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(54) Title of the Present Invention

HUMANIZED ANTI-EPIDERMAL GROWTH FACTOR RECEPTOR ANTIBODY AND APPLICATION THEREOF

(57) Abstract

The present invention relates to a humanized anti-epidermal growth factor receptor antibody. The present invention further relates to a nucleic acid molecule encoding the antibody, a vector, a host cell and a composition including the nucleic acid molecule, and a detection reagent and kit. The present invention further relates to application of the antibody to the preparation of drugs for the prevention/treatment of diseases related to an epidermal growth factor or an epidermal growth factor receptor, as well as to the preparation of the detection reagent or kit. The antibody molecule of the present invention has lower immunogenicity and higher target binding activity, resulting in stronger biological activity and anti-tumor activity in vivo and in vitro. It can be expected to have lower side effect and better curative effect in clinical application.

CN 103772504 A

1. An anti-epidermal growth factor receptor antibody or an antigen-binding part thereof, wherein the sequence of a heavy chain CDR1 thereof is selected from sequences shown in SEQ ID NO: 1 and SEQ ID NO: 39 or derivative sequences thereof, and the sequence of CDR3 is selected from sequences shown in SEQ ID NO: 2, SEQ ID NO: 3, and SEQ ID NO: 40 or derivative sequences thereof; and/or

the sequence of a light chain CDR3 thereof is selected from sequences shown in SEQ ID NO: 9, SEQ ID NO: 10, and SEQ ID NO: 41 or derivative sequences thereof.

2. The antibody or the antigen-binding part thereof according to claim 1, wherein the sequence of a heavy chain variable region CDR are selected from sequences shown in (1) to (4) below or derivative sequences thereof:

(1) the sequence of CDR1 is a sequence shown in SEQ ID NO: 1, the sequence of CDR2 is a sequence shown in SEQ ID NO: 4, and the sequence of CDR3 is a sequence shown in SEQ ID NO: 2;

(2) the sequence of CDR1 is a sequence shown in SEQ ID NO: 1, the sequence of CDR2 is a sequence shown in SEQ ID NO: 4, and the sequence of CDR3 is a sequence shown in SEQ ID NO: 40;

(3) the sequence of CDR1 is a sequence shown in SEQ ID NO: 39, the sequence of CDR2 is a sequence shown in SEQ ID NO: 4, and the sequence of CDR3 is a sequence shown in SEQ ID NO: 3; and

(4) The sequence of CDR1 is a sequence shown in SEQ ID NO: 39, the sequence of CDR2 is a sequence shown in SEQ ID NO: 4, the sequence of CDR3 is a sequence shown in SEQ ID NO: 40; and/or

the sequence of a light chain variable region CDR thereof is selected from sequences shown in (5) ~ (8) below or derivative sequences thereof:

(5) the sequence of CDR1 is a sequence shown in SEQ ID NO: 11, the sequence of CDR2 is a sequence shown in SEQ ID NO: 12, and the sequence of CDR3 is a sequence shown in SEQ ID NO: 9;

(6) the sequence of CDR1 is a sequence shown in SEQ ID NO: 11, the sequence of CDR2 is a sequence shown in SEQ ID NO: 12, and the sequence of CDR3 is a sequence shown in SEQ ID NO: 41;

(7) the sequence of CDR1 is a sequence shown in SEQ ID NO: 11, the sequence of CDR2 is a sequence shown in SEQ ID NO: 12, and the sequence of CDR3 is a sequence shown in SEQ ID NO: 41; and

(8) the sequence of CDR1 is a sequence shown in SEQ ID NO: 11, the sequence of CDR2 is a sequence shown in SEQ ID NO: 12, and the sequence of CDR3 is a sequence shown in SEQ ID NO: 10.

3. The antibody or the antigen-binding part thereof according to claim 1 or 2, wherein the sequence of a heavy chain FR1 thereof is selected from a sequence shown in SEQ ID NO: 19 to SEQ ID NO: 22 or a derivative sequence thereof, the sequence of FR2 is selected from a sequence shown in SEQ ID NO: 23 to SEQ ID NO: 25 or a derivative sequence thereof, and the sequence of FR3 is selected from a sequence shown in SEQ ID NO: 26 to SEQ ID NO: 29 or a derivative sequence thereof, and the sequence of FR4 is a sequence shown in SEQ ID NO: 30 or a derivative sequence thereof; and/or

the sequence of a light chain FR1 thereof is selected from a sequence shown in SEQ ID NO: 31 to SEQ ID NO: 32 or a derivative sequence thereof, the sequence of FR2 is selected from a sequence shown in SEQ ID NO: 33 to SEQ ID NO: 34 or a derivative sequence thereof, the sequence of FR3 is selected from a sequence shown in SEQ ID NO: 35 to SEQ ID NO: 36 or a derivative sequence thereof, and the sequence of FR4 is selected from a sequence shown in SEQ ID NO: 37 to SEQ ID NO: 38 or a derivative sequence thereof.

4. The antibody or the antigen-binding part thereof according to claim 3, wherein the sequence of a heavy chain variable region FR thereof is selected from sequences shown in (9) to (12) below or derivative sequences thereof:

(9) the sequence of FR1 is a sequence shown in SEQ ID NO: 19, the sequence of FR2 is a sequence shown in SEQ ID NO: 23, the sequence of FR3 is a sequence shown in SEQ ID NO: 26, and the sequence of FR4 is a sequence shown in SEQ ID NO: 30;

(10) the sequence of FR1 is a sequence shown in SEQ ID NO: 20, the sequence of FR2 is a sequence shown in SEQ ID NO: 24, the sequence of FR3 is a sequence shown in SEQ ID NO: 27, and the sequence of FR4 is a sequence shown in SEQ ID NO: 30;

(11) the sequence of FR1 is a sequence shown in SEQ ID NO: 21, the sequence of FR2 is a sequence shown in SEQ ID NO: 23, the sequence of FR3 is a sequence shown in SEQ ID NO: 28, and the sequence of FR4 is a sequence shown in SEQ ID NO: 30; and

(12) the sequence of FR1 is a sequence shown in SEQ ID NO: 22, the sequence of FR2 is a sequence shown in SEQ ID NO: 25, the sequence of FR3 is a sequence shown in SEQ ID NO: 29, and the sequence of FR4 is a sequence shown in SEQ ID NO: 30; and/or

the sequence of a light chain variable region FR thereof is selected from sequences shown in (13) to (16) below or derivative sequences thereof:

(13) the sequence of FR1 is a sequence shown in SEQ ID NO: 31, the sequence of FR2 is a sequence shown in SEQ ID NO: 33, the sequence of FR3 is a sequence shown in SEQ ID NO: 35, and the sequence of FR4 is a sequence shown in SEQ ID NO: 37;

(14) the sequence of FR1 is a sequence shown in SEQ ID NO: 31, the sequence of FR2 is a sequence shown in SEQ ID NO: 33, the sequence of FR3 is a sequence shown in SEQ ID NO: 35, and the sequence of FR4 is a sequence shown in SEQ ID NO: 38;

(15) the sequence of FR1 is a sequence shown in SEQ ID NO: 32, the sequence of FR2 is a sequence shown in SEQ ID NO: 34, the sequence of FR3 is a sequence shown in SEQ ID NO: 36, and the sequence of FR4 is a sequence shown in SEQ ID NO: 38; and

(16) the sequence of FR1 is a sequence shown in SEQ ID NO: 31, the sequence of FR2 is a sequence shown in SEQ ID NO: 33, the sequence of FR3 is a sequence shown in SEQ ID NO: 35, and the sequence of FR4 is a sequence shown in SEQ ID NO: 37.

5. The antibody or the antigen-binding part thereof according to any one of claims 1-4, further comprising a human IgG1 heavy chain constant region and a human kappa light chain constant region.

6. The antibody or the antigen-binding part thereof according to any one of claims 1-4, wherein the antigen-binding part is a single-chain antibody (scFv), a chimeric antibody, a double antibody, an scFv-Fc bivalent molecule, a dAb, and a complementarity-determining region (CDR) fragment, a Fab fragment, a Fab' fragment, an Fv, or an $F(ab')_2$ fragment.

7. An isolated nucleic acid molecule, encoding the antibody or the antigen-binding part thereof according to any one of claims 1-6.

8. A vector, comprising the nucleic acid molecule according to claim 7.

9. A host cell, comprising the nucleic acid molecule according to claim 7 or the vector according to claim 8.

10. A composition, comprising the antibody or the antigen-binding part thereof according to any one of claims 1-6, and optionally one or more pharmaceutically acceptable excipients, wherein preferably, the excipients are selected from protease inhibitors, pharmaceutical carriers, diluents, and adjuvants.

11. Application of the antibody or the antigen-binding part thereof according to any one of claims 1-6, or the nucleic acid molecule according to claim 7, or the vector according to claim 8, or the host cell according to claim 9 to the preparation of a drug for the prevention/treatment of diseases related to the epidermal growth factor or epidermal growth factor receptor, wherein preferably, the diseases related to epidermal growth factor or epidermal growth factor receptor are those caused by overexpression of the epidermal growth factor or epidermal growth factor receptor, and more preferably, the diseases caused by overexpression of the epidermal growth factor or epidermal growth factor receptor are malignant tumors.

12. A detection reagent or kit, comprising the antibody or the antigen-binding part thereof according to any one of claims 1-6, wherein

preferably, the antibody or the antigen-binding part thereof further contains a detectable label; or

preferably, the detection reagent or kit further contains a second antibody that specifically recognizes the antibody or the antigen-binding part thereof; or

preferably, the second antibody further contains a detectable label.

13. Application of the antibody or the antigen-binding part thereof according to any one of claims 1-6 to the preparation of a detection reagent or kit, wherein the detection reagent or kit is used for detecting an epidermal growth factor or epidermal growth factor receptor, or for diagnosing diseases related to an epidermal growth factor or epidermal growth factor receptor.

HUMANIZED ANTI-EPIDERMAL GROWTH FACTOR RECEPTOR ANTIBODY AND APPLICATION THEREOF

Technical Field

[0001] The present invention relates to a humanized antibody, and in particular to a humanized anti-epidermal growth factor receptor antibody. The present invention further relates to application of the antibody to the preparation of drugs for the prevention or treatment of diseases associated with high activity or high expression of an epidermal growth factor. Compared with a human-mouse chimeric antibody, the humanized anti-epidermal growth factor antibody involved in the present invention further reduces the immunogenicity of the antibody while enhancing the antigen recognition activity and anti-tumor functions both in vivo and in vitro, offering better clinical application prospects.

Background

[0002] An epidermal growth factor receptor (EGFR, also known as HERI, c-ErbB1) is a cell surface receptor of an epidermal growth factor family, and is a transmembrane glycoprotein (Jorissen RN, Walker F, Pouliot N, et al. Epidermal growth factor receptor: mechanisms of activation and signaling. Exp Cell Res, 2003; 284:31-53), composed of 1,186 amino acid residues and with a molecular weight of 170 kD. EGFR belongs to the type I tyrosine kinase receptor ErbB subfamily (ErbB 1-4) and has tyrosine kinase activity. EGFR is stably expressed in many epithelial tissues, including skin and hair follicles. Abnormal expression or activation due to receptor mutation of the epidermal growth factor receptor can lead to carcinogenesis. Many solid tumors have been found to overexpress the epidermal growth factor receptor, such as colorectal cancer, head and neck cancer, lung cancer, ovarian cancer, cervical cancer, bladder cancer and esophageal cancer (Olayioye MA, Neve RM, Lane HA, et al. The ErbB Signaling network: receptor heterodimerization in development and cancer. The EMBOJ, 2000; 19: 3159-3167). Growth factors such as a transforming growth factor alpha and an epidermal growth factor are endogenous ligands of the epidermal growth factor receptor. These ligands bind to the epidermal growth factor receptor to activate the intracellular tyrosine protein kinase activity of the receptor and initiate multiple downstream signal transduction pathways, thereby regulating the growth and differentiation of normal cells, increasing the invasiveness of tumor cells, promoting angiogenesis, and inhibiting tumor cell apoptosis (Ciardiello F, Tortora G. A novel approach in the treatment of cancer: targeting the epidermal growth factor receptor. Clin Cancer Res, 2001; 7: 2958-2970). The high expression of the epidermal growth factor receptor in tumors and the important role thereof in tumor cell growth and differentiation make the epidermal growth factor receptor become a promising target for tumor therapy.

[0003] The therapeutic antibody against the epidermal growth factor receptor was initially derived from a murine antibody M225, obtained by immunizing mice with the epidermal carcinoma A431 highly expressed by the epidermal growth factor receptor. M225 specifically binds to the epidermal growth factor receptor, blocking its binding with ligands, thereby inhibiting the activity of tyrosine protein kinase of the epidermal growth factor receptor (Goldstein et al., 1995, Clin. Cancer Res. 1: 1311-1318). As foreign proteins, murine antibodies can repeatedly stimulate the human body, potentially causing hypersensitivity reactions, known as human anti-mouse antibody responses. Murine antibodies may be neutralized by anti-antibodies in the body, leading to rapid clearance and even severe allergic reactions that can be fatal. Moreover, murine monoclonal antibodies lack the immunological effect functions of human antibodies, such as antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC), resulting in poor curative effect. Therefore, the human-mouse chimeric antibody C225 (Erbitux or Cetuximab, ImClone (now in Eli Lilly) company) uses variable regions of human IgG1k and murine anti-M225, reducing the immunogenicity of the murine-derived monoclonal antibody and retaining specific affinity for the epidermal growth factor receptor. It can block the binding of ligands such as EGF and TGFa to the epidermal growth factor receptor and inhibit its phosphorylation and downstream signal transduction, thereby suppressing tumor cell growth, inducing apoptosis, and reducing the production of matrix metalloproteinases and vascular endothelial growth factor. The U.S. FDA approved Erbitux for the treatment of colorectal cancer in 2004, and approved it for the treatment of head and neck cancer in 2006. Currently, there are more clinical trials for other tumor indications. Compared with other chemotherapy drugs, antibody drugs have strong specificity and small side effect, achieving better curative effect in clinical practice. However, the human-

mouse chimeric Erbitux monoclonal antibody still has immunogenicity, with clinical reports indicating an immunogenicity rate of 5%, which easily leads to human anti-antibody responses to cause immune complex-mediated antibody clearance, and side effects on the skin and mucous membranes (An Z, et al., 2009, Therapeutic Monoclonal Antibody, p313).
[0004] Panitumumab (Amgen Inc.) is a fully humanized monoclonal antibody prepared by a transgenic mouse technology, with no murine protein sequences, targeting the epidermal growth factor receptor (EGFR). It was approved by the FDA in September 2006 for use in combination with fluoropyrimidine, oxaliplatin and irinotecan, or after chemotherapy, for the treatment of EGFR-positive metastatic colorectal cancer. In 2006, the FDA approved its use as a monotherapy for the treatment of chemotherapy-resistant metastatic colorectal cancer (mCRC). However, panitumumab is an IgG2 subtype antibody, and compared with IgG1, the CDC activity and ADCC biological activities of IgG2 are significantly reduced. Additionally, the stability of IgG2 subtype antibodies is relatively poor, which may be the main reason why the fully humanized antibody panitumumab does not show a significant advantage over the chimeric antibody cetuximab in clinical effect.
[0005] Therefore, there is an urgent need in the field for humanized anti-epidermal growth factor receptor antibodies with lower immunogenicity and higher biological activity, to further improve curative effect and reduce side effects.

Summary

[0006] Based on murine antibodies, the inventors have surprisingly obtained a humanized epidermal growth factor receptor antibody with lower immunogenicity and higher biological activity using an antibody humanization technology. The present invention is completed based on the above findings and specifically includes the following aspects:
[0007] A first aspect of the present invention relates to an anti-epidermal growth factor receptor antibody or an antigen-binding part thereof, where the sequence of a heavy chain CDR1 thereof is selected from sequences shown in SEQ ID NO: 1 and SEQ ID NO: 39 or derivative sequences thereof, and the sequence of CDR3 is selected from sequences shown in SEQ ID NO: 2, SEQ ID NO: 3, and SEQ ID NO: 40 or derivative sequences thereof; and/or
[0008] the sequence of a light chain CDR3 thereof is selected from sequences shown in SEQ ID NO: 9, SEQ ID NO: 10, and SEQ ID NO: 41 or derivative sequences thereof.
[0009] In the antibody or the antigen-binding part thereof according to any one in the first aspect of the present invention, the sequences of a heavy chain CDR2 and light chains CDR1 and CDR2 thereof can be the corresponding parts of a murine, human, or human-mouse chimeric anti-epidermal growth factor receptor antibody.
[0010] In the antibody or the antigen-binding part thereof according to any one in the first aspects of the present invention, the sequence of its heavy chain and light chain FR can be the corresponding parts of a murine, human, or human-mouse chimeric anti-epidermal growth factor receptor antibody.
[0011] In the examples of the present invention, the sequence of a heavy chain variable region CDR of the antibody or the antigen-binding part thereof is selected from sequences shown in (1) to (4) below or derivative sequences thereof:
[0012] (1) the sequence of CDR1 is a sequence shown in SEQ ID NO: 1, the sequence of CDR2 is a sequence shown in SEQ ID NO: 4, and the sequence of CDR3 is a sequence shown in SEQ ID NO: 2;
[0013] (2) the sequence of CDR1 is a sequence shown in SEQ ID NO: 1, the sequence of CDR2 is a sequence shown in SEQ ID NO: 4, and the sequence of CDR3 is a sequence shown in SEQ ID NO: 40;
[0014] (3) the sequence of CDR1 is a sequence shown in SEQ ID NO: 39, the sequence of CDR2 is a sequence shown in SEQID NO: 4, and the sequence of CDR3 is a sequence shown in SEQ ID NO: 3; and
[0015] (4) the sequence of CDR1 is a sequence shown in SEQ ID NO: 39, the sequence of CDR2 is a sequence shown in SEQ ID NO: 4, and the sequence of CDR3 is a sequence shown in SEQ ID NO: 40; and/or
[0016] the sequence of a light chain variable region CDR thereof is selected from sequences shown in (5) to (8) below or derivative sequences thereof:
[0017] (5) the sequence of CDR1 is a sequence shown in SEQ ID NO: 11, the sequence of CDR2 is a sequence shown in SEQ ID NO: 12, and the sequence of CDR3 is shown in SEQ ID NO: 9;

[0018] (6) The sequence of CDR1 is a sequence shown in SEQ ID NO: 11, the sequence of CDR2 is a sequence shown in SEQ ID NO: 12, and the sequence of CDR3 is a sequence shown in SEQ ID NO: 41;
[0019] (7) the sequence of CDR1 is a sequence shown in SEQ ID NO: 11, the sequence of CDR2 is a sequence shown in SEQ ID NO: 12, and the sequence of CDR3 is a sequence shown in SEQ ID NO: 41; and
[0020] (8) the sequence of CDR1 is a sequence shown in SEQ ID NO: 11, the sequence of CDR2 is a sequence shown in SEQ ID NO: 12, and the sequence of CDR3 is a sequence shown in SEQ ID NO: 10.
[0021] In the antibody or the antigen-binding part thereof according to anyone in the first aspect of the present invention, the sequence of the heavy chain FR1 thereof is selected from sequences shown in SEQ ID NO: 19 to SEQ ID NO: 22 or derivative sequences thereof, the sequence of FR2 is selected from sequences shown in SEQ ID NO: 23 to SEQ ID NO: 25 or derivative sequences thereof, the sequence of FR3 is selected from sequences shown in SEQ ID NO: 26 to SEQ ID NO: 29 or derivative sequences thereof, and the sequence of FR4 is a sequence shown in SEQ ID NO: 30 or a derivative sequence thereof; and/or
[0022] the sequence of the light chain FR1 thereof is selected from sequences shown in SEQ ID NO: 31 to SEQ ID NO: 32 or derivative sequences thereof, the sequence of FR2 is selected from sequences shown in SEQ ID NO: 33 to SEQ ID NO: 34 or derivative sequences thereof, the sequence of FR3 is selected from sequences shown in SEQ ID NO: 35 to SEQ ID NO: 36 or derivative sequences thereof, and the sequence of FR4 is selected from sequences shown in SEQ ID NO: 37 to SEQ ID NO: 38 or derivative sequences thereof.
[0023] In the examples of the present invention, the sequence of the heavy chain variable region FR of the antibody or an antigen-binding part thereof is selected from sequences shown in (9) to (12) below or derivative sequences thereof:
[0024] (9) the sequence of FR1 is a sequence shown in SEQ ID NO: 19, the sequence of FR2 is a sequence shown in SEQ ID NO: 23, the sequence of FR3 is a sequence shown in SEQ ID NO: 26, and the sequence of FR4 is a sequence shown in SEQ ID NO: 30;
[0025] (10) the sequence of FR1 is a sequence shown in SEQ ID NO: 20, the sequence of FR2 is a sequence shown in SEQ ID NO: 24, the sequence of FR3 is a sequence shown in SEQ ID NO: 27, and the sequence of FR4 is a sequence shown in SEQ ID NO: 30;
[0026] (11) the sequence of FR1 is a sequence shown in SEQ ID NO: 21, the sequence of FR2 is a sequence shown in SEQ ID NO: 23, the sequence of FR3 is a sequence shown in SEQ ID NO: 28, and the sequence of FR4 is a sequence shown in SEQ ID NO: 30; and
[0027] (12) the sequence of FR1 is a sequence shown in SEQ ID NO: 22, the sequence of FR2 is a sequence shown in SEQ ID NO: 25, the sequence of FR3 is a sequence shown in SEQ ID NO: 29, and the sequence of FR4 is a sequence shown in SEQ ID NO: 30; and/or
[0028] the sequence of a light chain variable region FR thereof is selected from sequences shown in (13) to (16) below or derivative sequences thereof:
[0029] (13) the sequence of FR1 is a sequence shown in SEQ ID NO: 31, the sequence of FR2 is a sequence shown in SEQ ID NO: 33, the sequence of FR3 is a sequence shown in SEQ ID NO: 35, and the sequence of FR4 is a sequence shown in SEQ ID NO: 37;
[0030] (14) the sequence of FR1 is a sequence shown in SEQ ID NO: 31, the sequence of FR2 is a sequence shown in SEQ ID NO: 33, the sequence of FR3 is a sequence shown in SEQ ID NO: 35, and the sequence of FR4 is a sequence shown in SEQ ID NO: 38;
[0031] (15) the sequence of FR1 is a sequence shown in SEQ ID NO: 32, the sequence of FR2 is a sequence shown in SEQ ID NO: 34, the sequence of FR3 is a sequence shown in SEQ ID NO: 36, and the sequence of FR4 is a sequence shown in SEQ ID NO: 38; and
[0032] (16) the sequence of FR1 is a sequence shown in SEQ ID NO: 31, the sequence of FR2 is a sequence shown in SEQ ID NO: 33, the sequence of FR3 is a sequence shown in SEQ ID NO: 35, and the sequence of FR4 is a sequence shown in SEQ ID NO: 37.
[0033] In a specific example of the present invention, the sequence of the heavy chain variable region of the antibody or the antigen-binding part thereof is a sequence shown in SEQ ID NO: 5, and the sequence of the light chain variable region is a sequence shown in SEQ ID NO: 13.
[0034] In another specific example of the present invention, the sequence of the heavy chain variable region of

the antibody or the antigen-binding part thereof is a sequence shown in SEQ ID NO: 6, and the sequence of the light chain variable region is a sequence shown in SEQ ID NO: 14.

[0035] In another specific example of the present invention, the sequence of the heavy chain variable region of the antibody or the antigen-binding part thereof is a sequence shown in SEQ ID NO: 7, and the sequence of the light chain variable region is a sequence shown in SEQ ID NO: 15.

[0036] In another specific example of the present invention, the sequence of the heavy chain variable region of the antibody or the antigen-binding part thereof is a sequence shown in SEQ ID NO: 8, and the sequence of the light chain variable region is a sequence shown in SEQ ID NO: 16.

[0037] The antibody or the antigen-binding part thereof according to anyone in the first aspect of the present invention further includes a human IgG1 heavy chain constant region and a human kappa light chain constant region.

[0038] In the present invention, the IgG1 heavy chain constant region includes various allotypes, such as G1m(f), G1m(z), G1m(z, a), or G1m(z, a, x). In the examples of the present invention, the IgG1 heavy chain constant region is of the G1m(z) type.

[0039] In the present invention, the kappa light chain constant region includes various allotypes, such as Km1, Km1, 2, or Km3. In the examples of the present invention, the kappa light chain constant region is of the Km3 type.

[0040] In the antibody or the antigen-binding part thereof according to anyone in the first aspect of the present invention, the antigen-binding part includes a single-chain antibody (scFv), a chimeric antibody, a double antibody, a scFv-Fc bivalent molecule, dAb, a complementarity-determining region (CDR) fragment, a Fab fragment, a Fd fragment, a Fab' fragment, a Fy and a $F(ab')_2$ fragment.

[0041] A second aspect of the present invention relates to an isolated nucleic acid molecule encoding the antibody or the antigen-binding part according to anyone in the first aspect of the present invention.

[0042] A third aspect of the present invention relates to a vector including the nucleic acid molecule according to anyone in the second aspect of the present invention.

[0043] In the present invention, the vector can be a cloning vector or an expression vector. The expression vector is, for example, a prokaryotic expression vector, a eukaryotic expression vector, a phage vector or a viral vector, all of which are commonly used vectors in the field. The prokaryotic expression vector is, for example, PET28a, pGEM Ex1, pGEM7ZF(+), or pBAD24, etc. The eukaryotic expression vector is, for example, pBudCE4.1, pcDNA3.1, pCMV-Tag2B, or pGL3 basic, etc. In the examples of the present invention, the eukaryotic expression vector is pcDNA3.1, and the viral vector is, for example, a retrovirus, lentivirus, adenovirus and adeno-associated virus. In the examples of the present invention, the viral vector is an adenoviral vector.

[0044] A fourth aspect of the present invention relates to a host cell including the nucleic acid molecule according to the second aspect of the present invention or the vector according to the third aspect of the present invention.

[0045] In the present invention, the host cell can be a prokaryotic cell or a eukaryotic cell. The eukaryotic cell is, for example, a mammalian cell. The host cell can be obtained by introducing/transfecting the aforementioned nucleic acid molecule or vector into the prokaryotic or eukaryotic cell.

[0046] In the present invention, the prokaryotic cell can be, for example, Escherichia coli. The eukaryotic cells can be, for example, a yeast cell, an insect cell (such as Sf21), or a mammalian cell. The mammalian cell can be, for example, an NSO cell or a CHO cell. In one example of the present invention, the host cell is a eukaryotic cell. In one example of the present invention, the eukaryotic cell is a CHO-S cell.

[0047] In the present invention, any type of transfection method known in the art can be used to obtain host cells transfected with specific nucleic acids or vectors. For example, nucleic acids can be introduced into cells via electroporation or microinjection. Alternatively, lipid transfection reagents such as FuGENE6, X-tremeGENE and LipofectAmine can be used. Alternatively, nucleic acids can be introduced into cells via appropriate viral vectors based on retrovirus, lentivirus, adenovirus, and adeno-associated virus.

[0048] A fifth aspect of the present invention relates to a composition including the antibody or the antigen-binding part thereof according to anyone in the first aspect of the present invention, and optionally one or more pharmaceutically acceptable excipients. Preferably, the excipients are selected from protease inhibitors, pharmaceutical carriers, diluents, and adjuvants.

[0049] A sixth aspect of the present invention relates to application of the antibody or the antigen-binding part thereof according to anyone in the first aspect of the present invention, or the nucleic acid according to the second aspect, the vector according to the third aspect, or the host cell according to the fourth aspect to the preparation of a drug for the prevention/ treatment of diseases related to the epidermal growth factor or epidermal growth factor receptor.

[0050] In the present invention, the epidermal growth factor or epidermal growth factor receptor-related diseases refer to diseases caused by the overexpression of the epidermal growth factor or epidermal growth factor receptor. In the examples of the present invention, the epidermal growth factor or epidermal growth factor receptor-related diseases refer to malignant tumors, primarily those occurring in epithelial tissues. These include, but are not limited to, colorectal cancer, liver cancer, head and neck cancer, lung cancer, ovarian cancer, cervical cancer, vaginal cancer, bladder cancer, esophageal cancer, oral cancer, skin cancer, breast cancer, prostate cancer, and pancreatic cancer.

[0051] A seventh aspect of the present invention relates to a detection reagent or kit including the antibody or the antigen-binding part thereof according to anyone in the first aspects of the present invention.

[0052] Preferably, the antibody or the antigen-binding part thereof may further include a detectable label; or

[0053] Preferably, the detection reagent or kit may further include a second antibody, which specifically recognizes the antibody or the antigen-binding part thereof; or

[0054] Preferably, the second antibody may further include a detectable label.

[0055] An eighth aspect of the present invention relates to application of the antibody or the antigen-binding part thereof according to any one in the first aspect of the present invention to the preparation of a detection reagent or kit, where the detection reagent or kit is used for detecting the epidermal growth factor or epidermal growth factor receptor, or for diagnosing diseases related to the epidermal growth factor or epidermal growth factor receptor.

[0056] The following provides a further description of the present invention.

[0057] An objective of the present invention is to change the CDR region and/or framework region of the antibody for existing anti-epidermal growth factor receptor antibodies by using a de-immunization antibody humanization technology, so as to obtain anti-epidermal growth factor receptor antibodies with lower immunogenicity and higher biological activity.

[0058] In one example of the present invention, the sequence of the heavy chain variable region of the murine anti-M225 is first analyzed. Based on the FR1, FR2, FR3, and FR4 sequences of its heavy chain variable region, a comparison is performed on the human antibody gene sequence library to find out the corresponding sequences of the human germline antibodies (which have lower immunogenicity than mature antibodies) that are similar to the FR1, FR2, FR3, and FR4 sequences of the murine anti-M225 heavy chain variable region. Then, the affinity of the similar sequences for binding to HLA-DR molecules is analyzed by an in silico method, and the framework sequence with the lowest affinity is selected, thereby ultimately determining the humanized sequences of FR1, FR2, FR3, and FR4 of the heavy chain variable region. Based on this framework structure, a computer molecular model is used to analyze the three-dimensional space structure of the variable region of the murine M225 antibody to analyze the corresponding framework amino acid residues of the murine M225 antibody required to be retained to support the CDR conformation. For the deimmunogenization of CDR1 and CDR3 with higher affinity for binding to HLA-DR molecules, molecular docking is used to analyze the binding mode of the target site of the epidermal growth factor receptor antigen murine M225 antibody and its surrounding amino acid residues and the three-dimensional space of the humanized heavy chain variable region. By calculating electrostatic forces, salvation, van der Waals forces, and entropy values, amino acids in the CDR1 and CDR3 sequences that can be optimized are selected to reduce the affinity for binding to HLA-DR molecules and to improve the binding of the antibody CDR3 region to the epitope of the epidermal growth factor receptor antigen.

[0059] In another example of the present invention, the sequence of the light chain variable region of the murine anti-M225 antibody is analyzed. The human antibody gene sequence library (NCBI Ig BLAST) is compared based on the FR1, FR2, FR3, and FR4 sequences of its light chain variable region to find out the corresponding sequences of human germline antibody variable regions similar to the FR1, FR2, FR3, and FR4 sequences of the murine anti-M225 light chain variable region. Subsequently, the affinity of the similar sequences binding to HLA-DR molecules is analyzed through in silico, and the framework sequence with the lowest affinity is selected, thereby ultimately determining the humanized sequences of FR1, FR2, FR3, and FR4 of the light chain variable region. Based on this framework structure, a computer molecular model is used to analyze the three-dimensional space structure of the variable region of the murine 225 antibody to analyze the framework amino acid residues of the murine M225 antibody required to be retained to support the CDR conformation. For the deimmunogenization of CDR1 and CDR3 with high affinity for binding the HLA-DR molecule, molecular docking is used to analyze the binding mode of the target site of the epidermal growth factor receptor antigen murine M225 antibody and its surrounding amino acid residues and the three-dimensional space of the humanized heavy chain variable region. By calculating electrostatic forces, salvation, van der Waals forces, and entropy values, the amino acids in the CDR1 and CDR3 sequences that can be optimized are selected to reduce the affinity for HLA-DR molecule binding and to improve the binding of the antibody CDR3 region to the epitope of the epidermal growth factor receptor antigen.

[0060] In the examples of the present invention, the human IgG1 heavy chain constant region Fc fragment and the light chain constant region kappa fragment are obtained separately from normal human B cells, and are respectively connected with the aforementioned heavy chain variable region and light chain variable region to construct antibody heavy chain and light chain expression vectors. The expression vectors are co-transfected into mammalian cells for expression and purification to obtain humanized anti-epidermal growth factor receptor antibodies BA03, BB03, BC03, and BD03.

[0061] In the examples of the present invention, the antibody has higher activity compared with Erbitux in terms of affinity for the epidermal growth factor receptor on the cell surface, blocking the binding activity of the EGF ligand to the epidermal growth factor receptor on the cell surface, inhibiting the phosphorylation activity of the epidermal growth factor receptor on the cell surface, and ADCC effect, while also having lower immunogenicity.

[0062] In the examples of the present invention, the antibody exhibits inhibitory effects on the growth of various tumor cells, such as epidermal cancer, lung cancer, colon cancer, pancreatic cancer and breast cancer, and shows a significant effect in inhibiting tumor growth at the animal level.

[0063] In the present invention, unless otherwise specified, the scientific and technical terms used herein have the meanings commonly understood by those skilled in the art. Furthermore, the terms and laboratory procedures related to protein and nucleic acid chemistry, molecular biology, cell and tissue culture, microbiology, and immunology used herein are terms and conventional procedures widely used in the corresponding fields. At the same time, to better understand the present invention, definitions and explanations of relevant terms are provided below.

[0064] In the present invention, the term “antibody” refers to an immunoglobulin molecule typically composed of two pairs of identical polypeptide chains (each pair having one 'light' (L) chain and one “heavy” (H) chain). Antibody light chains can be classified as kappa and lambda light chains. Heavy chains can be classified as gamma, mu, alpha, delta, or epsilon, and the isotypes of antibodies are defined as IgM, IgD, IgG, IgA, and IgE, respectively. Within the light and heavy chains, the variable region and the constant region are connected by ”J” regions of about 12 or more amino acids, and the heavy chains further include ’D’ regions of about 3 or more amino acids. Each heavy chain includes a heavy chain variable region (V_H) and a heavy chain constant region (C_H). The heavy chain constant region includes 3 structural domains (C_H1, C_H2, and C_H3). Each light chain includes a light chain variable region (V_L) and a light chain constant region (C_L). The light chain constant region includes one structural domain C. The constant region of the antibody can mediate the binding of immunoglobulin to host tissues or factors, including various cells of an immune system (e.g., effector cells) and a first component of a classical complement system (Clq). The V_H and V_L regions can be further subdivided into regions with high variability (called complementarity-determining regions (CDR)), interspersed with more conserved regions called framework regions (FR). Each V_H and V_L include 3 CDRs and 4 FRs arranged from the amino terminus to the carboxyl terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. The variable regions (V_H and V_L) of each heavy chain/light chain pair respectively form the antibody binding site. The allocation of amino acids to each region or domain follows the Kabat Sequences of Proteins of Immunological Interest (National Institutes of Health, Bethesda, Md. (1987 and 1991)), or the definitions by Chothia & Lesk (1987) J. Mol. Biol. 196: 901-917; Chothia et al. (1989) Nature 342: 878-883. The term “antibody” is not limited by any specific method for antibody production. For example, it includes, in particular, recombinant antibodies, monoclonal antibodies and polyclonal antibodies. Antibodies can be of different isotypes, such as IgG (e.g., IgG1, IgG2, IgG3, or IgG4 subclasses), IgA1, IgA2, IgD, IgE, or IgM antibodies.

[0065] In the present invention, the term ”antigen-binding part” of an antibody refers to one or more parts of a full-length antibody, which retain the ability to bind to the same antigen (e.g., ERCC1) as the antibody binds, and compete with the intact antibody for specific binding to the antigen. See generally, Fundamental Immunology, Ch.7 (Paul, W., ed., 2nd version., Raven Press, N. Y. (1989), which is incorporated herein by reference in its entirety for all purposes. The antigen-binding part can be produced by a recombinant DNA technology or by enzymatic or chemical cleavage of the intact antibody. In some cases, the antigen-binding part includes Fab, Fab', F(ab')$_2$, Fd, Fv, dAb, and complementarity-determining region (CDR) fragments, single-chain antibodies (e.g., scFv), chimeric antibodies, double antibodies, and such polypeptides that include at least a part of an antibody sufficient to confer antigen-binding specificity to the polypeptide.

[0066] In the present invention, the term “Fd fragment” refers to an antibody fragment composed of V_H and C_H1 structural domains; the term “Fv fragment” refers to an antibody fragment composed of the V_L and V_H structural domains of a single arm of an antibody; the term “dAb fragment” refers to an antibody fragment composed of the V_H structural domain (Ward et al., Nature 341: 544-546 (1989)); the term “Fab fragment” refers to an antibody fragment composed of V_L, V_H, C_L, and C_H1 structural domains; and the term “F(ab')$_2$ fragment” refers to an antibody fragment including two Fab fragments connected by a disulfide bridge in the hinge region.

[0067] In some cases, the antigen-binding part of the antibody is a single-chain antibody (e.g., scFv), where the V_L and V_H structural domains are paired to form a monovalent molecule through a linker enabling the structural domains to produce a single polypeptide chain (see, for example, Bird et al., Science 242: 423-426 (1988) and Huston et al., Proc. Natl. Acad. Sci. USA 85: 5879-5883 (1988)). Such scFv molecules may have a general structure: NH_2-V_L-linker-V_H-COOH or NH_2-V_H-linker-V_L-COOH. Suitable existing technology linkers include repeated GGGGS amino acid sequences or their variants. For example, linkers with the amino acid sequence (GGGGS)$_4$ can be used, but their variants can also be used (Holliger et al. (1993), Proc. Natl. Acad. Sci. USA 90: 6444-6448). Other linkers that can be used in the present invention are described by Alfthan et al. (1995), Sci. USA Protein Eng. 8: 725-731, Choi et al. (2001), Eur. J. Immunol. 31: 94-106, Hu et al. (1996), Cancer Res. 56: 3055-3061, Kipriyanov et al. (1999), J. Mol. Biol. 293: 41-56, and Roovers et al. (2001), Cancer Immunol.

[0068] In some cases, the antibody is a double antibody, i.e., a bivalent antibody, where the V_H and V_L domains are expressed on a single polypeptide chain, but use a linker that is too short to allow pairing between the two domains on the same chain, thereby forcing the structural domains to pair with complementary domains on another chain and producing two antigen-binding sites (see, for example, Holliger P. et al., Proc. Natl. Acad. Sci. USA 90: 6444-6448 (1993), and Poljak R. J. et al., Structure 2: 1121-1123(1994)).

[0069] In the present invention, the term “derived sequence” refers to a sequence that is highly homologous to the corresponding fragment of the antibody or the antigen-binding part of the present invention and still retains similar EGFR binding activity and immunogenicity to the corresponding fragment of the antibody or the antigen-binding part of the present invention.

[0070] Conventional techniques known to those skilled in the art (e.g., recombinant DNA technology or enzymatic or chemical cleavage methods) can be used to obtain the antigen-binding part (such as the antibody fragment) of the antibody from a given antibody (e.g., monoclonal antibody 2E12), and the antigen-binding part of the antibody can be specifically screened in the same manner as for the intact antibody.

[0071] In the present invention, the 20 standard amino acids and their abbreviations follow conventional usage. Refer to Immunology-A Synthesis (2nd Edition, E.S. Golub and D.R. Gren, Eds., Sinauer Associates, Sunderland, Mass. (1991)), which is incorporated herein by reference.

[0072] In the present invention, the nucleic acids include DNA and RNA.

[0073] Beneficial effects of the present invention

[0074] The present invention targets the marketed chimeric human-mouse antibody cetuximab, utilizing a de-immunization humanization technology to change the framework regions of the antibody and parts of the immunogenic CDR regions, forming a novel sequence of humanized anti-epidermal growth factor receptor IgG1 subtype antibody molecules. The obtained antibody has lower immunogenicity and higher target binding than cetuximab, thereby achieving stronger biological activity and anti-tumor activity both in vitro and in vivo, with expected lower side effects and better curative effect in clinical applications.

Brief Description of the Drawings

[0075] FIG. 1 shows the comparison of the affinity of an antibody for an epidermal growth factor receptor on a cell surface;

[0076] FIG. 2 shows the comparison of an antibody blocking the binding of an EGF ligand to an epidermal growth factor receptor on a cell surface, with the vertical axis representing an OD450 values;

[0077] FIG. 3 shows the comparison of antibody inhibition of epidermal growth factor receptor phosphorylation on the surface of A431 cells;

[0078] FIG. 4 shows the measurement of antibody-dependent cell-mediated cytotoxicity (ADCC) activity;

[0079] FIG. 5 shows the dendritic cell-T cell activation assay for measuring the in vitro immunogenicity of the antibody; and

[0080] FIG. 6 shows the inhibitory effect of an antibody on the growth of human epidermal carcinoma A431 in a mouse tumor model.

Detailed Description

[0081] The following will provide a detailed description of the examples of the present invention in conjunction with examples. However, those skilled in the art will understand that the following examples are merely illustrative of the present invention and should not be construed as limiting the scope of the present invention. For conditions not specified in the examples, conventional conditions or those recommended by the manufacturer are followed. Reagents or instruments used without specified manufacturers are all conventional products available for purchase on the market.

[0082] Example 1: Design of the sequence of the heavy chain variable region of the humanized monoclonal antibody against epidermal growth factor receptor

[0083] The following is a sequence of the heavy chain variable region of the mouse anti-M225, with the underlined parts representing the FR1, FR2, FR3, and FR4 framework sequences FI:

<u>QVQLKQSGPGLVQPSQSLSITCTVSGFSLT</u>NYGVH<u>WVRQSPGKGLEWLG</u>VIWSGGNTDYNTPFTS<u>RLSINKDNSKSQVFFKMNSLQSNDTAIYYCAR</u>ALTYYDYEFAY<u>WGQGTLVTVSA</u> (SEQ ID NO: 17).

[0084] Based on the comparison between the FR1, FR2, FR3, and FR4 sequences of the heavy chain variable region and a human antibody gene sequence library, the corresponding sequences of human germline antibody variable regions that are similar to the FR1, FR2, FR3, and FR4 sequences of the mouse anti-M225 heavy chain variable region were found out. Then, NetMHCIIpan software or self-developed software was used to analyze the affinity of similar sequences with HLA-DR molecules through in silico methods, the framework sequence with the lowest affinity was selected to ultimately establish the FR1, FR2, FR3, and FR4 sequences of the humanized heavy chain variable region. Based on this framework structure, a computer molecular model was used to analyze the three-dimensional space structure of the variable region of the murine 225 antibody and analyze the murine 225 antibody corresponding framework amino acid residues L29, L48, and L67 required to be retained to support the CDR conformation. For the de-immunization of CDR1 and CDR3 with higher affinity for HLA-DR molecules, molecular docking was used to analyze the binding mode of the target site of the epidermal growth factor receptor antigen murine M225 antibody and its surrounding amino acid residues and the three-dimensional space of the humanized heavy chain variable region. By calculating electrostatic forces, salvation, van der Waals forces, and entropy values (Sircar et al. SnugDock: paratope structural optimization during antibody-antigen docking compensates for errors in antibody homology models, PLoS Comput Biol. 2010 Jan 22; 6), amino acids in the CDR1 and CDR3 sequences that can be optimized were selected. The 33rd position Gly in CDR1 was optimized to amino acid Asp, and the 100th position Thr in CDR3 was optimized to amino acid Asp, to reduce the affinity for binding to HLA-DR molecules and to improve the binding of the antibody CDR3 region to the epidermal growth factor receptor antigen epitope. The newly designed heavy chain CDR1 is: NYDVH (SEQ ID NO: 1); and the newly designed heavy chain CDR3 sequences are ALDYYDYEFAY (SEQ ID NO: 2) and ALTYYDYDFAY (SEQ ID NO: 3). Due to the low affinity of CDR2 for HLA-DR molecule binding, the original CDR2 sequence of the murine anti-M225 is retained (its sequence is VIWSGGNTDYNTPFTS, SEQ ID NO: 4). Therefore, by integrating and optimizing the framework sequence and CDR sequence, four novel anti-epidermal growth factor receptor humanized antibodies were obtained. The variable region sequence of the heavy chain of the source monoclonal antibody is:

[0085]

QVQLQESGPGLVKPSETLSLTCTVSGFSLSNYDVHWVRQAPGKGLEWLGVIWSGGNTDYNTPFTSRLTISVDTSKNQFSLKLSSVTAADTAVYYCARALDYYDYEFAYWGQGTLVTVSS (SEQ ID NO: 5);

[0086]
QVQLQESGPGLVKPSETLSLTCTVSGFSLTNYDVHWIRQPPGKGLEWIGVIWSGGNTDYNTPFTSRVTISVDTSKNQFSLKLSSVTAADTAVYYCARALTYYDYEFAYWGQGTLVTVSS (SEQ ID NO: 6);

[0087]
EVQLVESGGGLVQPGGSLRLSCAASGFSLTNYGVHWVRQAPGKGLEWLGVIWSGGNTDYNTPFTSRLTISKDNSKNTLYLQMNSLRAEDTAVYYCARALTYYDYDFAYWGQGTLVTVSS (SEQ ID NO: 7);

[0088]
QVQLKESGPGLVAPSQSLSITCTVSGFSLTNYGVHWVRQPPGKGLEWLGVIWSGGNTDYNTPFTSRLSISKDNSKSQVFLRMYSLQTDDTARYYCARALTYYDYEFAYWGQGTLVTVSS (SEQ ID NO: 8).

[0089] The serial number of each segment of sequence in the above sequences is as follows:

[0090] QVQLQESGPGLVKPSETLSLTCTVSGFSLS (SEQ IDNO: 19)

[0091] QVQLQESGPGLVKPSETLSLTCTVSGFSLT (SEQ IDNO: 20)

[0092] EVQLVESGGGLVQPGGSLRLSCAASGFSLT (SEQ ID NO: 21)

[0093] QVQLKESGPGLVAPSQSLSITCTVSGFSLT (SEQ ID NO: 22)

[0094] WVRQAPGKGLEWLG (SEQ ID NO: 23)

[0095] WIRQPPGKGLEWIG (SEQ ID NO: 24)

[0096] WVRQPPGKGLEWLG (SEQ ID NO: 25)

[0097] RLTISVDTSKNQFSLKLSSVTAADTAVYYCAR (SEQ ID NO: 26)

[0098] RVTISVDTSKNQFSLKLSSVTAADTAVYYCAR (SEQ ID NO: 27)

[0099] RLTISKDNSKNTLYLQMNSLRAEDTAVYYCAR (SEQ ID NO: 28)

[0100] RLSISKDNSKSQVFLRMYSLQTDDTARYYCAR (SEQ ID NO: 29)

[0101] WGQGTLVTVSS (SEQ ID NO: 30)

[0102] NYGVH (SEQ ID NO: 39)

[0103] ALTYYDYEFAY (SEQ ID NO:40).

[0104] Example 2 Design of the sequence of the variable region of the humanized monoclonal antibody light chain against epidermal growth factor receptor

[0105] The following is a sequence of the variable region of the light chain of the mouse anti-M225, with the underlined parts being the FR1, FR2, FR3, and FR4 framework sequences:

[0106] DILLTQSPVILSVSPGERVSFSCRASQSIGTNIHWYQQRTNGSPRLLIKYASESISGIPSRFSGSGSGTDFTLSINSVESEDIADYYCQQNNNVPTTFGAGTKLELK (SEQ ID NO: 18).

[0107] Similarly, the sequence of the light chain variable region of the mouse anti-225 antibody was analyzed. Based on the comparison between the FR1, FR2, FR3, and FR4 sequences of its light chain variable region and the human antibody gene sequence library, the corresponding sequences of the human germline antibody variable regio that are similar to the FR1, FR2, FR3, and FR4 sequences of the mouse anti-M225 light chain variable region were found out. Then, NetMHCIIpan software or self-developed software was used to perform in silico analysis of the affinity of binding the selected similar sequences to HLA-DR molecules, and the framework sequence with the lowest affinity was selected to ultimately establish the humanized sequences of FR1, FR2, FR3, and FR4 of the light chain variable region. Based on this framework structure, a computer molecular model was used to analyze the spatial structure of the variable region of the murine 225 antibody to analyze the murine antibody corresponding framework amino acid residues 158 required to be retained to support the CDR conformation. For the de-immunization of CDR3 with higher affinity for HLA-DR molecule binding, molecular docking was used to analyze the binding mode of the target site of the epidermal growth factor receptor antigen murine M225 antibody and its surrounding amino acid residues and the three-dimensional space of the humanized heavy chain variable region. By calculating electrostatic forces, solution mediation, salvation, van der Waals forces, and entropy values (Sircar et al., SnugDock: paratope structural optimization during docking compensates for errors in antibody homology models, PLoS Comput Biol. 2010 Jan 22; 6), amino acids in the CDR3 sequence that can be optimized were selected, Asn at position 93 in CDR3 was optimized to amino acid Glu, and Thr at position 97 was optimized to amino acid

Ser, to reduce the affinity for binding to HLA-DR molecules and to improve the binding of the antibody CDR3 region to the positively charged amino acids adjacent to the epidermal growth factor receptor antigen epitope. The newly designed light chain CDR3 is: QQNNEWPTS (SEQ ID NO: 9) and QQNNDWPTT (SEQ ID NO: 10). Since the light chain CDR1 and CDR2 have lower affinity for binding to HLA-DR molecules, the original mouse anti-M225 CDR1 (with sequence RASQSIGTNTH, SEQ ID NO: 11) and CDR2 sequence (with sequence YASESIS, SEQ ID NO: 12) are retained. Therefore, by integrating the optimized framework sequence and CDR sequence, the sequences of the obtained novel anti-surface epidermal growth factor receptor humanized monoclonal antibody light chain variable region are:

[0108] EIVLTQSPDFQSVTPKEKVTITCRASQSIGTNIHWYQQKPDQSPKLLIKYASESISGIPSRFSGSGSGT DFTLTINSLEAEDAATYYCQQNNEWPTSFGQGTKLEIK (SEQ ID NO: 13);

[0109] EIVLTQSPDFQSVTPKEKVTITCRASQSIGTNIHWYQQKPDQSPKLLIKYASESISGIPSRFSGSGSGT DFTLTINSLEAEDAATYYCQQNNNWPTTFGAGTKLEIK (SEQ ID NO: 14)

[0110] DIVLTQSPATLSVTPGESVSFSCRASQSIGTNIHWYQQKSHESPRLLIKYASESISGIPSRFSGSGSGT DFTLSINSVETEDFGMYYCQQNNNWPTTFGAGTKLEIK (SEQ ID NO: 15)

[0111] EIVLTQSPDFQSVTPKEKVTITCRASQSIGTNIHWYQQKPDQSPKLLIKYASESISGIPSRFSGSGSGT DFTLTINSLEAEDAATYYCQQNNDWPTTFGQGTKLEIK (SEQ ID NO: 16).

[0112] The serial number of each segment of sequence in the above sequences are as follows:

[0113] EIVLTQSPDFQSVTPKEKVTITC (SEQ ID NO: 31)

[0114] DIVLTQSPATLSVTPGESVSFSC (SEQ ID NO: 32)

[0115] WYQQKPDQSPKLLIK (SEQ ID NO: 33)

[0116] WYQQKSHESPRLLIK (SEQ ID NO: 34)

[0117] GIPSRFSGSGSGTDFTLTINSLEAEDAATYYC (SEQ ID NO: 35)

[0118] GIPSRFSGSGSGTDFTLSINSVETEDFGMYYC (SEQ ID NO: 36)

[0119] FGQGTKLEIK (SEQ ID NO: 37)

[0120] FGAGTKLEIK (SEQ ID NO: 38)

[0121] QQNNNWPTT (SEQ ID NO: 41).

[0122] The humanized antibodies obtained according to the above examples have the following combinations:

[0123] Table 1. Humanized anti-EGFR antibody combinations

[0124]

Antibody name	**Antibody heavy chain variable region**	**Antibody light chain variable region**
BA03	**SEQ ID NO: 5**	**SEQ ID NO: 13**
BB03	**SEQ ID NO: 6**	**SEQ ID NO: 14**
BC03	**SEQ ID NO: 7**	**SEQ ID NO: 15**
BD03	**SEQ ID NO: 8**	**SEQ ID NO: 16**

[0125] Example 3 Preparation of humanized monoclonal antibody against epidermal growth factor receptor

[0126] (1) According to the heavy chain variable region sequence and light chain variable region sequence determined in Example 1 and 2, PCR primer oligonucleotide fragments of the heavy chain and light chain variable region sequences were respectively designed, combined and coded, and necessary restriction sites were introduced during synthesis. PCR primer oligonucleotide fragments are generally about 54 bases long, with approximately 18 bases overlapping between adjacent oligonucleotide fragments. Equal amount of each PCR primer fragment was mixed to perform an overlapping extension PCR reaction.

[0127] PCR reaction system: dNTPs 0.2 μM (final concentration); each PCR primer fragment 1 μl; 10x buffer 3 μl; cloned pfu (Invitrogen) 1 μl: add water to 30 μl.

[0128] PCR reaction conditions: 94°C 3 min → (94°C 30 s → 56°C 30 s → 72°C 1 min) x 30 → 72°C 10 min

[0129] After the PCR product was separated by 1% agarose gel electrophoresis, the target fragment was recovered and purified. After EcoRI digestion, the heavy chain variable region sequence and the light chain variable region sequence of the antibody were cloned into the pCR-BluntII-TOPO (Invitrogen) vector, respectively, and then were transformed into Escherichia coli TOP10 (Invitrogen) and screened on LB/Kanamycin plates. Ten white plaques were inoculated into an LB liquid medium including Kanamycin for culture. Plasmids were extracted using aa QIAGEN plasmid extraction kit (QIAquick PCR purification kit) and sequenced to determine the heavy chain and light chain variable region sequences.

[0130] (2) Construct expression vector

[0131] RNA was isolated from normal human B cells, and the human IgG1 heavy chain constant region IgG1Fc fragment and light chain constant region kappa fragment were obtained by the RT-PCR method. During RT-PCR, the required restriction sites were introduced and pre-constructed into the pcDNA3.1 (Invitrogen) expression vector to obtain the pcDNA3.1-Fc and pcDNA3.1-kappa expression vectors, respectively. After transformation with DH5 bacteria, plasmid extraction and sequencing were performed to determine positive clones.

[0132] The sequences of the human IgG1 heavy chain constant region IgG1Fc fragment are:

[0133]

ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVT
VPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPE
VTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVITVLHQDWLNGKEYKCKVSN
KALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPP
VLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 42)

[0134] The sequence of the light chain constant region κ fragment is:

[0135]

RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLS
STLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC (SEQ ID NO: 43)

[0136] The heavy chain variable region of the antibody was excised from the positive clone of pCR-BluntII-TOPO by using Eco47III/NheI, and cloned into the pcDNA3.1-Fc expression vector, allowing the heavy chain variable region to connect with the constant region. The light chain variable region of the antibody was excised from the positive clone of pCR-BluntII-TOPO by using AscI/BsiWI, and cloned into the pcDNA3.1-kappa expression vector, allowing the light chain variable region to connect with the constant region (refer to Morrison SL. Cloning, expression, and modification of antibody V regions. Curr Protoc Immunol. 2002 May; Chapter 2: Unit 2.12.). After transformation with DH5 bacteria, plasmid extraction and sequencing were performed to determine positive clones. The sequencing results were consistent with the recorded coding sequence of the antibody, thereby obtaining the expression vectors for the antibody heavy chain and light chain, respectively.

[0137] (3) Transfection of CHO cells and specific antibody expression

[0138] Cell line and culture conditions: CHO-S cells (purchased from Invitrogen) were cultured in 1x CD-CHO (purchased from GIBCO), 1x HT (purchased from GIBCO), 8 mM glutamine (purchased from GIBCO), at 37°C in an 8% CO_2 incubator. The antibody heavy chain expression vector and light chain expression vector obtained in step (2) were co-transfected into CHO-S cells by using the DMRIE-C transfection reagent kit (purchased from Invitrogen), following the instructions provided in the kit manual. On the third day after transfection, the cells were cultured in the above medium with the addition of 500 µg/ml G418 (purchased from GIBCO) and 12.5 µg/ml puromycin (purchased from Sigma) for pressurization and screening. After about 14 days of pressurization, the selected positive polyclones were cultured in a six-well plate, and the specific antibody expression levels were detected by using the direct ELISA method (coated with EGFR-Fc from expressed 293T cells). The positive polyclones with the highest expression rates were selected and cultured in large batches for 7 days, and the culture supernatant was collected by centrifugation. After purification using a Protein A affinity chromatography column

(purchased from GE), the antibodies were dialyzed to PBS and filtered through a 0.22 μm membrane, and the purified antibodies BA03, BB03, BCO3, and BD03 were obtained for use in the following examples.
[0139] Example 4 Flow cytometry detection of antibody affinity activity with epidermal growth factor receptor on cell surface
[0140] A431 cells (ATCC) were used to detect the specific affinity of the expressed antibody to the epidermal growth factor receptor. A431 cells bound to the product antibody at different concentrations, at 4°C for 1 hour, and were washed three times with PBS, 100-fold diluted FITC-labeled anti-human Fcy specific secondary antibody (purchased from The Jackson Laboratory) was added, at 4°C for 1 hour. After washing three times with PBS, a flow cytometer was used to detect the bound fluorescent signal (FIG. 1). The EC50 (half-maximal effective concentration) value was calculated by using Prism software based on the mean fluorescence intensity, and results are shown in Table 2. The results indicate that the humanized antibodies BA03 and BD03 of the present invention have higher binding activity than Erbitux (purchased from Biotech Pharmaceuticals Co., Ltd.), while BB03 and BC03 are similar to Erbitux. The binding activity of all four antibodies is stronger than that of Nimotuzumab (purchased from Merck KgaA).
[0141] Table 2 EC50 Values of Antibody Binding Activity with EGFR
[0142]

Antibody	EC50 (μg/ml)
BA03	0.21
BB03	0.25
BC03	0.32
BD03	0.23
Erbitux	0.28
Nimotuzumab	0.42

[0143] Example 5 Antibody blocking EGF binding to cell surface epidermal growth factor receptor
[0144] The A431 cell (ATCC) Cell-ELISA method (please refer to the Bioo Scientific MaxDiscovery in Cell ELISA kit manual) was used to detect the ability of the expressed antibody to block EGF binding to the cell surface epidermal growth factor receptor. After incubating different concentrations of antibody with A431 cells at 4°C for 1 hour, a biotin-labeled recombinant human epidermal growth factor EGF (purchased from R&D) was added and washed three times with PBS. Then HRP-labeled avidin was used to detect the biotin-labeled recombinant human epidermal growth factor that binds to the epidermal growth factor receptor on the cell surface (FIG. 2). Based on the measured OD450 values, Prism software was used to calculate the IC50 (half maximal inhibitory concentration) values, as shown in Table 3. The results indicate that the humanized antibodies BA03 and BD03 of the present invention have stronger activity in blocking the ligand from binding to the epidermal growth factor receptor on the cell surface compared to Erbitux, while BB03 and BC03 are similar to Erbitux. The blocking activity of the four antibodies is stronger than that of Nimotuzumab.
[0145] Table 3 IC50 values for antibody blocking activity of EGF binding to EGFR
[0146]

Antibody	IC50 (μg/ml)
BA03	2.31

BB03	4.38
BC03	3.59
BD03	2.91
Erbitux	3.25
Nimotuzumab	8.25

[0147] Example 6 Antibody inhibition of epidermal growth factor receptor phosphorylation on A431 cell surface

[0148] 5x10^5 cells/well of A431 cells were inoculated in a 6-well plate and cultured overnight with complete medium (DMEM + 10% FBS). 2 ml DMEM was used for culture, starving the cells for 16-18 hours. 1 ml of pre-warmed fresh medium (DMEM, without 10% FBS) was used for culture for 1-2 hours. 5 μg/ml of anti-epidermal growth factor receptor antibody was added to A431 cells 60 minutes before stimulation with 50 ng/ml recombinant EGF (epidermal growth factor, purchased from R&D Systems). The cells were washed 3 times with pre-cooled 1x PBS, 250 μl of cell lysis buffer (purchased from Cell Signalling) was added for lysis, centrifugation was performed at 14,000 rpm, 4°C, for 15 minutes, the lysate was collected to quantify protein by using a BCA assay kit. Through the western blot method, the phosphorylation of the epidermal growth factor receptor was measured with an anti-pEGFR (pY1068) antibody (purchased from Abnova), and the total epidermal growth factor receptor content was measured with an anti-total epidermal growth factor receptor antibody (purchased from Abnova). The results indicate that the humanized antibody BA03 of the present invention has stronger activity in inhibiting the phosphorylation of the epidermal growth factor receptor on the surface of A431 cells compared with Erbitux. Both are stronger than Nimotuzumab (FIG. 3).

[0149] Example 7 In vitro antibody-dependent cell-mediated cytotoxicity (ADCC) experiment

[0150] Blood obtained from normal volunteers was inhaled into heparinized syringes, mixed with an equal volume of Ca^{2+}/Mg^{2+}-free HBSS (Life Technologies), layered over a lymphocyte gradient separation agent (Life Technologies), and centrifuged at 800 g for 30 minutes. The PBMC at the interface was collected, washed in HEPES-buffered saline, and resuspended in an assay medium, that is, a RPMI 1640 medium (Life Technologies) including 1% heat-inactivated FBS (HyClone Laboratories, Logan, UT), 2 nM L-glutamine, 10 mM HEPES, and 50 mg/mL gentamicin. Ramos cells (ATCC) (10^4 cells/well) placed in 50 mL of analytical buffer and various concentrations of antibodies (in 50 mL of assay buffer) were added into a round-bottom 96-well culture plate. This mixture was pre-cultured at 37°C for 30 minutes. Then 50 μL of 5 x 10^5 effector cells were distributed into each well and incubated at 37°C for 4 hours. The experiment was performed by using an effector cell (PBMC) to target cell (Ramos cell) ratio of 40:1. The culture plate was centrifuged at 250 g for 10 minutes, and then the supernatant was collected. The cytotoxicity detection kit (Boehringer Mannheim, Indianapolis, IN) and the manufacturer's instructions were used to detect lactate dehydrogenase activity in the supernatant, and the percentage of toxicity was calculated based on the average absorbance of the samples. Specific lysis rate = (average experimental release - average spontaneous release) / (average maximum release - average spontaneous release). The results are shown in FIG. 4. The humanized antibodies BA03 and BD03 of the present invention have higher ADCC activity than Erbitux (cetuximab) at 0.l μg/ml and l μg/ml, both higher than Erbitux ($p < 0.05$, Student's t-test), while BB03 and BC03 are similar to Erbitux.

[0151] Example 8 Dendritic cell-T cell activation experiment to determine in vitro immunogenicity of antibody

[0152] Blood obtained from normal volunteers (n = 12) was inhaled into heparinized syringes, mixed with the same volume of HBSS not including Ca^{2+}/Mg^{2+} (Life Technologies), layered on a lymphocyte gradient separation agent (Life Technologies), and centrifuged at 800 g for 30 minutes. The peripheral blood mononuclear cells at the interface were collected, washed in HEPES buffered saline, and resuspended in an assay medium RPMI 1,640 medium (Life Technologies). The obtained peripheral blood mononuclear cells continued adherent culture in 50 ml of serum-free AIM V medium (Gibco) with 1:100 diluted beta-mercaptoethanol for 2 hours to harvest monocytes. 800 units/ml of GM-CSF (Endogen) and 500 units/ml of IL-4 (Endogen) were added to continue culture for 5 days. Then 0.2 units/ml of TNF α (Endogen) and a final concentration of 50 units/ml of IL-1α (Endogen) were added to continue culture for 2 days. On the 7th day, 50 mg/ml of Mitomycin C was added for an hour to stop the further differentiation of dendritic cells, and then centrifugation was performed at 600 g to collect the differentiated mature dendritic cells. The differentiated mature dendritic cells were inoculated at 2 x 10^4 cells/well (100 μl) in a round-bottom 96-well culture plate. $CD4^+$Cellect Kit (Biotex) was used for negative selection to collect human $CD4^+$T cells in AIMV medium including 2% human serum. In a 96-well culture plate including 100 μl of dendritic cells (1 x 10^4/ml), 10 μl of detection antibody at a concentration of 5 mM was added (DMSO was added in the control group), then 100 μl of $CD4^+$T cells (1 x 10^5/ml) were added to each well. The proliferation quantity of activated T cells was measured by using the alamarBlue reagent (refer to the alamarBlue Assay Kit manual from Invitrogen for the measurement method). The results were converted into T-cell activation index (calculation method: T-cell activation index = T-cell proliferation number in the antibody group / T-cell proliferation number in the DMSO group, refer to the literature Stickler MM et al. A human dendritic cell-based method to identify CD4+ T-cell epitopes in potential protein allergens. Environ Health Perspect. 2003 February; 111 (2): 251-254.) The results are shown in FIG. 5. The four humanized antibodies of the present invention have lower immunogenicity than the chimeric antibody Erbitux, with BA03 and BB03 showing a significant reduction compared to Erbitux ($p < 0.05$, Student's t-test).

[0153] Example 9 The in vitro anti-tumor activity of BA03 on tumor cells of lung cancer, colon cancer, pancreatic cancer, and breast cancer

[0154] For the in vitro anti-tumor activity of BA03 on tumor cells of lung cancer, colon cancer, pancreatic cancer and breast cancer, the ADCC activity and in vitro growth inhibition effect on each tumor cell were discussed, respectively. The method for measuring ADCC activity is the same as in Example 7. The method for measuring in vitro growth inhibition is as follows: 300-2,000 different tumor cells per well (the inoculation cell density determined by the characteristics of different tumor cells) were inoculated in a 96-well plate, BA03 at the final centration of 10 μg/ml and IgG1 control antibody (purchased from R&D Systems) were added in a complete medium to culture for 5-10 days. Then, alamarBlue was added to measure cell activity (refer to the alamarBlue Assay Kit manual from Invitrogen for the measurement method). Compared with IgG1, the relative cell viability (% ADCC) and cell growth inhibition rate were calculated. Each tumor cell line growth inhibition experiment was repeated at least twice (three replicates). After the antibody was added to the tumor cells, the PE fluorescence-labeled secondary antibody (PE-labeled anti-human IgG1, Southern Biotechnology), and then FACS was used to measure the EGFR expression level. As shown in Table 4, the humanized antibody BA03 of the present invention has a significant inhibitory effect on the growth of various lung cancer, colon cancer, pancreatic cancer, and breast cancer tumor cells (Table 4).

[0155] Table 4: In vitro activity of BA03 on different tumor cells

[0156]

Tumor type	**Tumor cell line**	**EGFR expression level**	**% ADCC**	**Tumor cell growth Inhibition rate**

[0157]

Lung cancer	**H460**	+	**14%**	+
	A549	++	**28%**	++
	H292	++	**18%**	++
Colon cancer	**DLD-1**	++	**27%**	+
	HT-29	++	**29%**	+/-
Pancreatic cancer	**BXPC3**	++	**17%**	++
	HPAC	+	**15%**	+
Breast cancer	**BT474**	+/-	**3%**	+/-
	SKBr3	+	**9%**	+/-
	BT20	+++	**22%**	++
	MDA-MB468	+++	**18%**	+
	HCC1937	++	**21%**	++

[0158] Inhibition ratio: +/-5-10%，+10-20%，++20-40%

[0159] Example 10 Animal pharmacodynamics experiment

[0160] Balb/c nude mice were administrated with a subcutaneous injection of human skin basal cell carcinoma A431 cells (5 x 10^6 cells/0.1 ml/per mouse) on day 0. The size of the tumor was measured with a caliper, and the tumor volume was calculated by using the formula (length x width^2) / 2. When the average tumor size reached 100 mm^3 (61-158 mm^3), the tumor-bearing mice were randomly divided into 3 groups based on tumor size. Intraperitoneal administration was performed at 5 mg/kg every Monday and Thursday for two weeks, for a total of 5 doses. Saline (served as a negative control). As shown in FIG. 6, the tumor volume in the saline control group was rapidly increased to > 1,500 mm^3 within three weeks. The growth of tumors in mice treated with the humanized antibody BA03 of the present invention and Erbitux was significantly inhibited compared to the control group ($p < 0.001$, Student's t-test). However, the humanized BA03 antibody has better anti-tumor activity than Erbitux ($p < 0.05$, Student's t-test).

[0161] Although the specific examples of the present invention have been described in detail, those skilled in the art will understand. Based on all the teachings disclosed, various modifications and substitutions can be made to those details, and these changes are within the scope of protection of the present invention. All the scope of the present invention is given by the appended claims and any equivalents thereof.

[0001]

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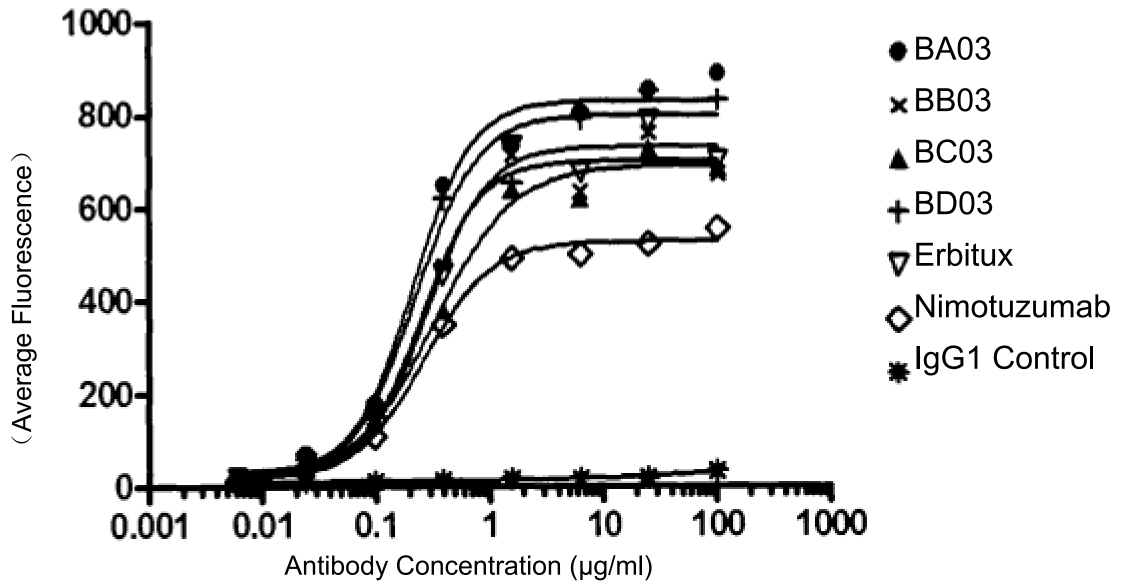
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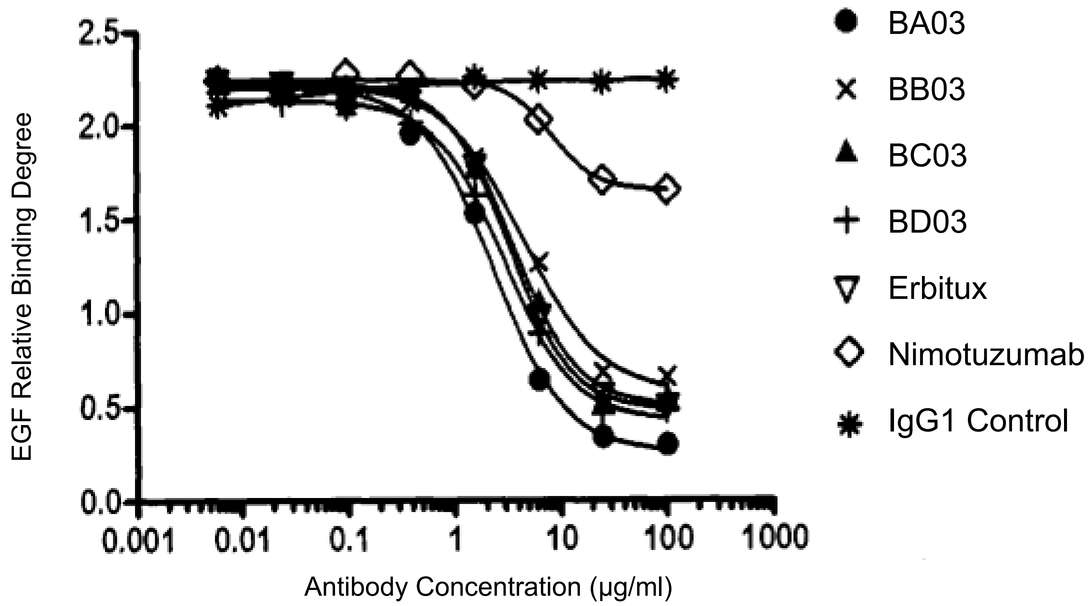
IgG1 Control

FIG. 1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10
100
1000
Antibody Concentration (μg/ml)
BA03
BB03
BC03
BD03
Erbitux
Nimotuzumab
IgG1 Control

FIG. 2

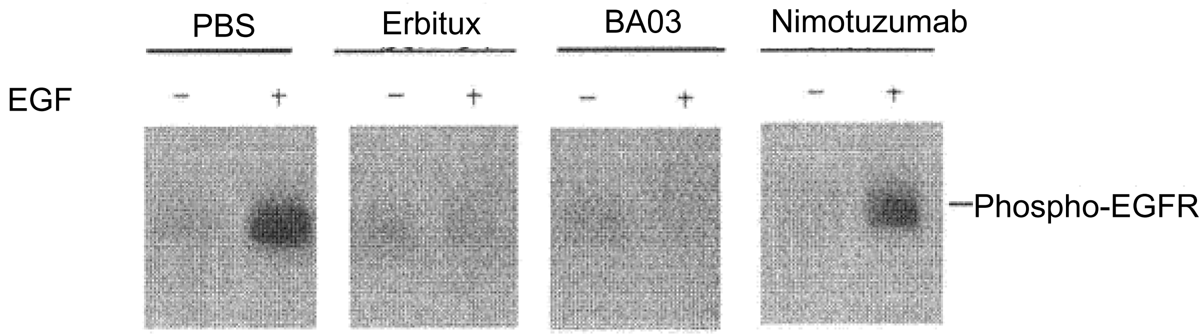
PBS
Erbitux
BA03
Nimotuzumab
EGF
−
+
Phospho-EGFR

FIG. 3

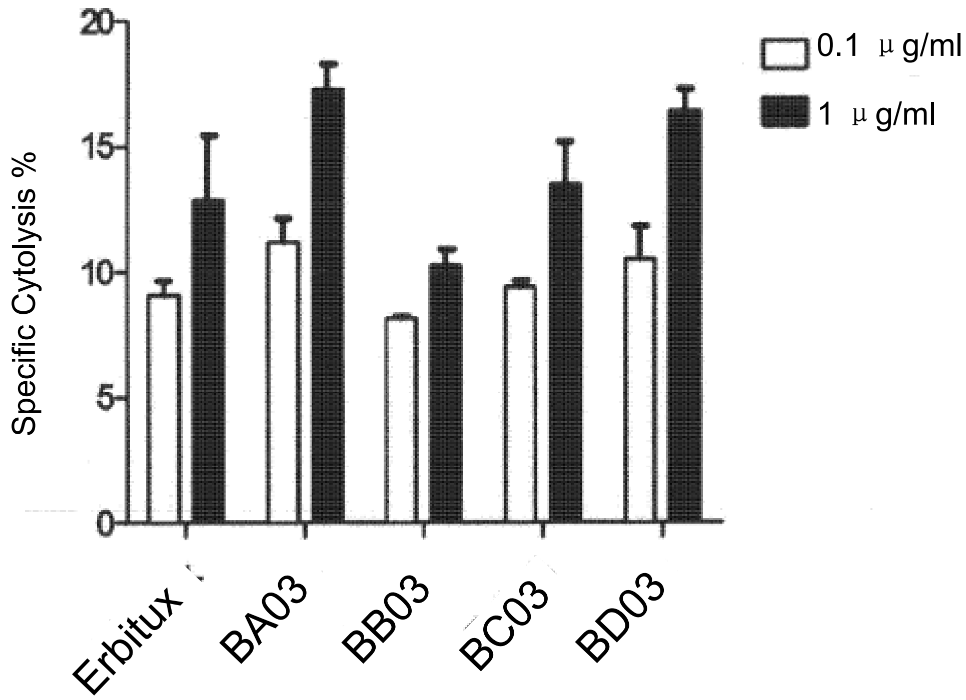
20
15
10
5
0
Specific Cytolysis %
0.1 μg/ml
1 μg/ml
Erbitux
BA03
BB03
BC03
BD03

FIG. 4

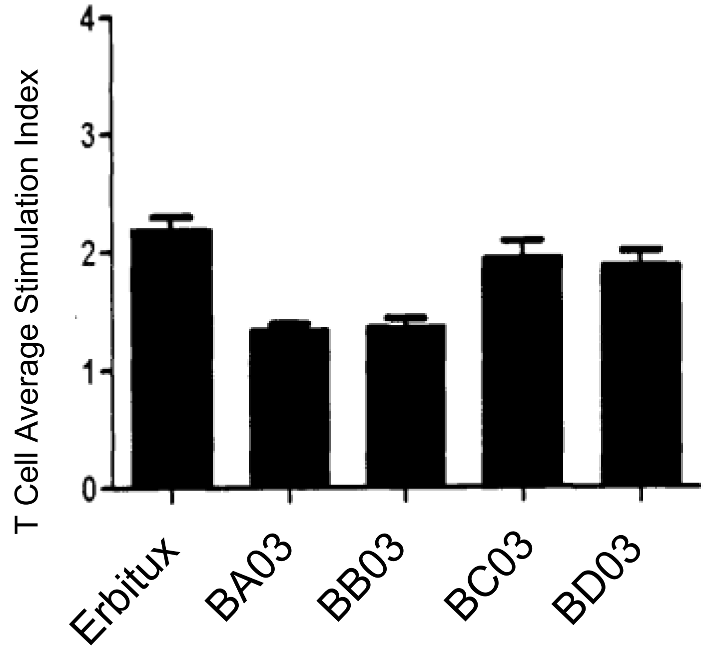
T Cell Average Stimulation Index
0
1
2
3
4
Erbitux
BA03
BB03
BC03
BD03

FIG. 5

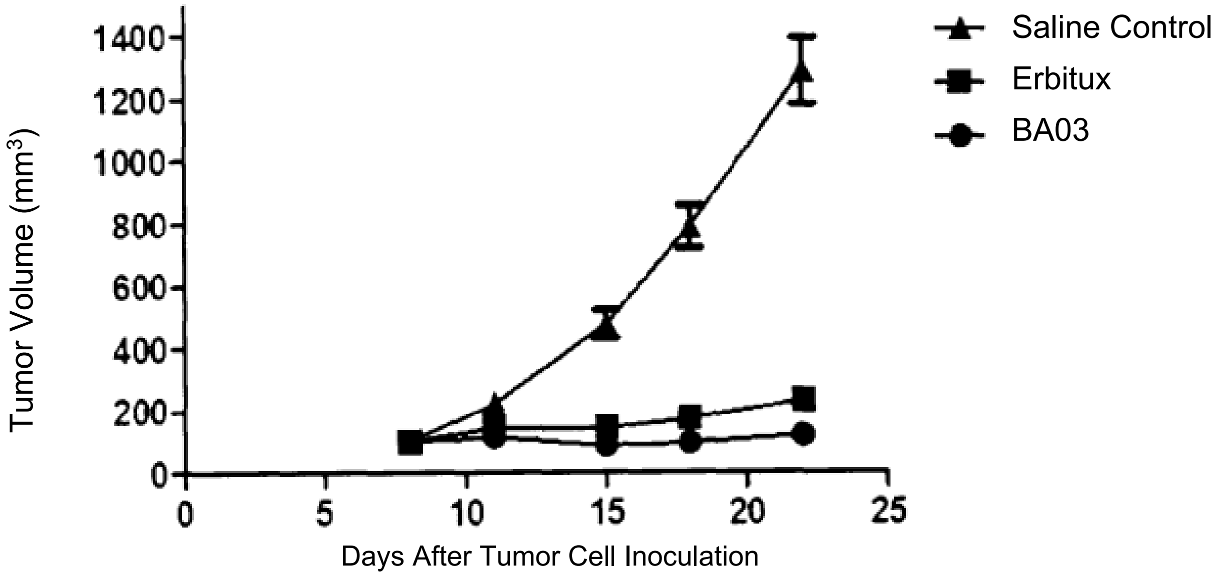
Tumor Volume (mm³)
0
200
400
600
800
1000
1200
1400
0
5
10
15
20
25
Days After Tumor Cell Inoculation
Saline Control
Erbitux
BA03

FIG. 6

MORNINGSIDE
A Questel Company

TRANSLATION CERTIFICATION

Date: January 5, 2025

To whom it may concern:

I, Aselin, a translator fluent in the Chinese Simplified and English (US) languages, on behalf of Morningside, do solemnly and sincerely declare that the following is, to the best of my knowledge and belief, a true and correct translation of the document(s) listed below in a form that best reflects the intention and meaning of the original text.

The document is designated as:

- BA03_CN103772504A (163176812.1)

Aselin

Signature

Aselin

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