

# **TUMOR-SPECIFIC ANTI-EGFR ANTIBODY AND APPLICATION THEREOF**

## **Technical field**

5 The present invention relates to the field of immunology, and in particular, the present invention relates to tumor-specific anti-EGFR antibodies and uses thereof.

## **Background**

10 EGFR is overexpressed or mutated in many tumors, and it is undoubtedly a very important scientific issue on how to selectively recognize these over-expressed or mutated EGFR. Until now, antibodies against EGFR287-302 epitope are believed to achieve the purpose of recognizing EGFR, EGFRvIII and de4 EGFR overexpressed on the surface of tumors, instead of EGFR in normal cells. Unfortunately, antibodies against this epitope still have side effects such as rashes in clinical trials (<http://meetinglibrary.asco.org/content/115945-132>), suggesting that targeting this epitope may  
15 identify EGFR in normal cells (such as keratinocytes).

Therefore, it is very urgent to screen anti-EGFR antibodies with higher tumor-specificity. Highly tumor-specific antibodies, whether for tumor imaging diagnosis, individual diagnosis or tumor targeting therapy, have a very large potential value.

## **Summary of the invention**

20 The object of the present invention is to provide tumor-specific anti-EGFR antibodies and uses thereof.

In a first aspect of the present invention, an antibody specifically recognizing EGFRvIII expressed or EGFR overexpressed by tumor cells is provided, wherein the antibody comprises a  
25 light chain variable region and a heavy chain variable region,

CDR1 of the light chain variable region has an amino acid sequence selected from a group consisting of SEQ ID NO: 41, SEQ ID NO: 47, SEQ ID NO: 55;

CDR2 of the light chain variable region has an amino acid sequence selected from a group consisting of SEQ ID NO: 42, SEQ ID NO: 53;

30 CDR3 of the light chain variable region has an amino acid sequence selected from a group consisting of SEQ ID NO: 43, SEQ ID NO: 48, SEQ ID NO: 54, SEQ ID NO: 56, SEQ ID NO: 57;

CDR1 of the heavy chain variable region has the amino acid sequence of SEQ ID NO: 44;

CDR2 of the heavy chain variable region has an amino acid sequence selected from a group consisting of SEQ ID NO: 45, SEQ ID NO: 49, SEQ ID NO: 51, SEQ ID NO: 52;

35 CDR3 of the heavy chain variable region has an amino acid sequence selected from a group consisting of SEQ ID NO: 46, SEQ ID NO: 50.

In a preferred embodiment, the antibody includes:

antibody (a), wherein the light chain variable region thereof has CDR1 of SEQ ID NO: 41, CDR2 of SEQ ID NO: 42, CDR3 of SEQ ID NO: 43, or the heavy chain variable region thereof has CDR1 of SEQ ID NO: 44, CDR2 of SEQ ID NO: 45, CDR3 of SEQ ID NO: 46,

5 antibody (b), wherein the light chain variable region thereof has CDR1 of SEQ ID NO: 47, CDR2 of SEQ ID NO: 42, CDR3 of SEQ ID NO: 48, or the heavy chain variable region thereof has CDR1 of SEQ ID NO: 44, CDR2 of SEQ ID NO: 49, CDR3 of SEQ ID NO: 50,

antibody (c), wherein the light chain variable region thereof has CDR1 of SEQ ID NO: 41, CDR2 of SEQ ID NO: 42, CDR3 of SEQ ID NO: 48, or the heavy chain variable region thereof  
10 has CDR1 of SEQ ID NO: 44, CDR2 of SEQ ID NO: 51, CDR3 of SEQ ID NO: 50,

antibody (d), wherein the light chain variable region thereof has CDR1 of SEQ ID NO: 41, CDR2 of SEQ ID NO: 42, CDR3 of SEQ ID NO: 43, or the heavy chain variable region thereof has CDR1 of SEQ ID NO: 44, CDR2 of SEQ ID NO: 52, CDR3 of SEQ ID NO: 50,

antibody (e), wherein the light chain variable region thereof has CDR1 of SEQ ID NO: 41, CDR2 of SEQ ID NO: 42, CDR3 of SEQ ID NO: 43, or the heavy chain variable region thereof  
15 has CDR1 of SEQ ID NO: 44, CDR2 of SEQ ID NO: 45, CDR3 of SEQ ID NO: 50,

antibody (f), wherein light chain variable region thereof has CDR1 of SEQ ID NO: 41, CDR2 of SEQ ID NO: 53, CDR3 of SEQ ID NO: 54, or the heavy chain variable region thereof has CDR1 of SEQ ID NO: 44, CDR2 of SEQ ID NO: 51, CDR3 of SEQ ID NO: 50,

20 antibody (g), wherein the light chain variable region has CDR1 of SEQ ID NO: 41, CDR2 of SEQ ID NO: 42, CDR3 of SEQ ID NO: 54, or the heavy chain variable region thereof has CDR1 of SEQ ID NO: 44, CDR2 of SEQ ID NO: 51, CDR3 of SEQ ID NO: 50,

antibody (h), wherein the light chain variable region thereof has CDR1 of SEQ ID NO: 55, CDR2 of SEQ ID NO: 42, CDR3 of SEQ ID NO: 56, or the heavy chain variable region thereof  
25 has CDR1 of SEQ ID NO: 44, CDR2 of SEQ ID NO: 45, CDR3 of SEQ ID NO: 50,

antibody (i), wherein the light chain variable region thereof has CDR1 of SEQ ID NO: 41, CDR2 of SEQ ID NO: 53, CDR3 of SEQ ID NO: 56, or the heavy chain variable region thereof has CDR1 of SEQ ID NO: 44, CDR2 of SEQ ID NO: 52, CDR3 of SEQ ID NO: 50,

antibody (j), wherein the light chain variable region thereof has CDR1 of SEQ ID NO: 41, CDR2 of SEQ ID NO: 42, CDR3 of SEQ ID NO: 56, or the heavy chain variable region thereof  
30 has CDR1 of SEQ ID NO: 44, CDR2 of SEQ ID NO: 52, CDR3 of SEQ ID NO: 50,

antibody (k), wherein the light chain variable region thereof has CDR1 of SEQ ID NO: 41, CDR2 of SEQ ID NO: 42, CDR3 of SEQ ID NO: 57, or the heavy chain variable region thereof has CDR1 of SEQ ID NO: 44, CDR2 of SEQ ID NO: 52, CDR3 of SEQ ID NO: 50; or

35 antibody (l) which recognizes the same antigenic determinant as that recognized by the antibody according to any one of (a) to (k).

In another preferred embodiment, the antibody specifically recognizing EGFRvIII expressed or EGFR overexpressed by tumor cells can be: single chain antibody (scFV), monoclonal antibody, domain antibody, Fab fragment, Fd fragment, Fv fragment, F (ab')<sub>2</sub> fragment and a derivative thereof, or other forms of antibody; preferably single chain antibody.

In another preferred embodiment, the antibody specifically recognizing EGFRvIII expressed or EGFR overexpressed by tumor cells is humanized, fully humanized, chimeric or murine.

In another preferred embodiment, the amino acid sequence of the heavy chain variable region of the antibody is shown in positions 124 to 239 of SEQ ID NO: 13; or the amino acid sequence of the light chain variable region of the antibody is shown in positions 1-108 of SEQ ID NO: 13;

The amino acid sequence of the heavy chain variable region of the antibody is shown in positions 124 to 239 of SEQ ID NO: 59; or the amino acid sequence of the light chain variable region of the antibody is shown in positions 1-108 of SEQ ID NO: 59;

The amino acid sequence of the heavy chain variable region of the antibody is shown in positions 124 to 239 of SEQ ID NO: 61; or the amino acid sequence of the light chain variable region of the antibody is shown in positions 1-108 of SEQ ID NO: 61;

The amino acid sequence of the heavy chain variable region of the antibody is shown in positions 124 to 239 of SEQ ID NO: 63; or the amino acid sequence of the light chain variable region of the antibody is shown in positions 1-108 of SEQ ID NO: 63;

The amino acid sequence of the heavy chain variable region of the antibody is shown in positions 124 to 239 of SEQ ID NO: 65; or the amino acid sequence of the light chain variable region of the antibody is shown in positions 1-108 of SEQ ID NO: 65;

The amino acid sequence of the heavy chain variable region of the antibody is shown in positions 124 to 239 of SEQ ID NO: 67; or the amino acid sequence of the light chain variable region of the antibody is shown in positions 1-108 of SEQ ID NO: 67;

The amino acid sequence of the heavy chain variable region of the antibody is shown in positions 124 to 239 of SEQ ID NO: 69; or the amino acid sequence of the light chain variable region of the antibody is shown in positions 1-108 of SEQ ID NO: 69;

The amino acid sequence of the heavy chain variable region of the antibody is shown in positions 124 to 239 of SEQ ID NO: 71; or the amino acid sequence of the light chain variable region of the antibody is shown in positions 1-108 of SEQ ID NO: 71;

The amino acid sequence of the heavy chain variable region of the antibody is shown in positions 124 to 239 of SEQ ID NO: 73; or the amino acid sequence of the light chain variable region of the antibody is shown in positions 1-108 of SEQ ID NO: 73;

The amino acid sequence of the heavy chain variable region of the antibody is shown in

positions 124 to 239 of SEQ ID NO: 75; or the amino acid sequence of the light chain variable region of the antibody is shown in positions 1-108 of SEQ ID NO: 75; or

The amino acid sequence of the heavy chain variable region of the antibody is shown in positions 124 to 239 of SEQ ID NO: 77; or the amino acid sequence of the light chain variable region of the antibody is shown in positions 1-108 of SEQ ID NO: 77.

In another preferred embodiment, the antibody is antibody (a); more preferably, the amino acid sequence of the heavy chain variable region of the antibody is shown in positions 124 to 239 of SEQ ID NO: 13; or the amino acid sequence of the light chain variable region of the antibody is shown in positions 1-108 of SEQ ID NO: 13.

In another aspect of the invention, a nucleic acid encoding the antibody as described above is provided.

In another aspect of the present invention, an expression vector is provided, comprising the nucleic acid. In another preferred embodiment, the expression vector is a PH / DHFR vector.

In another aspect of the present invention, a host cell is provided, comprising the expression vector or having the nucleic acid integrated into its genome. In another preferred embodiment, the host cell is a eukaryotic host cell or prokaryotic host cell; preferably a eukaryotic host cell, more preferably Chinese hamster ovary cell (CHO).

In another aspect of the invention, the use of any one of the above described antibodies is provided for the manufacture of a targeting drug, antibody drug conjugate, or multi-functional antibody specifically targeting tumor cells expressing EGFRvIII or over-expressing EGFR; or an agent for diagnosis of tumors that express EGFRvIII or overexpress EGFR; or is used to prepare a chimeric antigen receptor modified immune cell; preferably, the immune cell includes: T lymphocyte, NK cell or NKT lymphocyte.

In another aspect of the present invention, a multi-functional immunoconjugate is provided, comprising: any one of the above described antibodies; and a functional molecule linked thereto (including covalently linked, conjugated, attached, adsorbed); the functional molecule is selected from a group consisting of a molecule that targets a tumor surface marker, a tumor-suppressing molecule, a molecule that targets a surface marker of an immune cell, or a detectable label.

In a preferred embodiment, the molecule that targets the tumor surface marker is an antibody or ligand that binds to a tumor surface marker; or the tumor-suppressing molecule is an anti-tumor cytokine or an anti-tumor toxin; preferably, the cytokines include but are not limited to: IL-12, IL-15, IFN-beta, TNF-alpha.

In another preferred embodiment, in the multi-functional immunoconjugate, the detectable label includes a fluorescent label and a chromogenic label.

In another preferred embodiment, in the multi-functional immunoconjugate, the molecule targeting the surface marker of the immune cell is an antibody or ligand that binds to an immune

cell surface marker; preferably, the immune cell surface markers include, but are not limited to: CD3, CD16, CD28.

In another preferred embodiment, in the multi-functional immunoconjugate, the molecule that targets the surface marker of the immune cell is an antibody that binds to a T cell surface marker, which can form a T-cell-engaging bifunctional antibody with any one of the above described antibody (bispecific T cell engager, BiTE).

In another preferred embodiment, in the multi-functional immunoconjugate, the antibody that binds to the immune cell surface marker is an anti-CD3 antibody. In another preferred embodiment, the anti-CD3 antibody is a single chain antibody (scFV), a monoclonal antibody, a Fab fragment, an Fd fragment, an Fv fragment, an F(ab')<sub>2</sub> fragment and a derivative thereof, antibody; preferably single chain antibody. In another preferred embodiment, the anti-CD3 antibody is humanized, fully human, chimeric or murine.

In another preferred embodiment, the multi-functional immunoconjugate is a fusion polypeptide, and further comprises a linker peptide (linker) between any one of the above described antibodies and the functional molecule linked thereto.

In another preferred embodiment, the linker peptide has the sequence (GlyGlyGlyGlySer)<sub>n</sub>, wherein n is an integer from 1 to 5; more preferably, n = 3.

In another preferred embodiment, the multi-functional immunoconjugate is administered in a form of polypeptide or in the manner of gene administration.

In another aspect of the invention, a nucleic acid encoding the multi-functional immunoconjugate is provided.

In another aspect of the invention, the use of any one of the above described multi-functional immunoconjugate is provided, for the preparation of an antineoplastic agent or an agent for diagnosis of tumors that express EGFRvIII or overexpress EGFR; or for the preparation of chimeric antigen receptor modified immune cells. Preferably, the immune cells include T lymphocyte, NK cell or NKT lymphocyte.

In another aspect of the present invention, a chimeric antigen receptor comprising any one of the above described antibodies is provided, and the chimeric antigen receptor is expressed on the surface of an immune cell and comprises: any one of the above described antibodies, a transmembrane region and an intracellular signal region, which are sequentially linked; and the intracellular signal region is selected from a group consisting of intracellular signal region sequences of CD3 $\zeta$ , Fc $\epsilon$ RI $\gamma$ , CD27, CD28, CD137 and CD134, or a combination thereof.

In a preferred embodiment, the transmembrane region comprises a transmembrane region of CD8 or CD28.

In another preferred embodiment, the immune cells include T lymphocyte, NK cell or NKT cell.

In another preferred embodiment, the chimeric antigen receptor comprises the following sequentially linked antibody, transmembrane region and intracellular signal region:

Any one of the above described antibodies, CD8 and CD3 $\zeta$ ;

Any one of the above described antibodies, CD8, CD137 and CD3 $\zeta$ ;

5 Any one of the above described antibodies, a transmembrane region of a CD28 molecule, an intracellular signal region of a CD28 molecule, and CD3 $\zeta$ ; or

Any one of the above described antibodies, a transmembrane region of a CD28 molecule, an intracellular signal region of a CD28 molecule, CD137 and CD3 $\zeta$ .

10 In another preferred embodiment, the antibody is a single chain antibody or a domain antibody.

In another preferred embodiment, the chimeric antigen receptor comprises:

SEQ ID NO: 36 or the amino acid sequence shown in positions 285-601; or

SEQ ID NO: 37 or the amino acid sequence shown in positions 285-702; or

SEQ ID NO: 38 or the amino acid sequence shown in positions 285-744; or

15 SEQ ID NO: 39 or the amino acid sequence shown in positions 285-749; or

SEQ ID NO: 40 or the amino acid sequence shown in positions 285-791.

In another aspect of the invention, a nucleic acid encoding any one of the above described chimeric antigen receptors is provided. In another preferred embodiment, the nucleic acid encoding the chimeric antigen receptor comprises:

20 SEQ ID NO: 31 or the nucleotide sequence shown in positions 966-1916; or

SEQ ID NO: 32 or the nucleotide sequence shown in positions 966-2219; or

SEQ ID NO: 33 or the nucleotide sequence shown in positions 966-2345; or

SEQ ID NO: 34 or the nucleotide sequence shown in positions 966-2360; or

SEQ ID NO: 35 or the nucleotide sequences shown in positions 966-2486.

25 In another aspect of the present invention, an expression vector comprising the above described nucleic acid is provided.

In another preferred embodiment, the expression vector is derived from lentiviral plasmid pWPT (or pWPT-eGFP).

30 In another aspect of the present invention, a virus comprising the above described vector is provided.

Use of any one of the above described chimeric antigen receptors or an encoding nucleic acid thereof, or an expression vector or virus comprising the nucleic acid is provided, for the preparation of genetically modified immune cells that target tumor cells that express EGFRvIII or overexpress EGFR.

35 In another aspect of the present invention, a genetically modified immune cell is provided, which is transduced with the nucleic acid, or the expression vector or the virus; or expresses the

chimeric antigen receptor at its surface.

In a preferred embodiment, the immune cell further carries an exogenous encoding sequence for cytokine; preferably, the cytokine includes: IL-12, IL-15 or IL-21.

5 In another preferred embodiment, the immune cell further expresses another chimeric antigen receptor that does not contain CD3 $\zeta$ , but contains the intracellular signaling domain of CD28, the intracellular signaling domain of CD137, or a combination of both.

In another preferred embodiment, the immune cell further expresses a chemokine receptor; preferably, the chemokine receptor includes CCR2.

10 In another preferred embodiment, the immune cell further expresses siRNA that can reduce PD-1 expression or a protein that can block PD-L1.

In another preferred embodiment, the immune cell further expresses a safety switch; preferably, the safety switch includes iCaspase-9, Truncated EGFR or RQR8.

15 In another aspect of the invention, the use of said genetically modified immune cells is provided for the preparation of a tumor-inhibiting drug, and said tumor is the tumor that expresses EGFRvIII or overexpresses EGFR.

In another aspect of the invention, a pharmaceutical composition (including medicament or diagnostic reagent) is provided, comprising:

any one of the above described antibodies or a nucleic acid encoding the antibody; or

any one of the above described immunoconjugates or a nucleic acid encoding the conjugate;

20 or

any one of the above described chimeric antigen receptors or a nucleic acid encoding the chimeric antigen receptor; or

any one of the above described genetically modified immune cells.

25 Other aspects of the invention will be apparent to a person skilled in the art in view of the disclosure herein.

### **Description of drawings**

Figure 1. Antibodies 7B3 and Y022 can specifically bind to antigens EGFRvIII and N1N2-806 (phage ELISA assay).

30 Figure 2. Binding curve of antibody 7B3 vs antigen EGFRvIII.

Figure 3. Binding curve of antibody Y022 vs antigen EGFRvIII.

Figure 4. Electrophorogram of purification of three scFv-Fc fusion antibodies.

Figure 5. Detection of single-chain antibodies scFv-Y022-Fc, scFv-806-Fc and scFv-C225-Fc for their ability to bind to cell surface EGFR by FACS.

35 Figure 6. Structure diagram of pH-Y022/CD3 expression vector.

Figure 7. SDS-PAGE detection of single-chain bifunctional antibodies Y022/CD3, 806/CD3

and C225/CD3.

Figure 8. Detection of Y022/CD3 single chain bifunctional antibody for its antigen binding specificity by FACS.

Figure 9. Cytotoxicity plots of single-chain bifunctional antibodies.

5 Figure 10. Schematic illustration of the ligation order of various parts of the chimeric antigen receptor.

### **Modes for carrying out the invention**

10 After intensive research and screening, the present inventors obtained an antibody that specifically recognizes EGFRvIII or over-expressed EGFR in tumor cells and scarcely recognizes EGFR in normal cells. The antibody of the present invention can be used to prepare various targeting anti-tumor drugs and drugs for diagnosis of tumors.

### **Anti-EGFR antibody**

15 The present inventors further conducted screening and amino acid mutations based on the humanized antibodies obtained in the previous stage, and found an anti-EGFR antibody capable of targeting EGFR of tumor cells with higher specificity, which selectively binds to a tumor overexpressing EGFR or EGFRvIII, while does not bind to EGFR on normal cells.

20 Antibodies of the invention may be intact immunoglobulin molecules or antigen-binding fragments, including but not limited to Fab fragments, Fd fragments, Fv fragments, F (ab')<sub>2</sub> fragments, complementarity determining region (CDR) fragments, single-chain antibody (scFv), domain antibody, bivalent single chain antibody, single chain phage antibody, bispecific diabody, triple chain antibody, quadruple chain antibody.

25 The antigen-binding properties of an antibody can be described by three specific regions located in variable regions of the heavy and light chains, termed complementarity determining regions (CDRs), which divide the variable regions into four framework regions (FR), and the amino acid sequences of four FRs are relatively conservative, not directly involved in binding reaction. These CDRs form a loop structure, in which  $\beta$ -folds formed by the FRs are located close to each other in space and the antigen binding site of the antibody is constituted by CDRs on the heavy chain and CDRs on the corresponding light chain. It is possible to determine which amino acids make up FR or CDR regions by comparing the amino acid sequences of the same type of antibody. The CDR regions are sequences of immunologically interesting proteins and the CDR regions of the antibodies of the invention are brand new. The antibody may comprise two, three, 30 four, five, or all six of the CDR regions disclosed herein.

35 Another aspect of the invention includes functional variants of the antibodies described herein. If the variant is capable of competing with the parental antibody for specific binding to

SEQ ID NO: 1 and its ability to recognize EGFRvIII or overexpressed EGFR in tumor cells is close to that of the specific antibodies provided in Examples of the present invention. The functional variants may have conservative sequence modifications, including nucleotide and amino acid substitutions, additions and deletions. These modifications can be introduced by standard techniques known in the art, such as directed mutagenesis and random PCR-mediated mutagenesis, and can include both natural and non-natural nucleotides and amino acids. Preferably, modification of the sequence occurs on a region outside the CDR region of the antibody.

### **Immunoconjugate**

In the present invention, a multifunctional immunoconjugate is also provided, comprising the antibodies described herein and further comprising at least one functional molecule of other type. The functional molecule is selected from, but not limited to, a molecule that targets a tumor surface marker, a tumor-suppressing molecule, a molecule that targets a surface marker of an immune cell, or a detectable label. The antibody and the functional molecule may form a conjugate by covalent attachment, coupling, attachment, cross-linking, or the like.

As a preferred mode, the immunoconjugate may comprise an antibody of the invention and at least one molecule that targets a tumor surface marker or a tumor-suppressing molecule. The tumor-suppressing molecule may be anti-tumor cytokines or anti-tumor toxins. Preferably, the cytokines include but are not limited to IL-12, IL-15, IFN-beta, TNF-alpha. The molecules that target tumor surface markers, for example, can act synergistically with the antibodies of the invention to more precisely target tumor cells.

As a preferred mode, the immunoconjugate may comprise an antibody of the present invention and a detectable label. Such detectable labels include, but are not limited to, fluorescent labels, chromogenic labels such as enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, radioactive materials, positron-emitting metals and non-radioactive paramagnetic metal ion. More than one marker can also be included. The label used to label the antibody for the purpose of detection and / or analysis and / or diagnosis depends on the used particular detection / analysis / diagnosis technique and / or method, eg, immunohistochemical staining (tissue) samples, flow cytometry, and the like. Suitable labels for detection / analysis / diagnosis techniques and / or methods known in the art are well known to those skilled in the art.

As a preferred mode, the immunoconjugate may comprise an antibody of the invention as well as a molecule that targets a surface marker of an immune cell. The molecule that targets

surface markers of immune cells can recognize immune cells and carry the antibodies of the invention to the immune cells, so that the antibodies of the invention can target the immune cells to the tumor cells and thus trigger immunocyte for specifically killing tumor.

As a means of chemically generating an immunoconjugate by conjugation, either directly or indirectly (eg, by a linker), the immunoconjugate can be produced as a fusion protein comprising an antibody of the invention and other suitable proteins. The fusion protein can be produced by a method known in the art, for example recombinantly produced by constructing and subsequently expressing the nucleic acid molecule which comprises the nucleotide sequence encoding the antibody in frame with a nucleotide sequence encoding a suitable label.

In another aspect of the invention, a nucleic acid molecule encoding at least one antibody of the invention, a functional variant, or an immunoconjugate thereof is provided. Once obtaining the relevant sequence, the recombination method can be used to obtain the relevant sequence in large quantities. This is usually done by cloning it into a vector, transferring it to a cell, and then isolating the relevant sequence from the proliferating host cells by conventional methods.

The present invention also relates to vectors comprising the appropriate DNA sequences described above as well as appropriate promoters or control sequences. These vectors can be used to transform an appropriate host cell to enable expression of the protein. The host cell may be a prokaryotic cell, such as a bacterial cell; or a lower eukaryotic cell, such as a yeast cell; or a higher eukaryotic cell, such as a mammalian cell.

### **Chimeric antigen receptor and genetically modified immune cell**

In the present invention, a chimeric antigen receptor expressed on the surface of an immune effector cell (immune cell) is provided, wherein the chimeric antigen receptor comprises sequentially linked: extracellular binding region, transmembrane region and intracellular signal region, and the extracellular binding region comprises the antibody of the invention. By expressing the chimeric antigen receptor on the surface of immune effector cells, immune effector cells can have a highly specific cytotoxic effect on tumor cells that express EGFRvIII or overexpress EGFR.

As used herein, "immune cells" and "immune effector cells" are used interchangeably and include: T lymphocytes, NK cells or NKT cells, and the like.

As a preferred embodiment of the present invention, the antibody contained in the chimeric antigen receptor is a single chain antibody, which is connected to CD8 or the transmembrane region of CD28 through the hinge region of CD8, and the transmembrane region is immediately followed by the intracellular signal region.

The invention also includes nucleic acids encoding the chimeric antigen receptors. The present invention also relates to variants of the above described polynucleotides, which encode a polypeptide, or a fragment, analog and derivative of the polypeptide having the same amino acid sequence as the present invention.

5 The transmembrane region of the chimeric antigen receptor may be selected from the transmembrane region of a protein such as CD8 or CD28. The human CD8 protein is a heterodimer composed of two chains,  $\alpha\beta$  or  $\gamma\delta$ . In one embodiment of the invention, the transmembrane region is selected from the transmembrane region of CD8a or CD28. In addition, the CD8 $\alpha$  hinge is a flexible region so that CD8 or CD28 and the transmembrane region as well as the hinge region are  
10 used to connect the target recognition domain scFv of the chimeric antigen receptor CAR to the intracellular signal region .

The intracellular signal region may be selected from a group consisting of intracellular signal region of CD3 $\zeta$ , Fc $\epsilon$ RI $\gamma$ , CD28, CD137, CD134 protein, and combinations thereof. The CD3 molecule consists of five subunits, in which CD3 $\zeta$  subunit (also known as CD3 zeta, abbreviated as  
15 Z) contains 3 ITAM motifs that are important signal transduction regions in TCR-CD3 complex. CD3 $\delta$ Z is a truncated CD3 $\zeta$  sequence without ITAM motif and is generally constructed in the present invention as a negative control. Fc $\epsilon$ RI $\gamma$  is mainly distributed on the surface of mast cells and basophils, which contains an ITAM motif, which is similar to CD3 $\zeta$  in structure, distribution and function. In addition, as mentioned above, CD28, CD137 and CD134 are co-stimulatory  
20 signaling molecules. The co-stimulatory effect of their intracellular signaling segments upon binding to the respective ligands results in the continued proliferation of immune effector cells, primarily T lymphocytes, and increase in the level of cytokines such as IL-2 and IFN- $\gamma$  secreted by immune effector cells, and the survival period and anti-tumor effect of CAR immune effector cells *in vivo* are increased.

25 The chimeric antigen receptor of the present invention can be sequentially linked as follows:  
The antibody of the invention, CD8 and CD3 $\zeta$ ;  
The antibody of the invention, CD8, CD137 and CD3 $\zeta$ ;  
The antibody of the invention, the transmembrane region of CD28 molecule, the intracellular  
signal region of CD28 molecule and CD3  $\zeta$  ; or

30 The antibodies of the invention, the transmembrane region of CD28 molecule, the intracellular signal region of CD28 molecule, CD137 and CD3 $\zeta$ .

And combinations thereof, wherein CD28a in the relevant chimeric antigen receptor protein represents the transmembrane region of CD28 molecule and CD28b represents the intracellular signal region of CD28 molecule. The various chimeric antigen receptors described above are  
35 collectively referred to as scFv (EGFR)-CAR.

The present invention also provides a vector comprising the above-mentioned nucleic acid

encoding a chimeric antigen receptor protein expressed on the surface of an immune effector cell. In a specific embodiment, the vector used in the present invention is a lentiviral plasmid vector pWPT-eGFP. This plasmid belongs to the third generation of self-inactivating lentiviral vector system. The system has three plasmids, packaging plasmid psPAX2 encoding protein Gag / Pol,  
5 encoding Rev protein; envelope plasmid PMD2.G encoding VSV-G protein; and empty vector pWPT-eGFP, which can be used for recombinant introduction of a nucleic acid sequence of interest, i.e., a nucleic acid encoding CAR. In the empty vector pWPT-eGFP, the expression of enhanced green fluorescent protein (eGFP) is regulated by elongation factor-1 $\alpha$  (EF-1 $\alpha$ ) promoter. While in the recombinant expression vector pWPT-eGFP-F2A-CAR containing the nucleic acid sequence  
10 encoding CAR, co-expression of eGFP and CAR is achieved by ribosomal skipping sequence 2A (abbreviated as F2A) from food-and-mouth disease virus (FMDV).

The invention also includes viruses comprising the vectors described above. The viruses of the invention include packaged infectious viruses as well as viruses to be packaged that contain the necessary components for packaging into infectious viruses. Other viruses known in the art that  
15 can be used to transduce exogenous genes into immune effector cells and their corresponding plasmid vectors are also useful in the present invention.

The present invention further includes a genetically modified T lymphocyte, which is transduced with a nucleic acid of the present invention or transduced with the above-mentioned recombinant plasmid containing the nucleic acid of the present invention or a viral system  
20 containing the plasmid. Conventional nucleic acid transduction methods in the art, including non-viral and viral transduction methods, can be used in the present invention. Non-viral transduction methods include electroporation and transposon methods. Recently, nucleofector nuclear transfection instrument developed by Amaxa can directly introduce foreign genes into nucleus to achieve highly efficient transduction of target genes. In addition, compared with  
25 conventional electroporation, the transduction efficiency of transposon system based on Sleeping Beauty system or PiggyBac transposon was significantly improved. The combination of nucleofector transfection instrument and SB Sleeping Beauty transposon system has been reported [Davies JK., et al. Combining CD19 redirection and alloanergization to generate tumor-specific human T cells for allogeneic cell therapy of B-cell malignancies. Cancer Res, 2010, 70(10):  
30 OF1-10.], and high transduction efficiency and site-directed integration of target genes can be achieved by this method. In one embodiment of the invention, the transduction method of a T lymphocyte modified by a chimeric antigen receptor gene is a transduction method based on a virus such as a retrovirus or a lentivirus. The method has the advantages of high transduction efficiency and stable expression of exogenous gene, and the time for in vitro culturing T  
35 lymphocytes to clinical level can be shorten. The transduced nucleic acid is expressed on the surface of the transgenic T lymphocytes by transcription, translation. In vitro cytotoxicity assay

performed on various cultured tumor cells demonstrated that the immune effector cells of the present invention have highly specific tumor cell killing effects (also known as cytotoxicity). Therefore, the nucleic acid encoding a chimeric antigen receptor protein of the present invention, a plasmid comprising the nucleic acid, a virus comprising the plasmid, and a transgenic immune effector cells transfected with the nucleic acid, plasmid or virus described above can be effectively used in tumor immunotherapy.

The immune cells of the present invention may also carry exogenous encoding sequences for cytokines, including but not limited to IL-12, IL-15 or IL-21. These cytokines have immunomodulatory or antitumor activity, enhance the function of effector T cells and activated NK cells, or directly exert anti-tumor effects. Therefore, those skilled in the art will understand that the use of these cytokines will help the immune cells to function better.

In addition to the chimeric antigen receptor described above, the immune cells of the present invention may also express another chimeric antigen receptor, which does not contain CD3 $\zeta$ , but contains intracellular signaling domain of CD28 and intracellular signal domain of CD137, or a combination of both.

The immune cells of the present invention may also express chemokine receptors; the chemokine receptors include, but are not limited to, CCR2. A skilled person will understand that the CCR2 chemokine receptor can competitively bind CCR2 in the body and is beneficial for blocking the metastasis of the tumor.

The immune cells of the present invention may also express siRNAs that can reduce PD-1 expression or PD-L1-blocking proteins. A skilled person will understand that competitive blocking of the interaction between PD-L1 and its receptor PD-1 will facilitate the recovery of anti-tumor T-cell responses, thereby inhibiting tumor growth.

The immune cells of the present invention may also express a safety switch; preferably, the safety switch includes iCaspase-9, Truncated EGFR or RQR8.

### **Pharmaceutical composition**

The antibodies, immunoconjugates comprising the antibodies, and genetically modified immune cells of the present invention can be used in the preparation of a pharmaceutical composition or diagnostic reagent. In addition to an effective amount of the antibody, immunological conjugate, or immune cell, the composition may further comprise a pharmaceutically acceptable carrier. The term "pharmaceutically acceptable" means that when the molecular entities and compositions are properly administered to animals or humans, they do not cause adverse, allergic or other untoward reactions.

Specific examples of some of the substances which may be used as pharmaceutically acceptable carriers or components thereof are sugars, such as lactose, dextrose and sucrose;

starches, such as corn starch and potato starch; cellulose and its derivatives, such as carboxymethylcellulose sodium, ethylcellulose and methylcellulose; gum tragacanth; malt; gelatin; talc; solid lubricants such as stearic acid and magnesium stearate; calcium sulfate; vegetable oils, such as peanut oil, cottonseed oil, sesame oil, olive oil, corn oil and cocoa butter; polyhydric  
5 alcohols such as propylene glycol, glycerin, sorbitol, mannitol and polyethylene glycol; alginic acid; emulsifiers such as Tween®; wetting agents such as sodium lauryl sulfate; coloring agents; flavoring agents; tablets, stabilizers; antioxidants; preservatives; pyrogen-free water; isotonic saline solutions; and phosphate buffers and the like.

The composition of the present invention can be prepared into various dosage forms as  
10 needed, and the dosage to be administered to a patient can be determined by a physician according to factors, such as type, age, body weight, and general disease condition of a patient, mode of administration, and the like. For example, injection or other treatment may be used.

The present invention is further described below with reference to specific embodiments. It  
15 should be understood that these examples are only for illustrating the present invention and are not intended to limit the scope of the present invention. Experimental procedures in the following examples where no specific conditions are indicated are generally carried out in accordance with the conditions described in customary conditions such as those compiled by J. Sambrook et al., Molecular Cloning Experiments Guide, Third Edition, Science Press, 2002, or according to the  
20 manufacturer Suggested conditions.

### **Example 1. Construction of Affinity Mature Library of Single Chain Antibody 7B3**

The single chain antibody 7B3 is a humanized antibody fragment that specifically recognizes  
a cryptic epitope (<sup>287</sup>CGADSYEMEEDGVRKC<sup>302</sup> (SEQ ID NO: 1)) formed from the amino acid  
25 sequence of positions 287-302 of EGFR exposed in tumor cells. The nucleotide sequences of VL and VH genes were obtained from the sequences SEQ ID NO: 14 and SEQ ID NO: 13 as shown in patent application 201210094008.x and linked in the order of VL7B3-linker-VH7B3.

Nucleotide sequence (717 base pairs, SEQ ID NO: 2) of single chain antibody 7B3:

GATATTCAGATGACCCAGAGCCCGAGCAGCTGAGCGCGAGCGTGGGCGACCGTGTGACC  
30 ATTACCTGCCATGCGAGCCAGGATATTAACAGCAACATTGGCTGGCTGCAGCAGAAACCG  
GGCAAAGCGTTTAAAGGCCTGATTTATCATGGCAAAACCTGGAAGATGGCGTGCCGAGC  
CGTTTTAGCGGCAGCGGCAGCGGCACCGATTTTACCCTGACCATTAGCAGCCTGCAGCCG  
GAAGATTTTGCACCTATTATTGCGTTCAGTACGCCAGTCCCATATACATTTGGCCAG  
GGCACCAAAGTGAAATTAACGTGGTGGAGGCGGTTTCAGGCGGAGGTGGCTCTGGCGGT  
35 GCGGATCGATGTGCAGCTGGTGAAAGCGGCGGCGGCTGGTGCAGCCGGGCGGCAGC  
CTGCGTCTGAGCTGCGCGGTGAGCGGTATAGCATTACCAGCGATTATGCGTGGAACCTGG  
ATTCGTCAGGCGCCGGGCAAAGGCCTGGAATGGCTGGGCTATATTAGCTATCGTGGCCGC  
ACCAGCTATAACCCGAGCCTGAAAAGCCGTATTAGCATTACCCGTGATAACAGCAAAAAC

ACCTTTTCTGCAGCTGAACAGCCTGCGTGCGGAAGATACCGCGGTGTATTATTGCGCG  
CGCCTGGGACGCGGCTCCGCTACTGGGGCCAGGGCACCTGGTGACCGTGAGCAGC

The amino acid sequence of the single chain antibody 7B3 (239 amino acids, SEQ ID NO: 3; underlined area was 7B3 VL CDR1, CDR2, CDR3, 7B3 VH CDR1, CDR2, CDR3, respectively):

DIQMTQSPSSLSASVGDRVTITCHASQDINSNIGWLQQKPGKAFKGLIYHGK<sup>1</sup>NLEDGVPSRFSGSGSGTDFLT<sup>2</sup>ISSLQP  
EDFATYYCVQYAQFPYTFGQGTKVEIKRGGGSGGGGSGGGSDVQLVESGGGLVQPGGSLRLSCAVSGYSITS<sup>3</sup>DYAWN<sup>4</sup>W  
IRQAPGKGLEWLGYISYRGRTSYNPSLKS<sup>5</sup>RISITRDNSKNTFFLQLNSLRAEDTAVYYCARLGRGFRYWGQGT<sup>6</sup>LVTVSS

For enhancing the ability of 7B3 single-chain antibody to bind EGFR, some amino acids in CDR3 of light chain and CDR3 of heavy chain were randomly mutated and corresponding mature libraries were constructed.

### 1. Construction of 7B3 Light Chain CDR3 Affinity Mature Library

By sequence alignment and analysis of 7B3 single-chain antibody, part of the amino acids in the third CDR region of 7B3 light chain were selected and randomized mutations were introduced by primers to construct a light-chain affinity mature library.

To prepare a DNA fragment encoding the 7B3 mutant library, two DNA fragments were respectively obtained by PCR using plasmid pCantab 5E-7B3 (inserting 7B3 into *sfi*I/*Not*I site of pCantab 5E-7B3) as a template, followed by splicing through bypass PCR method. Specifically, the following procedure was used: for synthesizing genes, PCR reactions were performed in a volume of 50  $\mu$ l each using plasmid pCantab 5E-7B3 as a template with a final concentration of 0.2  $\mu$ M for each primer and 5  $\mu$ l of 10  $\times$  KOD Plus buffer, 4  $\mu$ l dNTPs (dATP, dCTP, dGTP and dTTP, 2 mM each), 2  $\mu$ l 25 mM MgSO<sub>4</sub> and 1 U KOD Plus (from Takara) were added and the PCR procedure was started in a thermal cycler after making up the volume with water. The reaction was firstly heated to 94°C for 5 minutes and then incubated for 25 cycles of 94°C for 30 seconds, 56°C for 30 seconds and 68°C for 30 seconds, and finally, at 68°C for 10 minutes. The first fragment was amplified using primers pC7B3fw (SEQ ID NO: 4, ATAACAGGCCCCAGCCGGCCATGGATATTCAGATGACCCAGAG) and LR3re (SEQ ID NO: 5, CACTTTGGTGCCCTGGCCAAATGTMNNTGGGNNMNNMNNMNNCTGMNNGCAATA ATAGGTCGCAAAATC) and the second fragment was amplified using primer LR3f2fw (SEQ ID NO: 6, ACATTTGGCCAGGGCACCAAAG) and pC7B3re (SEQ ID NO: 7, ATAAATGCGGCC GCGCTGCTCACGGTCAC).

Expected PCR products were identified by analytical agarose gel electrophoresis and purified from samples by Wizard SV Gel and PCR Clean-up Kit (available from Promega). The two fragments were added in equimolar ratio to a second round of bridge PCR as a template and the reaction system still used KOD Plus system mentioned above. The reaction was firstly heated to 94°C for 5 minutes and then incubated for 10 cycles, each cycling reaction conditions were 94°C for 30 seconds, 60°C for 30 seconds and 68°C for 30 seconds, and finally, at 68°C for 10 minutes. Subsequently, primers pC7B3fw and pC7B3re were directly added to the reaction system at a final



KOD Plus system mentioned above. The reaction was firstly heated to 94°C for 5 minutes and then incubated for 10 cycles, each cycling reaction conditions were 94°C for 30 seconds, 60°C for 30 seconds and 68°C for 30 seconds, and finally, at 68°C for 10 minutes. Subsequently, primers HR3f1fw and HR3f2re were directly added to the reaction system at a final concentration of 0.2 μM, and the PCR program was started. The reaction was firstly heated to 94°C for 5 minutes and then incubated for 25 cycles of 94°C for 30 seconds, 56°C for 30 seconds and 68°C for 30 seconds, and finally, at 68°C for 10 minutes. The expected PCR products were separated by preparative agarose gel electrophoresis and purified by Wizard SV Gel and PCR Clean-up kits according to the manufacturer's instructions.

In the library, complete DNA fragments contained *sfiI* and *NotI* restriction enzyme recognition sites at each end, and was digested by restriction endonuclease *sfiI* / *NotI* for restriction digestion and inserted into phagemid vector pCANTAB 5E digested by the same two enzymes. Ligation products were isolated and desalted using Wizard SV Gel and PCR Clean-up Kit for electrotransformation. For electrotransformation, a home-made competent *E. coli* ER2738 was used with electroporation cuvette and electroporation instrument Gene Pulser II. A library containing  $6.0 \times 10^9$  mutants was finally confirmed.

### **Example 2. Screening against EGFRvIII by using 7B3 affinity maturation library**

To obtain 7B3 mutants with higher affinity, four rounds of screening were performed using light chain and heavy chain mutant libraries, respectively, as follows: a corresponding phage library was obtained from the above library through infection of helper phage M13KO7. The phage library was incubated with the biotin-labeled antigen EGFRvIII (purchased from Shanghai raygene biotechnology Co., LTD) for 2 hours at room temperature and then incubated with 2% (w/v) BSA (bovine serum albumin, purchased from Shanghai Bioengineering)-blocked streptavidin magnetic beads MyOne Cl (from Invitrogen) at room temperature for 30 minutes. The beads were then washed with PBST (containing 0.1% Tween-20) buffer to remove phage not specifically bound or with weaker binding capacity. Strongly binding phages were eluted from magnetic beads with glycine-HCl (pH 2.2), neutralized with Tris neutralizing solution (pH 9.1), and then used to infect *E. coli* ER2738 in the mid-logarithmic growth phase for the next round of screening.

In the above described four rounds of screening, the amounts of magnetic beads were 50 μl, 25 μl, 10 μl and 10 μl, the concentrations of biotin-labeled antigen EGFRvIII were 10 nM, 1 nM, 0.5 nM and 0.1 nM, respectively, and PBST was used for washing for 10, 10, 15 and 20 times. From the second round of screening, 50-, 500-, and 1000-fold excess of unlabeled antigen EGFRvIII, respectively, was added as a competitor prior to elution to remove mutants with weaker binding capacity.

For the production of phage displaying 7B3 single chain antibody mutants on the surface, the strain in glycerol obtained in Example 1 was inoculated into 400 ml of 2YT / ampicillin medium to bring the cell density to  $OD_{600} = 0.1$ , and at 37°C and 200 rpm, cultured with shaking until the cell density reached  $OD_{600} = 0.5$ .  $10^{12}$  pfu of M13KO7 helper phage was used in infection and incubated at 30°C and 50 rpm for 30 minutes. After adding 50 mg/l kanamycin and shaking at 37°C and 200 rpm for 30 minutes, the pellet was separated by centrifugation (15 minutes, 1600 × g, 4°C) and resuspended in 400 ml 2YT/ampicillin/Kanamycin medium and cultured for 16 hours at 37°C with shaking at 200 rpm. Finally, the pellet was separated by centrifugation (5000 rpm, 4°C for 20 minutes) and discarded. The supernatant was filtered through a 0.45 µm filter and 1/4 volume of 20% (w/v) PEG 8000, 2.5 M NaCl solution was added and incubated in an ice bath for 1 hour for precipitating phage pellets. The pellet was then centrifuged (20 min, 8000 × g, 4°C) and the supernatant was discarded. The phage was resuspended in 25 ml of prechilled PBS (137 mM NaCl, 2.7 mM KCl, 8 mM  $Na_2HPO_4$ , 2 mM  $KH_2PO_4$ ) and centrifuged (5 minutes, 20000 × g, 4°C). 1/4 volume of 20% (w/v) PEG 8000, 2.5 M NaCl solution was added to the supernatant and incubated in an ice bath for 30 minutes for precipitating the phage particles again. The pellet was obtained by centrifugation (30 min at 20000 × g at 4°C), resuspended in 2 ml of prechilled PBS again, kept on ice for 30 min and centrifuged (30 min, 17000 xg, 4°C). The supernatant was mixed with 4% (w/v) BSA in PBS at 1: 1, placed on a rotary mixer and incubated for 30 minutes at room temperature, and then used directly in screening.

### **Example 3. Identification of 7B3 mutants specifically binding to EGFRvIII**

After four rounds of screening against EGFRvIII antigen, 96 clones were randomly selected from the clones obtained in the fourth round of screening and analyzed for their combination with antigens EGFRvIII and N1N2-806 (purchased from Shanghai raygene biotechnology Co., LTD) using single phage ELISA (enzyme-linked immunosorbent assay), where N1N2-806 is a fusion protein of N1N2 domain of M13 phage PIII protein and amino acids at positions 287-302 of EGFR. For this purpose, each single colony was inoculated into 300 µl of 2YT/ampicillin medium (containing 2% glucose) in a 96-well deep-well plate and cultured with shaking at 37°C and 250 rpm for 16 hours. 20 µl of culture was inoculated into 500 µl of 2YT/ampicillin medium (containing 0.1% glucose) and shaken at 37°C and 250 rpm for 1.5 hours. To prepare the helper phage solution, 75 µl of M13KO7 (titer of  $3 \times 10^{12}$  pfu / ml) was taken and mixed into 15 ml of 2YT medium and added into a plate at 50 µl/well. Incubation was performed at 37°C and 150 rpm for 30 minutes, and then 50 µl/well of prepared kanamycin solution (180 µl of 50 mg/ml kanamycin taken and added into 15 ml of 2YT medium) was added and cultured at 37°C and 250 rpm for 16 hours with shaking. Finally, the cells were precipitated by centrifugation (30 minutes at 5000 × g, 4°C) and the supernatant was transferred to a new 96-well deep-well plate.

To perform single-phase ELISA, 100 ng/well of antigen EGFRvIII, N1N2-806 and negative control proteins BSA and N1N2 (purchased from Shanghai raygene biotechnology Co., LTD) were used on 96-well MediSorp ELISA plate (purchased from Nunc) at 50 µl/well, and coated overnight at 4°C. Each well was blocked with PBST containing 2% BSA (w/v). The wells were then washed with PBST for three times. Then, each phage solution prepared above was added to each well of the plate at 100 µl/well. After incubation for 2 hours at 37°C, it was washed for three times with PBST. To detect bound phage, anti-M13 antibody superoxide dismutase conjugate (purchased from GE Healthcare) was diluted at 1: 5000 in PBST and 100 µl was added to each well. After incubating at 37°C for 1 hour, the wells were rinsed for three times with PBST and then rinsed for three times with PBS. Finally, 50 µl of TMB substrate was added into the wells and developed for 10 minutes at room temperature, followed by addition of 50 µl of 2M H<sub>2</sub>SO<sub>4</sub> per well to stop the color reaction. Extinction values were measured at 450 nm with an enzyme-linked immunosorbent (Bio-Rad).

Clones with stronger signal of binding antigen instead of BSA in ELISA were selected and used in subsequent evaluation and sequencing analysis. The antibodies obtained from the light chain affinity maturation library were then further combined with the antibodies obtained from Example 2 in the heavy chain affinity maturation library on light chain variable region sequence and heavy chain variable region sequence and the resulting antibodies are also capable of specifically binding antigens EGFRvIII and N1N2-806, instead of the control proteins BSA and N1N2.

From the crystal structure of the antibody and antigenic determinant, the binding of combined antibodies obtained from 7B3 light and heavy chain mutations and EGFR<sub>287-302</sub> was structurally analyzed, and finally, some amino acid positions were selected for further mutation for higher affinity and stability. All of the altered amino acid positions include S31 in the light chain CDR1 region, V89, A92, Q93, F94 and Y96 in the light chain CDR3 region, S182 in the heavy chain CDR2 region, L222, R224, G225, F226 and R227 in the heavy chain CDR3 region. Based on the sequences of combined antibodies after mutagenesis of the light and heavy chains, the mutation sites were further introduced to obtain the antibody Y022. Compared with the parental antibody 7B3, Y022 contains 12 amino acid mutation sites (S31V, V89N, A92E, Q93N, F94I, Y96L, S182Q, L222M, R224K, G225N, F226W, and R227D). As shown in Figure 1, in a single phase ELISA assay, Y022 was able to specifically bind to antigens EGFRvIII and N1N2-806 without binding to control proteins BSA and N1N2.

Nucleotide sequence of single chain antibody Y022 (717 bases; SEQ ID NO: 12):

GATATTCAGATGACCCAGAGCCCGAGCAGCCTGAGCGGAGCGTGGGCGACCGTGTGACC  
ATTACCTGCCATGCGAGCCAGGATATTAACGTGAACATTGGCTGGCTGCAGCAGAAACCG  
GGCAAAGCGTTTAAAGGCCTGATTTATCATGGCAAAACCTGGAAGATGGCGTGCCGAGC  
CGTTTGTAGCGGCAGCGGCAGCGGCACCGATTTTACCCTGACCATTAGCAGCCTGCAGCCG

GAAGATTTTGGCAGCTATTATTGCAATCAGTATGAAAATATCCCACTGACATTTGGCCAG  
GGCACCAAAGTGGAAATTAACGTGGTGGAGGCGGTTTCAGGCGGAGGTGGCTCTGGCGGT  
GGCGGATCGGATGTGCAGCTGGTGGAAAGCGGCGGCGCCTGGTGCAGCCGGGCGGCAGC  
CTGCGTCTGAGCTGCGCGGTGAGCGGCTATAGCATTACCAGCGATTATGCGTGGAAGTGG  
ATTCGTCAGGCGCCGGGCAAAGGCCTGGAATGGCTGGGCTATATTAGCTATCGTGGCCGC  
ACCCAGTATAACCCGAGCCTGAAAAGCCGTATTAGCATTACCCGTGATAACAGCAAAAAC  
ACCTTTTCTGTCAGCTGAACAGCCTGCGTGCAGGAAGATACCGCGGTGATTATTGCGCG  
CGCATGGGTAAGAATTGGGATTACTGGGGCCAGGGCACCCCTGGTGACCGTGAGCAGC

Amino acid sequence of single chain antibody Y022 (239 amino acids; SEQ ID NO:

13):

DIQMTQSPSSLSASVGRVTITCHASQDINVNIGWLQKPGKAFKGLIYHGKNLEDGVPS  
RFGSGSGTDFTLTISSLQPEDFATYYCNQYENIPLTFGQGTKVEIKRGGGSGGGSGG  
GGSDVQLVESGGGLVQPGGSLRLSCAVSGYSITSDYAWNWRQAPGKGLEWLGYSYRGR  
TQYNPSLKSRLITRDNSKNTFFLQLNSLRAEDTAVYYCARMGKNWDYWGQGLTVTVSS

Wherein the light chain is in positions 1-108 and the light chain CDR1 sequence: HASQDINVNIG (SEQ ID NO: 41), CDR2 sequence: HGKNLED (SEQ ID NO: 42), CDR3 sequence: NQYENIPLT (SEQ ID NO: 43).

Wherein the heavy chain is in positions 124-239 and the heavy chain CDR1 sequence: GYSITSDYAWN (SEQ ID NO: 44), CDR2 sequence: YISYRGRTQYNPSLKS (SEQ ID NO: 45), CDR3 sequence: MGKNWDY (SEQ ID NO: 46).

Since it was screened in the mutant library constructed previously and subjected to site-directed mutagenesis, the nucleotide sequence of Y022 was contained in pCantab 5E, named as pCantab 5E-Y022 plasmid.

The inventors also obtained 10 additional antibody clones with significantly improved affinity and stability using the same method as for the production of antibody Y022, namely M14, M15, M25, M26, S7, S8, S17, S22, S23 and S29. Compared with the parent antibody 7B3, all single-chain antibodies contained amino acid mutation sites as shown in Table 1.

Table 1

| Antibody | amino acid mutation site                                                     |
|----------|------------------------------------------------------------------------------|
| Y022     | S31V, V89N, A92E, Q93N, F94I, Y96L, S182Q, L222M, R224K, G225N, F226W, R227D |
| M14      | V89N, A92E, Q93N, F94N, Y96I, S182N                                          |
| M15      | S31V, V89N, A92E, Q93N, F94N, Y96I                                           |
| M25      | S31V, V89N, A92E, Q93N, F94I, Y96L, S182R                                    |
| M26      | S31V, V89N, A92E, Q93N, F94I, Y96L, S182Q                                    |
| S7       | S31V, K53T, V89N, A92E, Q93N, F94N, Y96I                                     |
| S8       | S31V, A44S, V89N, A92E, Q93N, F94N, Y96I                                     |
| S17      | S31T, V89N, A92E, Q93N, F94N, Y96L, S182Q                                    |

|     |                                                 |
|-----|-------------------------------------------------|
| S22 | S31V, K53T, V89N, A92E, Q93N, F94N, Y96L, S182R |
| S23 | S31V, A44S, V89N, A92E, Q93N, F94N, Y96L, S182R |
| S29 | S31V, V89N, A92E, Q93N, Y96L, S182R             |

**Nucleotide sequence of single chain antibody M14 (717 bases; SEQ ID NO: 58):**

GATATTCAGATGACCCAGAGCCCGAGCAGCCTGAGCGCGAGCGTGGGCGACCGTGTGACC  
ATTACCTGCCATGCGAGCCAGGATATTAACAGCAACATTGGCTGGCTGCAGCAGAAACCG  
GGCAAAGCGTTTAAAGGCCTGATTTATCATGGCAAAACCTGGAAGATGGCGTGCCGAGC  
CGTTTTAGCGGCAGCGGCAGCGGCACCGATTTTACCCTGACCATTAGCAGCCTGCAGCCG  
GAAGATTTTGCACCTATTATTGCAATCAGTATGAAAATAACCAATTACATTTGGCCAG  
GGCACCAAAGTGAAATTAACGTGGTGGAGGCGGTTTCAGGCGGAGGTGGCTCTGGCGGT  
GGCGGATCGGATGTGCAGCTGGTGAAAGCGGCGGCGCCTGGTGCAGCCGGGCGGCAGC  
CTGCGTCTGAGCTGCGCGGTGAGCGGCTATAGCATTACCAGCGATTATGCGTGAACTGG  
ATTCGTCAGGCGCCGGGCAAAGGCCTGGAATGGCTGGGCTATATTAGCTATCGTGGCCGC  
ACCAACTATAACCCGAGCCTGAAAAGCCGTATTAGCATTACCCGTGATAACAGCAAAAAC  
ACCTTTTTCTGCAGCTGAACAGCCTGCGTGCGGAAGATACCGCGGTGTATTATTGCGCG  
CGCCTGGGACGCGGCTTCCGCTACTGGGGCCAGGGCACCTGGTGACCGTGAGCAGC

**Amino acid sequence of single chain antibody M14 (239 amino acids; SEQ ID NO:**

59):

DIQMTQSPSSLSASVGRVITTHASQDINSNIGWLQKPGKAFKGLIYHGKNLEDGVPS  
RFSGSGSGTDFTLTISLQPEDFATYYCNQYENNPITFGQGTKVEIKRGGGSGGGSGG  
GGSDVQLVESGGGLVQPGGSLRLSCAVSGYSITSDYAWNWRQAPGKGLEWLGYSISYRGR  
TNYNPSLKSRIITRDNSKNTFFLQLNSLRAEDTAVYYCARLGRGFRYWQGTTLTVSS

Amino acid sequences of M14 light chain CDR1(HASQDINSNIG), CDR2(HGKNLED), CDR3(NQYENNPIT) and heavy chain CDR1 (GYSITSDYAWN), CDR2 (YISYRGRTNYNPSLKS), CDR3 (LGRGFRY) are SEQ ID NO: 47, 42, 48, 44, 49, 50, respectively.

**Nucleotide sequence of single chain antibody M15 (717 bases; SEQ ID NO: 60):**

GATATTCAGATGACCCAGAGCCCGAGCAGCCTGAGCGCGAGCGTGGGCGACCGTGTGACC  
ATTACCTGCCATGCGAGCCAGGATATTAACGTGAACATTGGCTGGCTGCAGCAGAAACCG  
GGCAAAGCGTTTAAAGGCCTGATTTATCATGGCAAAACCTGGAAGATGGCGTGCCGAGC  
CGTTTTAGCGGCAGCGGCAGCGGCACCGATTTTACCCTGACCATTAGCAGCCTGCAGCCG  
GAAGATTTTGCACCTATTATTGCAATCAGTATGAAAATAACCAATTACATTTGGCCAG  
GGCACCAAAGTGAAATTAACGTGGTGGAGGCGGTTTCAGGCGGAGGTGGCTCTGGCGGT  
GGCGGATCGGATGTGCAGCTGGTGAAAGCGGCGGCGCCTGGTGCAGCCGGGCGGCAGC  
CTGCGTCTGAGCTGCGCGGTGAGCGGCTATAGCATTACCAGCGATTATGCGTGAACTGG  
ATTCGTCAGGCGCCGGGCAAAGGCCTGGAATGGCTGGGCTATATTAGCTATCGTGGCCGC  
ACCAGCTATAACCCGAGCCTGAAAAGCCGTATTAGCATTACCCGTGATAACAGCAAAAAC  
ACCTTTTTCTGCAGCTGAACAGCCTGCGTGCGGAAGATACCGCGGTGTATTATTGCGCG  
CGCCTGGGACGCGGCTTCCGCTACTGGGGCCAGGGCACCTGGTGACCGTGAGCAGC

**Amino acid sequence of single chain antibody M15 (239 amino acids; SEQ ID NO: 61):**

DIQMTQSPSSLSASVGDRVTITCHASQDINVNIGWLQQKPGKAFKGLIYHGKNLEDGVPS  
 RFSGSGSGTDFTLTISSLQPEDFATYYCNQYENNPITFGQGTKVEIKRGGGSGGGSGG  
 GGSVDQLVESGGGLVQPGGSLRLSCAVSGYSITSDYAWNWRQAPGKGLEWLG~~YISYRGR~~  
 TSYNPSLKSRLSITRDNSKNTFFLQLNSLRAEDTAVYYCARLGRGFRYWGQGLTVTVSS

Amino acid sequences of M15 light chain CDR1 (HASQDINVNIG), CDR2 (HGKNLED), CDR3 (NQYENNPIT) and heavy chain CDR1 (GYSITSDYAWN), CDR2 (YISYRGR~~TSYNPSLKS~~), CDR3 (LGRGFRY) are SEQ ID NO: 41, 42, 48, 44, 51, 50, respectively.

Nucleotide sequence of single chain antibody M25 (717 bases; SEQ ID NO: 62):

GATATTAGATGACCCAGAGCCGAGCAGCCTGAGCGGAGCGTGGGCGACCGTGTGACC  
 ATTACCTGCCATGCGAGCCAGGATATTAACGTGAACATTGGCTGGCTGCAGCAGAAACCG  
 GGCAAAGCGTTTAAAGGCCTGATTTATCATGGCAAAAACCTGGAAGATGGCGTGCCGAGC  
 CGTTTATAGCGGCAGCGGCAGCGCACCGATTTACCCTGACCATAGCAGCCTGCAGCCG  
 GAAGATTTTGCACCTATTATTGCAATCAGTATGAAAATATCCACTGACATTGGCCAG  
 GGCACCAAAGTGAAATTAACCGTGGTGGAGGCGGTTCAGGCGAGGTGGCTCTGGCGGT  
 GGCGGATCGGATGTGCAGCTGGTGGAAAGCGGCGGCGCCTGGTGCAGCCGGGCGGCAGC  
 CTGCGTCTGAGCTGCGCGGTGAGCGGCTATAGCATTACCAGCGATTATGCGTGAACTGG  
 ATTCGTCAGGCGCGGGCAAAGGCCTGGAATGGCTGGGCTATATTAGCTATCGTGCCGC  
 ACCCGCTATAACCCGAGCCTGAAAAGCCGTATTAGCATTACCCGTGATAACAGCAAAAAC  
 ACCTTTTCTGACGTGAACAGCCTGCGTGCAGGAAGATACCGCGGTGATTATTGCGCG  
 CGCCTGGGACGCGGCTCCGCTACTGGGGCCAGGGCACCTGGTGACCGTGAGCAGC

Amino acid sequence of single chain antibody M25 (239 amino acids; SEQ ID NO: 63):

DIQMTQSPSSLSASVGDRVTITCHASQDINVNIGWLQQKPGKAFKGLIYHGKNLEDGVPS  
 RFSGSGSGTDFTLTISSLQPEDFATYYCNQYENIPLTFGQGTKVEIKRGGGSGGGSGG  
 GGSVDQLVESGGGLVQPGGSLRLSCAVSGYSITSDYAWNWRQAPGKGLEWLG~~YISYRGR~~  
 TRYNPSLKSRLSITRDNSKNTFFLQLNSLRAEDTAVYYCARLGRGFRYWGQGLTVTVSS

Amino acid sequences of M25 light chain CDR1 (HASQDINVNIG), CDR2 (HGKNLED), CDR3 (NQYENIPLT) and heavy chain CDR1 (GYSITSDYAWN), CDR2 (YISYRGR~~TRYNPSLKS~~), CDR3 (LGRGFRY) are SEQ ID NO: 41, 42, 43, 44, 52, 50, respectively.

Nucleotide sequence of single chain antibody M26 (717 bases; SEQ ID NO: 64):

GATATTAGATGACCCAGAGCCGAGCAGCCTGAGCGGAGCGTGGGCGACCGTGTGACC  
 ATTACCTGCCATGCGAGCCAGGATATTAACGTGAACATTGGCTGGCTGCAGCAGAAACCG  
 GGCAAAGCGTTTAAAGGCCTGATTTATCATGGCAAAAACCTGGAAGATGGCGTGCCGAGC  
 CGTTTATAGCGGCAGCGGCAGCGCACCGATTTACCCTGACCATAGCAGCCTGCAGCCG  
 GAAGATTTTGCACCTATTATTGCAATCAGTATGAAAATATCCACTGACATTGGCCAG  
 GGCACCAAAGTGAAATTAACCGTGGTGGAGGCGGTTCAGGCGAGGTGGCTCTGGCGGT  
 GGCGGATCGGATGTGCAGCTGGTGGAAAGCGGCGGCGCCTGGTGCAGCCGGGCGGCAGC  
 CTGCGTCTGAGCTGCGCGGTGAGCGGCTATAGCATTACCAGCGATTATGCGTGAACTGG  
 ATTCGTCAGGCGCGGGCAAAGGCCTGGAATGGCTGGGCTATATTAGCTATCGTGCCGC  
 ACCCAGTATAACCCGAGCCTGAAAAGCCGTATTAGCATTACCCGTGATAACAGCAAAAAC  
 ACCTTTTCTGACGTGAACAGCCTGCGTGCAGGAAGATACCGCGGTGATTATTGCGCG  
 CGCCTGGGACGCGGCTCCGCTACTGGGGCCAGGGCACCTGGTGACCGTGAGCAGC

Amino acid sequence of single chain antibody M26 (239 amino acids; SEQ ID NO: 65):

DIQMTQSPSSLSASVGDRVTITCHASQDINVNIGWLQQKPGKAFKGLIYHGKNLEDGVPS  
 RFSGSGSGTDFTLTISSLQPEDFATYYCNQYENIPLTFGQGTKVEIKRGGGSGGGSGG  
 GGSVDQLVESGGGLVQPGGSLRLSCAVSGYSITSDYAWNWRQAPGKGLEWLG~~YISYRGR~~

TQYNPSLKSRLSITRDNSKNTFFLQLNSLRAEDTAVYYCARLGRGFRYWQGTLTVSS

Amino acid sequences of M26 light chain CDR1 (HASQDINVNIG), CDR2 (HGKNLED), CDR3 (NQYENIPLT) and heavy chain CDR1 (GYSITSDYAWN), CDR2 (YISYRGRTQYNPSLKS), CDR3 (LGRGFRY) are SEQ ID NO: 41, 42, 43, 44, 45, 50, respectively.

Nucleotide sequence of single chain antibody S7 (717 bases; SEQ ID NO: 66):

GATATTCAGATGACCCAGAGCCCGAGCAGCCTGAGCGCGAGCGTGGGCGACCGTGTGACC  
ATTACCTGCCATGCGAGCCAGGATATTAACGTGAACATTGGCTGGCTGCAGCAGAAACCG  
GGCAAAGCGTTTAAAGGCCTGATTTATCATGGCACCAACCTGGAAGATGGCGTGCCGAGC  
CGTTTTAGCGGCAGCGGCAGCGGCACCGATTTACCCTGACCATTAGCAGCCTGCAGCCG  
GAAGATTTTGCACCTATTATTGCAATCAGTATGAAAATAACCAATTACATTTGGCCAG  
GGCACCAAAGTGAAAATTAAACGTGGTGGAGGCGGTTCAGGCGGAGGTGGCTCTGGCGGT  
GGCGGATCGGATGTGCAGCTGGTGGAAAGCGGCGGCGCCTGGTGCAGCCGGGCGGCAGC  
CTGCGTCTGAGCTGCGCGGTGAGCGGCTATAGCATTACCAGCGATTATGCGTGAACTGG  
ATTCGTCAGGCGCCGGGCAAAGGCCTGGAATGGCTGGGCTATATTAGCTATCGTGCCGC  
ACCAGCTATAACCCGAGCCTGAAAAGCCGTATTAGCATTACCCGTGATAACAGCAAAAAC  
ACCTTTTCTGAGCTGAACAGCCTGCGTGCGGAAGATACCGCGGTGTATTATTGCGCG  
CGCCTGGGACGCGGCTTCCGCTACTGGGGCCAGGGCACCTGGTGACCGTGAGCAGC

Amino acid sequence of single chain antibody S7 (239 amino acids; SEQ ID NO: 67):

DIQMTQSPSSLSASVGRVTITCHASQDINVNIGWLQKPGKAFKGLIYHGKNLEDGVPS  
RFGSGSGTDFTLTISLQPEDFATYYCNQYENNPITFGQGTKVEIKRGGGSGGGSGG  
GGSDVQLVESGGGLVQPGGSLRLSCAVSGYSITSDYAWNWRQAPGKGLEWLGYISYRGR  
TSYNPSLKSRLSITRDNSKNTFFLQLNSLRAEDTAVYYCARLGRGFRYWQGTLTVSS

Amino acid sequences of S7 light chain CDR1 (HASQDINVNIG), CDR2 (HGTNLED), CDR3 (NQYENNPIT) and heavy chain CDR1 (GYSITSDYAWN), CDR2 (YISYRGRTSYNPSLKS), CDR3 (LGRGFRY) are SEQ ID NO: 41, 53, 54, 44, 51, 50, respectively.

Nucleotide sequence of single chain antibody S8 (717 bases; SEQ ID NO: 68):

GATATTCAGATGACCCAGAGCCCGAGCAGCCTGAGCGCGAGCGTGGGCGACCGTGTGACC  
ATTACCTGCCATGCGAGCCAGGATATTAACGTGAACATTGGCTGGCTGCAGCAGAAACCG  
GGCAAAAGCTTTAAAGGCCTGATTTATCATGGCAAAACCTGGAAGATGGCGTGCCGAGC  
CGTTTTAGCGGCAGCGGCAGCGGCACCGATTTACCCTGACCATTAGCAGCCTGCAGCCG  
GAAGATTTTGCACCTATTATTGCAATCAGTATGAAAATAACCAATTACATTTGGCCAG  
GGCACCAAAGTGAAAATTAAACGTGGTGGAGGCGGTTCAGGCGGAGGTGGCTCTGGCGGT  
GGCGGATCGGATGTGCAGCTGGTGGAAAGCGGCGGCGCCTGGTGCAGCCGGGCGGCAGC  
CTGCGTCTGAGCTGCGCGGTGAGCGGCTATAGCATTACCAGCGATTATGCGTGAACTGG  
ATTCGTCAGGCGCCGGGCAAAGGCCTGGAATGGCTGGGCTATATTAGCTATCGTGCCGC  
ACCAGCTATAACCCGAGCCTGAAAAGCCGTATTAGCATTACCCGTGATAACAGCAAAAAC  
ACCTTTTCTGAGCTGAACAGCCTGCGTGCGGAAGATACCGCGGTGTATTATTGCGCG  
CGCCTGGGACGCGGCTTCCGCTACTGGGGCCAGGGCACCTGGTGACCGTGAGCAGC

Amino acid sequence of single chain antibody S8 (239 amino acids; SEQ ID NO: 69):

DIQMTQSPSSLSASVGRVTITCHASQDINVNIGWLQKPGKSFKGLIYHGKNLEDGVPS  
RFGSGSGTDFTLTISLQPEDFATYYCNQYENNPITFGQGTKVEIKRGGGSGGGSGG  
GGSDVQLVESGGGLVQPGGSLRLSCAVSGYSITSDYAWNWRQAPGKGLEWLGYISYRGR  
TSYNPSLKSRLSITRDNSKNTFFLQLNSLRAEDTAVYYCARLGRGFRYWQGTLTVSS

Amino acid sequences of S8 light chain CDR1 (HASQDINVNIG), CDR2 (HGKNLED), CDR3 (NQYENNPIT) and heavy chain CDR1 (GYSITSDYAWN), CDR2

(YISYRGRTSYNPSLKS), CDR3 (LGRGFRY) are SEQ ID NO: 41, 42, 54, 44, 51, 50, respectively.

Nucleotide sequence of single chain antibody S17 (717 bases; SEQ ID NO: 70):

5 GATATTCAGATGACCCAGAGCCCGAGCAGCCTGAGCGGAGCGTGGGCGACCGTGTGACC  
ATTACCTGCCATGCGAGCCAGGATATTAACACCAACATTGGCTGGCTGCAGCAGAAACCG  
GGCAAAGCGTTTAAAGGCCTGATTTATCATGGCAAAACCTGGAAGATGGCGTGCCGAGC  
CGTTTACGGCAGCGGCAGCGGCACCGATTTACCCTGACCATTAGCAGCCTGCAGCCG  
GAAGATTTTGCACCTATTATTGCAATCAGTATGAAAATAACCCACTGACATTTGGCCAG  
10 GGCACCAAAGTGAAAATTAACGTGGTGGAGGCGGTTTCAGGCGGAGGTGGCTCTGGCGGT  
GGCGGATCGGATGTGCAGCTGGTGAAAAGCGGCGGCGCCTGGTGCAGCCGGGCGGCAGC  
CTGCGTCTGAGCTGCGCGGTGAGCGGCTATAGCATTACCAGCGATTATGCGTGGAAGTGG  
ATTCGTCAGGCGCCGGGCAAAGGCCTGGAATGGCTGGGCTATATTAGCTATCGTGCCGC  
ACCCAGTATAACCCGAGCCTGAAAAGCCGTATTAGCATTACCCGTGATAACAGCAAAAAC  
ACCTTTTCTGCAGCTGAACAGCCTGCGTGCGGAAGATACCGCGGTGTATTATTGCGCG  
15 CGCCTGGGACGCGGCTTCCGCTACTGGGGCCAGGGCACCTGGTGACCGTGAGCAGC

Amino acid sequence of single chain antibody S17 (239 amino acids; SEQ ID NO: 71):

DIQMTQSPSSLSASVGRVTITCHASQDINTNIGWLQKPGKAFKGLIYHGKNLEDGVP  
RFGSGSGTDFTLTISSLQPEDFATYYCNQYENNPLTFGQGTKVEIKRGGGSGGGSGG  
20 GGSVDVQLVESGGGLVQPGGSLRLSCAVSGYSITSDYAWNWRQAPGKGLEWLG  
TQYNPSLKSRLISITRDNKNTFFLQLNSLRAEDTAVYYCARLGRGFRYWGQGLTVTVSS

Amino acid sequences of S17 light chain CDR1 (HASQDINTNIG), CDR2 (HGKNLED), CDR3 (NQYENNPLT) and heavy chain CDR1 (GYSITSDYAWN), CDR2 (YISYRGRTQYNPSLKS), CDR3 (LGRGFRY) are SEQ ID NO: 55, 42, 56, 44, 45, 50, respectively.

25 Nucleotide sequence of single chain antibody S22 (717 bases; SEQ ID NO: 72):

GATATTCAGATGACCCAGAGCCCGAGCAGCCTGAGCGGAGCGTGGGCGACCGTGTGACC  
ATTACCTGCCATGCGAGCCAGGATATTAACGTGAACATTGGCTGGCTGCAGCAGAAACCG  
GGCAAAGCGTTTAAAGGCCTGATTTATCATGGCACCAACCTGGAAGATGGCGTGCCGAGC  
30 CGTTTACGGCAGCGGCAGCGGCACCGATTTACCCTGACCATTAGCAGCCTGCAGCCG  
GAAGATTTTGCACCTATTATTGCAATCAGTATGAAAATAACCCACTGACATTTGGCCAG  
GGCACCAAAGTGAAAATTAACGTGGTGGAGGCGGTTTCAGGCGGAGGTGGCTCTGGCGGT  
GGCGGATCGGATGTGCAGCTGGTGAAAAGCGGCGGCGCCTGGTGCAGCCGGGCGGCAGC  
CTGCGTCTGAGCTGCGCGGTGAGCGGCTATAGCATTACCAGCGATTATGCGTGGAAGTGG  
ATTCGTCAGGCGCCGGGCAAAGGCCTGGAATGGCTGGGCTATATTAGCTATCGTGCCGC  
35 ACCCGCTATAACCCGAGCCTGAAAAGCCGTATTAGCATTACCCGTGATAACAGCAAAAAC  
ACCTTTTCTGCAGCTGAACAGCCTGCGTGCGGAAGATACCGCGGTGTATTATTGCGCG  
CGCCTGGGACGCGGCTTCCGCTACTGGGGCCAGGGCACCTGGTGACCGTGAGCAGC

Amino acid sequence of single chain antibody S22 (239 amino acids; SEQ ID NO: 73):

DIQMTQSPSSLSASVGRVTITCHASQDINVNIGWLQKPGKAFKGLIYHGNTLEDGVP  
RFGSGSGTDFTLTISSLQPEDFATYYCNQYENNPLTFGQGTKVEIKRGGGSGGGSGG  
40 GGSVDVQLVESGGGLVQPGGSLRLSCAVSGYSITSDYAWNWRQAPGKGLEWLG  
TRYNPSLKSRLISITRDNKNTFFLQLNSLRAEDTAVYYCARLGRGFRYWGQGLTVTVSS

Amino acid sequences of S22 light chain CDR1 (HASQDINVNIG), CDR2 (HGNTLED), CDR3 (NQYENNPLT) and heavy chain CDR1 (GYSITSDYAWN), CDR2 (YISYRGRTQYNPSLKS), CDR3 (LGRGFRY) are SEQ ID NO: 41, 53, 56, 44, 52, 50, respectively.

Nucleotide sequence of single chain antibody S23 (717 bases; SEQ ID NO: 74):

5 GATATTCAGATGACCCAGAGCCCGAGCAGCCTGAGCGCGAGCGTGGGCGACCGTGTGACC  
ATTACCTGCCATGCGAGCCAGGATATTAACGTGAACATTGGCTGGCTGCAGCAGAAACCG  
GGCAAAAGCTTTAAAGGCCTGATTTATCATGGCAAAACCTGGAAGATGGCGTGCCGAGC  
CGTTTTAGCGGCAGCGGCAGCGGCACCGATTTACCCTGACCATTAGCAGCCTGCAGCCG  
GAAGATTTTGCACCTATTATTGCAATCAGTATGAAAATAACCCACTGACATTTGGCCAG  
GGCACCAAAGTGAAATTAACGTGGTGGAGGCGGTTTCAGGCGGAGGTGGCTCTGGCGGT  
GGCGGATCGGATGTGCAGCTGGTGAAAGCGGCGGCGCCTGGTGCAGCCGGGCGGCAGC  
10 CTGCGTCTGAGCTGCGCGGTGAGCGGCTATAGCATTACCAGCGATTATGCGTGGAATGG  
ATTCGTCAGGCGCCGGGCAAAGGCCTGGAATGGCTGGGCTATATTAGCTATCGTGGCCGC  
ACCCGCTATAACCCGAGCCTGAAAAGCCGTATTAGCATTACCCGTGATAACAGCAAAAC  
ACCTTTTTCCTGCAGCTGAACAGCCTGCGTGCGGAAGATACCGCGGTGTATTATTGCGCG  
CGCCTGGGACGCGGCTTCCGCTACTGGGGCCAGGGCACCTGGTGACCGTGAGCAGC

Amino acid sequence of single chain antibody S23 (239 amino acids; SEQ ID NO: 75):

15 DIQMTQSPSSLSASVGRVTITCHASQDINVNIGWLQKPGKSFKGLIYHGKNLEDGVPVS  
RFGSGSGTDFTLTISSLQPEDFATYYCNQYENNPLTFGQGTKVEIKRGGGSGGGSGG  
GGSDVQLVESGGGLVQPGGSLRLSCAVSGYSITSDYAWNWRQAPGKGLEWLGYISYRGR  
TRYNPSLKSRISITRDNKNTFFLQLNSLRAEDTAVYYCARLGRGFRYWGQGLTVTVSS

20 Amino acid sequences of S23 light chain CDR1 (HASQDINVNIG), CDR2 (HGKNLEDG),  
CDR3 (NQYENNPLT) and heavy chain CDR1 (GYSITSDYAWN), CDR2  
(YISYRGRTRYNPSLKS), CDR3 (LGRGFRY) are SEQ ID NO: 41, 42, 56, 44, 52, 50,  
respectively.

Nucleotide sequence of single chain antibody S29 (717 bases; SEQ ID NO: 76):

25 GATATTCAGATGACCCAGAGCCCGAGCAGCCTGAGCGCGAGCGTGGGCGACCGTGTGACC  
ATTACCTGCCATGCGAGCCAGGATATTAACGTGAACATTGGCTGGCTGCAGCAGAAACCG  
GGCAAAGCGTTTAAAGGCCTGATTTATCATGGCAAAACCTGGAAGATGGCGTGCCGAGC  
CGTTTTAGCGGCAGCGGCAGCGGCACCGATTTACCCTGACCATTAGCAGCCTGCAGCCG  
GAAGATTTTGCACCTATTATTGCAATCAGTATGAAAATTTCCCACTGACATTTGGCCAG  
GGCACCAAAGTGAAATTAACGTGGTGGAGGCGGTTTCAGGCGGAGGTGGCTCTGGCGGT  
30 GGCGGATCGGATGTGCAGCTGGTGAAAGCGGCGGCGCCTGGTGCAGCCGGGCGGCAGC  
CTGCGTCTGAGCTGCGCGGTGAGCGGCTATAGCATTACCAGCGATTATGCGTGGAATGG  
ATTCGTCAGGCGCCGGGCAAAGGCCTGGAATGGCTGGGCTATATTAGCTATCGTGGCCGC  
ACCCGCTATAACCCGAGCCTGAAAAGCCGTATTAGCATTACCCGTGATAACAGCAAAAC  
ACCTTTTTCCTGCAGCTGAACAGCCTGCGTGCGGAAGATACCGCGGTGTATTATTGCGCG  
35 CGCCTGGGACGCGGCTTCCGCTACTGGGGCCAGGGCACCTGGTGACCGTGAGCAGC

Amino acid sequence of single chain antibody S29 (239 amino acids; SEQ ID NO: 77):

40 DIQMTQSPSSLSASVGRVTITCHASQDINVNIGWLQKPGKAFKGLIYHGKNLEDGVPVS  
RFGSGSGTDFTLTISSLQPEDFATYYCNQYENFPLTFGQGTKVEIKRGGGSGGGSGG  
GGSDVQLVESGGGLVQPGGSLRLSCAVSGYSITSDYAWNWRQAPGKGLEWLGYISYRGR  
TRYNPSLKSRISITRDNKNTFFLQLNSLRAEDTAVYYCARLGRGFRYWGQGLTVTVSS

Amino acid sequences of S23 light chain CDR1 (HASQDINVNIG), CDR2 (HGKNLED),  
CDR3 (NQYENFPLT) and heavy chain CDR1 (GYSITSDYAWN), CDR2  
(YISYRGRTRYNPSLKS), CDR3 (LGRGFRY) are SEQ ID NO: 41, 42, 57, 44, 52, 50,  
respectively.

#### Example 4. Expression and purification of antibody

Genes of each antibody were inserted into NdeI/XhoI site of the expression vector pET22B(+), antibody proteins were recombinantly produced in *E. coli* BL21 (DE3) and purified by nickel columns using polypeptides with carboxy-terminal fused 6 × histidine. In particular, to prepare the antibody protein, each single colony was inoculated into 5 ml of 2xYT/ampicillin medium and cultured with shaking at 37°C and 220 rpm for 16 hours. 1 ml of this preculture was used to inoculate 100 ml of 2 × YT / ampicillin medium and cultured with shaking at 37°C and 220 rpm until the cell density reached OD<sub>600</sub> = 0.5. After induction of foreign gene expression by 1 mM α-D-isopropylthiogalactoside (IPTG), the culture was shaken for 6 hours at 30°C and 220 rpm. The cells were then precipitated by centrifugation (15 minutes at 3500 x g, 4°C) and resuspended in 35 ml breaking buffer (50 mM PB, 300 mM NaCl, 2 M urea, 0.5% Triton X-100, pH 8.0). After sonication, the sample was shaken at room temperature for 30 minutes to completely lyse cell debris. Inclusion body pellets were then collected by centrifugation (15 minutes, 10,000 xg, 4°C) and 20 ml denaturing buffer (50 mM PB, 300 mM NaCl, 8 M urea, 10 mM imidazole, pH 8.0) was added and shaken for one hour at room temperature. The pellets were removed by centrifugation (15 min, 10,000 xg, 4°C), the lysate was collected and the protein was purified with 5 ml HisTrap HP purification column (commercially available from GE Healthcare). The purity of the purified antibody protein was analyzed by SDS polyacrylamide gel electrophoresis and the protein concentration was determined by the BCA method.

#### **Example 5. Binding activity assay of Antibody**

Binding activity of an antibody to antigen EGFRvIII was determined by concentration gradient ELISA assay. For this purpose, the antigen EGFRvIII was diluted with 0.1 M NaHCO<sub>3</sub> (pH 9.6) coating solution and each well was coated with 200 ng at 50 µl/well overnight at 4°C and blocked with PBST containing 2% (w/v) BSA for 2 hours at room temperature. The plate was then rinsed for three times with PBST. Subsequently, 100 µl of each antibody protein solution in PBST containing a series of concentrations (initial concentration of 50 ng/well, 18 nM diluted until to 1: 81) was added to each well plate and each sample was assayed using parallel three-well analysis. After incubation for 2 hours at 37°C, plate was rinsed for three times with PBST followed by adding 100 µl/well of a 1: 2000 dilution of mouse anti-His-tag antibody (available from Santa cruz) for 1 hour at 37°C. To test the bound antibody, HRP-labeled goat anti-mouse antibody (purchased from Santa Cruz) was diluted in PBST at a 1: 15,000 dilution and 100 µl per well was added and incubated at 37°C for 1 hour. For detection, wells were rinsed for three times with PBST followed by rinsing for three times with PBS and finally TMB was added for development for 15 mins. The chromogenic reaction was stopped with 50 µl of 2 M H<sub>2</sub>SO<sub>4</sub> per well and extinction value was measured at 450 nm using enzyme-linked immunoassay (Bio-Rad). The absorbance values obtained were evaluated using Sigma Plot software and the binding strength of an antibody was

calculated. For this purpose, the extinction value measured in each case was plotted against the corresponding antibody concentration and the resulting curve was fitted using the following non-linear regression.

$$y = \frac{a * x}{(b + x)}$$

Wherein the binding/dissociation equilibrium identified between the immobilized antigen and the antibody protein is:

x = concentration of antibody protein;

y = concentration of antigen / antibody complex (indirectly measured by absorbance after color reaction);

a = total concentration of immobilized antigen;

b = dissociation constant ( $K_D$ ).

The binding curve obtained for antibody 7B3 in concentration-gradient ELISA assay is exemplarily shown in Figure 2 with  $K_D$  of about 22.4 nM; the binding curve of antibody Y022 to EGFRvIII is shown in Figure 3 with apparent  $K_D$  of about 2.7 nM.

## **Example 6. Activity Analysis on Binding of Y022 to Cell Surface EGFR**

### **1. Expression and purification of scFv-Y022-Fc, scFv-806-Fc and scFv-C225-Fc fusion antibody**

According to a standard scheme, scFv-Y022 fragment was amplified from the resulting clones using the primer pair V5-Y022-F (SEQ ID NO: 14, ACAGTGCTAGCAGATATTCAGATGACCCAG) and V5-Y022-R (SEQ ID NO: 15, AAGAATGCGGCCGCTGCTCACGGTCACCAG); ScFv-806 was amplified using the primer pair V5-806-F (SEQ ID NO: 16, ACAGTGCTAGCAGACATCCTGATGACCCAAT) and V5-806-R (SEQ ID NO: 17, AAGAATGCGGCCGCTGCAGAGACAGTGACCAG) and pH-806/CD3 (see 201210094008.X) as the template; scFv-C225 fragment was cloned using the primer pairs V5-C225-F (SEQ ID NO: 18, ACAGTGCTAGCAGACATCTTGCTGACTCAG) and V5-C225-R (SEQ ID NO: 19, AAGAATGCGGCCGCTGCAGAGACAGTGACCAG) and C225 (VL-linker-VH) DNA fragment (sequence thereof was determined according to SEQ ID NO: 10 and SEQ ID NO: 12 in US20090099339A1 and obtained through whole-genome synthesis by Shanghai raygene biotechnology Co., LTD) as the template; and the amplified product was digested by NheI/NotI (purchased from NEB), linked with NheI/NotI-digested vector plasmid pCMV-V5-Fc (in the vector, the Fc fragment of human IgG1 was fused downstream to the multiple cloning site, abbreviated as V5-Fc, purchased from Shanghai raygene biotechnology Co., LTD) with T4 DNA ligase (purchased from NEB) and transformed into host strain TOP10. Clones were selected and the positive clones were identified by PCR and confirmed by sequencing, so as to obtain V5-scFv-Y022-Fc, V5-scFv-806-Fc and V5-scFv-C225-Fc eukaryotic expression

plasmids, respectively.

The above expression plasmids were respectively transfected into well-growing HEK-293F cells, cultured at 37°C, 5% CO<sub>2</sub>, 125 rpm shaker for 7 days, and centrifuged at 4000 rpm for 10min. The precipitate was removed, the supernatant was collected and filtered with 0.45 μm membrane.

The sample was affinity-purified with protein A (from GE) affinity column to finally obtain the purified antibody-Fc fusion proteins scFv-Y022-Fc, scFv-806-Fc and scFv-C225-Fc. Results are shown in Fig 4.

## 2. Detection of binding ability of single-chain scFv-Y022-Fc, scFv-806-Fc and scFv-C225-Fc to cell-surface EGFR by FACS

The binding capacities of each of single-chain antibodies scFv-Y022-Fc, scFv-806-Fc and scFv-C225-Fc to the following cell lines were analyzed by fluorescence activated cell sorter (FACS) (BD, FACS Calibur).

Specific methods are as follows:

1) tumor cells in logarithmic growth phase as listed in Table 2 were inoculated into a 6cm dish with a inoculation cell density of about 90%, and incubated at 37°C incubator overnight.

2) Cells were digested with 10 mM EDTA and cells were collected by centrifugation at 200 g for 5 min. Cells were resuspended in 1% phosphate buffered saline (NBS PBS) containing calf serum at a concentration of  $1 \times 10^6$  to  $1 \times 10^7$  / mL, and added in a flow tube in an amount of 100 μl/tube.

3) Cells were centrifuged at 200g × 5min, and the supernatant was discarded.

4) Antibodies to be tested, scFv-Y022-Fc, scFv-806-Fc and scFv-C225-Fc, were added respectively. And PBS was used as a negative control. The final concentration of antibody was 20μg / ml, 100μl was added to each tube and placed in an ice bath for 45 minutes.

5) 2ml of 1% NBS PBS was added to each tube and centrifuged at 200 g × 5min for two times.

6) Supernatant was discarded and FITC fluorescent-labeled goat anti-human antibody (from Shanghai Kangcheng Bio-engineering Company) at a dilution of 1:50 was added, and 100ul was added to each tube and placed in an ice bath for 45 minutes.

7) 2ml of 1% NBS PBS was added to each tube and centrifuged at 200 g × 5 min for two times.

8) Supernatant was discarded, resuspended in 300ul of 1% NBS PBS and detected by flow cytometry.

9) Data was analyzed by using flow cytometry data analysis software WinMDI 2.9.

Tbale 2

| Name of tumor cell | Source | Properties of cell | Expression of EGFR |
|--------------------|--------|--------------------|--------------------|
|--------------------|--------|--------------------|--------------------|

|              |                           |                                    |                          |
|--------------|---------------------------|------------------------------------|--------------------------|
| U87          | ATCC                      | Glioma cell line                   | Low-expressed EGFR       |
| U87-EGFR     | Shanghai Cancer Institute | EGFR-transfected U87 cell line     | over-expressed EGFR      |
| U87-EGFRvIII | Shanghai Cancer Institute | EGFRvIII-transfected U87 cell line | over-expressed EGFRvIII  |
| A431         | ATCC                      | Vaginal epithelial cancer          | over-expressed EGFR      |
| CAL 27       | ATCC                      | Tongue cancer cell line            | over-expressed EGFR      |
| MDA-MB-468   | ATCC                      | Breast cancer cell line            | over-expressed EGFR      |
| RWPE-1       | ATCC                      | Prostate normal epithelial cells   | normally-expressed EGFR  |
| K2           | Shanghai Cancer Institute | Human primary keratinocytes        | normallyr-expressed EGFR |

Results are shown in FIG. 5, single chain antibody Y022 of the present invention can, with different degrees, bind to U87-EGFR exogenously overexpressing EGFR (Construction method can be found in Wang H., et al., Identification of an Exon 4-Deletion Variant of Epidermal Growth Factor Receptor with Increased Metastasis-Promoting Capacity. Neoplasia, 2011, 13, 461-471) and U87-EGFRvIII overexpressing EGFRvIII (construction method can be found in WO/2011/035465), A431, CAL27, MDA-MB-468 endogenously overexpressing EGFR, and especially strongly bind to U87-EGFRvIII and A431 cells, but its binding ability was not as high as that of single-chain antibody 806. Binding ability of the single-chain antibody C225 to these cells is very strong. These single-chain antibodies have little binding to U87 cells.

In addition, both Y022 and 806 single-chain antibodies almost did not bind to glioma cell line U87 cells. Especially, single chain antibody Y022 also does not bind to Normal prostate epithelial cells RWPE-1 and human primary keratinocyte K2, whereas single-chain antibody 806 binds to both of these normal cells with different degrees.

These results indicate that the single chain antibody Y022 specifically binds to tumor cells overexpressing EGFR as well as EGFRvIII, while little binding to normal EGFR-expressing cells.

#### **Example 7. Construction of Expression Vector Containing Nucleotide Sequence Encoding Y022 / CD3 Single Chain Bifunctional Antibodies**

PCR amplification was performed using the pCantab 5E-Y022 plasmid obtained in Example 3 as a template and a primer pair, the forward primer pH7B3f2\_fw (SEQ ID NO: 20, GATATTCAGATGACCCAGAGCCCGAGCAG) and the reverse primer pH7B3f2\_re (SEQ ID NO: 21, AATAGGATCCACCACCTCCGCTGCTCACGGTCAC) to obtain DNA fragment of Y022 scFv. Another DNA fragment containing the pH vector signal peptide sequence was obtained by PCR using pH-7B3/CD3 plasmid (see 201210094008.X Example 3 and Figure 2) as a

template and the forward primer pH7B3f1\_fw (SEQ ID NO: 22 , CCATTGACGCAAATGGGCGGTAGG) and reverse primer pH7B3f1\_re (SEQ ID NO: 23, CTGCTCGGGCTCTGGGTCATCTGAATATC). The two fragments were mixed in equimolar ratio for fragment splicing and PCR. The splicing conditions were: denaturation: 94°C for 4 min; denaturation: 94°C for 40 s; annealing: 60°C for 40 s; extension: 68°C for 140 s for 5 cycles, and then the total extension of 68°C, 10min. And then DNA polymerase and forward primer pH7B3f1\_fw and reverse primer pH7B3f2\_re were supplemented, 30 cycles of amplification were performed, and amplification conditions were: 94°C, 4min; denaturation: 94°C, 40s; annealing: 60°C, 40s; 68°C for 140 s for 30 cycles and then total extension 68°C for 10 min.

The amplified sequence was digested with the restriction endonuclease NheI / BamHI and double-digested according to the reaction conditions recommended by the enzyme supplier (New England Biolabs, NEB). The expression vector pH (see 201210094008.X Example 3 and Figure 2) was also similarly digested with the restriction enzyme NheI / BamHI. The double-digested Y022 scFv fragment and pH vector fragment were then ligated with T4 DNA ligase following the reaction conditions recommended by the enzyme supplier (NEB). The nucleotide sequence encoding for Y022 single chain antibody polypeptide thus obtained was cloned into a vector, and was transcribed together with the nucleotide sequence already contained in the vector encoding CD3 single-chain antibody polypeptide into an mRNA, which finally translated into Y022/CD3 single-chain bifunctional antibody polypeptide. The new plasmid was named as pH-Y022/CD3, and the detailed structure was shown in Figure 6.

#### **Example 8. Expression and purification of single chain bifunctional antibody Y022/CD3, pH-806/CD3 and pH-C225/CD3**

The expression vectors pH-Y022/CD3, pH-806/CD3 and pH-C225/CD3 (see 201210094008.X) were transfected into Chinese hamster ovary (CHO) cells according to the procedure of FreeStyle MAX Reagent Transfection Reagent (from Invitrogen). And then the stable clones were screened according to OptiCHO™ protein expression kit (from Invitrogen). Stable clones of CHO cells transfected with each of the above expression vectors were cultured in shake flasks at 37°C for 7 days at 130 rpm, and the used medium was CD OptiCHO (from Gibco). The culture supernatant was obtained by centrifugation and then stored at -20°C.

Protein purification was performed using a histidine affinity column (His Trap HP column, available from GE Healthcare) according to the manufacturer's method steps. Specifically, the column was equilibrated with buffer A (20 mM sodium phosphate pH 7.4, 0.4 M NaCl) and then the cell culture supernatant (500 mL of the supernatant) was added to the column (1 mL) with a flow rate of 3 ml / min after dialysis against PBS. The column was then washed with 5 volumes of buffer A and 10 volumes of buffer A containing 50 mM imidazole to remove the impurity protein.

The bound protein of interest was eluted with the same buffer A supplemented with 250 mM imidazole. All purification steps were performed at 4°C.

Purified single-chain bifunctional antibodies were detected by reducing SDS-PAGE. As shown in FIG. 7, the molecular weights of these antibody molecules were all around 60 kD, which corresponded to the molecular weight of the single-chain bifunctional antibody calculated from the amino acid sequences.

#### **Example 9. Analysis of antigen binding specificity of single chain bifunctional antibody such as Y022/CD3**

The binding capacities of single chain bifunctional antibody Y022/CD3 to EGFR were analyzed by fluorescence activated cell sorter (FACS) (BD, FACS Calibur).

Specific methods are as follows:

1. Tumor cells in logarithmic growth phase as listed in Table 2 were inoculated into a 6cm dish with inoculation cell density of about 90%, and incubated at 37°C incubator overnight.

2. Cells were digested with 10 mM EDTA and cells were collected by centrifugation at 200 g for 5 min. Cells were resuspended in 1% phosphate buffered saline (NBS PBS) containing calf serum at a concentration of  $1 \times 10^6$  to  $1 \times 10^7$  / mL, and added in a flow tube in an amount of 100  $\mu$ l/tube.

3. Cells were centrifuged at  $200g \times 5min$ , and the supernatant was discarded.

4. Antibody Y022/CD3 to be tested was added. And irrelevant antibody NGR/CD3 was used as a negative control. The final concentration of antibody was 5  $\mu$ g/ml, 100 $\mu$ l was added to each tube and placed in an ice bath for 45 minutes.

5. 2ml of 1% NBS PBS was added to each tube and centrifuged at  $200 g \times 5min$  for two times.

6. Supernatant was discarded and mouse anti-his tag antibody (from Shanghai Genomics Technology Co., Ltd.) at a dilution of 1:50 was added, and 100 $\mu$ l was added to each tube and placed in an ice bath for 45 minutes.

7. 2ml of 1% NBS PBS was added to each tube and centrifuged at  $200 g \times 5 min$  for two times.

8. Supernatant was discarded and FITC fluorescent-labeled goat anti-mouse antibody (from Shanghai Kangcheng Bio-engineering Company) at a dilution of 1:50 was added, and 100 $\mu$ l was added to each tube and placed in an ice bath for 45 minutes.

9. 2ml of 1% NBS PBS was added to each tube and centrifuged at  $200 g \times 5 min$  for two times.

10. Supernatant was discarded, resuspended in 300 $\mu$ l of 1% NBS PBS and detected by flow cytometry.

11. Data was analyzed by using flow cytometry data analysis software WinMDI 2.9.

Results are shown in FIG. 8, the bifunctional antibody Y022/CD3 of the present invention can bind to U87-EGFR, U87-EGFRvIII and A431 cells, however, hardly bind to U87 and human keratinous epithelial cells. These results indicate that Y022/CD3 can specifically bind to tumor cells expressing mutant human EGFR and overexpressing EGFR, but not to tissues that normally express EGFR.

In addition, Y022/CD3 can also bind to human peripheral blood mononuclear cells (PBMCs) or Jurkat cells (human peripheral blood leukemia T cells, CD3 positive) as shown in the figure, suggesting that the bifunctional antibody of the present invention can specifically bind to CD3 antigen of T cell surface.

The expression plasmids were constructed according to the methods mentioned in Examples 7 and 8, respectively (Y022 in Examples 7 and 8 were replaced with other mutated forms of antibodies), and M14/CD3, M15/CD3, M25/CD3, M26/CD3, S7/CD3, S8/CD3, S17/CD3, S22/CD3, S23/CD3, S29/CD3 were expressed and purified. According to the method of this example, the binding abilities of these antibodies to U87-EGFRvIII overexpressing EGFRvIII and to CAL27 cells endogenously overexpressing EGFR were determined respectively. The above antibodies were able to bind both of these cells, and their mean fluorescence intensity (MFI) values are shown in Table 13.

Table 13

| Antibody | U87MG-EGFRvIII | CAL 27 |
|----------|----------------|--------|
| PBS      | 1              | 3.11   |
| M14      | 36.52          | 28.39  |
| M15      | 37.86          | 29.43  |
| M25      | 36.52          | 24.14  |
| M26      | 41.42          | 24.58  |
| S7       | 42.17          | 27.88  |
| S8       | 38.54          | 29.96  |
| S17      | 31.62          | 25.03  |
| S22      | 31.34          | 24.58  |
| S23      | 32.2           | 29.69  |
| S29      | 34.6           | 25.71  |

#### **Example 10. Biological Activity Analysis of Single-chain Bifunctional Antibody, such as Y022/CD3 - Cytotoxicity to Various Tumor Cells**

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy human-donated blood following standard procedures using Ficoll (from Biochrom) density gradient centrifugation. After centrifugation, the cells were washed with phosphate buffered saline (PBS) at a concentration of 0.1 M and then resuspended in RPMI 1640 complete medium (Gibco) and the cell concentration was adjusted to  $5 \times 10^5/\text{mL}$ . PBMCs served as effector cells in cytotoxicity

experiments. Different tumor cells act as target cells. The target cell concentration was adjusted to  $5 \times 10^4$ /mL with RPMI 1640 complete medium. The same volume of target cells and effector cells were mixed such that the effector cell: target cell (E: T) ratio was 10: 1.

The mixed cell suspension was added to a 96-well plate in a volume of 75  $\mu$ L/well. Then 25  $\mu$ L of the following reagent serially diluted ten times from 1000 ng / mL to 0.1 ng / mL was added to each well:

(1) Y022/CD3 Single chain bifunctional antibody (BiTe);

(2) RPMI 1640 complete medium (background);

(3) NGR/CD3 Single chain bifunctional antibody (negative control, NGR was a neovascular targeting peptide that has no cross-binding site with EGFR, and prepared according to a conventional method)

After incubation for 40 hours in a 37°C, 5% CO<sub>2</sub> incubator, the cytotoxicity of the antibody was tested using CytoTox 96® Non-Radioactive Cytotoxicity Assay kit (from Promega) according to the manufacturer's instructions.

The CytoTox 96® Non-Radioactive Cytotoxicity Assay is a colorimetric based assay that can replace 51Cr release assay. CytoTox 96® Assay measures lactate dehydrogenase (LDH) quantitatively. LDH is a stable cytosolic enzyme that is released upon lysis of cells and is released in the same way as radioactive 51Cr is released. The supernatant with released LDH medium can be detected by a 30-minute coupled enzyme reaction in which LDH converts a tetrazolium salt (INT) to a red formazan. The amount of red product produced is proportional to the number of lysed cells.

Five EGFR-associated tumor cells as listed in Table 3 below were used to analyze T cell tumors killing ability mediated by the bifunctional antibody Y022/CD3 of the present invention and the NGR/CD3 single-chain bifunctional antibody that is not associated with EGFR as a control, respectively.

Tumor cell killing rate (i.e., cytotoxicity%) was calculated based on the following formula provided in the manual of CytoTox 96® non-radioactive cytotoxicity assay G1780:

$$\text{cytotoxicity\%} = \frac{[(\text{experiment} - \text{effector cell spontaneously} - \text{target cell spontaneously})/(\text{target cell maximum} - \text{target cell spontaneously})] \times 100}{1}$$

wherein:

"Experiment" refers to LDH release produced in the experimental wells, in which the antibody / effector / target cells were added,

"effector cell spontaneously" refers to LDH release produced by effector cells spontaneously,

"target cell spontaneously" refers to LDH release produced by cells that are not treated by other factors,

"target cell maximum" refers to LDH release resulting from complete lysis of target cells after

treatment with 0.8% Triton X-100,

"target cell maximum - target cell spontaneously" refers to LDH release resulting from complete lysis of cells after external treatment.

Table 3

| Tumor cell line | 1000 ng/ml<br>Cytotoxicity% of Y022/CD3 | 1000 ng/ml<br>Cytotoxicity% of NGR/CD3 |
|-----------------|-----------------------------------------|----------------------------------------|
| U87             | 2.0                                     | 3.4                                    |
| U87-EGFR        | 32.1                                    | 3.7                                    |
| U87-EGFRvIII    | 66.2                                    | 6.3                                    |
| A431            | 48.7                                    | 5.2                                    |
| K2              | 5.1                                     | 4.5                                    |

5 The results of Table 3 above show that all of the tumor cells expressing mutant EGFR and / or overexpressing EGFR, such as U87-EGFRvIII, U87-EGFR and A431, will be specifically killed by T-cells directed by the bifunctional antibody Y022/CD3.

Specifically, in the above tumor cell group treated with Y022/CD3, the minimum specific cytotoxicity was 32.1% and the maximum was 66.2%. While the cytotoxicity of Y022/CD3 to cells expressing low levels of EGFR, U87, and human primary keratinocytes was very low at 3.4% and 4.5%, respectively, which were significantly lower than those to the above-mentioned mutant cells expressing EGFR and / or overexpressing EGFR.

More specifically, cytotoxicity% of Y022/CD3 and control antibody NGR/CD3 at various concentrations to each tumor is shown in Tables 4-8 below.

Table 4

| ng/ml | U87     |          |
|-------|---------|----------|
|       | NGR/CD3 | Y022/CD3 |
| 1000  | 3.4±1.2 | 2.0±1.3  |
| 100   | 4.8±1.1 | 1.6±3.2  |
| 10    | 4.3±1.5 | 2.5±2.3  |
| 1     | 5.2±2.1 | 0.5±1.2  |
| 0.1   | 5.4±2.2 | 0.2±1.7  |

Table 5

| ng/ml | U87-EGFR |          |
|-------|----------|----------|
|       | NGR/CD3  | Y022/CD3 |
| 1000  | 3.7±2.6  | 32.1±3.1 |
| 100   | 4.9±1.7  | 21.7±4.4 |
| 10    | 4.3±2.7  | 12.6±3.2 |
| 1     | 3.3±1.9  | 6.3±2.6  |
| 0.1   | 0.7±1.2  | 5.1±2.0  |

Table 6

| ng/ml | U87-EGFRvIII |          |
|-------|--------------|----------|
|       | NGR/CD3      | Y022/CD3 |
| 1000  | 6.3±1.3      | 66.2±5.8 |
| 100   | 7.4±2.4      | 52.5±4.5 |
| 10    | 6.5±0.8      | 33.6±3.2 |

|     |         |          |
|-----|---------|----------|
| 1   | 4.7±2.1 | 25.3±2.9 |
| 0.1 | 2.6±1.4 | 6.7±2.3  |

Table 7

| ng/ml | A431    |          |
|-------|---------|----------|
|       | NGR/CD3 | Y022/CD3 |
| 1000  | 5.2±2.9 | 48.7±4.3 |
| 100   | 5.6±2.7 | 35.3±5.1 |
| 10    | 3.7±2.4 | 22.7±3.3 |
| 1     | 1.3±0.6 | 10.8±4.4 |
| 0.1   | 1.5±1.1 | 4.3±2.1  |

Table 8

| ng/ml | K2      |          |
|-------|---------|----------|
|       | NGR/CD3 | Y022/CD3 |
| 1000  | 4.5±2.2 | 5.1±1.1  |
| 100   | 4.1±2.8 | 3.2±1.2  |
| 10    | 3.5±2.4 | 1.7±1.0  |
| 1     | 2.1±1.8 | 2.7±1.2  |
| 0.1   | 4.3±2.9 | 2.1±1.3  |

In addition, *in vitro* toxicity analysis was performed by the same method on the following expressed and purified BiTe: M14 / CD3, M15 / CD3, M25 / CD3, M26 / CD3, S7 / CD3, S8 / CD3, S17 / CD3, S22 / S29 / CD3, and the results are shown in Figure 9.

As can be seen in Figure 9, tumor cells expressing mutated EGFR and / or overexpressing EGFR, such as U87-EGFRvIII, U87-EGFR, and CAL27, can be killed by T cells directed by the bifunctional specific antibodies M14 / CD3, M15 / CD3, M25 / M26 / CD3, S7 / CD3, S8 / CD3, S17 / CD3, S22 / CD3, S23 / CD3, S29 / CD3 to different degrees. While for U87, a cell that expresses low levels of EGFR, there is little killing effect.

#### **Example 11. Construction of lentiviral plasmid expressing the chimeric antigen receptor protein encoded by the nucleic acid of the present invention and virus packaging**

Construction of the chimeric antigen receptor, and the connection order of chimeric antigen receptor exemplified in the present invention, is shown in Table 9 and FIG. 10.

Table 9

| chimeric antigen receptor | Extracellular binding region - transmembrane region - intracellular signal region 1 - intracellular signal region 2 and the like | Description                |
|---------------------------|----------------------------------------------------------------------------------------------------------------------------------|----------------------------|
| Y022-δZ                   | scFv(EGFR)-CD8-CD3δzeta                                                                                                          | Negative control           |
| Y022-Z                    | scFv(EGFR)-CD8-CD3 zeta                                                                                                          | 1 <sup>st</sup> generation |
| Y022-BBZ                  | scFv(EGFR)-CD8-CD137-CD3 zeta                                                                                                    | 2 <sup>nd</sup> generation |
| Y022-28Z                  | scFv(EGFR)-CD28a-CD28b-CD3 zeta                                                                                                  | 2 <sup>nd</sup> generation |
| Y022-28BBZ                | scFv(EGFR)-CD28a-CD28b-CD137-CD3 zeta                                                                                            | 3 <sup>rd</sup> generation |

**Note:** CD28a represents the transmembrane region of CD28 molecule and CD28b represents the intracellular signaling region of CD28 molecule.

## **1. Amplification of nucleic acid fragments**

### **(1) Amplification of scFv sequences**

Y022 scFv was obtained by PCR using pCantab 5E-Y022 plasmid as a template with a forward primer (SEQ ID NO: 24, comprising part of the sequence of CD8 signal peptide) and a reverse primer (SEQ ID NO: 25, comprising part of the sequence of CD8 hinge).

SEQ ID NO: 24 (TGCTCCACGCCGCCAGGCCGGATATTCAGATGACCCAG)

SEQ ID NO: 25 (CGCGGCGCTGGCGTCGTGGTGCTGCTCACGGTCAC)

### **(2) Nucleic acid sequences of other parts of the chimeric antigen receptor**

The nucleic acid sequences of other parts of the anti-EGFRvIII chimeric antigen receptor protein except for Y022 scFv were respectively obtained by PCR using the sequences SEQ ID NO: 26, 27, 28, 29 and 30 disclosed in Patent Application No. 201310164725.X as templates.

Specifically, the eGFP-F2A-CD8sp sequence was obtained by PCR amplification using SEQ ID NO: 27 plasmid contained in Patent Application No. 201310164725.X as a template and primer pairs (SEQ ID NOs: 26 and 27). CD8-CD3 $\delta$  zeta ( $\delta$ Z) was obtained by PCR amplification using SEQ ID NO: 26 plasmid in the patent application CN201310164725.X as a template and primer pairs (SEQ ID NOs: 28 and 29). The CD8-CD3 zeta (Z), CD8-CD137-CD3 zeta (BBZ), CD28a-CD28b-CD3 zeta (28Z) and CD28a-CD28b-CD137-CD3 zeta (28BBZ) were obtained by PCR amplification respectively using SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29 and SEQ ID NO: 30 in the patent application CN201310164725.X as templates and primer pairs (SEQ ID NO: 28, 30).

SEQ ID NO: 26 (TGCAGTAGTCGCCGTGAAC)

SEQ ID NO: 27 (CGGCCTGGCGGCGTGGAGCA)

SEQ ID NO: 28 (ACCACGACGCCAGCGCCGCGACCAC)

SEQ ID NO: 29 (GAGGTCGACCTACGCGGGGGCGTCTGCGCTCCTGCTGAACTTCACTCT)

SEQ ID NO: 30 (GAGGTCGACCTAGCGAGGGGGCAGGGCCTGCATGTGAAG)

## **2. Splicing of nucleic acid fragments**

eGFP-F2A-CD8sp nucleic acid fragment obtained as described above and equimolar Y022 scFv nucleic acid fragment and equimolar CD8-CD3 $\delta$  zeta ( $\delta$ Z) or CD8-CD3 zeta (Z) or CD8-CD137-CD3 zeta (BBZ) or CD28a-CD28b-CD3 zeta (28Z) or CD28a-CD28b-CD137-CD3 zeta (28BBZ) nucleic acid fragments were subjected to three-segment splicing and PCR as shown in Figure 9 under the following conditions: Pre-denaturation: 94°C for 4 min; denaturation: 94°C for 40 s; annealing: 60°C for 40 s; extension: 68°C for 140 s for 5 cycles and then total extension 68°C for 10 min. DNA polymerase and forward primer (SEQ ID NO: 24) and reverse primer (reverse primer corresponding to CD8-CD3 $\delta$  zeta was SEQ ID NO: 29, and other is SEQ ID NO:

30) were supplemented, and then PCR was performed for 30 cycles with the amplification conditions: Pre-denaturation: 94°C for 4 min; denaturation: 94°C for 40 s; annealing: 60°C for 40 s; extension: 68°C for 140 s for 30 cycles and then total extension 68°C for 10 min. The amplified fragments were referred to as (Table 2):

eGFP-F2A-Y022 scFv- $\delta$ Z (SEQ ID NO: 31),  
eGFP-F2A-Y022 scFv-Z (SEQ ID NO: 32),  
eGFP-F2A-Y022 scFv-BBZ (SEQ ID NO: 33),  
eGFP-F2A-Y022 scFv-28Z (SEQ ID NO: 34),  
eGFP-F2A-Y022 scFv-28BBZ (SEQ ID NO: 35).

### 3. Construction of lentiviral plasmid vector

By way of example, the vector system used for the lentiviral plasmid vectors constructed below belongs to self-inactivating lentiviral vector system of the third generation, which has three plasmids, namely, packaging plasmid psPAX2 encoding protein Gag/Pol, encoding Rev protein (from addgene); envelope plasmid PMD2.G encoding VSV-G protein (from addgene); and the recombinant expression vector encoding the gene of interest CAR based on empty vector pWPT-eGFP (from addgene).

In the empty vector pWPT-eGFP, the expression of enhanced green fluorescent protein (eGFP) is regulated by elongation factor-1 $\alpha$  (EF-1 $\alpha$ ) promoter. After inserting the constructs constructed as described in this example into an empty vector, a recombinant expression vector encoding CAR of the target gene was formed, wherein co-expression of eGFP and target gene CAR is achieved ribosomal skipping sequence from foot and mouth disease virus (FMDV, F2A). F2A is a core sequence of 2A (or "self-cleaving polypeptide 2A") from foot-and-mouth disease virus possessing the "self-shearing" function of 2A that enables upstream and downstream gene co-expression. 2A provides an effective and viable strategy for constructing polycistronic vectors for gene therapy due to its high shearing efficiency, high upstream and downstream gene expression balance and short sequence itself. Especially in immunotherapy based on chimeric antigen receptor gene modified T lymphocytes, this sequence is frequently used to achieve the co-expression of the target gene with GFP or eGFP. The expression of CAR can be indirectly detected by detecting GFP or eGFP.

In this example, a lentiviral expression vector co-expressing eGFP and specific CAR linked by F2A was constructed, collectively referred to as pWPT-eGFP-F2A-CAR. The target gene eGFP-F2A-CAR (see 2 in Example 7, the component after F2A is abbreviated as CAR) obtained in the above step 2 was double-digested by MluI and SalI restriction enzymes and ligated into the same double digested pWPT vector to construct a lentiviral vector expressing each chimeric antigen receptor. The constructed vector was identified by MluI and SalI digestion and sequenced correctly, which was ready for lentivirus packaging. As mentioned above, eGFP-F2A-CAR was

transcribed into one mRNA but eventually translated into two peptide chains of eGFP and anti-EGFRvIII chimeric antigen receptors, where the anti-EGFRvIII chimeric antigen receptor will be localized under the guidance of CD8 $\alpha$  signal peptide on the cell membrane.

The vectors containing the desired CARs are as follows (the components following F2A may be abbreviated as CAR):

pWPT-eGFP-F2A-Y022 scFv- $\delta$ Z;

pWPT-eGFP-F2A-Y022 scFv-Z;

pWPT-eGFP-F2A-Y022 scFv-BBZ;

pWPT-eGFP-F2A-Y022 scFv-28Z;

pWPT-eGFP-F2A-Y022 scFv-28BBZ.

5 eGFP-F2A-CAR polypeptide sequences were respectively obtained through the above construction, which are named as:

eGFP-F2A-Y022 scFv- $\delta$ Z (SEQ ID NO: 36);

eGFP-F2A-Y022 scFv-Z (SEQ ID NO: 37);

eGFP-F2A-Y022 scFv-BBZ (SEQ ID NO: 38);

eGFP-F2A-Y022 scFv-28Z (SEQ ID NO: 39);

eGFP-F2A-Y022 scFv-28BBZ (SEQ ID NO: 40).

#### **4. Plasmid-transfected 293T packaging lentivirus**

HEK-293T cells (ATCC: CRL-11268) cultured at passage 6 to passage 10 were seeded at a density of  $6 \times 10^6$  in 10 cm dishes and cultured overnight at 37°C in 5% CO<sub>2</sub> for transfection. The medium was DMEM (available from PAA) containing 10% fetal bovine serum (purchased from PAA).

Transfection steps are as follows:

4.1 Preparation of liquid A: dissolving 10  $\mu$ g of mock control or 10  $\mu$ g of each of the desired gene plasmids pWPT-eGFP-F2A-CAR with 7.5  $\mu$ g of packaging plasmid PAX2: and 3  $\mu$ g of envelope plasmid pMD2.G into 800  $\mu$ L of serum-free DMEM medium and mixing well.

4.2 Preparation of liquid B: dissolving 60 $\mu$ g PEI (polyethylenimine, purchased from Polysciences) in 800 $\mu$ L serum-free DMEM medium, mixing gently and incubating at room temperature for 5min.

4.3 Formation of transfection complex: adding liquid A into liquid B and gently mixing, vortexing or gently mixing immediately after addition, incubating at room temperature for 20min.

4.4 Adding 1.6 ml of the transfection complex into HEK-293T cells dropwise, and after 4-5 h, changing to DMEM with 2% FBS for transfected 293T cells.

In the next day after transfection, the transfection efficiency (that is, the proportion of green fluorescent cells) was observed: ~ 80% of the positive transfection efficiency represents the successful transfection experiments. After 72 h of transfection, the virus was collected by filtration

using a 0.45  $\mu\text{m}$  filter (available from Millipore Corporation) and centrifuged at 28,000 rpm using a Beckman Optima L-100XP ultracentrifuge for 2 hours at 4°C. The supernatant was discarded and the resulting pellet was centrifuged at 1/10 ~ 1/50 stock solution of AIM-V (purchased from Invitrogen) and resuspend at 100  $\mu\text{L}/\text{tube}$  in -80°C for virus titration or infection of T lymphocytes.

## 5. Determination of Lentiviral titers packaged with mock or eGFP-F2A-CAR

On the first day, 293T cells were inoculated at  $1 \times 10^5 / \text{mL}$  in 96-well culture plates, 100  $\mu\text{L}/\text{well}$ , and cultured at 37°C, 5%  $\text{CO}_2$ , and the culture medium was DMEM containing 10% fetal bovine serum. On the next day, 50  $\mu\text{L}/\text{well}$  of culture supernatant was discarded, 50  $\mu\text{L}/\text{well}$  of fresh medium was supplemented, and polybrene at final concentration of 6  $\mu\text{g} / \text{mL}$  was contained. The culture was incubated for 30 min at 37°C with 5%  $\text{CO}_2$ . 10  $\mu\text{L}/\text{well}$  of virus stock or 1  $\mu\text{L}/\text{well}$  of virus concentrate was added (3-fold diluted, 6 gradients, two replicate wells) and incubated at 37°C in 5%  $\text{CO}_2$ . 48h after infection, eGFP was detected by flow cytometry, cells with 5 to 20% of the positive rate are appropriate to calculate the titer ( $\text{U} / \text{mL}$ ) = positive rate  $\times$  dilution times  $\times 100 \times 10^4$ . The titers of virus comprising the above-mentioned mock empty vector control and each eGFP-F2A-CAR packaged in the PEI transfection method were both about 0.5 to  $1 \times 10^7 \text{U}/\text{mL}$ , and the detected virus titer after concentration was about  $0.5 \sim 1 \times 10^8 \text{U}/\text{mL}$ .

## Example 12. T cells infected by Recombinant lentivirus

Human peripheral blood mononuclear cells were obtained from healthy human peripheral blood by density gradient centrifugation (supplied by Shanghai Blood Center) and added in AIM-V lymphocyte medium (purchased from Invitrogen) at a density of about  $2 \times 10^6 / \text{mL}$  and added. The magnetic beads coated with anti-CD3 and CD28 antibodies (Invitrogen) were added in a 1: 1 ratio of cells to magnetic beads, and recombinant human IL-2 (purchased from Shanghai Huaxin Biotechnology Co., Ltd. ) at a final concentration of 300U/mL was added for stimulation and culture for 48 h. And then T cells were infected with the above recombinant lentivirus (MOI  $\approx 15$ ). Infected T cells were detected by flow cytometry on day 8 of culture for the expression of different chimeric antigen receptors. Since eGFP was co-expressed with CAR, the detected eGFP-positive cells were positive cells expressing chimeric antigen receptors. Using uninfected T lymphocytes as a negative control, the positive rates of virus-infected T cells expressing different chimeric antigen receptors are shown in Table 10. The positive rate results show that a certain positive rate of CAR T cells can be obtained by lentivirus infection.

Table 10

| T cells transfected by following CARs | eGFP positive rate of CAR T cells |
|---------------------------------------|-----------------------------------|
| Y022- $\delta\text{Z}$ (Mock)         | 66%                               |
| Y022-Z                                | 58%                               |
| Y022-BBZ                              | 53%                               |
| Y022-28Z                              | 54%                               |

|            |     |
|------------|-----|
| Y022-28BBZ | 52% |
|------------|-----|

T cells were infected with viruses that had different chimeric antigen receptors packaged, respectively, and then subcultured at a cell density of  $5 \times 10^5$  / ml quaque die alterna, counted, and supplemented with IL-2 (final concentration of 300 U / ml). On the 11th day of culture, about 100 ~ 1000 times of amplification was obtained, indicating that the T cells expressing different chimeric antigen receptors can be expanded in a certain amount *in vitro*, which ensures subsequent *in vitro* toxicity tests and *in vivo* experiments.

### Example 13. *in vitro* antitumor activity of CAR-Y022

*In vitro* toxicity experiments used the following materials:

The target cells were U87, U87-EGFR, U87-EGFRvIII, A431, CAL 27, MDA-MB-468, RWPE-1 cells and human primary keratinocyte K2 as shown in Table 5, respectively. Effector cells were T lymphocytes (CAR T cells) cultured for 12 days *in vitro*, which were detected chimeric antigen receptor positive by FACS.

Effective target ratios were 3: 1, 1: 1 and 1: 3, respectively. The number of target cells was 10000 / well, and each group had 5 replicate wells. Detection time was 18h.

Each experimental group and each control group are listed as follows:

Each experimental group: each target cell + CAR T lymphocytes expressing different chimeric antigen receptors;

Control group 1: target cells with maximum LDH release;

Control group 2: target cells with spontaneous LDH release;

Control group 3: effector cells with spontaneous LDH release.

Detection method: CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega) is used, which is a colorimetric based assay that can replace 51Cr release assay. CytoTox 96® Assay measures lactate dehydrogenase (LDH) quantitatively. LDH is a stable cytosolic enzyme that is released upon lysis of cells and is released in the same way as radioactive 51Cr is released. The supernatant with released LDH medium can be detected by a 30-minute coupled enzyme reaction in which LDH converts a tetrazolium salt (INT) to a red formazan. The amount of red product produced is proportional to the number of lysed cells. Details can be found in instructions of CytoTox 96 non-radioactive cytotoxicity detection kit.

Cytotoxicity is calculated as:

Cytotoxicity % = [(experiment group – control group 2 – control group 3)/(control group 1 – control group 2)]×100

Specifically, as shown in Table 11 and Table 12, compared with 806-CAR T, Y022-28Z CAR T and Y022-28BBZ CAR T expressing chimeric antigen receptors of the present invention at different effector target ratios showed a significantly killing effects on cells highly expressing EGFR and EGFRvIII and a effector target ratio gradient dependency, that is, the higher the effector

target ratio, the stronger the cytotoxic effects. Effector-target-ratio-dependency data further demonstrate the specific cytotoxic effects of CAR T cells expressing chimeric antigen receptors of the invention on cells that highly express EGFR and its variants.

It is noteworthy that Y022-CAR T has almost no killing effect on RWPE-1 cells that normally express EGFR and human primary keratinocytes K2. At the effector target ratio of 3: 1, Cytotoxicity of the chimeric antigen receptor Y022-28BBZ CAR T-lymphocyte to RWPE-1 cells and human primary keratinocytes K2 was 12% and 2%, respectively. Cytotoxicity of Y022-28Z CAR T lymphocytes to RWPE-1 cells and human primary keratinocytes K2 was 8% and 3%. In contrast, 806-CAR T had different degrees of cytotoxicity on both of these cells. The cytotoxicity of 806-28BBZ CAR T lymphocytes to RWPE-1 cells and human primary keratinocytes K2 were 25% and 22%, respectively, and the cytotoxicity of 806-28Z CAR T lymphocytes to RWPE-1 cells and human primary keratinocytes K2 was 15% and 13%, respectively.

In addition, CAR T, as a negative control, transfected with a virus containing the mock plasmid (carrying scFv-Y022- $\delta$ Z) showed very low cytotoxic effects on the above cell lines.

The above results indicate that the chimeric antigen receptor Y022-CAR T, which is constructed from a single chain antibody against EGFR and its variants, can selectively kill tumor cells that highly express EGFR and its variant (EGFRvIII), while hardly kill cells normally expressing EGFR. In addition, from the cytotoxicity data, CAR T of the third generation (Y022-28BBZ) was more cytotoxic to target cells than the second generation (Y022-28Z) CART.

Table 11

| Cytotoxicity % | Y022-28BBZ<br>Different effector<br>target ratio |     |     | Y022-28Z<br>Different effector target<br>ratio |     |     | mock<br>Different effector target<br>ratio |     |     |
|----------------|--------------------------------------------------|-----|-----|------------------------------------------------|-----|-----|--------------------------------------------|-----|-----|
|                | 3:1                                              | 1:1 | 1:3 | 3:1                                            | 1:1 | 1:3 | 3:1                                        | 1:1 | 1:3 |
| U87            | 2                                                | 4   | 5   | 4                                              | 3   | 1   | -4                                         | -3  | 2   |
| U87-EGFR       | 35                                               | 17  | 6   | 25                                             | 16  | 7   | -1                                         | 5   | 1   |
| U87-EGFRvIII   | 73                                               | 42  | 19  | 65                                             | 31  | 9   | 3                                          | 0.3 | 1   |
| A431           | 67                                               | 37  | 12  | 45                                             | 24  | 5   | 9                                          | 7   | 4   |
| CAL27          | 70                                               | 49  | 15  | 50                                             | 23  | 8   | 7                                          | 5   | 5   |
| MDA-MB-468     | 57                                               | 45  | 14  | 41                                             | 23  | 1   | 6                                          | 8   | 7   |
| RWPE-1         | 12                                               | 7   | 3   | 8                                              | 6   | 4   | 3                                          | 2   | 0.2 |
| K2             | 11                                               | 2   | 0.3 | 3                                              | 0.6 | 2   | -3                                         | -1  | 2   |

Table 12

| Cytotoxicity % | 806-28BBZ<br>Different<br>effector target<br>ratio |     |     | 806-28Z<br>Different<br>effector target<br>ratio |     |     | mock<br>Different<br>effector target<br>ratio |     |     |
|----------------|----------------------------------------------------|-----|-----|--------------------------------------------------|-----|-----|-----------------------------------------------|-----|-----|
|                | 3:1                                                | 1:1 | 1:3 | 3:1                                              | 1:1 | 1:3 | 3:1                                           | 1:1 | 1:3 |
| U87            | 3                                                  | 4   | 2   | 5                                                | 0.6 | 2   | 1                                             | 3   | -3  |
| U87-EGFR       | 68                                                 | 49  | 18  | 55                                               | 36  | 12  | 3                                             | 7   | 4   |
| U87-EGFRvIII   | 83                                                 | 51  | 23  | 75                                               | 42  | 15  | 6                                             | 5   | 2   |

|            |    |    |    |    |    |    |     |    |   |
|------------|----|----|----|----|----|----|-----|----|---|
| A431       | 75 | 48 | 16 | 56 | 35 | 12 | 7   | 7  | 5 |
| CAL27      | 81 | 57 | 25 | 65 | 33 | 18 | 3   | 6  | 2 |
| MDA-MB-468 | 62 | 49 | 32 | 51 | 32 | 16 | 6   | 9  | 7 |
| RWPE-1     | 29 | 17 | 10 | 15 | 10 | 7  | -2  | -3 | 2 |
| K2         | 27 | 15 | 2  | 13 | 9  | 4  | 0.2 | 1  | 3 |

All references mentioned in the present application are incorporated herein by reference, as if each reference was individually incorporated by reference. In addition, it should be understood that after reading the above teachings of the present invention, those skilled in the art can make various modifications or changes to the present invention, and such equivalent forms also fall within the scope of the appended claims of the present application.

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## **Claims**

1. An antibody specifically recognizing EGFRvIII expressed or EGFR overexpressed by tumor cells, wherein the antibody comprises a light chain variable region and a heavy chain variable region, and

CDR1 of the light chain variable region has an amino acid sequence selected from a group consisting of SEQ ID NO: 41, SEQ ID NO: 47, SEQ ID NO: 55;

CDR2 of the light chain variable region has an amino acid sequence selected from a group consisting of SEQ ID NO: 42, SEQ ID NO: 53;

CDR3 of the light chain variable region has an amino acid sequence selected from a group consisting of SEQ ID NO: 43, SEQ ID NO: 48, SEQ ID NO: 54, SEQ ID NO: 56, SEQ ID NO: 57;

CDR1 of the heavy chain variable region has the amino acid sequence of SEQ ID NO: 44;

CDR2 of the heavy chain variable region has an amino acid sequence selected from a group consisting of SEQ ID NO: 45, SEQ ID NO: 49, SEQ ID NO: 51, SEQ ID NO: 52;

CDR3 of the heavy chain variable region has an amino acid sequence selected from a group consisting of SEQ ID NO: 46, SEQ ID NO: 50.

2. The antibody of claim 1, wherein the antibody includes:

antibody (a), wherein the light chain variable region thereof has CDR1 of SEQ ID NO: 41, CDR2 of SEQ ID NO: 42, CDR3 of SEQ ID NO: 43, or the heavy chain variable region thereof has CDR1 of SEQ ID NO: 44, CDR2 of SEQ ID NO: 45, CDR3 of SEQ ID NO: 46,

antibody (b), wherein the light chain variable region thereof has CDR1 of SEQ ID NO: 47, CDR2 of SEQ ID NO: 42, CDR3 of SEQ ID NO: 48, or the heavy chain variable region thereof has CDR1 of SEQ ID NO: 44, CDR2 of SEQ ID NO: 49, CDR3 of SEQ ID NO: 50,

antibody (c), wherein the light chain variable region thereof has CDR1 of SEQ ID NO: 41, CDR2 of SEQ ID NO: 42, CDR3 of SEQ ID NO: 48, or the heavy chain variable region thereof has CDR1 of SEQ ID NO: 44, CDR2 of SEQ ID NO: 51, CDR3 of SEQ ID NO: 50,

antibody (d), wherein the light chain variable region thereof has CDR1 of SEQ ID NO: 41, CDR2 of SEQ ID NO: 42, CDR3 of SEQ ID NO: 43, or the heavy chain variable region thereof has CDR1 of SEQ ID NO: 44, CDR2 of SEQ ID NO: 52, CDR3 of SEQ ID NO: 50,

antibody (e), wherein the light chain variable region thereof has CDR1 of SEQ ID NO: 41, CDR2 of SEQ ID NO: 42, CDR3 of SEQ ID NO: 43, or the heavy chain variable region thereof has CDR1 of SEQ ID NO: 44, CDR2 of SEQ ID NO: 45, CDR3 of SEQ ID NO: 50,

antibody (f), wherein light chain variable region thereof has CDR1 of SEQ ID NO: 41, CDR2 of SEQ ID NO: 53, CDR3 of SEQ ID NO: 54, or the heavy chain variable region thereof has CDR1 of SEQ ID NO: 44, CDR2 of SEQ ID NO: 51, CDR3 of SEQ ID NO: 50,

antibody (g), wherein the light chain variable region has CDR1 of SEQ ID NO: 41, CDR2 of

SEQ ID NO: 42, CDR3 of SEQ ID NO: 54, or the heavy chain variable region thereof has CDR1 of SEQ ID NO: 44, CDR2 of SEQ ID NO: 51, CDR3 of SEQ ID NO: 50,

antibody (h), wherein the light chain variable region thereof has CDR1 of SEQ ID NO: 55, CDR2 of SEQ ID NO: 42, CDR3 of SEQ ID NO: 56, or the heavy chain variable region thereof has CDR1 of SEQ ID NO: 44, CDR2 of SEQ ID NO: 45, CDR3 of SEQ ID NO: 50,

antibody (i), wherein the light chain variable region thereof has CDR1 of SEQ ID NO: 41, CDR2 of SEQ ID NO: 53, CDR3 of SEQ ID NO: 56, or the heavy chain variable region thereof has CDR1 of SEQ ID NO: 44, CDR2 of SEQ ID NO: 52, CDR3 of SEQ ID NO: 50,

antibody (j), wherein the light chain variable region thereof has CDR1 of SEQ ID NO: 41, CDR2 of SEQ ID NO: 42, CDR3 of SEQ ID NO: 56, or the heavy chain variable region thereof has CDR1 of SEQ ID NO: 44, CDR2 of SEQ ID NO: 52, CDR3 of SEQ ID NO: 50,

antibody (k), wherein the light chain variable region thereof has CDR1 of SEQ ID NO: 41, CDR2 of SEQ ID NO: 42, CDR3 of SEQ ID NO: 57, or the heavy chain variable region thereof has CDR1 of SEQ ID NO: 44, CDR2 of SEQ ID NO: 52, CDR3 of SEQ ID NO: 50; or

antibody (l) which recognizes the same antigenic determinant as that recognized by the antibody according to any one of (a) to (k).

3. The antibody of claim 2, wherein the amino acid sequence of the heavy chain variable region of the antibody is shown in positions 124 to 239 of SEQ ID NO: 13; or the amino acid sequence of the light chain variable region of the antibody is shown in positions 1-108 of SEQ ID NO: 13;

The amino acid sequence of the heavy chain variable region of the antibody is shown in positions 124 to 239 of SEQ ID NO: 59; or the amino acid sequence of the light chain variable region of the antibody is shown in positions 1-108 of SEQ ID NO: 59;

The amino acid sequence of the heavy chain variable region of the antibody is shown in positions 124 to 239 of SEQ ID NO: 61; or the amino acid sequence of the light chain variable region of the antibody is shown in positions 1-108 of SEQ ID NO: 61;

The amino acid sequence of the heavy chain variable region of the antibody is shown in positions 124 to 239 of SEQ ID NO: 63; or the amino acid sequence of the light chain variable region of the antibody is shown in positions 1-108 of SEQ ID NO: 63;

The amino acid sequence of the heavy chain variable region of the antibody is shown in positions 124 to 239 of SEQ ID NO: 65; or the amino acid sequence of the light chain variable region of the antibody is shown in positions 1-108 of SEQ ID NO: 65;

The amino acid sequence of the heavy chain variable region of the antibody is shown in positions 124 to 239 of SEQ ID NO: 67; or the amino acid sequence of the light chain variable region of the antibody is shown in positions 1-108 of SEQ ID NO: 67;

The amino acid sequence of the heavy chain variable region of the antibody is shown in

positions 124 to 239 of SEQ ID NO: 69; or the amino acid sequence of the light chain variable region of the antibody is shown in positions 1-108 of SEQ ID NO: 69;

The amino acid sequence of the heavy chain variable region of the antibody is shown in positions 124 to 239 of SEQ ID NO: 71; or the amino acid sequence of the light chain variable region of the antibody is shown in positions 1-108 of SEQ ID NO: 71;

The amino acid sequence of the heavy chain variable region of the antibody is shown in positions 124 to 239 of SEQ ID NO: 73; or the amino acid sequence of the light chain variable region of the antibody is shown in positions 1-108 of SEQ ID NO: 73;

The amino acid sequence of the heavy chain variable region of the antibody is shown in positions 124 to 239 of SEQ ID NO: 75; or the amino acid sequence of the light chain variable region of the antibody is shown in positions 1-108 of SEQ ID NO: 75; or

The amino acid sequence of the heavy chain variable region of the antibody is shown in positions 124 to 239 of SEQ ID NO: 77; or the amino acid sequence of the light chain variable region of the antibody is shown in positions 1-108 of SEQ ID NO: 77.

4. The antibody of claim 2, wherein the antibody is antibody (a); more preferably, the amino acid sequence of the heavy chain variable region of the antibody is shown in positions 124 to 239 of SEQ ID NO: 13; or the amino acid sequence of the light chain variable region of the antibody is shown in positions 1-108 of SEQ ID NO: 13.

5. A nucleic acid encoding the antibody of any one of claims 1-4.

6. An expression vector comprising the nucleic acid of claim 5.

7. A host cell, comprising the expression vector of claim 6 or having the nucleic acid of claim 5 integrated into its genome.

8. Use of the antibody of any one of claims 1-4 for the manufacture of a targeting drug, antibody drug conjugate, or multi-functional antibody specifically targeting tumor cells expressing EGFRvIII or over-expressing EGFR; or

for the manufacture of an agent for diagnosis of tumors that express EGFRvIII or overexpress EGFR; or

for the manufacture of a chimeric antigen receptor modified immune cell; preferably, the immune cell includes: T lymphocyte, NK cell or NKT lymphocyte.

9. A multi-functional immunoconjugate, comprising:

The antibody of any one of claims 1-4; and

a functional molecule linked thereto; and the functional molecule is selected from a group consisting of a molecule that targets a tumor surface marker, a tumor-suppressing molecule, a molecule that targets a surface marker of an immune cell, a detectable label.

10. The multi-functional immunoconjugate of claim 9, wherein the molecule that targets the tumor surface marker is an antibody or ligand that binds to a tumor surface marker; or

the tumor-suppressing molecule is an anti-tumor cytokine or an anti-tumor toxin; preferably, the cytokines include but are not limited to: IL-12, IL-15, IFN-beta, TNF-alpha.

11. The multi-functional immunoconjugate of claim 9, wherein the detectable label includes a fluorescent label and a chromogenic label.

12. The multi-functional immunoconjugate of claim 9, wherein the molecule targeting the surface marker of the immune cell is an antibody or ligand that binds to an immune cell surface marker; preferably, the immune cell surface markers include: CD3, CD16, CD28.

13. The multi-functional immunoconjugate of claim 12, wherein the molecule that targets the surface marker of the immune cell is an antibody that binds to a T cell surface marker, which forms a T-cell-engaging bifunctional antibody with the antibody of any one of claims 1-4.

14. The multi-functional immunoconjugate of claim 13, wherein the antibody that binds to the immune cell surface marker is an anti-CD3 antibody.

15. The multi-functional immunoconjugate of claim 9, wherein the multi-functional immunoconjugate is a fusion polypeptide, and further comprises a linker peptide between the antibody of any one of claims 1-4 and the functional molecule linked thereto.

16. A nucleic acid encoding the multi-functional immunoconjugate of any one of claims 9-15.

17. Use of the multi-functional immunoconjugate of any one of claims 9-15 for the preparation of an antineoplastic agent, or

for the preparation of an agent for diagnosis of tumors that express EGFRvIII or overexpress EGFR; or

for the preparation of chimeric antigen receptor modified immune cells; preferably, the immune cells include T lymphocyte, NK cell or NKT lymphocyte.

18. A chimeric antigen receptor expressed on the surface of an immune cell comprising the antibody of any one of claims 1-4, wherein the chimeric antigen receptor comprises: the antibody of any one of claims 1-4, a transmembrane region and an intracellular signal region, which are sequentially linked.

19. The chimeric antigen receptor of claim 18, wherein the intracellular signal region is selected from a group consisting of intracellular signal region sequences of CD3 $\zeta$ , Fc $\epsilon$ RI $\gamma$ , CD27, CD28, CD137 and CD134, or a combination thereof.

20. The chimeric antigen receptor of claim 19, wherein the transmembrane region comprises a transmembrane region of CD8 or CD28.

21. The chimeric antigen receptor of claim 19, wherein the immune cells include T lymphocyte, NK cell or NKT cell.

22. The chimeric antigen receptor of claim 19, wherein the chimeric antigen receptor comprises the following sequentially linked antibody, transmembrane region and intracellular

signal region:

Antibody of any one of claims 1-4, CD8 and CD3 $\zeta$ ;

Antibody of any one of claims 1-4, CD8, CD137 and CD3 $\zeta$ ;

5 Antibody of any one of claims 1-4, a transmembrane region of a CD28 molecule, an intracellular signal region of a CD28 molecule, and CD3 $\zeta$ ; or

Antibody of any one of claims 1-4, a transmembrane region of a CD28 molecule, an intracellular signal region of a CD28 molecule, CD137 and CD3 $\zeta$ .

23. The chimeric antigen receptor of claim 19, wherein the antibody is a single chain antibody or a domain antibody.

10 24. The chimeric antigen receptor of claim 19, wherein the chimeric antigen receptor comprises:

SEQ ID NO: 36 or the amino acid sequence shown in positions 285-601; or

SEQ ID NO: 37 or the amino acid sequence shown in positions 285-702; or

SEQ ID NO: 38 or the amino acid sequence shown in positions 285-744; or

15 SEQ ID NO: 39 or the amino acid sequence shown in positions 285-749; or

SEQ ID NO: 40 or the amino acid sequence shown in positions 285-791.

25. A nucleic acid encoding the chimeric antigen receptor of any one of claims 18-24.

26. An expression vector comprising the nucleic acid of claim 25.

27. A virus comprising the vector of claim 26.

20 28. Use of the chimeric antigen receptor of any one of claims 18-24 or the nucleic acid of claim 25, or the expression vector of claim 26, or the virus of claim 27, for the preparation of genetically modified immune cells that target tumor cells that express EGFRvIII or overexpress EGFR.

25 29. A genetically modified immune cell, wherein it is transduced with the nucleic acid of claim 25, or the expression vector of claim 26 or the virus of claim 27; or

it expresses the chimeric antigen receptor of any one of claims 18-24 at its surface.

30. The immune cell of claim 29, wherein it further carries an exogenous encoding sequence for cytokine; preferably, the cytokine includes: IL-12, IL-15 or IL-21

30 31. The immune cell of claim 29, wherein it further expresses another chimeric antigen receptor that does not contain CD3 $\zeta$ , but contains the intracellular signaling domain of CD28, the intracellular signaling domain of CD137, or a combination of both.

32. The immune cell of claim 29, wherein it further expresses a chemokine receptor; preferably, the chemokine receptor includes CCR2.

35 33. The immune cell of claim 29, wherein it further expresses siRNA that can reduce PD-1 expression or a protein that can block PD-L1.

34. The immune cell of claim 29, wherein it further expresses a safety switch; preferably, the

safety switch includes iCaspase-9, Truncated EGFR or RQR8.

35. Use of the genetically modified immune cells of any one of claims 29-34 for the preparation of a tumor-inhibiting drug, and said tumor is the tumor that expresses EGFRvIII or overexpresses EGFR.

5        36. A pharmaceutical composition, comprising:

The antibody of any one of claims 1-4 or a nucleic acid encoding the antibody; or

The immunoconjugate of any one of claims 9-15 or a nucleic acid encoding the conjugate; or

The chimeric antigen receptor of any one of claims 18-24 or a nucleic acid encoding the chimeric antigen receptor; or

10        The genetically modified immune cell of any one of claims 29-34.