CHARACTERIZATION OF CXCL-16 AS A TUMOR ASSOCIATED MARKER OF COLORECTAL CANCER

[Fig. 4]

Colorectal cancer (n = 43)

Normal (n = 11)

Abstract: Disclosed herein is a colorectal cancer marker for diagnosing colorectal cancer based on CXCL-16 overexpression in tissues, cells or bodily fluids of colorectal cancer patients. Disclosed also is the use of the colorectal cancer marker in the development of therapeutic agents for cancer and in the diagnosis and treatment of colorectal cancer.
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[DESCRIPTION]

[invention Title]

CHARACTERIZATION OF CXCL-16 AS A TUMOR ASSOCIATED MARKER OF COLORECTAL CANCER

[Technical Field]

The present invention relates to methods of identifying the clinical importance and biological functions of CXC chemokine ligand 16 (CXCL-16) in patients having colorectal cancer and of exploring its potential as a diagnostic marker of colorectal cancer.

[Background Art]

Colorectal cancer is common in many Western countries, and this is associated with diets high in animal fat and meat. It is the second most common form of cancer and the second most important cause of cancer-related death. The incidence of colorectal cancer is also rising in Asia, including Korea and Japan, where colorectal cancer was less common than in the Western world, owing to the Westernization of the diet. A recent survey (2002), conducted by the Korean National Statistical Office, showed that colorectal cancer is the fourth most common cause of cancer death in Korea, after cancer of the lung, stomach and liver. Various methods including fecal occult blood test and colonoscopy are currently used for
screening colorectal cancer and increase the rates of detection of early-stage cancer. However, there are few biological markers which can be used for prognosis and to determine suitable therapy for colorectal cancer. Since to date no method capable of accurately diagnosing and staging cancer has been found, a colorectal cancer diagnosis is obtained through a physical examination, or using a variety of screening methods, including histological examination, cytological examination, endoscopy, tumor marker detection, imaging test and nuclear medical examination. A tumor marker test is used to screen for and diagnose a specific type of cancer by detecting a tumor marker in blood, tissue and urine specimens. However, since many tumor markers are elevated or detected in biological specimens from individuals not afflicted with cancer, a tumor marker test is not reliable enough to detect and diagnose cancer.

Colorectal cancer is typically screened using common traditional procedures, a guaiac-based fecal occult blood test (FOBT) and colonoscopy. With the recent advances in molecular biology, cyclooxygenase 2 and SFRP2 methylation are becoming known as novel markers for screening colorectal cancer. Carcinoembryonic antigen (CEA) is a glycoprotein that was first identified in 1965 and is detected in colon carcinoma and embryonic colonic mucosa. CEA is considered to be a fetal antigen that is normally present in embryonic and fetal digestive tissues between two and six months and disappears
after birth. However, it may reappear in adults who develop colorectal carcinoma and several other neoplastic conditions, including gastrointestinal, pancreatic and lung carcinomas. Due to its reappearance in malignant tissues, CEA is deemed valuable as a marker to help diagnose cancer, to monitor patients who have undergone surgery, or to monitor patients' response to treatment. When the serum CEA level is high before surgery in colorectal cancer or stomach cancer, patients are expected to have a high risk of recurrence. Thus, the CEA level seems to be a valuable prognostic indicator. Since the CEA level is increased in colorectal cancer and also in several other types of cancer in the pancreas (60-90%), stomach (40-60%), lung (60-75%), breast (20-50%), and the like, it is commonly used as a broad-spectrum tumor marker. In particular, in colorectal cancer, according to the degree of tumor invasion and spread, CEA levels are elevated in 20-40% of tumors limited to the bowel wall and in 80-90% of tumors metastasized to the liver and other organs. When the cancer has metastasized to the liver, CEA levels sharply rise. Thus, CEA levels are monitored to determine the prognosis of patients having colorectal cancer or to follow up on patients after surgery. In addition to in malignant tumors, Elevated CEA levels occur in various benign diseases including liver cirrhosis, alcoholic hepatitis, pancreatitis, and ulcerative colitis. Colorectal cancer can be sometimes associated with known risk factors. These include increasing age and smoking, which is modifiable.
As another tumor marker, carbohydrate antigen 19-9 (CA 19-9) was defined initially by a monoclonal antibody isolated from mice immunized with a human colorectal carcinoma cell line, which expresses an antigen that is strongly positive in pancreatic ductal adenocarcinoma. The CA 19-9 antigen is positive in pancreatic cancer (84%), gastric cancer (35%), liver cancer (22%) and gallbladder cancer (69%), and is elevated to a high concentration of 1,000 U/ml or more in 40-50% of cancer of the pancreas and bile duct. Thus, the CA 19-9 antigen is considered useful for the early diagnosis of cancer and to help diagnose pancreatic cancer. Gastric and colorectal carcinomas are positive for CA 19-9, especially when they have metastasized to the liver. The antigen is also found in normal tissues of the pancreas and in non-cancerous cells surrounding biliary, gastric mucosal and salivary epithelial carcinomas.

CXC chemokine ligand 16 (CXCL-16) functions as a low-density lipoprotein (LDL) receptor, a scavenger receptor and a chemokine. CXCL-16 is expressed on the cell membrane. It also occurs as a soluble molecule. The inventors of this application examined the biological functions played by CXCL-16 in colorectal cancer and evaluated its potential as a diagnostic and prognostic marker of colorectal cancer. cDNA microarray analysis and RT-PCR for CXCL-16 RNA levels using tissues from normal subjects and colorectal cancer patients revealed that CXCL-16 is more highly expressed in colorectal carcinoma tissues than non-tumorous or normal colorectal tissues. Western blot
and immunodot assays for serum CXCL-1-6 protein levels using an antibody against CXCL-1β showed higher levels of CXCL-1β circulating in the serum of colorectal cancer patients compared to in the serum of normal subjects. These results indicated that CXCL-1β is useful as a marker for the diagnosis of colorectal cancer and that colorectal cancer can be diagnosed readily and simply through the detection of the marker in sera, suggesting that CXCL-1-6 is highly valuable as a marker for the diagnosis and prognosis of colorectal cancer, thereby leading to the present invention.

[Disclosure]

[Technical Problem]

It is therefore an object of the present invention to provide a method of diagnosing colorectal cancer and a diagnostic kit for colorectal cancer, the method and kit based on employing a CXCL-1β gene, a fragment thereof, or an antibody thereagainst.

It is another object of the present invention to provide a small interfering RNA (siRNA) molecule for preventing or treating the incidence or metastasis of colorectal cancer, the siRNA molecule having a nucleotide sequence complementary to mRNA derived from a CXCL-1β gene or a fragment thereof.

It is a further object of the present invention to provide a method of screening a cancer repressor by determining the ability of a candidate compound to promote or inhibit the action
of a protein encoded by the above gene.

[Technical Solution]

In order to identify the clinical importance and biological functions of CXCL-16, the potential of CXCL-1β as a diagnostic and prognostic marker for colorectal cancer was evaluated by measuring its RNA and protein levels in tissues and blood samples from patients having colorectal cancer. cDNA microarray analysis, RT-PCR and Western blotting using tissues from colorectal cancer patients showed that CXCL-16 is more highly expressed in both RNA and protein levels in colorectal carcinoma tissues than nontumorous colorectal tissues. Also, RT-PCR using cell lines revealed that CXCL-1β is strongly up-regulated in the colorectal carcinoma cell lines SW620, COLO205, DLD1 and HCT116. The analysis of clinical serum samples for CXCL-16 distribution showed that high levels of CXCL-16 are secreted by colorectal cancer.

These results indicated that CXCL-16 is useful for the early diagnosis and prognosis of colorectal cancer and for the monitoring of response to treatment, thereby leading to the present invention.

In one aspect, the present invention relates to a diagnostic marker of colorectal cancer comprising a CXCL-16-encoding nucleic acid or a CXCL-16 protein.

The term "diagnosis" as used herein refers to the
identification of the presence or properties of colorectal cancer conditions. With respect to the objects of the present invention, the diagnosis indicates the identification of the incidence of colorectal cancer by detecting the expression of a diagnostic marker for colorectal cancer.

The term "marker", as used herein, is intended to indicate a substance that is able to diagnose colorectal cancer by distinguishing cancerous cells from normal cells. The marker is a nucleic acid marker for the CXCL-16 gene or a polypeptide marker for the CXCL-16 protein, and its expression is increased in colorectal carcinoma cells compared to normal cells.

The human colorectal cancer-specific CXCL-16 gene according to the present invention has a full-length nucleotide sequence of 1,644 bp, as shown in SEQ ID No. 1, and is translated into a polypeptide of 184 amino acids, corresponding to a molecular mass of about 20 kDa.

Real-time RT-PCR showed that the CXCL-16 gene is expressed at higher levels in colorectal carcinoma tissues than in normal (nontumorous) tissues, indicating that the CXCL-16 gene is a tumorigenic/metastatic gene causing colorectal cancer. Thus, the colorectal cancer gene of the present invention is deemed to be involved in colorectal oncogenesis, and is useful in the diagnosis of colorectal cancer, generation of transgenic animals, screening of anticancer agents specific to colorectal cancer and siRNA gene therapy, as well as for controlling human life span.
In another aspect, the present invention relates to a composition for diagnosing colorectal cancer comprising an agent detecting expression levels of the CXCL-1β gene or protein.

The expression levels of the CXCL-1β marker gene in biological samples may be determined by measuring mRNA or protein levels. The mRNA or protein isolation from a biological sample may be carried out using a known process, and may be quantitatively assessed using various analytical methods.

The term "the measurement of mRNA expression levels", as used herein, refers to a process of assessing the presence and expression levels of mRNA of the CXCL-16 marker gene in biological samples so as to detect the CXCL-16 marker gene, in which the amount of mRNA is measured. Analytical methods for detecting mRNA levels include, but are not limited to, RT-PCR, competitive RT-PCR, real-time RT-PCR, RNase protection assay (RPA), Northern blotting, and DNA chip assay.

With the detection methods, a patient suspected of having colorectal cancer may be compared with a normal control for mRNA expression levels of the CXCL-1β marker gene, and the patient's suspected colorectal cancer may be diagnosed by determining whether expression levels of mRNA from the CXCL-16 marker gene have significantly increased.

The term "the measurement of protein expression levels", as used herein, is a process of assessing the presence and expression levels of proteins coded by the CXCL-1β marker gene.
in biological samples, in which the amount of the protein product of the marker gene is measured using an antibody binding specifically to the protein.

Analytical methods for measuring the protein level include, but are not limited to, Western blotting, enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), radioimmunodiffusion, ouchterlony immunodiffusion, rocket Immunoelectrophoresis, immunohistostaining, immunoprecipitation assay, complement fixation assay, FACS, and protein chip assay.

Thus, in one detailed aspect, the present invention provides a composition for detecting a diagnostic marker of colorectal cancer comprising primer sequences complementary to mRNA of the CXCL-16 gene.

The "primer" refers to a short nucleic acid sequence having a free 3' hydroxyl group, which is able to form base-pairing interaction with a complementary template, and serves as a starting point for replicating the template strand. A primer is able to initiate DNA synthesis in the presence of a reagent for polymerization and four different nucleoside triphosphates at suitable buffers and temperature. The primer may have additional properties that do not change the ability of the primer to serve as an origin for DNA synthesis. The primers may be chemically synthesized using a phosphoramidite solid support method or other widely known methods. These nucleic acid sequences may also be modified using any means known in the art.

In the practice of the present invention, the composition
for detecting a diagnostic marker of colorectal cancer includes a pair of primers specific to the CXCL-I6 gene. Primers for amplifying CXCL-16 (SEQ ID No. 1) are preferably represented by SEQ ID Nos. 2 and 3.

In a further aspect, the present invention provides a composition for detecting a diagnostic marker of colorectal cancer, comprising an antibody specific to the CXCL-1β protein.

The term "antibody" as used herein refers to a specific protein molecule that is directed against an antigenic determinant. With respect to the objects of the present invention, an "antibody" binds specifically to the CXCL-16 marker protein, and includes all of polyclonal antibodies, monoclonal antibodies and recombinant antibodies.

Antibody production using the colorectal cancer marker protein identified as described above may be easily carried out using techniques widely known in the art.

Polyclonal antibodies may be produced using a method widely known in the art, which includes injecting the CXCL-16 protein antigen into an animal and collecting blood samples from the animal to obtain sera containing antibodies. Such polyclonal antibodies may be prepared from certain animal hosts, such as goats, rabbits and pigs. Monoclonal antibodies may be prepared by a method widely known in the art, such as a hybridoma method (see Kohler and Milstein (1976) European Journal of Immunology 6:511-519), or a phage antibody library technique (Clackson et al., Nature, 352:624-628, 1991; Marks et
The hybridoma method employs cells from an immunologically suitable host animal injected with the CXCL-1β protein as an antigen, such as mice, and a carcinoma or myeloma cell line as another group. Cells of the two groups are fused with each other using a method widely known in the art, for example, using polyethylene, and antibody-producing cells are propagated using a standard tissue culture method. After uniform cell colonies are obtained through subcloning using a limited dilution technique, hybridomas capable of producing an antibody specific for the CXCL-1β protein are cultivated in large scale in vitro or in vivo according to a standard technique.

The phage antibody library method includes constructing a phage antibody library in vitro by obtaining genes for antibodies to the CXCL-1β protein and expressing them in fusion protein form on the surface of phages, and isolating monoclonal antibodies binding to the CXCL-1β protein from the library.

Antibodies prepared using the above methods are isolated and purified using gel electrophoresis, dialysis, ion exchange chromatography, affinity chromatography, and the like.

In addition, the antibodies of the present invention include complete forms having two full-length light chains and two full-length heavy chains, as well as functional fragments of antibody molecules. The functional fragments of antibody molecules refer to fragments retaining at least an antigen-binding function, and include Fab, F(ab')₂, F(ab')₂, F(ab')₂, Fv.
In yet another aspect, the present invention provides a kit for diagnosing colorectal cancer comprising the composition for diagnosing colorectal cancer according to the present invention. Preferably, the kit for diagnosing colorectal cancer further includes a composition, solution or apparatus which includes one or more different kinds of constituents suitable for analytical methods.

In one detailed aspect, the diagnostic kit may be featured by comprising essential elements required for performing RT-PCR. A RT-PCR kit includes a pair of primers specific for each marker gene. The primer is a nucleotide having a sequence specific to a nucleic acid sequence of each marker gene, and is about 7 bp to 50 bp in length, more preferably about 10 bp to 30 bp in length. Also, the RT-PCR kit may include primers specific to a nucleic acid sequence of a control gene. The RT-PCR kit may further include test tubes or other suitable containers, reaction buffers (varying in pH and magnesium concentrations), deoxynucleotides (dNTPs), enzymes such as Taq-polymerase and reverse transcriptase, DNAse, RNAse inhibitor, DEPC-treated water, and sterile water.

In another detailed aspect, the diagnostic kit may be featured by comprising essential elements required for performing a DNA chip assay. A DNA chip kit may include a base plate, onto which genes or fragments thereof, cDNA, or oligonucleotides are attached, and reagents, agents and enzymes for preparing fluorescent probes. Also, the base plate may
include cDNA or oligonucleotides corresponding to a control gene or fragments thereof.

In a further detailed aspect, the diagnostic kit may be featured by comprising an antibody specifically binding to the CXCL-16 protein. The diagnostic kit is preferably an ELISA kit. The ELISA kit may also include an antibody specific to a control protein. The ELISA kit may further include reagents capable of detecting bound antibodies, for example, a labeled secondary antibody, chromophores, enzymes conjugated with an antibody and their substrates, or other substances capable of binding to the antibodies.

In still another aspect, the present invention provides a method of diagnosing colorectal cancer using the composition for diagnosing colorectal cancer or the kit for diagnosing colorectal cancer.

In one detailed aspect, the present invention provides a method of diagnosing colorectal cancer comprising measuring mRNA levels of the CXCL-16 gene in a biological sample from a patient suspected of having colorectal cancer using primers specific to the CXCL-16 gene; and comparing the mRNA levels of the sample from the patient with those of a normal control sample to ascertain whether there is an increase in mRNA level.

The term "biological sample", as used herein, refers to tissues, cells and others, in which a difference in mRNA or protein expression levels of the CXCL-16 marker gene can be detected. Examples of the biological samples include, but are
not limited to, urine, whole blood, plasma and sera.

Analytical methods for measuring mRNA levels include, but are not limited to, RT-PCR, competitive RT-PCR, real-time RT-PCR, RNase protection assay (RPA), Northern blotting, and DNA chip assay.

With the detection methods, a patient suspected of having colorectal cancer may be compared with a normal control for mRNA expression levels of the CXCL-16 gene, and the patient's suspected colorectal cancer may be diagnosed by determining whether expression levels of mRNA from the colorectal cancer marker gene have significantly increased.

The measurement of mRNA expression levels is preferably carried out using RT-PCR with primers specific to a gene used as a colorectal cancer marker, or using a DNA chip.

RT-PCR is a method capable of simply diagnosing the incidence of colorectal cancer by electrophoresing RT-PCR products and analyzing patterns and thicknesses of bands in order to determine the expression and levels of mRNA from a gene used as a diagnostic marker of colorectal cancer while comparing the mRNA expression and levels with those of a control (FIGS. 1 and 2). Alternatively, the measurement of mRNA expression levels is carried out using a DNA chip in which the colorectal cancer marker gene or nucleic acid fragments thereof are anchored at high density to a glass-like base plate. A cDNA probe labeled with a fluorescent substance either at its end or in the interior thereof is prepared using mRNA isolated from a
sample, and is hybridized with the DNA chip. The DNA chip is then read to determine the presence or expression levels of the gene, thereby diagnosing the incidence of colorectal cancer.

In another detailed aspect, the present invention provides a method of diagnosing colorectal cancer comprising detecting protein levels by contacting an antibody specific to the CXCL-I6 protein with a biological sample from a patient suspected of having colorectal cancer to form antigen-antibody complexes, and comparing the amount of antigen-antibody complexes formed in the biological sample from the patient with that in a normal control sample to ascertain whether there is an increase in protein level.

The isolation of proteins from a biological sample may be achieved using a known process, and protein levels may be measured using a variety of methods.

Analytical methods for measuring protein levels include, but are not limited to, ELISA, radioimmunoassay (RIA), sandwich assay, and Western blot or immunoblot assay on a polyacrylamide gel. The methods enable the detection of expression of the above protein in fluid samples from subjects.

With the analytical methods, a patient suspected of having colorectal cancer is compared with a normal control with respect to the amount of formed antigen-antibody complexes, and the patient's suspected colorectal cancer is diagnosed if a significant increase in expression levels of the CXCL-1ß protein is observed.
The term "antigen-antibody complexes", as used herein, refers to binding products of the CXCL-Iβ protein to an antibody specific thereto. The amount of formed antigen-antibody complexes may be quantitatively determined by measuring the signal size of a detection label. Such a detection label may be selected from the group consisting of enzymes, fluorescent substances, ligands, luminescent substances, microparticles, redox molecules and radioactive isotopes, but the present invention is not limited thereto. Examples of enzymes available as detection labels include, but are not limited to, β-glucuronidase, β-D-glucosidase, β-D-galactosidase, urease, peroxidase or alkaline phosphatase, acetylcholinesterase, glucose oxidase, and hexokinase. Examples of the fluorescent substances include, but are not limited to, fluorescein, phycocyanin, and fluorescamine. Examples of the ligands include, but are not limited to, biotin derivatives. Examples of luminescent substances include, but are not limited to, luciferin. Examples of the microparticles include, but are not limited to, colloidal gold. Examples of the redox molecules include, but are not limited to, quinone, 1,4-benzoquinone, and hydroquinone. Examples of the radioactive isotopes include, but are not limited to, 3H and 14C.

Preferably, the protein expression levels are measured using ELISA. Examples of ELISA include direct sandwich ELISA using a labeled antibody recognizing an antigen bound to an antibody immobilized on a solid support; and indirect sandwich
ELISA, in which a captured antigen bound to an antibody immobilized on a solid support is detected by first adding an antigen-specific antibody, and then a secondary labeled antibody which binds the antigen-specific antibody. More preferably, the protein expression levels are detected using sandwich ELISA, where a sample reacts with an antibody immobilized on a solid support, and the resulting antigen-antibody complexes are detected by adding a labeled antibody specific for the antigen, followed by enzymatic development, or by adding first an antigen-specific antibody and then a secondary labeled antibody which binds to the antigen-specific antibody, followed by enzymatic development. The incidence of colorectal cancer may be diagnosed by measuring the degree to which the CXCL-16 marker protein and an antibody thereto form complexes.

In addition, the measurement of the protein expression levels is preferably carried out using a protein chip in which one or more antibodies to the CXCL-16 marker are arrayed and immobilized at predetermined positions of a base plate at high density. Using a method of analyzing a sample using a protein chip, proteins are isolated from the sample and hybridized with the protein chip to form antigen-antibody complexes. The protein chip is then read to determine the presence or expression levels of the proteins, thereby diagnosing the incidence of colorectal cancer.

Further, the measurement of protein expression levels is preferably achieved using Western blotting using an antibody to
the CXCL-I 6 protein. Total proteins are isolated from a sample, electrophoresed to separate them according to size, transferred onto a nitrocellulose membrane, and reacted with an antibody. The amount of antigen-antibody complexes produced is detected using a labeled antibody, thereby diagnosing the incidence of colorectal cancer (FIG. 3).

The detection methods comprise comparing the expression level of a maker gene in cells in which colorectal cancer develops with that in a normal control. mRNA or protein levels may be expressed as an absolute or relative difference in the amount of the CXCL-I 6 marker protein.

In still another aspect, the present invention provides a composition for treating or preventing colorectal cancer or inhibiting the metastasis of the cancer comprising a substance suppressing the expression of the CXCL-16 gene. The composition preferably includes an antisense gene or small interfering RNA (siRNA) against the CXCL-16 gene as a substance suppressing CXCL-I 6 gene expression.

As used herein, the term "antisense gene" refers to a polynucleotide that has a sequence complementary to a partial or full-length sequence of mRNA transcribed from the CXCL-I 6 gene of SEQ ID No. 1 or a fragment thereof. A specific type of cancer developed by the expression of an oncogene can be treated by introducing a DNA sequence, capable of binding to mRNA from the oncogene and suppressing its translation, into a patient.
siRNA molecules to the CXCL-I6 gene are short double-stranded RNA (dsRNA) fragments of about 21 to 30 nucleotides. The endonuclease Dicer recognizes double-stranded RNA and chops it up into small fragments (called small interfering RNA (siRNA)). When introduced into cells, a double-stranded siRNA against the CXCL-16 gene interacts with the target mRNA and directs its cleavage via RNA interference, resulting in CXCL-Iβ gene silencing. The composition according to the present invention is administered into a patient using a common method in order to suppress the expression of a tumorigenic and/or metastatic gene. For example, Filleur et al. reported positive results on the silencing of gene expression by administering siRNAs through low-volume intravenous injection without the use of any facilitated delivery system (Filleur et al., Cancer Res., 63(14):3919-22, 2003). As a strategy to enhance the in vivo absorption and stability of siRNAs, Chien et al. described the use of a liposome to deliver siRNAs through intravenous injection. Delivery efficiency was estimated to be approximately seven times better than with traditional liposomes, and toxicity was lower (Chien et al., Cancer Gene Ther., 12(3):321-8, 2005).

In addition to one or more effective ingredients selected from the antisense gene and siRNA against CXCL-I6 gene, the pharmaceutical composition of the present invention may include at least one pharmaceutically acceptable carrier. Examples of the pharmaceutically acceptable carrier include saline solution,
sterile water, Ringer's solution, buffered saline solution, dextrose solution, maltodextrin solution, glycerol, ethanol, liposome, and a mixture of two or more thereof. If necessary, the composition may further include other conventional additives, such as antioxidants and buffers. Also, the composition may additionally include diluents, dispersants, surfactants, binders and lubricants in order to be formulated into injection formulations, such as aqueous solution, suspension and emulsion, pills, capsules, granules or tablets.

The carrier may be used in the form of being coupled to a target-specific antibody or other ligands in order for the effective ingredient to act in a target-specific manner. Furthermore, the composition may be preferably formulated depending on its components, using a suitable method known in the art, for example, the method described in Remington's Pharmaceutical Science (latest edition), Mack Publishing Company, Easton PA.

The administration route for the pharmaceutical composition of the present invention is not particularly restricted, but, according to the intended use, the composition may be administered orally or via parenteral routes, for example, intravenous, subcutaneous, intraperitoneal or topical. The specific therapeutically effective dose level for any particular patient may vary depending on a variety of factors, including the patient's weight, age, gender, general health status and diet, the time of administration, route of
administration, rate of excretion of the composition, and severity of the illness. The composition may be administered in a daily dosage ranging from about 0.1 to 100 mg/kg, and preferably 0.5 to 10 mg/kg. The daily dosage can be given in a single dose or in several divided doses.

The present invention is not limited to particular formulations, but the pharmaceutical composition is preferably formulated into an injection preparation.

In addition, the present invention enables the establishment of a cell line capable of continuously proliferating and harboring a reporter system using a promoter of the CXCL-16 oncogene. Such a cell line may be useful in anticancer drug screening through the screening of small molecules.

[Advantageous Effects]

The present invention is characterized by identifying the nature of the CXCL-16 protein and utilizing the protein in the diagnosis and treatment of colorectal cancer. In accordance with the present invention, the CXCL-16 protein is markedly upregulated in cells and tissues from colorectal cancer patients, and is also present in higher levels in bodily fluids of colorectal cancer patients than in those of normal individuals. The cancer-specific upregulation of CXCL-16 may facilitate the diagnosis of colorectal cancer, is applicable to the treatment of colorectal cancer, and is useful in studies...
for the involvement of CXCL-1β in oncogenesis. Thus, CXCL-1β has potential use as a diagnostic and therapeutic agent for colorectal cancer.

[Description of Drawings]

FIG. 1 shows the results of RT-PCR for CXCL-16 expression in tumorous (T) and nontumorous (NT) tissues from colorectal cancer patients;

FIG. 2 shows CXCL-16 expression levels in colorectal carcinoma cell lines;

FIG. 3 shows the results of Western blotting for CXCL-1β expression in serum samples from colorectal cancer patients; and

FIG. 4 shows the results of an Direct Immunodot assay for CXCL-16 expression in serum samples from normal individuals and colorectal cancer patients.

[Best Mode]

A better understanding of the present invention may be obtained through the following examples which are set forth to illustrate, but are not to be construed as the limit of the present invention.

EXAMPLE 1: Preparation of tissue samples

For a cDNA microarray assay, tumorous and normal
nontumorous tissue samples from twenty colorectal cancer patients were obtained from the Samsung Medical Center, Korea, and frozen in liquid nitrogen. Total RNA was isolated using an RNeasy midi kit (Qiagen, Hilden, Germany).

**EXAMPLE 2: Evaluation of CXCL-16 expression in colorectal carcinoma tissues**

A cDNA microarray assay was performed to find colorectal cancer-associated genes. CXCL-16 gene expression was assessed for twenty colorectal carcinoma tissues and normal tissues. As a result, a significant increase in CXCL-16 gene expression was seen in colorectal carcinoma tissues.

In addition, one dozen pairs of tumorous (T) and nontumorous (NT) tissue samples were prepared and assessed for RNA levels using RT-PCR. In brief, oligonucleotides corresponding to the CXCL-1β gene were designed using Primer3 software (http://frodo.wi.mit.edu/). RT-PCR was carried out using a first-strand cDNA mixture (5 µg RNA) as a template.

Primers for RT-PCR were as follows.

<table>
<thead>
<tr>
<th>Primer start</th>
<th>.end</th>
<th>Direction</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>(R) 970..989</td>
<td></td>
<td>Forward</td>
<td>ctcactcgtccccataagaaac (SEQ ID No. 2)</td>
</tr>
<tr>
<td>(B) 1146..1165</td>
<td></td>
<td>Reverse</td>
<td>tcttgacacacatagaa (SEQ ID No. 3)</td>
</tr>
</tbody>
</table>

Primer annealing was carried out at 50°C.
CXCL-16 mRNA expression was normalized against β-actin as an internal control.

Consistent with the results of the cDNA microarray assay in Example 2-1, CXCL-16 mRNA expression was markedly increased in colorectal carcinoma tissues compared to normal tissues (FIG. 1).

EXAMPLE 3: Evaluation of CXCL-16 expression in colorectal carcinoma cell lines and patient sera

Western blotting was carried out using a polyclonal antibody against CXCL-16 in order to compare CXCL-16 protein expression levels between colorectal carcinoma cell lines. Colorectal carcinoma cells and gastric carcinoma cells were cultured and harvested. The cells were then lysed with a lysis buffer (1% Triton X-100, 150 mM NaCl, 100 mM KCl, 20 mM HEPES (pH 7.9), 10 mM EDTA, 1 mM sodium orthovanadate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 M PMSF). The cell lysates were electrophoresed to be separated according to size, and transferred onto a nitrocellulose membrane (Bio-Rad, CA, USA). The blot was blocked in 5% non-fat dry milk plus 0.1% Tween 20 in PBS for 2 hrs at room temperature. The blocked blot was incubated in an antibody against CXCL-16 (1:5,000 diluted) for 2 hrs, and then in a horseradish peroxidase (HRP)-conjugated secondary antibody (1:5,000 diluted) for 1 hr. The blot was then developed using an enhanced chemiluminescence assay kit.
As shown in FIG. 3, colorectal carcinoma cell lines were found to express CXCL-16 at high levels, whereas lower expression levels of CXCL-16 were detected in gastric carcinoma cell lines, SNU216 and SNU16. These results indicate that the CXCL-1β protein is expressed in colorectal cancer. In addition, Western blotting using serum samples from colorectal cancer patients showed that the CXCL-16 protein is present in the sera of colorectal cancer patients (FIG. 3).

**EXAMPLE 4: Evaluation of CXCL-16 protein expression in patient sera using ELISA**

CXCL-16 protein levels were assessed using ELISA of serum samples collected from colorectal cancer patients and normal individuals. A CXCL-16 standard and serum samples were added to a microtiter plate, and incubated with HRP-conjugated anti-CXCL-16 antibody (monoclonal). A TMB substrate solution was added to each well for color development.

CXCL-16 protein levels in patient sera were determined using the ELISA assay. Dilutions of sera from normal individuals and colorectal cancer patients were used. After the TMB reaction was stopped, absorbance was measured at 450 nm. As a result, elevated CXCL-16 levels were found in cases of colorectal cancer compared to normal samples (FIG. 4). Taken together, CXCL-16 has potential as a diagnostic and prognostic marker for colorectal cancer, and the determination
of CXCL-16 protein levels in blood is highly useful in the detection of colorectal cancer.
[CLAIMS]

[Claim 1]  
A composition for diagnosing colorectal cancer comprising an agent detecting levels of mRNA or protein corresponding to CXC chemokine ligand 16 (CXCL-16) gene.

[Claim 2]  
The composition for diagnosing colorectal cancer according to claim 1, wherein the agent detecting the levels of mRNA corresponding to the gene comprises a primer binding specifically to the CXCL-16 gene.

[Claim 3]  
The composition for diagnosing colorectal cancer according to claim 2, wherein the primer is composed of a pair of sequences of SEQ ID Nos. 2 and 3.

[Claim 4]  
The composition for diagnosing colorectal cancer according to claim 1, wherein the agent detecting the protein levels comprises an antibody specific to CXC chemokine ligand 16 (CXCL-16) protein.

[Claim 5]  
A diagnostic kit for colorectal cancer comprising the composition according to any one of claims 1 to 4.
[Claim β]
The diagnostic kit for colorectal cancer according to claim 5, which is a RT-PCR kit, a DNA chip kit, or a protein chip kit.

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[Claim 7]
A method of diagnosing colorectal cancer, comprising:
(a) measuring levels of mRNA corresponding to CXC chemokine ligand 16 (CXCL-1β) gene in a biological sample from a patient suspected of having colorectal cancer; and
(b) comparing the levels of mRNA of the biological sample from the patient with those of a normal control sample to ascertain whether there is an increase in mRNA level.

[Claim 8]
A method of diagnosing colorectal cancer, comprising:
(a) contacting an antibody specific to CXC chemokine ligand 16 (CXCL-16) protein with a biological sample from a patient suspected of having colorectal cancer to form antigen-antibody complexes; and
(b) comparing an amount of antigen-antibody complexes formed in the biological sample from the patient with an amount of antigen-antibody complexes formed in a normal control sample to ascertain whether there is an increase in protein level.
[Claim 9]

The method of diagnosing colorectal cancer according to claim 8, wherein the biological sample is a bodily fluid from the patient.

[Claim 10]

A composition for preventing or treating colorectal cancer or inhibiting metastasis of the cancer, comprising an antisense gene or siRNA against CXC chemokine ligand 16 (CXCL-16) gene.
**INTERNATIONAL SEARCH REPORT**

**PCT/ISA/210 (second sheet) (July 2008)**

### A. CLASSIFICATION OF SUBJECT MATTER

*C12Q 1/68(2006.01)*

According to International Patent Classification (IPC) or to both national classification and IPC.

### B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 8 A61K 38/17, C12Q 1/68

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

NCBI PubMed, eKIPASS, "colorectal cancer, marker, diagnosis, CXCL16, etc."

### C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category*</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No</th>
</tr>
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<tbody>
<tr>
<td>X</td>
<td>Shozo Hojo, et al., 'High-level expression of CXCL16 by tumor cells correlates with a good prognosis and increased tumor-infiltrating lymphocytes in colorectal cancer', In Cancer Research, 15 May 2007, Vol 67(10), pp 4725-4731 - see the whole document, especially Table 1, Figures 1-4</td>
<td>1-6</td>
</tr>
<tr>
<td>A</td>
<td>US 2007/02381 15 A1 (Michael B Dwinell, et al, US) 11 October 2007 - see the whole document, especially Table 1</td>
<td>1-6</td>
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<td>A</td>
<td>Joseph Kim, et al., 'Chemokine receptor CXCR4 expression in colorectal cancer patients increases the risk for recurrence and for poor survival', In Journal of Clinical Oncology, 20 April 2005, Vol 23(12), pp 2744-2753 - see the whole document</td>
<td>1-6</td>
</tr>
<tr>
<td>A</td>
<td>Seungkoo Lee, et al., 'Differential expression in normal adenoma-carcinoma sequence suggests complex molecular carcinogenesis in colon', In Oncology Reports, 2006, Vol 16(4), pp 747-754 - see the whole document, especially Tables 1-IV</td>
<td>1-6</td>
</tr>
</tbody>
</table>

Further documents are listed in the continuation of Box C

See patent family annex

---

Date of the actual completion of the international search: 24 JULY 2008 (24.07.2008)

Date of mailing of the international search report: 24 JULY 2008 (24.07.2008)

Name and mailing address of the ISA/KR

Korean Intellectual Property Office
Government Complex-Daejeon, 139 Seonsa-ro, Seogu, Daejeon 302-701, Republic of Korea

Facsimile No 82-42-472-7140

Authorized officer: SHIN, Kyeong A

Telephone No 82-42-481-5589

Form PCT/ISA/210 (second sheet) (July 2008)
<table>
<thead>
<tr>
<th>Box No.</th>
<th>Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)</th>
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<tr>
<td>1</td>
<td>With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of</td>
</tr>
<tr>
<td>a</td>
<td>type of material</td>
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<td>□ a sequence listing</td>
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<td>□ table(s) related to the sequence listing</td>
</tr>
<tr>
<td>b</td>
<td>format of material</td>
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<td></td>
<td>□ on paper</td>
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<td>□ in electronic form</td>
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<td>c</td>
<td>time of filing/furnishing</td>
</tr>
<tr>
<td></td>
<td>□ contained in the international application as filed</td>
</tr>
<tr>
<td></td>
<td>□ filed together with the international application in electronic form</td>
</tr>
<tr>
<td></td>
<td>□ furnished subsequently to this Authority for the purposes of search</td>
</tr>
<tr>
<td>2</td>
<td>In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished</td>
</tr>
<tr>
<td>3</td>
<td>Additional comments</td>
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</table>
**INTERNATIONAL SEARCH REPORT**

**Box No. II  Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons

<table>
<thead>
<tr>
<th>No.</th>
<th>Claims No(s)</th>
<th>Remarks</th>
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<tbody>
<tr>
<td>1</td>
<td>7-9</td>
<td>because they relate to subject matter not required to be searched by this Authority, namely</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Claims 7-9 of the present invention pertain to diagnostic methods of the human or animal body. Thus this International Search Authority is not required, under Article 17(2)(a)(i) of the PCT and Rule 39 (iv) of the Regulations under the PCT, to search</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically</td>
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<td></td>
<td>Claim 10 does not meet the requirements of PCT Article 6 in that the matter for which protection is sought is not clearly defined. The constitutions of &quot;antisense gene or siRNA&quot; are so unclear and too broad to meaningful search possible</td>
</tr>
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<td>3</td>
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<td>because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6 4(a)</td>
</tr>
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</table>

**Box No. III  Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows

<table>
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<tr>
<th>No.</th>
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<tbody>
<tr>
<td>1</td>
<td>As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims</td>
</tr>
<tr>
<td>2</td>
<td>As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee</td>
</tr>
<tr>
<td>3</td>
<td>As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos</td>
</tr>
<tr>
<td>4</td>
<td>No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims, it is covered by claims Nos</td>
</tr>
</tbody>
</table>

**Remark on Protest**

- The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee
- The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation
- No protest accompanied the payment of additional search fees

International application No PCT/KR2007/005964

Form PCT/ISA/210 (continuation of first sheet (2)) (July 2008)
<table>
<thead>
<tr>
<th>Patent document cited in search report</th>
<th>Publication date</th>
<th>Patent family member(s)</th>
<th>Publication date</th>
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<tr>
<td>US 20070238115 A1</td>
<td>11.10.2007</td>
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<td>WO 2007032631 A1</td>
<td>22.03.2007</td>
<td>KR 20070030084 A</td>
<td>15.03.2007</td>
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