refers to molecules that inhibit the function of the

polypeptide of the present invention by binding thereto. Moreover, a compound isolated by this screening is a candidate for compounds which inhibit the *in vivo* interaction of the polypeptide of the present invention with molecules (including DNAs and proteins).

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 polypeptide of the present invention, culturing the cells in the presence of a test compound, and determining the speed of cell proliferation, measuring the cell cycle and such, as well as by measuring the colony forming activity as described in the Examples.

The compound isolated by the above screenings is a candidate for drugs which inhibit the activity of the polypeptide of the present invention and can be applied to the treatment of diseases associated with the polypeptide of the present invention, for example, cell proliferative diseases including cancer. More particularly, when the biological activity of CXADRL1 or GCUD1 protein is used as the index, compounds screened by the present method serve as a candidate for drugs for the treatment of gastric, colorectal, or liver cancer. On the other hand, when the biological activity of RNF43 protein is used as the index, compounds screened by the present method serve as a candidate for drugs for the treatment of colorectal, lung, gastric, or liver cancer.

Moreover, compound in which a part of the structure of the compound inhibiting the activity of CXADRL1, GCUD1, or RNF43 protein is converted by addition, deletion and/or replacement are also included in the compounds obtainable by the screening method of the present invention.

In a further embodiment of the method for screening a compound for treating a cell proliferative disease of the present invention, the method utilizes the binding ability of RNF43 to NOTCH2 or STRIN. The RNF43 protein of the present invention was revealed to associated with NOTCH2 and STRIN. These findings suggest that the RNF43 protein of the present invention exerts the function of cell proliferation via its binding to molecules, such as NOTCH2 and STRIN. Thus, it is expected that the inhibition of the binding between the RNF43 protein and NOTCH2 or STRIN leads to the suppression of cell proliferation, and compounds inhibiting the binding serve as pharmaceuticals for treating cell proliferative disease such as cancer. Preferably, the cell proliferative disease treated by the compound screened by the present method is colorectal, lung, gastric, or liver cancer.

This screening method includes the steps of: (a) contacting a polypeptide of the present invention with NOTCH2 or STRIN in the presence of a test compound; (b) detecting the binding between the polypeptide and NOTCH2 or STRIN; and (c) selecting the compound that inhibits the binding between the polypeptide and NOTCH2 or STRIN.

The RNF43 polypeptide of the present invention, and NOTCH2 or STRIN to be used for the screening may be a recombinant polypeptide or a protein derived from the nature, or may also be a partial peptide thereof so long as it retains the binding ability to each other. The RNF43 polypeptide, NOTCH2 or STRIN to be used in the screening can be, for example, a purified polypeptide, a soluble protein, a form bound to a carrier, or a fusion protein fused with other polypeptides.

Any test compound, 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 and natural compounds, can be used.

As a method of screening for compounds that inhibit the binding between the RNF43 protein and NOTCH2 or STRIN, many methods well known by one skilled in the art can be used. Such a screening can be carried out as an *in vitro* assay system, for example, in acellular system. More specifically, first, either the RNF43 polypeptide, or NOTCH2 or STRIN is bound to a support, and the other protein is added together with a test sample thereto. Next, the mixture is incubated, washed, and the other protein bound to the support is detected and/or measured.

In the same way, a compound interfering the association of CXADRL1 and AIP1 can be isolated by the present invention. It is expected that the inhibition of the binding between the CXADRL1 and AIP1 leads to the suppression of cell proliferation, and compounds inhibiting the binding serve as pharmaceuticals for treating cell proliferative disease such as cancer.

Examples of supports that may be used for binding proteins include insoluble polysaccharides, such as agarose, cellulose, and dextran; and synthetic resins, such as polyacrylamide, polystyrene, and silicon; preferably commercial available beads and plates (e.g., multi-well plates, biosensor chip, etc.) prepared from the above materials may be used. When using beads, they bay be filled into a column.

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

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

In the present invention, a biosensor using the surface plasmon resonance

phenomenon may be used as a mean for detecting or quantifying the bound protein. When such a biosensor is used, the interaction between the proteins can be observed real-time as a surface plasmon resonance signal, using only a minute amount of polypeptide and without labeling (for example, BIAcore, Pharmacia). Therefore, it is possible to evaluate the binding between the RNF43 polypeptide and NOTCH2 or STRIN using a biosensor such as BIAcore.

Alternatively, either the RNF43 polypeptide, or NOTCH2 or STRIN, may be labeled, and the label of the bound protein may be used to detect or measure the bound protein. Specifically, after pre-labeling one of the proteins, the labeled protein is contacted with the other protein in the presence of a test compound, and then, bound proteins are detected or measured according to the label after washing.

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 a protein in the present method. When the protein is labeled with radioisotope, the detection or measurement can be carried out by liquid scintillation. Alternatively, proteins labeled with enzymes can be detected or measured by adding a substrate of the enzyme to detect the enzymatic change of the substrate, such as generation of color, with absorptiometer. Further, in case where a fluorescent substance is used as the label, the bound protein may be detected or measured using fluorophotometer.

Furthermore, the binding of the RNF43 polypeptide and NOTCH2 or STRIN can be also detected or measured using antibodies to the RNF43 polypeptide and NOTCH2 or STRIN. For example, after contacting the RNF43 polypeptide immobilized on a support with a test compound and NOTCH2 or STRIN, the mixture is incubated and washed, and detection or measurement can be conducted using an antibody against NOTCH2 or STRIN. Alternatively, NOTCH2 or STRIN may be immobilized on a support, and an antibody against RNF43 may be used as the antibody.

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 RNF43 polypeptide, NOTCH2, or STRIN, may be used as a primary antibody to be detected with a secondary antibody that is labeled with a labeling substance. Furthermore, the antibody bound to the protein in the screening of the present invention may be detected or measured using protein G or protein A column.

Alternatively, in another embodiment of the screening method of the present

invention, a two-hybrid system utilizing cells may be used ("MATCHMAKER Two-Hybrid system", "Mammalian MATCHMAKER Two-Hybrid Assay Kit", "MATCHMAKER one-Hybrid system" (Clontech); "HybriZAP Two-Hybrid Vector System" (Stratagene); the references "Dalton and Treisman, Cell 68: 597-612 (1992)", "Fields and Sternglanz, Trends Genet 10: 286-92 (1994)").

In the two-hybrid system, the RNF43 polypeptide of the invention is fused to the SRF-binding region or GAL4-binding region and expressed in yeast cells. The NOTCH2 or STRIN binding to the RNF43 polypeptide of the invention is fused to the VP16 or GAL4 transcriptional activation region and also expressed in the yeast cells in the existence of a test compound. When the test compound does not inhibit the binding between the RNF43 polypeptide and NOTCH2 or STRIN, the binding of the two activates a reporter gene, making positive clones detectable.

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

The compound isolated by the screening is a candidate for drugs which inhibit the activity of the RNF43 protein of the present invention and can be applied to the treatment of diseases associated with the RNF43 protein, for example, cell proliferative diseases such as cancer, more particularly colorectal, lung, gastric, or liver cancer. Moreover, compounds in which a part of the structure of the compound inhibiting the binding between the RNF43 protein and NOTCH2 or STRIN is converted by addition, deletion, substitution and/or insertion are also included in the compounds obtainable by the screening method of the present invention.

When administrating the compound isolated by the methods of the invention as a pharmaceutical for humans and other mammals, such as mice, rats, guinea-pigs, rabbits, chicken, cats, dogs, sheep, pigs, cattle, monkeys, baboons, chimpanzees, for treating a cell proliferative disease (e.g., cancer) the isolated compound can be directly administered or can be formulated into a dosage form using known pharmaceutical preparation methods. For example, according to the need, the drugs can be taken orally, as sugarcoated tablets, capsules, elixirs and microcapsules, or non-orally, in the form of injections of sterile solutions or suspensions with water or any other pharmaceutically acceptable liquid. For example, the compounds can be mixed with pharmacologically acceptable carriers or medium, specifically, sterilized water, physiological saline, plant-oil, 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 ingredients in these preparations makes a suitable dosage within the indicated range acquirable.

Examples of additives that can be mixed to tablets and capsules are, 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; flavoring agents such as peppermint, Gaultheria adenothrix oil and cherry. When the unit dosage form is a capsule, a liquid carrier, such as oil, can also be further included in the above ingredients. Sterile composites for injections can be formulated following normal drug implementations using vehicles such as distilled water used for injections.

Physiological saline, glucose, and other isotonic liquids including adjuvants, such as D-sorbitol, D-mannnose, D-mannitol, and sodium chloride, can be used as aqueous solutions for injections. These can be used in conjunction with suitable solubilizers, such as alcohol, specifically ethanol, polyalcohols such as propylene glycol and polyethylene glycol, non-ionic surfactants, such as Polysorbate 80 (TM) and HCO-50.

Sesame oil or Soy-bean oil can be used as a oleaginous liquid and may be used in conjunction with benzyl benzoate or benzyl alcohol as a solubilizers 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, phenol; and an anti-oxidant. The prepared injection may be filled into a suitable ampule.

Methods well known to one skilled in the art may be used to administer the inventive pharmaceutical compound to patients, for example as intraarterial, intravenous, percutaneous injections and also as intranasal, transbronchial, intramuscular or oral administrations. The dosage and method of administration vary according to the body-weight and age of a patient and the administration method; however, one skilled in the art can routinely select them. If said compound is encodable by a DNA, the DNA can be inserted into a vector for gene therapy and the vector administered to perform the therapy. The dosage and method of administration vary according to the body-weight, age, and symptoms of a patient but one skilled in the art can select them suitably.

For example, although there are some differences according to the symptoms, the dose of a compound that binds with the polypeptide of the present invention and regulates its activity is about 0.1 mg to about 100 mg per day, preferably about 1.0 mg to about 50 mg per day and more preferably about 1.0 mg to about 20 mg per day, when administered orally to a normal adult (weight 60 kg).

When administering parenterally, in the form of an injection to a normal adult (weight 60 kg), although there are some differences according to the patient, target organ, symptoms and method of administration, it is convenient to intravenously inject a dose of about 0.01 mg to about 30 mg per day, preferably about 0.1 to about 20 mg per day and

more preferably about 0.1 to about 10 mg per day. Also, in the case of other animals too, it is possible to administer an amount converted to 60kgs of body-weight.

Moreover, the present invention provides a method for treating or preventing a cell proliferative disease, such as cancer, using an antibody against the polypeptide of the present invention. According to the method, a pharmaceutically effective amount of an antibody against the polypeptide of the present invention is administered. Since the expression of the CXADRL1, GCUD1, and RNF43 protein are up-regulated in cancer cells, and the suppression of the expression of these proteins leads to the decrease in cell proliferating activity, it is expected that cell proliferative diseases can be treated or prevented by binding the antibody and these proteins. Thus, an antibody against the polypeptide of the present invention are administered at a dosage sufficient to reduce the activity of the protein of the present invention, which is in the range of 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.

Alternatively, an antibody binding to a cell surface marker specific for tumor cells can be used as a tool for drug delivery. For example, the antibody conjugated with a cytotoxic agent is administered at a dosage sufficient to injure tumor cells.

The present invention also relates to a method of inducing anti-tumor immunity comprising the step of administering CXADRL1, GCUD1, or RNF43 protein or an immunologically active fragment thereof, or a polynucleotide encoding the protein or fragments thereof. The CXADRL1, GCUD1, or RNF43 protein or the immunologically active fragments thereof are useful as vaccines against cell proliferative diseases. In some cases the proteins or fragments thereof may be administered in a form bound to the T cell recepor (TCR) or presented by an antigen presenting cell (APC), such as macrophage, dendritic cell (DC), or B-cells. Due to the strong antigen presenting ability of DC, the use of DC is most preferable among the APCs.

In the present invention, vaccine against cell proliferative disease refers to a substance that has the function to induce anti-tumor immunity upon inoculation into animals. According to the present invention, polypeptides comprising the amino acid sequence of SEQ ID NO: 80, 97, or 108 were suggested to be HLA-A24 or HLA-A*0201 restricted epitopes peptides that may induce potent and specific immune response against colorectal, lung, gastric, or liver cancer cells expressing RNF43. According to the present invention, polypeptides comprising the amino acid sequence of SEQ ID NO:124 was suggested to be HLA-A*0201 restricted epitopes peptides that may induce potent and specific immune response against colorectal, gastric, or liver cancer cells expressing

CXADRL1. According to the present invention, polypeptides comprising the amino acid sequence of SEQ ID NO: 164 was suggested to be HLA-A*0201 restricted epitopes peptides that may induce potent and specific immune response against colorectal, gastric, or liver cancer cells expressing GCUD1. Thus, the present invention also encompasses method of inducing anti-tumor immunity using polypeptides comprising the amino acid sequence of SEQ ID NO: 80, 97, 108, 124 or 164. 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 decided to have anti-tumor immunity inducing effect. The induction of the anti-tumor immunity by a protein can be detected by observing *in vivo* or *in vitro* the response of the immune system in the host against the protein.

For example, a method for detecting the induction of cytotoxic T lymphocytes is well known. A foreign substance that enters the living body is presented to T cells and B cells by the action of antigen presenting cells (APCs). T cells that respond to the antigen presented by APC in antigen specific manner differentiate into cytotoxic T cells (or cytotoxic T lymphocytes; CTLs) due to stimulation by the antigen, and then proliferate (this is referred to as activation of T cells). Therefore, CTL induction by a certain peptide can be evaluated by presenting the peptide to T cell by APC, and detecting the induction of CTL. Furthermore, APC has the effect of activating CD4+ T cells, CD8+ T cells, macrophages, eosinophils, and NK cells. Since CD4+ T cells 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 CTL using dendritic cells (DCs) as APC is well known in the art. DC is a representative APC having the strongest CTL inducing action among APCs. In this method, the test polypeptide is initially contacted with DC, and then this DC is contacted with T cells. Detection of T cells having cytotoxic effects against the cells of interest after the contact with DC shows that the test polypeptide has an activity of inducing the cytotoxic T cells. Activity of CTL against tumors can be detected, for example, using the lysis of ^{51}Cr-labeled tumor cells as the indicator. Alternatively, the method of 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 DC, peripheral blood mononuclear cells (PBMCs) may also be used as the APC. The induction of CTL is reported that the it can be enhanced by culturing PBMC in the presence of GM-CSF and IL-4. Similarly, CTL has been shown to be induced by culturing PBMC in the presence of keyhole limpet hemocyanin (KLH) and IL-7.

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

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

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 those antibodies, the polypeptide can be determined to have an ability to induce anti-tumor immunity.

Anti-tumor immunity is induced by administering the vaccine of this invention, and the induction of anti-tumor immunity enables treatment and prevention of cell proliferating diseases, such as gastric, colorectal, lung, and liver cancers. Therapy against cancer or prevention of the onset of cancer includes any of the steps, such as inhibition of the growth of cancerous cells, involution of cancer, and suppression of occurrence of cancer. Decrease in mortality of individuals having cancer, decrease 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

analyses.

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

When using APC or CTL as the vaccine 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 polypeptide *ex vivo*, and following the induction of APC or CTL, the cells may be administered to the subject. APC can be also induced by introducing a vector encoding the polypeptide into PBMCs *ex vivo*. APC or CTL induced in vitro can be cloned prior to administration. By cloning and growing cells having high activity of damaging target cells, cellular immunotherapy can be performed more effectively. Furthermore, APC and CTL isolated in this manner may be used for cellular immunotherapy not only against individuals from whom the cells are derived, but also against similar types of tumors from other individuals.

Furthermore, a pharmaceutical composition for treating or preventing a cell proliferative disease, such as cancer, comprising a pharmaceutically effective amount of the polypeptide of the present invention is provided. The pharmaceutical composition may be used for raising anti tumor immunity. The normal expression of CXADRL1, restricted to testis and ovary; normal expression of GCUD1 is restricted to testis, ovary, and brain; and normal expression of RNF43 is restricted to fetus, more specifically to fetal lung and kidney. Therefore, suppression of these genes may not adversely affect other organs. Thus, the CXADRL1 and GCUD1 polypeptides are preferable for treating cell proliferative disease, especially gastric, colorectal, or liver cancer; and RNF43 polypeptide is also preferable for treating cell proliferative disease, especially colorectal, lung, gastric, and liver cancers. Furthermore, since peptide fragments of RNF43 comprising the amino acid sequences of SEQ ID NO: 80, 97, and 108, respectively, were revealed to induce immune response against RNF43, polypeptides comprising the amino acid sequence of SEQ ID NO: 80, 97, or 108 are preferable examples of polypeptides that can be used in a

pharmaceutical composition for treating or preventing cell proliferative disease, especially colorectal, lung, gastric, and liver cancers. Furthermore, since peptide fragments of CXADRL1 comprising the amino acid sequences of SEQ ID NO: 124, respectively, were revealed to induce immune response against CXADRL1, polypeptides comprising the amino acid sequence of SEQ ID NO: 124 is preferable examples of polypeptide that can be used in a pharmaceutical composition for treating or preventing cell proliferative disease, especially colorectal, lung, gastric, and liver cancers. Furthermore, since peptide fragments of GCUD1 comprising the amino acid sequences of SEQ ID NO: 164, respectively, was revealed to induce immune response against GCUD1, polypeptides comprising the amino acid sequence of SEQ ID NO: 164 is preferable examples of polypeptides that can be used in a pharmaceutical composition for treating or preventing cell proliferative disease, especially colorectal, lung, gastric, and liver cancers. In the present invention, the polypeptide or fragment thereof is administered at a dosage sufficient to induce anti-tumor immunity, which is in the range of 0.1 mg to 10 mg, preferably 0.3mg to 5mg, more preferably 0.8mg to 1.5 mg. The administrations are repeated. For example, 1mg of the peptide or fragment thereof may be administered 4 times in every two weeks for inducing the anti-tumor immunity.

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

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. Any patents, patent applications, and publications cited herein are incorporated by reference.

Best Mode for Carrying out the Invention

The present invention is illustrated in details by following Examples, but is not restricted to these Examples.

1. Materials and Methods

(1) Patients and tissue specimens

All gastric and colorectal cancer tissues, as well as corresponding non-cancerous tissues were obtained with informed consent from surgical specimens of patients who underwent surgery.

(2) Genome-wide cDNA microarray

In-house genome-wide cDNA microarray comprising 23040 genes were used in this study. DNase I treated total RNA extracted from microdissected tissue was amplified with Ampliscribe T7 Transcription Kit (Epicentre Technologies) and labeled during reverse transcription with Cy-dye (Amersham) (RNA from non-cancerous tissue with Cy5 and RNA from tumor with Cy3). Hybridization, washing, and detection were carried out as described previously (Ono et al., Cancer Res. 60: 5007-11 (2000)), and fluorescence intensity of Cy5 and Cy3 for each target spot was measured using Array Vision software (Amersham Pharmacia). After subtraction of background signal, duplicate values were averaged for each spot. Then, all fluorescence intensities on a slide were normalized to adjust the mean Cy5 and Cy3 intensity of 52 housekeeping genes for each slide. Genes with intensities below 25,000 fluorescence units for both Cy3 and Cy5 were excluded from further investigation, and those with Cy3/Cy5 signal ratios > 2.0 were selected for further evaluation.

(3) Cell lines

Human embryonic kidney 293 (HEK293) were obtained TaKaRa. COS7 cell, NIH3T3 cell, human cervical cancer cell line HeLa, human gastric cancer cell lines MKN-1 and MKN-28, human hepatoma cell line Alexander, and human colon cancer cell lines, LoVo, HCT116, DLD-1 and SW480, were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Human hepatoma cell line SNU475 and human colon cancer cell lines, SNUC4 and SNUC5, were obtained from the Korea cell-line bank. All cells were grown in monolayers in appropriate media: Dulbecco's modified Eagle's medium for COS7, NIH3T3, HEK293, and Alexander; RPMI1640 for MKN-1, MKN-28, SNU475, SNUC4, DLD-1 and SNUC5; McCoy's 5A medium for HCT116; Leibovitz's L-15 for SW480; HAM's F-12 for LoVo; and Eagle's minimum essential medium for HeLa (Life Technologies, Grand Island, NY). All media were supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic solution (Sigma). A human gastric cancer cell lines St-4 was kindly provided by Dr. Tsuruo in Cancer Institure in Japan. St-4 cells were grown in monolayers in RPMI1640 supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic solution (Sigma).

T2 cells (HLA-A*0201) and EHM (HLA-A3/3), human B-lymphoblastoid cell lines, were generous gifts from Prof. Shiku (Univ. Mie). HT29 (colon carcinoma cell line; HLA-A24/01), WiDR (colon carcinoma cell line; HLA-A24/01), and HCT116 (colon carcinoma cell line; HLA-A02/01), DLD-1 (colon carcinoma cell line; HLA-A24/01), SNU475 (hepatocellular carcinoma cell line; HLA-A*0201), MKN45 (gastric cancer cell line; HLA-A2 negative), MKN74 (gastric cancer cell line; HLA-A2 negative) were also purchased from ATCC. TISI cells (HLA-A24/24) were generous gifts from Takara Shuzo

Co, Ltd. (Otsu, Japan). RT-PCR examinations revealed strong *CXADRL1* expression in SNU475 and MKN74. RT-PCR examinations revealed strong *GCUD1* expression in SNU475 and MKN45.

(4) RNA preparation and RT-PCR

Total RNA was extracted with Qiagen RNeasy kit (Qiagen) or Trizol reagent (Life Technologies) according to the manufacturers' protocols. Ten-microgram aliquots of total RNA were reversely transcribed for single-stranded cDNAs using poly dT_{12-18} primer (Amersham Pharmacia Biotech) with Superscript II reverse transcriptase (Life Technologies). Each single-stranded cDNA preparation was diluted for subsequent PCR amplification by standard RT-PCR experiments carried out in 20μl volumes of PCR buffer (TaKaRa). Amplification was conducted under following conditions: denaturing for 4 min at 94°C, followed by 20 (for GAPDH), 35 (for CXADRL1), 30 (for GCUD1), 30 (for RNF43) cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 45 s, in GeneAmp PCR system 9700 (Perkin-Elmer, Foster City, CA). Primer sequences were; for GAPDH: forward, 5'-ACAACAGCCTCAAGATCATCAG (SEQ ID NO: 7) and reverse, 5'-GGTCCACCACTGACACGTTG (SEQ ID NO: 8); for CXADRL1: forward, 5'-AGCTGAGACATTTGTTCTCTTG (SEQ ID NO: 9) and reverse: 5'-TATAAACCAG CTGAGTCCAGAG (SEQ ID NO: 10); for GCUD1 forward: 5'-TTCCCGATATCAACATCTACCAG (SEQ ID NO: 11) reverse: 5'-AGTGTGTGACCTCAATAAGGCAT (SEQ ID NO: 12), for RNF43 forward; 5'-CAGGCTTTGGACGCACAGGACTGGTAC-3' (SEQ ID NO: 13) and reverse; 5'-CTTTGTGATCATCCTGGCTTCGGTGCT-3' (SEQ ID NO: 14).

(5) Northern-blot analysis

Human multiple-tissue blots (Clontech, Palo Alto, CA) were hybridized with ^{32}P-labeled PCR products of CXADRL1, GCUD1, or RNF43. Pre-hybridization, hybridization and washing were performed according to the supplier's recommendations. The blots were autoradiographed with intensifying screens at –80°C for 24 to 72 h.

(6) 5' rapid amplification of cDNA ends (5' RACE)

5' RACE experiments were carried out using Marathon cDNA amplification kit (Clontech) according to the manufacturer's instructions. For the amplification of the 5' part of CXADRL1, gene-specific reverse primers (5'-GGTTGAGATTTAAGTTCTCAAA-3' (SEQ ID NO: 15)) and the AP-1 primer supplied with the kit were used. The cDNA template was synthesized from human testis mRNA (Clontech). The PCR products were cloned using TA cloning kit (Invitrogen) and their sequences were determined with ABI PRISM 3700 DNA sequencer (Applied

Biosystems).

(7) Construction of plasmids expressing CXADRL1, GCUD1, and FLJ20315

The entire coding regions of CXADRL1, GCUD1, and RNF43 were amplified by RT-PCR using gene specific primer sets; for CXADRL1, 5'-AGTTAAGCTTGCCGGGATGACTTCTCAGCGTTCCCCTCTGG-3' (SEQ ID NO: 16) and 5'-ATCTCGAGTACCAAGGACCCGGCCCGACTCTG-3' (SEQ ID NO: 17), for GCUD1 5'-GCGGATCCAGGATGGCTGCTGCAGCTCCTCCAAG-3' (SEQ ID NO: 18) and 5'-TAGAATTCTTAAAGAACTTAATCTCCGTGTCAACAC-3' (SEQ ID NO: 19), for RNF43, 5'-TGCAGATCTGCAGCTGGTAGCATGAGTGGTG-3' (SEQ ID NO: 20) and 5'-GAGGAGCTGTGTGAACAGGCTGTGTGAGATGT-3' (SEQ ID NO: 21). The PCR products were cloned into appropriate cloning site of either pcDNA3.1 (Invitrogen), or pcDNA3.1myc/His (Invitrogen) vector.

(8) Immunoblotting

Cells transfected with pcDNA3.1myc/His-CXADRL1, pcDNA3.1myc/His-GCUD1, pcDNA3.1myc/His-RNF43 or pcDNA3.1myc/His-LacZ were washed twice with PBS and harvested in lysis buffer (150 mM NaCl, 1% Triton X-100, 50 mM Tris-HCl pH 7.4, 1mM DTT, and 1X complete Protease Inhibitor Cocktail (Boehringer)). Following homogenization, the cells were centrifuged at 10,000xg for 30 min, the supernatant were standardized for protein concentration by the Bradford assay (Bio-Rad). Proteins were separated by 10% SDS-PAGE and immunoblotted with mouse anti-myc (SANTA CRUZ) antibody. HRP-conjugated goat anti-mouse IgG (Amersham) served as the secondary antibody for the ECL Detection System (Amersham).

(9) Immunohistochemical staining

Cells transfected with pcDNA3.1myc/His-CXADRL1, pcDNA3.1myc/His-GCUD1, pcDNA3.1myc/His-RNF43 or pcDNA3.1myc/His-LacZ were fixed with PBS containing 4% paraformaldehyde for 15 min, then made permeable with PBS containing 0.1% Triton X-100 for 2.5 min at RT. Subsequently the cells were covered with 2% BSA in PBS for 24 h at 4°C to block non-specific hybridization. Mouse anti-myc monoclonal antibody (Sigma) at 1:1000 dilution was used as the primary antibody, and the reaction was visualized after incubation with Rhodamine-conjugated anti-mouse secondary antibody (Leinco and ICN). Nuclei were counter-stained with 4',6'-diamidine-2'-phenylindole dihydrochloride (DAPI). Fluorescent images were obtained under an ECLIPSE E800 microscope.

(10) Colony Formation assay

Cells transfected with plasmids expressing each gene or control plasmids were incubated with an appropriate concentration of geneticin for 10 to 21 days. The cells

were fixed with 100% methanol and stained by Giemsa solution. All experiments were carried out in duplicate.

(11) Establishment of cells over-expressing CXADRL1, or RNF43

NIH3T3, COS7, and LoVo cells transfected with either pcDNA3.1myc/His-CXADRL1, pcDNA3.1myc/His-RNF43, pcDNA3.1myc/His-LacZ or control plasmids, respectively, were maintained in media containing appropriate concentration of geneticin. Two weeks after the transfection, surviving single colonies were selected, and expression of each gene was examined by semi-quantitative RT-PCR.

(12) Examination on the effect of anti-sense oligonucleotides on cell growth

Cells plated onto 10-cm dishes ($2X10^5$ cells/dish) were transfected either with plasmid, or synthetic S-oligonucleotides of CXADRL1, GCUD1, or RNF43 using LIPOFECTIN Reagent (GIBCO BRL). Then the cells were cultured with the addition of an appropriate concentration of geneticin for six to twelve days. The cells were then fixed with 100% methanol and stained by Giemsa solution. Sequences of the S-oligonucleotides were as follows:

CXADRL1-S4, 5'-TCTGCACGGTGAGTAG-3' (SEQ ID NO: 22);
CXADRL1-AS4, 5'-CTACTCACCGTGCAGA-3' (SEQ ID NO: 23);
CXADRL1-S5, 5'-TTCTGTAGGTGTTGCA-3' SEQ ID NO: 24);
CXADRL1-AS5, 5'-TGCAACACCTACAGAA-3' (SEQ ID NO: 25);
GCUD1-S5, 5'-CTTTTCAGGATGGCTG-3' (SEQ ID NO: 26);
GCUD1-AS5, 5'-CAGCCATCCTGAAAAG-3' (SEQ ID NO: 27);
GCUD1-S8, 5'-AGGTTGAGGTAAGCCG-3' (SEQ ID NO: 28);
GCUD1-AS8, 5'-CGGCTTACCTCAACCT-3' (SEQ ID NO: 29);
RNF43-S1, 5'-TGGTAGCATGAGTGGT-3' (SEQ ID NO; 30); and
RNF43-AS1, 5'-ACCACTCATGCTACCA-3' (SEQ ID NO: 31).

(13) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Cells plated at a density of $5X10^5$ cells/100 mm dish were transfected in triplicate with sense or antisense S-oligonucleotides designated to suppress the expression of CXADRL1, GCUD1 or RNF43. Seventy-two hours after transfection, the medium was replaced with fresh medium containing 500 μg/ml of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Sigma) and the plates were incubated for four hours at 37°C. Subsequently, the cells were lysed by the addition of 1 ml of 0.01 N HCl/10%SDS and the absorbance of lysates was measured with ELISA plate reader at a test wavelength of 570 nm (reference, 630 nm). The cell viability was represented by the absorbance compared to that of control cells.

(14) Construction of psiH1BX3.0

Since H1RNA gene was reported to be transcribed by RNA polymerase III, which produce short transcripts with uridines at the 3' end, a genomic fragment of H1RNA gene containing its promoter region was amplified by PCR using a set of primers [5'-TGGTAGCCAAGTGCAGGTTATA-3' (SEQ ID NO: 32), and 5'-CCAAAGGGTTTCTGCAGTTTCA-3' (SEQ ID NO: 33)] and human placental DNA as a template. The products were purified and cloned into pCR2.0 plasmid vector using TA cloning kit (Invitrogen) according to the supplier's protocol. The *Bam*HI and *Xho*I fragment containing the H1RNA gene was purified and cloned into pcDNA3.1(+) plasmid at the nucleotide position from 1257 to 56, which plasmid was amplified by PCR with a set of primers, 5'-TGCGGATCCAGAGCAGATTGTACTGAGAGT-3' (SEQ ID NO: 34) and 5'-CTCTATCTCGAGTGAGGCGGAAAGAACCA-3' (SEQ ID NO: 35), and then digested with *Bam*HI and *Xho*I. The ligated DNA was used as a template for PCR with primers,
5'- TTTAAGCTTGAAGACCATTTTTGGAAAAAAAAAAAAAAAAAAAAAC-3' (SEQ ID NO: 36) and 5'-TTTAAGCTTGAAGACATGGGAAAGAGTGGTCTCA-3' (SEQ ID NO: 37). The product was digested with *Hin*dIII, and subsequently self-ligated to produce psiH1BX3.0 vector plasmid. As the control, psiH1BX-EGFP was prepared by cloning double-stranded oligonucleotides of
5'- CACCGAAGCAGCACGACTTCTTCTTCAAGAGAGAAGAAGTCGTGCTGCTTC -3' (SEQ ID NO: 38) and
5'- AAAAGAAGCAGCACGACTTCTTCTCTCTTGAAGAAGAAGTCGTGCTGCTTC -3' (SEQ ID NO: 39) into the *Bbs*I site of the psiH1BX vector.

(15) Examination on the gene silencing effect of RNF43-, or CXADRL1-siRNAs

A plasmid expressing either RNF43-siRNA or CXADRL1-siRNA was prepared by cloning of double-stranded oligonucleotides into psiH1BX3.0 vector.
Oligonucleotides used as RNF43 siRNAs were:
5'- TCCCGTCACCGGATCCAACTCAGTTCAAGAGACTGAGTTGGATCCGGTGA C-3' (SEQ ID NO: 40) and
5'-AAAAGTCACCGGATCCAACTCAGTCTCTTGAACTGAGTTGGATCCGGTGAC- 3' (SEQ ID NO: 41) as siRNA16-4;
5'- TCCCGCTATTGCACAGAACGCAGTTCAAGAGACTGCGTTCTGTGCAATAGC- 3' (SEQ ID NO: 42) and
5'-AAAAGCTATTGCACAGAACGCAGTCTCTTGAACTGCGTTCTGTGCAATAGC-3' (SEQ ID NO: 43) as siRNA1834;
5'- TCCCCAGAAAGCTGTTATCAGAGTTCAAGAGACTCTGATAACAGCTTTCTG- 3' (SEQ ID NO: 44) and

5'-AAAACAGAAAGCTGTTATCAGAGTCTCTTGAACTCTGATAACAGCTTTCTG-3' (SEQ ID NO: 45) as siRNA1; 5'- TCCCTGAGCCACCTCCAATCCACTTCAAGAGAGTGGATTGGAGGTGGCTCA-3' (SEQ ID NO: 46) and 5'-AAAATGAGCCACCTCCAATCCACTCTCTTGAAGTGGATTGGAGGTGGCTCA-3' (SEQ ID NO: 47) as siRNA14; 5'- TCCCCTGCACGGACATCAGCCTATTCAAGAGATAGGCTGATGTCCGTGCAG-3' (SEQ ID NO: 48) and 5'-AAAACTGCACGGACATCAGCCTATCTCTTGAATAGGCTGATGTCCGTGCAG-3' (SEQ ID NO: 49) as siRNA15. Oligonucleotides used as CXADRL1- siRNAs were: 5'-TCCCGTGTCAGAGAGCCCTGGGATTCAAGAGATCCCAGGGCTCTCTGACAC-3' (SEQ ID NO: 50) and 5'-AAAAGTGTCAGAGAGCCCTGGGATCTCTTGAATCCCAGGGCTCTCTGACAC-3' (SEQ ID NO: 51) as siRNA#1; 5'-TCCCCCTCAATGTCATTTGGATGTTCAAGAGACATCCAAATGCAATTGAGG-3' (SEQ ID NO: 52) and 5'-AAAACCTCAATGTCATTTGGATGTCTCTTGAACATCCAAATGCAATTGAGG-3' (SEQ ID NO: 53) as siRNA#2; 5'-TCCCTGTCATTTGGATGGTCACTTTCAAGAGAAGTGACCATCCAAATGACA-3' (SEQ ID NO: 54) and 5'-AAAATGTCATTTGGATGGTCACTTCTCTTGAAAGTGACCATCCAAATGACA-3' (SEQ ID NO: 55) as siRNA#3; 5'-TCCCTGCCAACCAACCTGAACAGTTCAAGAGACTGTTCAGGTTGGTTGGCA-3' (SEQ ID NO: 56) and 5'-AAAATGCCAACCAACCTGAACAGTCTCTTGAACTGTTCAGGTTGGTTGGCA-3' (SEQ ID NO: 57) as siRNA#4; 5'-TCCCCCAACCTGAACAGGTCATCTTCAAGAGAGATGACCTGTTCAGGTTGG-3' (SEQ ID NO: 58) and 5'-AAAACCAACCTGAACAGGTCATCTCTCTTGAAGATGACCTGTTCAGGTTGG-3' (SEQ ID NO: 59) as siRNA#5; 5'-TCCCCCTGAACAGGTCATCCTGTTTCAAGAGAACAGGATGACCTGTTCAGG-3' (SEQ ID NO: 60) and 5'-AAAACCTGAACAGGTCATCCTGTTCTCTTGAAACAGGATGACCTGTTCAGG-3' (SEQ ID NO: 61) as siRNA#6; and 5'-TCCCCAGGTCATCCTGTATCAGGTTCAAGAGACCTGATACAGGATGACCTG-3' (SEQ ID NO: 62) and 5'-AAAACAGGTCATCCTGTATCAGGTCTCTTGAACCTGATACAGGATGACCTG-3' (SEQ ID NO: 63) as CXADRL-siRNA#7. psiH1BX-RNF43, psiH1BX-CXADRL1, or psiH1BX-mock plasmids were transfected into SNUC4 or St-4 cells using FuGENE6 reagent (Roche) according to the supplier's recommendations. Total RNA was extracted from the cells 48 hours after the transfection.

(16) Construction of recombinant amino- and carboxyl-terminal regions of RNF43 protein

The amino- and carboxyl-terminal regions of *RNF43* was amplified by RT-PCR using following sets of primers: 5'-GAAGATCTGCAGCGGTGGAGTCTGAAAG-3'

(SEQ ID NO: 64) and 5'-GGAATTCGGACTGGGAAAATGAATCTCCCTC-3' (SEQ ID NO: 65) for the amino-terminal region; and 5'-GGAGATCTCCTGATCAGCAAGTCACC-3' (SEQ ID NO: 66) and 5'-GGAATTCCACAGCCTGTTCACACAGCTCCTC-3' (SEQ ID NO: 67) for the carboxyl-terminal region. The products were digested with *Bam*HI-*Eco*RI and cloned into the *Bam*HI-*Eco*RI site of pET-43.1a(+) vector (Novagen). The plasmids were transfected into *E. coli* BL21*trxB*(DE3)pLysS cells (Stratagene). Recombinant RNF43 protein was extracted from cells cultured at 25 °C for 16 h after the addition of 0.2 mM IPTG.

(17) Yeast two-hybrid experiment

Yeast two-hybrid assay was performed using MATCHMAKER GAL4 Two-Hybrid System 3 (Clontech) according to the manufacturer's protocols. The entire coding sequence of *RNF43* was cloned into the *Eco*R I-*BamH* I site of pAS2-1 vector as a bait for screening human-testis cDNA library (Clontech). To confirm the interaction in yeast, pAS2-RNF43 was used as bait vector, pACT2-NOTCH2 and pACT2-STRIN as prey vector.

We cloned the cytoplasmic region of CXADRL1 into the EcoRI site of pAS2-1 vector as a bait for screening a human testis cDNA library (Clontech). To confirm interaction in yeast, we used pAS2-CXADRL1 for bait vector, and pACT2-AIP1 for prey vector.

(18) Preparation of CXADRL specific antibody

Anti-CXADRL antisera was prepared by immunization with synthetic polypeptides of CXADRL1 encompassing codons from 235 to 276 for Ab-1, from 493 to 537 for Ab-2, or from 70 to 111 for Ab-3. Sera were purified using recombinant His-tagged CXADRL1 protein prepared in *E. coli* transfected with pET-CXADRL plasmid. Protein extracted from cells expressing Flag-tagged CXADRL1 was further separated by 10% SDS-PAGE and immunoblotted with either anti-CXADRL1 sera or anti-Flag antibody. HRP-conjugated goat anti-rabbit IgG or HRP-conjugated sheep anti-mouse IgG antibody served as the secondary antibody, respectively, for ECL Detection System (Amersham Pharmacia Biotech, Piscataway, NJ). Immunoblotting with anti-CXADRL antisera showed a 50 kD band of FLAG-tagged CXADRL1, which pattern was identical to that detected with anti-FLAG antibody.

(19) Preparation of recombinant GCUD1 protein

To generate an antibody specific against GCUD1, recombinant GCUD1 protein was prepared. The entire coding region of *GCUD1* was amplified by RT-PCR with a set

of primers, 5'-GCGGATCCAGGATGGCTGCAGCTCCTCCAAG-3' (SEQ ID NO: 68) and 5'-CTGAATTCACTTAAAGAACTTAATCTCCGTGTCAACAC-3' (SEQ ID NO: 69). The product was purified, digested with *Bam*H1 and *Eco*R1, and cloned into an appropriate cloning site of pGEX6P-2. The resulting plasmid was dubbed pGEX-GCUD1. pGEX-GCUD1 plasmid was transformed into *E. coli* DH10B. The production of the recombinant protein was induced by the addition of IPTG, and the protein was purified with Glutathione Sepharose™ 4B (Amersham Pharmacia) according to the manufacturers' protocols.

(20) Preparation of GCUD1 specific antibody

Polyclonal antibody against GCUD1 was purified from sera. Proteins from cells transfected with plasmids expressing Flag-tagged GCUD1 were separated by 10% SDS-PAGE and immunoblotted with anti-GCUD1 or anti-Flag antibody. HRP-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA) or HRP-conjugated anti-Flag antibody served as the secondary antibody, respectively, for ECL Detection System (Amersham Pharmacia Biotech, Piscataway, NJ). Immunoblotting with the anti-GCUD1 antibody showed a 47 kD band of FLAG-tagged GCUD1, which pattern was identical to that detected with the anti-FLAG antibody.

(21) Statistical analysis

The data were subjected to analysis of variance (ANOVA) and the Scheffé's F test.

(22) Preparation of peptides

9mer and 10mer peptides of RNF43, *CXADRL1* or *GCUD1* that bind to HLA-A24 or HLA-A*0201 molecule were predicted with the aide of binding prediction soft (http://bimas.dcrt.nih.gov/cgi-bin/molbio/ken_parker_comboform). These peptides were synthesized by Mimotopes, San Diego, LA according to the standard solid phase synthesis method and purified by reversed phase HPLC. The purity (>90%) and the identity of the peptides were determined by analytical HPLC and mass spectrometry analysis, respectively. Peptides were dissolved in dimethylsulfoxide (DMSO) at 20 mg/ml and stored at -80 ºC.

(23) In vitro CTL Induction

Monocyte-derived dendritic cells (DCs) were used as antigen-presenting cells (APCs) to induce CTL responses against peptides presented on HLA. DCs were generated in vitro as described elsewhere (Nukaya et al., Int J Cancer 80: 92-7 (1999); Tsai et al., J Immunol 158: 1796-802 (1997)). Specifically, peripheral blood mononuclear cells (PBMCs) were isolated from a healthy volunteer with HLA-A*0201 or HLA-A24 using Ficoll-Plaque (Pharmacia) solution, and monocyte fraction of PBMCs were separated by adherence to a plastic tissue culture flask (Becton Dickinson). This monocyte fraction was cultured for seven days in AIM-V medium (Invitrogen) containing

2% heat-inactivated autologous serum (AS), 1000 U/ml of GM-CSF (provided by Kirin Brewery Company), and 1000 U/ml of IL-4 (Genzyme) to obtain DCs fraction. The 20 μg/ml of candidate peptides were pulsed onto this DC enriched cell population in the presence of 3 μg/ml of β2-microglobulin for 4 h at 20°C in AIM-V. These peptide-pulsed antigen presenting cells were then irradiated (5500 rads) and mixed at a 1:20 ratio with autologous CD8+ T cells, obtained by positive selection with Dynabeads M-450 CD8 (Dynal) and Detachabead (Dynal). These cultures were set up in 48-well plates (Corning); each well contained $1.5x10^4$ peptide-pulsed antigen presenting cells, $3x10^5$ CD8+ T cells and 10 ng/ml of IL-7 (Genzyme) in 0.5 ml of AIM-V with 2% AS. Three days later, these cultures were supplemented with IL-2 (CHIRON) to a final concentration of 20 IU/ml. On day 7 and 14, the T cells were further restimulated with the autologous peptide-pulsed antigen presenting cells which were prepared each time in the same manner as described above. Lymphoid cells in the culture on day 21 were harvested and tested for cytotoxicity against peptide-pulsed TISI or T2 cells.

(24) CTL Expansion

Cultured lymphoid cells with proved significant cytotoxicity against peptide-pulsed TISI or T2 were further expanded in culture using a method similar to that described by Riddell, et al. (Walter et al., N Engl J Med 333:1038-1044, 1995; Riddel et al., Nature Med. 2:216-223, 1996). 5×10^4 of lymphoid cells were resuspended in 25 ml of AIM-V supplemented with 5% AS containing 25×10^6 irradiated (3300 rads) PBMC, 5×10^6 irradiated (8000 rads) EHM cells, and 40 ng/ml of anti-CD3 monoclonal antibody (Pharmingen). One day after initiating the cultures, 120 IU/ml of IL-2 were added to the cultures. The cultures comprised fresh AIM-V supplemented with 5% AS and 30 IU/ml of IL-2 on days 5, 8 and 11.

(25) Establishment of CTL clones

Some of the lymphoid cells with potent cytotoxicity were used to obtain CTL clones. The cell suspensions were diluted to density of 0.3, 1, and 3 CTLs/lymphoid cells per well in 96 round-bottom microtiter plate (Nalge Nunc International). These cells were cultured in 150μl/well of AIM-V supplemented with 5%AS containing 7×10^4 cells/well of allogenic PBMCs, 1×10^4 cells/well of EHM, 30ng/ml of anti-CD3 antibody, and 125 U/ml of IL-2. 10 days later, 50 μl /well of IL-2 was added to the medium to a final concentration of 125 U/ml. Cytotoxic activity of cultured CTLs was tested on day 14, and CTL clones were expanded using the same method as described above.

(26) Cytotoxicity Assay

Target cells were labeled with 100 μCi of $Na_2{}^{51}CrO_4$ (Perkin Elmer Life Sciences) for 1 h at 37 °C in a CO_2 incubator. When peptide-pulsed targets were used, target cells were

incubated with the addition of 20μg/ml of the peptide for 16 h at 37 °C before the labeling with $Na_2{}^{51}CrO_4$. Target cells were rinsed and mixed with effectors at a final volume of 0.2 ml in round-bottom microtiter plates. The plates were centrifuged (4 minutes at 800 x g) to increase cell-to-cell contact and placed in a CO_2 incubator at 37°C. After 4 h of incubation, 0.1 ml of the supernatant was collected from each well and the radioactivity was determined with a gamma counter. In case of evaluating cytotoxicity against target cells that endogenously express *RNF43* or *CXADRL1* or *GCUD1*, the cytolytic activity was tested in the presence of a 30-fold excess of unlabeled K562 cells to reduced any non-specific lysis due to NK-like effectors. Antigen specificity was confirmed by the cold target inhibition assay, which utilized unlabeled TISI or T2 cells that were pulsed with peptide (20 μ g/ml for 16 hrs at 37 ℃) to compete for the recognition of 51Cr-labeled HT29 or SNU475 cells. The MHC restriction was examined by blocking assay, measuring the inhibition of the cytotoxicity by anti HLA-class I (W6/32) antibody and anti HLA-class II antibody, anti CD4 antibody and anti CD8 antibody (DAKO).

The percentage of specific cytotoxicity was determined by calculating the percentage of specific $^{51}Cr^-$ release by the following formula: {(cpm of the test sample release - cpm of the spontaneous release)/(cpm of the maximum release - cpm of the spontaneous release)} × 100. Spontaneous release was determined by incubating the target cells alone in the absence of effectors, and the maximum release was obtained by incubating the targets with 1N HCl. All determinants were done in duplicate, and the standard errors of the means were consistently below 10% of the value of the mean.

2. Results

(1) Identification of two novel human genes, CXADRL1 and GCUD1, commonly up-regulated in gastric cancers

By means of a genome-wide cDNA microarray containing 23040 genes, expression profiles of 20 gastric cancers were compared with corresponding non-cancerous mucosae. Among commonly up-regulated genes detected in the microarray analysis, a gene with an in-house accession number of A5928 corresponding to an EST, Hs.6658 of UniGene cluster (http://www.ncbi.nlm.nih.gov/UniGene/), was found to be over-expressed in a range between 4.09 and 48.60 (Figure 1a). Since an open reading frame of this gene encoded a protein approximately 37% identical to that of CXADR (coxsackie and adenovirus receptor), this gene was dubbed CXADRL1 (coxsackie and adenovirus receptor like 1). *CXADRL1* was also up-regulated in 6 of 6 colorectal cancer cases and 12 out of 20 HCC cases. Furthermore, a gene with an in-house accession number of C8121, corresponding to KIAA0913 gene product (Hs.75137) of UniGene cluster was also focused

due to its significantly enhanced expression in nine of twelve gastric cancer tissues compared with the corresponding non-cancerous gastric mucosae by microarray (Figure 1b). This gene with the in-house accession number C8121 was dubbed GCUD1 (up-regulated in gastric cancer). *GCUD1* was also up-regulated in 5 of 6 colorectal cancer cases , 1 out of 6 HCC cases, 1 out of 14 lung cancer(squeamous cell carcinoma) cases, 1 out of 13 testicular seminomas cases. To clarify the results of the cDNA microarray, expression of these transcripts in gastric cancers was examined by semi-quantitative RT-PCR to confirm an increased expression of CXADRL1 in all of the 10 tumors (Figure 1c) and elevated expression of GCUD1 in seven of nine cancers (Figure 1d).

(2) Isolation and structure of a novel gene *CXADRL1*

Multiple-tissue northern-blot analysis using a PCR product of CXADRL1 as a probe revealed the expression of a 3.5-kb transcript in testis and ovary (Figure 2a). Since A5928 was smaller than the gene detected on the Northern blot, 5'RACE experiments were carried out to determine the entire coding sequence of the *CXADRL1* gene. The putative full-length cDNA consisted of 3423 nucleotides, with an open reading frame of 1296 nucleotides (SEQ ID NO: 1) encoding a 431-amino-acid protein (SEQ ID NO: 2) (GenBank Accession number: AB071618). The first ATG was flanked by a sequence (CCCGGGATGA) (SEQ ID NO: 70) that was consistent with the consensus sequence for the initiation of translation in eukaryotes, with an in-frame stop codon upstream. Using the BLAST program to search for homologies in the NCBI (the National Center for Biotechnology Information) databases, a genomic sequence with the GenBank accession number AC068984 was identified, which sequence had been assigned to chromosomal band 3q13. Comparison of the cDNA and the genomic sequence revealed that CXADRL1 consisted of 7 exons (Figure 2b).

A search for protein motifs using the Simple Modular Architecture Research Tool (SMART, http://smart.embl-heidelberg.de) revealed that the predicted protein contained two immunogloblin domains (codons 29-124 and 158-232) and a transmembrane domain (codons 246-268), suggesting that CXADRL1 might belong to the immunogloblin super family.

(3) Effect of CXADRL1 on cell growth

A colony-formation assay was performed by transfecting NIH3T3 cells with a plasmid expressing CXADRL1 (pcDNA3.1myc/His-CXADRL1). Cells transfected with pcDNA3.1myc/His-CXADRL1 produced markedly more colonies than mock–transfected cells (Figure 3a). To further investigate this growth-promoting effect of CXADRL1, NIH3T3 cells that stably expressed exogenous CXADRL1 were established (Figure. 3b). The growth rate of NIH3T3-CXADRL1 cells was significantly higher than that of parental

NIH3T3 cells in culture media containing 10% FBS (Figure 3c).

(4) Suppression of CXADRL1 expression in human gastric cancer cells by antisense S-oligonucleotides

Six pairs of control and antisense S-oligonucleotides corresponding to *CXADRL1* were transfected into MKN-1 gastric cancer cells, which had shown the highest level of CXADRL1 expression among the examined six gastric cancer cell lines. Six days after transfection, viability of transfected cells was measured by MTT assay. Viable cells transfected with antisense S-oligonucleotides (CXADRL1-AS4 or –AS5) were markedly fewer than those transfected with control S-oligonucleotides (CXADRL1-S4 or –S5) (Figure 4). Consistent results were obtained in three independent experiments.

(5) Construction of plasmids expressing CXADRL1 siRNAs and their effect on the growth of gastric cancer cells

Plasmids expressing various CXADRL1-siRNA were constructed and examined for their effect on *CXADRL1* expression. Among the constructed siRNAs, psiH1BX-CXADRL7 significantly suppressed the expression of *CXADRL1* in St-4 cells (Figure 5A). To test whether the suppression of *CXADRL1* may result in growth suppression of colon cancer cells, St-4 cells were transfected with psiH1BX-CXADRL7 or mock vector. The number of viable cells transfected with psiH1BX-CXADRL7 was fewer than the number of viable control cells (Figure 5B and 5C).

(6) Preparation of anti-CXADRL1 antibody

To examine the expression and explore the function of CXADRL1, anti-sera against CXADRL1 was prepared. Immunoblotting with anti-CXADRL1 detected a 50 kD band of FLAG-tagged CXADRL1, which was almost identical by size to that detected with anti-FLAG antibody (Figure 6).

(7) Identification of a CXADRL1-interacting protein by yeast two-hybrid screening system.

To clarify the function of CXADRL1, we searched for CXADRL1-interacting proteins using yeast two-hybrid screening system. Among the positive clones identified, C-terminal region of nuclear AIP1 (atrophin interacting protein 1) interacted with CXADRL1 by simultaneous transformation using pAS2.1-CXADRL1 and pACT2-AIP1 (Figure 7) in the yeast cells. The positive clones contained codons between 808 and 1008, indicating that responsible region for the interaction in AIP1 is within this region.

(8) Prediction of candidate peptides derived from CXADRL1

Table 1 show the candidate peptides (SEQ ID NOs: 115-154) in order of high binding affinity. Forty peptides in total were selected and examined as described below.

Table1 Prediction of candidate peptides derived from CXADRL1

HLA-A*0201 9 mer

Rank	sequence	Score	Position
1	YLWEKLDNT	1314.7	176
2	LLLLSLHGV	1006.2	11
3	I NLNVI WM	49.262	53
4	WM/TPLSNA	37.961	59
5	CLVNNLPDI	23.995	120
6	SLHGVAASL	21.362	15
7	VI I I FCI AL	18.975	252
8	LI NLNVI WM	14.69	52
9	AVLPCTFTT	13.993	40
10	ALSSGLYQC	11.426	207
11	VMSRSNGSV	11.101	384
12	SI FI NNTQL	10.868	104
13	KVHRNTDSV	10.437	327
14	RI GAVPVMV	9.563	413
15	NI GVTGLTV	9.563	132
16	SI YANGTHL	9.399	356
17	LLCSSEEGI	8.691	163
18	LLSLHGVAA	8.446	13
19	I I FCI ALI L	7.575	254
20	TMPATNVSI	7.535	97

HLA-A*0201 10 mer

Rank	sequence	Score	Position
1	YLWEkLDNTL	3344	176
2	LINLnVIWMV	280.45	52
3	ALSSgLYQCV	104.33	207
4	ALININVIWM	62.845	51
5	ILLCsSEEGI	32.155	162
6	VLPCtFTTSA	32.093	41
7	LLLSIHGVAA	31.249	12
8	SIYAnGTHLV	30.603	356
9	QLSDtGTYQC	20.369	111
10	GLYQcVASNA	15.898	211
11	PLLLISLHGV	13.022	10
12	IQVArGQPAV	11.988	32
13	FINNtQLSDT	10.841	106
14	LVPGqHKTLV	10.346	364
15	NLPDiGGRNI	8.555	124
16	VLVPpSAPHC	8.446	140
17	AVIIiFCIAL	7.103	251
18	VIIIfCIALI	5.609	252
19	ILGAfFYWRS	5.416	261
20	GLTVIVPPSA	4.968	137

(9) Stimulation of the T cells and establishment of CTL clones using the candidate peptides

Lymphoid cells were cultured using these candidate peptides derived from CXADRL1 in the manner described in "Materials and Methods". Resulting lymphoid cells showing detectable cytotoxic activity were expanded, and CTL clone was established. CTL clone was propagated from the CTL lines described above using limiting dilution methods. CTL clone induced with CXADRL1-207 (ALSSGLYQC) (SEQ ID NO: 124) showed the higher cytotoxic activities against the target pulsed with peptides when compared with those against targets not pulsed with any peptides. Cytotoxic activity of this CTL clone was shown in Figure 8. This CTL clone had very potent cytotoxic activity against the peptide-pulsed target without showing any cytotoxic activity against the target not pulsed with any peptides.

(10) Cytotoxic activity against tumor cell lines endogenously expressing CXADRL1 as a target

The CTL clones raised against predicted peptides were examined for their ability to recognize and kill the tumor cells endogenously expressing CXADRL1. Figure 9 shows the results of CTL Clone 75 raised against CXADRL1-207 (ALSSGLYQC) (SEQ ID NO: 124). CTL Clone 75 showed potent cytotoxic activity against SNU475 which expresses

CXADRL1 and HLA-A*0201, however it did not show against MKN74 which expressed CXADRL1 but not HLA-A*0201, and did not show against SNU-C4 which expressed HLA-A*0201 but not CXADRL1.

(11) Specificity of the established CTLs

Cold target inhibition assay was also performed to confirm the specificity of CXADRL1-207 CTL Clone. SNU475 cells labeled by ^{51}Cr were used as a hot target, while T2 cells pulsed with CXADRL1-207 (SEQ ID NO: 124) were used without ^{51}Cr labeling as a cold target. Specific cell lysis against SNU475 cells was significantly inhibited, when T2 pulsed with CXADRL1-207 (SEQ ID NO: 124) was added in the assay at various ratios (Figure 10). Theses results were indicated as a percentage of specific lysis at the E/T ratio of 20.

Regarding CXADRL1-207 CTL clone, to examine the characteristics of these CTL clones, antibodies against HLA-Class I, HLA-Class II, CD4 and CD8 were tested for their capacity to inhibit the cytotoxic activity. The cytotoxicity of CTL clone against SNU475 cells was significantly inhibited when anti-HLA-Class I antibody and anti-CD8 antibody were used (Figure 11), indicating that the CTL clone recognize the CXADRL1 derived peptide in a HLA-Class I and CD8 manner.

(12) Expression and characterization of novel human gene *GCUD1*

Multi-tissue northern blot analysis using GUCD1 cDNA as a probe showed a 5.0-kb transcript expressed specifically in testis, ovary, and brain (Figure 12). Although the nucleotide sequence of KIAA0913 (GenBank Accession Number: XM-014766), corresponding to GCUD1, consisted of 4987 nucleotides, RT-PCR experiments using testis, ovary and cancer tissues revealed a transcript that consisted of 4987 nucleotides containing an open reading frame of 1245 nucleotides (SEQ ID NO: 3) (GenBank Accession Number: AB071705). Furthermore, the genomic sequence corresponding to *GUCD1* was searched in genomic databases to find a draft sequence assigned to chromosomal band 7p14 (GenBank Accession Number: NT-007819). Comparison between the cDNA sequence and the genomic sequence revealed that the *GUCD1* gene consisted of 8 exons.

(13) Subcellular localization of GCUD1

The entire coding region corresponding to *GCUD1* was cloned into pCDNA3.1myc/His vector and the construct was transiently transfected into COS7 cell. Immunocytochemical staining of the COS7 cell revealed that the tagged-GCUD1 protein was present in the cytoplasm (Figure 13).

(14) Effect of GCUD1 on cell growth

To analyze the effect of GCUD1 on cell growth, a colony-formation assay was conducted by transfecting NIH3T3 cells with a plasmid expressing GCUD1

(pcDNA3.1myc/His-GCUD1). Compared with a control plasmid (pcDNA3.1myc/His-LacZ), pcDNA3.1myc/His-GCUD1 induced markedly more colonies in NIH3T3 cells (Figure 14). This result was confirmed by three independent experiments.

(15) Growth suppression of gastric cancer cells by antisense S-oligonucleotides designated to reduce expression of GCUD1

To test whether the suppression of GCUD1 may result in cell death of gastric cancer cells, various antisense S-oligonucleotides designed to suppress the expression of GCUD1 were synthesized. Six days after transfection of the respective antisense S-oligonucleotides, viability of transfected cells was measured by MTT assay. Viable cells transfected with antisense S-oligonucleotides (GCUD1-AS5 or -AS8) were markedly fewer than those transfected with control S-oligonucleotides (GCUD1-S5 or -S8) in MKN-28 cells (Figure 15). This result was confirmed by three independent experiments. Similar result was observed with MKN-1 cells.

(16) Preparation of anti-GCUD1 antibody

To examine the expression and explore the function of GCUD1, anti-sera against GCUD1 was prepared. Recombinant protein of GCUD1 was extracted and purified from bacterial cells expressing GST-GCUD1 fusion protein (Figure 16). The recombinant protein was used for immunization of three rabbits. Immunoblotting with anti-GCDU1 sera but not pre-immune sera showed a 47 kD band of FLAG-tagged GCUD1, which was almost identical by size to that detected with anti-FLAG antibody (Figure 17).

(17) Prediction of candidate peptides derived from GCUD1

Table 2 (GCUD1) shows the candidate peptides (SEQ ID NOs: 155-194) in order of high binding affinity. Forty peptides in total were selected and examined as described below.

Table. 2 Prediction of candidate peptides derived from GCUD1

HLA-A*0201 9 mer

Rank	sequence	Score	Position
1	SIFKPFIFV	369.77	303
2	WLWGAEMGA	189.68	75
3	IMISRPAWL	144.26	68
4	LLGMDLVRL	83.527	107
5	FIFVDDVKL	49.993	308
6	VCIDSEFFL	31.006	265
7	KPFIFVDDV	25.18	306
8	IVDRDEAWV	22.761	159
9	TLRDKASGV	21.672	257
10	KMDAEHPEL	21.6	196
11	ALDVIVSLL	19.653	126
12	YAQSQGWWT	19.639	207
13	KLRSTMLEL	13.07	367
14	YLIVDRDEA	11.198	157
15	AAPPSYCFV	7.97	3
16	GMDLVRLGL	6.171	109
17	KVTEGVRCI	6.026	179
18	CIDSEFFLT	4.517	266
19	TVQTMMNTL	4.299	250
20	EMGANEHGV	3.767	80

HLA-A*0201 10 mer

Rank	sequence	Score	Position
1	FIFVdDVKLV	374.37	308
2	LIVDrDEAWV	366.61	158
3	FLTTaSGVSV	319.94	272
4	TMLEIEKQGL	234.05	371
5	ALLGmDLVRL	181.79	106
6	AIMIsRPAWL	59.775	67
7	GVCIdSEFFL	59.628	264
8	KLVPkTQSPC	17.388	315
9	FNFSeVFSPV	14.682	220
10	YISIdQVPRT	10.841	56
11	GEGEfNFSEV	10.535	216
12	WAAEkVTEGV	8.927	175
13	VLPQnRSSPC	8.446	281
14	AAAPpSYCFV	7.97	2
15	TMMNtLRDKA	6.505	253
16	EVGDIFYDCV	5.227	397
17	AEMGaNEHGV	5.004	79
18	GLVVfGKNSA	4.968	20
19	QLSLtTKMDA	4.968	190
20	RSIFkPFIFV	4.745	302

(18) Stimulation of the T cells and establishment of CTL clones using the candidate peptides

Lymphoid cells were cultured using these candidate peptides derived from GCUD1 in the manner described in "Materials and Methods". Resulting lymphoid cells showing detectable cytotoxic activity were expanded, and CTL clones were established. CTL clones were propagated from the CTL lines described above using limiting dilution methods. CTL clones induced with GCUD1-196 (KMDAEHPEL) (SEQ ID NO: 164) and GCUD1-272 (FLTTASGVSV) (SEQ ID NO: 177) showed the higher cytotoxic activities against the target pulsed with peptides when compared with those against targets not pulsed with any peptides. Cytotoxic activity of these CTL clones was shown in Figure 18. Each CTL clone had very potent cytotoxic activity against the peptide-pulsed target without showing any cytotoxic activity against the target not pulsed with any peptides.

(19) Cytotoxic activity against tumor cell lines endogenously expressing GCUD1 as a target

The CTL clones raised against predicted peptides were examined for their ability to recognize and kill the tumor cells endogenously expressing GCUD1. Figure 19 shows the results of CTL Clone 23 raised against GCUD1-196 (SEQ ID NO: 164). CTL Clone 23

showed potent cytotoxic activity against SNU475 which expresses GCUD1 and HLA-A*0201, however it did not show against MKN45 which expressed GCUD1 but not HLA-A*0201.

(20) Specificity of the established CTLs

Cold target inhibition assay was also performed to confirm the specificity of GCUD1-196 CTL Clone. SNU475 cells labeled by ^{51}Cr were used as a hot target, while T2 cells pulsed with GCUD1-196 were used without ^{51}Cr labeling as a cold target. Specific cell lysis against SNU475 cells was significantly inhibited, when T2 pulsed with GCUD1-196 (SEQ ID NO: 164) was added in the assay at various ratios (Figure 20). Theses results were indicated as a percentage of specific lysis at the E/T ratio of 20.

Regarding GCUD1-196 (SEQ ID NO: 164) CTL clone, to examine the characteristics of these CTL clones, antibodies against HLA-Class ~~I,~~ HLA-Class II, CD4 and CD8 were tested for their capacity to inhibit the cytotoxic activity. The cytotoxicity of CTL clone against SNU475 cells was significantly inhibited when anti-HLA-Class I antibody and anti-CD8 antibody were used (Figure 21), indicating that the CTL clone recognize the GCUD1 derived peptide in a HLA-Class I and CD8 manner.

(21) Identification of gene *FLJ20315* commonly up-regulated in human colon cancer

Expression profiles of 11 colon cancer tissues were compared with non-cancerous mucosal tissues of the colon corresponding thereto using the cDNA microarray containing 23040 genes. According to this analysis, expression levels of a number of genes that were frequently elevated in cancer tissues compared to corresponding non-cancerous tissues. Among them, a gene with an in-house accession number of B4469 corresponding to an EST (FLJ20315), Hs.18457 in UniGene cluster (http://www.ncbi.nlm.nih.gov/UniGene/), was up-regulated in the cancer tissues compared to the corresponding non-cancerous mucosae at a magnification range between 1.44 and 11.22 (Figure 22a). *FLJ20315* was also up-regulated in 6 out of 18 gastric cancer cases, 12 out of 20 HCC cases, 11 out of 22 lung cancer(adenocarcinoma) cases, 2 out of 2 testicular seminomas cases and 3 out of 9 prostate cancer cases. To clarify the results of the microarray, the expression of these transcripts in additional colon cancer samples were examined by semi-quantitative RT-PCR to confirm the increase of FLJ20315 expression in 15 of the 18 tumors (Figure 22b).

(22) Expression and Structure of RNF43

Additional homology searches of the sequence of *FLJ20315* in public databases using BLAST program in National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/) identified ESTs including XM_097063, BF817142 and a genomic sequence with a GenBank Accession Number NT-010651 assigned to

chromosomal band 17pter-p13.1. As a result, an assembled sequence of 5345 nucleotides containing an open reading frame of 2352 nucleotides (SEQ ID NO: 5) encoding a 783-amino-acid protein (SEQ ID NO: 6) (GenBank Accession Number: AB081837) was obtained. The gene was dubbed RNF43 (Ring finger protein 43). The first ATG was flanked by a sequence (AGCATGC) that agreed with the consensus sequence for initiation of translation in eukaryotes, and by an in-frame stop codon upstream. Comparison of the cDNA and the genomic sequence revealed that this gene consisted of 11 exons. Northern-blot analysis using human adult Multiple-Tissue northern-blots with a PCR product of RNF43 as a probe failed to detect any band (data not shown). However, a 5.2 kb-transcript was detected to be expressed in fetal lung and fetal kidney using a human fetal tissue northern-blot with the same PCR product as a probe (Figure 23a). A search for protein motifs with the Simple Modular Architecture Research Tool (SMART, http://smart.embl-heidelberg.de) revealed that the predicted protein contained a Ring finger motif (codons 272-312) (Figure 23b).

(23) Subcellular localization of myc-tagged RNF43 protein

To investigate the subcellular localization of RNF43 protein, a plasmid expressing myc-tagged RNF43 protein (pDNAmyc/His-RNF43) was transiently transfected into COS7 cells. Western blot analysis using extracts from the cells and anti-myc antibody revealed a 85.5-KDa band corresponding to the tagged protein (Figure 24a). Subsequent immunohistochemical staining of the cells with the same antibody indicated the protein to be mainly present in the nucleus (Figure 24b). Similar subcellular localization of RNF43 protein was observed in SW480 human colon cancer cells.

(24) Effect of RNF43 on cell growth

A colony-formation assay was conducted by transfecting NIH3T3 cells with a plasmid expressing RNF43 (pcDNA-RNF43). Cells transfected with pcDNA-RNF43 produced significantly more number of colonies than control cells (Figure 25a). Increased activity of colony formation by RNF43 was also shown in SW480 cells wherein the endogenous expression of RNF43 was very low (data not shown). To further investigate this growth-promoting effect, COS7 cells that stably express exogenous RNF43 (COS7-RNF43) were established (Figure 25b). The growth rate of COS7-RNF43 cells was significantly higher than that of COS7-mock cells in culture media containing 10% FBS (Figure 25c).

(25) Growth suppression of colon cancer cells by antisense S-oligonucleotides designated to reduce expression of RNF43

To test whether the suppression of RNF43 expression may result in growth retardation and/or cell death of colon cancer cells, five pairs of control and antisense

S-oligonucleotides corresponding to RNF43 were synthesized and transfected into LoVo colon cancer cells, which shown a higher level of RNF43 expression among the examined 11 colon cancer cell lines. Among the five antisense S-oligonucleotides, RNF43-AS1 significantly suppressed the expression of RNF43 compared to control S-oligonucleotides (RNF43-S1) 12 hours after transfection (Figure 26a). Six days after transfection, number of surviving cells transfected with RNF43-AS1 was significantly fewer than those transfected with RNF43-S1 suggesting that the suppression of RNF43 expression reduced growth and/or survival of transfected cells (Figure 26b). Consistent results were obtained in three independent experiments.

(26) Construction of plasmids expressing RNF43 siRNAs and their effect on growth of colon cancer cells

In mammalian cells, small interfering RNA (siRNA) composed of 20 or 21-mer double-stranded RNA (dsRNA) with 19 complementary nucleotides and 3' terminal complementary dimmers of thymidine or uridine, has been recently shown to have a gene specific gene silencing effect without inducing global changes in gene expression. Therefore, plasmids expressing various RNF43-siRNAs were constructed to examine their effect on *RNF43* expression. Among the various RNF43-siRNAs, psiH1BX-RNF16-4 and psiH1BX-RNF1834 significantly suppressed the expression of *RNF43* in SNUC4 cells (Figure 27A). To test whether the suppression of *RNF43* results in growth suppression of colon cancer cells, SNUC4 cells were transfected with psiH1BX-RNF16-4, psiH1BX-RNF1834 or mock vector. In line with the data of antisense S-oligonucleotides, the number of viable cells transfected with psiH1BX-RNF16-4 or psiH1BX-RNF1834 was fewer than the number of viable control cells (Figure 27B, 27C).

(27) Secretion of Flag-tagged RNF43 protein in culture media of COS7 cells with exogenous Flag-tagged RNF43 protein

Since a search for protein motifs with amino acid sequence of RNF43 using Simple Modular Architecture Research Tool (SMART, http://smart.embl-heidelberg.de) predicted a signal peptide and a ring finger domain, secretion of the RNF43 protein was examined. Plasmid expressing Flag-tagged RNF43 (pFLAG-RNF43) or Myc-tagged RNF43 (pcDNA3.1-Myc/His-RNF43), or mock vector was transfected into COS7 cells, and the cells were cultured in media supplemented with 0.5 % of bovine calf serum for 48 hours . Western blot analysis with anti-Flag antibody or anti-Myc antibody detected secreted Flag-tagged RNF43 or Myc-tagged protein in the media containing cells transfected with pFLAG-RNF43 or pcDNA3.1-Myc/His-RNF43, respectively, but not in the media containing cells with mock vector (Figure 28A and 28B).

(28) Effect of cultured media of cells transfected with pFLAG-RNF43 on NIH3T3 cells

Since exogenous expression of RNF43 conferred growth promoting effect on NIH3T3 cells, secreted Flag-tagged RNF43 was examined whether it also has a proliferative effect on NIH3T3 cells. NIH3T3 cells were cultured without the change of media, or with conditioned media of cells transfected with mock-transfected cells, or those with pFlag-RNF43. As expected, cells transfected with either pFlag-RNF43 or pcDNA3.1-Myc/His-RNF43 cultured in conditioned media showed a significantly higher growth rate compared to non-treated cells or mock-vector transfected cells cultured in conditioned media (Figure 29A and 29B). These data suggest that RNF43 may exert its growth promoting effect in an autocrining manner.

(29) Preparation of recombinant amino- and carboxyl-terminal RNF43 protein

To generate a specific antibody against RNF43, a plasmid expressing Nus-tagged RNF43 protein was constructed (Figure 30A). Upon transformation of the plasmid into *E. coli* BL21trxB(DE3)pLysS cell, production of a recombinant protein in the bacterial extract with the expected size was observed by SDS-PAGE (Figure 30B and 30C).

(30) Identification of RNF43-interacting proteins by yeast two-hybrid screening system

To clarify the oncogenic mechanism of RNF43, RNF43-interacting proteins were searched using yeast two-hybrid screening system. Among the identified positive clones, NOTCH2 or STRIN interacted with RNF43 by simultaneous transformation of an yeast cell with pAS2.1-RNF43 and pACT2-NOTCH2 (Figure 31B), or pAS2.1-RNF43 and pACT2-STRIN (Figure 32B). The regions responsible for the interaction in NOTCH2 and STRIN are indicated in Figure 31A and Figure 32A, respectively.

(31) Prediction of HLA-A24 binding peptides derived from RNF43

The amino acid sequence of RNF43 was scanned for peptides with a length of 9 or 10 amino acids which peptides bind to HLA-A24 using the binding prediction soft (http://bimas.dcrt.nih.gov/cgi-bin/molbio/ken_parker_comboform). Table 3 shows the predicted peptides (SEQ ID NOs: 71-90) in order of high binding affinity. Twenty peptides in total were selected and examined as described below.

Table 3

Predicted *RNF43* peptides binding to HLA-A24

(http://bimas.dcrt.nih.gov/cgi-bin/molbio/ken_parker_comboform)

Start position	AA sequence (9mers)	Binding affinity *1	Start position	AA sequence (10mers)	Binding affinity
RNF43-331	SYQEPGRRL	360	RNF43-449	SYCTERSGYL	200
RNF43-350	HYHLPAAYL	200	RNF43-350	HYHLPAAYLL	200
RNF43-639	LFNLQKSSL	30	RNF43-718	CYSNSQPVWL	200
RNF43-24	GFGRTGLVL	20	RNF43-209	IFVIILASVL	36
RNF43-247	RYQASCRQA	15	RNF43-313	VFNITEGDSF	15
RNF43-397	RAPGEQQRL	14	RNF43-496	TFCSSLSSDF	12
RNF43-114	RAPRPCLSL	12	RNF43-81	KLMQSHPLYL	12
RNF43-368	RPPRPGPFL	12	RNF43-54	KMDPTGKLNL	9
RNF43-45	KAVIRVIPL	12	RNF43-683	HYTPSVAYPW	8
RNF43-721	NSQPVWLCL	10	RNF43-282	GQELRVISCL	4

In the table, start position indicates the location of amino acids from the N-terminus of RNF43.

(32) Stimulation of T cells using the predicted peptides

CTLs against these peptides derived from RNF43 were generated according to the method described in the "Materials and Methods". Resulting CTLs showing detectable cytotoxic activity were expanded, and CTL lines were established.

The cytotoxic activities of CTL lines induced by 9 mer-peptide (SEQ ID NOs: 71-80) stimulation are shown in Table 4.

Table 4 Cytotoxicity of CTL lines (9mer)

Start position	AA sequences	Binding affinity	Cytotoxicity ×20 Pep(+)	Cytotoxicity ×20 Pep(-)	Cytotoxicity ×2 Pep(+)	Cytotoxicity ×2 Pep(-)	Established CTL clones
RNF43-331	SYQEPGRRL	360.0	2%	1%	0%	0%	
RNF43-350	HYHLPAAYL	200.0	26%	17%	5%	4%	
RNF43-639	LFNLQKSSL	30.0	42%	33%	7%	5%	1
RNF43-24	GFGRTGLVL	20.0	8%	9%	1%	2%	
RNF43-247	RYQASCRQA	15.0	71%	82%	28%	16%	
RNF43-397	RAPGEQQRL	14.4	41%	32%	15%	15%	
RNF43-114	RAPRPCLSL	12.0	23%	26%	6%	9%	
RNF43-368	RPPRPGPFL	12.0	1%	0%	0%	0%	
RNF43-45	KAVIRVIPL	12.0	NE				
RNF43-721	NSQPVWLCL	10.0	68%	0%	26%	0%	13

NE:No establishment of CTL lines

CTL lines stimulated with RNF43-350 (HYHLPAAYL) (SEQ ID NO: 72), RNF43-639 (LFNLQKSSL) (SEQ ID NO: 73), and RNF43-721 (NSQPVWLCL) (SEQ ID NO: 80) showed higher cytotoxic activities on the target that were pulsed with peptides compared with those on target that was not pulsed with any of the peptides. Starting from these CTLs, one CTL clone was established with RNF43-639 and 13 CTL clones were established with RNF43-721.

The CTL line stimulated with RNF43-721 showed a potent cytotoxic activity on the peptide-pulsed target without showing any significant cytotoxic activity on target that was not pulsed with any of the peptides (Figure 33).

The results obtained by examining the cytotoxic activity of CTL lines stimulated with the 10 mer-peptides (SEQ ID NOs: 81-90) are shown in Table 5.

Table 5 Cytotoxicity of CTL lines (10mer)

Start position	AA sequences	Binding affinity	Cytotoxicity ×20 Pep(+)	Cytotoxicity ×20 Pep(-)	Cytotoxicity ×2 Pep(+)	Cytotoxicity ×2 Pep(-)	Established CTL clones
RNF43-449	SYCTERSGYL	200.0	1%	1%	0%	0%	
RNF43-350	HYHLPAAYLL	200.0	NE				
RNF43-718	CYSNSQPVWL	200.0	NE				
RNF43-209	IFVLILASVL	36.0	Not synthesis				
RNF43-313	VFNLTEGDSF	15.0	Not synthesis				
RNF43-496	TFCSSLSSDF	12.0	8%	9%	0%	0%	
RNF43-81	KLMQSHPLYL	12.0	10%	5%	2%	-3%	
RNF43-54	KMDPTGKLNL	9.6	5%	2%	0%	-1%	
RNF43-683	HYTPSVAYPW	8.4	0%	0%	0%	-1%	—
RNF43-282	GQELRVISCL	8.4	NE				

NE:No establishment of CTL lines

CTL lines stimulated with RNF43-81 (KLMQSHPLYL) (SEQ ID NO: 87) or RNF43-54 (KMDPTGKLNL) (SEQ ID NO: 88) showed modest cytotoxic activity on the peptide-pulsed target compared with that on the target that was not pulsed with any of the peptides.

(33) Establishment of CTL clones

CTL clones were propagated from the CTL lines described above using the limiting dilution method. 13 CTL clones against RNF43-721 and 1 CTL clone against RNF43-639 were established (see Table 4 supra). The cytotoxic activity of RNF43-721 CTL clones is shown in Figure 34. Each CTL clone had a very potent cytotoxic activity on the peptide-pulsed target without showing any cytotoxic activity on the target that was not pulsed with any of the peptides.

(34) Cytotoxic activity against colorectal cancer cell lines endogenously expressing RNF43 as target

The CTL clones raised against predicted peptides were examined for their ability to recognize and kill tumor cells that endogenously express RNF43. Figure35 shows the results of CTL Clone 45 raised against RNF43-721. CTL Clone 45 showed a potent cytotoxic activity on HT29 and WiDR both expressing RNF43 and HLA-A24. On the other hand, CTL Clone 45 did not show any cytotoxic activity on either HCT116 (expressing RNF43 but not HLA-A24) or TISI (expressing HLA-A24 but not RNF43). Moreover, CTL Clone 45 did not show any cytotoxic activity on irrelevant peptide pulsed

TISI and SNU-C4 that express RNF43 but little HLA-A24 (data not shown).

(35) Characterization of established CTLs

A cold target inhibition assay was performed to confirm the specificity of RNF43-721 CTL Clone. HT29 cells labeled by ^{51}Cr were used as a hot target, while TISI cells pulsed with RNF43-721 without ^{51}Cr labeling were used as a cold target. When TISI pulsed with RNF43-721 was added in the assay at various ratios, specific cell lysis against the HT29 cell target was significantly inhibited, (Figure 36). This result is indicated as a percentage of specific lysis at the E/T ratio of 20. To examine the characteristics of the CTL clone raised against RNF43 peptide, antibodies against HLA-Class I, HLA-Class II, CD3, CD4 and CD8 were tested for their ability to inhibit the cytotoxic activity of the CTL clone. The cytotoxicity of the CTL clone on the WiDR cell target was significantly inhibited when anti-HLA-Class I, CD3 and CD8 antibodies were used (Figure 37). The result indicates that the CTL clone recognizes the RNF43 derived peptide via HLA-Class I, CD3 and CD8.

(36) Homology analysis of RNF43-721 peptide

The CTL clones established against RNF43-721 showed a very potent cytotoxic activity. This result may indicate that the sequence of RNF43-721 is homologous to the peptides derived from other molecules which are known to sensitize human immune system. To exclude this possibility, homology analysis of RNF43-721 was performed using BLAST (http://www.ncbi.nlm.nih.gov/blast/blast.cgi). No sequence completely matching or highly homologous to RNF43-721 was found among the molecules listed in BLAST (Table 6).

Table 6

Homology analysis of RNF43-721

(BLAST http://www.ncbi.nlm.nih.gov/blast/blast.cgi)

Identification(9/9)	0
Identification(8/9)	0
Identification(7/9)	0
Identification(6/9)	2

These results indicate that the sequence of RNF43-721 is unique and there is little possibility for the CTL clones established with RNF43-721 to raise immunologic response to other molecules.

(37) Modification of RNF43-721 to improve the efficacy of epitope presentation

To improve the efficacy of peptide presentation, RNF43-721 peptide were modified at amino acid alternations on the anchor site. The modification was expected to improve the binding affinity of the peptide to the HLA Class I molecule. Table 7 demonstrates a better binding affinity to HLA-A24 molecule of RNF43-721 with alternations of amino acids at position 2 (SEQ ID NOs: 91 and 92).

Table 7

Predicted binding capacities of the peptides modified from the RNF43-721 native peptide

Sequence	Score	Rank*
NSQPVWLCL	10.08	10
NFQPVWLCL	50.40	3
NYQPVWLCL	504.00	1

*** In HLA-A24 restricted 9mer peptides**

(38) Prediction of candidate peptides derived from RNF43

Table 8 shows candidate peptides (SEQ ID NOs: 87, and 93-111) in order of high binding affinity.

Table 8

RNF43:Prediction of epitope peptides (HLA-A*0201)

9mer

No	Starting position	Sequences	Score
1	60	KLNLTLEGV	274.3
2	8	QLAALWPWL	199.7
3	82	LMQSHPLYL	144.2
4	358	LLGPSRSAV	118.2
5	11	ALWPWLLMA	94.8
6	15	WLLMATLQA	84.5
7	200	WILMTVVGT	40.1
8	171	KLMEFVYKN	34.5
9	62	NLTLEGVFA	27.3
10	156	GLTWPVVLI	23.9

10mer

No	Starting position	Sequences	Score
11	81	KLMQSHPLYL	1521.5
12	357	YLLGPSRSAV	1183.7
13	202	LMTVVGTIFV	469.6
14	290	CLHEFHRNCV	285.1
15	500	SLSSDFDPLV	264.2
16	8	QLAAIWPWLL	160.2
17	11	ALWPWLLMAT	142.2
18	7	LQLAALWPWL	127.3
19	726	WLCLTPRQPL	98.2
20	302	WLHQHRTCPL	98.2

Twenty peptides in total were selected and examined as described below.

(39) Stimulation of T cells using candidate peptides

Lymphoid cells were cultured using the candidate peptides derived from RNF43 according to the method described in the "Materials and Methods". Resulting lymphoid cells showing detectable cytotoxic activity were expanded, and CTL lines were established. The cytotoxic activities of CTL lines induced by the stimulation using 9 mer-peptides (SEQ ID NOs: 93-102) are shown in Table 9.

Table 9

Cytotoxicity of CTL lines (HLA-A*0201 9mer)

Start position	AA sequences	Binding affinity	Cytotoxicity(%) ×20 Pep(+)	×20 Pep(-)	×2 Pep(+)	×2 Pep(-)
RNF43-60	KLNLTLEGV	274.3	-2.1	0.2	-1.6	0.0
RNF43- 8	QLAALWPWL	199.7	3.5	0.0	0.0	1.0
RNF43-82	LMQSHPLYL	144.2	1.7	1.2	0.0	-0.4
RNF43-358	LLGPSRSAV	118.2	-0.4	-0.7	0.0	-0.8
RNF43-11	ALWPWLLMA	94.8	90.2	1.5	45.4	1.3
RNF43-15	WLLMATLQA	84.5	-0.2	0.0	-0.4	-0.9
RNF43-200	WILMTVVGT	40.1	Not Synthesis			
RNF43-171	KLMEFVYKN	34.5	2.6	0.0	1.1	-0.5
RNF43-62	NLTLEGVFA	27.3	Not Synthesis			
RNF43-156	GLTWPVVLI	23.9	-0.4	0.7	-0.5	-0.3

NE:No establishment of CTL lines

CTL lines induced with RNF43-11-9 (ALWPWLLMA) (SEQ ID NO: 97) showed higher cytotoxic activities on the target pulsed with peptides compared with those on the target that was not pulsed with any of the peptides. Starting from these CTLs, four CTL clone were established with RNF43-11-9. The CTL line stimulated with RNF43-11-9 showed a potent cytotoxic activity on the peptide-pulsed target without showing any significant cytotoxic activity on the target that was not pulsed with any of the peptides (Figure 38A).

The results of examination on the cytotoxic activity of CTL lines induced with the 10 mer-peptides (SEQ ID NOs: 87, and 103-111) are shown in Table 10.

Table 10

Cytotoxicity of CTL lines (HLA-A*0201 10mer)

Start position	AA sequences	Binding affinity	Cytotoxicity(%) ×20 Pep(+)	×20 Pep(-)	×2 Pep(+)	×2 Pep(-)
RNF43-81	KLMQSHPLYL	1521.5	18.0	27.6	6.3	8.3
RNF43-357	YLLGPSRSAV	1183.7	18.2	15.4	3.7	3.0
RNF43-202	LMTVVGTIFV	469.6	Not Synthesis			
RNF43-290	CLHEFHRNCV	285.1	9.6	9.7	2.7	3.7
RNF43-500	SLSSDFDPLV	264.2	NE			
RNF43-8	QLAAIWPWLL	160.2	6.7	9.0	1.1	1.3
RNF43-11	ALWPWLLMAT	142.2	91.5	27.1	40.5	4.3
RNF43-7	LQLAALWPWL	127.3	NE			
RNF43-726	WLCLTPRQPL	98.2	NE			
RNF43-302	WLHQHRTCPL	98.2	7.4	6.1	1.5	2.2

NE:No establishment of CTL lines

CTL lines induced with RNF43-11-10 (ALWPWLLMAT) (SEQ ID NO: 108) showed a higher cytotoxic activity on the peptide-pulsed target compared with that on the target that was not pulsed with any of the peptides (Figure 38B).

(40) Establishment of CTL clones

CTL clones were propagated from the CTL lines described above using the limiting dilution method. Four CTL clones against RNF43-11-9 were established (see Table 9 supra). The cytotoxic activity of RNF43 peptides-derived CTL clones is shown in Figure 39A and 39B. Each CTL clone had a very potent cytotoxic activity on the peptide-pulsed target without showing any cytotoxic activity on the target that was not pulsed with any of the peptides.

(41) Cytotoxic activity against colorectal cancer cell lines endogenously expressing RNF43 as targets

The CTL clones raised against the predicted peptides were examined for their ability to recognize and kill tumor cells that endogenously express RNF43. Figure 40A and 40B show the results obtained for the CTL clones raised against RNF43 derived peptides. The CTL Clones showed a potent cytotoxic activity on DLD-1 which expresses RNF43 and HLA-A*0201, but none on HT29 which expresses RNF43 but not

HLA-A*0201.

(42) Specificity of CTL clones

A cold target inhibition assay was performed to confirm the specificity of RNF43-5-11(9mer) CTL Clone. HCT116 cells labeled with ^{51}Cr were used as a hot target, while T2 cells pulsed with RNF43-5 without ^{51}Cr labeling were used as a cold target. Specific cell lysis of the HCT-116 cell target was significantly inhibited, when T2 pulsed with RNF43-5 was added in the assay at various ratios (Figure 41).

Industrial Applicability

The expression of novel human genes *CXADRL1* and *GCUD1* is markedly elevated in gastric cancer as compared to non-cancerous stomach tissues. On the other hand, the expression of novel human gene RNF43 is markedly elevated in colorectal cancers as compared to non-cancerous mucosal tissues. Accordingly, these genes may serve as a diagnostic marker of cancer and the proteins encoded thereby may be used in diagnostic assays of cancer.

The present inventors have also shown that the expression of novel protein CXADRL1, GCUD1, or RNF43 promotes cell growth whereas cell growth is suppressed by antisense oligonucleotides or small interfering RNAs corresponding to the *CXADRL1*, *GCUD1*, or *RNF43* gene. These findings suggest that each of CXADRL1, GCUD1, and RNF43 proteins stimulate oncogenic activity. Thus, each of these novel oncoproteins is useful targets for the development of anti-cancer pharmaceuticals. For example, agents that block the expression of CXADRL1, GCUD1, or RNF43, or prevent its activity may find therapeutic utility as anti-cancer agents, particularly anti-cancer agents for the treatment of gastric and colorectal cancers. Examples of such agents include antisense oligonucleotides, small interfering RNAs, and antibodies that recognize CXADRL1, GCUD1, or RNF43.

Furthermore, the present inventors have shown that CXADRL1 interacts with AIP1. It is expected that the cell proliferating activity of CXADRL1 is regulated by its binding to AIP1. Thus, agents that inhibit the activity of the formation of a complex composed of CXADRL1 and AIP1 may also find utility in the treatment and prevention of cancer, specifically colorectal, lung, gastric, and liver cancers. Alternatively, the present inventors have shown that RNF43 interacts with NOTCH2 or STRIN. It is expected that the cell proliferating activity of RNF43 is regulated by its binding to NOTCH2 or STRIN. Thus, agents that inhibit the activity of the formation of a complex composed of RNF43 and NOTCH2 or STRIN may also find utility in the treatment and prevention of cancer, specifically colorectal, lung, gastric, and liver cancers.

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

CLAIMS

1. A small interfering RNA against the polynucleotide encoding the polypeptide consisting of the amino acid sequence of SEQ ID NO: 6, wherein the sense strand of said small interfering RNA is the nucleotide sequence of SEQ ID NO: 112 as the target sequence.

2. An in vitro method for diagnosing colorectal cancer, said method comprising the steps of:

(a) detecting the expression level of the gene encoding the amino acid sequence of SEQ ID NO: 6 in a biological sample of specimen; and

(b) relating an elevation of the expression level to colorectal cancer.

3. The method of claim 2, wherein the expression level is detected by any one of the method selected from the group consisting of:
   (a) detecting the mRNA encoding the amino acid sequence of SEQ ID NO: 6,
   (b) detecting the protein consisting of the amino acid sequence of SEQ ID NO: 6, and
   (c) detecting the biological activity of the protein consisting of the amino acid sequence of SEQ ID NO: 6.
4. A method of screening for a compound for treating colorectal cancer, said method comprising the steps of:
   (a) contacting a test compound with a polypeptide selected from the group consisting of:
   (1) a polypeptide consisting of the amino acid sequence of SEQ ID NO: 6;
   (2) a polypeptide that consists of the amino acid sequence of SEQ ID NO: 6 in which one or more amino acids are substituted, deleted, inserted, and/or added and that has a cell-proliferating activity equivalent to a protein consisting of the amino acid sequence of SEQ ID NO: 6; and
   (3) a polypeptide encoded by a polynucleotide that hybridizes under stringent conditions to a polynucleotide consisting of the nucleotide sequence of SEQ ID NO: 5, wherein the polypeptide has a cell-proliferating activity equivalent to a polypeptide consisting of the amino acid sequence of SEQ ID NO: 6;
   (b) detecting the binding activity between the polypeptide and the test compound; and
   (c) selecting a compound that binds to the polypeptide.
5. A method of screening for a compound for treating colorectal cancer, said method comprising the steps of:
   (a) contacting a candidate compound with a cell expressing one or more polynucleotides consisting of the nucleotide sequence of SEQ ID NO: 5; and
   (b) selecting a compound that reduces the expression level of one or more polynucleotides consisting of the nucleotide sequence of SEQ ID NO: 5 in comparison with the expression level detected in the absence of the test compound.
6. A method of screening for a compound for treating a colorectal cancer, said method comprising the steps of:
   (a) contacting a test compound with a polypeptide selected from the group consisting of:
   (1) a polypeptide consisting of the amino acid sequence of SEQ ID NO: 6;
   (2) a polypeptide that consists of the amino acid sequence of SEQ ID NO: 6 in which one or more amino acids are substituted, deleted, inserted, and/or added and that has a cell-proliferating activity equivalent to a protein consisting of the amino acid sequence of SEQ ID NO: 6; and

(3) a polypeptide encoded by a polynucleotide that hybridizes under stringent conditions to a polynucleotide consisting of the nucleotide sequence of SEQ ID NO: 5, wherein the polypeptide has a cell-proliferating activity equivalent to a polypeptide consisting of the amino acid sequence of SEQ ID NO: 6;

(b) detecting the cell-proliferating activity of the polypeptide of step (a); and

(c) selecting a compound that suppresses the cell-proliferating activity of the polypeptide in comparison with the cell- proliferating activity detected in the absence of the test compound.

7. A method of screening for a compound for treating a cell proliferative disease, said method comprising the steps of:

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

(1) a polypeptide consisting of the amino acid sequence of SEQ ID NO: 6;

(2) a polypeptide that consists of the amino acid sequence of SEQ ID NO: 6 in which one or more amino acids are substituted, deleted, inserted, and/or added and that has a biological activity equivalent to a protein consisting of the amino acid sequence of SEQ ID NO: 6;

(3) a polypeptide encoded by a polynucleotide that hybridizes under stringent conditions to a polynucleotide consisting of the nucleotide sequence of SEQ ID NO: 5, wherein the polypeptide has a biological cell-proliferating activity equivalent to a polypeptide consisting of the amino acid sequence of SEQ ID NO: 6;

with NOTCH2 or STRIN in the existence of a test compound;

(b) detecting the binding between the polypeptide and NOTCH2 or STRIN; and

(c) selecting the test compound that inhibits the binding between the polypeptide and NOTCH2 or STRIN.

8. A method of screening for a compound for treating colorectal cancer, said method comprising the steps of:

a) contacting a candidate compound with a cell into which a vector comprising the transcriptional regulatory region of a marker gene and a reporter gene that is expressed under the control of the transcriptional regulatory region has been introduced, wherein the marker gene consists of the nucleotide sequence of SEQ ID:NO 5,

b) measuring the activity of said reporter gene; and

c) selecting a compound that reduces the expression level of said reporter gene as compared to a control.

9. A composition for treating colorectal cancer, said composition comprising a pharmaceutically effective amount of small interfering RNA

against a polynucleotide encoding a polypeptide that consists of the amino acid sequence of SEQ ID NO: 6 as an active ingredient, and a pharmaceutically acceptable carrier, wherein the sense strand of said small interfering RNA is the nucleotide sequence of SEQ ID NO: 112 as the target sequence.

10. Use of an antisense polynucleotide or small interfering RNA against a polynucleotide encoding a polypeptide selected from the group consisting of:

(1) a polypeptide consisting of the amino acid sequence of SEQ ID NO: 6;

(2) a polypeptide that consists of the amino acid sequence of SEQ ID NO: 6 in which one or more amino acids are substituted, deleted, inserted, and/or added and that has a cell-proliferating activity equivalent to a protein consisting of the amino acid sequence of SEQ ID NO: 6; and

(3) a polypeptide encoded by a polynucleotide that hybridizes under stringent conditions to a polynucleotide consisting of the nucleotide sequence of SEQ ID NO: 5, wherein the polypeptide has a cell-proliferating activity equivalent to a polypeptide consisting of the amino acid sequence of SEQ ID NO: 6

for the preparation of a pharmaceutical composition for treating colorectal cancer.

11. Use of an antibody against a polypeptide selected from the group consisting of:

(a) a polypeptide that consists of the amino acid sequence of SEQ ID NO: 6;

(b) a polypeptide that consists of the amino acid sequence of SEQ ID NO: 6 in which one or more amino acids are substituted, deleted, inserted, and/or added and that has a cell-proliferating activity equivalent to a protein consisting of the amino acid sequence of SEQ ID NO: 6; and

(c) a polypeptide encoded by a polynucleotide that hybridizes under stringent conditions to a polynucleotide consisting of the nucleotide sequence of SEQ ID NO: 5, wherein the polypeptide has a cell-proliferating activity equivalent to a polypeptide consisting of the amino acid sequence of SEQ ID NO: 6

for the preparation of a pharmaceutical composition for treating colorectal cancer.

12. Use of a polypeptide selected from the group consisting of (a)-(c), or a polynucleotide encoding the polypeptide:

(a) a polypeptide consisting of the amino acid sequence of SEQ ID NO: 6 or fragment thereof;

(b) a polypeptide that consists of the amino acid sequence of SEQ ID NO: 6 in which one or more amino acids are substituted, deleted , inserted, and/or added and that has a cell-proliferating activity equivalent to a protein consisting of the amino acid sequence of SEQ ID NO: 6, or fragment thereof;

(c) a polypeptide encoded by a polynucleotide that hybridizes under stringent conditions to a polynucleotide consisting of the nucleotide sequence of SEQ ID NO: 5, wherein the polypeptide has a cell-proliferating activity equivalent to a polypeptide consisting of the amino acid sequence of SEQ ID NO: 6, or fragment thereof

for the preparation of a pharmaceutical composition for treating or preventing colorectal cancer.

13. The use of claim 12, wherein the polypeptide is selected from the group of polypeptides

consisting of the amino acid sequence of SEQ ID NO: 80, 97, and 108.

14. Use of a polypeptide consisting of the amino acid sequence of SEQ ID NO: 80, 97, or 108, or a polynucleotide encoding said polypeptide or vector comprising said polynucleotide for the preparation of a pharmaceutical composition for inducing an anti tumor immunity.

15. Use of a polypeptide consisting of the amino acid sequence SEQ ID NO: 80, 97, or 108, or a polynucleotide encoding said polypeptide or vector comprising said polynucleotide for the preparation of the antigen presenting cells.

16. A pharmaceutical composition for treating or preventing colorectal cancer, said composition comprising a pharmaceutically effective amount of a polypeptide consisting of the amino acid sequence selected from the group of SEQ ID NO: 80, 97, and 108.

17. The pharmaceutical composition of claim 16, wherein the polynucleotide is incorporated in expression vector.

18. A substantially pure polypeptide consisting of the amino acid sequence of SEQ ID NO: 80, 97 or 108.

ABSTRACT

GENES AND POLYPEPTIDES RELATING TO HUMAN COLON CANCERS

The present application provides novel human genes RNF43 whose expression is markedly elevated in colorectal cancers, as well as CXADRL1 and GCUD1 whose expression is markedly elevated in gastric cancers compared to corresponding non-cancerous tissues. The genes and polypeptides encoded by the genes can be used, for example, in the diagnosis of a cell proliferative disease, and as target molecules for developing drugs against the disease.

SEQUENCE LISTING

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JAPAN AS REPRESENTED BY THE PRESIDENT OF THE UNIVERSITY OF TOKYO

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Ser Ala Ala Leu Ile Asn Leu Asn Val Ile Trp Met Val Thr Pro Leu
    50                  55                  60

tcc aat gcc aac caa cct gaa cag gtc atc ctg tat cag ggt gga cag      477
Ser Asn Ala Asn Gln Pro Glu Gln Val Ile Leu Tyr Gln Gly Gly Gln
65                  70                  75                  80

atg ttt gat ggt gcc ccc cgg ttc cac ggt agg gta gga ttt aca ggc      525
Met Phe Asp Gly Ala Pro Arg Phe His Gly Arg Val Gly Phe Thr Gly
                85                  90                  95

acc atg cca gct acc aat gtc tct atc ttc att aat aac act cag tta      573
Thr Met Pro Ala Thr Asn Val Ser Ile Phe Ile Asn Asn Thr Gln Leu
            100                 105                 110

tca gac act ggc acc tac cag tgc ctg gtc aac aac ctt cca gac ata      621
Ser Asp Thr Gly Thr Tyr Gln Cys Leu Val Asn Asn Leu Pro Asp Ile
        115                 120                 125

ggg ggc agg aac att ggg gtc acc ggt ctc aca gtg tta gtt ccc cct      669
Gly Gly Arg Asn Ile Gly Val Thr Gly Leu Thr Val Leu Val Pro Pro
    130                 135                 140

tct gcc cca cac tgc caa atc caa gga tcc cag gat att ggc agc gat      717
Ser Ala Pro His Cys Gln Ile Gln Gly Ser Gln Asp Ile Gly Ser Asp
145                 150                 155                 160

gtc atc cta ctc tgt agc tca gag gaa ggc att cct cga cca act tac      765
Val Ile Leu Leu Cys Ser Ser Glu Glu Gly Ile Pro Arg Pro Thr Tyr
                165                 170                 175

ctt tgg gag aag tta gac aat acc ctc aaa cta cct cca aca gct act      813
Leu Trp Glu Lys Leu Asp Asn Thr Leu Lys Leu Pro Pro Thr Ala Thr
            180                 185                 190

cag gac cag gtc cag gga aca gtc acc atc cgg aac atc agt gcc ctg      861
Gln Asp Gln Val Gln Gly Thr Val Thr Ile Arg Asn Ile Ser Ala Leu
        195                 200                 205

tct tca ggt ttg tac cag tgc gtg gct tct aat gct att gga acc agc      909
Ser Ser Gly Leu Tyr Gln Cys Val Ala Ser Asn Ala Ile Gly Thr Ser
    210                 215                 220
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acc tgt ctt ctg gat ctc cag gtt att tca ccc cag ccc agg aac att      957
Thr Cys Leu Leu Asp Leu Gln Val Ile Ser Pro Gln Pro Arg Asn Ile
225                 230                 235                 240

gga cta ata gct gga gcc att ggc act ggt gca gtt att atc att ttt     1005
Gly Leu Ile Ala Gly Ala Ile Gly Thr Gly Ala Val Ile Ile Ile Phe
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tgc att gca cta att tta ggg gca ttc ttt tac tgg aga agc aaa aat     1053
Cys Ile Ala Leu Ile Leu Gly Ala Phe Phe Tyr Trp Arg Ser Lys Asn
            260                 265                 270

aaa gag gag gaa gaa gaa gaa gaa att cct aat gaa ata aga gag gat gat  1101
Lys Glu Glu Glu Glu Glu Glu Ile Pro Asn Glu Ile Arg Glu Asp Asp
        275                 280                 285

ctt cca ccc aag tgt tct tct gcc aaa gca ttt cac act gag att tcc     1149
Leu Pro Pro Lys Cys Ser Ser Ala Lys Ala Phe His Thr Glu Ile Ser
    290                 295                 300

tcc tcg gac aac aac aca cta acc tct tcc aat gcc tac aac agt cga     1197
Ser Ser Asp Asn Asn Thr Leu Thr Ser Ser Asn Ala Tyr Asn Ser Arg
305                 310                 315                 320

tac tgg agc aac aat cca aaa gtt cat aga aac aca gat tca gtc agc     1245
Tyr Trp Ser Asn Asn Pro Lys Val His Arg Asn Thr Asp Ser Val Ser
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cac ttc agt gac ttg ggc caa tct ttc tct ttc cac tca ggc aat gcc     1293
His Phe Ser Asp Leu Gly Gln Ser Phe Ser Phe His Ser Gly Asn Ala
            340                 345                 350

aac ata cca tcc att tat gct aat ggg acc cat ctg gtc ccg ggt caa     1341
Asn Ile Pro Ser Ile Tyr Ala Asn Gly Thr His Leu Val Pro Gly Gln
        355                 360                 365

cat aag act ctg gta gtg aca gcc aac aga ggg tca tca cca cag gtg     1389
His Lys Thr Leu Val Val Thr Ala Asn Arg Gly Ser Ser Pro Gln Val
    370                 375                 380

atg tcc agg agc aat ggc tca gtc agt agg gag cct cgg cct cca cac     1437
Met Ser Arg Ser Asn Gly Ser Val Ser Arg Glu Pro Arg Pro Pro His
385                 390                 395                 400

act cat tcc tac acc atc agc cac gca aca ctg gaa cga att ggt gca     1485
Thr His Ser Tyr Thr Ile Ser His Ala Thr Leu Glu Arg Ile Gly Ala
                405                 410                 415
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420 425 430

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ggtggggtgg ggagtgctgg gaaagaaaca cttccttata attatattag taaaatgcac 1653

aaagaagaag gcagtgctgt tacttggcca ctaagatgtg taaaatggac tgaaatgctc 1713

catcatgaag acttgcttcc ccaccaaaga tgtcctggga ttctgctgga tctcaaagat 1773

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Gln Val Ala Arg Gly Gln Pro Ala Val Leu Pro Cys Thr Phe Thr Thr
35 40 45

Ser Ala Ala Leu Ile Asn Leu Asn Val Ile Trp Met Val Thr Pro Leu
50 55 60

Ser Asn Ala Asn Gln Pro Glu Gln Val Ile Leu Tyr Gln Gly Gly Gln
65 70 75 80

Met Phe Asp Gly Ala Pro Arg Phe His Gly Arg Val Gly Phe Thr Gly
85 90 95

Thr Met Pro Ala Thr Asn Val Ser Ile Phe Ile Asn Asn Thr Gln Leu
100 105 110

Ser Asp Thr Gly Thr Tyr Gln Cys Leu Val Asn Asn Leu Pro Asp Ile
115 120 125

Gly Gly Arg Asn Ile Gly Val Thr Gly Leu Thr Val Leu Val Pro Pro
130 135 140

Ser Ala Pro His Cys Gln Ile Gln Gly Ser Gln Asp Ile Gly Ser Asp
145 150 155 160

Val Ile Leu Leu Cys Ser Ser Glu Glu Gly Ile Pro Arg Pro Thr Tyr
165 170 175

Leu Trp Glu Lys Leu Asp Asn Thr Leu Lys Leu Pro Pro Thr Ala Thr
180 185 190

Gln Asp Gln Val Gln Gly Thr Val Thr Ile Arg Asn Ile Ser Ala Leu
195 200 205

Ser Ser Gly Leu Tyr Gln Cys Val Ala Ser Asn Ala Ile Gly Thr Ser
210 215 220

Thr Cys Leu Leu Asp Leu Gln Val Ile Ser Pro Gln Pro Arg Asn Ile
225 230 235 240

Gly Leu Ile Ala Gly Ala Ile Gly Thr Gly Ala Val Ile Ile Ile Phe
245 250 255

Cys Ile Ala Leu Ile Leu Gly Ala Phe Phe Tyr Trp Arg Ser Lys Asn
260 265 270

Lys Glu Glu Glu Glu Glu Glu Ile Pro Asn Glu Ile Arg Glu Asp Asp
275 280 285

Leu Pro Pro Lys Cys Ser Ser Ala Lys Ala Phe His Thr Glu Ile Ser
290 295 300

Ser Ser Asp Asn Asn Thr Leu Thr Ser Ser Asn Ala Tyr Asn Ser Arg
305 310 315 320

Tyr Trp Ser Asn Asn Pro Lys Val His Arg Asn Thr Asp Ser Val Ser
325 330 335

His Phe Ser Asp Leu Gly Gln Ser Phe Ser Phe His Ser Gly Asn Ala
340 345 350

Asn Ile Pro Ser Ile Tyr Ala Asn Gly Thr His Leu Val Pro Gly Gln
355 360 365

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His Lys Thr Leu Val Val Thr Ala Asn Arg Gly Ser Ser Pro Gln Val
    370                 375                 380

Met Ser Arg Ser Asn Gly Ser Val Ser Arg Glu Pro Arg Pro Pro His
385                 390                 395                 400

Thr His Ser Tyr Thr Ile Ser His Ala Thr Leu Glu Arg Ile Gly Ala
                405                 410                 415

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Met Ala Ala Ala Pro Pro Ser Tyr Cys Phe Val Ala Phe Pro Pro Arg
1               5                   10                  15

gct aag gat ggt ctg gtg gta ttt ggg aaa aat tca gcc cgg ccc aga      155
Ala Lys Asp Gly Leu Val Val Phe Gly Lys Asn Ser Ala Arg Pro Arg
            20                  25                  30

gat gaa gtg caa gag gtt gtg tat ttc tcg gct gct gat cac gaa ccg      203
Asp Glu Val Gln Glu Val Val Tyr Phe Ser Ala Ala Asp His Glu Pro
        35                  40                  45

gag agc aag gtt gag tgc act tac att tca atc gac caa gtt cca agg      251
Glu Ser Lys Val Glu Cys Thr Tyr Ile Ser Ile Asp Gln Val Pro Arg
    50                  55                  60

acc tat gcc ata atg ata agc aga ccc gcc tgg ctc tgg gga gca gaa      299
Thr Tyr Ala Ile Met Ile Ser Arg Pro Ala Trp Leu Trp Gly Ala Glu
65                  70                  75                  80

atg gga gcc aat gaa cat gga gtg tgc ata gcc aat gaa gcc atc aac      347
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Met Gly Ala Asn Glu His Gly Val Cys Ile Ala Asn Glu Ala Ile Asn
                85                  90                  95

acc aga gag cca gct gcc gag ata gaa gcc ttg ctg ggg atg gat ctg      395
Thr Arg Glu Pro Ala Ala Glu Ile Glu Ala Leu Leu Gly Met Asp Leu
            100                 105                 110

gtc agg ctt ggt tta gaa aga ggg gaa aca gct aaa gaa gcc tta gat      443
Val Arg Leu Gly Leu Glu Arg Gly Glu Thr Ala Lys Glu Ala Leu Asp
        115                 120                 125

gtc att gtc tcc ttg ttg gaa gaa cat gga caa ggt ggg aat tac ttt      491
Val Ile Val Ser Leu Leu Glu Glu His Gly Gln Gly Gly Asn Tyr Phe
    130                 135                 140

gaa gat gca aac tcc tgc cac agc ttc caa agt gca tat ctg att gtg      539
Glu Asp Ala Asn Ser Cys His Ser Phe Gln Ser Ala Tyr Leu Ile Val
145                 150                 155                 160

gat cgt gat gaa gcc tgg gtg ctc gag acc ata ggg aag tac tgg gct      587
Asp Arg Asp Glu Ala Trp Val Leu Glu Thr Ile Gly Lys Tyr Trp Ala
                165                 170                 175

gcc gag aaa gtc aca gag gga gtg agg tgc att tgc agt cag ctt tcg      635
Ala Glu Lys Val Thr Glu Gly Val Arg Cys Ile Cys Ser Gln Leu Ser
            180                 185                 190

ctc acc act aag atg gat gca gag cat ccg gaa ctc agg agt tac gct      683
Leu Thr Thr Lys Met Asp Ala Glu His Pro Glu Leu Arg Ser Tyr Ala
        195                 200                 205

cag agc caa ggt tgg tgg acg gga gag ggc gag ttc aat ttt tcc gaa      731
Gln Ser Gln Gly Trp Trp Thr Gly Glu Gly Glu Phe Asn Phe Ser Glu
    210                 215                 220

gtc ttt tct cca gtt gag gat cat cta gac tgc ggt gct ggc aaa gac      779
Val Phe Ser Pro Val Glu Asp His Leu Asp Cys Gly Ala Gly Lys Asp
225                 230                 235                 240

agc tta gaa aaa caa gaa gaa agc atc aca gtg cag act atg atg aac      827
Ser Leu Glu Lys Gln Glu Glu Ser Ile Thr Val Gln Thr Met Met Asn
                245                 250                 255

acc tta cgg gac aaa gcc agc gga gtg tgc ata gac tct gag ttt ttc      875
Thr Leu Arg Asp Lys Ala Ser Gly Val Cys Ile Asp Ser Glu Phe Phe
            260                 265                 270

ctc acc aca gcc agt gga gtg tct gtc ctg ccg cag aat aga agc tct      923
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Leu Thr Thr Ala Ser Gly Val Ser Val Leu Pro Gln Asn Arg Ser Ser
        275                 280                 285

ccg tgc att cac tac ttc act gga acc cct gat cct tcc agg tcc ata      971
Pro Cys Ile His Tyr Phe Thr Gly Thr Pro Asp Pro Ser Arg Ser Ile
    290                 295                 300

ttc aag cct ttc atc ttt gtt gat gac gta aaa ctt gtc ccc aaa aca     1019
Phe Lys Pro Phe Ile Phe Val Asp Asp Val Lys Leu Val Pro Lys Thr
305                 310                 315                 320

cag tct ccc tgt ttt ggg gat gac gac cct gcc aaa aag gag cct cgg     1067
Gln Ser Pro Cys Phe Gly Asp Asp Asp Pro Ala Lys Lys Glu Pro Arg
                325                 330                 335

ttc cag gag aaa cca gac cgc cgg cat gag ctg tac aaa gcc cac gag     1115
Phe Gln Glu Lys Pro Asp Arg Arg His Glu Leu Tyr Lys Ala His Glu
            340                 345                 350

tgg gca cgt gcc atc atc gaa agt gac cag gag caa ggt cgc aag ctg     1163
Trp Ala Arg Ala Ile Ile Glu Ser Asp Gln Glu Gln Gly Arg Lys Leu
        355                 360                 365

agg agc acc atg ctg gag ctg gag aag caa ggc ctg gaa gcc atg gaa     1211
Arg Ser Thr Met Leu Glu Leu Glu Lys Gln Gly Leu Glu Ala Met Glu
    370                 375                 380

gaa atc ctg acc agc tcc gag cca ctg gac cct gcg gaa gtg ggg gac     1259
Glu Ile Leu Thr Ser Ser Glu Pro Leu Asp Pro Ala Glu Val Gly Asp
385                 390                 395                 400

ctt ttc tat gac tgt gtt gac acg gag att aag ttc ttt aag tga         1304
Leu Phe Tyr Asp Cys Val Asp Thr Glu Ile Lys Phe Phe Lys
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35 40 45

Glu Ser Lys Val Glu Cys Thr Tyr Ile Ser Ile Asp Gln Val Pro Arg
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Thr Tyr Ala Ile Met Ile Ser Arg Pro Ala Trp Leu Trp Gly Ala Glu
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Met Gly Ala Asn Glu His Gly Val Cys Ile Ala Asn Glu Ala Ile Asn
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Val Arg Leu Gly Leu Glu Arg Gly Glu Thr Ala Lys Glu Ala Leu Asp
115 120 125

Val Ile Val Ser Leu Leu Glu Glu His Gly Gln Gly Gly Asn Tyr Phe
130 135 140

Glu Asp Ala Asn Ser Cys His Ser Phe Gln Ser Ala Tyr Leu Ile Val
145 150 155 160

Asp Arg Asp Glu Ala Trp Val Leu Glu Thr Ile Gly Lys Tyr Trp Ala
165 170 175

Ala Glu Lys Val Thr Glu Gly Val Arg Cys Ile Cys Ser Gln Leu Ser
180 185 190

Leu Thr Thr Lys Met Asp Ala Glu His Pro Glu Leu Arg Ser Tyr Ala
195 200 205

Gln Ser Gln Gly Trp Trp Thr Gly Glu Gly Glu Phe Asn Phe Ser Glu
210 215 220

Val Phe Ser Pro Val Glu Asp His Leu Asp Cys Gly Ala Gly Lys Asp
225 230 235 240

Ser Leu Glu Lys Gln Glu Glu Ser Ile Thr Val Gln Thr Met Met Asn
245 250 255

Thr Leu Arg Asp Lys Ala Ser Gly Val Cys Ile Asp Ser Glu Phe Phe
260 265 270

Leu Thr Thr Ala Ser Gly Val Ser Val Leu Pro Gln Asn Arg Ser Ser
275 280 285

Pro Cys Ile His Tyr Phe Thr Gly Thr Pro Asp Pro Ser Arg Ser Ile
290 295 300

Phe Lys Pro Phe Ile Phe Val Asp Asp Val Lys Leu Val Pro Lys Thr
305 310 315 320

Gln Ser Pro Cys Phe Gly Asp Asp Asp Pro Ala Lys Lys Glu Pro Arg
325 330 335

Phe Gln Glu Lys Pro Asp Arg Arg His Glu Leu Tyr Lys Ala His Glu
340 345 350

Trp Ala Arg Ala Ile Ile Glu Ser Asp Gln Glu Gln Gly Arg Lys Leu
355 360 365

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

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tcacaagcaa agttggaaat tccaaagaga agtggtgaga tctttactag tcacagtgaa 240

gatgggagaa aatgacatac ctgcagcaga tgtgggctga aaatatcctc ttctctgccc 300

aatcaggaat gctacctgtt tttgggaata aactttagag aaaggaaggg ccaaaactac 360

gacttggctt tctgaaacgg aagcataaat gttcttttcc tccatttgtc tggatctgag 420

aacctgcatt tggtattagc tagtggaagc agtatgtatg gttgaagtgc attgctgcag 480

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Met Ser Gly Gly His Gln Leu Gln Leu Ala Ala Leu Trp Pro
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Trp Leu Leu Met Ala Thr Leu Gln Ala Gly Phe Gly Arg Thr Gly Leu
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gta ctg gca gca gcg gtg gag tct gaa aga tca gca gaa cag aaa gct 626
Val Leu Ala Ala Ala Val Glu Ser Glu Arg Ser Ala Glu Gln Lys Ala
35 40 45

att atc aga gtg atc ccc ttg aaa atg gac ccc aca gga aaa ctg aat 674
Ile Ile Arg Val Ile Pro Leu Lys Met Asp Pro Thr Gly Lys Leu Asn
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ctc act ttg gaa ggt gtg ttt gct ggt gtt gct gaa ata act cca gca 722
Leu Thr Leu Glu Gly Val Phe Ala Gly Val Ala Glu Ile Thr Pro Ala
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Glu Gly Lys Leu Met Gln Ser His Pro Leu Tyr Leu Cys Asn Ala Ser
80 85 90

gat gac gac aat ctg gag cct gga ttc atc agc atc gtc aag ctg gag 818
Asp Asp Asp Asn Leu Glu Pro Gly Phe Ile Ser Ile Val Lys Leu Glu
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Ser Pro Arg Arg Ala Pro Arg Pro Cys Leu Ser Leu Ala Ser Lys Ala
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Arg Met Ala Gly Glu Arg Gly Ala Ser Ala Val Leu Phe Asp Ile Thr
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gag gat cga gct gct gct gag cag ctg cag cag ccg ctg ggg ctg acc 962
Glu Asp Arg Ala Ala Ala Glu Gln Leu Gln Gln Pro Leu Gly Leu Thr
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Trp Pro Val Val Leu Ile Trp Gly Asn Asp Ala Glu Lys Leu Met Glu
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Gly Thr Ile Phe Val Ile Ile Leu Ala Ser Val Leu Arg Ile Arg Cys
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Arg Pro Arg His Ser Arg Pro Asp Pro Leu Gln Gln Arg Thr Ala Trp
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gcc atc agc cag ctg gcc acc agg agg tac cag gcc agc tgc agg cag 1250
Ala Ile Ser Gln Leu Ala Thr Arg Arg Tyr Gln Ala Ser Cys Arg Gln
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Ala Arg Gly Glu Trp Pro Asp Ser Gly Ser Ser Cys Ser Ser Ala Pro
255                 260                 265                 270

gtg tgt gcc atc tgt ctg gag gag ttc tct gag ggg cag gag cta cgg      1346
Val Cys Ala Ile Cys Leu Glu Glu Phe Ser Glu Gly Gln Glu Leu Arg
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gtc att tcc tgc ctc cat gag ttc cat cgt aac tgt gtg gac ccc tgg      1394
Val Ile Ser Cys Leu His Glu Phe His Arg Asn Cys Val Asp Pro Trp
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tta cat cag cat cgg act tgc ccc ctc tgc gtg ttc aac atc aca gag      1442
Leu His Gln His Arg Thr Cys Pro Leu Cys Val Phe Asn Ile Thr Glu
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gga gat tca ttt tcc cag tcc ctg gga ccc tct cga tct tac caa gaa      1490
Gly Asp Ser Phe Ser Gln Ser Leu Gly Pro Ser Arg Ser Tyr Gln Glu
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cca ggt cga aga ctc cac ctc att cgc cag cat ccc ggc cat gcc cac      1538
Pro Gly Arg Arg Leu His Leu Ile Arg Gln His Pro Gly His Ala His
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Tyr His Leu Pro Ala Ala Tyr Leu Leu Gly Pro Ser Arg Ser Ala Val
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gct cgg ccc cca cgg cct ggt ccc ttc ctg cca tcc cag gag cca ggc      1634
Ala Arg Pro Pro Arg Pro Gly Pro Phe Leu Pro Ser Gln Glu Pro Gly
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atg ggc cct cgg cat cac cgc ttc ccc aga gct gca cat ccc cgg gct      1682
Met Gly Pro Arg His His Arg Phe Pro Arg Ala Ala His Pro Arg Ala
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cca gga gag cag cag cgc ctg gca gga gcc cag cac ccc tat gca caa      1730
Pro Gly Glu Gln Gln Arg Leu Ala Gly Ala Gln His Pro Tyr Ala Gln
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ggc tgg gga atg agc cac ctc caa tcc acc tca cag cac cct gct gct      1778
Gly Trp Gly Met Ser His Leu Gln Ser Thr Ser Gln His Pro Ala Ala
415                 420                 425                 430

tgc cca gtg ccc cta cgc cgg gcc agg ccc cct gac agc agt gga tct      1826
Cys Pro Val Pro Leu Arg Arg Ala Arg Pro Pro Asp Ser Ser Gly Ser
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gcc agt gac tcc agc tca ggg ccc tgt cat ggc tct tcc agt gac tct      1922
Ala Ser Asp Ser Ser Ser Gly Pro Cys His Gly Ser Ser Ser Asp Ser
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gtg gtc aac tgc acg gac atc agc cta cag ggg gtc cat ggc agc agt      1970
Val Val Asn Cys Thr Asp Ile Ser Leu Gln Gly Val His Gly Ser Ser
    480                 485                 490

tct act ttc tgc agc tcc cta agc agt gac ttt gac ccc cta gtg tac      2018
Ser Thr Phe Cys Ser Ser Leu Ser Ser Asp Phe Asp Pro Leu Val Tyr
495                 500                 505                 510

tgc agc cct aaa ggg gat ccc cag cga gtg gac atg cag cct agt gtg      2066
Cys Ser Pro Lys Gly Asp Pro Gln Arg Val Asp Met Gln Pro Ser Val
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acc tct cgg cct cgt tcc ttg gac tcg gtg gtg ccc aca ggg gaa acc      2114
Thr Ser Arg Pro Arg Ser Leu Asp Ser Val Val Pro Thr Gly Glu Thr
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cag gtt tcc agc cat gtc cac tac cac cgc cac cgg cac cac cac tac      2162
Gln Val Ser Ser His Val His Tyr His Arg His Arg His His His Tyr
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aaa aag cgg ttc cag tgg cat ggc agg aag cct ggc cca gaa acc gga      2210
Lys Lys Arg Phe Gln Trp His Gly Arg Lys Pro Gly Pro Glu Thr Gly
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gtc ccc cag tcc agg cct cct att cct cgg aca cag ccc cag cca gag      2258
Val Pro Gln Ser Arg Pro Pro Ile Pro Arg Thr Gln Pro Gln Pro Glu
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cca cct tct cct gat cag caa gtc acc gga tcc aac tca gca gcc cct      2306
Pro Pro Ser Pro Asp Gln Gln Val Thr Gly Ser Asn Ser Ala Ala Pro
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tcg ggg cgg ctc tct aac cca cag tgc ccc agg gcc ctc cct gag cca      2354
Ser Gly Arg Leu Ser Asn Pro Gln Cys Pro Arg Ala Leu Pro Glu Pro
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gcc cct ggc cca gtt gac gcc tcc agc atc tgc ccc agt acc agc agt      2402
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aaa agg cgg ggg ggt ccc tcc gag ccc acc cct ggc tct cgg ccc cag      2498
Lys Arg Arg Gly Gly Pro Ser Glu Pro Thr Pro Gly Ser Arg Pro Gln
655                 660                 665                 670

gat gca act gtg cac cca gct tgc cag att ttt ccc cat tac acc ccc      2546
Asp Ala Thr Val His Pro Ala Cys Gln Ile Phe Pro His Tyr Thr Pro
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agt gtg gca tat cct tgg tcc cca gag gca cac ccc ttg atc tgt gga      2594
Ser Val Ala Tyr Pro Trp Ser Pro Glu Ala His Pro Leu Ile Cys Gly
            690                 695                 700

cct cca ggc ctg gac aag agg ctg cta cca gaa acc cca ggc ccc tgt      2642
Pro Pro Gly Leu Asp Lys Arg Leu Leu Pro Glu Thr Pro Gly Pro Cys
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tac tca aat tca cag cca gtg tgg ttg tgc ctg act cct cgc cag ccc      2690
Tyr Ser Asn Ser Gln Pro Val Trp Leu Cys Leu Thr Pro Arg Gln Pro
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ctg gaa cca cat cca cct ggg gag ggg cct tct gaa tgg agt tct gac      2738
Leu Glu Pro His Pro Pro Gly Glu Gly Pro Ser Glu Trp Ser Ser Asp
735                 740                 745                 750

acc gca gag ggc agg cca tgc cct tat ccg cac tgc cag gtg ctg tcg      2786
Thr Ala Glu Gly Arg Pro Cys Pro Tyr Pro His Cys Gln Val Leu Ser
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gcc cag cct ggc tca gag gag gaa ctc gag gag ctg tgt gaa cag gct      2834
Ala Gln Pro Gly Ser Glu Glu Glu Leu Glu Glu Leu Cys Glu Gln Ala
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ccgtttcctg ctccagctcc agaccttgtc tgcagaaaac atctgcagtg cagcaaatcc  3070

atgtccagcc aggcaaccag ctgctgcctg tggcgtgtgt gggctggatc ccttgaaggc  3130
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Ala Ala Ala Val Glu Ser Glu Arg Ser Ala Glu Gln Lys Ala Ile Ile
35 40 45

Arg Val Ile Pro Leu Lys Met Asp Pro Thr Gly Lys Leu Asn Leu Thr
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Leu Glu Gly Val Phe Ala Gly Val Ala Glu Ile Thr Pro Ala Glu Gly
65 70 75 80

Lys Leu Met Gln Ser His Pro Leu Tyr Leu Cys Asn Ala Ser Asp Asp
85 90 95

Asp Asn Leu Glu Pro Gly Phe Ile Ser Ile Val Lys Leu Glu Ser Pro
100 105 110

Arg Arg Ala Pro Arg Pro Cys Leu Ser Leu Ala Ser Lys Ala Arg Met
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Ala Gly Glu Arg Gly Ala Ser Ala Val Leu Phe Asp Ile Thr Glu Asp
130 135 140

Arg Ala Ala Ala Glu Gln Leu Gln Gln Pro Leu Gly Leu Thr Trp Pro
145 150 155 160

Val Val Leu Ile Trp Gly Asn Asp Ala Glu Lys Leu Met Glu Phe Val
165 170 175

Tyr Lys Asn Gln Lys Ala His Val Arg Ile Glu Leu Lys Glu Pro Pro
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Ala Trp Pro Asp Tyr Asp Val Trp Ile Leu Met Thr Val Val Gly Thr
195 200 205

Ile Phe Val Ile Ile Leu Ala Ser Val Leu Arg Ile Arg Cys Arg Pro
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Arg His Ser Arg Pro Asp Pro Leu Gln Gln Arg Thr Ala Trp Ala Ile
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Ser Gln Leu Ala Thr Arg Arg Tyr Gln Ala Ser Cys Arg Gln Ala Arg
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Gly Glu Trp Pro Asp Ser Gly Ser Ser Cys Ser Ser Ala Pro Val Cys
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Ala Ile Cys Leu Glu Glu Phe Ser Glu Gly Gln Glu Leu Arg Val Ile
275 280 285

Ser Cys Leu His Glu Phe His Arg Asn Cys Val Asp Pro Trp Leu His
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Gln His Arg Thr Cys Pro Leu Cys Val Phe Asn Ile Thr Glu Gly Asp
305 310 315 320

Ser Phe Ser Gln Ser Leu Gly Pro Ser Arg Ser Tyr Gln Glu Pro Gly
325 330 335

Arg Arg Leu His Leu Ile Arg Gln His Pro Gly His Ala His Tyr His
340 345 350

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Pro Pro Arg Pro Gly Pro Phe Leu Pro Ser Gln Glu Pro Gly Met Gly
370 375 380

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