DIAGNOSIS AND TREATMENT OF CANCER USING ANTI-EREG ANTIBODY

Technical Field

The present invention relates to methods for diagnosing and treating cancer, cell proliferation inhibitors, and anticancer agents.

Background Art

A member of the epidermal growth factor (hereinafter referred to as EGF) family purified by Toyoda *et al.* was named "epiregulin (EREG)". EREG is known to function as a cancer growth inhibitor that induces morphological changes of HeLa cells (Non-Patent Document 1). The amino acid sequence of mouse-derived EREG (mature protein) purified by Toyoda *et al.* consists of 46 amino acid residues, and shares a sequence identity of about 24% to 50 % with other members of the EGF family. Mouse EREG showed low affinity to the EGF receptor on A431 cells (a human epithelial carcinoma cell line). The cloning and expression analysis of the human EREG gene by Toyoda *et al.* showed that, while other members of the EGF family are expressed ubiquitously in human tissues, the EREG expression is detectable in macrophages, placenta, and various types of cancer cells (Non-Patent Document 2). In addition, the soluble form of EREG was shown to have proliferation-suppressing effect on several types of cancer cells (WO94/29340).

Takahashi *et al.* showed that the activation of Erk (MPK3) and p38 (MAPK14) in differentiated arterial vascular smooth muscle cells (hereinafter referred to as VSMC) from rats induces dedifferentiation of the cells. Furthermore, it was demonstrated that EREG secreted by VSMC acts as an autocrine and/or paracrine differentiation factor. Unsaturated lysophosphatidic acid and PDGFB homodimer, which may act as differentiation factors of VSMC, rapidly up-regulated the mRNA expression of EREG in an Erk- and p38 MAPK-dependent manner. Reverse transcriptase polymerase chain reaction (hereinafter referred to as RT-PCR) analysis, and immunohistochemical or immunohistochemistry (hereinafter referred to as IHC) analysis revealed localized EREG expression in atherosclerotic arteries and balloon-injured rat arteries. From these results, Takahashi and others speculated that EREG might be involved in the progression of vascular remodeling such as atherosclerosis (Non-Patent Document 3).

Minn *et al.* identified several gene clusters related to lung metastasis of breast cancer based on *in vivo* selection, transcriptome analysis, functional analysis, and clinical research, and showed that EREG is one of the genes (Non-Patent Document 4).

Furthermore, Shirasawa *et al.* showed that EREG is expressed not only in keratinocytes but also in tissue macrophages, and that EREG-knockout mice develop chronic dermatitis.

Examinations in the analysis of these mice revealed that EREG plays an important role in immunity- and inflammatory-related responses of keratinocytes and macrophages at the boundary with the external environment (Non-Patent Document 5).

As described above, the connection between EREG and dermatitis, cancer metastasis, and atherosclerosis has been indicated. However, there are still no specific descriptions on the effect of EREG-binding antibodies that have neutralizing activity and cytotoxic activity on EREG-expressing cancer cells.

[Non-Patent Document 1] J. Biol. Chem. 270: 7495-7500, 1995

[Non-Patent Document 2] Biochem. J. 326: 69-75, 1997

[Non-Patent Document 3] Circulation 108: 2524-2529, 2003

[Non-Patent Document 4] Nature 436: 518-524, 2005

[Non-Patent Document 5] Proc. Nat. Acad. Sci. 101: 13921-13926, 2004

Summary

In a first aspect, there is provided a use of an antibody that binds to an EREG protein, and which has cell proliferation inhibitory activity against cells expressing the EREG protein for the preparation of a composition for the treatment of cancer.

In a second aspect, there is provided a use of a minibody produced from the antibody set forth as defined above for the preparation of a composition for the treatment of cancer.

Disclosure of the Invention

[Problems to be Solved by the Invention]

An objective of the present invention is to provide anti-EREG antibodies and uses thereof. More specifically, an objective of the present invention is to provide novel methods for diagnosing and treating cancer using anti-EREG antibodies, novel cell proliferation inhibitors and anticancer agents comprising anti-EREG antibodies, and novel anti-EREG antibodies.

[Means for Solving the Problems]

The present inventors discovered that EREG is highly expressed in cancer cells such as colon cancer cells. Furthermore, when complement-dependent cytotoxicity (CDC) activity and antibody-dependent cell-mediated cytotoxicity (ADCC) activity of anti-EREG antibodies were measured, the anti-EREG antibodies were found to have CDC activity and ADCC activity in EREG-expressing cells. The present inventors also showed that the anti-EREG antibodies have proliferation-suppressing effect on cancer cell lines via neutralization. Furthermore, from the above-mentioned findings, the present inventors discovered that the anti-EREG antibodies were effective for diagnosis, prevention, and treatment of various types of primary and metastatic cancers, and thereby completed the present invention. More specifically, the present inventors completed the present invention by discovering that EREG is useful as a tool for treating or diagnosing cancers in which the EREG expression is enhanced, including colon cancer, lung adenocarcinoma, pancreatic cancer, stomach cancer, and kidney cancer.

The present invention provides pharmaceutical compositions comprising an antibody that binds to an EREG protein as an active ingredient. The present invention also provides cell proliferation inhibitors comprising an antibody that binds to an EREG protein as an active ingredient. The present invention further provides anticancer agents comprising an antibody

that binds to an EREG protein as an active ingredient. Preferably, the antibody that binds to an EREG protein has cytotoxic activity. More preferably, the antibody also has neutralizing activity. In a preferred embodiment of the present invention, the cancers that can be targeted for treatment are colon cancer, lung adenocarcinoma, pancreatic cancer, stomach cancer, and kidney cancer. Anticancer agents comprising an anti-EREG antibody of the present invention are useful for treatment of these cancers which are primary or metastatic cancers and have elevated expression of EREG. Particularly preferred targets for treatment of the present invention are primary colon cancer, metastatic colon cancer, and pancreatic cancer.

Furthermore, the present invention provides pharmaceutical compositions comprising an antibody that binds to an EREG protein and a pharmaceutically acceptable carrier. Pharmaceutical compositions of the present invention are useful for treating and/or preventing cancers that have elevated expression of EREG. That is, the present invention relates to the use of an antibody that binds to an EREG protein for the manufacturing of pharmaceutical compositions for treating and/or preventing cancer.

In another embodiment, the present invention provides methods for inducing cell injury in cells that express an EREG protein by contacting EREG-expressing cells with an antibody that binds to an EREG protein. The present invention also provides methods for suppressing proliferation of cells that express an EREG protein by contacting EREG protein-expressing cells with an antibody that binds to an EREG protein. The antibody that binds to an EREG protein preferably has cytotoxic activity. Cells that express an EREG protein are preferably cancer cells.

Furthermore, in another embodiment, the present invention provides antibodies that bind to an EREG protein and have cytotoxic activity in cells that express the EREG protein. Preferably, the cytotoxic activity is ADCC activity. Preferably, the cytotoxic activity is CDC activity. The present invention also provides antibodies to which a cytotoxic substance is binding. In the present invention, the cytotoxic substances that may be binding to the antibody include chemotherapeutic agents, radioisotopes, and toxic peptides. Preferably, in the present invention, antibody itself has cytotoxic activity.

The present invention further provides antibodies that bind to an EREG protein, and which have cytotoxic activity and neutralizing activity against EREG protein-expressing cells.

In another embodiment, the present invention provides the use of an EREG protein as a cancer diagnostic marker.

Furthermore, in another embodiment, the present invention provides methods for diagnosing cancer, which comprises detecting an EREG protein using an antibody that binds to the EREG protein. In the methods of the present invention, preferably, the extracellular region of the EREG protein is detected. Preferably, the methods of the present invention are carried

out using an antibody that recognizes the EREG protein. Preferably, the EREG protein in blood, serum, or plasma, or EREG protein isolated from cells is detected in the methods of the present invention.

In another embodiment, the present invention provides methods for diagnosing cancer which comprise the following steps of:

(a) collecting a sample from a subject; and

(b) using an antibody that binds to EREG protein to detect the EREG protein contained in the collected sample.

In the present invention, any sample can be used as the above-mentioned sample as long as it can be collected from the subject. In one embodiment, blood sample collected from a subject is used. A preferred blood sample in the present invention is serum. In another embodiment, samples collected surgically or by biopsy from a subject may be used. The methods of diagnosis can be used for any cancer as long as it is a cancer in which the target cancer cells express an EREG protein. Cancers that are preferred in the present invention are colon cancer, lung adenocarcinoma, pancreatic cancer, stomach cancer, and kidney cancer. Based on the present invention, both primary and metastatic foci of these cancers can be diagnosed. Cancers that are particularly preferred are primary colon cancer, metastatic colon cancer, and pancreatic cancer.

In the present invention, the step of collecting a sample from a subject can also be expressed as the step of providing a sample collected from a subject.

Furthermore, in another embodiment, the present invention provides methods for diagnosing cancer, which comprise the steps of: (1) administering to a subject a radioisotope-labeled antibody that binds to an EREG protein; and (2) detecting accumulation of the radioisotope. In a certain embodiment, the radioisotope is a positron-emitting nuclide. A preferred positron-emitting nuclide of the present invention can be selected, for example, from the group consisting of ^{11}C, ^{13}N, ^{15}O, ^{18}F, ^{45}Ti, ^{55}Co, ^{64}Cu, ^{66}Ga, ^{68}Ga, ^{76}Br, ^{89}Zr, and ^{124}I.

Furthermore, in another embodiment, the present invention provides methods for diagnosing cancer, in which the expression of a gene encoding the EREG protein is detected.

Furthermore, in another embodiment, the present invention provides diagnostic agents and kits to be used in the methods for diagnosis of the present invention.

In addition, the present invention provides methods of screening for candidate compounds as cancer therapeutic agents. In the present invention, compounds can be selected as a candidate cancer therapeutic agent by using, for example, the EREG expression level as an index. Alternatively, compound can be selected as a candidate cancer therapeutic agent by using the neutralizing effect against cell proliferation stimulating activity of EREG as an indicator.

More specifically, the present invention provides the following:

[1] a pharmaceutical composition comprising as an active ingredient an antibody that binds to an EREG protein;

[2] a cell proliferation inhibitor comprising as an active ingredient an antibody that binds to an EREG protein;

[3] an anticancer agent comprising as an active ingredient an antibody that binds to an EREG protein;

[4] the anticancer agent of [3], wherein the antibody that binds to an EREG protein is an antibody that has cell proliferation inhibitory activity;

[5] the anticancer agent of [3] or [4], wherein the antibody that binds to an EREG protein is an antibody of any of (1) to (57) below:

(1) an antibody comprising an H chain having the amino acid sequence of SEQ ID NO: 2 as CDR1, the amino acid sequence of SEQ ID NO: 4 as CDR2, and the amino acid sequence of SEQ ID NO: 6 as CDR3;

(2) an antibody comprising the H chain of (1), wherein the H chain has the amino acid sequence of positions 117 to 452 in the amino acid sequence of SEQ ID NO: 8 as CH;

(3) an antibody comprising the H chain of (1), wherein the H chain has the amino acid sequence of positions 117 to 446 in the amino acid sequence of SEQ ID NO: 10 as CH;

(4) an antibody comprising an L chain having the amino acid sequence of SEQ ID NO: 12 as CDR1, the amino acid sequence of SEQ ID NO: 14 as CDR2, and the amino acid sequence of SEQ ID NO: 16 as CDR3;

(5) an antibody comprising the L chain of (4), wherein the L chain has the amino acid sequence of positions 107 to 213 in the amino acid sequence of SEQ ID NO: 18 as CL;

(6) an antibody comprising the L chain of (4), wherein the L chain has the amino acid sequence of positions 107 to 213 in the amino acid sequence of SEQ ID NO: 20 as CL;

(7) an antibody comprising the H chain of (1) and the L chain of (4);

(8) an antibody comprising the H chain of (2) and the L chain of (5);

(9) an antibody comprising the H chain of (3) and the L chain of (6);

(10) an antibody comprising an H chain having the amino acid sequence of SEQ ID NO: 49 as CDR1, the amino acid sequence of SEQ ID NO: 51 as CDR2, and the amino acid sequence of SEQ ID NO: 53 as CDR3;

(11) an antibody comprising the H chain of (10), wherein the H chain has CH derived from mouse IgG1;

(12) an antibody comprising the H chain of (10), wherein the H chain has the amino acid sequence of positions 117 to 446 in the amino acid sequence of SEQ ID NO: 10 as CH;

(13) an antibody comprising an L chain having the amino acid sequence of SEQ ID NO: 55 as

CDR1, the amino acid sequence of SEQ ID NO: 57 as CDR2, and the amino acid sequence of SEQ ID NO: 59 as CDR3;

(14) an antibody comprising the L chain of (13), wherein the L chain has CL derived from the mouse κ chain;

(15) an antibody comprising the L chain of (13), wherein the L chain has the amino acid sequence of positions 107 to 213 in the amino acid sequence of SEQ ID NO: 20 as CL;

(16) an antibody comprising the H chain of (10) and the L chain of (13);

(17) an antibody comprising the H chain of (11) and the L chain of (14);

(18) an antibody comprising the H chain of (12) and the L chain of (15);

(19) an antibody comprising an H chain having the amino acid sequence of SEQ ID NO: 61 as CDR1, the amino acid sequence of SEQ ID NO: 63 as CDR2, and the amino acid sequence of SEQ ID NO: 65 as CDR3;

(20) an antibody comprising the H chain of (19), wherein the H chain has CH derived from mouse IgG1;

(21) an antibody comprising the H chain of (19), wherein the H chain has the amino acid sequence of positions 117 to 446 in the amino acid sequence of SEQ ID NO: 10 as CH;

(22) an antibody comprising an L chain having the amino acid sequence of SEQ ID NO: 67 as CDR1, the amino acid sequence of SEQ ID NO: 69 as CDR2, and the amino acid sequence of SEQ ID NO: 71 as CDR3;

(23) an antibody comprising the L chain of (22), wherein the L chain has CL derived from the mouse κ chain;

(24) an antibody comprising the L chain of (22), wherein the L chain has the amino acid sequence of positions 107 to 213 in the amino acid sequence of SEQ ID NO: 20 as CL;

(25) an antibody comprising the H chain of (19) and the L chain of (22);

(26) an antibody comprising the H chain of (20) and the L chain of (23);

(27) an antibody comprising the H chain of (21) and the L chain of (24);

(28) an antibody comprising an H chain having the amino acid sequence of SEQ ID NO: 73 as CDR1, the amino acid sequence of SEQ ID NO: 75 as CDR2, and the amino acid sequence of SEQ ID NO: 77 as CDR3;

(29) an antibody comprising the H chain of (28), wherein the H chain has CH derived from mouse IgG1;

(30) an antibody comprising the H chain of (28), wherein the H chain has the amino acid sequence of positions 117 to 446 in the amino acid sequence of SEQ ID NO: 10 as CH;

(31) an antibody comprising an L chain having the amino acid sequence of SEQ ID NO: 79 as CDR1, the amino acid sequence of SEQ ID NO: 81 as CDR2, and the amino acid sequence of SEQ ID NO: 83 as CDR3;

(32) an antibody comprising the L chain of (31), wherein the L chain has CL derived from the mouse κ chain;

(33) an antibody comprising the L chain of (31), wherein the L chain has the amino acid sequence of positions 107 to 213 in the amino acid sequence of SEQ ID NO: 20 as CL;

(34) an antibody comprising the H chain of (28) and the L chain of (31);

(35) an antibody comprising the H chain of (29) and the L chain of (32);

(36) an antibody comprising the H chain of (30) and the L chain of (33);

(37) an antibody comprising an H chain having the amino acid sequence of SEQ ID NO: 85 as CDR1, the amino acid sequence of SEQ ID NO: 87 as CDR2, and the amino acid sequence of SEQ ID NO: 89 as CDR3;

(38) an antibody comprising the H chain of (37), wherein the H chain has CH derived from mouse IgG1;

(39) an antibody comprising the H chain of (37), wherein the H chain has the amino acid sequence of positions 117 to 446 in the amino acid sequence of SEQ ID NO: 10 as CH;

(40) an antibody comprising an L chain having the amino acid sequence of SEQ ID NO: 91 as CDR1, the amino acid sequence of SEQ ID NO: 93 as CDR2, and the amino acid sequence of SEQ ID NO: 95 as CDR3;

(41) an antibody comprising the L chain of (40), wherein the L chain has CL derived from the mouse κ chain;

(42) an antibody comprising the L chain of (40), wherein the L chain has the amino acid sequence of positions 107 to 213 in the amino acid sequence of SEQ ID NO: 20 as CL;

(43) an antibody comprising the H chain of (37) and the L chain of (40);

(44) an antibody comprising the H chain of (38) and the L chain of (41);

(45) an antibody comprising the H chain of (39) and the L chain of (42);

(46) an antibody comprising an H chain having the amino acid sequence of SEQ ID NO: 97 as CDR1, the amino acid sequence of SEQ ID NO: 99 as CDR2, and the amino acid sequence of SEQ ID NO: 101 as CDR3;

(47) an antibody comprising the H chain of (46), wherein the H chain has CH derived from mouse IgG1;

(48) an antibody comprising the H chain of (46), wherein the H chain has the amino acid sequence of positions 117 to 446 in the amino acid sequence of SEQ ID NO: 10 as CH;

(49) an antibody comprising an L chain having the amino acid sequence of SEQ ID NO: 103 as CDR1, the amino acid sequence of SEQ ID NO: 105 as CDR2, and the amino acid sequence of SEQ ID NO: 107 as CDR3;

(50) an antibody comprising the L chain of (49), wherein the L chain has CL derived from the mouse κ chain;

(51) an antibody comprising the L chain of (49), wherein the L chain has the amino acid sequence of positions 107 to 213 in the amino acid sequence of SEQ ID NO: 20 as CL;
(52) an antibody comprising the H chain of (46) and the L chain of (49);
(53) an antibody comprising the H chain of (47) and the L chain of (50);
(54) an antibody comprising the H chain of (48) and the L chain of (51);
(55) an antibody comprising one or more amino acid substitutions, deletions, additions, and/or insertions in the antibody of any of (1) to (54), which has equivalent activity as the antibody of any of (1) to (54);
(56) an antibody that binds to the same EREG protein epitope as the antibody of any of (1) to (55); and
(57) a minibody of the antibody of any of (1) to (56);
[6] the anticancer agent of any one of [3] to [5], wherein the antibody that binds to EREG protein recognizes Ala at position 29 to Ser at position 69, or Val at position 63 to Leu at position 108, in the amino acid sequence of SEQ ID NO: 21;
[7] the anticancer agent of any one of [3] to [6], wherein the cancer is any cancer selected from the group consisting of colon cancer, lung adenocarcinoma, pancreatic cancer, stomach cancer, and kidney cancer;
[8] the anticancer agent of [7], wherein the cancer is primary cancer;
[9] the anticancer agent of [7], wherein the cancer is metastatic cancer;
[10] an antibody that binds to an EREG protein, and which has cell proliferation inhibitory activity against cells expressing the EREG protein;
[11] the antibody of [10], wherein the cell proliferation inhibitory activity is cytotoxic activity;
[12] the antibody of [11], wherein the cytotoxic activity is ADCC activity;
[13] the antibody of [11], wherein the cytotoxic activity is CDC activity;
[14] the antibody of any one of [10] to [13], which additionally has neutralizing activity against the EREG protein;
[15] The antibody of [10], wherein the cell proliferation inhibitory activity is neutralizing activity;
[16] a minibody produced from the antibody of [10];
[17] the antibody of any one of [10] to [16], wherein a chemotherapeutic agent or a toxic peptide is bound to the antibody;
[18] an antibody that binds to an EREG protein, wherein any one cytotoxic substance selected from the group consisting of a chemotherapeutic agent, a toxic peptide, and a radioisotope is bound to the antibody;
[19] the antibody of any one of [10] to [18], which is an antibody of any of (1) to (57) below:
(1) an antibody comprising an H chain having the amino acid sequence of SEQ ID NO: 2 as

CDR1, the amino acid sequence of SEQ ID NO: 4 as CDR2, and the amino acid sequence of SEQ ID NO: 6 as CDR3;

(2) an antibody comprising the H chain of (1), wherein the H chain has the amino acid sequence of positions 117 to 452 in the amino acid sequence of SEQ ID NO: 8 as CH;

(3) an antibody comprising the H chain of (1), wherein the H chain has the amino acid sequence of positions 117 to 446 in the amino acid sequence of SEQ ID NO: 10 as CH;

(4) an antibody comprising an L chain having the amino acid sequence of SEQ ID NO: 12 as CDR1, the amino acid sequence of SEQ ID NO: 14 as CDR2, and the amino acid sequence of SEQ ID NO: 16 as CDR3;

(5) an antibody comprising the L chain of (4), wherein the L chain has the amino acid sequence of positions 107 to 213 in the amino acid sequence of SEQ ID NO: 18 as CL;

(6) an antibody comprising the L chain of (4), wherein the L chain has the amino acid sequence of positions 107 to 213 in the amino acid sequence of SEQ ID NO: 20 as CL;

(7) an antibody comprising the H chain of (1) and the L chain of (4);

(8) an antibody comprising the H chain of (2) and the L chain of (5);

(9) an antibody comprising the H chain of (3) and the L chain of (6);

(10) an antibody comprising an H chain having the amino acid sequence of SEQ ID NO: 49 as CDR1, the amino acid sequence of SEQ ID NO: 51 as CDR2, and the amino acid sequence of SEQ ID NO: 53 as CDR3;

(11) an antibody comprising the H chain of (10), wherein the H chain has CH derived from mouse IgG1;

(12) an antibody comprising the H chain of (10), wherein the H chain has the amino acid sequence of positions 117 to 446 in the amino acid sequence of SEQ ID NO: 10 as CH;

(13) an antibody comprising an L chain having the amino acid sequence of SEQ ID NO: 55 as CDR1, the amino acid sequence of SEQ ID NO: 57 as CDR2, and the amino acid sequence of SEQ ID NO: 59 as CDR3;

(14) an antibody comprising the L chain of (13), wherein the L chain has CL derived from the mouse κ chain;

(15) an antibody comprising the L chain of (13), wherein the L chain has the amino acid sequence of positions 107 to 213 in the amino acid sequence of SEQ ID NO: 20 as CL;

(16) an antibody comprising the H chain of (10) and the L chain of (13);

(17) an antibody comprising the H chain of (11) and the L chain of (14);

(18) an antibody comprising the H chain of (12) and the L chain of (15);

(19) an antibody comprising an H chain having the amino acid sequence of SEQ ID NO: 61 as CDR1, the amino acid sequence of SEQ ID NO: 63 as CDR2, and the amino acid sequence of SEQ ID NO: 65 as CDR3;

(20) an antibody comprising the H chain of (19), wherein the H chain has CH derived from mouse IgG1;
(21) an antibody comprising the H chain of (19), wherein the H chain has the amino acid sequence of positions 117 to 446 in the amino acid sequence of SEQ ID NO: 10 as CH;
(22) an antibody comprising an L chain having the amino acid sequence of SEQ ID NO: 67 as CDR1, the amino acid sequence of SEQ ID NO: 69 as CDR2, and the amino acid sequence of SEQ ID NO: 71 as CDR3;
(23) an antibody comprising the L chain of (22), wherein the L chain has CL derived from the mouse κ chain;
(24) an antibody comprising the L chain of (22), wherein the L chain has the amino acid sequence of positions 107 to 213 in the amino acid sequence of SEQ ID NO: 20 as CL;
(25) an antibody comprising the H chain of (19) and the L chain of (22);
(26) an antibody comprising the H chain of (20) and the L chain of (23);
(27) an antibody comprising the H chain of (21) and the L chain of (24);
(28) an antibody comprising an H chain having the amino acid sequence of SEQ ID NO: 73 as CDR1, the amino acid sequence of SEQ ID NO: 75 as CDR2, and the amino acid sequence of SEQ ID NO: 77 as CDR3;
(29) an antibody comprising the H chain of (28), wherein the H chain has CH derived from mouse IgG1;
(30) an antibody comprising the H chain of (28), wherein the H chain has the amino acid sequence of positions 117 to 446 in the amino acid sequence of SEQ ID NO: 10 as CH;
(31) an antibody comprising an L chain having the amino acid sequence of SEQ ID NO: 79 as CDR1, the amino acid sequence of SEQ ID NO: 81 as CDR2, and the amino acid sequence of SEQ ID NO: 83 as CDR3;
(32) an antibody comprising the L chain of (31), wherein the L chain has CL derived from the mouse κ chain;
(33) an antibody comprising the L chain of (31), wherein the L chain has the amino acid sequence of positions 107 to 213 in the amino acid sequence of SEQ ID NO: 20 as CL;
(34) an antibody comprising the H chain of (28) and the L chain of (31);
(35) an antibody comprising the H chain of (29) and the L chain of (32);
(36) an antibody comprising the H chain of (30) and the L chain of (33);
(37) an antibody comprising an H chain having the amino acid sequence of SEQ ID NO: 85 as CDR1, the amino acid sequence of SEQ ID NO: 87 as CDR2, and the amino acid sequence of SEQ ID NO: 89 as CDR3;
(38) an antibody comprising the H chain of (37), wherein the H chain has CH derived from mouse IgG1;

(39) an antibody comprising the H chain of (37), wherein the H chain has the amino acid sequence of positions 117 to 446 in the amino acid sequence of SEQ ID NO: 10 as CH;
(40) an antibody comprising an L chain having the amino acid sequence of SEQ ID NO: 91 as CDR1, the amino acid sequence of SEQ ID NO: 93 as CDR2, and the amino acid sequence of SEQ ID NO: 95 as CDR3;
(41) an antibody comprising the L chain of (40), wherein the L chain has CL derived from the mouse κ chain;
(42) an antibody comprising the L chain of (40), wherein the L chain has the amino acid sequence of positions 107 to 213 in the amino acid sequence of SEQ ID NO: 20 as CL;
(43) an antibody comprising the H chain of (37) and the L chain of (40);
(44) an antibody comprising the H chain of (38) and the L chain of (41);
(45) an antibody comprising the H chain of (39) and the L chain of (42);
(46) an antibody comprising an H chain having the amino acid sequence of SEQ ID NO: 97 as CDR1, the amino acid sequence of SEQ ID NO: 99 as CDR2, and the amino acid sequence of SEQ ID NO: 101 as CDR3;
(47) an antibody comprising the H chain of (46), wherein the H chain has CH derived from mouse IgG1;
(48) an antibody comprising the H chain of (46), wherein the H chain has the amino acid sequence of positions 117 to 446 in the amino acid sequence of SEQ ID NO: 10 as CH;
(49) an antibody comprising an L chain having the amino acid sequence of SEQ ID NO: 103 as CDR1, the amino acid sequence of SEQ ID NO: 105 as CDR2, and the amino acid sequence of SEQ ID NO: 107 as CDR3;
(50) an antibody comprising the L chain of (49), wherein the L chain has CL derived from the mouse κ chain;
(51) an antibody comprising the L chain of (49), wherein the L chain has the amino acid sequence of positions 107 to 213 in the amino acid sequence of SEQ ID NO: 20 as CL;
(52) an antibody comprising the H chain of (46) and the L chain of (49);
(53) an antibody comprising the H chain of (47) and the L chain of (50);
(54) an antibody comprising the H chain of (48) and the L chain of (51);
(55) an antibody comprising one or more amino acid substitutions, deletions, additions, and/or insertions in the antibody of any of (1) to (54), which has equivalent activity as the antibody of any of (1) to (54);
(56) an antibody that binds to the same EREG protein epitope as the antibody of any of (1) to (55); and
(57) a minibody of the antibody of any of (1) to (56);
[20] the antibody of any one of [10] to [19], wherein the antibody recognizes Ala at position 29

to Ser at position 69, or Val at position 63 to Leu at position 108, in the amino acid sequence of SEQ ID NO: 21;

[21] use of an EREG protein or an EREG mRNA, or both, as a cancer diagnostic marker;

[22] a method for diagnosis of cancer, comprising combining an EREG protein with an antibody that binds to the EREG protein;

[23] a method for diagnosis of cancer, comprising the steps of:

(a) collecting a sample from a subject; and

(b) detecting an EREG protein contained in the collected sample using an antibody that binds to the EREG protein;

[24] a method for diagnosis of cancer, comprising the steps of: (1) administering to a subject a radioisotope-labeled antibody that binds to an EREG protein; and (2) detecting accumulation of said radioisotope;

[25] the method of diagnosis of [22], wherein the radioisotope is a positron-emitting nuclide;

[26] the method of diagnosis of [25], wherein the positron-emitting nuclide is any one nuclide selected from the group consisting of ^{11}C, ^{13}N, ^{15}O, ^{18}F, ^{45}Ti, ^{55}Co, ^{64}Cu, ^{66}Ga, ^{68}Ga, ^{76}Br, ^{89}Zr, and ^{124}I;

[27] a method for diagnosis of cancer, comprising detecting the expression of a gene encoding an EREG protein;

[28] the method of diagnosis of any one of [22] to [27], wherein the cancer is any cancer selected from the group consisting of colon cancer, lung adenocarcinoma, pancreatic cancer, stomach cancer, and kidney cancer;

[29] the method of diagnosis of [28], wherein the cancer is primary cancer;

[30] the method of diagnosis of [28], wherein the cancer is metastatic cancer;

[31] a diagnostic agent for use in a method for diagnosis of cancer, which comprises an antibody that binds to an EREG protein;

[32] a kit for use in a method for diagnosis of cancer, which comprises an antibody that binds to an EREG protein, and a biological sample comprising an EREG protein;

[33] a method of screening for a candidate compound as cancer therapeutic agent, which comprises the steps of:

(1) contacting EREG-expressing cells with a test compound;

(2) measuring the EREG expression level in the EREG-expressing cells; and

(3) selecting a compound that lowers the EREG expression level compared to a control as a candidate therapeutic agent for cancer;

[34] the method of [33], wherein the EREG expression level is evaluated by the level of the EREG protein secreted into a culture supernatant;

[35] a method of screening for a candidate compound as cancer therapeutic agent, which

comprises the steps of:

(1) culturing cells that proliferate in an EREG-dependent manner, in the presence of EREG and a test compound;

(2) measuring the cell proliferation level; and

(3) selecting a test compound that suppresses cell proliferation compared to a control as a candidate therapeutic agent for cancer.

[Effects of the Invention]

The EREG gene expression was confirmed to be significantly elevated in cancer cells based on gene expression analysis of various cancer tissues and cancer cell lines. On the other hand, the EREG expression is very low in normal cells. Therefore, EREG is useful as a specific marker for cancer detection.

The cytotoxic effect of anti-EREG antibodies against EREG-expressing cells was confirmed in the present invention. While the EREG expression is very low in normal cells, it is elevated in cancer cells. This supports the possibility that cancer cell-specific cytotoxic effect could be obtained *in vivo*, for example, by administering an anti-EREG antibody.

Furthermore, in the present invention, it was confirmed that EREG-dependent cell proliferation is neutralized by anti-EREG antibodies. Therefore, in a preferred embodiment, in addition to the cytotoxic effect, anti-EREG antibodies inhibit cancer cell proliferation by neutralizing the cell proliferation effect of EREG.

Brief Description of the Drawings

Fig. 1 shows the levels of EREG mRNA transcript in normal tissues and cancer tissues. The vertical axis indicates the signal intensity of Probe ID: 205767_at HG-U133A. The intensity is relative to the mean value of the expression scores of all the genes on GeneChip U133A, which was set to 100.

Fig. 2 shows the levels of EREG mRNA transcript in cancer cell lines. The vertical axis indicates the signal intensity of Probe ID: 205767_at HG-U133A. The intensity is relative to the mean value of the expression scores of all the genes on GeneChip U133A, which was set to 100.

Fig. 3 shows the binding of anti-EREG monoclonal antibodies to EREG-DG cells by flow cytometry. The vertical axis indicates the cell number (fluorescent count), and the horizontal axis indicates the fluorescence intensity value.

Fig. 4 shows the binding of anti-EREG monoclonal antibodies to the DLD1 colon cancer cell line by flow cytometry. The vertical axis indicates the cell number (fluorescent count), and the horizontal axis indicates the fluorescence intensity value.

Fig. 5 shows the CDC activity of anti-EREG monoclonal antibodies against the DLD1 colon cancer cell line. The vertical axis indicates the chromium release rate (%), and the horizontal axis indicates the name of antibody.

Fig. 6 shows the ADCC activity of anti-EREG monoclonal antibodies against the DLD1 colon cancer cell line. The vertical axis indicates the chromium release rate (%), and the horizontal axis indicates the name of antibody.

Fig. 7 shows the dependence of proliferation of EGFR-BAF cells on human EREG. The vertical axis indicates the absorbance at 450 nm (reference wavelength of 655 nm), and the horizontal axis indicates the EREG concentration (ng/mL).

Fig. 8 shows the dependence of proliferation of the AsPC1 pancreatic cancer cell line on human EREG. The vertical axis indicates the absorbance at 450 nm (reference wavelength of 655 nm), and the horizontal axis indicates the EREG concentration (ng/mL).

Fig. 9 shows the dependence of proliferation of the DLD1 colon cancer cell line on human EREG. The vertical axis indicates the absorbance at 450 nm (reference wavelength of 655 nm), and the horizontal axis indicates the EREG concentration (ng/mL).

Fig. 10 shows the neutralizing activity of anti-human EREG monoclonal antibodies in EGFR-BAF cells. The vertical axis indicates the cell proliferation rate (absorbance at each antibody concentration/absorbance of the control x 100%), and the horizontal axis indicates the antibody concentration (μg/mL).

Fig. 11 shows the neutralizing activity of anti-human EREG monoclonal antibodies in the AsPC1 pancreatic cancer cell line. The vertical axis indicates the cell proliferation rate (absorbance at each antibody concentration/absorbance of the control x 100%), and the horizontal axis indicates the antibody concentration (μg/mL).

Fig. 12 shows the binding of chimeric antibodies, which were reconstituted by identifying the sequence of variable region, to EREG.

Fig. 13 shows the ADCC-inducing activity of chimeric antibodies against cancer cell lines.

Fig. 14 shows the binding of the chimeric antibodies to EREG orthologues.

Fig. 15 shows the result of comparing the activation of the EGFR receptor by recombinant soluble EREG and EGF.

Fig. 16 shows the activation of the EGF receptor by coculturing cells forcedly expressing EREG. This activation is suppressed by anti-EREG antibodies.

Fig. 17 shows the EGF receptor activation by coculturing cells forcedly expressing EREG, and suppression of this activation by anti-EREG antibodies.

Fig. 18 shows the activation of the EGFR receptor by coculturing DLD-1 cells, and suppression of this activation by addition of anti-EREG antibodies.

Fig. 19 shows the detection of the soluble EREG protein by sandwich ELISA using anti-EREG antibodies.

Fig. 20 shows the result of immunostaining with an anti-EREG mouse monoclonal antibody (EP27).

Fig. 21-1 shows the results of sandwich ELISA for antigenic proteins using mouse anti-EREG antibodies and human chimeric anti-EREG antibodies.

Fig. 21-2 is the continuation of Fig. 22-1.

Fig. 21-3 is the continuation of Fig. 22-2.

Fig. 22 shows the result which was derived from the sandwich ELISA data, by clustering analysis of the antibody binding fingerprints.

Mode for Carrying Out the Invention

EREG is a membrane-bound epidermal growth factor protein. Its amino acid sequence and its encoding gene sequence are disclosed in GenBank Accession Number NP_001423 (SEQ ID NO: 22) and NM_001432 (SEQ ID NO: 21), respectively. In the present invention, the EREG protein refers to both the full-length protein and fragments thereof. "Fragments" refers to polypeptides comprising any region of the EREG protein, and may not have the function of the naturally-occurring EREG protein. Without being limited thereto, an example of the fragments is a fragment comprising the extracellular region of the EREG protein. Positions 29 to 122 in the amino acid sequence of SEQ ID NO: 22 correspond to the extracellular region of the EREG protein. Positions 123 to 140 in the amino acid sequence of SEQ ID NO: 22 correspond to the transmembrane region.

In the present invention, it was shown by analyses of clinical samples and cancer cell lines that the EREG geneis highly and frequently expressed in primary colon cancer, metastatic colon cancer, lung adenocarcinoma, pancreatic cancer, stomach cancer, and kidney cancer tissues. Furthermore, it was also shown that EREG protein was highly expressed in cancer cell lines. That is, the EREG protein is useful as a diagnostic marker for cancer.

Detection of EREG gene expression

In the present invention, methods for diagnosing cancer comprise the step of detecting the EREG gene expression. In one embodiment of the methods of the present invention, the expression of EREG protein is detected.

In the present invention, detection includes quantitative and qualitative detection. Examples of the qualitative detection include the following measurements:

measurement only for the presence or absence of the EREG protein;

measurement to see whether or not the EREG protein is present above a certain amount; and

measurement that compares the amount of the EREG protein with that of other samples (for example, a control sample).

On the other hand, examples of quantitative detection include measurement of the EREG protein concentration, and measurement of the amount of the EREG protein.

Test samples of the present invention are not particularly limited as long as they are samples that may contain an EREG protein. Specifically, samples collected from the body of organisms such as mammals are preferred. Samples collected from humans are more preferred. Specific examples of the test samples include blood, interstitial fluid, plasma, extravascular fluid, cerebrospinal fluid, synovial fluid, pleural fluid, serum, lymphatic fluid, saliva, and urine. Preferred samples are blood samples. Blood samples include serum, plasma, and whole blood. Of these blood samples, serum is preferred. Test samples obtained from samples such as immobilized specimens of tissue or cells collected from the body of an organism, and cell culture solution are also included in the test samples of the present invention.

The cancers that are diagnosed by the present invention are not particularly limited and may be any cancer. Specific examples include colon cancer, metastatic colon cancer, lung adenocarcinoma, pancreatic cancer, stomach cancer, and kidney cancer. In the present invention, both primary and metastatic foci of these cancers can be diagnosed. Primary colon cancer, metastatic colon cancer, and pancreatic cancer are particularly preferable in the present invention.

In the present invention, cancer is detected when an EREG protein is detected in a test sample. More specifically, if the amount of the EREG protein detected in a test sample is larger than that in a negative control or a healthy individual, it is determined that the subject has cancer or is at high risk of being affected with cancer in the future. That is, the present invention relates to methods for detecting cancer which comprise the steps of:

(1) detecting the EREG expression in a biological sample collected from a subject; and

(2) determining that the subject has cancer if the EREG expression detected in step (1) is higher than that of a control.

In the present invention, the control includes a negative control and a biological sample from a healthy individual. A negative control can be obtained by collecting biological samples from healthy individuals, and mixing the biological samples as necessary. The EREG expression in a control can be detected together with the EREG expression in the biological sample of a subject. Alternatively, the EREG expression in biological samples of many healthy individuals can be detected in advance, and a standard expression level in healthy individuals can be determined statistically. More specifically, for example, the mean value ± 2 x standard deviation (S.D.), or mean value ± 3 x standard deviation (S.D.) may be used as the standard value. Statistically, 80% of the healthy individuals are within ± 2 x standard deviation (S.D.)

from the mean value, and 90% of the healthy individuals are within ± 3 x standard deviation (S.D.) from the mean value.

Alternatively, the EREG expression level in a control can be determined using an ROC curve. A receiver operating characteristic (ROC) curve is a graph with detection sensitivity on the vertical axis, and false positive rate (*i.e.*, "1-specificity") on the horizontal axis. In the present invention, an ROC curve can be obtained by plotting the changes of sensitivity to false positive rate, as a result of continuously varying the standard values for determining the EREG expression level in a biological sample.

The "standard value" used for obtaining an ROC curve is a numerical value temporarily used for statistical analysis. In general, "standard values" for obtaining an ROC curve are continuously varied within a range that covers all selectable standard values. For example, the ~~standard values can be varied between the minimum and maximum values of EREG measured in~~ the population analyzed.

Based on the ROC curve obtained, standard values that are expected to yield the desired detection sensitivity and accuracy can be selected. Standard values that are statistically determined by an ROC curve or such are also called "cut-off values". In step (2) of the above methods of cancer detection based on cut-off values, the EREG expression detected in step (1) is compared to the cut-off value. Detection of a higher EREG expression in step (1) than the cut-off value means detection of cancer in the subject.

In the present invention, the EREG expression level can be determined by any method. More specifically, the EREG expression level can be determined by evaluating the amount of EREG mRNA, the amount of EREG protein, and the biological activity of EREG protein. The amount of EREG mRNA and EREG protein can be determined by the methods described herein. Alternatively, one can evaluate the cell proliferation-inducing activity as a biological activity of EREG, using cells whose proliferation is induced in an EREG-dependent manner.

Subjects in the present invention may be any animal species that express an EREG protein. For example, many non-human mammals such as monkeys, cattle, sheep, mice, dogs, cats, and hamsters are known to express EREG protein. Therefore, these animals are included in the subjects of the present invention. Subjects that are particularly preferred are humans. As a matter of course, when a non-human animal is used as a subject, the EREG protein for the animal species is detected.

Preferred embodiments of the diagnostic methods of the present invention include methods of cancer diagnosis that comprise the step of detecting an EREG protein on a section of immobilized tissue or cells obtained from a patient affected with an aforementioned cancer. Furthermore, other embodiments of the present invention include diagnostic methods comprising the step of detecting cell-released EREG protein in the blood. In particular, the present

invention is preferably a method of cancer diagnosis that comprises the step of detecting a fragment comprising the extracellular domain of the EREG protein present in the blood.

Methods for detecting the EREG protein contained in a test sample are not particularly limited. An immunological method that uses an anti-EREG antibody for detection is preferred. The immunological methods that may be used include:

radioimmunoassay (RIA);
enzyme immunoassay (EIA);
fluorescence immunoassay (FIA);
luminescence immunoassay (LIA);
immunoprecipitation (IP);
turbidimetric immunoassay (TIA);
Western blotting (WB);
immunohistochemical staining (IHC); and
single radial immunodiffusion (SRID).

Of the above techniques, enzyme immunoassay is a preferred immunological assay. More specifically, enzyme-linked immunosorbent assay (ELISA) is a preferred enzyme immunoassay. An embodiment of ELISA is, for example, sandwich ELISA. The above-mentioned immunological methods such as ELISA are methods known to those skilled in the art.

The following method is, for example, a common detection method that uses an anti-EREG antibody. After an anti-EREG antibody is immobilized onto a support, the support is blocked to avoid non-specific binding of proteins to the support. For example, bovine serum albumin (BSA), gelatin, or albumin is used for blocking. Various methods for binding antibodies to a support are known. For example, synthetic resins such as polystyrene resins physically adsorb antibodies. Alternatively, antibodies may be chemically bound to a support into which functional groups are introduced. Bifunctional linkers can be used to bind antibodies chemically.

Then, a test sample is added to the support for incubation. During the incubation, the anti-EREG antibodies bound to the support bind with the EREG protein in the test sample. Subsequently, EREG bound to the support via the anti-EREG antibody is detected. Prior to detection of EREG bound to the support, the support may be washed. EREG bound to the support may be detected, for example, using a second antibody that recognizes EREG. The second antibody can be labeled with a labeling substance. Alternatively, the second antibody can be labeled indirectly by using a third antibody (secondary antibody) that recognizes the second antibody. As described above, the EREG protein in a test sample can be detected by qualitatively or quantitatively detecting the EREG protein bound to the anti-EREG antibody on

the support. Several specific examples are further described below.

In the present invention, the following materials may be utilized as a support to immobilize an anti-EREG antibody:

insoluble polysaccharides: agarose, cellulose, and such;

synthetic resins: silicon resin, polystyrene resin, polyacrylamide resin, nylon resin, polycarbonate resin, and such; and

insoluble support such as glass.

Such a support is used in the form of beads or plates. In the case of beads, a column or the like filled with beads can be used. In the case of a plate, a multi-well plate (96-well multi-well plate, or such), or a biosensor chip can be used. For binding an anti-EREG antibody to a support, an anti-EREG antibody can be bound to a support by commonly used methods such as chemical bonding or physical adsorption. Commercially available supports can be suitably used.

Binding of an anti-EREG antibody to an EREG protein is generally performed in a buffer. For example, phosphate buffer, Tris buffer, citric acid buffer, borate buffer, carbonate buffer, or such is used as the buffer. Furthermore, incubation can be suitably carried out using commonly used conditions, such as incubation at a temperature between 4°C and room temperature for one to 24 hours. So long as the binding between the EREG protein and anti-EREG antibody is not interrupted, anything can be used for washing after incubation, and for example, a buffer containing a surfactant such as Tween 20 or such can be suitably used.

In the method of detecting an EREG protein in the present invention, a control sample can be suitably prepared in addition to the test sample in which the EREG protein content will be detected. The control sample includes, for example, a negative control sample containing no EREG protein and a positive control sample containing the EREG protein. In this case, by comparing the results obtained from a negative control sample containing no EREG protein with the results obtained from a positive control sample containing the EREG protein, the presence or absence of the EREG protein in the test sample can be confirmed. Furthermore, after preparing a series of control samples with stepwise changes in concentration, and obtaining detection results for each control sample as a numerical value, a standard curve can be obtained based on the values of the EREG protein concentration and their corresponding measured values. The EREG protein contained in a test sample can be quantitatively determined according to the standard curve, and the result of measuring the EREG protein contained in the test sample.

In a preferred embodiment of detection of the EREG protein bound to a support via an anti-EREG antibody, a method that uses an anti-EREG antibody labeled with a labeling substance is used. For example, the EREG protein can be detected by contacting a test sample with the anti-EREG antibody immobilized onto a support, washing it as necessary, and then

adding a labeled antibody that specifically recognizes the EREG protein.

Anti-EREG antibodies can be labeled by generally known methods. A labeling substance known to those skilled in the art such as fluorescent dyes, enzymes, coenzymes, chemiluminescent substances, and radioactive substances can be used as the labeling substance. Specifically, the following labeling substances are used for labeling antibodies:

radioisotopes: ^{32}P, ^{14}C, ^{125}I, ^{3}H, ^{131}I, and such;

fluorescent dyes: fluorescein, rhodamine, dansyl chloride, umbelliferone, and such;

enzymes: luciferase, peroxidase, alkaline phosphatase, β-galactosidase, β-glucosidase, horseradish peroxidase, glucoamylase, lysozyme, saccharide oxidase, micro peroxidase, and such; and

binding affinity substances: biotin, and such.

When using biotin as a labeling substance, biotin-labeled antibodies can be detected using avidin bound to an enzyme such as alkaline phosphatase. For binding a labeling substance to an anti-EREG antibody, known methods such as the glutaraldehyde method, maleimide method, pyridyl disulfide method, or periodic acid method can be used.

Methods for detecting labeling substances are also known. For example, when detecting an anti-EREG antibody labeled with a radioactive substance, the radioactivity can be detected by a liquid scintillation counter. An anti-EREG antibody labeled with an enzyme can be detected by adding a substrate to the labeled anti-EREG antibody, and then detecting the substrate's enzymatic change. Combination of an enzyme that catalyzes color reaction or luminescence reaction with a substrate is known. Specific examples of a substrate for peroxidase detection include 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 1,2-phenylenediamine (ortho-phenylenediamine), and 3,3',5,5'-tetramethylbenzidine (TMB). When these coloring substrates are used, the reaction can be traced by an absorption photometer. When the substrate is a fluorescence emitting substance, enzymatic change of the substrate can be detected by a spectrofluorometer.

In the present invention, a particularly preferred embodiment of the methods for detecting the EREG protein is, for example, a method that uses a biotin-labeled anti-EREG antibody and avidin. Specifically, avidin or streptavidin bound to an enzyme can be used to detect the biotin-labeled anti-EREG antibody. Avidin and streptavidin bound to an enzyme such as alkaline phosphatase or peroxidase are known.

In the present invention, another embodiment of the methods for detecting the EREG protein includes a method that uses one or more types of primary antibodies that specifically recognize the EREG protein, and one or more types of secondary antibodies that specifically recognize the primary antibodies.

When using a secondary antibody, an anti-EREG antibody derived from a species

different from that of the antibody bound to a support (solid-phase antibody) is used as the liquid-phase antibody. The liquid-phase antibody binds to the EREG protein trapped by the solid-phase antibody, and the secondary antibody binds to the liquid-phase antibody. The secondary antibody can bind to the liquid-phase antibody, but cannot bind to the solid-phase antibody. Therefore, the amount of the secondary antibody remaining on the support depends on the amount of the liquid-phase antibody bound to the support via the EREG protein. As a result of the above-mentioned operation, the EREG protein in a test sample can be detected by qualitatively or quantitatively detecting the secondary antibodies bound. In this case, the secondary antibody can be suitably labeled with a labeling substance.

In the present invention, another embodiment of the methods for detecting the EREG protein is, for example, a detection method that uses aggregation reaction. In this method, the EREG protein can be detected using a carrier adsorbed with an anti-EREG antibody. Any carrier may be used for adsorbing the antibody, as long as it is insoluble and stable, and does not cause non-specific reactions. For example, latex particles, bentonite, collodion, kaolin, or immobilized sheep erythrocytes can be used. Latex particles have excellent homogeneity and stability, and thus are preferred carriers. Latex particles that can be used are, for example, polystyrene latex particles, styrene-butadiene copolymer latex particles, or polyvinyl toluene latex particles. Alternatively, latex particles into which functional groups such as carboxyl groups have been introduced are also known. For example, polystyrene latex particles are preferred latex particles.

When mixed with an antibody, latex particles that have a hydrophobic surface physically adsorb the antibody. Alternatively, if the latex particles have functional groups, the antibody can be bound chemically. Antibody-bound particles are mixed with a sample, and this is stirred for a given period of time. Since the degree of particle aggregation becomes larger as the concentration of EREG protein in the sample increases, the EREG protein can be detected by assessing the degree of aggregation with the naked eye. Furthermore, the EREG protein can also be detected by measuring the increase in turbidity or scattered light caused by aggregation using a spectrophotometer or such.

In the present invention, another embodiment of the methods for detecting the EREG protein includes, for example, a method that uses a biosensor utilizing the surface plasmon resonance phenomenon. The use of a biosensor utilizing the surface plasmon resonance phenomenon enables real-time observation of protein-protein interactions as surface plasmon resonance signals without the need of protein labeling. For example, by using a biosensor such as BIAcore (Biacore), binding between the EREG protein and an anti-EREG antibody can be detected. Specifically, a test sample is contacted with a sensor chip onto which an anti-EREG antibody is immobilized, and the EREG protein that binds to the anti-EREG antibody can be

detected as a change in resonance signals.

EREG is a membrane protein whose expression is elevated specifically in cancer cells. Therefore, cancer cells or cancer tissues can be detected by anti-EREG antibodies. For example, cancer cells contained in cells or tissues collected from a living body are detected by immunohistochemical analysis using anti-EREG antibodies. Alternatively, cancer tissues in a living body can be detected with anti-EREG antibodies. More specifically, the present invention relates to methods of cancer detection which comprise the steps of: (1) administering to a subject a radioisotope-labeled antibody that binds to an EREG protein; and (2) detecting accumulation of the radioisotope. In order to trace the antibody administered into a living body, the antibody may be labeled for detection. For example, the behavior of antibodies labeled with a fluorescent substance, luminescent substance, or radioisotope can be traced *in vivo*. Antibodies labeled with a fluorescent substance or a luminescent substance can be observed using an endoscope or a laparoscope. When using a radioisotope, the localization of an antibody can be imaged by tracing the radioactivity of the radioisotope. In the present invention, the localization of an anti-EREG antibody *in vivo* demonstrates the presence of cancer cells.

A positron-emitting nuclide can be used as a radioisotope for antibody labeling for the detection of cancer in a living body. For example, antibodies can be labeled with positron-emitting nuclides such as ^{18}F, ^{55}Co, ^{64}Cu, ^{66}Ga, ^{68}Ga, ^{76}Br, ^{89}Zr, and ^{124}I. Anti-EREG antibodies can be labeled with these positron-emitting nuclides by known methods (Acta Oncol. 32, 825-830, 1993).

After administering an anti-EREG antibody labeled with a positron-emitting nuclide to humans or animals, radiation emitted by the radioactive nuclide is measured from outside the body using PET (positron emission tomography scanner), and then converted into an image by computed tomography methods. PET is an instrument for non-invasively obtaining data on the *in vivo* behavior of drugs and such. Radiation intensity can be quantitatively converted into an image as signal intensity using PET. By using PET as described above, antigenic molecules that are highly expressed in a particular cancer can be detected without collecting samples from patients. In addition to the above-mentioned nuclides, anti-EREG antibodies can be radiolabeled with short-lived nuclides using positron-emitting nuclides such as ^{11}C, ^{13}N, ^{15}O, ^{18}F, and ^{45}Ti.

The use of a medical cyclotron for production of short-lived nuclides using the above-mentioned nuclides, techniques for producing short-lived radiolabeled compounds, and such, are currently under research and development. Anti-EREG antibodies can be labeled with various radioisotopes using such techniques. Anti-EREG antibodies administered to patients gather at primary foci and metastatic foci according to the specificity of the anti-EREG

antibodies at each site of the pathological tissue. If the anti-EREG antibodies are labeled with positron-emitting nuclides, the presence of primary foci and metastatic foci can be detected from the localization of their radioactivity by detecting radioactivity. For use in such diagnostic purpose, emission activity values of 25-4000 keV gamma particles or positrons can be suitably used. Furthermore, therapeutic effects can be expected by selecting a suitable nuclide and administering it in large quantities. To obtain anticancer effect by radiation, nuclides that provide emission values of 70-700 keV gamma particles or positrons can be used.

In another embodiment of the methods of the present invention, the expression of EREG mRNA is detected. In the present invention, detection includes quantitative and qualitative detection. Examples of qualitative detection include the following measurement techniques:
simple measurement for the presence or absence of EREG mRNA;
measurement to see whether or not the EREG mRNA is present above a certain amount; and
measurement that compares the amount of EREG mRNA to that of other samples (for example, a control sample).

On the other hand, quantitative detection includes, for example, measurement of the EREG mRNA concentration, and measurement of the amount of EREG mRNA.

Any sample that may contain EREG mRNA may be used as a test sample of the present invention. Samples collected from the body of an organism such as mammal are preferred, and samples collected from humans are more preferred. Specific examples of the test samples include blood, interstitial fluid, plasma, extravascular fluid, cerebrospinal fluid, synovial fluid, pleural fluid, serum, lymphatic fluid, saliva, and urine. Preferred samples are blood samples. Blood samples include serum, plasma, and whole blood. Test samples of the present invention also include samples obtained from test samples, such as cell culture solutions and specimens of immobilized tissues or cells collected from the body of an organism.

The cancer for diagnosis is not particularly limited. Specific examples include colon cancer, lung adenocarcinoma, pancreatic cancer, stomach cancer, and kidney cancer. In the present invention, both primary and metastatic foci of these cancers can be diagnosed. Primary colon cancer, metastatic colon cancer, and pancreatic cancer are particularly preferable.

Subjects in the present invention may be any animal species that expresses the EREG protein. For example, many non-human mammals such as monkeys, cattle, sheep, mice, dogs, cats, and hamsters are known to express EREG. Particularly suitable subjects are humans. When a non-human animal species is used as a subject, the EREG mRNA of the animal species is detected.

Specific embodiments of the detection method are described below. First, a sample is prepared from a subject. Then, EREG mRNAs included in the sample are detected. In the present invention, it is also possible to detect cDNAs synthesized from mRNAs. In the present

invention, detection of the EREG mRNA or EREG-encoding cDNA in a test sample means detection of cancer in the patient. For example, if a greater amount of EREG mRNA or EREG-encoding cDNA is detected in the test sample than in a negative control or a healthy individual, it can be determined that the subject has cancer or is at high risk of being affected by cancer in the future.

Methods of mRNA detection are known. Specifically, for example, the Northern blotting method, RT-PCR method, DNA array method, and such may be used in the present invention.

The detection methods of the present invention described above can be automated using various automatic testing devices. Through automation, large quantities of sample can be examined at a time.

A further objective of the present invention is to provide diagnostic agents or kits for cancer diagnosis which comprise reagents for detecting the EREG protein in a test sample. The diagnostic agents of the present invention comprise at least an anti-EREG antibody. When the diagnostic agents or kits of the present invention are based on an EIA method such as ELISA, a carrier for immobilizing the antibody may be included. Alternatively, a carrier-bound antibody may be provided. If the diagnostic agents or kits of the present invention are based on an aggregation method that uses a carrier such as latex, they may include an antibody-adsorbed carrier.

Kits for cancer diagnosis can be produced by combining the diagnostic agents for cancer of the present invention with another element used for detecting EREG. More specifically, the present invention relates to kits for cancer diagnosis which comprise an EREG-binding antibody and a control sample comprising a biological sample containing EREG. The kits of the present invention may additionally comprise a reagent for immunoassay of EREG. For example, as regents for ELISA, chromogenic substrates for detecting enzymatic labels or washing solutions for washing the solid phase can be combined. In addition, instructions that describe the measurement operation can be attached to the kits.

Furthermore, the present invention provides diagnostic agents or kits for cancer diagnosis which comprise a reagent for detecting EREG mRNA or EREG-encoding cDNA in a test sample. The diagnostic agents of the present invention contain at least an EREG-encoding DNA (SEQ ID NO: 21; a DNA consisting of the nucleotide sequence of NM_001423) or an oligonucleotide comprising at least 15 nucleotides complementary to the complementary strand of the DNA.

Herein, the term "complementary strand" refers to the other strand with respect to one of the strands of a double-stranded nucleic acid consisting of A:T (U in the case of RNA) and G:C base pairs. In addition, "complementary" refers not only to cases of completely

complementary sequences within a region of at least 15 consecutive nucleotides, but also to cases of at least 70%, preferably at least 80%, more preferably 90%, and even more preferably 95% homology or higher in a nucleotide sequence. Homology may be determined using an algorithm described herein.

Such oligonucleotides can be used as probes or primers for detecting or amplifying EREG-encoding DNA, and probes or primers for detecting the expression of these DNAs. Furthermore, the probes can be used in the form of a DNA array substrate.

When such oligonucleotides are used as primers, their lengths are normally 15 bp to 100 bp. The preferred lengths of the primers are 17 bp to 30 bp. Any primer that can amplify at least a portion of the EREG-encoding DNA or a complementary strand thereof may be used as a primer. Furthermore, when they are used as primers, their 3'-end regions can be made to be complementary, and for the 5' ends, restriction enzyme recognition sequences or tags can be added.

Furthermore, any oligonucleotide that hybridizes specifically to at least a portion of the EREG-encoding DNA, or to a complementary strand thereof can be used as a probe. The nucleotide sequence of the probe for mRNA detection is selected from the nucleotide sequences complementary to the sense strand of EREG. The chain length of the probe is usually at least 15 bp or longer.

In the present invention, oligonucleotides can be made into probes by suitable labeling. Methods for labeling oligonucleotides are known. For example, oligonucleotides can be labeled by phosphorylating the 5' ends of the oligonucleotides, using T4 polynucleotide kinase with ^{32}P-labeled ATP as a substrate. Alternatively, examples of the methods include methods that incorporate a labeled substrate nucleotide using a DNA polymerase and a random hexamer oligonucleotide or such as a primer (random priming methods, etc.). In random priming methods, Klenow enzyme or such is used as a DNA polymerase. Furthermore, nucleotides can be labeled with a radioisotope such as ^{32}P, a fluorescent dye, biotin, digoxin, or the like.

The oligonucleotides of the present invention can be produced using, for example, a commercially available oligonucleotide synthesizer. The probes may be produced as double-stranded DNA fragments obtained by restriction enzyme treatment or the like.

In the diagnostic agents or kits mentioned above, sterilized water, physiological saline, vegetable oil, surfactants, lipids, solubilizers, buffers, protein stabilizers (BSA, gelatin, or such), preservatives, blocking solutions, reaction solution, reaction-stopping solution, reagents for treating samples, and such may be combined as necessary, in addition to the oligonucleotides and antibodies, which are the active ingredients.

Production of anti-EREG antibodies

The anti-EREG antibodies used in the present invention may be derived from any origin, and may be of any type and in any form, as long as they specifically bind to an EREG protein. Specifically, known antibodies such as non-human animal antibodies (for example, mouse antibodies, rat antibodies, and camel antibodies), human antibodies, chimeric antibodies, and humanized antibodies can be used. In the present invention, the antibodies used may be monoclonal antibodies or polyclonal antibodies. Monoclonal antibodies are preferred.

Anti-EREG antibodies to be used in the present invention can be obtained as polyclonal or monoclonal antibodies using known techniques. In particular, monoclonal antibodies derived from a mammal are preferable as the anti-EREG antibodies for use in the present invention. The monoclonal antibodies derived from a mammal include antibodies produced by hybridoma, and antibodies produced by a host transformed by genetic engineering techniques with an expression vector containing an antibody gene.

A monoclonal antibody-producing hybridoma can be prepared essentially by using the following known technique. First, immunization is performed using the EREG protein as a sensitizing antigen according to a general immunization method. Immunocytes that are obtained from the immunized animals are then fused to known parent cells by a general cell fusion method to obtain hybridomas. Furthermore, hybridomas that produce an anti-EREG antibody can be selected by screening for cells that produce the antibodies of interest using a general screening method.

Specifically, monoclonal antibodies are prepared as follows. First, the EREG gene can be expressed to obtain EREG protein for use as the sensitizing antigen for antibody production. The nucleotide sequence of the EREG gene is disclosed as GenBank Accession No. NM_001432 (SEQ ID NO: 21) and such. Specifically, the EREG-encoding gene sequence is inserted into a known expression vector, and it is used to transform an appropriate host cell. Then, the human EREG protein of interest can be purified by a known method from the host cell or its culture supernatant. Alternatively, a purified naturally-derived EREG protein may be similarly used. Furthermore, as used in the present invention, a fusion protein produced by fusing a desired partial peptide of an EREG protein with another polypeptide may be used as an immunogen. For example, Fc fragments of antibodies, peptide tags, or such can be used to produce a fusion protein for use as an immunogen. A vector that expresses the fusion protein can be produced by fusing the desired genes encoding two or more types of polypeptide fragments in frame, and inserting the fused genes into an expression vector as described above. Methods for producing fused proteins are described in Molecular Cloning 2nd ed. (Sambrook, J. *et al.*, Molecular Cloning 2nd ed., 9.47-9.58, Cold Spring Harbor Lab. press, 1989).

The EREG protein purified in this manner can be used as a sensitizing antigen for immunization of mammals. A partial peptide of EREG can also be used as a sensitizing antigen.

For example, the following peptides can be used as a sensitizing antigen:
peptides obtained from the amino acid sequence of human EREG by chemical synthesis;
peptides obtained by inserting a portion of the EREG gene into an expression vector and expressing it; and
peptides obtained by degrading the EREG protein with a protease.

There are no limitations on the region or size of the partial EREG peptides used. The preferred region can be selected from the amino acid sequence composing the extracellular domain of EREG (positions 29 to 122 in the amino acid sequence of SEQ ID NO: 22). The number of amino acids constituting a peptide to be used as the sensitizing antigen is preferably at least three or more, for example, five or more, or six or more. More specifically, a peptide comprising 8 to 50 residues, or preferably 10 to 30 residues can be used as a sensitizing antigen.

The mammal to be immunized with the sensitizing antigen is not particularly limited. To obtain monoclonal antibodies by the cell fusion method, the animal to be immunized is preferably selected in consideration of the compatibility with the parent cells to be used for cell fusion. Generally, rodents are preferred as the animal to be immunized. Specifically, mice, rats, hamsters, or rabbits can be used as the animal to be immunized. In addition, monkeys and such may be used as the animal to be immunized.

The above-described animals can be immunized with a sensitizing antigen according to a known method. For example, as a general method, immunization can be performed by injecting a mammal intraperitoneally or subcutaneously with a sensitizing antigen. Specifically, the sensitizing antigen is administered to mammals several times every four to 21 days. The sensitizing antigen is diluted at an appropriate dilution with PBS (Phosphate-Buffered Saline), physiological saline, or such, and then used for immunization. Furthermore, the sensitizing antigen may be administered together with an adjuvant. For example, the sensitizing antigen can be prepared by mixing with a Freund's complete adjuvant, and then emulsifying it. Furthermore, an appropriate carrier can be used for immunization using the sensitizing antigen. Particularly when a partial peptide with a small molecular weight is used as a sensitizing antigen, the sensitizing antigen peptide is desirably bound to a carrier protein such as albumin or keyhole limpet hemocyanin, and then used for immunization.

Mammals are immunized as described, and after an increase in the amount of desired antibody in the serum is confirmed, immunocytes are collected from the mammals and subjected to cell fusion. A particularly preferred immunocyte is a splenocyte.

A mammalian myeloma cell is used as a cell to be fused with the above-mentioned immunocyte. The myeloma cells preferably comprise a suitable selection marker for screening. A selection marker confers characteristics to cells for their survival (or failure to survive) under a specific culturing condition. Hypoxanthine-guanine phosphoribosyltransferase deficiency

(hereinafter abbreviated as HGPRT deficiency), and thymidine kinase deficiency (hereinafter abbreviated as TK deficiency) are known as selection markers. Cells having HGPRT or TK deficiency have hypoxanthine-aminopterin-thymidine sensitivity (hereinafter abbreviated as HAT sensitivity). HAT-sensitive cells cannot carry out DNA synthesis in a HAT selection medium, and are thus killed. However, when the cells are fused with normal cells, they can continue to synthesize DNA using the salvage pathway of the normal cells, and therefore they can grow in the HAT selection medium.

HGPRT-deficient and TK-deficient cells can be selected in a medium containing 6-thioguanine or 8-azaguanine (hereinafter abbreviated as 8AG), and 5'-bromodeoxyuridine, respectively. Normal cells are killed since they incorporate these pyrimidine analogs into their DNA. However, cells that are deficient in these enzymes can survive in the selection medium, since they cannot incorporate these pyrimidine analogs. Alternatively, a selection marker referred to as G418 resistance provides resistance to 2-deoxystreptamine-type antibiotics (gentamycin analogs) from the neomycin-resistance gene. Various types of myeloma cells thata re suitable for cell fusion are known. For example, myeloma cells including the following cells can be used to produce the monoclonal antibodies of the present invention:

P3 (P3x63Ag8.653) (J. Immunol. (1979) 123, 1548-1550);

P3x63Ag8U.1 (Current Topics in Microbiology and Immunology (1978) 81, 1-7);

NS-1 (Kohler. G. and Milstein, C. Eur. J. Immunol. (1976) 6, 511-519);

MPC-11 (Margulies. D.H. *et al.*, Cell (1976) 8, 405-415);

SP2/0 (Shulman, M. *et al.*, Nature (1978) 276, 269-270);

FO (de St. Groth, S. F. *et al.*, J. Immunol. Methods (1980) 35, 1-21);

S194 (Trowbridge, I. S. J. Exp. Med. (1978) 148, 313-323); and

R210 (Galfre, G. *et al.*, Nature (1979) 277, 131-133).

Cell fusion of the above-mentioned immunocytes with myeloma cells is essentially performed according to a known method, for example, the method of Kohler and Milstein *et al.* (Kohler. G. and Milstein, C., Methods Enzymol. (1981) 73, 3-46).

More specifically, the above-mentioned cell fusion can be performed in a standard nutritional culture medium in the presence of, for example, a cell-fusion accelerator. A cell-fusion accelerator may be, for example, polyethylene glycol (PEG), Sendai virus (HVJ), or the like. If desired, an auxiliary agent such as dimethylsulfoxide can be added to further enhance fusion efficiency.

The ratio of immunocytes to myeloma cells used can be established at one's discretion. For example, the number of immunocytes is preferably set to one to ten times of that of myeloma cells. As a medium to be used for the above-mentioned cell fusion, for example, RPMI1640 medium and MEM medium, which are appropriate for the growth of the above-mentioned

myeloma cell line, or other standard media that are used for this type of cell culture can be used. Moreover, a serum supplement solution such as fetal calf serum (FCS) can be added to the media.

Cell fusion is performed by thoroughly mixing predetermined amounts of the above-mentioned immunocytes and myeloma cells in the above-mentioned medium, adding and mixing with a PEG solution pre-heated to approximately 37°C, so as to form the desired fused cells (hybridomas). In the cell fusion method, for example, PEG with an average molecular weight of approximately 1000 to 6000 can generally be added at a concentration of 30 to 60% (w/v). Subsequently, the agent for cell fusion or the like which is unfavorable for the growth of hybridomas can be removed by successively adding an appropriate medium such as those listed above, removing the supernatant after centrifugation, and repeating these steps.

Hybridomas obtained in this manner can be selected using a selection medium appropriate for the selection markers carried by myelomas used for cell fusion. For example, cells that have HGPRT and TK deficiencies can be selected by culturing them in a HAT medium (a medium containing hypoxanthine, aminopterin, and thymidine). More specifically, when HAT-sensitive myeloma cells are used for cell fusion, cells that successfully fuse with normal cells can be selectively grown in the HAT medium. Culturing using the above-mentioned HAT medium is continued for a sufficient period of time to kill the cells other than the hybridoma of interest (non-fused cells). More specifically, the hybridoma of interest can be selected, typically by culturing for several days to several weeks. Subsequently, hybridomas that produce the antibody of interest can be screened and monocloned by carrying out a standard limiting dilution method. Alternatively, an EREG-recognizing antibody can be prepared using the method described in International Patent Publication No. WO 03/104453.

An antibody of interest can be suitably screened and singly cloned by a screening method based on known antigen-antibody reaction. For example, the antigen is bound to a carrier such as polystyrene beads or the like, or a commercially available 96-well microtiter plate, followed by reaction with the culture supernatant of the hybridomas. Then, after the carrier is washed, it is reacted with an enzyme-labeled secondary antibody or the like. If the antibody of interest that reacts with the sensitizing antigen is contained in the culture supernatant, the secondary antibody will bind to the carrier via the antibody. Ultimately, the presence of the antibody of interest in the culture supernatant can be determined by detecting secondary antibodies bound to the carrier. Hybridomas producing desired antibodies that can bind to the antigen can be cloned by the limiting dilution method or the like. Antigens used for immunization as well as an operably equivalent EREG protein can be suitably used in this case. For example, the extracellular domain of EREG or an oligopeptide comprising a partial amino acid sequence composing this region may be used as the antigen.

In addition to the above-mentioned method where hybridomas are obtained by immunizing non-human animals with an antigen, an antibody of interest can be obtained by antigen sensitization of human lymphocytes. More specifically, first, human lymphocytes are sensitized with the EREG protein *in vitro*. Then, immunosensitized lymphocytes are fused with a suitable fusion partner. For example, human-derived myeloma cells that have infinite division potential can be used as a fusion partner (see Japanese Patent Publication Kokoku Publication No. (JP-B) H01-59878 (examined, approved Japanese patent application published for opposition)). Anti-EREG antibodies obtained by this method are human antibodies that have binding activity to an EREG protein.

Alternatively, anti-EREG human antibodies can also be obtained by administering an EREG protein that serves as an antigen to a transgenic animal having a complete human antibody gene repertoire. Antibody-producing cells of the immunized animal can be immortalized by cell fusion with a suitable fusion partner, or by treatment such as Epstein-Barr virus infection. Human antibodies against the EREG protein can be isolated from the immortalized cells obtained in this manner (see International Patent Publication Nos. WO 94/25585, WO 93/12227, WO 92/03918, and WO 94/02602). Furthermore, cells that produce an antibody having the reaction specificity of interest can be cloned by cloning the immortalized cells. When a transgenic animal is used as the animal to be immunized, the immune system of this animal recognizes human EREG as a foreign substance. Therefore, human antibodies against human EREG can be readily obtained.

The monoclonal antibody-producing hybridomas produced in this manner can be passaged and cultured in a standard medium. Alternatively, the hybridomas can be stored for a long period in liquid nitrogen.

The hybridomas can be cultured according to a standard method, and the monoclonal antibody of interest can be obtained from the culture supernatants. Alternatively, the hybridomas can be grown by administering them to a compatible mammal, and monoclonal antibodies can be obtained as its ascites. The former method is suitable for obtaining highly purified antibodies.

In the present invention, an antibody encoded by an antibody gene cloned from antibody-producing cells can be used. The cloned antibody gene can be incorporated into a suitable vector and then introduced into a host to express the antibody. Methods for isolating an antibody gene, introducing the gene into a vector, and transforming host cells have been established (see for example, Vandamme, A. M. *et al.*, Eur. J. Biochem. (1990) 192, 767-775).

For example, a cDNA encoding the variable region (V region) of an anti-EREG antibody can be obtained from hybridoma cells producing the anti-EREG antibody. Usually, in order to accomplish this, first, total RNA is extracted from the hybridoma. For example, the

following methods can be used as methods for extracting mRNA from cells:
the guanidine ultracentrifugation method (Chirgwin, J. M. *et al.*, Biochemistry (1979) 18, 5294-5299); and
the AGPC method (Chomczynski, P. *et al.*, Anal. Biochem. (1987) 162, 156-159).

The extracted mRNA can be purified using an mRNA purification kit (GE Healthcare Bio-Sciences) or the like. Alternatively, kits for directly extracting total mRNA from cells, such as the QuickPrep mRNA Purification Kit (GE Healthcare Bio-Sciences), are also commercially available. Total RNA can be obtained from the hybridoma by using such kits. A cDNA encoding the antibody V region can be synthesized from the obtained mRNA using reverse transcriptase. cDNA can be synthesized using the AMV Reverse Transcriptase First-strand cDNA Synthesis Kit (SEIKAGAKU CORPORATION) or the like. To synthesize and amplify cDNA, the 5'-Ampli FINDER RACE Kit (Clontech) and the 5'-RACE method using PCR (Frohman, M. A. *et al.*, Proc. Natl. Acad. Sci. USA (1988) 85, 8998-9002; Belyavsky, A. *et al.*, Nucleic Acids Res. (1989) 17, 2919-2932) can be used. Furthermore, in the process of such cDNA synthesis, appropriate restriction enzyme sites, which will be described later, can be introduced into both ends of the cDNA.

The cDNA fragment of interest is purified from the obtained PCR product, and then ligated to a vector DNA. The recombinant vector is prepared in this manner and introduced into *Escherichia coli* or the like, and after colonies are selected, the desired recombinant vector can be prepared from the *E. coli* that formed the colonies. Whether or not the recombinant vector has the cDNA nucleotide sequence of interest can be confirmed by a known method, such as the dideoxynucleotide chain termination method.

In order to obtain genes encoding the variable regions, the PCR method that uses primers for amplifying the variable region genes can be used. First, a cDNA library is obtained by synthesizing cDNAs using the extracted mRNAs as templates. It is convenient to use a commercially available kit for cDNA library synthesis. In practice, since the amount of mRNA obtainable from only a small number of cells is extremely minute, the yield of such mRNA from direct purification is low. Therefore, purification is usually performed after adding a carrier RNA that does not contain an antibody gene. Alternatively, when a certain amount of RNA can be extracted, efficient extraction can be accomplished by using just the RNA of antibody-producing cells. For example, addition of carrier RNA may not be required when RNA is extracted from ten or more, 30 or more, or preferably 50 or more antibody-producing cells.

The antibody genes are amplified by the PCR method using the obtained cDNA library as a template. The primers used for amplification of the antibody genes by the PCR method are known. For example, primers for human antibody gene amplification can be designed based on

the disclosure of an article (J. Mol. Biol. (1991) 222, 581-597) and the like. The nucleotide sequences of these primers vary depending on the subclass of immunoglobulin. Therefore, when a cDNA library of an unknown subclass is used as the template, various possibilities need to be considered when performing the PCR method.

More specifically, when the objective is to obtain genes encoding human IgG, one may use primers capable of amplifying genes encoding γ1 to γ5 for the heavy chain, and genes encoding the κ chain and λ chain for the light chain. To amplify genes of the IgG variable region, generally, a primer that anneals to the portion corresponding to the hinge region is used as the 3'-end primer. Meanwhile, a primer corresponding to each subclass can be used as the 5'-end primer.

PCR products obtained by the primers for gene amplification of the heavy chain and light chain subclasses are made into independent libraries. Using the libraries synthesized in this manner, immunoglobulins comprising a combination of heavy and light chains can be reconstituted. The antibodies of interest can be screened by using the activity of the reconstituted immunoglobulins to bind to EREG as an index.

For example, when the objective is to obtain antibodies against EREG, it is more preferable that the binding of the antibodies to EREG is specific. For instance, it is possible to screen for antibodies that bind to EREG as described below:

(1) contacting EREG with an antibody comprising the V region encoded by a cDNA obtained from a hybridoma;

(2) detecting the binding between EREG and the antibody; and

(3) selecting an antibody that binds to EREG.

Methods for detecting the binding between an antibody and EREG are known. Specifically, a test antibody is reacted with EREG immobilized onto a support, and then this is reacted with a labeled antibody that recognizes the test antibody. If the labeled antibody on the support is detected after washing, this proves that the test antibody binds to EREG. For labeling, enzymatically active proteins such as peroxidase or β-galactosidase, or fluorescent substances such as FITC can be used. In order to evaluate the binding activity of the antibody, specimens of immobilized EREG-expressing cells can be used.

Alternatively, for an antibody screening method that uses the binding activity as an index, a phage vector-based panning method may be used. When the antibody genes are obtained as libraries of the heavy-chain and light-chain subclasses as described above, phage vector-based screening methods are advantageous. Genes encoding variable regions of the heavy and light chains can be made into a single-chain Fv (scFv) gene by linking the genes via suitable linker sequences. Phages expressing an scFv on their surface can be obtained by inserting a gene encoding the scFv into a phage vector. DNA encoding an scFv having the

binding activity of interest can be collected by contacting the phage with an antigen of interest, and then collecting antigen-bound phage. scFv having the binding activity of interest can be concentrated by repeating this operation as necessary.

An antibody-encoding polynucleotide of the present invention may encode a full-length antibody or a portion of the antibody. "A portion of an antibody" refers to any portion of an antibody molecule. Hereinafter, the term "antibody fragment" may be used to refer to a portion of an antibody. A preferred antibody fragment of the present invention comprises the complementarity determination region (CDR) of an antibody. More preferably, an antibody fragment of the present invention comprises all of the three CDRs that constitute a variable region.

Once a cDNA encoding the V region of an anti-EREG antibody of interest is obtained, this cDNA is digested with restriction enzymes that recognize the restriction enzyme sites inserted to both ends of the cDNA. A preferred restriction enzyme recognizes and digests a nucleotide sequence that is less likely to appear in the nucleotide sequence constituting the antibody gene. Furthermore, to insert a single copy of the digested fragment into a vector in the correct direction, a restriction enzyme that provides sticky ends is preferred. A cDNA encoding the anti-EREG antibody V region, which has been digested as described above, is inserted into a suitable expression vector to obtain the antibody expression vector. In this step, a chimeric antibody can be obtained by fusing a gene encoding the antibody constant region (C region) with the above-mentioned gene encoding the V region in frame. Herein, "chimeric antibody" refers to an antibody whose constant and variable regions are derived from different origins. Therefore, in addition to interspecies chimeric antibodies such as mouse-human chimeric antibodies, human-human intraspecies chimeric antibodies are also included in the chimeric antibodies of the present invention. A chimeric antibody expression vector can also be constructed by inserting the aforementioned V-region gene into an expression vector into which a constant region gene has been introduced.

More specifically, for example, the restriction enzyme recognition sequence for a restriction enzyme that digests the aforementioned V-region gene can be placed at the 5' end of an expression vector carrying a DNA encoding a desired antibody constant region (C region). The chimeric antibody expression vector is constructed by digesting the two genes using the same combination of restriction enzymes, and fusing them in frame.

To produce an anti-EREG antibody for use in the present invention, the antibody gene can be incorporated into an expression vector so that it is expressed under the regulation of an expression control region. The expression regulatory region for antibody expression includes, for example, an enhancer or a promoter. Then, by transforming suitable host cells with this expression vector, recombinant cells that express the anti-EREG antibody-encoding DNA can be

obtained.

To express an antibody gene, a DNA encoding the antibody heavy chain (H-chain) and a DNA encoding the antibody light chain (L-chain) can be incorporated separately into expression vectors. An antibody molecule comprising the H chain and L chain can be expressed by simultaneously transfecting (co-transfecting) the H-chain and L-chain-incorporated vectors into the same host cell. Alternatively, DNAs encoding the H chain and L chain can be incorporated into a single expression vector to transform a host cell with the vector (see International Patent Publication No. WO 94/11523).

Many combinations of hosts and expression vectors for isolating an antibody gene and then introducing the gene into an appropriate host to produce the antibody are known. Any of these expression systems can be applied to the present invention. When using eukaryotic cells as a host, animal cells, plant cells, and fungal cells can be used. More specifically, animal cells that may be used in the present invention are, for example, the following cells:

(1) mammalian cells such as CHO, COS, myeloma, baby hamster kidney (BHK), HeLa, and Vero cells;

(2) amphibian cells such as Xenopus oocytes; and

(3) insect cells such as sf9, sf21, Tn5.

In addition, as a plant cell system, an antibody gene expression system using cells derived from the *Nicotiana* genus such as *Nicotiana tabacum* is known. Callus-cultured cells can be used to transform plant cells.

Furthermore, the following cells can be used as fungal cells; yeasts: the *Saccharomyces* genus, for example, *Saccharomyces cerevisiae*, and the *Pichia* genus, for example, *Pichia pastoris*; and filamentous fungi: the *Aspergillus* genus, for example, *Aspergillus niger*.

Antibody gene expression systems that utilize prokaryotic cells are also known. For example, when using bacterial cells, *E. coli* cells, *Bacillus subtilis* cells, and such may be used in the present invention.

Expression vectors comprising the antibody genes of interest are introduced into these cells by transformation. By culturing the transformed cells *in vitro*, the desired antibodies can be obtained from the transformed cell culture.

In addition to the above host cells, transgenic animals can also be used to produce a recombinant antibody. That is, the antibody can be obtained from an animal into which the gene encoding the antibody of interest is introduced. For example, the antibody gene can be inserted in frame into a gene that encodes a protein produced inherently in milk to construct a fused gene. Goat β-casein or such can be used, for example, as the protein secreted in milk. A DNA fragment containing the fused gene inserted with the antibody gene is injected into a goat embryo, and then this embryo is introduced into a female goat. Desired antibodies can be

obtained as a protein fused with the milk protein from milk produced by the transgenic goat born from the goat that received the embryo (or progeny thereof). To increase the volume of milk containing the desired antibody produced by the transgenic goat, hormones can be used on the transgenic goat as necessary (Ebert, K. M. *et al.*, Bio/Technology (1994) 12, 699-702).

Animal-derived antibody C regions can be used for the C regions of a recombinant antibody of the present invention. For example, Cγ1, Cγ2a, Cγ2b, Cγ3, Cμ, Cδ, Cα1, Cα2, and Cε can be used for the mouse antibody H-chain C-region, and Cκ and Cλ can be used for the L-chain C-region. In addition to mouse antibodies, antibodies of animals such as rats, rabbits, goat, sheep, camels, and monkeys can be used as animal antibodies. Their sequences are known. Furthermore, the C region can be modified to improve the stability of the antibodies or their production.

In the present invention, when administering antibodies to humans, genetically recombinant antibodies that have been artificially modified for the purpose of reducing xenoantigenicity against humans, or the like can be used. Examples of the genetically recombinant antibodies include chimeric antibodies and humanized antibodies. These modified antibodies can be produced using known methods.

A chimeric antibody is an antibody whose variable regions and constant regions are of different origins. For example, an antibody comprising the heavy-chain and light-chain variable regions of a mouse antibody and the heavy-chain and light-chain constant regions of a human antibody is a mouse-human interspecies chimeric antibody. A recombinant vector expressing a chimeric antibody can be produced by ligating a DNA encoding a mouse antibody variable region to a DNA encoding a human antibody constant region, and then inserting it into an expression vector. The recombinant cells that have been transformed with the vector are cultured, and the incorporated DNA is expressed to obtain the chimeric antibody produced in the culture. Human C regions are used for the C regions of chimeric antibodies and humanized antibodies.

For example, Cγ1, Cγ2, Cγ3, Cγ4, Cμ, Cδ, Cα1, Cα2, and Cε can be used as an H-chain C region. Cκ and Cλ can be used as an L-chain C region. The amino acid sequences of these C regions and the nucleotide sequences encoding them are known. Furthermore, the human antibody C region can be modified to improve the stability of an antibody or its production.

Generally, a chimeric antibody consists of the V region of an antibody derived from a non-human animal, and a C region derived from a human antibody. On the other hand, a humanized antibody consists of the complementarity determining region (CDR) of an antibody derived from a non-human animal, and the framework region (FR) and C region derived from a human antibody. Since the antigenicity of a humanized antibody in human body is reduced, a humanized antibody is useful as an active ingredient for therapeutic agents of the present

invention.

For example, mouse-human chimeric antibodies that can be obtained by linking the amino acid sequences constituting the human constant regions and the variable regions of an anti-EREG monoclonal antibody (B3#18) prepared based on the present invention are preferred monoclonal antibodies in the present invention. More specifically, the present invention provides mouse-human chimeric monoclonal antibodies comprising H and L chains having the following amino acid sequences.

H chain: the amino acid sequence of positions 1 to 446 in the amino acid sequence of SEQ ID NO: 10 (the signal sequence of positions -19 to -1 is removed)

L chain: the amino acid sequence of positions 1 to 213 in the amino acid sequence of SEQ ID NO: 20 (the signal sequence of positions -20 to -1 is removed)

Furthermore, other preferred examples of the chimeric antibodies of the present invention include the following antibodies.

An antibody comprising an H chain having the sequence in which the signal sequence (-19 to -1) has been removed from the amino acid sequence of SEQ ID NO: 129, and an L chain having the sequence in which the signal sequence (-19 to -1) has been removed from the amino acid sequence of SEQ ID NO: 131.

An antibody comprising an H chain having the sequence in which the signal sequence (-19 to -1) has been removed from the amino acid sequence of SEQ ID NO: 133, and an L chain having the sequence in which the signal sequence (-19 to -1) has been removed from the amino acid sequence of SEQ ID NO: 135.

An antibody comprising an H chain having the sequence in which the signal sequence (-19 to -1) has been removed from the amino acid sequence of SEQ ID NO: 137, and an L chain having the sequence in which the signal sequence (-19 to -1) has been removed from the amino acid sequence of SEQ ID NO: 139.

An antibody comprising an H chain having the sequence in which the signal sequence (-19 to -1) has been removed from the amino acid sequence of SEQ ID NO: 141, and an L chain having the sequence in which the signal sequence (-19 to -1) has been removed from the amino acid sequence of SEQ ID NO: 143.

An antibody comprising an H chain having the sequence in which the signal sequence (-19 to -1) has been removed from the amino acid sequence of SEQ ID NO: 145 and an L chain having a sequence in which the signal sequence (-19 to -1) has been removed from the amino acid sequence of SEQ ID NO: 147.

The antibody variable region generally comprises three complementarity-determining regions (CDRs) separated by four framework regions (FRs). CDR is a region that substantially determines the binding specificity of an antibody. The amino acid sequences of CDRs are

highly diverse. On the other hand, the FR-constituting amino acid sequences are often highly homologous even among antibodies with different binding specificities. Therefore, generally, the binding specificity of a certain antibody can be transferred to another antibody by CDR grafting.

A humanized antibody is also called a reshaped human antibody. Specifically, humanized antibodies prepared by grafting the CDR of a non-human animal antibody such as a mouse antibody to a human antibody and such are known. Common genetic engineering techniques for obtaining humanized antibodies are also known.

Specifically, for example, overlap extension PCR is known as a method for grafting a mouse antibody CDR to a human FR. In overlap extension PCR, a nucleotide sequence encoding a mouse antibody CDR to be grafted is added to the primers for synthesizing a human antibody FR. Primers are prepared for each of the four FRs. It is generally considered that when grafting a mouse CDR to a human FR, selecting a human FR that is highly homologous to a mouse FR is advantageous for maintaining the CDR function. That is, it is generally preferable to use a human FR comprising an amino acid sequence highly homologous to the amino acid sequence of the FR adjacent to the mouse CDR to be grafted.

Nucleotide sequences to be ligated are designed so that they will be connected to each other in frame. Human FRs are individually synthesized using the respective primers. As a result, products in which the mouse CDR-encoding DNA is attached to the individual FR-encoding DNAs are obtained. Nucleotide sequences encoding the mouse CDR of each product are designed so that they overlap with each other. Then, overlapping CDR regions of the products synthesized using a human antibody gene as the template are annealed for complementary strand synthesis reaction. By this reaction, human FRs are ligated through the mouse CDR sequences.

The full length of the V-region gene, in which three CDRs and four FRs are ultimately ligated, is amplified using primers that anneal to its 5' and 3' ends and which have suitable restriction enzyme recognition sequences. A vector for human antibody expression can be produced by inserting the DNA obtained as described above and a DNA that encodes a human antibody C region into an expression vector so that they will ligate in frame. After inserting this integration vector into a host to establish recombinant cells, the recombinant cells are cultured, and the DNA encoding the humanized antibody is expressed to produce the humanized antibody in the cell culture (see, European Patent Publication No. EP 239,400, and International Patent Publication No. WO 96/02576).

By qualitatively or quantitatively measuring and evaluating the antigen-binding activity of the humanized antibody produced as described above, one can suitably select human antibody FRs that allow CDRs to form a favorable antigen-binding site when ligated through the CDRs.

As necessary, amino acid residues in an FR may be substituted so that the CDRs of a reshaped human antibody form an appropriate antigen-binding site. For example, amino acid sequence mutations can be introduced into FRs by applying the PCR method used for fusing a mouse CDR with a human FR. More specifically, partial nucleotide sequence mutations can be introduced into primers that anneal to the FR sequence. Nucleotide sequence mutations are introduced into the FRs synthesized using such primers. Mutant FR sequences having the desired characteristics can be selected by measuring and evaluating the activity of the amino acid-substituted mutant antibody to bind to the antigen by the above-mentioned method (Sato, K. *et al.*, Cancer Res. 1993, 53, 851-856).

Methods for obtaining human antibodies are also known. For example, human lymphocytes are sensitized *in vitro* with a desired antigen or cells expressing a desired antigen. Then, by fusing the sensitized lymphocytes with human myeloma cells, desired human antibodies having the antigen-binding activity can be obtained (see JP-B H01-59878). U266 or such can be used as the fusion partner human myeloma cell.

Alternatively, a desired human antibody can be obtained by using a desired antigen to immunize a transgenic animal that comprises the entire repertoire of human antibody genes (see International Patent Publication Nos. WO 93/12227, WO 92/03918, WO 94/02602, WO 94/25585, WO 96/34096, and WO 96/33735). Furthermore, techniques to obtain human antibodies by panning a human antibody library are also known. For example, the V region of a human antibody is expressed as a single chain antibody (scFv) on the phage surface using a phage display method, and phages that bind to the antigen can be selected. By analyzing the genes of selected phages, the DNA sequences encoding the V regions of human antibodies that bind to the antigen can be determined. After determining the DNA sequences of scFvs that bind to the antigen, the V region sequence is fused in frame with the desired human antibody C region sequence, and this is inserted into a suitable expression vector to produce an expression vector. This expression vector can be introduced into suitable expression cells such as those described above, and the human antibody-encoding gene can be expressed to obtain the human antibodies. Such methods are well known (International Patent Publication Nos. WO 92/01047, WO 92/20791, WO 93/06213, WO 93/11236, WO 93/19172, WO 95/01438, and WO 95/15388).

The antibody used in the present invention is not limited to bivalent antibodies represented by IgG, but includes monovalent antibodies and multivalent antibodies represented by IgM, as long as it binds to the EREG protein. The multivalent antibody of the present invention includes a multivalent antibody that has the same antigen binding sites, and a multivalent antibody that has partially or completely different antigen binding sites. The antibody used in the present invention is not limited to the whole antibody molecule, but includes minibodies and modified products thereof, as long as they bind to the EREG protein.

A minibody contains an antibody fragment lacking a portion of a whole antibody (for example, whole IgG). As long as it has the ability to bind the EREG antigen, partial deletions of an antibody molecule are permissible. Antibody fragments of the present invention preferably contain a heavy-chain variable region (VH) and/or a light-chain variable region (VL). The amino acid sequence of VH or VL may have substitutions, deletions, additions, and/or insertions. Furthermore, as long as it has the ability to bind the EREG antigen, VH and/or VL can be partially deleted. The variable region may be chimerized or humanized. Specific examples of the antibody fragments include Fab, Fab', F(ab')2, and Fv. Specific examples of minibodies include Fab, Fab', F(ab')2, Fv, scFv (single chain Fv), diabody, and sc(Fv)2 (single chain (Fv)2). Multimers of these antibodies (for example, dimers, trimers, tetramers, and polymers) are also included in the minibodies of the present invention.

Fragments of antibodies can be obtained by treating an antibody with an enzyme to produce antibody fragments. Known enzymes that produce antibody fragments are, for example, papain, pepsin, and plasmin. Alternatively, genes encoding these antibody fragments can be constructed, introduced into expression vectors, and then expressed in appropriate host cells (see, for example, Co, M. S. *et al.*, J. Immunol. (1994) 152, 2968-2976; Better, M. and Horwitz, A. H., Methods in Enzymology (1989) 178, 476-496; Plueckthun, A. and Skerra, A., Methods in Enzymology (1989) 178, 476-496; Lamoyi, E., Methods in Enzymology (1989) 121, 652-663; Rousseaux, J. *et al.*, Methods in Enzymology (1989) 121, 663-669; and Bird, R. E. *et al.*, TIBTECH (1991) 9, 132-137).

Digestive enzymes cleave specific sites of an antibody fragment, and yield antibody fragments with the following specific structures. When genetic engineering techniques are used on such enzymatically obtained antibody fragments, any portion of the antibody can be deleted.
Papain digestion: F(ab)2 or Fab
Pepsin digestion: F(ab')2 or Fab'
Plasmin digestion: Facb

Therefore, minibodies of the present invention may be antibody fragments lacking any region, as long as they have binding affinity to EREG. Furthermore, according to the present invention, the antibodies desirably maintain their effector activity, particularly in the treatment of cell proliferative diseases such as cancer. More specifically, preferred minibodies of the present invention have both binding affinity to EREG and effector function. The antibody effector function includes ADCC activity and CDC activity. Particularly preferably, therapeutic antibodies of the present invention have ADCC activity and/or CDC activity as effector function.

A diabody refers to a bivalent antibody fragment constructed by gene fusion (Hollinger P. *et al.*, Proc. Natl. Acad. Sci. USA 90: 6444-6448 (1993); EP 404,097; WO 93/11161; and such). A diabody is a dimer composed of two polypeptide chains. Generally, in each

polypeptide chain constituting the dimer, VL and VH are linked by a linker within the same chain. The linker in a diabody is generally short enough to prevent binding between VL and VH. Specifically, the amino acid residues constituting the linker are, for example, five residues or so. Therefore, VL and VH that are encoded by the same polypeptide chain cannot form a single-chain variable region fragment, and form a dimer with another single chain variable region fragment. As a result, diabodies have two antigen binding sites.

scFv can be obtained by ligating the H-chain V region and L-chain V region of an antibody. In scFv, the H-chain V region and L-chain V region are ligated via a linker, preferably a peptide linker (Huston, J. S. *et al.*, Proc. Natl. Acad. Sci. U.S.A., 1988, 85, 5879-5883). The H-chain V region and L-chain V region of scFv may be derived from any of the antibodies described herein. The peptide linker for ligating the V regions is not particularly limited. For example, any single-chain peptide consisting of 3 to 25 residues or so can be used as the linker. More specifically, for example, peptide linkers described below or such can be used.

PCR methods such as those described above can be used for ligating the V regions. For ligation of the V regions by PCR methods, first, a whole DNA or a DNA encoding a desired partial amino acid sequence selected from the following DNAs can be used as a template:
a DNA sequence encoding the H chain or the H-chain V region of the above-mentioned antibody; and
a DNA sequence encoding the L chain or the L-chain V region of the above-mentioned antibody.

DNAs encoding the H-chain and L-chain V regions are individually amplified by PCR methods using a pair of primers that have sequences corresponding to the sequences of both ends of the DNA to be amplified. Then, a DNA encoding the peptide linker portion is prepared. The DNA encoding the peptide linker can also be synthesized using PCR. To the 5' end of the primers used, nucleotide sequences that can be ligated to each of the individually synthesized V-region amplification products are added. Then, PCR reaction is carried out using the "H-chain V region DNA", "peptide linker DNA", and "L-chain V region DNA", and the primers for assembly PCR.

The primers for assembly PCR consist of the combination of a primer that anneals to the 5' end of the "H-chain V region DNA" and a primer that anneals to the 3' end of the "L-chain V region DNA". That is, the primers for assembly PCR are a primer set that can amplify a DNA encoding the full-length sequence of scFv to be synthesized. On the other hand, nucleotide sequences that can be ligated to each V-region DNA are added to the "peptide linker DNA". Thus, these DNAs are ligated, and the full-length scFv is ultimately produced as an amplification product using the primers for assembly PCR. Once the scFv-encoding DNA is constructed, expression vectors containing the DNA, and recombinant cells transformed by these expression

vectors can be obtained according to conventional methods. Furthermore, the scFvs can be obtained by culturing the resulting recombinant cells and expressing the scFv-encoding DNA.

sc(Fv)2 is a minibody prepared by ligating two VHs and two VLs with linkers or such to form a single chain (Hudson et al., J. Immunol. Methods 1999; 231: 177-189). sc(Fv)2 can be produced, for example, by joining scFvs with a linker.

Moreover, antibodies in which two VHs and two VLs are arranged in the order of VH, VL, VH, and VL ([VH]-linker-[VL]-linker-[VH]-linker-[VL]), starting from the N-terminal side of a single chain polypeptide, are preferred.

The order of the two VHs and the two VLs is not particularly limited to the above-mentioned arrangement, and they may be placed in any order. Examples include the following arrangements:

[VL]-linker-[VH]-linker-[VH]-linker-[VL]
[VH]-linker-[VL]-linker-[VL]-linker-[VH]
[VH]-linker-[VH]-linker-[VL]-linker-[VL]
[VL]-linker-[VL]-linker-[VH]-linker-[VH]
[VL]-linker-[VH]-linker-[VL]-linker-[VH]

Any arbitrary peptide linker can be introduced by genetic engineering, and synthetic linkers (see, for example, those disclosed in Protein Engineering, 9(3), 299-305, 1996) or such can be used as linkers for linking the antibody variable regions. In the present invention, peptide linkers are preferable. The length of the peptide linkers is not particularly limited, and can be suitably selected by those skilled in the art according to the purpose. The length of amino acid residues composing a peptide linker is generally 1 to 100 amino acids, preferably 3 to 50 amino acids, more preferably 5 to 30 amino acids, and particularly preferably 12 to 18 amino acids (for example, 15 amino acids).

Any amino acid sequences composing peptide linkers can be used, as long as they do not inhibit the binding activity of scFv. Examples of the amino acid sequences used in peptide linkers include:

Ser
Gly-Ser
Gly-Gly-Ser
Ser-Gly-Gly
Gly-Gly-Gly-Ser (SEQ ID NO: 23)
Ser-Gly-Gly-Gly (SEQ ID NO: 24)
Gly-Gly-Gly-Gly-Ser (SEQ ID NO: 25)
Ser-Gly-Gly-Gly-Gly (SEQ ID NO: 26)
Gly-Gly-Gly-Gly-Gly-Ser (SEQ ID NO: 27)

Ser-Gly-Gly-Gly-Gly-Gly (SEQ ID NO: 28)
Gly-Gly-Gly-Gly-Gly-Gly-Ser (SEQ ID NO: 29)
Ser-Gly-Gly-Gly-Gly-Gly-Gly (SEQ ID NO: 30)
(Gly-Gly-Gly-Gly-Ser (SEQ ID NO: 31))n
(Ser-Gly-Gly-Gly-Gly (SEQ ID NO: 32))n

in which n is an integer of 1 or larger. The amino acid sequences of the peptide linkers can be selected appropriately by those skilled in the art according to the purpose. For example, n, which determines the length of the peptide linkers, is generally 1 to 5, preferably 1 to 3, more preferably 1 or 2.

Therefore, a particularly preferred embodiment of sc(Fv)2 in the present invention is, for example, the following sc(Fv)2:
[VH]-peptide linker (15 amino acids)-[VL]-peptide linker (15 amino acids)-[VH]-peptide linker (15 amino acids)-[VL]

Alternatively, synthetic chemical linkers (chemical crosslinking agents) can be used to link the V regions. Crosslinking agents routinely used to crosslink peptide compounds and such can be used in the present invention. For example, the following chemical crosslinking agents are known. These crosslinking agents are commercially available:
N-hydroxy succinimide (NHS);
disuccinimidyl suberate (DSS);
bis(sulfosuccinimidyl) suberate (BS3);
dithiobis(succinimidyl propionate) (DSP);
dithiobis(sulfosuccinimidyl propionate) (DTSSP);
ethylene glycol bis(succinimidyl succinate) (EGS);
ethylene glycol bis(sulfosuccinimidyl succinate) (sulfo-EGS);
disuccinimidyl tartrate (DST);
disulfosuccinimidyl tartrate (sulfo-DST);
bis[2-(succinimidoxycarbonyloxy)ethyl] sulfone (BSOCOES); and
bis[2-(sulfosuccinimidoxycarbonyloxy)ethyl] sulfone (sulfo-BSOCOES).

Usually, three linkers are required to link four antibody variable regions. The multiple linkers to be used may all be of the same type or different types. In the present invention, a preferred minibody is a diabody or an sc(Fv)2. Such minibody can be obtained by treating an antibody with an enzyme, such as papain or pepsin, to generate antibody fragments, or by constructing DNAs that encode these antibody fragments, introducing them into expression vectors, and then expressing them in appropriate host cells (see, for example, Co, M. S. *et al.*, J. Immunol. (1994) 152, 2968-2976; Better, M. and Horwitz, A. H., Methods Enzymol. (1989) 178, 476-496; Pluckthun, A. and Skerra, A., Methods Enzymol. (1989) 178, 497-515; Lamoyi, E.,

Methods Enzymol. (1986) 121, 652-663; Rousseaux, J. *et al.*, Methods Enzymol. (1986) 121, 663-669; and Bird, R. E. and Walker, B. W., Trends Biotechnol. (1991) 9, 132-137).

Any EREG-recognizing antibody can be used as the antibody of the present invention. Preferred antibodies include the antibodies of (1) to (57) below. These antibodies may be, for example, full-length antibodies, minibodies, animal antibodies, chimeric antibodies, humanized antibodies, or human antibodies:

(1) an antibody comprising an H chain having the amino acid sequence of SEQ ID NO: 2 (sequence of the B3#18 antibody H-chain CDR1) as CDR1, the amino acid sequence of SEQ ID NO: 4 (sequence of the B3#18 antibody H-chain CDR2) as CDR2, and the amino acid sequence of SEQ ID NO: 6 (sequence of the B3#18 antibody H-chain CDR3) as CDR3;

(2) an antibody comprising the H chain of (1), wherein the H chain has the amino acid sequence of positions 117 to 452 in the amino acid sequence of SEQ ID NO: 8 (sequence of the B3#18 antibody CH) as CH (H-chain constant region);

(3) an antibody comprising the H chain of (1), wherein the H chain has the amino acid sequence of positions 117 to 446 in the amino acid sequence of SEQ ID NO: 10 (sequence of the CH of the mouse-human chimeric DF151 antibody) as CH;

(4) an antibody comprising an L chain having the amino acid sequence of SEQ ID NO: 12 (sequence of the B3#18 antibody L-chain CDR1) as CDR1, the amino acid sequence of SEQ ID NO: 14 (sequence of the B3#18 antibody L-chain CDR2) as CDR2, and the amino acid sequence of SEQ ID NO: 16 (sequence of the B3#18 antibody L-chain CDR3) as CDR3;

(5) an antibody comprising the L chain of (4), wherein the L chain has the amino acid sequence of positions 107 to 213 in the amino acid sequence of SEQ ID NO: 18 (sequence of the B3#18 antibody CL) as CL (L-chain constant region);

(6) an antibody comprising the L chain of (4), wherein the L chain has the amino acid sequence of positions 107 to 213 in the amino acid sequence of SEQ ID NO: 20 (sequence of the CL of the mouse-human chimeric B3#18 antibody) as CL;

(7) an antibody comprising the H chain of (1) and the L chain of (4);

(8) an antibody comprising the H chain of (2) and the L chain of (5);

(9) an antibody comprising the H chain of (3) and the L chain of (6);

(10) an antibody comprising an H chain having the amino acid sequence of SEQ ID NO: 49 as CDR1, the amino acid sequence of SEQ ID NO: 51 as CDR2, and the amino acid sequence of SEQ ID NO: 53 as CDR3;

(11) an antibody comprising the H chain of (10), wherein the H chain has CH derived from mouse IgG1;

(12) an antibody comprising the H chain of (10), wherein the H chain has the amino acid sequence of positions 117 to 446 in the amino acid sequence of SEQ ID NO: 10 as CH;

(13) an antibody comprising an L chain having the amino acid sequence of SEQ ID NO: 55 as CDR1, the amino acid sequence of SEQ ID NO: 57 as CDR2, and the amino acid sequence of SEQ ID NO: 59 as CDR3;

(14) an antibody comprising the L chain of (13), wherein the L chain has CL derived from the mouse κ chain;

(15) an antibody comprising the L chain of (13), wherein the L chain has the amino acid sequence of positions 107 to 213 in the amino acid sequence of SEQ ID NO: 20 as CL;

(16) an antibody comprising the H chain of (10) and the L chain of (13);

(17) an antibody comprising the H chain of (11) and the L chain of (14);

(18) an antibody comprising the H chain of (12) and the L chain of (15);

(19) an antibody comprising an H chain having the amino acid sequence of SEQ ID NO: 61 as CDR1, the amino acid sequence of SEQ ID NO: 63 as CDR2, and the amino acid sequence of SEQ ID NO: 65 as CDR3;

(20) an antibody comprising the H chain of (19), wherein the H chain has CH derived from mouse IgG1;

(21) an antibody comprising the H chain of (19), wherein the H chain has the amino acid sequence of positions 117 to 446 in the amino acid sequence of SEQ ID NO: 10 as CH;

(22) an antibody comprising an L chain having the amino acid sequence of SEQ ID NO: 67 as CDR1, the amino acid sequence of SEQ ID NO: 69 as CDR2, and the amino acid sequence of SEQ ID NO: 71 as CDR3;

(23) an antibody comprising the L chain of (22), wherein the L chain CL derived from the mouse κ chain;

(24) an antibody comprising the L chain of (22), wherein the L chain has the amino acid sequence of positions 107 to 213 in the amino acid sequence of SEQ ID NO: 20 as CL;

(25) an antibody comprising the H chain of (19) and the L chain of (22);

(26) an antibody comprising the H chain of (20) and the L chain of (23);

(27) an antibody comprising the H chain of (21) and the L chain of (24);

(28) an antibody comprising an H chain having the amino acid sequence of SEQ ID NO: 73 as CDR1, the amino acid sequence of SEQ ID NO: 75 as CDR2, and the amino acid sequence of SEQ ID NO: 77 as CDR3;

(29) an antibody comprising the H chain of (28), wherein the H chain has CH derived from mouse IgG1;

(30) an antibody comprising the H chain of (28), wherein the H chain has the amino acid sequence of positions 117 to 446 in the amino acid sequence of SEQ ID NO: 10 as CH;

(31) an antibody comprising an L chain having the amino acid sequence of SEQ ID NO: 79 as CDR1, the amino acid sequence of SEQ ID NO: 81 as CDR2, and the amino acid sequence of

SEQ ID NO: 83 as CDR3;

(32) an antibody comprising the L chain of (31), wherein the L chain has CL derived from the mouse κ chain;

(33) an antibody comprising the L chain of (31), wherein the L chain has the amino acid sequence of positions 107 to 213 in the amino acid sequence of SEQ ID NO: 20 as CL;

(34) an antibody comprising the H chain of (28) and the L chain of (31);

(35) an antibody comprising the H chain of (29) and the L chain of (32);

(36) an antibody comprising the H chain of (30) and the L chain of (33);

(37) an antibody comprising an H chain having the amino acid sequence of SEQ ID NO: 85 as CDR1, the amino acid sequence of SEQ ID NO: 87 as CDR2, and the amino acid sequence of SEQ ID NO: 89 as CDR3;

(38) an antibody comprising the H chain of (37), wherein the H chain has CH derived from the mouse IgG1;

(39) an antibody comprising the H chain of (37), wherein the H chain has the amino acid sequence of positions 117 to 446 in the amino acid sequence of SEQ ID NO: 10 as CH;

(40) an antibody comprising an L chain having the amino acid sequence of SEQ ID NO: 91 as CDR1, the amino acid sequence of SEQ ID NO: 93 as CDR2, and the amino acid sequence of SEQ ID NO: 95 as CDR3;

(41) an antibody comprising the L chain of (40), wherein the L chain has CL derived from mouse κ chain;

(42) an antibody comprising the L chain of (40), wherein the L chain has the amino acid sequence of positions 107 to 213 in the amino acid sequence of SEQ ID NO: 20 as CL;

(43) an antibody comprising the H chain of (37) and the L chain of (40);

(44) an antibody comprising the H chain of (38) and the L chain of (41);

(45) an antibody comprising the H chain of (39) and the L chain of (42);

(46) an antibody comprising an H chain having the amino acid sequence of SEQ ID NO: 97 as CDR1, the amino acid sequence of SEQ ID NO: 99 as CDR2, and the amino acid sequence of SEQ ID NO: 101 as CDR3;

(47) an antibody comprising the H chain of (46), wherein the H chain has CH derived from mouse IgG1;

(48) an antibody comprising the H chain of (46), wherein the H chain has the amino acid sequence of positions 117 to 446 in the amino acid sequence of SEQ ID NO: 10 as CH;

(49) an antibody comprising an L chain having the amino acid sequence of SEQ ID NO: 103 as CDR1, the amino acid sequence of SEQ ID NO: 105 as CDR2, and the amino acid sequence of SEQ ID NO: 107 as CDR3;

(50) an antibody comprising the L chain of (49), wherein the L chain has

CL derived from the mouse κ chain;
(51) an antibody comprising the L chain of (49), wherein the L chain has the amino acid sequence of positions 107 to 213 in the amino acid sequence of SEQ ID NO: 20 as CL;
(52) an antibody comprising the H chain of (46) and the L chain of (49);
(53) an antibody comprising the H chain of (47) and the L chain of (50);
(54) an antibody comprising the H chain of (48) and the L chain of (51);
(55) an antibody comprising one or more amino acid substitutions, deletions, additions, and/or insertions in the antibody of any of (1) to (54), which has equivalent activity as the antibody of any of (1) to (54);
(56) an antibody that binds to the same EREG protein epitope as the antibody of any of (1) to (55); and
(57) a minibody of the antibody of any of (1) to (56).

An example of VH in the above-mentioned H chain of (1), which has the CDRs listed below, includes a VH having the amino acid sequence of SEQ ID NO: 44 (sequence of the B3#18 antibody VH):
CDR1: the amino acid sequence of SEQ ID NO: 2 (sequence of the B3#18 (EP27) antibody H-chain CDR1);
CDR2: the amino acid sequence of SEQ ID NO: 4 (sequence of the B3#18 (EP27) antibody H-chain CDR2); and
CDR3: the amino acid sequence of SEQ ID NO: 6 (sequence of the B3#18 (EP27) antibody H-chain CDR3).

An example of VL in the above-mentioned L chain of (4), which has the CDRs listed below, includes a VL having the amino acid sequence of SEQ ID NO: 46 (sequence of the B3#18 antibody VL):
CDR1: the amino acid sequence of SEQ ID NO: 12 (sequence of the B3#18 (EP27) antibody L-chain CDR1);
CDR2: the amino acid sequence of SEQ ID NO: 14 (sequence of the B3#18 (EP27) antibody L-chain CDR2); and
CDR3: the amino acid sequence of SEQ ID NO: 16 (sequence of the B3#18 (EP27) antibody L-chain CDR3).

An example of VH in the above-mentioned H chain of (10), which has the CDRs listed below, includes a VH having the amino acid sequence of SEQ ID NO: 109 (sequence of the C7 (EP03) VH):
CDR1: the amino acid sequence of SEQ ID NO: 49 (sequence of the C7 (EP03) antibody H-chain CDR1);
CDR2: the amino acid sequence of SEQ ID NO: 51 (sequence of the C7 (EP03) antibody

H-chain CDR2); and
CDR3: the amino acid sequence of SEQ ID NO: 53 (sequence of the C7 (EP03) antibody H-chain CDR3).

An example of VL in the above-mentioned L chain of (13), which has the CDRs listed below, includes a VL having the amino acid sequence of SEQ ID NO: 111 (sequence of the C7 (EP03) antibody VL):
CDR1: the amino acid sequence of SEQ ID NO: 55 (sequence of the C7 (EP03) antibody L-chain CDR1);
CDR2: the amino acid sequence of SEQ ID NO: 57 (sequence of the C7 (EP03) antibody L-chain CDR2); and
CDR3: the amino acid sequence of SEQ ID NO: 59 (sequence of the C7 (EP03) antibody L-chain CDR3).

An example of VH in the above-mentioned H chain of (19), which has the CDRs listed below, includes a VH having the amino acid sequence of SEQ ID NO: 113 (sequence of the #15 (EP08) VH):
CDR1: the amino acid sequence of SEQ ID NO: 61 (sequence of the #15 (EP08) antibody H-chain CDR1);
CDR2: the amino acid sequence of SEQ ID NO: 63 (sequence of the #15 (EP08) antibody H-chain CDR2); and
CDR3: the amino acid sequence of SEQ ID NO: 65 (sequence of the #15 (EP08) antibody H-chain CDR3).

An example of VL in the above-mentioned L chain of (22), which has the CDRs listed below, includes a VL having the amino acid sequence of SEQ ID NO: 115 (sequence of the #15 (EP08) antibody VL):
CDR1: the amino acid sequence of SEQ ID NO: 67 (sequence of the #15 (EP08) antibody L-chain CDR1);
CDR2: the amino acid sequence of SEQ ID NO: 69 (sequence of the #15 (EP08) antibody L-chain CDR2); and
CDR3: the amino acid sequence of SEQ ID NO: 71 (sequence of the #15 (EP08) antibody L-chain CDR3).

An example of VH in the above-mentioned H chain of (28), which has the CDRs listed below, includes a VH having the amino acid sequence of SEQ ID NO: 117 (sequence of the B2#30 (EP20) VH):
CDR1: the amino acid sequence of SEQ ID NO: 73 (sequence of the B2#30 (EP20) antibody H-chain CDR1);
CDR2: the amino acid sequence of SEQ ID NO: 75 (sequence of the B2#30 (EP20) antibody

H-chain CDR2); and
CDR3: the amino acid sequence of SEQ ID NO: 77 (sequence of the B2#30 (EP20) antibody H-chain CDR3).

An example of VL in the above-mentioned L chain of (31), which has the CDRs listed below, includes a VL having the amino acid sequence of SEQ ID NO: 119 (sequence of the B2#30 (EP20) antibody VL):
CDR1: the amino acid sequence of SEQ ID NO: 79 (sequence of the B2#30 (EP20) antibody L-chain CDR1);
CDR2: the amino acid sequence of SEQ ID NO: 81 (sequence of the B2#30 (EP20) antibody L-chain CDR2); and
CDR3: the amino acid sequence of SEQ ID NO: 83 (sequence of the B2#30 (EP20) antibody L-chain CDR3).

An example of VH in the above-mentioned H chain of (37), which has the CDRs listed below, includes a VH having the amino acid sequence of SEQ ID NO: 121 (sequence of the B3#8 (EP24) VH):
CDR1: the amino acid sequence of SEQ ID NO: 85 (sequence of the B3#8 (EP24) antibody H-chain CDR1);
CDR2: the amino acid sequence of SEQ ID NO: 87 (sequence of the B3#8 (EP24) antibody H-chain CDR2); and
CDR3: the amino acid sequence of SEQ ID NO: 89 (sequence of the B3#8 (EP24) antibody H-chain CDR3).

An example of VL in the above-mentioned L chain of (40), which has the CDRs listed below, includes a VL having the amino acid sequence of SEQ ID NO: 123 (sequence of the B3#8 (EP24) antibody VL):
CDR1: the amino acid sequence of SEQ ID NO: 91 (sequence of the B3#8 (EP24) antibody L-chain CDR1);
CDR2: the amino acid sequence of SEQ ID NO: 93 (sequence of the B3#8 (EP24) antibody L-chain CDR2); and
CDR3: the amino acid sequence of SEQ ID NO: 95 (sequence of the B3#8 (EP24) antibody L-chain CDR3).

An example of VH in the above-mentioned H chain of (46), which has the CDRs listed below, includes a VH having the amino acid sequence of SEQ ID NO: 125 (sequence of the B3#41 (EP29) VH):
CDR1: the amino acid sequence of SEQ ID NO: 97 (sequence of the B3#41 (EP29) antibody H-chain CDR1);
CDR2: the amino acid sequence of SEQ ID NO: 99 (sequence of the B3#41 (EP29) antibody

H-chain CDR2); and
CDR3: the amino acid sequence of SEQ ID NO: 101 (sequence of the B3#41 (EP29) antibody H-chain CDR3).

An example of VL in the above-mentioned L chain of (49), which has the CDRs listed below, includes a VL having the amino acid sequence of SEQ ID NO: 127 (sequence of the B3#41 (EP29) antibody VL):
CDR1: the amino acid sequence of SEQ ID NO: 103 (sequence of the B3#41 (EP29) antibody L-chain CDR1);
CDR2: the amino acid sequence of SEQ ID NO: 105 (sequence of the B3#41 (EP29) antibody L-chain CDR2); and
CDR3: the amino acid sequence of SEQ ID NO: 107 (sequence of the B3#41 (EP29) antibody L-chain CDR3).

The above-mentioned antibodies of (1) to (57) include not only monovalent antibodies but also multivalent antibodies with two or more valencies. Multivalent antibodies of the present invention include multivalent antibodies whose antigen binding sites are all the same and multivalent antibodies whose antigen binding sites are partially or completely different.

A preferred embodiment of the above-mentioned antibody of (55) is an antibody in which the CDR has not been modified. For example, among the above-mentioned antibodies of (55), a preferred embodiment of "an antibody having one or more amino acid substitutions, deletions, additions, and/or insertions in the antibody of (1) and having an activity equivalent to that of the antibody of (1)" is "an antibody having an activity equivalent to that of the antibody of (1) and having one or more amino acid substitutions, deletions, additions, and/or insertions in the antibody of (1), and also comprising an H chain having the amino acid sequence of SEQ ID NO: 2 as CDR1, the amino acid sequence of SEQ ID NO: 4 as CDR2, and the amino acid sequence of SEQ ID NO: 6 as CDR3". Preferred embodiments of other antibodies included in the above-mentioned antibody of (55) can be expressed in a similar manner.

A method of introducing mutations into polypeptides is one of the methods well known to those skilled in the art for preparing polypeptides that are functionally equivalent to a certain polypeptide. For example, those skilled in the art can prepare an antibody functionally equivalent to an antibody of the present invention by introducing appropriate mutations into the antibody using site-directed mutagenesis (Hashimoto-Gotoh, T. *et al.* (1995) Gene 152, 271-275; Zoller, MJ, and Smith, M. (1983) Methods Enzymol. 100, 468-500; Kramer, W. *et al.* (1984) Nucleic Acids Res. 12, 9441-9456; Kramer W, and Fritz HJ (1987) Methods. Enzymol. 154, 350-367; Kunkel, TA (1985) Proc. Natl. Acad. Sci. USA. 82, 488-492; Kunkel (1988) Methods Enzymol. 85, 2763-2766) and such. Amino acid mutations may also occur naturally. In this way, the antibodies of the present invention also comprise antibodies comprising amino acid

sequences with one or more amino acid mutations in the amino acid sequences of the antibodies of the present invention, and which are functionally equivalent to the antibodies of the present invention.

The number of amino acids that are mutated in such mutants is generally considered to be 50 amino acids or less, preferably 30 amino acids or less, and more preferably 10 amino acids or less (for example, 5 amino acids or less).

It is desirable that the amino acid residues are mutated into amino acids in which the properties of the amino acid side chains are conserved. For example, the following categories have been established depending on the amino acid side chain properties:
hydrophobic amino acids (A, I, L, M, F, P, W, Y, and V);
hydrophilic amino acids (R, D, N, C, E, Q, G, H, K, S, and T);
amino acids having aliphatic side chains (G, A, V, L, I, and P);
amino acids having hydroxyl-containing side chains (S, T, and Y);
amino acids having sulfur-containing side chains (C and M);
amino acids having carboxylic acid- and amide-containing side chains (D, N, E, and Q);
amino acids having basic side chains (R, K, and H); and
amino acids having aromatic ring-containing side chains (H, F, Y, and W)
(amino acids are represented by one-letter codes in parentheses).

Polypeptides comprising a modified amino acid sequence, in which one or more amino acid residues in a certain amino acid sequence is deleted, added, and/or substituted with other amino acids, are known to retain their original biological activities (Mark, D. F. *et al.*, Proc. Natl. Acad. Sci. USA (1984) 81, 5662-5666; Zoller, M. J. & Smith, M. Nucleic Acids Research (1982) 10, 6487-6500; Wang, A. *et al.*, Science 224, 1431-1433; Dalbadie-McFarland, G. *et al.*, Proc. Natl. Acad. Sci. USA (1982) 79, 6409-6413). That is, generally in an amino acid sequence constituting a certain polypeptide, the activity of the polypeptide is highly likely to be maintained when amino acids classified into the same group are mutually substituted. In the present invention, the above-mentioned substitution between amino acids within the same amino acid group is referred to as conservative substitution.

The present invention provides antibodies that bind to the same epitope as the anti-EREG antibodies disclosed in the present application. More specifically, the present invention relates to antibodies that recognize the same epitope as the antibody of any of (1) to (57) mentioned above, and uses thereof. Such antibodies can be obtained, for example, by the following method.

Whether a test antibody shares the epitope of a certain antibody can be confirmed by checking whether the two antibodies compete for the same epitope. Competition between antibodies can be detected by cross-blocking assay and such. For example, competitive ELISA

is a preferred cross-blocking assay.

Specifically, in a cross-blocking assay, the wells of a microtiter plate are coated with the EREG protein which is then pre-incubated with or without a candidate competing antibody, and an anti-EREG antibody of the present invention is then added. The amount of the anti-EREG antibody of the present invention bound to the EREG protein in the wells indirectly correlates with the binding ability of the candidate competing antibody (test antibody) that competes for binding to the same epitope. More specifically, the greater the affinity the test antibody has for the same epitope, the lower the activity of the anti-EREG antibody of the present invention to bind to the EREG protein-coated wells. In other words, the greater the affinity the test antibody has for the same epitope, the higher the activity of the test antibody to bind to the EREG protein-coated wells.

The amount of antibody bound to the wells can be easily measured by labeling the antibodies in advance. For example, a biotin-labeled antibody can be measured using an avidin-peroxidase conjugate and a suitable substrate. In particular, cross-blocking assays using enzyme labels such as peroxidase are called competitive ELISA assays. The antibodies can be labeled with other detectable or measurable labeling substances. More specifically, radiolabels and fluorescent labels are known.

Furthermore, when the test antibody comprises a constant region derived from a species different from that of the anti-EREG antibody of the present invention, measurement can be done for either one of the antibodies bound to the wells using a labeled antibody that recognizes its constant region. If the antibodies are derived from the same species but belong to different classes, the antibodies bound to the wells can be measured using antibodies that distinctively recognize individual classes.

If a candidate competing antibody can block binding of the EREG antibody by at least 20%, preferably by at least 20% to 50%, and even more preferably, by at least 50%, as compared to the binding activity obtained in a control experiment performed in the absence of the candidate competing antibody, the candidate competing antibody is either an antibody that binds substantially to the same epitope or one that competes for binding to the same epitope as an anti-EREG antibody of the present invention. Antibodies that bind to the same epitope as the anti-EREG antibodies include, for example, the above-mentioned antibody of (57), but are not limited thereto.

As described above, the above-mentioned antibodies of (1) to (57) include not only monovalent antibodies but also multivalent antibodies. Multivalent antibodies of the present invention include multivalent antibodies whose antigen binding sites are all the same and multivalent antibodies whose antigen binding sites are partially or completely different.

Antibodies bound to various types of molecules such as polyethylene glycol (PEG) can

also be used as modified antibodies. Moreover, chemotherapeutic agents, toxic peptides, or radioactive chemical substances can be bound to the antibodies. Such modified antibodies (hereinafter referred to as antibody conjugates) can be obtained by subjecting the obtained antibodies to chemical modification. Methods for modifying antibodies are already established in this field. Furthermore, as described below, such antibodies can also be obtained in the molecular form of a bispecific antibody designed using genetic engineering techniques to recognize not only EREG proteins, but also chemotherapeutic agents, toxic peptides, radioactive chemical compounds, or such. These antibodies are included in the "antibodies" of the present invention.

Chemotherapeutic agents that bind to anti-EREG antibodies to drive the cytotoxic activity include the following:

azaribine, anastrozole, azacytidine, bleomycin, bortezomib, bryostatin-1, busulfan, camptothecin, 10-hydroxycamptothecin, carmustine, celebrex, chlorambucil, cisplatin, irinotecan, carboplatin, cladribine, cyclophosphamide, cytarabine, dacarbazine, docetaxel, dactinomycin, daunomycin glucuronide, daunorubicin, dexamethasone, diethylstilbestrol, doxorubicin, doxorubicin glucuronide, epirubicin, ethinyl estradiol, estramustine, etoposide, etoposide glucuronide, floxuridine, fludarabine, flutamide, fluorouracil, fluoxymesterone, gemcitabine, hydroxyprogesterone caproate, hydroxyurea, idarubicin, ifosfamide, leucovorin, lomustine, mechlorethamine, medroxyprogesterone acetate, megestrol acetate, melphalan, mercaptopurine, methotrexate, mitoxantrone, mithramycin, mitomycin, mitotane, phenylbutyrate, prednisone, procarbazine, paclitaxel, pentostatin, semustine streptozocin, tamoxifen, taxanes, taxol, testosterone propionate, thalidomide, thioguanine, thiotepa, teniposide, topotecan, uracil mustard, vinblastine, vinorelbine, and vincristine.

In the present invention, preferred chemotherapeutic agents are low-molecular-weight chemotherapeutic agents. Low-molecular-weight chemotherapeutic agents are unlikely to interfere with antibody function even after binding to antibodies. In the present invention, low-molecular-weight chemotherapeutic agents usually have a molecular weight of 100 to 2000, preferably 200 to 1000. Examples of the chemotherapeutic agents demonstrated herein are all low-molecular-weight chemotherapeutic agents. The chemotherapeutic agents of the present invention include prodrugs that are converted to active chemotherapeutic agents *in vivo*. Prodrug activation may be enzymatic conversion or non-enzymatic conversion.

Furthermore, the antibodies can be modified using toxic peptides such as ricin, abrin, ribonuclease, onconase, DNase I, Staphylococcal enterotoxin-A, pokeweed antiviral protein, gelonin, diphtheria toxin, Pseudomonas exotoxin, Pseudomonas endotoxin, L-asparaginase, and PEG L-Asparaginase. In another embodiment, one or two or more of the low-molecular-weight chemotherapeutic agents and toxic peptides can be combined and used for antibody modification.

The bonding between an anti-EREG antibody and the above-mentioned low-molecular weight chemotherapeutic agent may be covalent bonding or non-covalent bonding. Methods for producing antibodies bound to these chemotherapeutic agents are known.

Furthermore, proteinaceous pharmaceutical agents or toxins can be bound to antibodies by gene recombination techniques. Specifically, for example, it is possible to construct a recombinant vector by fusing a DNA encoding the above-mentioned toxic peptide with a DNA encoding an anti-EREG antibody in frame, and inserting this into an expression vector. This vector is introduced into suitable host cells, the obtained transformed cells are cultured, and the incorporated DNA is expressed. Thus, an anti-EREG antibody bound to the toxic peptide can be obtained as a fusion protein. When obtaining an antibody as a fusion protein, the proteinaceous pharmaceutical agent or toxin is generally positioned at the C terminus of the antibody. A peptide linker can be positioned between the antibody and the proteinaceous pharmaceutical agent or toxin.

Furthermore, the antibody used in the present invention may be a bispecific antibody. A bispecific antibody refers to an antibody that carries variable regions that recognize different epitopes within the same antibody molecule. The bispecific antibody may have antigen-binding sites that recognize different epitopes on an EREG molecule. Two molecules of such a bispecific antibody can bind to one molecule of EREG. As a result, stronger cytotoxic action can be expected.

Alternatively, the bispecific antibody may be an antibody in which one antigen-binding site recognizes EREG, and the other antigen-binding site recognizes a cytotoxic substance. Specifically, cytotoxic substances include chemotherapeutic agents, toxic peptides, and radioactive chemical substances. Such a bispecific antibody binds to EREG-expressing cells, and at the same time, captures cytotoxic substances. This enables the cytotoxic substances to directly act on EREG-expressing cells. Therefore, bispecific antibodies that recognize cytotoxic substances specifically injure tumor cells and suppress tumor cell proliferation.

Furthermore, in the present invention, bispecific antibodies that recognize antigens other than EREG may be combined. For example, it is possible to combine bispecific antibodies that recognize non-EREG antigens that are specifically expressed on the surface of target cancer cells like EREG.

Methods for producing bispecific antibodies are known. For example, two types of antibodies recognizing different antigens may be linked to prepare a bispecific antibody. The antibodies to be linked may be half molecules each having an H chain or an L chain, or may be quarter molecules consisting of only an H chain. Alternatively, bispecific antibody-producing fused cells can be prepared by fusing hybridomas producing different monoclonal antibodies. Bispecific antibodies can also be prepared by genetic engineering techniques.

Antibody genes constructed as described above can be obtained through expression by known methods. In the case of mammalian cells, the antibody genes can be expressed by operably linking the antibody gene to be expressed with an effective commonly used promoter, and a polyA signal on the 3' downstream side of the antibody gene. An example of the promoter/enhancer is human cytomegalovirus immediate early promoter/enhancer.

Other promoters/enhancers that can be used for antibody expression include viral promoters/enhancers, or mammalian cell-derived promoters/enhancers such as human elongation factor 1α (HEF1α). Specific examples of viruses whose promoters/enhancers may be used include retrovirus, polyoma virus, adenovirus, and simian virus 40 (SV40).

When an SV40 promoter/enhancer is used, the method of Mulligan *et al.* (Nature (1979) 277, 108) may be utilized. An HEF1α promoter/enhancer can be readily used for expressing a gene of interest by the method of Mizushima *et al.* (Nucleic Acids Res. (1990) 18, 5322).

In the case of *E. coli*, the antibody gene to be expressed is operably linked to an effective commonly used promoter and a signal sequence for antibody secretion to express the gene. Examples of the promoter include the lacZ promoter and araB promoter. When the lacZ promoter is used, the method of Ward *et al.*, (Nature (1989) 341, 544-546; FASEB J. (1992) 6, 2422-2427) may be used. Alternatively, the araB promoter can be used for expressing a gene of interest by the method of Better *et al.* (Science (1988) 240, 1041-1043).

With regard to the signal sequence for antibody secretion, the pelB signal sequence (Lei, S. P. *et al.*, J. Bacteriol. (1987) 169, 4379) may be used for production in the periplasm of *E. coli*. After the antibody produced in the periplasm is isolated, the antibody structure is refolded by using a protein denaturant like guanidine hydrochloride or urea so that the antibody will have the desired binding activity.

The replication origin inserted into the expression vector includes, for example, those derived from SV40, polyoma virus, adenovirus, or bovine papilloma virus (BPV). In order to amplify the gene copy number in the host cell system, a selection marker can be inserted into the expression vector. Specifically, the following selection markers can be used:
the aminoglycoside transferase (APH) gene;
the thymidine kinase (TK) gene;
the *E. coli* xanthine guanine phosphoribosyltransferase (Ecogpt) gene;
the dihydrofolate reductase (dhfr) gene, etc.

Any expression system, for example, a eukaryotic cell system or a prokaryotic cell system can be used to produce antibodies used in the present invention. Examples of eukaryotic cells include animal cells such as established mammalian cell system, insect cell system, and filamentous fungus cells and yeast cells. Examples of prokaryotic cells include bacterial cells such as *E. coli* cells. Antibodies used in the present invention are preferably

expressed in mammalian cells. For example, mammalian cells such as CHO, COS, myeloma, BHK, Vero, or HeLa cells can be used.

Then, the transformed host cell is then cultured *in vitro* or *in vivo* to induce production of an antibody of interest. The host cells are cultured according to known methods. For example, DMEM, MEM, RPMI 1640, or IMDM can be used as the culture medium. A serum supplement solution such as fetal calf serum (FCS) can also be used in combination.

Antibodies expressed and produced as described above can be purified by using a single known method or a suitable combination of known methods generally used for purifying proteins. Antibodies can be separated and purified by, for example, appropriately selecting and combining affinity columns such as protein A column, chromatography column, filtration, ultrafiltration, salt precipitation, dialysis, and such (Antibodies A Laboratory Manual. Ed Harlow, David Lane, Cold Spring Harbor Laboratory, 1988).

Known means can be used to measure the antigen-binding activity of the antibodies (Antibodies A Laboratory Manual. Ed Harlow, David Lane, Cold Spring Harbor Laboratory, 1988). For example, an enzyme linked immunosorbent assay (ELISA), an enzyme immunoassay (EIA), a radioimmunoassay (RIA), or a fluoroimmunoassay can be used.

The antibodies used in the present invention may be antibodies with a modified sugar chain. It is known that the cytotoxic activity of an antibody can be increased by modifying its sugar chain. Known antibodies having modified sugar chains include the following:
antibodies with modified glycosylation (for example, WO 99/54342);
antibodies deficient in fucose attached to sugar chains (for example, WO 00/61739 and WO 02/31140);
antibodies having a sugar chain with bisecting GlcNAc (for example, WO 02/79255), etc.

The antibodies used in the present invention are preferably antibodies having cytotoxic activity.

In the present invention, the cytotoxic activity includes, for example, antibody-dependent cell-mediated cytotoxicity (ADCC) activity and complement-dependent cytotoxicity (CDC) activity. In the present invention, CDC activity refers to complement system-mediated cytotoxic activity. ADCC activity refers to the activity of injuring a target cell when a specific antibody attaches to its cell surface antigen. An Fcγ receptor-carrying cell (immune cell, or such) binds to the Fc portion of the antigen via the Fcγ receptor and the target cell is damaged.

An anti-EREG antibody can be tested to see whether it has ADCC activity or CDC activity using known methods (for example, Current Protocols in Immunology, Chapter 7. Immunologic studies in humans, Editor, John E. Coligan *et al.*, John Wiley & Sons, Inc., (1993) and the like).

First, specifically, effector cells, complement solution, and target cells are prepared.

(1) Preparation of effector cells

Spleen is removed from a CBA/N mouse or the like, and spleen cells are isolated in RPMI1640 medium (manufactured by Invitrogen). After washing in the same medium containing 10% fetal bovine serum (FBS, manufactured by HyClone), the cell concentration is adjusted to 5×10^6 /mL to prepare the effector cells.

(2) Preparation of complement solution

Baby Rabbit Complement (manufactured by CEDARLANE) is diluted 10-fold in a culture medium (manufactured by Invitrogen) containing 10% FBS to prepare a complement solution.

(3) Preparation of target cells

The target cells can be radioactively labeled by incubating cells expressing the EREG protein with 0.2 mCi of sodium chromate-^{51}Cr (manufactured by GE Healthcare Bio-Sciences) in a DMEM medium containing 10% FBS for one hour at 37°C. For EREG protein-expressing cells, one may use cells transformed with a gene encoding the EREG protein, primary colon cancer, metastatic colon cancer, lung adenocarcinoma, pancreatic cancer, stomach cancer, kidney cancer cells, colon cancer cells, esophageal cancer cells, stomach cancer cells, pancreatic cancer cells, or such. After radioactive labeling, cells are washed three times in RPMI1640 medium containing 10% FBS, and the target cells can be prepared by adjusting the cell concentration to 2×10^5 /mL.

ADCC activity or CDC activity can be measured by the method described below. In the case of ADCC activity measurement, the target cell and anti-EREG antibody (50 μL each) are added to a 96-well U-bottom plate (manufactured by Becton Dickinson), and reacted for 15 minutes on ice. Thereafter, 100 μL of effector cells are added and incubated in a carbon dioxide incubator for four hours. The final concentration of the antibody is adjusted to 0 or 10 μg/mL. After culturing, 100 μL of the supernatant is collected, and the radioactivity is measured with a gamma counter (COBRAII AUTO-GAMMA, MODEL D5005, manufactured by Packard Instrument Company). The cytotoxic activity (%) can be calculated using the obtained values according to the equation: (A - C) / (B - C) x 100, wherein A represents the radioactivity (cpm) in each sample, B represents the radioactivity (cpm) in a sample where 1% NP-40 (manufactured by Nacalai Tesque) has been added, and C represents the radioactivity (cpm) of a sample containing the target cells only.

Meanwhile, in the case of CDC activity measurement, 50 μL of target cell and 50 μL of an anti-EREG antibody are added to a 96-well flat-bottomed plate (manufactured by Becton Dickinson), and reacted for 15 minutes on ice. Thereafter, 100 μL of the complement solution is added, and incubated in a carbon dioxide incubator for four hours. The final concentration of

the antibody is adjusted to 0 or 3 μg/mL. After incubation, 100 μL of supernatant is collected, and the radioactivity is measured with a gamma counter. The cytotoxic activity can be calculated in the same way as in the ADCC activity determination.

On the other hand, in the case of measuring the cytotoxic activity of an antibody conjugate, 50 μL of target cell and 50 μL of an anti-EREG antibody conjugate are added to a 96-well flat-bottomed plate (manufactured by Becton Dickinson), and reacted for 15 minutes on ice. This is then incubated in a carbon dioxide incubator for one to four hours. The final concentration of the antibody is adjusted to 0 or 3 μg/mL. After culturing, 100 μL of supernatant is collected, and the radioactivity is measured with a gamma counter. The cytotoxic activity can be calculated in the same way as in the ADCC activity determination.

An antibody of the present invention having cytotoxic activity is more preferably an antibody that has neutralizing activity. Generally, "neutralizing activity" refers to the activity to inhibit the biological activity of a ligand such as viruses or toxins towards cells. More specifically, "substances having neutralizing activity" refers to substances that bind to the ligand or to a receptor binding to the ligand, and inhibit the binding between the ligand and the receptor. When the ligand binding of a receptor is blocked by neutralizing activity, the receptor-mediated biological activity cannot be exerted. Antibodies that have such neutralizing activity are generally referred to as neutralizing antibodies. The neutralizing activity can be measured by comparing the biological activities in the presence and absence of a test substance of which the neutralizing activity is to be evaluated, in the presence of the ligand of interest.

In the present invention, ligand binding of the EGF receptor, which is considered to be the main receptor of EREG, results in dimerization of the receptor, and activation of the intracellular tyrosine kinase domain of the receptor. The activated tyrosine kinase forms phosphorylated tyrosine-containing peptides by autophosphorylation, and the peptides associate with various signal transduction accessory molecules. They are mainly PLCγ (phospholipase Cγ), Shc, Grb2, and such. Of these accessory molecules, the former two are further phosphorylated by the EGF receptor tyrosine kinase. The main signal transduction pathway from the EGF receptor is the pathway in which phosphorylation is transduced in the order of Shc, Grb2, Sos, Ras, Raf/MAPK kinase/MAP kinase. Furthermore, an alternative pathway which is from PLCγ to PKC is considered to exist.

Since such intracellular signal cascades vary depending on the cell type, suitable molecules can be targeted in the target cell of interest, and the target molecules are not limited to the above-mentioned factors. Commercially available kits for measuring *in vivo* signal activation can be suitably used (for example, the protein kinase C activity assay system (GE Healthcare Bio-Sciences)).

Furthermore, *in vivo* signaling activation can be detected using as an index, the

transcription-inducing effect on a target gene present downstream of the *in vivo* signaling cascade. Changes in the transcriptional activity can be detected based on the principle of reporter assay. More specifically, a reporter gene such as green fluorescence protein (GFP) or luciferase is positioned downstream of the transcriptional factor or promoter region of the target gene, and the reporter activity is measured. The change in transcriptional activity can be measured based on the reporter activity.

Furthermore, since the EGF receptor usually functions to promote cell proliferation, *in vivo* signaling activation can be evaluated by measuring the proliferation activity of target cells. In the present invention, the neutralizing activity of a neutralizing antibody of the present invention is evaluated by assessing the cell proliferation activity. However, the present invention is not limited to this method, and the neutralizing activity can be assessed by suitably applying the aforementioned methods to selected target cells.

Specifically, for example, by measuring the below-mentioned cell proliferation activity, the neutralizing activity of an anti-EREG antibody can be evaluated or measured. For example, a method that measures the incorporation of [^{3}H]-labeled thymidine added to the medium by living cells as an index of DNA replication ability is used.

As a more convenient method, a dye exclusion method that measures under a microscope the ability of a cell to release a dye such as trypan blue to the outside of the cell, or the MTT method is used. The latter makes use of the ability of living cells to convert 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), which is a tetrazolium salt, to a blue formazan product. More specifically, a test antibody is added to the culture solution of a test cell, and after a certain period of time, the MTT solution is added to the culture solution, and this is left to stand for a certain time for MTT to be incorporated into the cell. As a result, MTT which is a yellow compound is converted to a blue compound by the action of succinate dehydrogenase in the mitochondria of the cell. After dissolving this blue product for coloration, absorbance is measured and used as an indicator for the number of viable cells.

Besides MTT, reagents such as MTS, XTT, WST-1, and WST-8 are commercially available (Nacalai Tesque, and such) and can be suitably used. Furthermore, methods that evaluate cell proliferation activity using cellular ATP or impedance of cell culture as an indicator are known. For activity measurements, a binding antibody that has the same isotype as the anti-EREG antibody but does not have the neutralizing activity can be used as a control antibody in the same manner as the anti-EREG antibody, and it can be determined that the activity is present when the anti-EREG antibody has a stronger neutralizing activity than the control antibody.

The cells whose proliferation is suppressed by the anti-EREG antibody are not particularly limited as long as they express an EREG protein. Preferred EREG-expressing cells

are, for example, cancer cells. Specifically, cells derived from colon cancer, lung adenocarcinoma, pancreatic cancer, stomach cancer, or kidney cancer are suitable as the EREG-expressing cells of the present invention. According to the present invention, effective cell proliferation-suppressing effect can be obtained for both primary and metastatic foci of these cancers. The more preferable cancer cells are cells of primary colon cancer, metastatic colon cancer, lung adenocarcinoma, pancreatic cancer, stomach cancer, and kidney cancer. Therefore, the anti-EREG antibody can be used for the purpose of treating or preventing cell proliferation-induced diseases such as colon cancer, lung adenocarcinoma, pancreatic cancer, stomach cancer, and kidney cancer. These cancers may be targets for the treatment or prevention regardless of whether they are primary foci or metastatic foci. More preferably, the anti-EREG antibody can be used for the purpose of treating and/or preventing primary colon cancer, metastatic colon cancer, or pancreatic cancer. Furthermore, among these cancers, cancers that proliferate in an EREG-dependent manner are preferred targets for the treatment and/or prevention of the present invention.

The present invention also provides polynucleotides encoding the antibodies of the present invention, and polynucleotides that hybridize under stringent conditions to these polynucleotides and encode antibodies having an activity equivalent to that of the antibodies of the present invention. The present invention also provides vectors containing these polynucleotides and transformants (including transformed cells) containing such vectors.

The polynucleotides of the present invention are polymers comprising multiple nucleotides or base pairs of deoxyribonucleic acids (DNA) or ribonucleic acids (RNA), and are not particularly limited, as long as they encode the antibodies of the present invention. The polynucleotides of the present invention may also contain non-natural nucleotides.

The polynucleotides of the present invention can be used to express antibodies using genetic engineering techniques. Furthermore, they can be used as probes in the screening of antibodies that are functionally equivalent to the antibodies of the present invention. Specifically, a DNA that hybridizes under stringent conditions to the polynucleotide encoding an antibody of the present invention, and encodes an antibody having an activity equivalent to that of the antibody of the present invention, can be obtained by techniques such as hybridization and gene amplification techniques (for example, PCR), using the polynucleotide encoding an antibody of the present invention, or a portion thereof, as a probe. Such DNAs are included in the polynucleotides of the present invention. Hybridization techniques are well known to those skilled in the art (Sambrook, J. *et al.*, Molecular Cloning 2nd ed., 9.47-9.58, Cold Spring Harbor Lab. press, 1989).

Conditions for hybridization include, for example, those with low stringency. Examples of conditions of low stringency include post-hybridization washing under conditions

of 0.1x SSC and 0.1% SDS at 42°C, and preferably under conditions of 0.1x SSC and 0.1% SDS at 50°C. More preferable hybridization conditions include those of high stringency. High stringency conditions include, for example, conditions of 5x SSC and 0.1% SDS at 65°C. Under these conditions, higher temperature can be expected to efficiently yield polynucleotides with high homology. However, several factors such as temperature and salt concentration can influence hybridization stringency, and those skilled in the art can suitably select these factors to achieve similar stringencies.

A functionally equivalent antibody encoded by a polynucleotide obtained by these hybridization and gene amplification techniques in the present invention usually has a high homology to the amino acid sequences of the antibodies of the present invention. The antibodies of the present invention also include antibodies that are functionally equivalent to and have high amino acid sequence homology to the antibodies of the present invention. The term "high homology" generally refers to amino acid identity of at least 50% or higher, preferably 75% or higher, more preferably 85% or higher, still more preferably 95% or higher. Polypeptide homology can be determined by the algorithm described in literature (Wilbur, W. J. and Lipman, D. J. Proc. Natl. Acad. Sci. USA (1983) 80, 726-730).

Pharmaceutical Compositions

In another aspect, the present invention provides pharmaceutical compositions comprising an antibody that binds to an EREG protein as an active ingredient. In addition, the present invention relates to a cell proliferation inhibitor, in particular an anticancer agent, comprising an antibody that binds to an EREG protein as an active ingredient. Cell proliferation inhibitors and anticancer agents of the present invention are preferably administered to a subject affected by cancer, or to a subject who is likely to be affected by cancer.

In the present invention, a cell proliferation inhibitor comprising as an active ingredient an antibody that binds to an EREG protein can also be described as a method for suppressing cell proliferation which comprises the step of administering an antibody that binds to an EREG protein to a subject, or as use of an antibody that binds to an EREG protein in the production of a cell proliferation inhibitor.

Furthermore, in the present invention, an anticancer agent comprising as an active ingredient an antibody that binds to an EREG protein can also be described as a method for preventing or treating cancer which comprises the step of administering an antibody that binds to an EREG protein to a subject, or as use of an antibody that binds to an EREG protein in the production of an anticancer agent.

In the present invention, the phrase "comprising an antibody that binds to EREG as an active ingredient" means comprising an anti-EREG antibody as the main active substance, and

does not limit the content percentage of the anti-EREG antibody.

The antibody included in the pharmaceutical composition of the present invention (for example, cell proliferation inhibitor and anticancer agent; same hereinafter) is not particularly limited as long as it binds to an EREG protein, and examples include antibodies described herein.

The pharmaceutical compositions of the present invention can be administered orally or parenterally to a patient. Preferably, the administration is parenteral administration. Specifically, the method of administration is, for example, administration by injection, transnasal administration, transpulmonary administration, or transdermal administration. Examples of administration by injection include systemic and local administrations of a pharmaceutical composition of the present invention by intravenous injection, intramuscular injection, intraperitoneal injection, subcutaneous injection, or such. A suitable administration method may be selected according to the age of the patient and symptoms. The dosage may be selected, for example, within the range of 0.0001 mg to 1000 mg per kg body weight in each administration. Alternatively, for example, the dosage for each patient may be selected within the range of 0.001 to 100,000 mg/body. However, the pharmaceutical composition of the present invention is not limited to these doses.

The pharmaceutical compositions of the present invention can be formulated according to conventional methods (for example, Remington's Pharmaceutical Science, latest edition, Mark Publishing Company, Easton, U.S.A), and may also contain pharmaceutically acceptable carriers and additives. Examples include, but are not limited to, surfactants, excipients, coloring agents, perfumes, preservatives, stabilizers, buffers, suspending agents, isotonization agents, binders, disintegrants, lubricants, fluidity promoting agents, and flavoring agents; and other commonly used carriers can be suitably used. Specific examples of the carriers include light anhydrous silicic acid, lactose, crystalline cellulose, mannitol, starch, carmellose calcium, carmellose sodium, hydroxypropyl cellulose, hydroxypropyl methyl cellulose, polyvinylacetal diethylaminoacetate, polyvinylpyrrolidone, gelatin, medium chain fatty acid triglyceride, polyoxyethylene hardened castor oil 60, saccharose, carboxymethyl cellulose, corn starch, inorganic salt, and such.

The present invention also provides methods for inducing damages in EREG-expressing cells and methods for suppressing cell proliferation by contacting EREG-expressing cells with antibodies that bind to the EREG protein.

More specifically, the present invention includes the following embodiments.

– A method for inducing cell injury in EREG-expressing cells, which comprises the step of contacting the EREG protein-expressing cells with an EREG protein-binding antibody.

– A method for suppressing the proliferation of EREG-expressing cells, which comprises the step

of contacting the EREG protein-expressing cells with an EREG protein-binding antibody.

– The aforementioned method, wherein the EREG protein-binding antibody has cytotoxic activity.

– The aforementioned method, wherein the EREG protein-binding antibody has neutralizing activity.

– The aforementioned method, wherein the EREG protein-binding antibody is an antibody of any of (1) to (57) below:

(1) an antibody comprising an H chain having the amino acid sequence of SEQ ID NO: 2 as CDR1, the amino acid sequence of SEQ ID NO: 4 as CDR2, and the amino acid sequence of SEQ ID NO: 6 as CDR3;

(2) an antibody comprising the H chain of (1), wherein the H chain has the amino acid sequence of positions 117 to 452 in the amino acid sequence of SEQ ID NO: 8 as CH;

(3) an antibody comprising the H chain of (1), wherein the H chain has the amino acid sequence of positions 117 to 446 in the amino acid sequence of SEQ ID NO: 10 as CH;

(4) an antibody comprising an L chain having the amino acid sequence of SEQ ID NO: 12 as CDR1, the amino acid sequence of SEQ ID NO: 14 as CDR2, and the amino acid sequence of SEQ ID NO: 16 as CDR3;

(5) an antibody comprising the L chain of (4), wherein the L chain has the amino acid sequence of positions 107 to 213 in the amino acid sequence of SEQ ID NO: 18 as CL;

(6) an antibody comprising the L chain of (4), wherein the L chain has the amino acid sequence of positions 107 to 213 in the amino acid sequence of SEQ ID NO: 20 as CL;

(7) an antibody comprising the H chain of (1) and the L chain of (4);

(8) an antibody comprising the H chain of (2) and the L chain of (5);

(9) an antibody comprising the H chain of (3) and the L chain of (6);

(10) an antibody comprising an H chain having the amino acid sequence of SEQ ID NO: 49 as CDR1, the amino acid sequence of SEQ ID NO: 51 as CDR2, and the amino acid sequence of SEQ ID NO: 53 as CDR3;

(11) an antibody comprising the H chain of (10), wherein the H chain has CH derived from mouse IgG1;

(12) an antibody comprising the H chain of (10), wherein the H chain has the amino acid sequence of positions 117 to 446 in the amino acid sequence of SEQ ID NO: 10 as CH;

(13) an antibody comprising an L chain having the amino acid sequence of SEQ ID NO: 55 as CDR1, the amino acid sequence of SEQ ID NO: 57 as CDR2, and the amino acid sequence of SEQ ID NO: 59 as CDR3;

(14) an antibody comprising the L chain of (13), wherein the L chain has CL derived from the mouse κ chain;

(15) an antibody comprising the L chain of (13), wherein the L chain has the amino acid sequence of positions 107 to 213 in the amino acid sequence of SEQ ID NO: 20 as CL;

(16) an antibody comprising the H chain of (10) and the L chain of (13);

(17) an antibody comprising the H chain of (11) and the L chain of (14);

(18) an antibody comprising the H chain of (12) and the L chain of (15);

(19) an antibody comprising an H chain having the amino acid sequence of SEQ ID NO: 61 as CDR1, the amino acid sequence of SEQ ID NO: 63 as CDR2, and the amino acid sequence of SEQ ID NO: 65 as CDR3;

(20) an antibody comprising the H chain of (19), wherein the H chain has CH derived from mouse IgG1;

(21) an antibody comprising the H chain of (19), wherein the H chain has the amino acid sequence of positions 117 to 446 in the amino acid sequence of SEQ ID NO: 10 as CH;

(22) an antibody comprising an L chain having the amino acid sequence of SEQ ID NO: 67 as CDR1, the amino acid sequence of SEQ ID NO: 69 as CDR2, and the amino acid sequence of SEQ ID NO: 71 as CDR3;

(23) an antibody comprising the L chain of (22), wherein the L chain has CL derived from the mouse κ chain;

(24) an antibody comprising the L chain of (22), wherein the L chain has the amino acid sequence of positions 107 to 213 in the amino acid sequence of SEQ ID NO: 20 as CL;

(25) an antibody comprising the H chain of (19) and the L chain of (22);

(26) an antibody comprising the H chain of (20) and the L chain of (23);

(27) an antibody comprising the H chain of (21) and the L chain of (24);

(28) an antibody comprising an H chain having the amino acid sequence of SEQ ID NO: 73 as CDR1, the amino acid sequence of SEQ ID NO: 75 as CDR2, and the amino acid sequence of SEQ ID NO: 77 as CDR3;

(29) an antibody comprising the H chain of (28), wherein the H chain has CH derived from mouse IgG1;

(30) an antibody comprising the H chain of (28), wherein the H chain has the amino acid sequence of positions 117 to 446 in the amino acid sequence of SEQ ID NO: 10 as CH;

(31) an antibody comprising an L chain having the amino acid sequence of SEQ ID NO: 79 as CDR1, the amino acid sequence of SEQ ID NO: 81 as CDR2, and the amino acid sequence of SEQ ID NO: 83 as CDR3;

(32) an antibody comprising the L chain of (31), wherein the L chain has CL derived from the mouse κ chain;

(33) an antibody comprising the L chain of (31), wherein the L chain has the amino acid sequence of positions 107 to 213 in the amino acid sequence of SEQ ID NO: 20 as CL;

(34) an antibody comprising the H chain of (28) and the L chain of (31);
(35) an antibody comprising the H chain of (29) and the L chain of (32);
(36) an antibody comprising the H chain of (30) and the L chain of (33);
(37) an antibody comprising an H chain having the amino acid sequence of SEQ ID NO: 85 as CDR1, the amino acid sequence of SEQ ID NO: 87 as CDR2, and the amino acid sequence of SEQ ID NO: 89 as CDR3;
(38) an antibody comprising the H chain of (37), wherein the H chain has CH derived from mouse IgG1;
(39) an antibody comprising the H chain of (37), wherein the H chain has the amino acid sequence of positions 117 to 446 in the amino acid sequence of SEQ ID NO: 10 as CH;
(40) an antibody comprising an L chain having the amino acid sequence of SEQ ID NO: 91 as CDR1, the amino acid sequence of SEQ ID NO: 93 as CDR2, and the amino acid sequence of SEQ ID NO: 95 as CDR3;
(41) an antibody comprising the L chain of (40), wherein the L chain has CL derived from the mouse κ chain;
(42) an antibody comprising the L chain of (40), wherein the L chain has the amino acid sequence of positions 107 to 213 in the amino acid sequence of SEQ ID NO: 20 as CL;
(43) an antibody comprising the H chain of (37) and the L chain of (40);
(44) an antibody comprising the H chain of (38) and the L chain of (41);
(45) an antibody comprising the H chain of (39) and the L chain of (42);
(46) an antibody comprising an H chain having the amino acid sequence of SEQ ID NO: 97 as CDR1, the amino acid sequence of SEQ ID NO: 99 as CDR2, and the amino acid sequence of SEQ ID NO: 101 as CDR3;
(47) an antibody comprising the H chain of (46), wherein the H chain has CH derived from mouse IgG1;
(48) an antibody comprising the H chain of (46), wherein the H chain has the amino acid sequence of positions 117 to 446 in the amino acid sequence of SEQ ID NO: 10 as CH;
(49) an antibody comprising an L chain having the amino acid sequence of SEQ ID NO: 103 as CDR1, the amino acid sequence of SEQ ID NO: 105 as CDR2, and the amino acid sequence of SEQ ID NO: 107 as CDR3;
(50) an antibody comprising the L chain of (49), wherein the L chain has CL derived from the mouse κ chain;
(51) an antibody comprising the L chain of (49), wherein the L chain has the amino acid sequence of positions 107 to 213 in the amino acid sequence of SEQ ID NO: 20 as CL;
(52) an antibody comprising the H chain of (46) and the L chain of (49);
(53) an antibody comprising the H chain of (47) and the L chain of (50);

(54) an antibody comprising the H chain of (48) and the L chain of (51);
(55) an antibody comprising one or more amino acid substitutions, deletions, additions, and/or insertions in the antibody of any of (1) to (54), which has equivalent activity as the antibody of any of (1) to (54);
(56) an antibody that binds to the same EREG protein epitope as the antibody of any of (1) to (55); and
(57) a minibody of the antibody of any of (1) to (56).
– The aforementioned method, wherein the EREG protein-expressing cells are cancer cells.

As described above, the above-mentioned antibodies of (1) to (57) include not only monovalent antibodies but also multivalent antibodies with two or more valencies. Multivalent antibodies of the present invention include multivalent antibodies whose antigen binding sites are all the same, and multivalent antibodies whose antigen binding sites are partially or completely different.

The EREG protein-binding antibodies are described above as EREG protein-binding antibodies included in the cell proliferation inhibitors of the present invention. Cells that are bound by the anti-EREG antibodies are not particularly limited as long as the cells are EREG-expressing cells. Preferred EREG-expressing cells of the present invention are cancer cells. More preferably, the cells are colon cancer, lung adenocarcinoma, pancreatic cancer, stomach cancer, and kidney cancer cells. Methods of the present invention can be applied to both primary and metastatic foci of these cancers. More preferred cancer cells are primary colon cancer, metastatic colon cancer, and pancreatic cancer cells.

In the present invention "contacting" is accomplished, for example, by adding an antibody to a culture solution of EREG-expressing cells cultured in a test tube. In this case, the antibody can be added in the form of, for example, a solution or a solid obtained by freeze-drying or the like. When adding the antibody as an aqueous solution, the aqueous solution used may purely contain only the antibody, or the solution may include, for example, the above-mentioned surfactants, excipients, coloring agents, perfumes, preservatives, stabilizers, buffers, suspending agents, isotonization agents, binders, disintegrants, lubricants, fluidity promoting agents, or flavoring agents. The concentration for addition is not particularly limited, but the final concentration in the culture that may be suitably used is preferably in the range of 1 pg/mL to 1 g/mL, more preferably 1 ng/mL to 1 mg/mL, and even more preferably 1 μg/mL to 1 mg/mL.

Furthermore, in another embodiment, "contacting" in the present invention is carried out by administration to a non-human animal to which an EREG-expressing cell has been transplanted into the body, or to an animal carrying cancer cells endogenously expressing EREG. The method of administration may be oral or parenteral administration. The method of

administration is particularly preferably parenteral administration, and specifically, the method of administration is, for example, administration by injection, transnasal administration, transpulmonary administration, or transdermal administration. Examples of administration by injection include systemic and local administrations of pharmaceutical compositions, cell proliferation inhibitors and anticancer agents of the present invention by intravenous injection, intramuscular injection, intraperitoneal injection, subcutaneous injection, or such. A suitable administration method may be selected according to the age of the test animal and symptoms. When administering as an aqueous solution, the aqueous solution used may purely contain only the antibody, or the solution may include, for example, the above-mentioned surfactants, excipients, coloring agents, perfumes, preservatives, stabilizers, buffers, suspending agents, isotonization agents, binders, disintegrants, lubricants, fluidity promoting agents, or flavoring agents. The dosage may be selected, for example, within the range of 0.0001 mg to 1000 mg per kg body weight in each administration. Alternatively, for example, the dosage for each patient may be selected within the range of 0.001 to 100,000 mg/body. However, the antibody dose of the present invention is not limited to these doses.

The following method is suitably used as a method for evaluating or measuring cell damage induced by contacting EREG-expressing cells with an anti-EREG antibody. Examples of a method for evaluating or measuring the cytotoxic activity in a test tube include methods for measuring the above-mentioned antibody-dependent cell-mediated cytotoxicity (ADCC) activity, complement-dependent cytotoxicity (CDC) activity, and such. Whether or not an anti-EREG antibody has ADCC activity or CDC activity can be measured by known methods (for example, Current protocols in Immunology, Chapter 7. Immunologic studies in humans, Editor, John E. Coligan *et al.*, John Wiley & Sons, Inc., (1993) and the like). For activity measurements, a binding antibody having the same isotype as anti-EREG antibody but not having any cell-damaging activity can be used as a control antibody in the same manner as the anti-EREG antibody, and it can be determined that the activity is present when the anti-EREG antibody shows a stronger cytotoxic activity than the control antibody.

The isotype of an antibody is defined by the sequence of its H chain constant region in the antibody amino acid sequence. The isotype of an antibody is ultimately determined *in vivo* by class switching that arises from genetic recombinations in chromosomes which occur during maturation of antibody-producing B-cells. Difference in isotype is reflected in the difference of physiological and pathological functions of antibodies. Specifically, for example, the strength of cytotoxic activity is known to be influenced by antibody isotype in addition to the expression level of the antigen. Therefore, when measuring the above-described cell damaging activity, an antibody of the same isotype as the test antibody is preferably used as the control.

To evaluate or measure cell damaging activity *in vivo*, for example, EREG-expressing

cancer cells are intradermally or subcutaneously transplanted to a non-human test animal, and then a test antibody is intravenously or intraperitoneally administered daily or at the interval of few days, starting from the day of transplantation or the following day. Cytotoxicity can be defined by daily measurement of tumor size. In a similar manner to the evaluation in a test tube, cytotoxicity can be determined by administering a control antibody having the same isotype, and observing that the tumor size in the anti-EREG antibody-administered group is significantly smaller than the tumor size in the control antibody-administered group. When using a mouse as the non-human test animal, it is suitable to use a nude (nu/nu) mouse whose thymus has been made genetically defective so that its T lymphocyte function is lost. The use of such a mouse can eliminate the participation of T lymphocytes in the test animals when evaluating or measuring the cytotoxicity of the administered antibody.

The same method used for measuring neutralizing activity mentioned above can be suitably applied as a method for evaluating or measuring the contact-mediated inhibitory effect of an anti-EREG antibody on the proliferation of EREG-expressing cells.

As a method for evaluating or measuring cell proliferation-inhibiting activity *in vivo*, the same method described above for evaluating or measuring cytotoxicity *in vivo* can be suitably used.

Screening methods

The present inventors showed that EREG secreted by cells induces the proliferation of cells growing in an EREG-dependent manner. Furthermore, it was also confirmed that the EREG expression is specifically enhanced in cancer cells. Therefore, cancer treatment can be accomplished by suppressing the EREG expression in EREG-expressing cells. More specifically, in the present invention, it is shown that compounds that suppress the EREG expression in cancer cells are useful as candidate compounds as cancer therapeutic agents. Based on these findings, the present invention provides methods of screening for candidate compounds as cancer therapeutic agents, which comprise the steps of:

(1) contacting EREG-expressing cells with a test compound;

(2) measuring the EREG expression level in the EREG-expressing cells; and

(3) selecting a compound that lowers the EREG expression compared to a control as a candidate therapeutic agent for cancer.

In the present invention, for example, EREG-expressing cancer cells may be used as the EREG-expressing cells. Specifically, for example, any cancer cell line showing enhanced EREG expression in Fig. 2 can be used for the screening methods of the present invention. More specifically, cells that may be used for the screening methods of the present invention include the following cancer cell lines. All the examples of cell lines listed below can be

obtained from a cell bank. Culturing conditions for these cell lines are already established. More specifically, the cells can be cultured, for example, under the conditions shown in Table 2.

Esophageal cancer cell line:	TE2
Stomach cancer cell lines:	GT3
	MKN45
	2M
	2MD3
Colon cancer cell lines:	CACO2
	DLD1
	hCT116
	LOVO
Liver cancer cell line:	Alexander
Pancreatic cancer cell lines:	Capan1
	Paca2
	PK-1
Kidney cancer cell lines:	Caki1
	Caki2
Lung cancer cell lines:	A549
	H157
	H1648
	H2009
	H23
	H2347
	H522

These cell lines are cultured in the presence of a test compound, and the EREG expression level in the cell lines is measured. The EREG expression level can be evaluated by measuring the amount of intracellular mRNA, or the amount of EREG protein secreted inside the cell, on the cell surface, or to the outside of the cell. Any method described herein may be used as a method for measuring EREG mRNA or EREG protein. The present inventors confirmed the cell proliferation-inducing effect of secreted EREG in cells that proliferate in an EREG-dependent manner. Therefore, a preferred embodiment of the present invention is a method of screening for candidate compounds as anti-cancer agents, which uses the amount of extracellularly secreted EREG as an index.

In the present invention, a test compound that lowers the EREG expression compared to a control is selected as the candidate compound of interest. As the control in the present invention, for example, the same cell line cultured in the absence of the test compound may be

used. Alternatively, for a control, one may use a cell line cultured in the presence of a compound whose effect on the EREG expression is already known. By using such a control, a compound having a greater effect than a certain compound can be selected.

In the present invention, one can screen for cancer therapeutic agents using the neutralizing effect of a test compound against EREG as an index. More specifically, the present invention relates to methods of screening for candidate compounds as cancer therapeutic agents, which comprise the steps of:

(1) culturing cells that proliferate in an EREG-dependent manner, in the presence of EREG and a test compound;

(2) measuring the cell proliferation level; and

(3) selecting a test compound that suppresses cell proliferation compared to a control as a candidate therapeutic agent for cancer.

In the present invention, "cells that proliferate in an EREG-dependent manner" refers to cells showing dose-dependent cell proliferation mediated by EREG. For example, in the Examples described below, the AsPC1 pancreatic cancer cell line shows dose-dependent cell proliferation mediated by EREG. Furthermore, according to findings by the present inventors, the EREG dependency can be conferred by expressing an EGF receptor/G-CSF receptor chimeric receptor. More specifically, mouse cells transformed with DNA encoding a chimeric receptor comprising the extracellular domain of EGF receptor and the intracellular domain of G-CSF receptor exhibit dose-dependent cell proliferation to human EREG. Therefore, cells in which the EGFR dependency has been artificially induced in this way can also be used for the screening methods of the present invention.

The proliferation level of cells proliferating in an EREG-dependent manner cultured in the presence of EREG and a test compound is then measured. Cell proliferation can be measured by any method. For example, the number of viable cells can be compared using a commercially available reagent for counting viable cells, as described in the Examples. Alternatively, systems for efficient, larger-scale screening are also commercially available. For example, a system that can evaluate the number of viable cells in each well using a multi-well plate is in practical use. Based on the result of comparing the viable cell number, test compounds that suppress the increase in cell number compared to a control can be selected as candidate compounds for cancer therapeutic agents.

In the screening methods of the present invention, for example, the same cell line cultured in the absence of a test compound can be used as a control. Alternatively, one can use a cell line cultured in the presence of a compound that has clear effect on the cell proliferation-inducing action depending on the EREG secretion as a control. By using such a control, one can select a compound that has greater effect than a certain compound.

Candidate compounds selected by screening methods of the present invention are useful as candidate compounds of anticancer therapeutic agent that work to suppress the EREG function. In the present invention, the cancer therapeutic effect of anti-EREG antibodies was confirmed. Therefore, compounds selected by the screening methods of the present invention can be expected to have similar effects as the antibodies. If necessary, candidate compounds selected by the screening methods of the present invention are further evaluated for their effect on other cancer cell lines or primary culture cells, and also for their effect such as toxicity against normal cells. Compounds that are useful as cancer therapeutic agents can be selected through such evaluation. A series of evaluation methods for cancer therapeutic agents are already established.

In the screening methods of the present invention, various naturally-occurring and artificially-synthesized compounds can be used as test compounds. For example, protein libraries and antibody libraries are preferred as the test compound libraries of the present invention. Alternatively, phage libraries presenting proteins or antibodies may be used as the test compounds. Furthermore, libraries of artificially-synthesized compounds such as combinatorial libraries may be used as the test compounds.

Examples

Herein below, the present invention will be specifically described with reference to the Examples, but it is not to be construed as being limited thereto.

[Example 1] Analysis of the human EREG gene expression in various types of cancers

1-1. GeneChip analysis of the human EREG gene expression

To search for a gene of which expression is enhanced specifically in cancer tissues such as colon cancer and pancreatic cancer tissues, comprehensive gene expression analysis was carried out for normal tissues, cancer tissues, and cancer cell lines using GeneChip U-133A (manufactured by Affymetrix).

Total RNAs were prepared from normal tissues, cancer tissues, and cancer cell lines shown in Tables 1 and 2 by conventional methods using ISOGEN (manufactured by Nippon Gene). Gene expression analysis was carried out using 10 μg each of these total RNAs, and subjecting them to transcript analysis on GeneChip U-133A (manufactured by Affymetrix). This method was carried out according to the Expression Analysis Technical Manual (manufactured by Affymetrix). For searching genes of which expression is enhanced in cancer tissues or cancer cells, the mean value of the expression scores of all the genes was normalized to 100.

We found that the expression of human EREG mRNA (probe ID: 205767_at HG-U133A) was enhanced in primary colon cancer, metastatic colon cancer, lung adenocarcinoma, and pancreatic cancer tissues, and colon cancer cell lines (CACO2, DLD1, HCT116, and LOVO), stomach cancer cell lines (2M and 2MD3), liver cancer cell line (Alexander), pancreatic cancer cell lines (Capan1 and Paca2), kidney cancer cell lines (Caki1 and Caki2), and primary colon cancer, metastatic colon cancer, lung adenocarcinoma, pancreatic cancer, stomach cancer, and kidney cancer cell lines (H157, H1648, H2009, H23, and H2347), while no remarkable expression of human EREG mRNA could be observed in the normal tissues examined (Figs. 1 and 2).

From the above finding, it became apparent that, while expression of the human EREG gene (probe ID: 205767_at HG-U133A) is very low in normal tissues, expression of the human EREG gene is enhanced in a wide variety of cancers including primary colon cancer, metastatic colon cancer, lung adenocarcinoma, pancreatic cancer, stomach cancer, kidney cancer, colon cancer, pancreatic cancer, stomach cancer and kidney cancer.

[Table 1]Tissues used for EREG gene expression analysis

Tissue	Source
Whole brain	Clontech 64020-1
Lung	Clinical sample, 1 case
Trachea	Clontech 64091-1
Heart	Ambion 7966
Kidney	Ambion 7976
Liver	Clinical sample (Surgery)
Pancreas	Ambion 7954
Stomach	Clinical sample (Surgery)
Small intestine	Ambion 7984
Large intestine	Ambion 7986
Bone marrow	Clontech 64106-1
Peripheral blood mononuclear cell	Clinical sample, 1 case
Testis	Clontech 64027-1
Prostate	Ambion 7988
Ovary	Ambion 7974
Skin	Stratagene 735031
Lung adenocarcinoma 1	Clinical sample, 1 case
Lung adenocarcinoma 2	Clinical sample, 1 case

Lung adenocarcinoma 3	Clinical sample, 1 case
Lung adenocarcinoma 4	Clinical sample, 1 case
Lung adenocarcinoma 5	Clinical sample, 1 case
Primary colon cancer 1	Clinical sample, 1 case
Primary colon cancer 2	Clinical sample, 1 case
Primary colon cancer 3	Clinical sample, 1 case
Primary colon cancer 4	Clinical sample, 1 case
Metastatic colon cancer 1	Clinical sample, 1 case
Metastatic colon cancer 2	Clinical sample, 1 case
Metastatic colon cancer 3	Clinical sample, 1 case
Metastatic colon cancer 4	Clinical sample, 1 case
Metastatic colon cancer 5	Clinical sample, 1 case
Metastatic colon cancer 6	Clinical sample, 1 case
Metastatic colon cancer 7	Clinical sample, 1 case
Pancreatic cancer 1	Clinical sample, 1 case
Pancreatic cancer 2	Clinical sample, 1 case
Pancreatic cancer 3	Clinical sample, 1 case
Pancreatic cancer 4	Clinical sample, 1 case

[Table 2]

Cell lines and culture conditions used for the EREG gene expression analysis

Type of cancer	Cell line	Medium	Serum (%)
Brain tumor	U251	DMEM	10
Breast cancer	MCF7	RPMI1640	10
Esophageal cancer	TE2	RPMI1640	10
Stomach cancer	AGS	RPMI1640	10
	GT3	DMEM	10
	KatoIII	RPMI1640:DMEM=1:1	10
	MKN45	RPMI1640	10
	MKN74	RPMI1640	10
	2M	DMEM	10
	2MD3	DMEM	10
Colon cancer	CACO2	DMEM	20
	DLD1	RPMI1640	10
	hCT116	McCoy5A	10
	LOVO	HamF12:DMEM=1:1	10
	SW480	RPMI1640	10
Liver cancer	Alexander	DMEM	10
	HepG2	DMEM	10
	HLE	DMEM	10
	HuH6	DMEM	10
	HuH7	DMEM	10
Pancreatic cancer	Capan1	DMEM	20
	KLM1	RPMI1640	10
	Panc1	RPMI1640	10
	Paca2	RPMI1640	10
	PK-1	RPMI1640	10
Kidney cancer	Caki1	RPMI1640	10
	Caki2	RPMI1640	10
Lung cancer	A549	DMEM	10
	Lu130	RPMI1640	10
	H1359	RPMI1640	10
	H157	RPMI1640	10
	H1648	HamF12:DMEM=1:1	10
	H2009	HamF12:DMEM=1:1	10
	H23	RPMI1640	10
	H2347	RPMI1640	10
	H522	RPMI1640	10
Cervical cancer	Hela	DMEM	10

[Example 2] Preparation of anti-EREG monoclonal antibody

2-1. Establishment of cells expressing the full-length human EREG

The full-length human EREG cDNA (NM_001432) comprising the sequence shown in SEQ ID NO: 21 was isolated by conventional methods. The gene fragment was cloned into an expression vector (pMCN) for mammalian cells, and the resulting vector was named hEREG/pMCN. pMCN enables induced expression of a foreign gene under the control of a

mouse CMV promoter (ACCESSION No. U68299), and is a vector into which a neomycin resistance gene has been incorporated. A CHO cell line that stably expresses full-length human EREG was established by introducing hEREG/pMCN into the CHO DG44 cell line (Invitrogen) by electroporation, and selecting the cells in the presence of 500 μg/mL of Geneticin. The cell line established was named EREG_DG.

2-2. Preparation of soluble human EREG/mouse IgG2a Fc-fusion protein (sEREG_mFc)

Soluble human EREG/mouse IgG2a Fc-fusion protein (hereinafter, sEREG_mFc) was prepared as an immunizing antigen for anti-EREG antibody production.

sEREG_mFc was constructed by fusion of the extracellular domain of human EREG (amino acids 7 to 122) to the mouse IgG2a constant region via the CpoI recognition sequence in the hinge region, and cloning into the pMCDN vector, which was prepared by inserting the DHFR gene into the pMCN expression vector. The vector constructed was named sEREG-mFc/pMCDN. The sequence represented by SEQ ID NO: 33 shows the nucleotide sequence of the sEREG-mFc gene, and the sequence represented by SEQ ID NO: 34 shows the amino acid sequence of sEREG-mFc. An sEREG-mFc-expressing CHO cell line was established by introducing sEREG-mFc/pMCDN into the CHO DG44 cell line (Invitrogen) by electroporation, and selecting the cells in the presence of 500 μg/mL of Geneticin.

Then, sEREG-mFc was purified from the culture supernatant. The culture supernatant was applied onto a HiTrap Protein G HP (Amersham, CAT# 17-0404-01) column, unbound materials were washed out with a binding buffer (20 mM sodium phosphate (pH 7.0)), and sEREG-mFc of interest was eluted with an elution buffer (0.1 M glycine-HCl (pH 2.7)). The eluate was immediately neutralized by collection into a tube containing a neutralization buffer (1 M Tris-HCl (pH 9.0)). Then, the eluted sEREG-mFc was separated by gel filtration using Superdex 200 HR 10/30 (Amersham) so that the solvent of this solution was replaced with PBS. The purified protein was quantified using DC protein assay (BIO-RAD), in which the protein concentration was converted using the included bovine IgG as a standard.

2-3. Preparation of anti-EREG monoclonal antibody

Balb/c mice and MRL/MpJ-Tnfrsf6<lpr>/Crlj mice (Charles River Japan) were used as the animals for immunization. Immunization was initiated at the 5th to 8th week. For the first immunization, sEREG-mFc was emulsified using Freund's complete adjuvant (FCA, Beckton Dickinson), and administered subcutaneously (100 μg/head). Two weeks later, sEREG-mFc was emulsified using Freund's incomplete adjuvant (FIA, Beckton Dickinson), and administered subcutaneously (50 μg/head). Subsequently, boosting immunization was performed at one-week intervals for two to three times, and for the final immunization, sEREG-mFc was

diluted with PBS, and then administered to the tail vein (50 μg/head).

At four days after the final immunization, spleen cells were extirpated and mixed with mouse myeloma cells P3-X63Ag8U1 (P3U1, purchased from ATCC) at 2:1 ratio, and cell fusion was carried out by gradual addition of PEG 1500 (Roche Diagnostics). RPMI1640 medium (GIBCO BRL) was added carefully to dilute PEG 1500, and then PEG 1500 was removed by centrifugation. Then, the cells were resuspended in RPMI1640 containing 10% FBS, 1x HAT media supplement (SIGMA), and 0.5x BM-Condimed H1 Hybridoma cloning supplement (Roche Diagnostics) (hereinafter referred to as HAT medium), and seeded into a 96-well culture plate at 200 μL/well. The screening was performed by evaluating the binding activity to human EREG_DG using a flow cytometer. Clones showing a positive binding activity obtained by this analysis were monocloned by the limiting dilution method.

Furthermore, monoclonal antibodies were obtained by immunizing the mice by the DNA inoculation method using the Helios gene gun system (Bio-Rad). The cartridge tubing was coated with gold-DNA (full-length human EREG expression vector) particles according to the Helios gene gun operation manual. 50 mg of 1.0-μm gold particles were weighed out, suspended and mixed in 0.1 mL of 0.05 M spermidine solution. 0.1 mL of 1 mg/mL plasmid solution was added to this and then vortexed. Subsequently, 0.1 mL of 1 M $CaCl_2$ was added, and this was left to stand for ten minutes. After brief centrifugation, the supernatant was removed. The pellet was suspended in ethanol, and then centrifuged. After repeating the ethanol dehydration step three times, the pellet was ultimately suspended in 6 mL of 0.05 mg/mL polyvinylpyrollidone-ethanol solution. The solution was drawn into the tubing for coating, and the tubing was coated, dried, and cut into 0.5-inch-long segments using a tube cutter.

DNA immunization was performed on four- to five-week-old MRL/MpJ-Tnfrsf6<lpr>/Crlj mice (approximately 200 psi helium pressure) at an interval of one to three times a week, and the anti-EREG antibody titer in the serum was monitored intermittently during this period. A cell line forcedly expressing EREG (5×10^6 cells/head) was administered intraperitoneally or to the tail vein of individuals that were confirmed to have an increased serum antibody titer. After rearing for two to three days, the spleen was extirpated, and mononuclear cells containing antibody-producing cells were isolated. Cell fusion and cloning was performed by the same method as in the case of immunization with sEREG-mFc.

The antibodies were purified from the culture supernatant of hybridomas cultured in a HAT medium supplemented with serum. The serum added to the medium was FBS (Ultra low IgG) (GIBCO BRL). The antibodies were purified from the culture supernatant using HiTrap Protein G HP by the same method as the sEREG_mFc purification method described in Example 2-2. The HiTrap Protein G HP eluate fractions were subjected to solvent substitution with PBS using a PD-10 column (Amersham), and then stored at 4°C. The purified antibodies were

quantified using DC protein assay (BIO-RAD), in which the protein concentration was calculated using the included bovine IgG as a standard.

As a result, clones B2#30 (IgG1, Kappa), B3#18 (IgG2b, Kappa), B3#41 (IgG1, Kappa), and such, which are shown in Table 3, were successfully isolated.

[Table 3]

The antibody subtypes and cross-species reactivity of the isolated anti-EREG mouse monoclonal antibodies, and the epitope sites recognized by them.

Antibody ID	Antibody name	Antibody subtype	Epitope	Mouse cross-reactivity	Simian cross-reactivity
EP02	C1	IgG2a/κ	ereg	+++	NA
EP03	C7	IgG1/κ	ereg	+++	+++
EP04	#6	IgG2b/κ	ereg	+	NA
EP05	#9	IgG1/κ	ereg	++	NA
EP06	#11	IgG1/κ	ereg	+	NA
EP07	#13	IgG1/κ	ereg	-	NA
EP08	#15	IgG1/κ	ereg	-	+++
EP09	#19	IgG1/κ	ereg	+	NA
EP10	#20	IgG2b/κ	ereg	++	NA
EP11	#24	IgG1/κ	ereg	+	NA
EP12	#26	IgG1/κ	ereg	-	NA
EP14	#31	IgG1/κ	ereg	++	NA
EP15	#32	IgG2b/κ	(ereg)	-	NA
EP16	#33	IgG1/κ	ereg	++	NA
EP17	#34	IgG1/κ	ereg	-	NA
EP18	#35	IgG2b/κ	ereg	-	NA
EP19	B2#15	IgG1/κ	ereg	++	NA
EP20	B2#30	IgG1/κ	ereg	+++	+++
EP22	B3#2	IgG1/κ	ereg	-	NA
EP23	B3#7	IgG1/κ	ereg	-	NA
EP24	B3#8	IgG1/κ	ereg	+++	+++
EP25	B3#10	IgG1/κ	Nterm	+++	NA
EP26	B3#13	IgG1/κ	ereg	-	NA
EP27	B3#18	IgG2b/κ	ereg	++	+++
EP29	B3#41	IgG1/κ	(ereg)	+++	+++
EP30	B3#44	IgG1/κ,λ	ereg	-	NA
EP31	B3#52	IgG1/κ	ereg	+++	NA
EP32	B3#61	IgG1/κ	Nterm	+++	NA
EP33	B3#62	IgG1/κ	ereg	+++	NA

*Note (ereg): Binds, but with low reactivity
NA: Not analyzed

2-4. Evaluation of binding activity by flow cytometry

Flow cytometry was used to evaluate the binding of the antibodies obtained by the above-mentioned methods to EREG_DG. Anti-EREG antibodies diluted to a suitable

concentration were added to EREG_DG suspended in an FACS Buffer (1% FBS/PBS) at a density of 1 x 10^5 cells/mL, and this was then allowed to react on ice for 60 minutes. After washing the cells once with the FACS Buffer, an FITC-labeled anti-mouse IgG antibody was added, and this was allowed to react on ice for 30 minutes. After the reaction was completed, the supernatant was removed by centrifugation. The cells were then resuspended in 150 μL of FACS Buffer, and then subjected to flow cytometric analysis.

FACS Calibur (Becton Dickinson) was used as the flow cytometer. The living cell population was selected by gating a histogram of forward scatter and side scatter. In addition to the binding activity to EREG_DG, the binding activity to the DLD1 human colon cancer cell line was also evaluated. As confirmed by the result shown in Fig. 2, the human EREG gene expression was similarly enhanced in the DLD1 human colon cancer cell line as in EREG_DG cells.

As shown in Fig. 3, anti-EREG monoclonal antibodies (B2#30, B3#18, and B3#41) produced by hybridomas bound strongly to EREG_DG, but not to the parental DG44 cells. These results revealed that these monoclonal antibodies specifically bind to EREG present on the cell membrane.

Furthermore, the above-described anti-EREG monoclonal antibodies of the present invention were confirmed to bind specifically to DLD1 which has enhanced human EREG gene expression (Fig. 4), as well as to EREG_DG.

Enhanced human EREG gene expression in bladder cancer (Thogersen, V. *et al.*, Cancer Res. 61:6227-6233, 2001), pancreatic cancer (Zhu. Z., *et al.*, Biochem. Biophys. Res. Commun. 273: 1019-1024, 2000), prostate cancer (Torring, N., *et al.*, Anticancer Res. 20: 91-95, 2000), colon cancer (Baba, I., *et al.*, Cancer Res. 60: 6886-6889, 2000), and such, has been reported. However, the present inventors showed for the first time that the enhanced human EREG gene expression correlates with the enhanced human EREG protein expression.

[Example 3] Measurement of complement-dependent cytotoxicity (CDC) activity and antibody-dependent cellular cytotoxicity (ADCC) activity induced by the anti-EREG monoclonal antibodies

3-1. Measurement for CDC activity by the anti-EREG monoclonal antibodies

The DLD1 human colon cancer cell line was used as the target cell. RPMI1640 medium (GIBCO BRL) containing 10% FBS was used to maintain DLD1 cells. 5 x 10^5 DLD1 cells were collected by centrifugation (1000 rpm, five minutes, 4°C). The cell pellet was suspended in approximately 200 μL of medium containing 3.7 MBq of Chromium-51 (Code No. CJS4, Amersham Pharmacia Biotech), and then cultured in a 5% carbon dioxide incubator for one hour at 37°C. These cells were washed three times with the medium, then adjusted to a cell

density of 1 x 10^4 cells/mL in the medium, and then dispensed into a 96-well flat-bottomed plate at 100 μL/well.

Then, an anti-EREG monoclonal antibody (B3#18_IgG2b) and a control mouse IgG2a antibody (Cat. No. 553453, BD Biosciences Pharmingen) diluted into the medium were added at 50 μL per well. The final concentration of the antibody was adjusted to 10 μg/mL. Then, baby rabbit complement (Cat. No. CL3441, Cederlane) diluted 5-fold in the medium was added at 50 μL/well, and then the plate was left to stand in a 5% carbon dioxide incubator for 1.5 hours at 37°C. Thereafter, this plate was centrifuged (1000 rpm for five minutes at 4°C), 100 μL of the supernatant was collected from each well of the plate, and the radioactivity of the collected supernatant was measured using a gamma counter (1480 WIZARD 3", Wallac). The specific chromium release rate was determined based on the following equation:

Specific chromium release rate (%) = (A - C) x 100/(B - C)

where

A represents the radioactivity (cpm) in each well;

B represents the mean value of radioactivity (cpm) in wells where 100 μL of 2% NP-40 solution (Nonidet P-40, Code No. 252-23, Nacalai Tesque) was added to 100 μL of the cells; and

C represents the mean value of radioactivity (cpm) in wells where 100 μL of the medium has been added to 100 μL of the cells. The measurements were conducted in duplicate, and the mean value and standard deviation were calculated for the specific chromium release rate of the supernatant derived from each well.

The anti-EREG monoclonal antibodies used in the experiment were confirmed to have CDC activity, as shown in Fig. 5. On the other hand, the control mouse IgG2a antibody did not show CDC activity at the same concentration.

3-2. Measurement for ADCC activity by the anti-EREG monoclonal antibodies

As in the case of CDC activity measurement, the DLD1 human colon cancer cell line was used for ADCC activity measurement. Specifically, the cells were cultured in a 96-well flat-bottomed plate, and then incubated with Chromium-51. Each well was washed with RPMI1640 medium (Invitrogen) containing 10% FBS, and then 100 μL of the medium was added. Then, 50 μL each of an anti-EREG monoclonal antibody (B2#30, B3#18, and B3#41) diluted into the medium was added to the wells of the plate. The antibody was added at a final concentration of 1 μg/mL. Then, 50 μL of an effector cell solution (1 x 10^7 cells/mL) was added to each well, then the plate was left to stand in a 5% carbon dioxide incubator for four hours at 37°C, and then the specific chromium release rate was determined. Effector cells were obtained by culturing the spleen cells of a Balb/c mouse (Charles River Japan) in a medium containing 50 ng/mL of recombinant human interleukin-2 (Cat. No. 200-02, Peprotech) for five

days, or by culturing the bone marrow cells from the same mouse in a medium containing 50 ng/mL of recombinant human interleukin-2 and 10 ng/mL of recombinant mouse GM-CSF (Cat. No. 315-03, Peprotech) for six days.

The results showed that all of the anti-EREG monoclonal antibodies used in the experiment induced ADCC activity against DLD1 (Fig. 6).

[Example 4] Measurement for neutralizing activity of the anti-EREG monoclonal antibodies on cell proliferation enhanced by human EREG

4-1. Establishment of a Ba/F3 cell line that expresses EGFR-mG-CSF chimeric receptor (EGFR_BAF)

Using standard methods, the human EGF receptor comprising the sequence shown in SEQ ID NO: 35 (GenBank Acc. No. NM_005228) (hereinafter referred to as "EGFR") was isolated, and then a vector that can express a chimeric receptor of human EGF receptor fused with mG-CSFR was prepared. In brief, the vector (pCV_hEGFR/mG-CSFR) expresses a chimeric receptor (hEGFR/mG-CSFR) comprising the extracellular domain (Met7 - Ser645) of human EGFR and the intracellular domain of mouse G-CSFR (NM_007782). The nucleotide sequence and amino acid sequence of the hEGFR/mG-CSFR chimeric receptor are shown in SEQ ID NOs: 39 and 40, respectively.

Fifteen μg of the linearized chimeric receptor (hEGFR/mG-CSFR) expression vector (pCV_hEGFR/mG-CSFR) obtained by digestion with PvuI was transfected into Ba/F3 cells by electroporation (Gene Pulser; BioRad) at a setting of 0.33 kV and 950 μFD. Transfected cells were selected in RPMI1640 medium containing 10% FBS (GIBCO), 200 μg/mL Geneticin, and recombinant human EREG (R&D Systems, Cat: 1195-EP/CF, 200 ng/mL) and EGFR_BAF cell line was isolated.

4-2. Evaluation of EREG-dependency of EGFR_BAF

An experiment was carried out to measure the human EREG concentration-dependent cell proliferation of EGFR_BAF isolated by the method described in 4-1. To remove human EREG from the culture, the cells were centrifuged and resuspended in RPMI1640 + 10% FBS medium, and then the cells were seeded into each well of a 96-well plate at a density of 2×10^4 cells/100 μL. Human EREG (R&D Systems) diluted into the medium was added to the cells at various concentrations (7.8 to 250 ng/mL), and then the cells were incubated under 5% CO_2 at 37°C. After incubation for three days, the Cell Count Reagent SF reagent (Nacalai Tesque) was added and coloring was developed for two hours according to the instructions, and the absorbance (450/655 nm) of the reaction solution was measured using Benchmark Plus (Bio-Rad).

Similarly, the human EREG-dependent proliferation of the AsPC1 human pancreatic cancer cell line and DLD1 human colon cancer cell line was examined in the same manner as described above. CHO-S-SFMII (GIBCO) was used as the medium.

The result showed that EGFR_BAF (Fig. 7) and AsPC1 (Fig. 8) proliferated in a human EREG concentration-dependent manner. However, DLD1 was found not to have human EREG dependency (Fig. 9).

4-3. Measurement for neutralizing activity of the anti-EREG monoclonal antibodies on human EREG-dependent cell proliferation

On the EGFR_BAF proliferation dependent on human EREG, neutralizing activity of the anti-EREG monoclonal antibodies was examined. EGFR_BAF was suspended in RPMI1640 + 10% FBS medium containing human EREG (final concentration of 125 ng/mL), and the cells were seeded into a 96-well plate at a density of 2×10^4 cells per well. An anti-EREG monoclonal antibody diluted into the medium was added to the cells at various concentrations (0.008 to 25 μg/mL), and then the cells were cultured in a 5% CO_2 incubator for three days at 37°C. After culturing, the Cell Count Reagent SF reagent (Nacalai Tesque) was added and coloring was developed for two hours. Then, the absorbance of the reaction solution at 450 nm was measured using Benchmark Plus (Bio-Rad) by setting the reference wavelength at 655 nm.

The cell proliferation rate was calculated by using the measured value at 0 μg/mL antibody concentration as the reference (OD value at each antibody concentration/OD value of the reference x 100 (%)). Anti-human EREG goat polyclonal antibody known to have inhibitory activity (R&D Systems, Cat: AF1195) was used as a positive control. The attached document of this antibody product indicates its neutralizing activity against the human EREG-dependent proliferation of the Balb/3T3 fibroblast cell line. However, the attached document does not describe any information regarding the proliferation-suppressing effect in cancer cell lines.

Furthermore, neutralizing activity of the anti-EREG monoclonal antibodies in AsPC1 as the target cell was measured by the same method as described above, and the measurement was carried out under the following three antibody concentration conditions: 25, 2.5, and 0.25 μg/mL.

The results are shown in Figures 10 and 11 respectively.

In both of the EGFR_BAF and AsPC1, B2#30 and B3#18 showed neutralizing activity, and B3#41 showed a very weak neutralizing activity. Furthermore, the neutralizing activity of the known anti-hEREG goat polyclonal antibody was found to be very weak, as compared to B2#30 and B3#18.

Accordingly, B2#30 and B3#18 were found to have neutralizing activity for EREG, and to suppress proliferation of the AsPC1 pancreatic cancer cell line.

The present study demonstrates that the anti-EREG monoclonal antibodies (B2#30 and B3#18) isolated in this study have cell-killing effect mediated by ADCC activity and CDC activity, as well as suppressing effect on human EREG-dependent cell proliferation (neutralizing activity), and that the antibodies can be used as antibody pharmaceuticals with a novel mechanism against cancer cells that have enhanced expression of the human EREG gene.

[Example 5] Nucleotide sequence determination for the anti-EREG monoclonal antibody variable regions

Nucleotide sequences of the antibody variable regions were determined for hybridoma antibody B3#18 (EP27) which showed neutralizing activity for EREG, as well as ADCC activity and CDC activity against the DLD1 cell line. The antibody variable-region sequences were amplified by the RT-PCR from total RNA of the B3#18 anti-EREG antibody-producing hybridomas. Total RNA was extracted from 1 x 10^7 hybridoma cells by the RNeasy Plant Mini Kit (QIAGEN).

A RACE library was constructed from 1 μg of total RNA using the SMART RACE cDNA Amplification Kit (CLONTECH). The reverse transcription reaction was performed for one hour and thirty minutes at 42°C. The 5'-end cDNA sequence was amplified with synthetic oligonucleotide MHC-IgG2b (SEQ ID NO: 41) which is complementary to the mouse IgG2b constant region sequence, or synthetic oligonucleotide kappa (SEQ ID NO: 42) which is complementary to the mouse κ chain constant region nucleotide sequence. The components of the PCR solution (in 50 μL) are as follows:

5 μL of 10x Advantage 2 PCR Buffer;

5 μL of 10x Universal Primer A Mix;

0.2 mM dNTPs (dATP, dGTP, dCTP, and dTTP);

1 μL of Advantage 2 Polymerase Mix (the above were manufactured by CLONTECH);

2.5 μL of reverse transcription reaction product; and

10 pmole of synthetic oligonucleotide MHC-IgG2b or kappa.

The PCR conditions are as follow.

An initial temperature of 94°C for 30 seconds, followed by five cycles of 94°C for five seconds and 72°C for three minutes;

Five cycles of 94°C for five seconds, 70°C for ten seconds, and 72°C for three minutes; and

25 cycles of 94°C for five seconds, 68°C for ten seconds, and 72°C for three minutes.

Finally, the reaction product was elongated at 72°C for seven minutes. Each PCR

product separated by an agarose gel was purified using the QIAquick Gel Extraction Kit (manufactured by QIAGEN), and then cloned into the pGEM-T Easy vector (manufactured by Promega). The nucleotide sequence of the clone was determined. For B3#18, the nucleotide sequence and amino acid sequence of the H-chain variable region are shown in SEQ ID NO: 43 and SEQ ID NO: 44, respectively, and the nucleotide sequence and amino acid sequence of the L-chain variable region are shown in SEQ ID NO: 45 and SEQ ID NO: 46, respectively.

The variable region sequences of C7 (EP03), #15 (EP08), B2#30 (EP20), B3#8 (EP24), and B3#41 (EP29) were determined by same methods. Synthetic oligonucleotide MHC-IgG1 (SEQ ID NO: 47, 5'-ccatggagttagtttgggcagcagatcc-3') which is complementary to the mouse IgG1 constant region sequence was used to amplify the heavy-chain variable regions. The determined variable-region nucleotide sequences are shown in SEQ ID NOs: 108 (EP03, heavy chain), 110 (EP03, light chain), 112 (EP08, heavy chain), 114 (EP08, light chain), 116 (EP20, heavy chain), 118 (EP20, light chain), 120 (EP24, heavy chain), 122 (EP24, light chain), 124 (EP29, heavy chain), and 126 (EP29, light chain). The translated sequences of the variable regions are shown in SEQ ID NOs: 109 (EP03, heavy chain), 111 (EP03, light chain), 113 (EP08, heavy chain), 115 (EP08, light chain), 117 (EP20, heavy chain), 119 (EP20, light chain), 121 (EP24, heavy chain), 123 (EP24, light chain), 125 (EP29, heavy chain), and 127 (EP29, light chain). The ID numbers for the variable-region sequences, and the CDR1, CDR2, and CDR3 amino acid sequences of each antibody are summarized in Table 4.

[Table 4]

The anti-EREG monoclonal antibody CDR sequences and their sequence ID numbers

		CDR1	CDR2	CDR3	Variable region nucleotide sequence ID number	Variable region translated sequence ID number
EP03	Heavy chain	NYWMN (SEQ ID NO:49)	RIHPSDSETHYNQKFKN (SEQ ID NO:51)	LYYYTMDY (SEQ ID NO:53)	(SEQ ID NO:108)	(SEQ ID NO:109)
	Light chain	KASQDINKNIA (SEQ ID NO:55)	YTSTLQP (SEQ ID NO:57)	LQYDNLPYT (SEQ ID NO:59)	(SEQ ID NO:110)	(SEQ ID NO:111)
EP08	Heavy chain	SYWMH (SEQ ID NO:61)	EINPYNGGTNYNEKFKS (SEQ ID NO:63)	WKLGTY (SEQ ID NO:65)	(SEQ ID NO:112)	(SEQ ID NO:113)
	Light chain	RASQEISGYLS (SEQ ID NO:67)	AASTLDS (SEQ ID NO:69)	LQYASYPRT (SEQ ID NO:71)	(SEQ ID NO:114)	(SEQ ID NO:115)
EP20	Heavy chain	VYSLH (SEQ ID NO:73)	VISTYYGDAIYNQKFKG (SEQ ID NO:75)	EGNGNPLDY (SEQ ID NO:77)	(SEQ ID NO:116)	(SEQ ID NO:117)
	Light chain	TASSSVSSSYLH (SEQ ID NO:79)	STSNLAS (SEQ ID NO:81)	HQYHRSPLT (SEQ ID NO:83)	(SEQ ID NO:118)	(SEQ ID NO:119)
EP24	Heavy chain	SYGVH (SEQ ID NO:85)	VIWRDGSTTYNSALKS (SEQ ID NO:87)	NWNGLMDY (SEQ ID NO:89)	(SEQ ID NO:120)	(SEQ ID NO:121)
	Light chain	RASQDISYYLN (SEQ ID NO:91)	YTSRLHS (SEQ ID NO:93)	QQGHTVPWT (SEQ ID NO:95)	(SEQ ID NO:122)	(SEQ ID NO:123)
EP27	Heavy chain	DTYIQ (SEQ ID NO:2)	RIDPLNGNTKYVPKFQG (SEQ ID NO:4)	SGTLFDF (SEQ ID NO:6)	(SEQ ID NO:43)	(SEQ ID NO:44)
	Light chain	KASQDIHKYIA (SEQ ID NO:12)	YTSTLQP (SEQ ID NO:14)	LQYDNLRT (SEQ ID NO:16)	(SEQ ID NO:45)	(SEQ ID NO:46)
EP29	Heavy chain	SYWMH (SEQ ID NO:97)	AIYPGNSDSYNQKFKG (SEQ ID NO:99)	VMAY (SEQ ID NO:101)	(SEQ ID NO:124)	(SEQ ID NO:125)
	Light chain	RASQDIGNSLN (SEQ ID NO:103)	ATSNLDS (SEQ ID NO:105)	LQYASSPWT (SEQ ID NO:107)	(SEQ ID NO:126)	(SEQ ID NO:127)

[Example 6] Confirmation of the EREG-binding activity of the recombinant chimeric antibodies

It was confirmed that the recombinant chimeric antibodies comprising the isolated antibody variable region and the human IgG1/IgK constant region sequences binds to EREG as follows. The sequence of each heavy-chain variable region was amplified by PCR, and then inserted into the heavy-chain cloning site in a human chimeric antibody expression vector. Then, a light-chain variable region sequence was amplified by PCR, and then inserted into the light-chain cloning site in the above-mentioned human chimeric antibody expression vector into which the heavy chain sequence has been introduced. In the mammalian cell expression vectors constructed, both the antibody heavy chain and light chain genes are designed to be transcribed under the control of mouse CMV promoters. The sequence ID numbers of the nucleotide sequences and translated sequences for each human chimeric antibody are summarized in Table 5.

[Table 5]

Sequence ID numbers of the human chimeric antibodies

		Nucleotide sequence ID number	Amino acid sequence ID number
Chimeric EP03	Heavy chain	SEQ ID NO:128	SEQ ID NO:129
	Light chain	SEQ ID NO:130	SEQ ID NO:131
Chimeric EP08	Heavy chain	SEQ ID NO:132	SEQ ID NO:133
	Light chain	SEQ ID NO:134	SEQ ID NO:135
Chimeric EP20	Heavy chain	SEQ ID NO:136	SEQ ID NO:137
	Light chain	SEQ ID NO:138	SEQ ID NO:139
Chimeric EP24	Heavy chain	SEQ ID NO:140	SEQ ID NO:141
	Light chain	SEQ ID NO:142	SEQ ID NO:143
Chimeric EP27	Heavy chain	SEQ ID NO:9	SEQ ID NO:10
	Light chain	SEQ ID NO:19	SEQ ID NO:20
Chimeric EP29	Heavy chain	SEQ ID NO:144	SEQ ID NO:145
	Light chain	SEQ ID NO:146	SEQ ID NO:147

DG44 cells were transformed by the electroporation method with a human chimeric antibody expression vector. Recombinant cell clones were selected on geneticin resistance, which is conferred by a selection marker in the human chimeric antibody expression vector. The antibody in the culture supernatant of the recombinant clones was quantified by sandwich ELISA using anti-human antibodies, and recombinant antibody-expressing cells were selected. Human chimeric antibodies were purified from the culture supernatant of the selected recombinant cells using a HiTrap Protein A column (Amersham Bioscience) according to the attached manual.

Nunc-Immuno plates, which was coated with sEREG-mFc and then blocked with a solution containing BSA, was used to analyze the binding of the purified chimeric antibodies to EREG. After the chimeric antibodies were added to bind, the plates were incubated for one hour, and then washed. Subsequently, an alkaline phosphatase-labeled anti-human IgG antibody (BIOSOURCE, AHI0305) was added and allowed to react. After washing, the bound chimeric antibody was quantified by adding a chromogenic reagent, Sigma 104. As shown in Fig. 12, the chimeric antibodies bound to EREG in a dose-dependent manner. From the above-mentioned results, the isolated antibody variable region sequences were confirmed to be truly those of the anti-EREG antibodies.

[Example 7] *In vitro* ADCC activity by chimeric anti-EREG antibodies

Fig. 13 shows *in vitro* ADCC activity by anti-EREG chimeric antibodies and human PBMC. Using a syringe injected with 200 μL of 1000 units/mL heparin solution (Novo Heparin for injection, 5000 units, Novo Nordisk), 50 mL of peripheral blood was collected from each healthy individual. This peripheral blood was diluted two-fold with PBS(-) and divided into four aliquots. For lymphocyte separation, they were added to Leucosep tubes (Cat. No. 227290, Greiner Bio-One) into which 15 mL of Ficoll-Paque PLUS (GE Healthcare) had been added in advance, and subjected to centrifugation. After centrifugation at 2150 rpm, room temperature for ten minutes, the monocyte fraction was collected. The cells were washed once with Dulbecco's Modified Eagle's Medium (manufactured by SIGMA) containing 10% FBS (hereinafter referred to as 10% FBS/D-MEM), and then the cell density was adjusted to 5 x 10^6/mL in 10% FBS/D-MEM to prepare a human PBMC solution.

DLD-1 and MIA-PaCa2 cells were Chromium-51-labeled by a method similar to that of Example 3. An antibody solution adjusted to each concentration was added to the target cells, and allowed to bind at room temperature for 15 minutes. Then, the human PBMC solution (5 x 10^5 cells/well) was added, and the cells were incubated in a 5% carbon dioxide incubator for approximately four hours. The radioactivity in the supernatant after incubation was measured using a gamma counter, and the chromium release rate was calculated by the method described in Example 3. ADCC activity was induced by all of the chimeric antibodies, and the EP20, EP27, and EP08 chimeric antibodies had particularly strong activity. The strengthness of ADCC induction by each of the antibodies showed a similar tendency in the MIA-PaCa2 pancreatic cancer cell line and the DLD-1 colon cancer cell line.

[Example 8] Binding to EREG orthologues and epitope analysis of anti-EREG antibodies

The binding of isolated antibodies to mouse EREG and simian EREG was analyzed. Full-length mouse EREG cDNA (NM_007950) and simian EREG cDNA (XM_001102069) were

amplified by PCR from cDNA libraries, and cloned. The isolated sequences are shown in SEQ ID NOs: 148 and 149, respectively. Soluble mouse EREG/mouse IgG2a Fc fusion protein (hereinafter, mouse EREG-mFc) and soluble simian EREG/mouse IgG2a Fc fusion protein (hereinafter, simian EREG-mFc) were prepared by the method described in Example 2. The mouse EREG-mFc and simian EREG-mFc sequences are shown in SEQ ID NOs: 150 and 151, respectively. Nunc-Immuno plates were coated with human EREG-mFc, mouse EREG-mFc, and simian EREG-mFc respectively, and then blocked with a solution containing BSA. Subsequently, the binding of the purified chimeric antibodies was analyzed. After addition of the chimeric antibodies, the plates were incubated for one hour and then washed. Subsequently, an alkaline phosphatase-labeled anti-human IgG antibody (BIOSOURCE, AHI0305) was added and allowed to react. After washing, the bound chimeric antibody was quantified by adding a chromogenic reagent, Sigma 104 (Fig. 14). Differences at the degree of saturation in antibody binding to EREG at the same antibody concentration were demonstrated by plotting the reactivity towards various types of EREG at the same antibody concentration as "human EREG-mFc" on the horizontal axis against "mouse EREG-mFc" on the vertical axis, or "human EREG-mFc" on the horizontal axis against "simian EREG-mFc" on the vertical axis. The plot along the horizontal axis indicates the binding selectivity towards human EREG, and this shows that EP08 binds to human EREG, but it does not bind to mouse EREG. The parabolic plot for EP27 indicates that EP27 binds to the mouse molecule, but a higher concentration of the antibody is required for the binding saturation. In other words, EP27 has a lower binding affinity for mouse EREG than for human EREG. An antibody that produces a diagonal plot means that the antibody has an equivalent affinity for EREG of non-human species as for human EREG. The result obtained by above analysis for hybridoma-producing mouse antibodies is summarized in Table 3. The result is represented by four levels of reactivity relative to the human molecule: no reactivity (-); very low reactivity (+); slightly lower reactivity (++); and equivalent reactivity (+++).

The binding towards partial fragments of the extracellular domain of human EREG was analyzed to determine antibody epitope. The N-terminal partial sequence fragment of ^{29}Ala to ^{69}Ser of human EREG of SEQ ID NO: 21 was expressed as a GST fusion protein and purified to homogeneity. The sequence of the obtained recombinant protein is shown in SEQ ID NO: 152. A soluble human EREG fragment (EGF domain; corresponding to ^{63}Val to ^{108}Leu of SEQ ID NO: 21) comprising a mature structure was purchased from R&D Systems (cat. no. 1195-EP/CF). Fragments of the EREG protein were immobilized onto an immuno plate, and the binding of the antibodies was examined by ELISA. The result is summarized in Table 3. Most of the antibodies bound to the soluble EREG fragment that comprises the EGF domain (indicated as "ereg" in Table 3). EP25 and EP32 bound to the N-terminal fragment ("Nterm"). All of the

isolated antibodies bound to either one of the peptide fragments, and none of the antibodies recognized the sequence beyond residue 109.

[Example 9] Activation of the EGF receptor signaling by EREG-expressing cells

Soluble EREG has been reported as a low-affinity ligand for EGF receptors (reference documents: Shelly *et al.* (1998) J. Biol. Chem. 273, 10496-505; Jones *et al.* (1999) FEBS Lett 447, 227-31). By comparison of the effects on EGFR_BaF, it was confirmed that EREG has a low activity (affinity) towards the EGF receptor, which is one-hundredth of the affinity or less of EGF (Fig. 15). EREG is not an abundant protein in the blood under physiological conditions. While increased EREG gene expression has been reported during malignant transformation, no reports show that the EREG protein was detected at a high concentration in the blood of cancer patients. The present inventors hypothesized that membrane-type EREG expressed on the cell membrane, rather than soluble EREG, stimulates a neighboring EGF receptor, and examined the hypothesis that a local activation mechanism may be present as follows. They examined whether proliferation of EGFR_BaF is not only stimulated by soluble EREG but by co-culturing EGFR_BaF with cells forcedly expressing EREG. EREG-expressing EREG_DG cells and DG44 cells (negative control) were seeded into a 96-well plate at 5×10^3 cells/well, and incubated for overnight with a medium containing FCS. On the following day, 1×10^4 EGFR_BaF cells or Ba/F3 cells (negative control) were added, and then the cells were incubated for three days. As shown in Fig. 16, the proliferation of EGFR_BaF was remarkably enhanced by co-culturing with EREG_DG cells which have forced expression of EREG. However proliferation of EGFR_BaF was not stimulated at all by co-culturing with DG44 cells which do not express EREG. The stimulated proliferation by EREG_DG was cancelled by addition of the EP20 anti-EREG antibody (10 μg/mL). The EGFR_BaF cell proliferation was quantified by adding the WST-8 reagent (Fig. 17). It was shown that proliferation of Ba/F3 cells was not stimulated by co-culturing with EREG_DG cells, and that the cell proliferation promoted by co-culturing of EGFR_BaF with EREG_DG could be suppressed completely by addition of the anti-EREG antibody.

GeneChip analysis showed that in addition to EREG, amphiregulin (a member of the EGF ligand family) mRNA expression was also enhanced in the DLD-1 colon cancer cells. In EGF receptor signaling, there is a redundancy among the ligand family molecules that activate the receptor, and thus theoretically, inhibition of one of the ligands is not equivalent to the receptor signaling inhibition. It was shown that by coculturing with DLD-1, proliferation of EGFR_BaF was remarkably enhanced as com pared to that of Ba/F3, and that this enhanced proliferation was cancelled by addition of the EP20 or EP27 anti-EREG neutralizing antibody (Fig. 18).

The soluble EREG protein in the culture supernatants of EREG_DG and DLD-1 cells was quantified by sandwich ELISA. An immuno plate was coated with mouse EP30 and subjected to blocking. Then, soluble recombinant human EREG (R&D Systems) used as a standard to calculate concentration and cell culture supernatant after three days of culturing was added for examination. After incubation, EREG protein was detected by addition of the chimerized EP20 antibody and an alkaline phosphatase-labeled anti-human IgG antibody in order. The detection of soluble EREG proteins using a standard is shown in Fig. 19. The concentration of EREG in the culture supernatant was lower than the detection limit (1 ng/mL). The A405/655 detection values are 0.157 for EREG_DG and 0.184 for DLD-1. The above-mentioned result shows that the EREG-mediated activation of EGF receptor signaling occurs between neighboring cells, and that membrane-type EREG is highly likely to be involved in the activation.

The result that the EREG-mediated EGF receptor activation occurs efficiently through intercellular interaction was discovered for the first time by the present invention. The finding that this activation can be suppressed by an anti-EREG antibody was also discovered for the first time.

[Example 10] Detection of EREG protein in colon tumors by immunohistological staining

Expression of the EREG protein in tumors and localization of the protein on the cancer cell membrane were evidenced by immunohistological staining. Immunohistological staining was performed using a tissue array prepared from clinical cancer tissues, and tissue specimens were prepared as follows. Tissues removed by surgery were fixed overnight in 4% paraformaldehyde, then washed for two hours with 0.01 M phosphate buffer solution, and then embedded in paraffin by the AMeX method (reference document: Sato *et al.* (1986) Am. J. Pathol. 125, 431-5). A 1.5-mm-diameter cancer tissue was excised from this block, transferred to a new block of approximately 2.5 x 3.0 cm, and different tissues were placed in an array to prepare a tissue array. The tissue array was sliced and deparaffinized, and the sections were stained immunohistochemically using the Ventana HX Discovery System (Ventana Medical Systems, Inc.). Fifty μg/mL of an anti-EREG mouse monoclonal antibody (EP27) was added to the sliced tissues and then allowed to react. A peroxidase-labeled anti-mouse IgG antibody was used as the secondary antibody, and then color development reaction was performed using DAB. Expression of the EREG protein and its localization to the cell membrane were detected in the primary colon cancer (Fig. 20). Meanwhile, the EREG protein was not detected at all in non-cancerous tissues under the same staining conditions. Detection of the EREG protein and localization of the protein on the membrane in cancer tissues were shown for the first time in the present invention.

[Example 11] Classification of anti-EREG monoclonal antibodies according to epitope recognition in sandwich ELISA

In Example 8, it was found that antibody recognition epitopes were mainly located in the mature EGF domain of EREG. More specifically, antibody epitopes were classified by identifying combinations of antibodies which could noncompetitively bind to the same antigen molecule in a sandwich ELISA. A mouse anti-EREG antibody was diluted to 1 μg/mL, and was immobilized onto Nunc-Immuno plates. After blocking, 50 μg/mL of a soluble human EREG fragment comprising a mature EGF domain (^{63}Val to ^{108}Leu of SEQ ID NO: 21) or 100 μg/mL of human EREG-mFc (SEQ ID NO: 34) was added at 100 μL/well, and therefore the antigen molecule was captured by the mouse antibody. After washing, an anti-EREG chimeric antibody was added (antibody concentration: 1, 0.33, 0.11, or 0.037 μg/mL), and the binding of the chimeric antibody to the captured antigen was quantified by addition of an alkaline phosphatase-labeled anti-human IgG antibody and a Sigma 104 chromogenic reagent. The obtained data (Fig. 21-1 to Fig. 21-3) were subjected to clustering analysis using the R statistical analysis software (URL http://www.R-project.org.), and the dendrogram is shown in Fig. 22. In Fig. 22, the farther the distance to the branching point on the horizontal axis is, the more different the reaction patterns in the sandwich ELISA system are, and the more different the modes of antigen recognition are. EP20 can bind to EREG simultaneously with many antibodies including EP27, and it turns out that the EP20 epitope is characteristic. Antibodies belonging to the cluster from EP29 to EP25 in Fig. 22 hardly allow simultaneous EREG-binding with other antibodies, and thus the antigen molecule is speculated to be deeply captured by these antibodies.

Industrial Applicability

The EREG protein-specific antibodies of the present invention can be used as diagnostic agents for colon cancer, lung adenocarcinoma, pancreatic cancer, stomach cancer, kidney cancer, or such. The diagnostic agents of the present invention are useful for diagnosing primary or metastatic cancers. More specifically, cancers can be detected by detecting the EREG protein or EREG-encoding mRNA contained in a biological sample collected from a patient. Alternatively, the presence of primary colon cancer, metastatic colon cancer, lung adenocarcinoma, pancreatic cancer, stomach cancer, or kidney cancer can be detected *in vivo* by detecting the localization of EREG-expressing cells *in vivo*.

Furthermore, anti-EREG antibodies having cytotoxic activity in the present invention are useful for treating or preventing cancers that express an EREG protein. More specifically, cytotoxic agents or cell proliferation inhibitors for various types of cancer cells such as cells of

colon cancer, lung adenocarcinoma, pancreatic cancer, stomach cancer, or kidney cancer, are provided by the present invention. Cytotoxic agents or cell proliferation inhibitors for cancer cells in the present invention may be applied to both primary and metastatic cancers.

Furthermore, according to the present invention, anti-EREG antibodies having cytotoxic activity can be used as therapeutic agents against various types of cancers such as colon cancer, lung adenocarcinoma, pancreatic cancer, stomach cancer, or kidney cancer. In the present invention, the anti-EREG antibodies are useful as therapeutic agents for both primary and metastatic cancers.

Additionally, genes encoding the antibodies of the present invention and recombinant cells transformed with these genes can be used to prepare recombinant antibodies that exhibit the above-mentioned effects or more preferable effects.

Furthermore, based on the findings of the present invention, candidate compounds can be screened to find useful cancer therapeutic agents. The present invention showed EREG-dependent proliferation of cancer. Therefore, one may consider compounds selected by the screening methods of the present invention as compounds that suppress EREG-dependent proliferation of cancer. Compounds selected by the present invention are useful as candidate compounds for evaluating the utility of anticancer agents by assessment of safety and cancer specificity.

CLAIMS

1. Use of an antibody that binds to an EREG protein, and which has cell proliferation inhibitory activity against cells expressing the EREG protein for the preparation of a composition for the treatment of cancer.

2. The use of claim 1, wherein the cell proliferation inhibitory activity is cytotoxic activity.

3. The use of claim 2, wherein the cytotoxic activity is ADCC activity.

4. The use of claim 2, wherein the cytotoxic activity is CDC activity.

5. The use of any one of claims 1 to 4, which additionally has neutralizing activity against the EREG protein.

6. The use of claim 1, wherein the cell proliferation inhibitory activity is neutralizing activity.

7. Use of a minibody produced from the antibody set forth in any one of claims 1 to 6 for the preparation of a composition for the treatment of cancer.

8. The use of any one of claims 1 to 7, wherein a chemotherapeutic agent or a toxic peptide is bound to the antibody set forth in any one of claims 1 to 6, or the minibody set forth in claim 7.

9. The use of any one of claims 1 to 8, wherein the antibody is an antibody of any of (1) to (32) below:
(1) an antibody comprising (i) an H chain having the amino acid sequence of SEQ ID NO: 2 as CDR1, the amino acid sequence of SEQ ID NO: 4 as CDR2, and the amino acid sequence of SEQ ID NO: 6 as CDR3, and (ii) an L chain having the amino acid sequence of SEQ ID NO: 12 as CDR1, the amino acid sequence of SEQ ID NO: 14 as CDR2, and the amino acid sequence of SEQ ID NO: 16 as CDR3;
(2) an antibody comprising the H chain of (1), wherein the H chain has the amino acid sequence of positions 117 to 452 in the amino acid sequence of SEQ ID NO: 8 as CH;
(3) an antibody comprising the H chain of (1), wherein the H chain has the amino acid sequence of positions 117 to 446 in the amino acid sequence of SEQ ID NO: 10 as CH;
(4) an antibody comprising the L chain of (1) or (2), wherein the L chain has the amino acid sequence of positions 107 to 213 in the amino acid sequence of SEQ ID NO: 18 as CL;
(5) an antibody comprising the L chain of (1) or (3), wherein the L chain has the amino acid sequence of positions 107 to 213 in the amino acid sequence of SEQ ID NO: 20 as CL;

(6) an antibody comprising (i) an H chain having the amino acid sequence of SEQ ID NO: 49 as CDR1, the amino acid sequence of SEQ ID NO: 51 as CDR2, and the amino acid sequence of SEQ ID NO: 53 as CDR3, and (ii) an L chain having the amino acid sequence of SEQ ID NO: 55 as CDR1, the amino acid sequence of SEQ ID NO: 57 as CDR2, and the amino acid sequence of SEQ ID NO: 59 as CDR3;

(7) an antibody comprising the H chain of (6), wherein the H chain has CH derived from mouse IgG1;

(8) an antibody comprising the H chain of (6), wherein the H chain has the amino acid sequence of positions 117 to 446 in the amino acid sequence of SEQ ID NO: 10 as CH;

(9) an antibody comprising the L chain of (6) or (7), wherein the L chain has CL derived from the mouse κ chain;

(10) an antibody comprising the L chain of (6) or (8), wherein the L chain has the amino acid sequence of positions 107 to 213 in the amino acid sequence of SEQ ID NO: 20 as CL;

(11) an antibody comprising (i) an H chain having the amino acid sequence of SEQ ID NO: 61 as CDR1, the amino acid sequence of SEQ ID NO: 63 as CDR2, and the amino acid sequence of SEQ ID NO: 65 as CDR3, and (ii) an L chain having the amino acid sequence of SEQ ID NO: 67 as CDR1, the amino acid sequence of SEQ ID NO: 69 as CDR2, and the amino acid sequence of SEQ ID NO: 71 as CDR3;

(12) an antibody comprising the H chain of (11), wherein the H chain has CH derived from mouse IgG1;

(13) an antibody comprising the H chain of (11), wherein the H chain has the amino acid sequence of positions 117 to 446 in the amino acid sequence of SEQ ID NO: 10 as CH;

(14) an antibody comprising the L chain of (11) or (12), wherein the L chain has CL derived from the mouse κ chain;

(15) an antibody comprising the L chain of (11) or (13), wherein the L chain has the amino acid sequence of positions 107 to 213 in the amino acid sequence of SEQ ID NO: 20 as CL;

(16) an antibody comprising (i) an H chain having the amino acid sequence of SEQ ID NO: 73 as

CDR1, the amino acid sequence of SEQ ID NO: 75 as CDR2, and the amino acid sequence of SEQ ID NO: 77 as CDR3, and (ii) an L chain having the amino acid sequence of SEQ ID NO: 79 as CDR1, the amino acid sequence of SEQ ID NO: 81 as CDR2, and the amino acid sequence of SEQ ID NO: 83 as CDR3;

(17) an antibody comprising the H chain of (16), wherein the H chain has CH derived from mouse IgG1;

(18) an antibody comprising the H chain of (16), wherein the H chain has the amino acid sequence of positions 117 to 446 in the amino acid sequence of SEQ ID NO: 10 as CH;

(19) an antibody comprising the L chain of (16) or (17), wherein the L chain has CL derived from the mouse κ chain;

(20) an antibody comprising the L chain of (16) or (18), wherein the L chain has the amino acid sequence of positions 107 to 213 in the amino acid sequence of SEQ ID NO: 20 as CL;

(21) an antibody comprising (i) an H chain having the amino acid sequence of SEQ ID NO: 85 as CDR1, the amino acid sequence of SEQ ID NO: 87 as CDR2, and the amino acid sequence of SEQ ID NO: 89 as CDR3, and (ii) an L chain having the amino acid sequence of SEQ ID NO: 91 as CDR1, the amino acid sequence of SEQ ID NO: 93 as CDR2, and the amino acid sequence of SEQ ID NO: 95 as CDR3;

(22) an antibody comprising the H chain of (21), wherein the H chain has CH derived from mouse IgG1;

(23) an antibody comprising the H chain of (21), wherein the H chain has the amino acid sequence of positions 117 to 446 in the amino acid sequence of SEQ ID NO: 10 as CH;

(24) an antibody comprising the L chain of (21) or (22), wherein the L chain has CL derived from the mouse κ chain;

(25) an antibody comprising the L chain of (21) or (23), wherein the L chain has the amino acid sequence of positions 107 to 213 in the amino acid sequence of SEQ ID NO: 20 as CL;

(26) an antibody comprising (i) an H chain having the amino acid sequence of SEQ ID NO: 97 as CDR1, the amino acid sequence of SEQ ID NO: 99 as CDR2, and the amino acid sequence of SEQ ID NO: 101 as CDR3, and (ii) an L chain having the amino acid sequence of SEQ ID NO: 103 as CDR1, the amino acid sequence of SEQ ID NO: 105 as CDR2, and the amino acid sequence of SEQ ID NO: 107 as CDR3;

(27) an antibody comprising the H chain of (26), wherein the H chain has CH derived from mouse IgG1;
(28) an antibody comprising the H chain of (26), wherein the H chain has the amino acid sequence of positions 117 to 446 in the amino acid sequence of SEQ ID NO: 10 as CH;
(29) an antibody comprising the L chain of (26) or (27), wherein the L chain has CL derived from the mouse κ chain;
(30) an antibody comprising the L chain of (26) or (28), wherein the L chain has the amino acid sequence of positions 107 to 213 in the amino acid sequence of SEQ ID NO: 20 as CL;
(31) an antibody comprising one or more amino acid substitutions, deletions, additions, and/or insertions in the antibody of any of (1) to (30), which has equivalent binding activity as the antibody of any of (1) to (30);
(32) an antibody that binds to the same EREG protein epitope as the antibody of any of (1) to (30).

10. The use of any one of claims 1 to 9, wherein the antibody or minibody recognizes Ala at position 29 to Ser at position 69, or Val at position 63 to Leu at position 108, in the amino acid sequence of SEQ ID NO: 21.

ABSTRACT

Methods that entail detection of an epiregulin (EREG) protein for cancer diagnosis are disclosed. In colon cancer, lung adenocarcinoma, pancreatic cancer, stomach cancer, and kidney cancer, the gene and protein expressions of EREG were frequently found to be elevated. Antibodies that recognize an EREG protein are used for diagnosing or treating cancer in the present invention. Pharmaceutical compositions, cell proliferation inhibitors, and anticancer agents containing an EREG-binding antibody as an active ingredient are also disclosed. Methods of inducing cell damage in EREG-expressing cells and methods of suppressing the proliferation of EREG-expressing cells by contacting the EREG-expressing cells with EREG-binding antibodies are also disclosed.