

Antigen-binding protein and use thereof

Cross-Reference to Related Application

The present application claims priority to the Chinese patent application with application number CN 202311743747.1 (filed on December 18, 2023). The entire contents of the patent application are incorporated into the present application by reference.

Technical Field

The present invention relates to a nanobody capable of specifically binding to HER3 or an antigen-binding fragment thereof, a polypeptide construct containing the nanobody or antigen-binding fragment thereof, and a nucleic acid molecule encoding the nanobody or antigen-binding fragment thereof or the polypeptide construct. Furthermore, the present invention relates to the use of the nanobody or antigen-binding fragment thereof in the treatment and diagnosis of diseases.

Background

Human epidermal growth factor receptor 3 (HER3, also known as ErbB3) is a member of the human epidermal growth factor receptor (HER) family, which also includes HER1/EGFR/ErbB1, HER2/ErbB2, and HER4/ErbB4. Each member consists of an extracellular domain capable of binding to ligand, a transmembrane domain, an intracellular kinase domain, and a C-terminal tail. The HER3 gene is located on the long arm of chromosome 12 (12q13.2) and encodes a 180 kDa protein. It is the only member of the HER family that lacks or has almost no tyrosine kinase activity, with its kinase activity being 1,000 times weaker than that of fully activated HER1. HER3 requires heterodimerization with other receptors, such as HER1, HER2, or HER4, to induce downstream C-terminal phosphorylation. Recent studies have found that HER3 is also co-expressed with other non-HER family receptor tyrosine kinases (RTKs) in cancer cells, forming heterodimers that activate oncogenic signaling pathways, particularly the PI3K/Akt, MAPK/ERK, and JAK/STAT pathways, as well as Src kinase.

Compared to other members of the HER family, HER3 alone does not induce carcinogenesis when overexpressed, but it can cooperate with other receptors to promote tumorigenesis, metastasis, and drug resistance. Currently, HER3 has been found to be overexpressed and/or hyperactivated in various cancers, including breast cancer, ovarian cancer, prostate cancer, liver cancer, kidney cancer, bladder cancer, pancreatic cancer, brain cancer, hematopoietic tissue tumor, retinoblastoma, melanoma, colon cancer, gastric cancer, head and neck cancer, lung cancer, among others. Overexpression of HER3 leads to resistance to multiple therapeutic agents, such as

resistance to EGFR/HER2-TKIs. Moreover, its high expression is associated with disease progression and/or poor prognosis.

HER3 is a promising target for cancer therapy, but to date, no HER3-targeted therapies have been approved for clinical use. Research on anti-tumor therapies targeting HER3 has primarily focused on monoclonal antibodies. Seribantumab and Patritumab are the most promising HER3-targeted monoclonal antibodies that have shown encouraging results in clinical trials so far, with multiple Phase I studies demonstrating good tolerability and safety.

Seribantumab (MM-121) is a fully human IgG2 monoclonal antibody capable of binding to HER3. It can block the binding of neuregulin (NRG) ligand to HER3, thereby inhibiting HER3 signaling that sustains tumor activation. Additionally, by blocking ErbB homodimerization or heterodimerization, it blocks downstream signaling pathways that drive cell growth, ultimately inducing tumor cell death.

Patritumab (U3-1287) is a fully human HER3 antibody capable of inhibiting the binding of HER3 to its ligand. In HER2-positive breast cancer, the overall response rate (ORR) of patritumab in combination with trastuzumab and paclitaxel was 39%. However, in a clinical study evaluating patritumab in combination with erlotinib for the treatment of non-small cell lung cancer, it failed to meet the expected clinical trial endpoint (NCT02134015).

HER3 lacks intrinsic kinase activity like other family members such as HER2, and there are no suitable biomarkers to reflect HER3 activation in patients, which poses significant challenges for drug development. The development of anti-HER3 targeted therapies still needs to continue. Traditional IgG antibodies have a molecular weight of approximately 150 kDa, but single-domain antibodies/nanobodies derived from alpacas, when fused with human Fc, have a molecular weight of only about 75 kDa, only half that of conventional antibodies. Compared to traditional monoclonal antibodies, they have a smaller structure and stronger ability to penetrate the blood-brain barrier, allowing them to infiltrate deeper into tumors and maximize the opportunity for antibody drugs to bind to tumor cells, thereby achieving better diagnostic and therapeutic effects for tumors.

Therefore, providing an HER3-targeting nanobody has promising prospects for the diagnosis and treatment of tumors.

Contents of the Invention

The inventors of the present application, through extensive research, have screened and obtained a series of anti-HER3 nanobodies and polypeptide constructs, which exhibit high binding

activity to HER3. In particular, the nanobody or polypeptide construct of the present invention possess internalization activity, enabling them to mediate the entry of toxins into cells through internalization, thereby exerting a cytotoxic effect on tumor cells. On this basis, humanized nanobodies or polypeptide constructs prepared from these nanobodies retain these remarkable activities. Furthermore, the nanobodies or polypeptide constructs are characterized by their small molecular weight and ease of production.

Based on this, the present application further provides a conjugate containing the nanobody or antigen-binding fragment thereof, a nucleic acid molecule encoding the nanobody or antigen-binding fragment thereof, a host cell containing it, and its related uses.

Nanobody or antigen-binding fragment thereof

In one aspect, the present invention provides a nanobody or antigen-binding fragment thereof capable of specifically binding to HER3. The nanobody described herein typically consists of 4 framework regions (FRs) and 3 complementarity-determining regions (CDRs), referred to as FR1, CDR1, FR2, CDR2, FR3, CDR3, and FR4, wherein the antigen-binding fragment comprises at least a portion of the nanobody, wherein the portion is sufficient to confer the ability to specifically bind HER3 to the fragment. In some embodiments, the nanobody described in the present invention may be truncated at the N-terminus or C-terminus to contain only a portion of FR1 and/or FR4, or to lack one or both of those framework regions, as long as it substantially retains antigen binding ability and specificity.

In some embodiments, the nanobody or antigen-binding fragment thereof comprises CDR1, CDR2, and/or CDR3 contained in a VHH as set forth in any one of SEQ ID NOs: 8-10.

In some embodiments, the CDRs are defined according to the Kabat, Chothia, or IMGT numbering system.

In some embodiments, the nanobody or antigen-binding fragment thereof comprises: CDR1 as set forth in SEQ ID NO: 1; CDR2 as set forth in SEQ ID NO: 2 or 7; and CDR3 as set forth in SEQ ID NO: 3; wherein the CDRs are defined according to the Kabat numbering system.

In some embodiments, the nanobody or antigen-binding fragment thereof comprises: CDR1 as set forth in SEQ ID NO: 4; CDR2 as set forth in SEQ ID NO: 5; and CDR3 as set forth in SEQ ID NO: 6; wherein the CDRs are defined according to the IMGT numbering system.

In some embodiments, the nanobody or antigen-binding fragment thereof comprises a frame region sequence of a camelid antibody.

In some embodiments, the nanobody or antigen-binding fragment thereof comprises the sequence as set forth in SEQ ID NO: 8, or a sequence having a sequence identity of at least 80%,

at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% as compared thereto, or a sequence having a substitution, deletion, or addition of one or more amino acids (e.g., a substitution, deletion, or addition of 1, 2, 3, 4, or 5 amino acids) as compared thereto. In some embodiments, the nanobody or antigen-binding fragment thereof comprises the sequence as set forth in SEQ ID NO: 8.

In some embodiments, the nanobody or antigen-binding fragment thereof is humanized, i.e., one or more of its frame regions have been substantially replaced by human frame regions.

In some embodiments, the nanobody or antigen-binding fragment thereof further comprises a heavy chain framework region of a human immunoglobulin (e.g., a heavy chain framework region comprised in an amino acid sequence encoded by a human heavy chain germline antibody gene), the heavy chain framework region optionally comprising one or more (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10) back mutations from human residues to camelid residues.

In some embodiments, the nanobody or antigen-binding fragment thereof comprises the sequence as set forth in SEQ ID NO: 9, or a sequence having a sequence identity of at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% as compared thereto, or a sequence having a substitution, deletion, or addition of one or more amino acids (e.g., a substitution, deletion, or addition of 1, 2, 3, 4, or 5 amino acids) as compared thereto. In some embodiments, the nanobody or antigen-binding fragment thereof comprises the sequence as set forth in SEQ ID NO: 9.

In some embodiments, the nanobody or antigen-binding fragment thereof comprises the sequence as set forth in SEQ ID NO: 10, or a sequence having a sequence identity of at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% as compared thereto, or a sequence having a substitution, deletion, or addition of one or more amino acids (e.g., a substitution, deletion, or addition of 1, 2, 3, 4, or 5 amino acids) as compared thereto. In some embodiments, the nanobody or antigen-binding fragment thereof comprises the sequence as set forth in SEQ ID NO: 10.

Polypeptide construct

In another aspect, the present invention also provides a polypeptide construct capable of specifically binding to HER3, which comprises the nanobody or antigen-binding fragment thereof of the present invention, and an immunoglobulin Fc domain.

As used herein, the Fc domain is also referred to as the Fc region, which refers to a portion of the heavy chain constant region comprising CH2 and CH3. In some embodiments, the Fc domain comprises a hinge, CH2, and CH3. When the Fc domain contains a hinge, the hinge regulates dimerization between two Fc-containing polypeptides. The Fc domain can be any antibody heavy chain constant region isotype. In some embodiments, the Fc domain is IgG1, IgG2, IgG3, or IgG4.

In some embodiments, the Fc domain comprised in the polypeptide construct of the present invention is a natural Fc region, which comprises an amino acid sequence consistent with an amino acid sequence of Fc region found in nature. For example, the Fc domain can be the natural sequence human IgG1 Fc region, the natural sequence human IgG2 Fc region, the natural sequence human IgG3 Fc region, or the natural sequence human IgG4 Fc region. The natural Fc region may have effector functions. Exemplary "effector functions" include binding to Fc receptors; C1q binding and complement-dependent cytotoxicity (CDC); antibody-dependent cell-mediated cytotoxicity (ADCC); phage activity; downregulation of cell surface receptors (e.g., B cell receptors); and B cell activation, etc. Functional alterations can be achieved by replacing at least one amino acid residue in the natural Fc region with a different residue or by chemical modification. For example, altering the antibody's affinity for effector ligands (e.g., FcR or complement C1q) can change (e.g., reduce or enhance) effector functions.

Therefore, in some embodiments, the Fc domain comprised in the polypeptide construct of the present invention may also be a variant Fc region, which may comprise a mutation of one or more (e.g., 1 to 10, e.g., 1 to 5) amino acids or a chemical modification as compared to the natural Fc region to alter one or more of the following properties of the antibody of the present invention: Fc receptor binding, antibody glycosylation, number of cysteine residues, effector cell function, or complement function, etc. In some embodiments, the Fc domain comprised in the polypeptide construct of the present invention has a reduced or removed effector function, for example, it is an Fc domain that comprises a LALA mutation.

In some embodiments, the immunoglobulin Fc domain is optionally linked via a peptide linker to the N-terminus and/or C-terminus (e.g., C-terminus) of the nanobody or antigen-binding fragment thereof.

In some embodiments, the immunoglobulin Fc domain is an Fc domain of IgG (e.g., an Fc domain of IgG1, IgG2, IgG3, or IgG4).

In some embodiments, the immunoglobulin Fc domain is an Fc domain of human immunoglobulin, for example, an Fc domain of human IgG (e.g., an Fc domain of human IgG1, IgG2, IgG3, or IgG4).

In some embodiments, the immunoglobulin Fc domain comprises the sequence as set forth in SEQ ID NO: 11, or a sequence having a sequence identity of at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% as compared thereto, or a sequence having a substitution, deletion, or addition of one or more amino acids (e.g., a substitution, deletion, or addition of 1, 2, 3, 4, or 5 amino acids) as compared thereto. In some embodiments, the immunoglobulin Fc domain comprises the sequence as set forth in SEQ ID NO: 11.

In some embodiments, the polypeptide construct comprises the sequence as set forth in any one of SEQ ID NOs: 12-14, or a sequence having a sequence identity of at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% as compared thereto, or a sequence having a substitution, deletion, or addition of one or more amino acids (e.g., a substitution, deletion, or addition of 1, 2, 3, 4, or 5 amino acids) as compared thereto.

Preparation of nanobody and polypeptide construct

The nanobody or polypeptide construct of the present invention can be prepared using various methods known in the art, such as by genetic engineering recombination techniques. For example, a DNA molecule encoding the nanobody or polypeptide construct of the present invention can be obtained by chemical synthesis or PCR amplification. The resulting DNA molecule is inserted into an expression vector and then transfected into a host cell. The transfected host cell is then cultured under specific conditions to express the antibody or polypeptide construct of the present invention.

In another aspect, the present invention also provides an isolated nucleic acid molecule, which encodes the nanobody or antigen-binding fragment thereof of the present invention, or the polypeptide construct of the present invention.

In another aspect, the present invention also provides a vector, which comprises the nucleic acid molecule of the present invention. In some embodiments, the vector is a cloning vector or an expression vector.

In another aspect, the present invention also provides a host cell, which comprises the nucleic acid molecule or vector of the present invention. Such host cell includes, but is not limited to, prokaryotic cell such as bacterial cell (e.g., *E. coli* cell), and eukaryotic cell such as fungal cell (e.g., yeast cell), insect cell, plant cell, and animal cell (e.g., mammalian cell, such as mouse cell, human cell, etc.).

In another aspect, the present invention also provides a method for preparing the nanobody or antigen-binding fragment thereof of the present invention or the polypeptide construct of the present invention, which comprises culturing the host cell of the present invention under a

condition that allows protein expression, and recovering the nanobody or antigen-binding fragment thereof or the polypeptide construct from a culture of the cultured host cell.

Bispecific or multispecific antibody

In another aspect, the present invention also provides a bispecific or multispecific antibody, which comprises the nanobody or antigen-binding fragment thereof of the present invention or the polypeptide construct of the present invention. The present invention also provides a use of the nanobody or antigen-binding fragment thereof or the polypeptide construct, or the nucleic acid molecule, vector or host cell encoding the same, as described in the present invention, in the manufacture of a bispecific or multispecific antibody.

In some embodiments, the bispecific or multispecific antibody is capable of specifically binding to HER3, and additionally specifically binding to one or more other targets.

In some embodiments, the bispecific or multispecific antibody further comprises at least one second antibody having a second binding specificity against a second target.

Conjugate

In another aspect, the present invention also provides a conjugate, which comprises the nanobody or antigen-binding fragment thereof of the present invention, or the polypeptide construct of the present invention, or the bispecific or multispecific antibody of the present invention, and a conjugated portion connected thereto.

In some embodiments, the conjugated portion is selected from therapeutic agents (e.g., cytotoxic agent, cytokine, toxin, or radionuclide).

In some embodiments, the conjugate is an antibody-drug conjugate (ADC).

Pharmaceutical composition

In another aspect, the present invention also provides a pharmaceutical composition, which comprises the nanobody or antigen-binding fragment thereof, the polypeptide construct, isolated nucleic acid molecule, vector, host cell, bispecific or multispecific antibody, or conjugate, as described in the present invention, and a pharmaceutically acceptable carrier and/or excipient.

In some embodiments, the pharmaceutical composition further comprises an additional pharmaceutically active agent, such as an antitumor agent.

In some embodiments, the nanobody or antigen-binding fragment thereof, polypeptide construct, isolated nucleic acid molecule, vector, host cell, bispecific or multispecific antibody, or conjugate, as described in the present invention, and the additional pharmaceutically active agent

may be provided as separate components or as mixed components in the pharmaceutical composition.

The nanobody or antigen-binding fragment thereof, polypeptide construct, isolated nucleic acid molecule, vector, host cell, bispecific or multispecific antibody, conjugate, or pharmaceutical composition, as described in the present invention, can be formulated into any dosage form known in the medical field, such as tablet, pill, suspension, emulsion, solution, gel, capsule, powder, granule, elixir, lozenge, suppository, injection (including solution for injection, sterile powder for injection, and concentrated solution for injection), inhaler, spray, etc. Preferred dosage form depends on the intended route of administration and therapeutic use. A preferred dosage form is an injection. Such injections may be sterile injection solutions. For example, a sterile solution for injection can be prepared by incorporating the required dose of the antibody or antigen-binding fragment of the present invention into a suitable solvent, and optionally, simultaneously incorporating other desired components (including, but not limited to, pH adjuster, surfactant, adjuvant, ionic strength enhancer, isotonic agent, preservative, diluent, or any combination thereof), followed by sterile filtration. Alternatively, the sterile solution for injection can be prepared as a sterile lyophilized powder (e.g., by vacuum drying or freeze-drying) for easy storage and use. Such sterile lyophilized powders can be dispersed in a suitable carrier prior to use.

In some exemplary embodiments, the pharmaceutically acceptable carrier and/or excipient comprises a sterile injectable liquid (e.g., an aqueous or non-aqueous suspension or solution). In some exemplary embodiments, such sterile injectable liquid is selected from the group consisting of water for injection (WFI), bacteriostatic water for injection (BWFI), sodium chloride solution (e.g., 0.9% (w/v) NaCl), glucose solution (e.g., 5% glucose), surfactant-containing solution (e.g., 0.01% polysorbate 20), pH buffer solution (e.g., phosphate buffered solution), Ringer's solution, and any combination thereof.

The pharmaceutical composition of the present invention may comprise a “therapeutically effective amount” or a “prophylactically effective amount” of the nanobody or antigen-binding fragment thereof, isolated nucleic acid molecule, vector, host cell, bispecific or multispecific antibody, or conjugate, as described in the present invention. The “prophylactically effective amount” refers to an amount sufficient to prevent, arrest, or delay the onset of disease. The “therapeutically effective amount” refers to an amount sufficient to cure or at least partially arrest a disease and complications thereof in a patient already suffering from the disease. The therapeutically effective amount may vary depending on factors such as the severity of the disease to be treated, the overall state of the patient's own immune system, the patient's general characteristics such as age, weight, and sex, the method of drug administration, and other concurrent treatments, etc.

Detection use

Kit

In another aspect, the present invention also provides a kit, which comprises the nanobody or antigen-binding fragment thereof of the present invention or the polypeptide construct of the present invention.

In some embodiments, the kit comprises a conjugate, in which the conjugate comprises the nanobody or antigen-binding fragment thereof of the present invention or the polypeptide construct of the present invention, and a detectable label, such as an enzyme (e.g., horseradish peroxidase or alkaline phosphatase), a chemiluminescent reagent (e.g., acrid ester, luminol and derivative thereof, or ruthenium derivative), a fluorescent dye (e.g., fluorescein or fluorescent protein), a radionuclide, or biotin, linked to the nanobody or antigen-binding fragment thereof or polypeptide construct.

In some embodiments, the kit comprises the nanobody or antigen-binding fragment thereof of the present invention or the polypeptide construct of the present invention, and a second antibody that specifically recognizes the nanobody or antigen-binding fragment thereof or polypeptide construct; optionally, the second antibody further comprises a detectable label, such as an enzyme (e.g., horseradish peroxidase or alkaline phosphatase), a chemiluminescent reagent (e.g., acrid esters, luminol and derivative thereof, or ruthenium derivative), a fluorescent dye (e.g., fluorescein or fluorescent protein), a radionuclide, or biotin.

Chimeric antigen receptor

In another aspect, the present application also provides a chimeric antigen receptor, which comprises an antigen-binding domain of the nanobody or antigen-binding fragment thereof as described above, or the polypeptide construct as described above.

In some embodiments, the antigen-binding domain is expressed by an immune effector cell (e.g., a T cell).

Isolated nucleic acid molecule

In another aspect, the present application also provides an isolated nucleic acid molecule, which encodes the chimeric antigen receptor as described above.

Vector

In another aspect, the present application also provides a vector, which comprises the isolated nucleic acid molecule as described above. In some embodiments, it is used to prepare a chimeric antigen receptor T cell.

Host cell

In another aspect, the present application also provides a host cell, which comprises the isolated nucleic acid molecule as described above or the vector as described above. In some embodiments, the host cell is an immune effector cell (e.g., a T cell or NK cell). In some embodiments, the host cell is a chimeric antigen receptor T cell (CAR-T).

Pharmaceutical use

In another aspect, the present invention also provides a use of the nanobody or antigen-binding fragment thereof, polypeptide construct, isolated nucleic acid molecule, vector, host cell, bispecific or multispecific antibody, conjugate, or pharmaceutical composition, as described in the present invention, in the manufacture of a medicament for prevention and/or treatment of a tumor in a subject.

In some embodiments, the tumor is an HER3-positive tumor.

In some embodiments, the medicament is used to inhibit the growth of an HER3-expressing tumor cell and/or kill the tumor cell.

In some embodiments, the medicament further comprises an additional pharmaceutically active agent.

In some embodiments, the additional pharmaceutically active agent is a drug with antitumor activity, such as an alkylating agent, mitotic inhibitor, antitumor antibiotic, antimetabolite, topoisomerase inhibitor, tyrosine kinase inhibitor, radionuclide agent, radiosensitizer, antiangiogenic agent, cytokine, molecularly targeted drug, immune checkpoint inhibitor, or oncolytic virus.

In some embodiments, the tumor is selected from the group consisting of solid tumors, such as gastric cancer, lung cancer, liver cancer, head and neck cancer, skin cancer, colorectal cancer, ovarian cancer, esophageal cancer, pancreatic cancer, cervical cancer, mesothelioma, breast cancer, colorectal cancer, or oral squamous cell carcinoma.

In some embodiments, the subject is a mammal, such as a human.

Method for prevention and/or treatment of tumor

In another aspect, the present invention also provides a method for preventing and/or treating a tumor in a subject, comprising: administering to the subject in need an effective amount of the nanobody or antigen-binding fragment thereof, polypeptide construct, isolated nucleic acid molecule, vector, host cell, bispecific or multispecific antibody, conjugate, or pharmaceutical composition, as described in the present invention.

In some embodiments, the tumor is an HER3-positive tumor.

In some embodiments, the tumor is selected from the group consisting of solid tumors, such as gastric cancer, lung cancer (e.g., non-small cell lung cancer), liver cancer, head and neck cancer, skin cancer, colorectal cancer, ovarian cancer, esophageal cancer, pancreatic cancer, cervical cancer, mesothelioma, breast cancer, colorectal cancer, or oral squamous cell carcinoma.

In some embodiments, the subject is a mammal, such as a human.

The nanobody or antigen-binding fragment thereof, polypeptide construct, bispecific or multispecific antibody, conjugate, or pharmaceutical composition, as described in the present invention, can be formulated into any dosage form known in the medical field, such as tablet, pill, suspension, emulsion, solution, gel, capsule, powder, granule, elixir, lozenge, suppository, injection (including solution for injection, sterile powder for injection, and concentrated solution for injection), inhaler, spray, etc. Preferred dosage form depends on the intended route of administration and therapeutic use. The nanobody or antigen-binding fragment thereof, polypeptide construct, bispecific or multispecific antibody, conjugate, or pharmaceutical composition, as described in the present invention, should be sterile and stable under manufacturing and storage conditions. A preferred dosage form is an injection. Such injection may be a sterile solution for injection. For example, a sterile solution for injection can be prepared by incorporating an appropriate dose of the nanobody or antigen-binding fragment, polypeptide construct, bispecific or multispecific antibody, conjugate, or pharmaceutical composition, as described in the present invention, and optionally, simultaneously incorporating other desired components (including, but not limited to, pH adjuster, surfactant, adjuvant, ionic strength enhancer, isotonic agent, preservative, diluent, or any combination thereof) into a suitable solvent, followed by sterile filtration. Alternatively, a sterile solution for injection can be prepared as a sterile lyophilized powder (e.g., by vacuum drying or freeze-drying) for easy storage and use. Such sterile lyophilized powder can be dispersed in a suitable carrier before use, such as water for injection (WFI), antibacterial water for injection (BWFI), sodium chloride solution (e.g., 0.9% (w/v) NaCl), glucose solution (e.g., 5% glucose), surfactant-containing solution (e.g., 0.01% polysorbate 20), pH buffer solution (e.g., phosphate buffered solution), Ringer's solution, and any combination thereof.

The nanobody or antigen-binding fragment thereof, polypeptide construct, bispecific or multispecific antibody, conjugate, or pharmaceutical composition, as described in the present invention can be administered by any suitable method known in the art, including but not limited to oral, buccal, sublingual, ocular, topical, parenteral, rectal, intrathecal, intracisternal (within cisterna of endoplasmic reticulum), groin, intrabladder, topical (e.g., powder, ointment, or drop), or nasal routes. However, for many therapeutic applications, the preferred route/mode of

administration is parenteral administration (e.g., intravenous injection or bolus, subcutaneous injection, intraperitoneal injection, intramuscular injection). Those skilled in the art will understand that the route and/or mode of administration will vary depending on the intended purpose. In certain embodiments, the nanobody or antigen-binding fragment thereof, polypeptide construct, bispecific or multispecific antibody, conjugate, or pharmaceutical composition of the present invention is administered by intravenous injection or bolus injection.

Detection method

In another aspect, the present invention also provides a method for detecting the presence or amount of HER3 in a sample, which comprises using the nanobody or antigen-binding fragment thereof, or polypeptide construct of the present invention. In some embodiments, the method is used for therapeutic purposes, diagnostic purposes, or non-therapeutic and non-diagnostic purposes.

In some embodiments, the method is an immunological assay, such as Western blotting, enzyme immunoassay (e.g., ELISA), chemiluminescent immunoassay, fluorescence immunoassay, or radioimmunoassay.

In some embodiments, the method comprises using a conjugate, wherein the conjugate comprises the nanobody or antigen-binding fragment thereof of the present invention or the polypeptide construct of the present invention, and a detectable label, such as an enzyme (e.g., horseradish peroxidase or alkaline phosphatase), a chemiluminescent reagent (e.g., acrid esters, luminol and derivative thereof, or ruthenium derivative), a fluorescent dye (e.g., fluorescein or fluorescent protein), a radionuclide, or biotin, that is linked to the nanobody or antigen-binding fragment thereof or polypeptide construct.

In some embodiments, the method comprises using the nanobody or antigen-binding fragment thereof of the present invention or the polypeptide construct of the present invention, and the method further comprises using a second antibody bearing a detectable label (e.g., an enzyme (e.g., horseradish peroxidase or alkaline phosphatase), a chemiluminescent reagent (e.g., acrid esters, luminol and derivative thereof, or ruthenium derivative), a fluorescent dye (e.g., fluorescein or fluorescent protein), a radionuclide, or biotin) to detect the nanobody or antigen-binding fragment thereof or polypeptide construct.

In some embodiments, the method comprises: (1) contacting the sample with the nanobody or antigen-binding fragment thereof of the present invention, the polypeptide construct of the present invention, or a conjugate, wherein the conjugate comprises the nanobody or antigen-binding fragment thereof of the present invention, or the polypeptide construct of the present invention, and a detectable label, such as an enzyme (e.g., horseradish peroxidase or alkaline

phosphatase), a chemiluminescent reagent (e.g., acrid esters, luminol and derivative thereof, or ruthenium derivative), a fluorescent dye (e.g., fluorescein or fluorescent protein), a radionuclide, or biotin, that is linked to the nanobody or antigen-binding fragment thereof or polypeptide construct; and (2) detecting the formation of an antigen-antibody immune complex or detecting the amount of the immune complex. The formation of the immune complex indicates the presence of HER3 or cells expressing HER3.

In some embodiments, the method is used to detect whether a tumor can be treated by an HER3-targeted antitumor therapy. In such embodiments, the sample is from a subject who has a tumor, is suspected of having a tumor, or is at risk of having a tumor. In some embodiments, the sample is a cell sample (e.g., a sample containing tumor cell) or a body fluid sample (e.g., blood) from a subject (e.g., a mammal, such as a human). In some embodiments, the presence of HER3 or HER3-expressing cells, and/or an increase in the amount of HER3 or HER3-expressing cells compared to a reference level (e.g., compared to a patient without tumor disease), indicates that the subject is suitable for an HER3-targeted antitumor therapy.

Use in manufacture of detection reagent

In another aspect, the present invention also provides a use of the nanobody or antigen-binding fragment thereof of the present invention or the polypeptide construct of the present invention in the manufacture of a detection reagent for detecting the presence or amount of HER3 in a sample or for detecting whether a tumor can be treated by an HER3-targeted antitumor therapy.

In some embodiments, the detection reagent detects the presence or amount of HER3 in a sample and optionally detects whether a tumor can be treated by an HER3-targeted antitumor therapy by the method of the present invention as described above.

In some embodiments, the sample is a cell sample (e.g., a sample containing tumor cells) or a body fluid sample (e.g., blood) from a subject (e.g., a mammal, such as a human).

Definitions of terms

In the present invention, unless otherwise stated, scientific and technical terms used herein have the meanings commonly understood by those skilled in the art. Furthermore, the virological, biochemical, and immunological laboratory procedures used herein are standard procedures widely used in the respective fields. Meanwhile, for a better understanding of the present invention, definitions and explanations of relevant terms are provided below.

When the terms “e.g.,” “such as,” “comprising,” “including,” “containing,” or variations thereof are used herein, these terms shall not be considered limiting terms but shall be construed as meaning “but not limited to” or “not limited to.”

Unless otherwise specified herein or clearly contradicted by the context, the terms “an,” “a,” “the,” and similar designations shall be construed to cover both the singular and plural in the context of describing the present invention (particularly in the context of the following claims).

As used herein, the term “camelid antibody” refers to an antibody against an antigen produced by an immunized or antigen-invaded *Camelidae* animal (including *Camel*, *Alpaca*, and *L. glama*). It is known to those skilled in the art that antibodies produced by *Camelidae* animals include “heavy-chain antibodies” (Camelid heavy-chain antibody, HCAb) that lack light chains. These antibodies consist solely of a variable domain of the heavy chain of HCAb (VHH) and two conventional CH2 and CH3 regions. The VHH domain, when cloned and expressed independently, exhibits excellent structural stability and antigen-binding activity. VHH is currently the smallest known unit capable of binding to a target antigen.

As used herein, the terms “nanobody” or “single-domain antibody” have the meanings commonly understood by those skilled in the art and are used interchangeably. They refer to antibody fragments composed of a single monomeric variable antibody domain (e.g., a single heavy chain variable region), typically derived from the variable region of heavy chain antibodies (e.g., camelid antibodies or shark antibodies). Typically, a nanobody consists of four framework regions and three complementarity-determining regions, with a structure of FR1-CDR1-FR2-CDR2-FR3-CDR3-FR4. Nanobodies may be truncated at the N-terminus or C-terminus to include only part of FR1 and/or FR4, or to lack one or both of those framework regions, as long as they substantially retain antigen binding ability and specificity.

As used herein, the term “antigen-binding fragment” of nanobody refers to a polypeptide comprising a fragment of nanobody that retains the ability to specifically bind to the same antigen bound by the nanobody, and/or competes with the nanobody for specific binding to the antigen, which is also referred to as an “antigen-binding fragment.” See generally Fundamental Immunology, Ch. 7 (Paul, W., ed., 2nd ed., Raven Press, N.Y. (1989), which is incorporated herein by reference in its entirety for all purposes. Antigen-binding fragments of the nanobody of the present invention can be produced by recombinant DNA techniques or by enzymatic or chemical cleavage of the nanobody of the present invention. In some embodiments, the “antigen-binding fragment” of nanobody may be truncated at the N-terminus or C-terminus compared to the full-length nanobody so as to comprise only a portion of FR1 and/or FR4, or lack one or both of those framework regions, as long as it substantially retains antigen binding ability and specificity.

Antigen-binding fragments of nanobodies can be obtained from a given nanobody (e.g., the nanobody provided by the present invention) using conventional techniques known to those skilled in the art (e.g., recombinant DNA technology or enzymatic or chemical cleavage), and the antigen-

binding fragments of the nanobody can be screened for specificity in the same manner as for the complete nanobody.

As used herein, unless the context explicitly states otherwise, the terms “single-domain antibody” or “nanobody” refer not only to the complete nanobody but also to the antigen-binding fragment of the nanobody.

As used herein, the term “complementarity-determining region” or “CDR” refers to the amino acid residues in the variable region of an antibody responsible for antigen binding. Nanobodies contain three CDRs, designated CDR1, CDR2, and CDR3. The precise boundaries of these CDRs can be defined according to various numbering systems known in the art, such as the Kabat numbering system (Kabat et al., *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md., 1991), the Chothia numbering system (Chothia & Lesk (1987) *J. Mol. Biol.* 196:901-917; Chothia et al. (1989) *Nature* 342:878-883), or the IMGT numbering system (Lefranc et al., *Dev.*). The CDRs of nanobodies are defined in *Comparat. Immunol.* 27:55-77, 2003. For a given nanobody, those skilled in the art will readily identify the CDRs as defined by various numbering systems. Furthermore, the correspondences between different numbering systems are well known to those skilled in the art (e.g., see Lefranc et al., *Dev. Comparat. Immunol.* 27:55-77, 2003). As used herein, the CDRs of nanobodies are preferably determined using the Kabat, Chothia, and/or IMGT numbering systems.

As used herein, the term “framework region” or “FR” residues refer to those amino acid residues in a variable region of the antibody other than the CDR residues defined above.

As used herein, the term “Fc domain” or “Fc region” refers to a fragment of the heavy chain constant region comprising CH2 and CH3. The Fc fragment of an antibody has a variety of different functions but does not participate in antigen binding. “Effector functions” mediated by the Fc region include Fc receptor binding; C1q binding and complement-dependent cytotoxicity (CDC); antibody-dependent cell-mediated cytotoxicity (ADCC); phagocytosis; down-regulation of cell surface receptors (e.g., B cell receptor); and B cell activation, among others. In some embodiments, the Fc region comprises a hinge, CH2, and CH3. When the Fc region comprises a hinge, the hinge regulates dimerization between two Fc-containing polypeptides. The Fc region can be of any antibody heavy chain constant region isotype, such as IgG1, IgG2, IgG3, or IgG4.

The Fc domain may comprise either a native Fc region or a variant Fc region. A native Fc region comprises an amino acid sequence that is consistent with the amino acid sequence of an Fc region found in nature. For example, native sequence human Fc regions include native sequence human IgG1 Fc region (non-A and A allotypes); native sequence human IgG2 Fc region; native sequence human IgG3 Fc region; and native sequence human IgG4 Fc region, as well as naturally

occurring variants thereof. A variant Fc region comprises an amino acid sequence that differs from the amino acid sequence of a native Fc region due to at least one amino acid modification. In some embodiments, the variant Fc region may exhibit altered effector functions compared to the native Fc region (e.g., Fc receptor binding, antibody glycosylation, the number of cysteine residues, effector cell function, or complement function).

As used herein, the term "humanized antibody" refers to a genetically engineered non-human antibody whose amino acid sequence has been modified to increase its homology with the sequence of a human antibody. Generally, all or part of the CDR regions of a humanized antibody are derived from a non-human antibody (donor antibody), while all or part of the non-CDR regions (e.g., framework regions (FR) of variable region and/or constant region) are derived from a human immunoglobulin (acceptor antibody). In certain embodiments, the CDR regions of a humanized antibody are derived from a non-human antibody (donor antibody), and all or part of the non-CDR regions (e.g., variable region FR and/or constant region) are derived from a human immunoglobulin (acceptor antibody). Humanized antibodies typically retain the desired properties of the donor antibody, including but not limited to antigen specificity, affinity, reactivity, and the like. In the present application, the donor antibody may be a camelid antibody with desired properties (e.g., antigen specificity, affinity, reactivity, etc.). To prepare humanized antibodies, the CDR regions of the immunized animal can be inserted into human framework sequences using methods known in the art. In the context of nanobodies, a humanized antibody may refer to a humanized VHH, i.e., a VHH in which one or more framework regions have been substantially replaced with human framework regions. In some cases, certain framework regions (FR) of human immunoglobulin may be substituted with corresponding non-human residues. Additionally, humanized VHH may contain residues not found in the original VHH or human framework sequences but included to further improve and optimize the performance of the VHH or VHH-containing polypeptides.

As used herein, the term "identity" refers to the degree of sequence matching between two polypeptides or between two nucleic acids. To determine the percent identity between two amino acid sequences or two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (e.g., gaps may be introduced in the first amino acid sequence or nucleic acid sequence to achieve optimal alignment with the second amino acid or nucleic acid sequence). The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences (i.e., percent identity = number of identical overlapping

positions / total number of positions $\times$ 100%). In certain embodiments, the two sequences are of the same length.

Determination of percent identity between two sequences can also be accomplished using a mathematical algorithm. A non-limiting example of a mathematical algorithm utilized for the comparison of two sequences is the algorithm of Karlin and Altschul, 1990, Proc. Natl. Acad. Sci. U.S.A. 87:2264-2268, as modified in Karlin and Altschul, 1993, Proc. Natl. Acad. Sci. U.S.A. 90:5873-5877. Such an algorithm is integrated into the NBLAST and XBLAST programs of Altschul et al., 1990, J. Mol. Biol. 215:403.

As used herein, the term "specific binding" refers to a non-random binding reaction between two molecules, such as the reaction between an antibody and its target antigen. The strength or affinity of a specific binding interaction can be expressed in terms of the equilibrium dissociation constant (K_D) of the interaction. In the present invention, the term " K_D " refers to the equilibrium dissociation constant of a particular antibody-antigen interaction, which is used to describe the binding affinity between the antibody and the antigen. The smaller the equilibrium dissociation constant, the tighter the antibody-antigen binding and the higher the affinity between the antibody and the antigen.

The specific binding properties between two molecules can be determined using methods well known in the art. One method involves measuring the rates of formation and dissociation of antigen-binding site/antigen complexes. Both the "association rate constant" (k_a or k_{on}) and the "dissociation rate constant" (k_{dis} or k_{off}) can be calculated based on concentration and the actual rates of association and dissociation (see Malmqvist M, Nature, 1993, 361:186-187). The ratio of k_{dis}/k_{on} equals the dissociation constant K_D (see Davies et al., Annual Rev Biochem, 1990; 59:439-473). The values of K_D , k_{on} , and k_{dis} can be measured using any effective method. In some embodiments, surface plasmon resonance (SPR) can be used to measure the dissociation constant in Biacore. Additionally, bio-layer interferometry or Kinexa can be used to measure the dissociation constant.

As used herein, the detectable label of the present invention can be any substance detectable by fluorescent, spectroscopic, photochemical, biochemical, immunological, electrical, optical, or chemical means. Such labels are well known in the art, and examples thereof include, but are not limited to, enzymes (e.g., horseradish peroxidase, alkaline phosphatase, β -galactosidase, urease, glucose oxidase, etc.), radionuclides (e.g., 3H , ^{125}I , ^{35}S , ^{14}C , or ^{32}P), fluorescent dyes (e.g., fluorescein isothiocyanate (FITC), fluorescein, tetramethylrhodamine isothiocyanate (TRITC), phycoerythrin (PE), Texas Red, rhodamine, quantum dots, or cyanine dye derivatives such as Cy7, Alexa 750), luminescent substances (e.g., chemiluminescent substances such as acridinium esters, luminol and derivatives thereof, ruthenium derivatives such as tris(bipyridine)ruthenium),

magnetic beads (e.g., Dynabeads®), calorimetric labels such as colloidal gold, or colored glass or plastic (e.g., polystyrene, polypropylene, latex, etc.) beads, and biotin for binding with avidin (e.g., streptavidin) modified with the aforementioned labels.

As used herein, the term "vector" refers to a nucleic acid delivery vehicle into which a polynucleotide can be inserted. When a vector enables the expression of the protein encoded by the inserted polynucleotide, it is referred to as an expression vector. A vector can be introduced into a host cell by transformation, transduction, or transfection, allowing the genetic material elements it carries to be expressed in the host cell. Vectors are well known to those skilled in the art and include, but are not limited to: plasmids; phagemids; cosmids; artificial chromosomes such as yeast artificial chromosome (YAC), bacterial artificial chromosome (BAC), or P1-derived artificial chromosome (PAC); bacteriophages such as λ phage or M13 phage; and animal viruses. Animal viruses that can be used as vectors include, but are not limited to, retroviruses (including lentivirus), adenoviruses, adeno-associated viruses, herpes viruses (e.g., herpes simplex virus), poxviruses, baculoviruses, papillomaviruses, and papovaviruses (e.g., SV40). A vector may contain multiple elements that control expression, including but not limited to promoter sequence, transcription initiation sequence, enhancer sequence, selection element, and reporter gene. Additionally, a vector may contain an origin of replication.

As used herein, the term "host cell" refers to a cell into which a vector can be introduced, including but not limited to, prokaryotic cells such as *Escherichia coli* or *Bacillus subtilis*, fungal cells such as yeast cells or *Aspergillus*, insect cells such as *S2 Drosophila* cells or *Sf9* cells, or animal cells such as fibroblasts, CHO cells, COS cells, NSO cells, HeLa cells, BHK cells, HEK 293 cells, or human cells.

As used herein, the term "prevention" refers to a method implemented to prevent or delay the occurrence of a disease or disorder or symptom (e.g., tumor) in a subject. As used herein, the term "treatment" refers to a method implemented to obtain a beneficial or desired clinical outcome. For the purposes of the present invention, the beneficial or desired clinical outcome includes, but is not limited to, alleviation of symptom, reduction of the extent of the disease, stabilization (i.e., not worsening) of the state of the disease, delay or slowing of the progression of the disease, amelioration or palliation of the state of the disease, and remission of symptom (whether partial or total), whether detectable or undetectable. In addition, "treatment" can also refer to prolonged survival compared to expected survival (if not receiving treatment).

As used herein, the terms "cancer" or "tumor" are used interchangeably and refer to a broad class of diseases characterized by the uncontrolled growth of abnormal cells in the body. Uncontrolled cell division can lead to the formation of malignant tumors or cells that invade adjacent tissues and may metastasize to distant sites of the body via the lymphatic system or

bloodstream. Cancer includes benign and malignant cancers, as well as dormant tumors or micrometastases. Cancer also includes hematologic malignancies.

Beneficial effects of the invention

The present application provides an anti-HER3 nanobody or polypeptide construct, which exhibits high binding activity to HER3. In particular, the nanobody or polypeptide construct of the invention possesses internalization activity and can mediate the entry of toxins into cells through internalization, thereby exerting a killing effect on tumor cells. On this basis, humanized nanobodies or polypeptide constructs prepared from the aforementioned nanobody or polypeptide construct still retain these outstanding activities. Moreover, due to its small molecular weight, the nanobody or polypeptide construct also offers advantages such as ease of production.

The embodiments of the present invention will be described in detail below with reference to the accompanying drawings and examples. However, those skilled in the art will understand that the following drawings and examples are for illustrative purposes only and are not intended to limit the scope of the invention. Various objects and advantages of the present invention will become apparent to those skilled in the art from the following detailed description of the drawings and preferred embodiments.

Brief Description of the Drawings

Fig. 1 shows the binding activities of candidate antibodies to human HER3 antigen detected by ELISA.

Fig. 2A shows the binding activities of 14 candidate antibodies to SK-BR-3 cells in the FACS experiment.

Fig. 2B shows the binding activities of 4 candidate antibodies to SK-BR-3 cells in the FACS experiment.

Fig. 3 shows the internalization percentage of the candidate antibodies in SK-BR-3 cells.

Fig. 4 shows the ELISA binding curves of the MHAB15-6 humanized antibodies to recombinant human HER3.

Fig. 5 shows the flow cytometry binding curves of the MHAB15-6 humanized antibodies to SK-BR-3 cells.

Fig. 6 shows the internalization percentages of the humanized antibodies in SK-BR-3 cells.

Fig. 7 shows the detection results for whether the antibody MHAB15-6 competitively binds to recombinant human HER3 antigen with Patritumab.

Fig. 8A shows the ELISA binding curves of the antibody MHAB15-6-5 to HER3 from different species.

Fig. 8B shows the ELISA binding curves of Isotype to HER3 from different species.

Fig. 9A shows the ELISA binding curves of the antibody MHAB15-6-5 to EGFR family proteins.

Fig. 9B shows the ELISA binding curve of Isotype to EGFR family proteins.

Information of sequences

Information on some of the sequences involved in the present invention is provided in Table 1 below.

Table 1: Information of sequences

SEQ ID NO:	Sequence name	Sequence
1	MHAB15-6-Kabat-CDR1 MHAB15-6-5-Kabat-CDR1 MHAB15-6-7-Kabat-CDR1	AYITN
2	MHAB15-6-Kabat-CDR2 MHAB15-6-5-Kabat-CDR2	TVTPGGSTIAADSVKG
3	MHAB15-6-Kabat-CDR3 MHAB15-6-5-Kabat-CDR3 MHAB15-6-7-Kabat-CDR3	IPPKR
4	MHAB15-6-IMGT-CDR1 MHAB15-6-5-IMGT-CDR1 MHAB15-6-7-IMGT-CDR1	GITLSAYI
5	MHAB15-6-IMGT-CDR2 MHAB15-6-5-IMGT-CDR2 MHAB15-6-7-IMGT-CDR2	VTPGGST
6	MHAB15-6-IMGT-CDR3 MHAB15-6-5-IMGT-CDR3 MHAB15-6-7-IMGT-CDR3	NTIPPKR
7	MHAB15-6-7-Kabat-CDR2	TVTPGGSTYYADSVKG
8	MHAB15-6 VHH	QVQLVESGGDLVQPGGSLRLSCAASGITLSAYITNW YRQVPGKQREL VATVTPGGSTIAADSVKGRFTISR DNTKNTVY LQMNILKPEDTAVYYCNTIPPKRWGQGT RVTVSS
9	MHAB15-6-5 VHH	QVQLVESGGGLVQPGGSLRLSCAASGITLSAYITNW VRQAPGKGREL VATVTPGGSTIAADSVKGRFTISR D

		NSKNTVYLQMNSLRPEDTAVYYCNTIPPKRWGQGT RVTVSS
10	MHAB15-6-7 VHH	QVQLVESGGGLVQPGGSLRLSCAASGITLSAYITNW VRQAPGKQREL VATVTPGGSTYYADSVKGRFTISR DNSKNTVYLQMNSLRPEDTAVYYCNTIPPKRWGQ GTRVTVSS
11	Fc	EPKSSDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTL MISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVH NAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEY KCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSR EEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENN YKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFSC SVMHEALHNHYTQKSLSLSPGK
12	MHAB15-6	QVQLVESGGDLVQPGGSLRLSCAASGITLSAYITNW YRQVPGKQREL VATVTPGGSTIAADSVKGRFTISR NTKNTVYLQMNLKPEDTAVYYCNTIPPKRWGQGT RVTVSSEPKSSDKTHTCPPCPAPEAAGGPSVFLFPPK PKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDG VEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLN GKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTL PPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQ PENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGN VFSCSVMHEALHNHYTQKSLSLSPGK
13	MHAB15-6-5	QVQLVESGGGLVQPGGSLRLSCAASGITLSAYITNW VRQAPGKGREL VATVTPGGSTIAADSVKGRFTISR NSKNTVYLQMNSLRPEDTAVYYCNTIPPKRWGQGT RVTVSSEPKSSDKTHTCPPCPAPEAAGGPSVFLFPPK PKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDG VEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLN GKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTL PPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQ PENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGN VFSCSVMHEALHNHYTQKSLSLSPGK
14	MHAB15-6-7	QVQLVESGGGLVQPGGSLRLSCAASGITLSAYITNW VRQAPGKQREL VATVTPGGSTYYADSVKGRFTISR DNSKNTVYLQMNSLRPEDTAVYYCNTIPPKRWGQ GTRVTVSSEPKSSDKTHTCPPCPAPEAAGGPSVFLFP PKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYV DGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDW LNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVY TLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNG QPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQG NVFSCSVMHEALHNHYTQKSLSLSPGK

Specific Models for Carrying Out the present invention

The present invention will now be described with reference to the following examples, which are intended to illustrate the present invention (but not limit it).

Unless otherwise specified, the molecular biology experimental methods and immunoassays used in the present invention are substantially in accordance with the methods described in J.

Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory Press, 1989, and F. M. Ausubel et al., A Concise Guide to Laboratory Molecular Biology, 3rd Edition, John Wiley & Sons, Inc., 1995; the use of restriction endonucleases is in accordance with the manufacturer's recommendations. Those skilled in the art will understand that the examples illustrate the present invention by way of example and are not intended to limit the scope sought to be protected by the present invention.

Example 1. Acquisition of anti-human HER3 nanobody sequences

1-1 Alpaca immunization and serum titer detection

A blank alpaca was immunized with Her3/ERBB3 Protein, Human (His Tag) antigen (KactusBio, Cat. No. HER-HM403) and Her3/ERBB3 Protein, Human (His Tag) antigen (ACRO, Cat. No. ER3-H5223).

10 mL of blood was collected before the initial immunization to serve as negative serum. The initial immunization was administered subcutaneously at multiple sites using a 1:1 mixture of complete Freund's adjuvant and 1 mg of HER3 antigen. Then, the second, third and fourth immunizations were administered subcutaneously at multiple sites at weeks 2, 4, and 6 using a 1:1 mixture of incomplete Freund's adjuvant and 0.5 mg of HER3 antigen, respectively. Further, the fifth and sixth immunizations were administered subcutaneously at multiple sites at weeks 8 and 10 using a 1:1 mixture of incomplete Freund's adjuvant and 1 mg of HER3 antigen, respectively. Furthermore, the seventh, eighth and ninth immunizations were administered subcutaneously at multiple sites at weeks 12, 14, and 16 using a 1:1 mixture of incomplete Freund's adjuvant and 0.5 mg of HER3 antigen, respectively. Before each of the third, fourth, fifth, sixth, seventh, eighth, and ninth immunizations, 10 mL of peripheral blood was collected, serum was separated, and immune responses were monitored using ELISA. Seven days after the ninth immunization, 50 mL of peripheral blood was collected for subsequent nanobody library construction.

1-2 Screening and identification of specific nanobodies

Peripheral blood was collected from immunized alpacas, and RNA was extracted to prepare cDNA samples. The VHH antibody-encoding genes were cloned by PCR to construct a single-domain antibody phage display library. Random clones were selected to assess the library capacity and diversity.

Using the Her3/ERBB2 Protein, Human, Recombinant, His Tag antigen (KactusBio, Cat. No. HER-HM403; ACRO, Cat. No. ER3-H5223), the single-domain antibody phage display library was subjected to panning enrichment experiments. After screening, individual monoclonal

colonies were selected for Phage-ELISA detection. Candidate clones with distinct amino acid sequences in the CDR regions were chosen, ultimately resulting in the selection of 25 unique sequences.

1-3 Unique sequence solubility verification and antibody expression

The plasmids of the aforementioned 25 positive clones were extracted and individually transformed into *E. coli Rosetta*. Expression was induced overnight with 0.4 mM IPTG at 20°C and 200 rpm. The next day, the cultures were centrifuged, and the pellets were resuspended in PBS, followed by sonication and centrifugation to obtain the supernatants from the 25 clones after lysis. The binding activity of the clarified supernatants from the 25 clones to human HER3 antigen was detected by ELISA (OD₄₅₀ values are shown in Table 2 below).

Fourteen unique sequences with strong binding activity to human HER3 were selected. The VHH sequences were integrated into the Fc (LALA) sequence (SEQ ID NO: 11) of Human IgG1, followed by transient expression in CHO cells to produce candidate antibodies for MHAB15 (the correspondence between the numbers in the present application and the selected unique sequences is shown in Table 3; and in the subsequent description of candidate antibodies for MHAB15, the numbers in the present application, i.e., MHAB15-1 to MHAB15-14, will be used). After expression, the antibodies were purified using Protein A and dissolved in PBS. The antibody solutions all had a purity of >95% (SEC, 280 nm), and an endotoxin level of <1 (EU/mg). The antibodies were aliquoted and stored at -80°C.

Table 2. OD₄₅₀ results of 25 clones

Unique Sequence	OD ₄₅₀	Unique Sequence	OD ₄₅₀
M26H3-2011	3.246	M29H3-2-112	3.041
M26H3-2049	0.116	M29H3-2-139	3.198
M26H3-3014	0.09	M29H3-2-140	0.106
M26H3-3039	0.217	M29H3-2-145	0.091
M26H3-3155	1.568	M29H3-3-12	0.078
M29H3-1-72	0.18	M29H3-3-21-1	0.53
M29H3-2-12	0.86	M29H3-3-32	0.117
M29H3-2-41	1.164	M29H3-3-33-1	0.1
M29H3-2-79	2.367	M29H3-3-37	0.082
M29H3-2-84	1.053	M29H3-3-52	0.103
M29H3-2-100	0.157	M29H3-3-149	0.157
M29H3-2-105	2.294	M29H3-3-177	0.105
M29H3-2-111	0.33		
Irrelevant control	0.073	1%M-PBS	0.079

Table 3. Number correspondence

Number in the present application	Unique Sequence	Number in the present application	Unique Sequence
MHAB15-1	M26H3-3155	MHAB15-8	M29H3-2-112
MHAB15-2	M29H3-2-41	MHAB15-9	M29H3-2-139
MHAB15-3	M29H3-2-79	MHAB15-10	M29H3-2-140
MHAB15-4	M29H3-2-84	MHAB15-11	M29H3-3-21-1
MHAB15-5	M29H3-2-100	MHAB15-12	M29H3-3-33-1
MHAB15-6	M29H3-2-105	MHAB15-13	M26H3-2049
MHAB15-7	M29H3-2-111	MHAB15-14	M29H3-3-52

Example 2. Detection of anti-human HER3 nanobody activity

2-1 ELISA binding assay

The binding ability of the MHAB15 candidate antibodies (Fc fusion protein of anti-human HER3 VHH) to the human HER3 antigen was detected using ELISA. The Human ErbB3/Her3 Protein, His Tag (MALIS verified) antigen (ACRO, Cat. No. ER3-H5223) was diluted to 0.5 µg/mL with PBS and added to an ELISA plate (Corning, Cat. No. 9018) at 100 µL/well. The plate was washed three times with PBST, followed by blocking with 1% BSA/PBST for 1 hour at room temperature. After blocking, the plate was washed five times with PBST, and the test antibodies (test antibody number: MHAB15, positive control antibody: Patritumab, with initial concentration of 10 µg/mL, serially diluted 4-fold) were added. Following incubation for 1 hour, the plate was washed seven times with PBST to remove unbound antibodies. Subsequently, Goat Anti-Human IgG Fc (HRP) (Abcam, Cat. No. ab97225), diluted at 1:50,000, was added and incubated for 30 minutes at room temperature. After washing away the excess secondary antibody, 1-Step™ Ultra TMB-ELISA Substrate Solution (Absin, Cat. No. 9178) was added at 100 µL/well, and the plate was incubated in the dark at room temperature for 15 minutes for color development. The reaction was then stopped by adding 100 µL of TMB Stop Solution (Absin, Cat. No. abs9472). The absorbance at wavelength 450 nm was measured using a microplate reader, and a four-parameter logistic curve was plotted using GraphPad.

All experimental results are expressed as mean ± SEM (standard error of the mean). Prism 6 (GraphPad) software was used for plotting and data analysis.

As shown in Fig. 1, the binding activity of the MHAB15 candidate antibodies to the human HER3 antigen was as follows: the EC₅₀ of all antibodies, MHAB15-1 to MHAB15-14, was lower than that of the positive control antibody Patritumab, demonstrating their superior binding activity.

2-2 FACS binding assay

In the first experiment, the binding of the candidate antibodies was detected using SK-BR-3 human breast cancer cells endogenously expressing human HER3. The tested antibodies were serially diluted (30000 ng/mL, 7500 ng/mL, 1875 ng/mL, 468.8 ng/mL, 117.2 ng/mL, 29.3 ng/mL, 7.3 ng/mL, and 1.8 ng/mL), and 100 μ L of diluted AF488 Anti-Human IgG (H+L) secondary antibody (Yesen, Cat: 33126ES60) was used to detect the binding of the tested antibodies. The fluorescence intensity (Mean Fluorescence Intensity, MFI) was measured by flow cytometry. In the second experiment, the top 4 candidate antibodies were selected to detect their binding using SK-BR-3 cells, and serially diluted (same as in the first experiment). Then, 100 μ L of diluted PE Goat anti-Human IgG Fc Secondary Antibody (Invitrogen, Cat: 12-4998-82) was used to detect the binding of the tested antibodies. The fluorescence intensity of the bound secondary antibody was measured by flow cytometry. All experimental results were expressed as mean $\pm$ SEM (standard error of the mean). Prism 6 (GraphPad) software was used for plotting and data analysis.

The FACS binding results are shown in Fig. 2A and Fig. 2B. MHAB15-6, MHAB15-1, MHAB15-2, and MHAB15-10 showed good binding activity, with MHAB15-6 showing the best.

2-3 Internalization assay

Internalization activity was assessed using SK-BR-3 cells. A cell suspension with a concentration of 1×10^6 cells/mL and test anti-HER3 antibody solutions at 10 μ g/mL were prepared using FACS buffer (PBS containing 2% FBS). The cell suspension was transferred into 1.5 mL EP tubes and centrifuged at 1200 rpm for 3 minutes at 4°C, after which the supernatant was discarded. Subsequently, 1.5 mL of the test antibody solution was added to the cells, and the mixture was incubated on ice for 30 minutes. Then, the cells were washed four times with pre-cooled FACS buffer at 200 μ L/well. The cells were then resuspended in 1.5 mL of complete culture medium, mixed thoroughly, and transferred to 96-well plate at 100 μ L/well. The plates were incubated at 4°C and 37°C respectively for 0, 1, 2, and 4 hours (in duplicate). After incubation, the cells were centrifuged at 1200 rpm for 3 minutes at 4°C, and 100 μ L of diluted AF488 Anti-Human IgG (H+L) (Yesen, Cat: 33126ES60) was added. The cells were incubated at 4°C for 30 minutes, and unbound secondary antibody was removed by washing. The mean fluorescence intensity (MFI) of the bound secondary antibody was measured using flow cytometry, and the internalization rate was calculated using the following formula:

$$\text{Internalization (\%)} = 100\% - \text{MFI}_T / \text{MFI}_{T0} * 100\%$$

All experimental results are expressed as mean $\pm$ SEM (standard error of the mean). Prism 6 (GraphPad) software was used for plotting and data analysis.

The internalization percentage curves at each time point are shown in Fig. 3. At 37°C, the MHAB15 candidate antibodies exhibited good internalization activity in SK-BR-3 cells, and their internalization activity was comparable to or slightly better than that of Patritumab.

2-4 Fab-ZAP assay for detection of antibody-induced cell killing

Human breast cancer SK-BR-3 cells were digested and then resuspended in complete culture medium (ATCC-formulated McCoy's 5a Medium Modified, Catalog No. 30-2007+10% FBS, Sigma-Aldrich, Cat. No. F8687+1% P/S, Gibco, Cat. No. 15140-122). The cell density was adjusted to 4E4 cells/mL, and 50 µL of the cell suspension was added to each well of a 96-well cell culture plate. The plate was then incubated in a 37°C cell culture incubator for 16 hours. The next day, a ZAP dilution solution containing 9 nM ZAP-Fab was prepared using the culture medium. Subsequently, the antibody was serially diluted with the ZAP dilution solution to create working solutions ranging from 4000 pM to 0.256 pM (7 concentration gradients, 1:5 dilution). After incubation at 37°C for 15 minutes, the working solutions were added to the cell culture plate, 50 µL per well, resulting in final antibody concentrations ranging from 2000 pM to 0.128 pM. After thoroughly mixing, the cell culture plate was placed in a 37°C cell culture incubator and incubated for an additional 120 hours. For the positive control, 7.5 µL of Triton-X 100 was added in advance to wells without antibody-Fab-ZAP treatment, followed by a 30-minute incubation. Wells without antibody-Fab-ZAP or Triton-X 100 treatment (NT) served as the negative control. Afterward, 20 µL of MTS (Promega, Cat: G3598B) was added to each well of the cell culture plate, and the plate was incubated in a 37°C cell culture incubator for 2 hours. The plate was then shaken for 10 seconds in a microplate reader, and the absorbance was measured at a wavelength of A490. The cell killing efficiency was calculated using the formula: killing rate % = 100% - (OD_{sample} - OD_{Triton-X100}) / (OD_{NT} - OD_{Triton-X100}) * 100%. Prism 6 (GraphPad) software was used for plotting and data analysis.

The internalization-mediated cell killing results showed that MHAB15-6-Fab-ZAP had good killing activity against SK-BR-3 cells, comparable to Patritumab. Based on the combined FACS binding and internalization results, MHAB15-6 would be humanized and further screened.

Table 4. EC₅₀ results

	MHAB15-1	MHAB15-2	MHAB15-6	MHAB15-10	Patritumab	Isotype
EC ₅₀ (pM)	~ 140.6	~ 18.84	~ 17.69	89.70	~ 16.07	N/A

Example 3. Humanization and activity detection of anti-human HER3 nanobodies

The VHH sequence of MHAB15-6 is set forth in SEQ ID NO: 8, with the CDR1 to CDR3 sequences defined by Kabat as set forth in SEQ ID NOs: 1-3, respectively, and the CDR1 to CDR3 sequences defined by IMGT as set forth in SEQ ID NOs: 4-6, respectively. Humanization of the MHAB15-6 VHH was performed, resulting in two humanized nanobodies, MHAB15-6-5 (VHH as set forth in SEQ ID NO: 9) and MHAB15-6-7 (VHH as set forth in SEQ ID NO: 10). The aforementioned VHH sequences were integrated into the Fc sequence of human IgG1 (SEQ ID NO: 11), and used for subsequent activity assays after purification and expression.

3-1 ELISA binding assay

The binding abilities of the MHAB15-6 humanized sequences to human HER3 antigen were detected using an ELISA method. Human ErbB3/Her3 Protein, His Tag (MALS verified) antigen (ACRO, Cat. No. ER3-H5223) was diluted to 0.5 $\mu\text{g/mL}$ with PBS and added to ELISA plates (Corning, Cat. No. 9018) at 100 $\mu\text{L/well}$, followed by incubation at 2°C to 8°C overnight. The plates were washed three times with PBST, blocked with 1% BSA/PBST for 1 hour at room temperature, and then washed five times with PBST. The MHAB15-6 humanized antibodies (antibody numbers: MHAB15-6-1 to MHAB15-6-8, positive control antibody: Patritumab, with initial concentration of 10 $\mu\text{g/mL}$, serially diluted 10-fold) were added. After incubation for 1 hour, the plates were washed seven times with PBST to remove unbound antibodies. Goat Anti-Human IgG Fc (HRP) (Abcam, Cat. No. ab97225), diluted at 1:50,000, was added and incubated for 30 minutes at room temperature. Following the removal of excess secondary antibody by washing, 1-Step™ Ultra TMB-ELISA Substrate Solution (Absin, Cat. No. 9178) was added at 100 $\mu\text{L/well}$ and incubated for 15 minutes at room temperature in the dark for color development. The reaction was then stopped by adding 100 μL of TMB Stop Solution (Absin, Cat. No. abs9472). The absorbance at 450 nm was measured using a microplate reader, and a four-parameter logistic curve was plotted using GraphPad.

All experimental results are expressed as mean $\pm$ SEM (standard error of the mean). Prism 6 (GraphPad) software was used for plotting and data analysis.

The ELISA binding results (Fig. 4) showed that the binding activity of each humanized antibody to the antigen was comparable to that of parental MHAB15-6.

3-2 FACS binding assay

The binding of the MHAB15-6 humanized antibodies was detected using SK-BR-3 cells. The test antibody was serially diluted (30000 ng/mL, 7500 ng/mL, 1875 ng/mL, 469 ng/mL, 117 ng/mL, 29 ng/mL, 7 ng/mL, and 1.8 ng/mL), and the binding of the test antibody was detected using PE Goat anti-Human IgG Fc Secondary Antibody (Invitrogen, Cat# 12-4998-82). The mean fluorescence intensity (MFI) of the bound secondary antibody was measured by flow cytometry.

All experimental results are expressed as mean $\pm$ SEM (standard error of the mean). Prism 6 (GraphPad) software was used for plotting and data analysis.

The binding curves are shown in Fig. 5. The binding activities of the MHAB15-6 humanized antibodies to SK-BR-3 cells were comparable to that of the parental MHAB15-6.

3-3 Internalization assay

Internalization activity was assessed using SK-BR-3 cells. A cell suspension with a concentration of 1×10^6 cells/mL and test anti-HER3 antibody solution at 10 μ g/mL were prepared using FACS buffer (PBS containing 2% FBS). The cell suspension was transferred into a 1.5 mL EP tube and centrifuged at 1200 rpm for 3 minutes at 4°C, after which the supernatant was discarded. Subsequently, 1.5 mL of the test antibody solution was added to the cells, and the mixture was incubated on ice for 30 minutes. Then, the cells were washed four times with pre-cooled FACS buffer at 200 μ L/well. The cells were then resuspended in 1.5 mL of complete culture medium, mixed thoroughly, and transferred to a 96-well plate at 100 μ L/well. The plates were incubated at 4°C and 37°C respectively for 0, 1, 2, and 4 hours (in duplicate). After incubation, the cells were centrifuged at 1200 rpm for 3 minutes at 4°C, and 100 μ L of diluted PE Goat anti-Human IgG Fc Secondary Antibody (Invitrogen, Cat. No. 12-4998-82) was added. The cells were incubated at 4°C for 30 minutes, and unbound secondary antibody was removed by washing. The mean fluorescence intensity (MFI) of the bound secondary antibody was measured using flow cytometry, and the internalization rate was calculated using the following formula:

$$\text{Internalization (\%)} = 100\% - \text{MFI}_T / \text{MFI}_{T0} * 100\%$$

The internalization percentage curve is shown in Fig. 6. The internalization results showed that at 37°C, the MHAB15-6 humanized antibodies exhibited good internalization activity in SK-BR-3 cells, and their internalization activities were comparable to that of the parental MHAB15-6.

3-4 Fab-ZAP assay for detection of antibody-induced internalization-mediated cell killing

Human breast cancer SK-BR-3 cells were digested and then resuspended in complete culture medium (ATCC-formulated McCoy's 5a Medium Modified, Catalog No. 30-2007+10% FBS, Sigma-Aldrich, Cat. No. F8687+1% P/S, Gibco, Cat. No. 15140-122). The cell density was adjusted to 4×10^4 cells/mL, and 50 μ L of the cell suspension was added to each well of a 96-well cell culture plate. The plate was then incubated in a 37°C cell culture incubator for 16 hours. The next day, a ZAP dilution solution containing 9 nM ZAP-Fab was prepared using the culture medium. Subsequently, the antibody was serially diluted with the ZAP dilution solution to create working solutions ranging from 4000 pM to 0.256 pM (7 concentration gradients, 1:5 dilution). After incubation at 37°C for 15 minutes, the working solutions were added to the cell culture plate,

50 μ L per well, resulting in final antibody concentrations ranging from 2000 pM to 0.128 pM. After thoroughly mixing, the cell culture plate was placed in a 37°C cell culture incubator and incubated for an additional 120 hours. For the positive control, 7.5 μ L of Triton-X 100 was added in advance to wells without antibody-Fab-ZAP treatment, followed by a 30-minute incubation. Wells without antibody-Fab-ZAP or Triton-X 100 treatment (NT) served as the negative control. Afterward, 20 μ L of MTS (Promega, Cat: G3598B) was added to each well of the cell culture plate, and the plate was incubated in a 37°C cell culture incubator for 2 hours. The plate was then shaken for 10 seconds in a microplate reader, and the absorbance was measured at a wavelength of A490. The cell killing efficiency was calculated using the formula: killing rate % = $100\% - (\text{OD}_{\text{Sample}} - \text{OD}_{\text{Triton-X100}}) / (\text{OD}_{\text{NT}} - \text{OD}_{\text{Triton-X100}}) * 100\%$.

All experimental results are expressed as mean $\pm$ SEM (standard error of the mean). Prism 6 (GraphPad) software was used for plotting and data analysis.

The internalization-mediated cell killing results showed that the humanized antibody MHAB15-6-5-Fab-ZAP had good killing activity against SK-BR-3 cells, and was superior to Patritumab.

Table 5. EC₅₀ results

	MHAB15-6-5	MHAB15-6	Patritumab	Isotype
EC ₅₀ (pM)	~ 15.88	~ 16.24	19.78	N/A

Example 4. Competitive ELISA binding assay

The ability of the MHAB15 candidate antibody, MHAB15-6, to competitively bind to the human HER3 antigen with Patritumab was detected using an ELISA method. Patritumab was diluted to 1 μ g/mL with PBS, added at 100 μ L/well to an ELISA plate (Corning, Cat. No. 9018), and incubated overnight at 2°C to 8°C. The plate was washed three times with PBST, blocked with 1% BSA/PBST at room temperature for 60 minutes, and then washed five times with PBST. Human HER3 Protein, His Tag (ACRO, Cat. No. ER3-H5223) was diluted to 0.4 μ g/mL with 1% BSA/PBST, while MHAB15-6, the positive control antibody Patritumab, and the negative control Isotype were diluted to 60 μ g/mL and subjected to a 3-fold serial dilution. First, 50 μ L of the serially diluted MHAB15-6, Patritumab, or Isotype was added, followed by 50 μ L of the diluted HER3 (His Tag) antigen, resulting in a total volume of 100 μ L/well, and incubated at room temperature for 60 minutes. The plate was washed seven times with PBST to remove unbound antibodies, and then Anti-His tag Antibody (HRP), Mouse Monoclonal (Sino Biological, Cat. No. 105327-MM02T-H), diluted at 1:12000, was added and incubated at room temperature for 60

minutes. After washing away the excess secondary antibody, 1-Step™ Ultra TMB-ELISA Substrate Solution (Absin, Cat. No. 9178) was added at 100 µL/well and incubated in the dark at room temperature for 15 minutes for color development. The reaction was then stopped by adding 100 µL of TMB Stop Solution (Absin, Cat. No. abs9472). The absorbance at a wavelength of 450 nm was measured using a microplate reader, and a four-parameter logistic curve was plotted using GraphPad.

All experimental results are expressed as mean $\pm$ SEM (standard error of the mean). Prism 6 (GraphPad) software was used for plotting and data analysis.

The ELISA binding results (Fig. 7) showed that MHAB15-6 and Patritumab competitively bound to the human HER3 antigen.

Example 5. ELISA cross-species reactivity assay

The binding ability of MHAB15-6-5 (anti-human HER3 nanobody) to HER3 antigens from different species (human, Rhesus/Cynomolgus, mouse, and rat) was detected using ELISA method. Human HER3, His Tag (ACRO, Cat. No. ER3-H5223); Rhesus/Cynomolgus HER3 Protein, His Tag (Sino Biological, Cat. No. 90043-K08H); Mouse HER3 Protein, His Tag (Sino Biological, Cat. No. 51003-M08H); and Rat HER3 Protein, His Tag (Sino Biological, Cat. No. 80111-R08H) were diluted to 0.5 µg/mL with PBS, respectively, added to microplates (Corning, Cat. No. 9018) at 100 µL/well, and incubated overnight at 4°C for coating. After washing three times with PBST, each well was blocked with 200 µL of 3% BSA/PBST for 1 hour at room temperature, and washed five times with PBST. The test antibody was added at an initial concentration of 10 µg/mL with 10-fold serial dilutions and incubated for 1 hour at room temperature. After incubation, the plates were washed seven times with PBST to remove unbound antibodies. Then, 100 µL of 1:50,000 diluted goat anti-human IgG Fc (HRP) (Abcam, Cat. No. ab97225) was added to each well and incubated for 30 minutes at room temperature. The plates were washed seven times with PBST to remove excess secondary antibody, 100 µL of 1-Step™ Ultra TMB-ELISA Substrate Solution (Absin, Cat. No. 9178) was added to each well and incubated for 15 min at room temperature in the dark for color development. The reaction was then stopped by adding 100 µL of TMB Stop Solution (Absin, Cat. No. abs9472) to each well. The absorbance at a wavelength of 450 nm was measured using a microplate reader, and four-parameter logistic curves were plotted using GraphPad software.

All experimental results are expressed as mean $\pm$ SEM (standard error of the mean). Prism (GraphPad) software was used for plotting and data analysis.

The results are shown in Fig. 8A and Fig. 8B, and Tables 6 and 7, which indicated that MHAB15-6-5 exhibited binding activity against human HER3, Rhesus/Cynomolgus HER3, mouse HER3, and rat HER3, while Isotype showed no binding activity against human HER3, Rhesus/Cynomolgus HER3, mouse HER3, and rat HER3.

Table 6. EC₅₀ results of MHAB15-6-5 binding to HER3 in different species

	Human	Rhesus/Cynomolgus	Mouse	Rat
EC ₅₀ (ng/ml)	4.235	4.642	5.447	5.127

Table 7. EC₅₀ results of Isotype binding to HER3 in different species

	Human	Rhesus/Cynomolgus	Mouse	Rat
EC ₅₀ (ng/ml)	N/A	N/A	N/A	N/A

Example 6. ELISA for EGFR family-specific binding

The binding ability of MHAB15-6-5 (anti-human HER3 nanobody) to EGFR family proteins (HER1, HER2, HER3, and HER4) was detected using an ELISA method. Human HER1, His Tag (Sino Biological, Cat. No. 10001-H08H); Human HER2, His Tag (ACRO, Cat. No. HE2-H5225); Human HER3 Protein, His Tag (ACRO, Cat. No. ER3-H5223); and Human HER4 Protein, His Tag (Sino Biological, Cat. No. 10363-H08H) were diluted to 0.5 µg/mL with PBS, added at 100 µL/well to a microplate (Corning, Cat. No. 9018), respectively, and then incubated overnight at 4°C for coating. The plate was washed three times with PBST. Then, 200 µL of 3% BSA/PBST was to each well, and incubated at room temperature for 1 hour for blocking. After washing five times with PBST, the test antibodies, initially at a concentration of 10 µg/mL and serially diluted 10-fold, were added. Following a 1-hour incubation at room temperature, the plate was washed seven times with PBST to remove unbound antibodies. Subsequently, 100 µL of a 1:50,000 diluted Goat Anti-Human IgG Fc (HRP) (Abcam, Cat. No. ab97225) was added to each well and incubated at room temperature for 30 minutes. After washing seven times with PBST to remove excess secondary antibody, 100 µL of 1-Step™ Ultra TMB-ELISA Substrate Solution (Absin, Cat. No. 9178) was added to each well and incubated at room temperature for 15 minutes in the dark for color development. The reaction was then stopped by adding 100 µL of TMB Stop Solution (Absin, Cat. No. abs9472) to each well. The absorbance at a wavelength of 450 nm was measured using a microplate reader, and four-parameter logistic curves were plotted using GraphPad.

All experimental results are expressed as mean ± SEM (standard error of the mean). Prism (GraphPad) software was used for plotting and data analysis.

The results are shown in Fig. 9A and Fig. 9B, and Table 8, which indicate that MHAB15-6-5 bound only to HER3 and had no binding activity against other members of the EGFR family, HER1, HER2, and HER4, while Isotype had no binding activity against EGFR family proteins. Therefore, the antibody of the present application exhibited outstanding selective binding activity to EGFR family proteins and specific binding activity to HER3.

Table 8. EC₅₀ results of MHAB15-6-5 binding to human HER1 to HER4

	Human HER1	Human HER2	Human HER3	Human HER4
EC ₅₀ (ng/ml)	N/A	N/A	4.657	N/A

Although specific embodiments of the present invention have been described in detail, those skilled in the art will understand that, based on the all teachings disclosed, the details may be modified and altered, and all such changes are within the scope of protection of the present invention. The entire scope of the present invention is given by the appended claims and any equivalents thereof.

What is claimed is:

1. A nanobody or antigen-binding fragment thereof capable of specifically binding to human epidermal growth factor receptor-3 (HER3), wherein the nanobody or antigen-binding fragment thereof comprises: CDR1, CDR2 and/or CDR3 contained in VHH as set forth in any one of SEQ ID NOs: 8-10;

preferably, the CDRs are defined according to the Kabat, Chothia or IMGT numbering system.

2. The nanobody or antigen-binding fragment according to claim 1, which comprises:

(1) CDR1 as set forth in SEQ ID NO: 1; CDR2 as set forth in SEQ ID NO: 2 or 7; and CDR3 as set forth in SEQ ID NO: 3; wherein the CDRs are defined according to the Kabat numbering system; or,

(2) CDR1 as set forth in SEQ ID NO: 4; CDR2 as set forth in SEQ ID NO: 5; and CDR3 as set forth in SEQ ID NO: 6; wherein the CDRs are defined according to the IMGT numbering system.

3. The nanobody or antigen-binding fragment according to claim 1 or 2, wherein the nanobody or antigen-binding fragment comprises the sequence as set forth in SEQ ID NO: 8, or a sequence having a sequence identity of at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% as compared thereto, or a sequence having a substitution, deletion, or addition of one or more amino acids (e.g., a substitution, deletion, or addition of 1, 2, 3, 4, 5, 6, 7, or 8 amino acids) as compared thereto.

4. The nanobody or antigen-binding fragment according to claim 1 or 2, wherein the nanobody or antigen-binding fragment is humanized;

preferably, the nanobody or antigen-binding fragment further comprises a heavy chain framework region of a human immunoglobulin (e.g., a heavy chain framework region contained in the amino acid sequence encoded by a human heavy chain germline antibody gene), the heavy chain framework region optionally comprises one or more (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10) back mutations from human residues to camelid residues.

5. The nanobody or antigen-binding fragment according to claim 4, wherein the nanobody or antigen-binding fragment comprises the sequence as set forth in SEQ ID NO: 9 or 10, or a sequence having a sequence identity of at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% as compared thereto, or a sequence having a substitution, deletion, or addition of one or more amino acids (e.g., a substitution, deletion, or addition of 1, 2, 3, 4, or 5 amino acids) as compared thereto.

6. A polypeptide construct capable of specifically binding to HER3, which comprises the nanobody or antigen-binding fragment according to any one of claims 1 to 5, and an immunoglobulin Fc domain;

preferably, the immunoglobulin Fc domain is optionally linked via a peptide linker to the N-terminus and/or C-terminus (e.g., C-terminus) of the nanobody or antigen-binding fragment;

preferably, the immunoglobulin Fc domain is a Fc domain of IgG, such as the constant region of a heavy chain of IgG1, IgG2, IgG3, or IgG4;

preferably, the immunoglobulin Fc domain comprises the sequence as set forth in SEQ ID NO: 11, or a sequence having a substitution, deletion, or addition of one or more amino acids, or any combination thereof (e.g., a sequence having a substitution, deletion, or addition of 1, 2, 3, 4, or 5 amino acids, or any combination thereof);

preferably, the polypeptide construct has a sequence as set forth in any one of SEQ ID NOs: 12-14.

7. An isolated nucleic acid molecule, which encodes the nanobody or antigen-binding fragment according to any one of claims 1 to 5 or the polypeptide construct according to claim 6.

8. A vector, which comprises the nucleic acid molecule according to claim 7; preferably, the vector is a cloning vector or an expression vector.

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

10. A method for preparing the nanobody or antigen-binding fragment thereof according to any one of claims 1 to 5 or the polypeptide construct according to claim 6, which comprises culturing the host cell according to claim 9 under a condition that allows protein expression, and recovering the nanobody or antigen-binding fragment thereof or the polypeptide construct from a culture of the cultured host cell.

11. A bispecific or multispecific antibody, which comprises the nanobody or antigen-binding fragment thereof according to any one of claims 1 to 5 or the polypeptide construct according to claim 6;

preferably, the bispecific or multispecific antibody is capable of specifically binding to HER3 and additionally specifically binding to one or more other targets;

preferably, the bispecific or multispecific antibody further comprises at least one second antibody having a second binding specificity against a second target.

12. A conjugate, which comprises the nanobody or antigen-binding fragment thereof according to any one of claims 1 to 5, the polypeptide construct according to claim 6, or the bispecific or multispecific antibody according to claim 11, and a conjugated portion connected thereto;

preferably, the conjugated portion is selected from therapeutic agents (e.g., cytotoxic agent, cytokine, toxin, or radionuclide);

preferably, the conjugate is an antibody-drug conjugate (ADC).

13. A pharmaceutical composition, which comprises the nanobody or antigen-binding fragment thereof according to any one of claims 1 to 5, the polypeptide construct according to claim 6, the isolated nucleic acid molecule according to claim 7, the vector according to claim 8, the host cell according to claim 9, the bispecific or multispecific antibody according to claim 11, or the conjugate according to claim 12, and a pharmaceutically acceptable carrier and/or excipient;

preferably, the pharmaceutical composition further comprises an additional pharmaceutically active agent, such as an antitumor agent.

14. A kit, which comprises the nanobody or antigen-binding fragment thereof according to any one of claims 1 to 5 or the polypeptide construct according to claim 6;

preferably, the kit comprises a conjugate, wherein the conjugate comprises the nanobody or antigen-binding fragment thereof according to any one of claims 1 to 5, or the polypeptide construct according to claim 6, and a detectable label that is linked to the nanobody or antigen-binding fragment thereof, or to the polypeptide construct; such as an enzyme (e.g., horseradish peroxidase or alkaline phosphatase), a chemiluminescent reagent (e.g., acridinium ester compound, luminol and derivative thereof, or ruthenium derivative), a fluorescent dye (e.g., fluorescein or fluorescent protein), a radionuclide, or biotin;

preferably, the kit comprises the nanobody or the antigen-binding fragment thereof according to any one of claims 1 to 5, or the polypeptide construct according to claim 6, and a second antibody capable of specifically recognizing the nanobody or the antigen-binding fragment thereof or the polypeptide construct; optionally, the second antibody further comprises a detectable label, such as an enzyme (e.g., horseradish peroxidase or alkaline phosphatase), a chemiluminescent reagent (e.g., acridinium ester compound, luminol and derivative thereof, or ruthenium derivative), a fluorescent dye (e.g., fluorescein or fluorescent protein), a radionuclide, or biotin.

15. A chimeric antigen receptor, which comprises the nanobody or antigen-binding fragment of any one according to claims 1 to 5 or the polypeptide construct according to claim 6;

preferably, the antigen-binding domain is expressed by an immune effector cell (e.g., a T cell).

16. An isolated nucleic acid molecule, which encodes the chimeric antigen receptor according to claim 15.

17. A vector, which comprises the isolated nucleic acid molecule according to claim 16; preferably, the vector is used for preparing a chimeric antigen receptor T cell.

18. A host cell, which comprises the isolated nucleic acid molecule according to claim 16 or the vector according to claim 17;

preferably, the host cell is an immune effector cell (e.g., a T cell or an NK cell);

preferably, the host cell is a chimeric antigen receptor T cell (CAR-T).

19. Use of the nanobody or antigen-binding fragment thereof according to any one of claims 1 to 5, the polypeptide construct according to claim 6, the isolated nucleic acid molecule according to claim 7, the vector according to claim 8, the host cell according to claim 9, the bispecific or multispecific antibody according to claim 11, the conjugate according to claim 12, or the pharmaceutical composition according to claim 13, or the kit according to claim 14, or the chimeric antigen receptor according to claim 15, in the manufacture of a medicament for prevention and/or treatment of a tumor in a subject;

preferably, the tumor expresses HER3;

preferably, the medicament is used to inhibit the growth of a tumor cell expressing HER3 and/or to kill the tumor cell;

preferably, the medicament further comprises an additional pharmaceutically active agent;

preferably, the additional pharmaceutically active agent is a drug with antitumor activity, such as an alkylating agent, a mitotic inhibitor, an antitumor antibiotic, an antimetabolite, a topoisomerase inhibitor, a tyrosine kinase inhibitor, a radionuclide, a radiosensitizer, an antiangiogenic agent, a cytokine, a molecularly targeted drug, an immune checkpoint inhibitor, or an oncolytic virus;

preferably, the tumor is selected from solid tumors, such as gastric cancer, lung cancer, liver cancer, head and neck cancer, skin cancer, colorectal cancer, ovarian cancer, esophageal cancer, pancreatic cancer, cervical cancer, mesothelioma, breast cancer, colorectal cancer, or oral squamous cell carcinoma;

preferably, the subject is a mammal, such as a human.

20. Use of the nanobody or antigen-binding fragment thereof according to any one of claims 1 to 5 or the polypeptide construct according to claim 6 in the manufacture of a kit for detecting whether a tumor can be treated by an HER3-targeted antitumor therapy, or for detecting the presence or amount of HER3 in a sample;

preferably, the sample is a cell sample (e.g., a sample containing tumor cells) or a body fluid sample (e.g., blood) from a subject (e.g., a mammal, such as a human).

21. A method for detecting the presence or amount of HER3 in a sample, which comprises using the nanobody or antigen-binding fragment thereof according to any one of claims 1 to 5 or the polypeptide construct according to claim 6;

preferably, the method is an immunological assay, such as Western blotting, enzyme immunoassay (e.g., ELISA), chemiluminescent immunoassay, fluorescence immunoassay, or radioimmunoassay.