(1) contacting an Epiregulin-expressing cell with an antibody comprising the V region encoded by a cDNA isolated from a hybridoma;

(2) detecting the binding of the antibody to the Epiregulin-expressing cell; and

(3) selecting an antibody that binds to the Epiregulin-expressing cell.

Methods for detecting the binding of an antibody to Epiregulin-expressing cells are known. Specifically, the binding of an antibody to Epiregulin-expressing cells can be detected by the above-described techniques such as FACS. Immobilized samples of Epiregulin-expressing cells are appropriately used to assess the binding activity of an antibody.

Preferred antibody screening methods that use the binding activity as an indicator also include panning methods using phage vectors. Screening methods using phage vectors are advantageous when the antibody genes are isolated from heavy-chain and light-chain subclass libraries from a polyclonal antibody-expressing cell population. Genes encoding the heavy-chain and light-chain variable regions can be linked by an appropriate linker sequence to form a single-chain Fv (scFv). Phages presenting scFv on their surface can be produced by inserting a gene encoding scFv into a phage vector. The phages are contacted with an antigen of interest. Then, a DNA encoding scFv having the binding activity of interest can be isolated by collecting phages bound to the antigen. This process can be repeated as necessary to enrich scFv having the binding activity of interest.

After isolation of the cDNA encoding the V region of the anti-Epiregulin antibody of interest, the cDNA is digested with restriction enzymes that recognize the restriction sites introduced into both ends of the cDNA. Preferred restriction enzymes recognize and cleave a nucleotide sequence that occurs in the nucleotide sequence of the antibody gene at a low frequency. Furthermore, a restriction site for an enzyme that produces a sticky end is preferably introduced into a vector to insert a single-copy digested fragment in the correct orientation. The cDNA encoding the V region of the ~~anti-IL-6R~~ anti-Epiregulin antibody is digested as described above, and this is inserted into an appropriate expression vector to construct an antibody expression vector. In this case, if a gene encoding the antibody constant region (C region) and a gene encoding the above V region are fused in-frame, a chimeric antibody is obtained. Herein, "chimeric antibody" means that the origin of the constant region is different from that of the variable region. Thus, in addition to mouse/human heterochimeric antibodies, human/human allochimeric antibodies are included in the chimeric antibodies of the present invention. A chimeric antibody expression vector can be constructed by inserting the above V region gene into an expression vector that already has the constant region. Specifically, for example, a recognition sequence for a restriction enzyme that excises the above V region gene can be appropriately placed on the 5' side of an expression vector carrying a DNA encoding a desired antibody constant region (C region). A chimeric antibody expression vector is constructed by fusing in frame the two genes

value for monkey Epiregulin (cEREG KD) to the KD value for human Epiregulin (hEREG KD) (cEREG KD/hEREG KD) can be determined by dividing this KD value by the KD value for human Epiregulin of SEQ ID NO: 34.

In the present invention, cEREG KD/hEREG KD is preferably less than 40. Furthermore, in a non-limiting embodiment, cEREG KD/hEREG KD is preferably less than 10. In another non-limiting embodiment, cEREG KD/hEREG KD is preferably less than 6, and in a different non-limiting embodiment, cEREG KD/hEREG KD is preferably less than 4.

Non-limiting examples of position(s) where amino acid(s) is/are altered to confer cross-species reactivity for binding to non-human animal Epiregulin and human Epiregulin provided by the present invention preferably include amino acid(s) at position(s) 33, 51, 54, 55, 56, 57, 58, 59, 60, 62, 65, 96, 97, and/or 98 as indicated by Kabat numbering in the heavy chain sequence of the anti-Epiregulin antibody of SEQ ID NO: 38. Furthermore, non-limiting examples of position(s) where amino acid(s) is/are altered to confer cross-species reactivity for binding to non-human animal Epiregulin and human Epiregulin provided by the present invention preferably include amino acid(s) at position(s) 24, 93, and/or 94 as indicated by Kabat numbering in the light chain sequence of the anti-Epiregulin antibody of SEQ ID NO: 29. A preferred example of the amino acid alteration includes substitution of Arg (R) for one or more of the above-mentioned amino acids.

Examples of a non-limiting embodiment of the heavy chain of an anti-Epiregulin antibody of the present invention conferred with cross-species reactivity include heavy chains selected from the group consisting of SEQ ID NOs: 115-127, 131-135, 137-140, and 142-150. Examples of a non-limiting embodiment of the light chain of a humanized anti-Epiregulin antibody of the present invention ~~with modified isoelectric point~~ conferred with cross-species reactivity include light chains selected from the group consisting of SEQ ID NOs: 128-130, 136, and 141.

Examples of a non-limiting embodiment of an anti-Epiregulin antibody of the present invention conferred with cross reactivity include anti-Epiregulin antibodies comprising heavy chains selected from the group consisting of SEQ ID NOs: 98, 115-127, 131-135, 137-140, and 142-150, and light chains selected from the group consisting of SEQ ID NOs: 29, 128-130, 136, and 141.

Neutralizing activity

An anti-Epiregulin antibody of the present invention is 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

damaging agents, antimitotic agents, and/or antimetabolites. DNA damaging agents may be alkylating agents, topoisomerase inhibitors and/or DNA intercalators. Preferred non-limiting examples of the growth inhibitors may include carboplatin (DNA alkylating agent), etoposide (inhibitor of topoisomerase II), doxorubicin (DNA intercalator), docetaxel (antimitotic agent), and Gemzar (gemcitabine, antimetabolite).

At least one below may be selected as the alkylating agent. That is, at least one alkylating agent selected from among chlorambucil, cyclophosphamide, ifosfamide, mechlorethamine, melphalan, uracil mustard, thiotepa, busulfan, carmustine, lomustine, streptozocin, carboplatin, cisplatin, satraplatin, oxaliplatin, altretamine, ET-743, XL119 (becatecarin), dacarbazine, chlormethine, bendamustine, trofosfamide, uramustine, fotemustine, nimustine, prednimustine, ranimustine, semustine, nedaplatin, triplatin tetranitrate, mannosulfan, treosulfan, temozolomide, carboquone, triaziquone, triethylenemelamine, and procarbazin can be used as the alkylating agent.

At least one below may be selected as the topoisomerase inhibitor. That is, at least one topoisomerase inhibitor selected from among doxorubicin (Doxil), daunorubicin, epirubicin, idarubicin, anthracenedione (Novantrone), mitoxantrone, mitomycin C, bleomycin, dactinomycin, plicatomycin, ilinotecan (Camptosar), camptothecin, rubitecan, belotecan, etoposide, teniposide, and topotecan (Hycamptin) can be used as the topoisomerase inhibitor.

At least one ~~topoisomerase inhibitor~~DNA intercalator selected from proflavine, doxorubicin (adriamycin), daunorubicin, dactinomycin, and thalidomide may be used as the DNA intercalator.

At least one below may be selected as the antimitotic agent. At least one ~~topoisomerase inhibitor~~antimitotic agent selected from among paclitaxel (Abraxane)/Taxol, docetaxel (Taxotere), BMS-275183, Xyotax, Tocosal, vinorlebine, vincristine, vinblastine, vindesine, vinzolidine, etoposide (VP-16), teniposide (VM-26), ixabepilone, larotaxel, ortataxel, tesetaxel, and ispinesib can be used as the antimitotic agent.

At least one below may be selected as the antimetabolite. At least one ~~topoisomerase inhibitor~~antimetabolite selected from among fluorouracil (5-FU), floxuridine (5-FUdR), methotrexate, Xeloda, Arranon, leucovorin, hydroxyurea, thioguanine (6-TG), mercaptopurine (6-MP), cytarabine, pentostatin, fludarabine phosphate, cladribine (2-CDA), asparaginase, gemcitabine, pemetrexed, bortezomib, aminopterin, raltitrexed, clofarabine, enocitabine, sapacitabine, azacitidine, and such can be used as the antimetabolite.

When an anti-Epiregulin antibody-drug conjugate is incorporated into a cell, the conjugate-linked growth inhibitor or cytotoxic substance such as a toxic peptide can induce cell death of the cell that incorporated this antibody. Therefore, the antibody to which the growth inhibitor or the cytotoxic substance such as a toxic peptide is linked preferably also has internalization activity. In the present invention, "antibody having internalization activity" refers

4×10^4, 5×10^4, 6×10^4, 7×10^4, 8×10^4, 9×10^4, 1×10^5, 2×10^5, 3×10^5, 4×10^5, 5×10^5, 6×10^5, 7×10^5, 8×10^5, 9×10^5, 1×10^6, 2×10^6, 3×10^6, 4×10^6, 5×10^6, 6×10^6, 7×10^6, 8×10^6, 9×10^6, 1×10^7, 2×10^7, 3×10^7, 4×10^7, 5×10^7, 6×10^7, 7×10^7, 8×10^7, 9×10^7, 1×10^8, 2×10^8, 3×10^8, 4×10^8, 5×10^8, 6×10^8, 7×10^8, 8×10^8, and 9×10^8 can be shown as a preferred example of the numerical value (threshold value) that can be set as the standard for selecting ~~controls~~ subjects who will receive the administration. The numerical values mentioned above may be those calculated to one significant figure; and in this case, for example, the EREG expression level of 1×10^3 calculated to one significant figure can be an EREG expression level having a numerical value of any one of 0.5×10^3, 0.6×10^3, 0.7×10^3, 0.8×10^3, 0.9×10^3, 1.0×10^3, 1.1×10^3, 1.2×10^3, 1.3×10^3, 1.4×10^3 if calculated to two significant figures.

Tissue samples

The term "tissue sample" used in the present invention refers to any biological sample that can be obtained from an individual, body fluid (for example, blood, serum, plasma, and spinal fluid), tissue culture, tissue sections, or such. Samples from subjects can be used as preferred examples of biological samples. Preferred samples from subjects are tissues obtained from subjects, and more preferably colon tissues from subjects. Biopsy, a known method, is preferably used as a method for collecting colon tissues. Known means can be used appropriately as the means for biopsy. Such examples include obtaining tissues by biopsy such as aspiration biopsy, clutch biopsy, sponge biopsy, biopsy for cytodiagnosis, endoscopic biopsy, fine-needle aspiration biopsy, needle biopsy, transbronchial lung biopsy, or obtaining tissues during surgery.

In the present invention, since the tissue samples are observed with transmitted light under a microscope, they are sliced to such an extent that the light used on the microscope is sufficiently transmitted through the tissue samples. Prior to slicing, the tissue samples are fixed. Specifically, the tissue samples are solidified by dehydrating or denaturing proteins in the tissues/cells to rapidly kill the cells constituting the tissues. The resulting tissues have a stabilized and insolubilized structure. First, the tissue samples to be fixed are cut into fragments having a size and a shape suitable for preparing paraffin-embedded sections using a cutting knife such as a scalpel. Subsequently, the fragments are immersed into a fixing solution, a reagent used for carrying out fixation. The fixing solution used is preferably formalin, or more preferably neutral buffered formalin. The concentration of the neutral buffered formalin is appropriately selected according to the characteristics or physical properties of the tissue samples. The concentration can be appropriately changed and used between 1% and 50%, preferably between 5% and 25%, and more preferably between 10% and 15%. The fixing solution into which the tissue preparations have been immersed is appropriately deaerated using a vacuum

Antibody name	Aggregate (%)	Tm (℃)	hEREG KD ratio	cEREG KD ratio	cEREG (nM) / hEREG (nM)	Immunogenicity score
cH-G1/cL-k	18.9	<70	1	1	40.9	800.2
H87-G1d/LB-k	1.0	76.2	0.74	0.99	35.1	589.4
H205-G1d/L53-k	1.0	74.3	1.08	0.23	5.5	516.1
H206-G1d/L53-k	1.7	72.9	0.82	0.11	5.2	606.8
H231-G1d/L53-k	2.2	73.3	1.15	0.27	6.0	541.7
H240-G1d/L53-k	1.3	74.7	1.34	0.28	5.4	515.6
H205-G1d/L73-k	1.8	74.5	0.85	0.12	3.1	519.0
H206-G1d/L73-k	3.6	72.9	0.49	0.08	3.4	609.7
H231-G1d/L73-k	4.0	73.2	0.70	0.12	3.5	544.6
H240-G1d/L73-k	2.8	74.9	0.91	0.15	3.6	518.5

(KD ratio represents a relative ratio when the value for cH-G1/cL-k = 1.)

[Example 7] ADCC activity of each test antibody using human peripheral blood mononuclear cells as effector cells

Human peripheral blood mononuclear cells (hereinafter referred to as human PBMC) were used as effector cells to measure the ADCC activity of each test antibody as hereinbelow. A mononuclear cells fraction collected from human peripheral blood was used for the effector cell of human origin. As a result, all humanized EP27 antibodies used in the test were found to induce ADCC against MIA PaCa-2 cells (Reference Example 7) (Fig. 5).

[Example 8] Measurement of the activity of anti-Epiregulin monoclonal antibodies to neutralize cell growth stimulation by human or monkey Epiregulin

Humanized EP27 antibody sequences with enhanced affinity to monkey Epiregulin were revealed in Example 5. To assess their activities in cells, the humanized EP27 antibodies were measured for their activities to neutralize cell growth stimulation by monkey Epiregulin and human Epiregulin. BAF_EGFR was used for the cells. All of the humanized EP27 antibodies evaluated this time were examined for their neutralizing activities which inhibit human and monkey Epiregulin-dependent EGFR_BAF cell growth (Reference Example 8), and all of the humanized EP27 antibodies showed enhanced neutralizing activities against monkey Epiregulin than the chimeric antibody (Figs. 6 and 7).

[Example 9] Drug efficacy test for the humanized EP27 antibodies using an *in vivo* model

The *in vivo* antitumor activity of the humanized EP27 antibodies, of which ~~intracellular activity~~activity in *in vitro* cells had been confirmed in Examples 7 and 8, was evaluated using a human tumor-grafted mouse model (Reference Example 14). The anti-tumor effects of each test antibody evaluated in MIA PaCa-2 and DLD-1 human cancer cell-transplanted mouse models were evaluated by

measuring the tumor volume on day 7 after the last day of sample administration. As a result, as shown in Fig. 8, when the cH-G1/cL-k antibody, H206-G1d/L73-k antibody, and H240-G1d/L73-k antibody were individually administered at ~~10 mg/kg~~both of 0.4 mg/kg and 2mg/kg, the excellent drug efficacy by the altered humanized EP27 antibodies was observed in the animal models as well.

[Example 10] Immunohistochemical staining using an anti-Epiregulin antibody

An immunohistochemical staining method that reflects the antigen expression level was established using an EP27 antibody. Immunohistochemical staining was carried out using paraffin-embedded tissue blocks prepared with tissues of scid mice transplanted with cell lines that are forced to express EREG at different expression levels, which are SKE-18 (estimated amount of antigen was 7.7×10^3), SKE-23 (estimated amount of antigen was 6.1×10^4), and SKE-4B2 (estimated amount of antigen was 2.9×10^5), and the host cell SK-HEP-1 (amount of antigen was 8.4×10^2). The EREG expression in these thinly sliced tissue sections prepared from these paraffin blocks was visualized by incubation using an EP27 antibody as the primary antibody, followed by reacting a rabbit anti-mouse IgG polyclonal antibody (Jackson Immunoresearch Laboratories) as the secondary antibody, and a polymer-HRP (Dako cytomation)-bound goat anti-rabbit IgG antibody as the tertiary antibody, and using diaminobenzidine as substrate. As shown in Fig. 10, gradation of staining is observed in the stained cells/tissues depending on the amount of EREG expression. Furthermore, the *in vitro* ADCC activity of the H240-G1d/L73-k antibody was confirmed to depend on the expression level in these cells (Fig. 11). In the above-mentioned investigation, a low-fucose antibody was used for the H240-G1d/L73-k antibody. The low-fucose antibody was produced by the method described in WO2004/065540. Herein below, the low-fucose antibody is also referred to as the humanized EP27 antibody Glycomab. The *in vitro* ADCC activity of the H240-G1d/L73-k antibody (humanized EP27 antibody Glycomab) was confirmed in SKE-15 and SKE-10 which have an estimated antigen level of 2.8×10^4, in addition to in the above-mentioned four cell lines of forced EREG expression. In a similar manner to Fig. 11, the *in vitro* ADCC activity of the H240-G1d/L73-k antibody in accordance with the EREG expression level in these cells was confirmed (Table 20). Furthermore, the *in vivo* tumor growth inhibition activity of the H240-G1d/L73-k antibody (humanized EP27 antibody Glycomab) in accordance with the expression level in these cells was confirmed (Fig. 12). That is, assessment of the EREG expression level by immunohistochemical staining confirmed a correlation between the expression level and drug efficacy.

[Table 20]

TOSOH) for the column, and using 50 mM sodium phosphate buffer (pH7.0) containing 300 mM NaCl as the mobile phase, the amount of aggregate was measured by gel filtration chromatography. A column connected to an Alliance system (manufactured by Waters) was equilibrated at a flow rate of 0.5 mL/min, and then 5-10 μg of antibody solution was injected into the column. Antibody elution was detected using an ultraviolet absorption detector (215 or 280 nm). From the obtained chromatogram, the proportion of aggregate peak area in the total peak area was calculated.

[Reference Example 10] Measurement of the thermal denaturation midpoint temperature (Tm) by a differential scanning calorimeter

A 20 mmol/L sodium acetate buffer (pH 6.0) containing 150 mmol/L sodium chloride was prepared as the external solution for dialysis, and dialysis was carried out for one whole day by soaking in this external solution a dialysis membrane enclosing an antibody solution of an equivalent amount of 50 to 100 μg antibody. An antibody solution prepared at 50 μg/mL to 100 μg/mL antibody concentration using the external solution for dialysis was used as the sample solution for Tm value measurements.

A suitable DSC equipment, for example, DSC-II (manufactured by Calorimetry Sciences Corporation) or MicroCal VP-DSC (manufactured by GE healthcare) can be used for this experiment. A sufficiently degassed sample solution and a reference solution (external solution for dialysis) were individually enclosed in the calorimeter cells, and subjected to sufficient thermal equilibration at 40°C. Next, a DSC scan was run from 40°C to 100°C with a scanning rate of approximately 1K to 2.5 K/min. The results of this measurement are given as the top of the denaturation peak as a function of temperature. The thermal denaturation midpoint temperature of the sample was calculated by assigning the peak of the Fab domain according to a non-patent document (Rodolfo *et al.*, Immunology Letters (1999), p47-52).

[Reference Example 11] pI measurements by isoelectric focusing

Using Phastsystem Cassette (Amersham Bioscience), a Phast-Gel Dry IEF (Amersham Bioscience) gel was swollen for about 30 minutes in a swelling solution having the composition described below.

[Table 22]

Bioscience) at 1 : 1 ratio, suspensions of MIA PaCa-2 and DLD-1 cells were prepared at 5 x 10^7 cells/mL. 100 μL of the cell suspension (5 x 10^6 cells/mouse) was transplanted subcutaneously in the abdominal region of SCID mice (female, 5 week old, CLEA Japan, Inc.) ~~to which 100 μL had been administered intraperitoneally~~. The tumor volume was calculated using Formula 5, and when the average tumor volume reached 130-330 mm^3, the mouse model was determined to be established.

[Formula 5]

Tumor volume = long diameter x short diameter x short diameter / 2

(3) Preparation of administration samples containing each test antibody

Administration samples each containing the cH-G1/cL-k antibody, H206-G1d/L7-3k antibody, or H240-G1d/L73-k antibody at 0.04 mg/mL (0.4 mg/kg administration group) or 0.2 mg/mL (2 mg/kg administration group) were prepared with physiological saline on the day of administration.

(4) Administration of the antibody-containing administration samples

The administration samples prepared in (3) was administered ~~at a dose of 10 mL/kg~~ through the tail vein to the mouse models prepared in (2) once per week for ~~17~~ 14 days starting from day ~~13~~ 14 post-MIA PaCa-2 cell transplantation, and once per week for 14 days starting from day 12 post-DLD cell transplantation. As a negative control, physiological saline was similarly administered at a dose of 10 mL/kg through the tail vein once per week for the respective durations. All groups had five animals in each group, and to each group, the respective test antibody-containing administration sample was administered.

(5) Evaluation of the antitumor effect of each test antibody

The antitumor effect of each test antibody was evaluated in the mouse model transplanted with human cancer cells. The tumor volume was measured on day 3 or day 7 after the last day of sample administration.