

NOVEL EGFR-BINDING MOLECULES AND IMMUNOCONJUGATES THEREOF

Field of the Invention

[0001] The present invention generally relates to antibodies, antigen-binding fragments thereof, polypeptides, and immunoconjugates that bind to EGFR. The present invention also relates to methods of using such EGFR-binding molecules for diagnosing and treating diseases, such as malignancies.

Background of the Invention

[0002] The epidermal growth factor receptor (EGFR or ErbB1 or HER1) is a transmembrane glycoprotein of 170 kDa that is encoded by the c-erbB1 proto-oncogene located in the 7q22 chromosome. EGFR is a member of the human epidermal growth factor receptor (HER) family of receptor tyrosine kinases (RTK) which includes HER2 (ErbB2), HER3 (ErbB3) and HER4 (ErbB4). These RTKs share a homologous structure that consists of a ligand-binding extracellular domain (ECD), a single span transmembrane domain and an intracellular domain that contain catalytic-kinase domain and a C-terminal tail. HER kinase signaling pathways are initiated by the binding of extracellular ligand that induces receptor homodimerization or heterodimerization with other HER kinase member and transphosphorylation of the intracellular regions. These events generate the initial signal leading to activation of numerous downstream signaling pathways that are critical for cell proliferation and survival.

[0003] EGFR is over-expressed in many malignant tumor types of epithelial cell origin such as head and neck, colorectal, lung, ovarian, renal, pancreatic, skin and other solid tumors. EGFR-mediated signaling pathways play a significant role in the progression of tumor growth and metastases, making EGFR a good target for tumor therapy (Baselga, *Oncologist*, 7:2-8 (2002), Yarden and Sliwkowski, *Nat Rev Mol Cell Biol*, 2:127-137 (2001)). At present, four EGFR targeting agents including two small molecules tyrosine kinase inhibitors (TKIs) (erlotinib (Tarceva from Genentech and OSI Pharmaceuticals) and gefitinib (Iressa from AstraZeneca and Teva Pharmaceuticals)) and two naked monoclonal antibodies cetuximab (Erbix from ImClone and BMS) and panitumumab (Vectibix from Amgen)) have been approved for treatment of colorectal cancer, pancreatic cancer, head and neck cancer, and non-small cell lung cancer (NSCLC). These anti-EGFR agents strongly inhibit EGFR activation and downstream signaling. The TKIs compete with ATP for binding to the EGFR's intracellular kinase domain (Baselga and

Arteaga, J Clin Oncol, 23:2445-2459 (20005)), whereas the two monoclonal antibodies compete with the EGFR ligands for binding to the receptor (Gill et al., J Biol Chem, 259:7755-7760 (1984), Goldstein et al., Clin Cancer Res, 1:1311-1318 (1995), Prewett et al., Clin Cancer Res, 4:2957-2966 (1998)).

[0004] Anti-EGFR therapies are not perfect. Inhibition of EGFR signaling is only effective in certain tumor type. For example, the efficacy of anti-EGFR antibodies is significantly reduced in colorectal cancer patients with KRAS, BRAF, PIK3CA and PTEN mutations (De Roock et al., Lancet Oncol, 11:753-762 (2010), Bardelli and Sienna, J Clin Oncol, 28: 1254-1261 (2010)). Additionally, the activity of small molecule EGFR inhibitors is limited to NSCLC patients with activating EGFR mutations (Linardou et al., Nat Rev Clin Oncol, 6: 352-366 (2009), Paz-Ares et al., J Cell Mol Med, 14: 51-69 (2009), Mok et al., Discov Med, 8: 227-231 (2009)). EGFR therapies also result in skin toxicity. EGFR expression in normal basal epithelial cells of the skin plays a crucial role in normal development and physiology of epidermis, and inhibition of EGFR signaling causes various skin toxicities including acneiform skin rash, skin dryness, pruritus, paronychia, hair abnormality, mucositis and increased growth of the eyelashes or facial hair (reviewed in Li and Perez-Soler, Targ Oncol 4:107-119 (2009)). Although rarely life-threatening, the skin toxicities cause significant physical and psycho-social discomfort that decrease the patient's life quality. Additionally, in around 10% of patients, the skin toxicity is so severe that it requires treatment interruption or discontinuation that impairs the clinical outcomes of EGFR inhibitors.

[0005] Accordingly, the need exists for improved anti-EGFR therapy which is harmless to normal tissues but still very effective in treating EGFR overexpressing malignant tumors, in particular, tumors that are resistant to the current EGFR therapies. To address this particular need, the present invention focuses on a unique novel class of EGFR antibodies that are very potent in killing EGFR expressing tumor cells but have no or little impact on normal epithelial cell growth. Furthermore, while the conjugation of the EGFR antibodies of the invention with cytotoxic agents potentiates the anti-tumor activity of these antibodies, it does not cause additional toxicity to normal epithelial cells.

BRIEF SUMMARY OF THE INVENTION

[0006] The present invention generally relates to antibodies, antigen-binding fragments thereof, polypeptides, and immunoconjugates that bind to EGFR. The present invention also relates to

methods of using such EGFR-binding molecules for diagnosing and treating diseases, such as malignancies.

[0007] Thus, in one embodiment the invention provides an antibody or antigen binding fragment thereof that specifically binds to human EGFR, wherein said antibody has at least one characteristic selected from the group consisting of: (a) inhibits at least 80% of epidermal growth factor (EGF) and transforming growth factor alpha (TGF α) binding to A431 cells at a concentration of 10 nM or higher, (b) causes at least 50% inhibition of H292 and HCC827 tumor cell proliferation at 30 nM or higher, and (c) does not inhibit more than 20% proliferation of keratinocytes and MCF-10A epithelial cells at 60 nM or lower. In another embodiment, the antibody has at least two characteristics selected from the group consisting of: (a) inhibits at least 80% of EGF and TGF α binding to A431 cells at a concentration of 10 nM or higher, (b) causes at least 50% inhibition of H292 and HCC827 tumor cell proliferation at 30 nM or higher, and (c) does not inhibit more than 20% proliferation of keratinocytes and MCF-10A epithelial cells at 60 nM or lower. In another embodiment, the antibody or antigen binding fragment thereof that specifically binds to human EGFR, wherein said antibody (a) inhibits at least 80% of EGF and TGF α binding to A431 cells at a concentration of 10 nM or higher, (b) causes at least 50% inhibition of H292 and HCC827 tumor cell proliferation at 30 nM or higher, and (c) does not inhibit more than 20% proliferation of keratinocytes and MCF-10A epithelial cells at 60 nM or lower.

[0008] In one embodiment, the invention provides an antibody which comprises (a) a VH sequence at least 90% identical to a reference VH sequence selected from the group consisting of SEQ ID NOs:19-23 and 69-73; and (b) a VL sequence at least 90% identical to a reference VL sequence selected from the group consisting of SEQ ID NOs:24-30 and 70. In another embodiment, the VH and VL sequences are at least 95% identical to the reference VH and VL sequences. In another embodiment, the VH and VL sequences are at least 99% identical to the reference VH and VL sequences.

[0009] In one embodiment, the invention provides an antibody or antigen binding fragment thereof which comprises (a) a VH sequence selected from the group consisting of SEQ ID NOs:19-23 and 69-73; and (b) a VL sequence selected from the group consisting of SEQ ID NOs:24-30 and 70. In another embodiment, the antibody or antigen binding fragment thereof comprises SEQ ID NO:19 and SEQ ID NO:24. In another embodiment, the antibody or antigen binding fragment thereof comprises SEQ ID NO:20 and SEQ ID NO:25. In another

embodiment, the antibody or antigen binding fragment thereof comprises SEQ ID NO:21 and SEQ ID NO:26. In another embodiment, the antibody or antigen binding fragment thereof comprises SEQ ID NO:21 and SEQ ID NO:27. In another embodiment, the antibody or antigen binding fragment thereof comprises SEQ ID NO:22 and SEQ ID NO:28. In another embodiment, the antibody or antigen binding fragment thereof comprises SEQ ID NO:23 and SEQ ID NO:29. In another embodiment, the antibody or antigen binding fragment thereof comprises SEQ ID NO:23 and SEQ ID NO:30. In another embodiment, the antibody or antigen binding fragment thereof comprises SEQ ID NO:69 and SEQ ID NO:70. In another embodiment, the antibody or antigen binding fragment thereof comprises SEQ ID NO:71 and SEQ ID NO:26. In another embodiment, the antibody or antigen binding fragment thereof comprises SEQ ID NO:71 and SEQ ID NO:27. In another embodiment, the antibody or antigen binding fragment thereof comprises SEQ ID NO:72 and SEQ ID NO:26. In another embodiment, the antibody or antigen binding fragment thereof comprises SEQ ID NO:72 and SEQ ID NO:27. In another embodiment, the antibody or antigen binding fragment thereof comprises SEQ ID NO:73 and SEQ ID NO:26. In another embodiment, the antibody or antigen binding fragment thereof comprises SEQ ID NO:73 and SEQ ID NO:27.

[0010] In one embodiment, the invention provides an antibody or antigen binding fragment thereof produced by hybridoma selected from the group consisting of ATCC Deposit Designation PTA-11331, deposited with the ATCC on October 6, 2010, ATCC Deposit Designation PTA-11332, deposited with the ATCC on October 6, 2010, and ATCC Deposit Designation PTA-11333, deposited with the ATCC on October 6, 2010. In another embodiment, the invention provides an antibody or antigen binding fragment thereof that specifically binds to the same EGFR epitope as an antibody selected from the group consisting of ATCC Deposit Designation PTA-11331, deposited with the ATCC on October 6, 2010, ATCC Deposit Designation PTA-11332, deposited with the ATCC on October 6, 2010, and ATCC Deposit Designation PTA-11333, deposited with the ATCC on October 6, 2010. In yet another embodiment, the invention provides an antibody or antigen binding fragment thereof that competitively inhibits binding of a reference antibody to human EGFR, wherein said reference antibody is selected from the group consisting of ATCC Deposit Designation PTA-11331, deposited with the ATCC on October 6, 2010, ATCC Deposit Designation PTA-11332, deposited with the ATCC on October 6, 2010, and ATCC Deposit Designation PTA-11333, deposited with the ATCC on October 6, 2010.

[0011] In one embodiment, the invention provides an antibody or antigen binding fragment thereof that specifically binds to the same EGFR epitope as an antibody selected from the group consisting of: (a) an antibody comprising the VH polypeptide of SEQ ID NO:19 and the VL polypeptide of SEQ ID NO:24; (b) an antibody comprising the VH polypeptide of SEQ ID NO:20 and the VL polypeptide of SEQ ID NO:25; (c) an antibody comprising the VH polypeptide of SEQ ID NO:21 and the VL polypeptide of SEQ ID NO:26; (d) an antibody comprising the VH polypeptide of SEQ ID NO:21 and the VL polypeptide of SEQ ID NO:27; (e) an antibody comprising the VH polypeptide of SEQ ID NO:22 and the VL polypeptide of SEQ ID NO:28; (f) an antibody comprising the VH polypeptide of SEQ ID NO:23 and the VL polypeptide of SEQ ID NO:29; (g) an antibody comprising the VH polypeptide of SEQ ID NO:23 and the VL polypeptide of SEQ ID NO:30; (h) an antibody comprising the VH polypeptide of SEQ ID NO:69 and the VL polypeptide of SEQ ID NO:70; (i) an antibody comprising the VH polypeptide of SEQ ID NO:71 and the VL polypeptide of SEQ ID NO: 26; (j) an antibody comprising the VH polypeptide of SEQ ID NO:71 and the VL polypeptide of SEQ ID NO:27; (k) an antibody comprising the VH polypeptide of SEQ ID NO:72 and the VL polypeptide of SEQ ID NO:26; (l) an antibody comprising the VH polypeptide of SEQ ID NO:72 and the VL polypeptide of SEQ ID NO:27; (m) an antibody comprising the VH polypeptide of SEQ ID NO:73 and the VL polypeptide of SEQ ID NO:26; and (n) an antibody comprising the VH polypeptide of SEQ ID NO:73 and the VL polypeptide of SEQ ID NO:27.

[0012] In one embodiment, the invention provides an antibody or antigen binding fragment thereof that competitively inhibits binding of a reference antibody to human EGFR, wherein said reference antibody is selected from the group consisting of: (a) an antibody comprising the VH polypeptide of SEQ ID NO:19 and the VL polypeptide of SEQ ID NO:24; (b) an antibody comprising the VH polypeptide of SEQ ID NO:20 and the VL polypeptide of SEQ ID NO:25; (c) an antibody comprising the VH polypeptide of SEQ ID NO:21 and the VL polypeptide of SEQ ID NO:26; (d) an antibody comprising the VH polypeptide of SEQ ID NO:21 and the VL polypeptide of SEQ ID NO:27; (e) an antibody comprising the VH polypeptide of SEQ ID NO:22 and the VL polypeptide of SEQ ID NO:28; (f) an antibody comprising the VH polypeptide of SEQ ID NO:23 and the VL polypeptide of SEQ ID NO:29; (g) an antibody comprising the VH polypeptide of SEQ ID NO:23 and the VL polypeptide of SEQ ID NO:30; (h) an antibody comprising the VH polypeptide of SEQ ID NO:69 and the VL polypeptide of SEQ ID NO:70; (i) an antibody comprising the VH polypeptide of SEQ ID NO:71 and the VL

polypeptide of SEQ ID NO:26; (j) an antibody comprising the VH polypeptide of SEQ ID NO:71 and the VL polypeptide of SEQ ID NO:27; (k) an antibody comprising the VH polypeptide of SEQ ID NO:72 and the VL polypeptide of SEQ ID NO:26; (l) an antibody comprising the VH polypeptide of SEQ ID NO:72 and the VL polypeptide of SEQ ID NO:27; (m) an antibody comprising the VH polypeptide of SEQ ID NO:73 and the VL polypeptide of SEQ ID NO:26; and (n) an antibody comprising the VH polypeptide of SEQ ID NO:73 and the VL polypeptide of SEQ ID NO:27.

[0013] In one embodiment, the invention provides an antibody or antigen binding fragment thereof that specifically binds to human EGFR, wherein the antibody or antigen binding fragment thereof comprises: (a) an immunoglobulin heavy chain variable region comprising CDR1, CDR2, and CDR3, which, with the exception of 1, 2, or 3 conservative amino acid substitutions, are respectively identical to the reference heavy chain CDR1 sequence of SEQ ID NO:1, the reference heavy chain CDR2 sequence of SEQ ID NO: 2, 4, 6, 63, or 64, and the reference heavy chain CDR3 sequence of SEQ ID NO: 3 or 5; and (b) an immunoglobulin a light chain variable region comprising CDR1, CDR2, and CDR3, which, with the exception of 1, 2, or 3 conservative amino acid substitutions, are respectively identical to the reference light chain CDR1 sequence of SEQ ID NO: 10, 13, or 14, the reference light chain sequence CDR2 of SEQ ID NO:11, and the reference light chain CDR3 sequence of SEQ ID NO: 12. In another embodiment, the antibody comprises: (a) an immunoglobulin heavy chain variable region comprising CDR1, CDR2, and CDR3, which are respectively identical to the reference heavy chain CDR1 sequence of SEQ ID NO:1, the reference heavy chain CDR2 sequence of SEQ ID NO: 2, 4, 6, 63, or 64, and the reference heavy chain CDR3 sequence of SEQ ID NO: 3 or 5; and (b) an immunoglobulin a light chain variable region comprising CDR1, CDR2, and CDR3, which are respectively identical to the reference light chain CDR1 sequence of SEQ ID NO: 10, 13, or 14, the reference light chain sequence CDR2 of SEQ ID NO:11, and the reference light chain CDR3 sequence of SEQ ID NO: 12.

[0014] In one embodiment, the invention provides an antibody or antigen binding fragment thereof that specifically binds to human EGFR, wherein the antibody or antigen binding fragment thereof comprises: (a) an immunoglobulin heavy chain variable region comprising CDR1, CDR2, and CDR3, which, with the exception of 1, 2, or 3 conservative amino acid substitutions, are respectively identical to the reference heavy chain CDR1 sequence of SEQ ID NO:1, the reference heavy chain CDR2 sequence of SEQ ID NO: 7, 8, or 9, and the reference

heavy chain CDR3 sequence of SEQ ID NO: 3; and (b) an immunoglobulin a light chain variable region comprising CDR1, CDR2, and CDR3, which, with the exception of 1, 2, or 3 conservative amino acid substitutions, are respectively identical to the reference light chain CDR1 sequence of SEQ ID NO: 15 or 16, the reference light chain CDR2 sequence of SEQ ID NO:17, and the reference light chain CDR3 sequence of SEQ ID NO: 18. In another embodiment, the antibody comprises: (a) an immunoglobulin heavy chain variable region comprising CDR1, CDR2, and CDR3, which are respectively identical to the reference heavy chain CDR1 sequence of SEQ ID NO:1, the reference heavy chain CDR2 sequence of SEQ ID NO: 7, 8, or 9, and the reference heavy chain CDR3 sequence of SEQ ID NO: 3; and (b) an immunoglobulin a light chain variable region comprising CDR1, CDR2, and CDR3, which are respectively identical to the reference light chain CDR1 sequence of SEQ ID NO: 15 or 16, the reference light chain CDR2 sequence of SEQ ID NO:17, and the reference light chain CDR3 sequence of SEQ ID NO: 18.

[0015] In one embodiment, the invention provides an antibody or antigen binding fragment thereof that specifically binds to human EGFR, wherein the antibody or antigen binding fragment thereof comprises: (a) an immunoglobulin heavy chain variable region comprising CDR1, CDR2, and CDR3, which, with the exception of 1, 2, or 3 conservative amino acid substitutions, are respectively identical to the reference heavy chain CDR1 sequence of SEQ ID NO:1, the reference heavy chain CDR2 sequence of SEQ ID NO: 65, 66, or 67, and the reference heavy chain CDR3 sequence of SEQ ID NO: 3; and (b) an immunoglobulin a light chain variable region comprising CDR1, CDR2, and CDR3, which, with the exception of 1, 2, or 3 conservative amino acid substitutions, are respectively identical to the reference light chain CDR1 sequence of SEQ ID NO: 68 or 13, the reference light chain sequence CDR2 of SEQ ID NO:11, and the reference light chain CDR3 sequence of SEQ ID NO: 12. In another embodiment, the antibody comprises: (a) an immunoglobulin heavy chain variable region comprising CDR1, CDR2, and CDR3, which are respectively identical to the reference heavy chain CDR1 sequence of SEQ ID NO:1, the reference heavy chain CDR2 sequence of SEQ ID NO: 65, 66, or 67, and the reference heavy chain CDR3 sequence of SEQ ID NO: 3; and (b) an immunoglobulin a light chain variable region comprising CDR1, CDR2, and CDR3, which are respectively identical to the reference light chain CDR1 sequence of SEQ ID NO: 68 or 13, the reference light chain sequence CDR2 of SEQ ID NO:11, and the reference light chain CDR3 sequence of SEQ ID NO: 12.

[0016] In one embodiment, the antibody or antigen binding fragment of the invention is murine, non-human, humanized, chimeric, resurfaced, or human. In another embodiment, the antibody or

antigen binding fragment thereof is a full length antibody. In another embodiment, the antibody or antigen binding fragment thereof is an antigen binding fragment. In another embodiment, the antibody or antigen binding fragment thereof comprises a Fab, Fab', F(ab')₂, Fd, single chain Fv or scFv, disulfide linked Fv, V-NAR domain, IgNar, intrabody, IgGΔCH₂, minibody, F(ab')₃, tetrabody, triabody, diabody, single-domain antibody, DVD-Ig, Fcab, mAb₂, (scFv)₂, or scFv-Fc.

[0017] The invention also provides a polypeptide comprising the VH and VL sequences described herein.

[0018] In one embodiment, the invention provides an antibody or polypeptide that binds both human and macaque EGFR with a substantially similar binding affinity. In one embodiment, the antibody or polypeptide binds to human and macaque EGFR with a K_d of about 1.0 to about 10 nM. In another embodiment, the antibody or polypeptide binds to human and macaque EGFR with a K_d of about 1.0 nM or better. In another embodiment, binding affinity is measured by flow cytometry, Biacore, or radioimmunoassay.

[0019] In one embodiment, the invention provides an isolated cell producing an antibody or antigen binding fragment thereof, or polypeptide described herein.

[0020] In one embodiment, the invention provides a method of making an antibody or antigen binding fragment thereof, or polypeptide described herein comprising (a) culturing a cell that expresses the antibody, antigen-binding fragment thereof, or polypeptide, and (b) isolating said antibody, antigen-binding fragment thereof, or polypeptide from said cultured cell. In one embodiment the cell is a eukaryotic cell.

[0021] In one embodiment, the invention provides an immunoconjugate having the formula (A) - (L) - (C), wherein: (A) is an antibody or antigen binding fragment thereof, or polypeptide described herein; (L) is a linker; and (C) is a cytotoxic agent; and wherein said linker (L) links (A) to (C). In another embodiment, the linker is selected from the group consisting of a cleavable linker, a non-cleavable linker, a hydrophilic linker, and a dicarboxylic acid based linker. In another embodiment, the linker is a non-cleavable linker. In another embodiment, the linker is selected from the group consisting: N-succinimidyl 4-(2-pyridyldithio)pentanoate (SPP); N-succinimidyl 4-(2-pyridyldithio)butanoate (SPDB) or N-succinimidyl 4-(2-pyridyldithio)-2-sulfobutanoate (sulfo-SPDB); N-succinimidyl 4-(maleimidomethyl) cyclohexanecarboxylate (SMCC); N-sulfosuccinimidyl 4-(maleimidomethyl) cyclohexanecarboxylate (sulfoSMCC); N-succinimidyl-4-(iodoacetyl)-aminobenzoate (SIAB); and N-succinimidyl-[(N-

maleimidopropionamido)-tetraethyleneglycol] ester (NHS-PEG4-maleimide). In a further embodiment, the linker is N-succinimidyl-[(N-maleimidopropionamido)-tetraethyleneglycol] ester (NHS-PEG4-maleimide).

[0022] In another embodiment, the immunoconjugate comprises a cytotoxic agent selected from the group consisting of a maytansinoid, maytansinoid analog, doxorubicin, a modified doxorubicin, benzodiazepine, taxoid, CC-1065, CC-1065 analog, duocarmycin, duocarmycin analog, calicheamicin, dolastatin, dolastatin analog, aristatin, tomaymycin derivative, and leptomycin derivative or a prodrug of the agent. In another embodiment, the cytotoxic agent is a maytansinoid. In a further embodiment, the cytotoxic agent is N(2')-deacetyl-N(2')-(3-mercapto-1-oxopropyl)-maytansine (DM1) or N(2')-deacetyl-N2-(4-mercapto-4-methyl-1-oxopentyl)-maytansine (DM4).

[0023] In one embodiment, the invention provides a pharmaceutical composition comprising an antibody or antigen binding fragment thereof, or polypeptide described herein, or an immunoconjugate described herein and a pharmaceutically acceptable carrier. In another embodiment, the pharmaceutical composition comprises a second anti-cancer agent.

[0024] In one embodiment, the invention provides a diagnostic reagent comprising an antibody or antigen binding fragment thereof, polypeptide, or immunoconjugate of the invention which is labeled. In one embodiment, the label is selected from the group consisting of a radiolabel, a fluorophore, a chromophore, an imaging agent and a metal ion.

[0025] In one embodiment, the invention provides a kit comprising an antibody or antigen binding fragment thereof, polypeptide, or immunoconjugate described herein.

[0026] In one embodiment, the invention provides a method for inhibiting the growth of a cell expressing EGFR comprising contacting the cell with an immunoconjugate or the pharmaceutical composition described herein. In another embodiment, the cell is a tumor cell.

[0027] In one embodiment, the invention provides a method for treating a patient having a neoplasm comprising administering to said patient a therapeutically effective amount of an immunoconjugate or pharmaceutical composition described herein. In another embodiment, the neoplasm is selected from the group consisting of: abdominal, bone, breast, digestive system, liver, pancreas, peritoneum, adrenal, parathyroid, pituitary, testicles, ovary, thymus, thyroid, eye, head and neck, central nervous system, peripheral nervous system, lymphatic system, pelvic, skin, soft tissue, spleen, thoracic region, and urogenital system. In another embodiment, the

method comprises administering a second anti-cancer agent to the subject. In a further embodiment, the second anti-cancer agent is a chemotherapeutic agent.

[0028] In one embodiment, the invention provides a method for treating a cell proliferative disorder in a patient comprising administering to said patient a therapeutically effective amount of an immunoconjugate or pharmaceutical composition described herein. In another embodiment, the cell proliferative disorder is selected from the group consisting of: adrenal cortex hyperplasia (Cushing's disease), congenital adrenal hyperplasia, endometrial hyperplasia, benign prostatic hyperplasia, breast hyperplasia, intimal hyperplasia, focal epithelial hyperplasia (Heck's disease), sebaceous hyperplasia, compensatory liver hyperplasia,, and any other cell proliferation disease, besides neoplasia.

[0029] In one embodiment, the invention provides an isolated polynucleotide comprising a sequence that encodes a polypeptide at least 90% identical to a sequence selected from the group consisting of SEQ ID NOs: 39-43, 77-80, and 82-84. In another embodiment, the sequence is at least 95% identical a sequence selected from the group consisting of SEQ ID NOs: 39-43, 77-80, and 82-84. In another embodiment, the sequence is at least 99% identical to a sequence selected from the group consisting of SEQ ID NOs: 39-43, 77-80, and 82-84.

[0030] In one embodiment, the invention provides an isolated polynucleotide which comprises a sequence that is at least 90% identical to SEQ ID NOs: 44-50 and 81. In another embodiment, the polynucleotide comprises a sequence that is at least 95% identical to SEQ ID NOs: 44-50 and 81. In another embodiment, the polynucleotide comprises a sequence that is at least 99% identical to SEQ ID NOs: 44-50 and 81. In another embodiment, the invention provides an isolated polynucleotide comprising a sequence selected from the group consisting of SEQ ID NOs:39-58 and 77-84. In another embodiment, the invention provides a vector comprising the polynucleotides described herein. In another embodiment, the invention provides a host cell comprising the vectors described herein.

BRIEF DESCRIPTION OF THE DRAWINGS/FIGURES

[0031] Figure 1 is a table describing the binding affinity of the indicated anti-EGFR antibodies to human EGFR (huEGFR) and monkey EGFR (moEGFR) antigen.

[0032] Figure 2 is a Western blot data depicting the effect of the indicated anti-EGFR antibodies in ligand-induced EGFR phosphorylation in MDA-MB468 cells (A) and in human primary keratinocytes (B).

[0033] Figure 3 is a Western blot data depicting the effect of the indicated anti-EGFR antibodies on EGFR phosphorylation in MDA-MB468 cells in absence of exogenous EGFR ligand.

[0034] Figure 4 is a line graph depicting the binding of biotinylated TGF α (A) and EGF (B) to the A431 cells in presence of the indicated antibodies.

[0035] Figure 5 is a line graph depicting the growth of human primary keratinocytes in presence of the indicated antibodies at various concentrations.

[0036] Figure 6 is a bar graph depicting the growth of MCF10A cells in the presence of 10 μ g/ml of the indicated antibodies.

[0037] Figure 7 is a line graph depicting the growth of HCC827 cells (A) and NCI-H292 cells (B) in the presence of the indicated antibodies.

[0038] Figure 8 is a line graph depicting the binding of biotinylated 528 antibody (A), biotinylated cetuximab (B), and biotinylated EGFR-7 antibody (C) to the MDA-MB468 cells in presence of the indicated competing antibodies at the indicated concentration.

[0039] Figure 9 is the alignment of the murine EGFR-7 variant light and heavy chain variable region sequences.

[0040] Figure 10 is tables depicting specific framework surface residue changes in resurfacing of EGFR-7 V_L (A) and V_H (B).

[0041] Figure 11 is tables depicting specific framework surface residue changes in resurfacing of EGFR-12 V_L (A) and V_H (B).

[0042] Figure 12 is alignment of the resurfaced sequences and murine counterparts of EGFR-7 V_L (A), EGFR-7 V_H (B), EGFR-12 V_L (C) and EGFR-12 V_H (D).

[0043] Figure 13 is tables depicting specific framework surface residue changes in CDR grafting of EGFR-7 V_L (A) and V_H (B).

[0044] Figure 14 is alignment of the CDR grafted sequences and the murine counterparts of EGFR-7 V_L (A) and EGFR-7 V_H (B).

[0045] Figure 15 shows line graphs depicting the binding competition between the murine antibody and its corresponding humanized antibody for EGFR-6 (A) and EGFR-7 (B).

[0046] Figure 16 shows line graphs depicting the ability of the murine antibody and its corresponding humanized antibody in inhibiting tumor cell growth.

[0047] Figure 17 shows a line graph depicting NK cell mediated ADCC activity of humanized EGFR-6 and EGFR-7R on A431 cells.

[0048] Figure 18 depicts the binding curves of the indicated antibody and the corresponding antibody-maytansinoid conjugate for EGFR-6 (A) and EGFR-7 (B).

[0049] Figure 19 shows line graphs depicting the cytotoxic activity of the indicated antibody and the corresponding antibody-maytansinoid conjugate in FaDu (A) and H292 (B) cell lines.

[0050] Figure 20 shows line graphs depicting the cytotoxic activity of the indicated antibody and the corresponding antibody-maytansinoid conjugate in H226 (A) and SCC-4 (B) cell lines.

[0051] Figure 21 shows a line graph depicting the growth of H292 tumor xenograft in mice treated with a single dose of the indicated antibodies and antibody-maytansinoid conjugates.

[0052] Figure 22 shows a line graph depicting the growth of FaDu tumor xenograft in mice treated with a single dose of the indicated antibodies and antibody-maytansinoid conjugates.

[0053] Figure 23 shows line graphs depicting the capacity of the indicated antibodies and conjugates in inhibiting the growth of human primary keratinocytes (A) and H292 tumor cells (B).

[0054] Figure 24 shows bar graphs depicting the amount of CXCL8, CXCL10, CCL5 produced by the human primary keratinocytes in presence of the indicated antibodies and antibody-maytansinoid-conjugates.

[0055] Figure 25 shows a line graph depicting the growth of H292 tumor xenograft in mice treated with a single 3 mg/kg dose of either the huEGFR-7R antibody or the huEGFR-7R-SMCC-DM1, or huEGFR-7R-PEG-MAL-DM1 antibody-maytansinoid conjugates.

[0056] Figure 26 shows a line graph depicting the growth of HSC2 tumor xenograft in mice treated with a single 5 mg/kg dose of either the huEGFR-7R antibody or the huEGFR-7R-SMCC-DM1, or huEGFR-7R-PEG-MAL-DM1 antibody-maytansinoid conjugates.

[0057] Figure 27 shows a line graph depicting the growth of FaDu tumor xenograft in mice treated with a single 5 mg/kg dose of either the huEGFR-7R antibody or the huEGFR-7R-SMCC-DM1, or huEGFR-7R-PEG-MAL-DM1 antibody-maytansinoid conjugates.

[0058] Figure 28 shows alignment of human and murine EGFR extracellular domain (ECD) sequences.

[0059] Figure 29 shows alignment of sequences of huEGFRdIII, muEGFRdIII and chEGFRdIII.

[0060] Figure 30 shows a line graph depicting the binding of huEGFR-7R antibody to the huEGFR, huEGFRdIII, muEGFRdIII and chEGFRdIII.

[0061] Figure 31 shows a line graph depicting the binding of huEGFR-6 antibody to the huEGFR, huEGFRdIII, muEGFRdIII and chEGFRdIII.

[0062] Figure 32 shows a line graph depicting the binding of muEGFR-7 antibody to the huEGFR, huEGFRdIII, muEGFRdIII and chEGFRdIII.

[0063] Figure 33 shows a line graph depicting the binding of muEGFR-6 antibody to the huEGFR, huEGFRdIII, muEGFRdIII and chEGFRdIII.

[0064] Figure 34 shows a line graph depicting the binding of muEGFR-12 antibody to the huEGFR, huEGFRdIII, muEGFRdIII and chEGFRdIII.

[0065] Figure 35 shows a line graph depicting the binding of muEGFR-13 antibody to the huEGFR, huEGFRdIII, muEGFRdIII and chEGFRdIII.

[0066] Figure 36 shows a line graph depicting the binding of cetuximab to the huEGFR, huEGFRdIII, muEGFRdIII and chEGFRdIII.

DETAILED DESCRIPTION OF THE INVENTION

[0067] The present invention provides a new class of EGFR binding molecules which partially inhibit EGFR signaling, have no effect on EGFR-positive normal epithelial cells, including primary keratinocytes, but are highly cytotoxic to EGFR-overexpressing tumor cells. Further, immunoconjugates of anti-EGFR antibodies potentiate the anti-tumor activity of the antibodies.

I. Definitions

[0068] To facilitate an understanding of the present invention, a number of terms and phrases are defined below.

[0069] As used herein, "epidermal growth factor receptor" or "EGFR" refers to the mature, tyrosine kinase cell surface receptor. The term "soluble EGFR" or "sEGFR" refers to a portion of EGFR containing the extracellular, ligand-binding domain of EGFR. More specifically, sEGFR contains amino acids 1-619 of mature EGFR (Ullrich et al., Human Epidermal Growth Factor cDNA Sequence and Aberrant Expression of the Amplified Gene in A-431 Epidermoid Carcinoma Cells, Nature, Vol. 309, 418-25 (1986)).

[0070] The phrase "EGFR mediated cancer" refers to a cancer characterized by epithelial tumors in which EGFR is abnormally activated to levels greater than in normal, corresponding epithelial tissue. These greater levels of EGFR activity promote tumor growth in many types of cancer. Such cancers include, but are not limited to, non-small cell lung cancer, breast cancer, colorectal cancer, head and neck cancers, and prostate cancer. Abnormal activation of EGFR can arise from overexpression of the receptor, gene amplification, activating mutations, overexpression of receptor ligands, and/or loss of regulators of EGFR activity.

[0071] The term "antibody" means an immunoglobulin molecule that recognizes and specifically binds to a target, such as a protein, polypeptide, peptide, carbohydrate, polynucleotide, lipid, or combinations of the foregoing through at least one antigen recognition site within the variable region of the immunoglobulin molecule. As used herein, the term "antibody" encompasses intact polyclonal antibodies, intact monoclonal antibodies, antibody fragments (such as Fab, Fab', F(ab')₂, and Fv fragments), single chain Fv (scFv) mutants, multispecific antibodies such as bispecific antibodies generated from at least two intact antibodies, chimeric antibodies, humanized antibodies, human antibodies, fusion proteins comprising an antigen determination portion of an antibody, and any other modified immunoglobulin molecule comprising an antigen recognition site so long as the antibodies exhibit the desired biological activity. An antibody can be of any the five major classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM, or subclasses (isotypes) thereof (e.g. IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2), based on the identity of their heavy-chain constant domains referred to as alpha, delta, epsilon, gamma, and mu, respectively. The different classes of immunoglobulins have different and well known subunit structures and three-dimensional configurations. Antibodies can be naked or conjugated to other molecules such as toxins, radioisotopes, etc.

[0072] A "blocking" antibody or an "antagonist" antibody is one which inhibits or reduces biological activity of the antigen it binds, such as EGFR. In some embodiments, blocking antibodies or antagonist antibodies substantially or completely inhibit the biological activity of the antigen. The biological activity can be reduced by 10%, 20%, 30%, 50%, 70%, 80%, 90%, 95%, or even 100%.

[0073] The phrase "ability to inhibit EGFR activation" with respect to an antibody as used herein, is intended to refer to an antibody whose binding to EGFR results in inhibition of human EGFR activation and the biological activity of human EGFR that occurs upon activation of the receptor. Measuring one or more indicators of EGFR biological activity as determined using either a cell proliferation assay, an apoptosis assay, a receptor binding assay, a receptor phosphorylation assay, or a mouse tumor model (see Examples) can assess an antibody's ability to inhibit EGFR activation.

[0074] The term "anti-EGFR antibody" or "an antibody that binds to EGFR" refers to an antibody that is capable of binding EGFR with sufficient affinity such that the antibody is useful as a diagnostic and/or therapeutic agent in targeting EGFR. Several anti-EGFR antibodies are

known in the art. For example, cetuximab (Ab 225) and 528 Ab are described in US Pat. No. 4,943,533, which is herein incorporated by reference.

[0075] The extent of binding of an anti-EGFR antibody to an unrelated, non-EGFR protein can be less than about 10% of the binding of the antibody to EGFR as measured, e.g., by a radioimmunoassay (RIA). In certain embodiments, an antibody that binds to EGFR has a dissociation constant (K_d) of $\leq 1 \mu\text{M}$, $\leq 100 \text{ nM}$, $\leq 10 \text{ nM}$, $\leq 1 \text{ nM}$, or $\leq 0.1 \text{ nM}$.

[0076] As used herein, the term "epithelial toxicity" refers to an abnormality or dysfunction of the epithelium, and can be manifested in a patient being treated for cancer by administration of an EGFR inhibitor by one or more symptoms or conditions selected from skin rash, diarrhea, corneal thinning, hair atrophy or loss, hair follicle dysplasia, degeneration, necrosis or inflammation, interfollicular epidermal hyperplasia, or a failure to heal or a delayed healing after injury, among other symptoms. In one embodiment, the epithelial toxicity is manifested as a skin toxicity such as acneform or macro-papular rash.

[0077] The term "antibody fragment" refers to a portion of an intact antibody and refers to the antigenic determining variable regions of an intact antibody. Examples of antibody fragments include, but are not limited to Fab, Fab', F(ab')₂, and Fv fragments, linear antibodies, single chain antibodies, and multispecific antibodies formed from antibody fragments.

[0078] A "monoclonal antibody" refers to a homogeneous antibody population involved in the highly specific recognition and binding of a single antigenic determinant, or epitope. This is in contrast to polyclonal antibodies that typically include different antibodies directed against different antigenic determinants. The term "monoclonal antibody" encompasses both intact and full-length monoclonal antibodies as well as antibody fragments (such as Fab, Fab', F(ab')₂, Fv), single chain (scFv) mutants, fusion proteins comprising an antibody portion, and any other modified immunoglobulin molecule comprising an antigen recognition site. Furthermore, "monoclonal antibody" refers to such antibodies made in any number of manners including but not limited to by hybridoma, phage selection, recombinant expression, and transgenic animals.

[0079] The term "humanized antibody" refers to forms of non-human (e.g. murine) antibodies that are specific immunoglobulin chains, chimeric immunoglobulins, or fragments thereof that contain minimal non-human (e.g., murine) sequences. Typically, humanized antibodies are human immunoglobulins in which residues from the complementary determining region (CDR) are replaced by residues from the CDR of a non-human species (e.g. mouse, rat, rabbit, hamster) that have the desired specificity, affinity, and capability (Jones et al., 1986, *Nature*, 321:522-525; Riechmann et al., 1988, *Nature*, 332:323-327; Verhoeyen et al., 1988, *Science*, 239:1534-1536).

In some instances, the Fv framework region (FR) residues of a human immunoglobulin are replaced with the corresponding residues in an antibody from a non-human species that has the desired specificity, affinity, and capability. The humanized antibody can be further modified by the substitution of additional residues either in the Fv framework region and/or within the replaced non-human residues to refine and optimize antibody specificity, affinity, and/or capability. In general, the humanized antibody will comprise substantially all of at least one, and typically two or three, variable domains containing all or substantially all of the CDR regions that correspond to the non-human immunoglobulin whereas all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody can also comprise at least a portion of an immunoglobulin constant region or domain (Fc), typically that of a human immunoglobulin. Examples of methods used to generate humanized antibodies are described in U.S. Pat. 5,225,539.

[0080] A "variable region" of an antibody refers to the variable region of the antibody light chain or the variable region of the antibody heavy chain, either alone or in combination. The variable regions of the heavy and light chain each consist of four framework regions (FR) connected by three complementarity determining regions (CDRs) also known as hypervariable regions. The CDRs in each chain are held together in close proximity by the FRs and, with the CDRs from the other chain, contribute to the formation of the antigen-binding site of antibodies. There are at least two techniques for determining CDRs: (1) an approach based on cross-species sequence variability (i.e., Kabat et al. *Sequences of Proteins of Immunological Interest*, (5th ed., 1991, National Institutes of Health, Bethesda Md.)); and (2) an approach based on crystallographic studies of antigen-antibody complexes (Al-lazikani et al (1997) *J. Molec. Biol.* 273:927-948)). In addition, combinations of these two approaches are sometimes used in the art to determine CDRs.

[0081] The Kabat numbering system is generally used when referring to a residue in the variable domain (approximately residues 1-107 of the light chain and residues 1-113 of the heavy chain) (e.g, Kabat et al., *Sequences of Immunological Interest*. 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)).

[0082] The amino acid position numbering as in Kabat, refers to the numbering system used for heavy chain variable domains or light chain variable domains of the compilation of antibodies in Kabat et al., *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991). Using this numbering system, the actual

linear amino acid sequence can contain fewer or additional amino acids corresponding to a shortening of, or insertion into, a FR or CDR of the variable domain. For example, a heavy chain variable domain can include a single amino acid insert (residue 52a according to Kabat) after residue 52 of H2 and inserted residues (e.g. residues 82a, 82b, and 82c, etc according to Kabat) after heavy chain FR residue 82. The Kabat numbering of residues can be determined for a given antibody by alignment at regions of homology of the sequence of the antibody with a "standard" Kabat numbered sequence. Chothia refers instead to the location of the structural loops (Chothia and Lesk J. Mol. Biol. 196:901-917 (1987)). The end of the Chothia CDR-H1 loop when numbered using the Kabat numbering convention varies between H32 and H34 depending on the length of the loop (this is because the Kabat numbering scheme places the insertions at H35A and H35B; if neither 35A nor 35B is present, the loop ends at 32; if only 35A is present, the loop ends at 33; if both 35A and 35B are present, the loop ends at 34). The AbM hypervariable regions represent a compromise between the Kabat CDRs and Chothia structural loops, and are used by Oxford Molecular's AbM antibody modeling software.

Loop	Kabat	AbM	Chothia
L1	L24-L34	L24-L34	L24-L34
L2	L50-L56	L50-L56	L50-L56
L3	L89-L97	L89-L97	L89-L97
H1	H31-H35B	H26-H35B (Kabat Numbering)	H26-H32..34
H1	H31-H35	H26-H35 (Chothia Numbering)	H26-H32
H2	H50-H65	H50-H58	H52-H56
H3	H95-H102	H95-H102	H95-H102

[0083] The term "human antibody" means an antibody produced by a human or an antibody having an amino acid sequence corresponding to an antibody produced by a human made using any technique known in the art. This definition of a human antibody includes intact or full-length antibodies, fragments thereof, and/or antibodies comprising at least one human heavy and/or light chain polypeptide such as, for example, an antibody comprising murine light chain and human heavy chain polypeptides.

[0084] The term "chimeric antibodies" refers to antibodies wherein the amino acid sequence of the immunoglobulin molecule is derived from two or more species. Typically, the variable region of both light and heavy chains corresponds to the variable region of antibodies derived

from one species of mammals (e.g. mouse, rat, rabbit, etc) with the desired specificity, affinity, and capability while the constant regions are homologous to the sequences in antibodies derived from another (usually human) to avoid eliciting an immune response in that species.

[0085] The term "epitope" or "antigenic determinant" are used interchangeably herein and refer to that portion of an antigen capable of being recognized and specifically bound by a particular antibody. When the antigen is a polypeptide, epitopes can be formed both from contiguous amino acids and noncontiguous amino acids juxtaposed by tertiary folding of a protein. Epitopes formed from contiguous amino acids are typically retained upon protein denaturing, whereas epitopes formed by tertiary folding are typically lost upon protein denaturing. An epitope typically includes at least 3, and more usually, at least 5 or 8-10 amino acids in a unique spatial conformation.

[0086] "Binding affinity" generally refers to the strength of the sum total of noncovalent interactions between a single binding site of a molecule (e.g., an antibody) and its binding partner (e.g., an antigen). Unless indicated otherwise, as used herein, "binding affinity" refers to intrinsic binding affinity which reflects a 1:1 interaction between members of a binding pair (e.g., antibody and antigen). The affinity of a molecule X for its partner Y can generally be represented by the dissociation constant (K_d). Affinity can be measured by common methods known in the art, including those described herein. Low-affinity antibodies generally bind antigen slowly and tend to dissociate readily, whereas high-affinity antibodies generally bind antigen faster and tend to remain bound longer. A variety of methods of measuring binding affinity are known in the art, any of which can be used for purposes of the present invention. Specific illustrative embodiments are described in the following.

[0087] "Or better" when used herein to refer to binding affinity refers to a stronger binding between a molecule and its binding partner. "Or better" when used herein refers to a stronger binding, represented by a smaller numerical K_d value. For example, an antibody which has an affinity for an antigen of "0.6 nM or better", the antibody's affinity for the antigen is <0.6 nM, i.e. 0.59 nM, 0.58 nM, 0.57 nM etc. or any value less than 0.6 nM.

[0088] By "specifically binds," it is generally meant that an antibody binds to an epitope via its antigen binding domain, and that the binding entails some complementarity between the antigen binding domain and the epitope. According to this definition, an antibody is said to "specifically bind" to an epitope when it binds to that epitope, via its antigen binding domain more readily than it would bind to a random, unrelated epitope. The term "specificity" is used herein to

qualify the relative affinity by which a certain antibody binds to a certain epitope. For example, antibody "A" may be deemed to have a higher specificity for a given epitope than antibody "B," or antibody "A" may be said to bind to epitope "C" with a higher specificity than it has for related epitope "D."

[0089] By "preferentially binds," it is meant that the antibody specifically binds to an epitope more readily than it would bind to a related, similar, homologous, or analogous epitope. Thus, an antibody which "preferentially binds" to a given epitope would more likely bind to that epitope than to a related epitope, even though such an antibody may cross-react with the related epitope.

[0090] An antibody is said to "competitively inhibit" binding of a reference antibody to a given epitope if it preferentially binds to that epitope to the extent that it blocks, to some degree, binding of the reference antibody to the epitope. Competitive inhibition may be determined by any method known in the art, for example, competition ELISA assays. An antibody may be said to competitively inhibit binding of the reference antibody to a given epitope by at least 90%, at least 80%, at least 70%, at least 60%, or at least 50%.

[0091] The phrase "substantially similar," or "substantially the same", as used herein, denotes a sufficiently high degree of similarity between two numeric values (generally one associated with an antibody of the invention and the other associated with a reference/comparator antibody) such that one of skill in the art would consider the difference between the two values to be of little or no biological and/or statistical significance within the context of the biological characteristic measured by said values (e.g., K_d values). The difference between said two values can be less than about 50%, less than about 40%, less than about 30%, less than about 20%, or less than about 10% as a function of the value for the reference/comparator antibody. The difference between two "substantially similar binding affinities" is generally less than about 10% as a function of the value for the reference/comparator antibody.

[0092] "Ligand-independent binding" as used herein denotes the ability of the EGFR binding agents to bind an epitope on human EGFR in the absence of ligand interaction with EGFR.

[0093] A polypeptide, antibody, polynucleotide, vector, cell, or composition which is "isolated" is a polypeptide, antibody, polynucleotide, vector, cell, or composition which is in a form not found in nature. Isolated polypeptides, antibodies, polynucleotides, vectors, cell or compositions include those which have been purified to a degree that they are no longer in a form in which they are found in nature. In some embodiments, an antibody, polynucleotide, vector, cell, or composition which is isolated is substantially pure.

[0094] As used herein, "substantially pure" refers to material which is at least 50% pure (i.e., free from contaminants), at least 90% pure, at least 95% pure, at least 98% pure, or at least 99% pure.

[0095] The term "immunoconjugate" or "conjugate" as used herein refers to a compound or a derivative thereof that is linked to a cell binding agent (i.e., an anti-EGFR antibody or fragment thereof) and is defined by a generic formula: C-L-A, wherein C = cytotoxin, L = linker, and A = cell binding agent or anti-EGFR antibody or antibody fragment. Immunoconjugates can also be defined by the generic formula in reverse order: A-L-C.

[0096] A "linker" is any chemical moiety that is capable of linking a compound, usually a drug, such as a maytansinoid, to a cell-binding agent such as an anti-EGFR antibody or a fragment thereof in a stable, covalent manner. Linkers can be susceptible to or be substantially resistant to acid-induced cleavage, light-induced cleavage, peptidase-induced cleavage, esterase-induced cleavage, and disulfide bond cleavage, at conditions under which the compound or the antibody remains active. Suitable linkers are well known in the art and include, for example, disulfide groups, thioether groups, acid labile groups, photolabile groups, peptidase labile groups and esterase labile groups. Linkers also include charged linkers, and hydrophilic forms thereof as described herein and known in the art.

[0097] The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals in which a population of cells are characterized by unregulated cell growth. Examples of cancer include, but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia. "Tumor" and "neoplasm" refer to one or more cells that result from excessive cell growth or proliferation, either benign (noncancerous) or malignant (cancerous) including pre-cancerous lesions. Examples of "cancer" or "tumorigenic" diseases which can be treated and/or prevented include neoplasms of the abdomen, bone, breast, digestive system, liver, pancreas, peritoneum, endocrine glands (adrenal, parathyroid, pituitary, testicles, ovary, thymus, thyroid), eye, head and neck, nervous system (central and peripheral), lymphatic system, pelvic, skin, soft tissue, spleen, thoracic region, and urogenital system.

[0098] The terms "cancer cell," "tumor cell," and grammatical equivalents refer to the total population of cells derived from a tumor or a pre-cancerous lesion, including both non-tumorigenic cells, which comprise the bulk of the tumor cell population, and tumorigenic stem cells (cancer stem cells). As used herein, the term "tumor cell" will be modified by the term "non-tumorigenic" when referring solely to those tumor cells lacking the capacity to renew and differentiate to distinguish those tumor cells from cancer stem cells.

[0099] The term "subject" refers to any animal (e.g., a mammal), including, but not limited to humans, non-human primates, rodents, and the like, which is to be the recipient of a particular treatment. Typically, the terms "subject" and "patient" are used interchangeably herein in reference to a human subject.

[00100] Administration "in combination with" one or more further therapeutic agents includes simultaneous (concurrent) and consecutive administration in any order.

[00101] The term "pharmaceutical formulation" refers to a preparation which is in such form as to permit the biological activity of the active ingredient to be effective, and which contains no additional components which are unacceptably toxic to a subject to which the formulation would be administered. The formulation can be sterile.

[00102] An "effective amount" of an antibody as disclosed herein is an amount sufficient to carry out a specifically stated purpose. An "effective amount" can be determined empirically and in a routine manner, in relation to the stated purpose.

[00103] The term "therapeutically effective amount" refers to an amount of an antibody or other drug effective to "treat" a disease or disorder in a subject or mammal. In the case of cancer, the therapeutically effective amount of the drug can reduce the number of cancer cells; reduce the tumor size; inhibit (i.e., slow to some extent or stop) cancer cell infiltration into peripheral organs; inhibit (i.e., slow to some extent or stop) tumor metastasis; inhibit, to some extent, tumor growth; and/or relieve to some extent one or more of the symptoms associated with the cancer. See the definition herein of "treating". To the extent the drug can prevent growth and/or kill existing cancer cells, it can be cytostatic and/or cytotoxic. A "prophylactically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired prophylactic result. Typically but not necessarily, since a prophylactic dose is used in subjects prior to or at an earlier stage of disease, the prophylactically effective amount will be less than the therapeutically effective amount.

[00104] The word "label" when used herein refers to a detectable compound or composition which is conjugated directly or indirectly to the antibody so as to generate a "labeled" antibody. The label can be detectable by itself (e.g. radioisotope labels or fluorescent labels) or, in the case of an enzymatic label, can catalyze chemical alteration of a substrate compound or composition which is detectable.

[00105] A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer, regardless of mechanism of action. Chemotherapeutic agents include, for example,

antagonists of CD20 such as Rituximab and cyclophosphamide, doxorubicin, vincristine, prednisone, fludarabine, etoposide, methotrexate, lenalidomide, chlorambucil, bentamustine and/or modified versions of such chemotherapeutics.

[00106] Terms such as "treating" or "treatment" or "to treat" or "alleviating" or "to alleviate" refer to both 1) therapeutic measures that cure, slow down, lessen symptoms of, and/or halt progression of a diagnosed pathologic condition or disorder and 2) prophylactic or preventative measures that prevent and/or slow the development of a targeted pathologic condition or disorder. Thus, those in need of treatment include those already with the disorder; those prone to have the disorder; and those in whom the disorder is to be prevented. In certain embodiments, a subject is successfully "treated" for cancer according to the methods of the present invention if the patient shows one or more of the following: a reduction in the number of or complete absence of cancer cells; a reduction in the tumor size; inhibition of or an absence of cancer cell infiltration into peripheral organs including, for example, the spread of cancer into soft tissue and bone; inhibition of or an absence of tumor metastasis; inhibition or an absence of tumor growth; relief of one or more symptoms associated with the specific cancer; reduced morbidity and mortality; improvement in quality of life; reduction in tumorigenicity, tumorigenic frequency, or tumorigenic capacity, of a tumor; reduction in the number or frequency of cancer stem cells in a tumor; differentiation of tumorigenic cells to a non-tumorigenic state; or some combination of effects.

[00107] "Polynucleotide," or "nucleic acid," as used interchangeably herein, refer to polymers of nucleotides of any length, and include DNA and RNA. The nucleotides can be deoxyribonucleotides, ribonucleotides, modified nucleotides or bases, and/or their analogs, or any substrate that can be incorporated into a polymer by DNA or RNA polymerase. A polynucleotide can comprise modified nucleotides, such as methylated nucleotides and their analogs. If present, modification to the nucleotide structure can be imparted before or after assembly of the polymer. The sequence of nucleotides can be interrupted by non-nucleotide components. A polynucleotide can be further modified after polymerization, such as by conjugation with a labeling component. Other types of modifications include, for example, "caps", substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications such as, for example, those with uncharged linkages (e.g., methyl phosphonates, phosphotriesters, phosphoamidates, cabamates, etc.) and with charged linkages (e.g., phosphorothioates, phosphorodithioates, etc.), those containing pendant moieties, such as,

for example, proteins (e.g., nucleases, toxins, antibodies, signal peptides, poly-L-lysine, etc.), those with intercalators (e.g., acridine, psoralen, etc.), those containing chelators (e.g., metals, radioactive metals, boron, oxidative metals, etc.), those containing alkylators, those with modified linkages (e.g., alpha anomeric nucleic acids, etc.), as well as unmodified forms of the polynucleotide(s). Further, any of the hydroxyl groups ordinarily present in the sugars can be replaced, for example, by phosphonate groups, phosphate groups, protected by standard protecting groups, or activated to prepare additional linkages to additional nucleotides, or can be conjugated to solid supports. The 5' and 3' terminal OH can be phosphorylated or substituted with amines or organic capping group moieties of from 1 to 20 carbon atoms. Other hydroxyls can also be derivatized to standard protecting groups. Polynucleotides can also contain analogous forms of ribose or deoxyribose sugars that are generally known in the art, including, for example, 2'-O-methyl-, 2'-O-allyl, 2'-fluoro- or 2'-azido-ribose, carbocyclic sugar analogs, .alpha.-anomeric sugars, epimeric sugars such as arabinose, xyloses or lyxoses, pyranose sugars, furanose sugars, sedoheptuloses, acyclic analogs and abasic nucleoside analogs such as methyl riboside. One or more phosphodiester linkages can be replaced by alternative linking groups. These alternative linking groups include, but are not limited to, embodiments wherein phosphate is replaced by P(O)S ("thioate"), P(S)S ("dithioate"), "(O)NR₂" ("amidate"), P(O)R, P(O)OR', CO or CH₂ ("formacetal"), in which each R or R' is independently H or substituted or unsubstituted alkyl (1-20 C) optionally containing an ether (--O--) linkage, aryl, alkenyl, cycloalkyl, cycloalkenyl or araldyl. Not all linkages in a polynucleotide need be identical. The preceding description applies to all polynucleotides referred to herein, including RNA and DNA.

[00108] The term "vector" means a construct, which is capable of delivering, and optionally expressing, one or more gene(s) or sequence(s) of interest in a host cell. Examples of vectors include, but are not limited to, viral vectors, naked DNA or RNA expression vectors, plasmid, cosmid or phage vectors, DNA or RNA expression vectors associated with cationic condensing agents, DNA or RNA expression vectors encapsulated in liposomes, and certain eukaryotic cells, such as producer cells..

[00109] The terms "polypeptide," "peptide," and "protein" are used interchangeably herein to refer to polymers of amino acids of any length. The polymer can be linear or branched, it can comprise modified amino acids, and it can be interrupted by non-amino acids. The terms also encompass an amino acid polymer that has been modified naturally or by intervention; for example, disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any

other manipulation or modification, such as conjugation with a labeling component. Also included within the definition are, for example, polypeptides containing one or more analogs of an amino acid (including, for example, unnatural amino acids, etc.), as well as other modifications known in the art. It is understood that, because the polypeptides of this invention are based upon antibodies, in certain embodiments, the polypeptides can occur as single chains or associated chains.

[00110] The terms "identical" or percent "identity" in the context of two or more nucleic acids or polypeptides, refer to two or more sequences or subsequences that are the same or have a specified percentage of nucleotides or amino acid residues that are the same, when compared and aligned (introducing gaps, if necessary) for maximum correspondence, not considering any conservative amino acid substitutions as part of the sequence identity. The percent identity can be measured using sequence comparison software or algorithms or by visual inspection. Various algorithms and software are known in the art that can be used to obtain alignments of amino acid or nucleotide sequences. One such non-limiting example of a sequence alignment algorithm is the algorithm described in Karlin et al, 1990, *Proc. Natl. Acad. Sci.*, 87:2264-2268, as modified in Karlin et al., 1993, *Proc. Natl. Acad. Sci.*, 90:5873-5877, and incorporated into the NBLAST and XBLAST programs (Altschul et al., 1991, *Nucleic Acids Res.*, 25:3389-3402). In certain embodiments, Gapped BLAST can be used as described in Altschul et al., 1997, *Nucleic Acids Res.* 25:3389-3402. BLAST-2, WU-BLAST-2 (Altschul et al., 1996, *Methods in Enzymology*, 266:460-480), ALIGN, ALIGN-2 (Genentech, South San Francisco, California) or Megalign (DNASTAR) are additional publicly available software programs that can be used to align sequences. In certain embodiments, the percent identity between two nucleotide sequences is determined using the GAP program in GCG software (e.g., using a NWSgapdna.CMP matrix and a gap weight of 40, 50, 60, 70, or 90 and a length weight of 1, 2, 3, 4, 5, or 6). In certain alternative embodiments, the GAP program in the GCG software package, which incorporates the algorithm of Needleman and Wunsch (*J. Mol. Biol.* (48):444-453 (1970)) can be used to determine the percent identity between two amino acid sequences (e.g., using either a Blossum 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5). Alternatively, in certain embodiments, the percent identity between nucleotide or amino acid sequences is determined using the algorithm of Myers and Miller (CABIOS, 4:11-17 (1989)). For example, the percent identity can be determined using the ALIGN program (version 2.0) and using a PAM120 with residue table, a gap length penalty of 12 and a gap

penalty of 4. Appropriate parameters for maximal alignment by particular alignment software can be determined by one skilled in the art. In certain embodiments, the default parameters of the alignment software are used. In certain embodiments, the percentage identity "X" of a first amino acid sequence to a second sequence amino acid is calculated as $100 \times (Y/Z)$, where Y is the number of amino acid residues scored as identical matches in the alignment of the first and second sequences (as aligned by visual inspection or a particular sequence alignment program) and Z is the total number of residues in the second sequence. If the length of a first sequence is longer than the second sequence, the percent identity of the first sequence to the second sequence will be longer than the percent identity of the second sequence to the first sequence.

[00111] As a non-limiting example, whether any particular polynucleotide has a certain percentage sequence identity (e.g., is at least 80% identical, at least 85% identical, at least 90% identical, and in some embodiments, at least 95%, 96%, 97%, 98%, or 99% identical) to a reference sequence can, in certain embodiments, be determined using the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, WI 53711). Bestfit uses the local homology algorithm of Smith and Waterman, *Advances in Applied Mathematics* 2: 482-489 (1981), to find the best segment of homology between two sequences. When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set such that the percentage of identity is calculated over the full length of the reference nucleotide sequence and that gaps in homology of up to 5% of the total number of nucleotides in the reference sequence are allowed.

[00112] In some embodiments, two nucleic acids or polypeptides of the invention are substantially identical, meaning they have at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, and in some embodiments at least 95%, 96%, 97%, 98%, 99% nucleotide or amino acid residue identity, when compared and aligned for maximum correspondence, as measured using a sequence comparison algorithm or by visual inspection. Identity can exist over a region of the sequences that is at least about 10, about 20, about 40-60 residues in length or any integral value therebetween, and can be over a longer region than 60-80 residues, for example, at least about 90-100 residues, and in some embodiments, the sequences are substantially identical over the full length of the sequences being compared, such as the coding region of a nucleotide sequence for example.

[00113] A "conservative amino acid substitution" is one in which one amino acid residue is replaced with another amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art, including basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine). For example, substitution of a phenylalanine for a tyrosine is a conservative substitution. In some embodiments, conservative substitutions in the sequences of the polypeptides and antibodies of the invention do not abrogate the binding of the polypeptide or antibody containing the amino acid sequence, to the antigen(s), i.e., the EGFR to which the polypeptide or antibody binds. Methods of identifying nucleotide and amino acid conservative substitutions which do not eliminate antigen binding are well-known in the art (see, e.g., Brummell et al., *Biochem.* 32: 1180-1 187 (1993); Kobayashi et al. *Protein Eng.* 12(10):879-884 (1999); and Burks et al. *Proc. Natl. Acad. Sci. USA* 94:412-417 (1997)).

[00114] As used in the present disclosure and claims, the singular forms "a," "an," and "the" include plural forms unless the context clearly dictates otherwise.

[00115] It is understood that wherever embodiments are described herein with the language "comprising," otherwise analogous embodiments described in terms of "consisting of" and/or "consisting essentially of" are also provided.

[00116] The term "and/or" as used in a phrase such as "A and/or B" herein is intended to include both "A and B," "A or B," "A," and "B." Likewise, the term "and/or" as used in a phrase such as "A, B, and/or C" is intended to encompass each of the following embodiments: A, B, and C; A, B, or C; A or C; A or B; B or C; A and C; A and B; B and C; A (alone); B (alone); and C (alone).

II. EGFR binding agents

[00117] Tumors often overexpress growth factor receptors that bind various ligands ligand and facilitate unrestricted tumor growth. One example of such growth factor receptors are the receptors of the Epidermal Growth Factor Receptor (EGFR) protein family.

[00118] Signal transduction through members of the Epidermal Growth Factor Receptor (EGFR) protein family is dependent upon the formation of homodimers or heterodimers triggered

by the binding of ligand. This receptor family is comprised of four membrane-bound proteins: EGFR, HER2/neu, HER3 and HER4. Overexpression of these proteins has been correlated with a poor prognosis in a number of types of cancer, including, but not limited to, breast, colon, ovarian, endometrial, gastric, pancreatic, prostate and salivary gland cancers. While a number of groups have developed strategies to target individual members of the EGFR protein family (e.g., HER2/neu or EGFR) to inhibit tumor growth, none of the treatments has been proven to ultimately cure these forms of cancer.

[00119] In accordance with this invention novel agents (e.g. antibodies) are provided that specifically bind to human EGFR. These novel agents partially inhibit EGFR signaling and have no effect on EGFR-positive normal epithelial cells, including primary keratinocytes. However, these agents are highly cytotoxic to EGFR-overexpressing tumor cells.

[00120] Thus, present invention provides agents that specifically bind human EGFR. These agents are referred to herein as "EGFR binding agents." In certain embodiments, the EGFR binding agents are antibodies, immunoconjugates or polypeptides. In some embodiments, the EGFR binding agents are humanized antibodies.

[0100] In certain embodiments, the EGFR -binding agents have one or more of the following effects: inhibit proliferation of tumor cells, reduce the tumorigenicity of a tumor by reducing the frequency of cancer stem cells in the tumor, inhibit tumor growth, increase survival, trigger cell death of tumor cells, differentiate tumorigenic cells to a non-tumorigenic state, or prevent metastasis of tumor cells.

[0101] In some embodiments, the EGFR-binding agents are capable of reducing tumor volume. The ability of a EGFR-binding agent to reduce tumor volume can be assessed, for example, by measuring a %T/C value, which is the median tumor volume of treated subjects divided by the median tumor volume of the control subjects. In some embodiments, treatment with a EGFR-binding agent results in a %T/C value that is less than about 55%, less than about 50%, less than about 45%, less than about 40%, less than about 35%, less than about 30%, less than about 25%, less than about 20%, less than about 15%, less than about 10%, or less than about 5%.

[0102] In certain embodiments, immunoconjugates or other agents that specifically bind human EGFR trigger cell death via a cytotoxic agent. For example, in certain embodiments, an antibody to a human EGFR antibody is conjugated to a maytansinoid that is activated in tumor cells expressing the EGFR by protein internalization. In certain alternative embodiments, the agent or antibody is not conjugated.

[0103] In certain embodiments, the EGFR-binding agents are capable of inhibiting tumor growth. In certain embodiments, the EGFR-binding agents are capable of inhibiting tumor growth *in vivo* (e.g., in a xenograft mouse model and/or in a human having cancer).

[0104] The EGFR-binding agents include EGFR antibodies EGFR-6, EGFR-7, and EGFR-12 and fragments, variants and derivatives thereof. The EGFR-binding agents also include EGFR-binding agents that specifically bind to the same EGFR epitope as the antibodies EGFR-6, EGFR-7, and EGFR-12. The EGFR-binding agents also include EGFR-binding agents that competitively inhibit the antibodies EGFR-6, EGFR-7, and EGFR-12.

[0105] The EGFR-binding agents also include EGFR-binding agents that comprise the heavy and light chain CDR sequences of EGFR-6, EGFR-7, and EGFR-12. The CDR sequences of EGFR-7 and EGFR-12, both murine and humanized are described in Tables 1 and 2 below.

Table 1: Variable heavy chain CDR amino acid sequences

Antibody	VH-CDR1	VH-CDR2	VH-CDR3
EGFR-7	TSYWMQ (SEQ ID NO:1)	TIYPGDGDTT (SEQ ID NO:2)	YDAPGYAMDY (SEQ ID NO:3)
EGFR-7 variant composite sequences	TSYWMQ (SEQ ID NO:1)	Xaa ₁ Xaa ₂ YPGDGD _{Xaa₃} Xaa ₄ ; Xaa ₁ = T or A Xaa ₂ = I or L Xaa ₃ = T or A Xaa ₄ = T, R, or S (SEQ ID NO:4)	YDAPGYXaa ₁ MDY Xaa ₁ = A or T (SEQ ID NO:5)
EGFR-7 Kabat HC CDR2		Xaa ₁ Xaa ₂ YPGDGD _{Xaa₃} Xaa ₄ Xaa ₅ QKFXaa ₆ G Xaa ₁ = T or A Xaa ₂ = I or L Xaa ₃ = T or A Xaa ₄ = T, R, or S Xaa ₅ = Y, T, or I Xaa ₆ = Q or K (SEQ ID NO:6)	
EGFR-12	TSYWMQ (SEQ ID NO:1)	TIYPGDGDTR (SEQ ID NO:7)	YDAPGYAMDY (SEQ ID NO:3)
Kabat HC (murine)		TIYPGDGDTRYIQKFKG (SEQ ID NO:8)	
Kabat HC (resurfaced)		TIYPGDGDTRYIQKFQG (SEQ ID NO:9)	
EGFR-6	TSYWMQ (SEQ ID NO:1)	ALYPGDGDAR (SEQ ID NO:65)	YDAPGYAMDY (SEQ ID NO:3)
Kabat HC (murine)		ALYPGDGDARYTQKFKG (SEQ ID NO:66)	
Kabat HC		ALYPGDGDARYTQKFQG (SEQ ID NO:67)	

(resurfaced)			
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Table 2: Variable light chain CDR amino acid sequences

Antibody	VL-CDR1	VL-CDR2	VL-CDR3
EGFR-7	KASQDINNYLA (murine and resurfaced v1.01) (SEQ ID NO:10)	YTSTLHP (SEQ ID NO:11)	LQYDNLLYT (SEQ ID NO:12)
	RASQDINNYLA (resurfaced v1.0 and CDR grafted) (SEQ ID NO:13)		
EGFR-7 variant composite sequences	Xaa ₁ ASQDINNYXaa ₂ A Xaa ₁ = K or R Xaa ₂ = L or I (SEQ ID NO:14)	YTSTLHP (SEQ ID NO:11)	LQYDNLLYT (SEQ ID NO:12)
EGFR-12	RASKSISKYLA (murine and resurfaced v1.01) (SEQ ID NO:15)		
	RASQSISKYLA (resurfaced v1.0) (SEQ ID NO:16)	SGSTLQS (SEQ ID NO:17)	QQHNEYPWT (SEQ ID NO:18)
EGFR-6 (murine)	KASQDINNYIA (SEQ ID NO: 68)	YTSTLHP (SEQ ID NO:11)	LQYDNLLYT (SEQ ID NO:12)
(resurfaced)	RASQDINNYLA (SEQ ID NO:13)		

[0106] The EGFR binding molecules can be antibodies or antigen binding fragments that specifically bind to EGFR that comprise the CDRs of EGFR-6, EGFR-7, and EGFR-12 with up to four (i.e. 0, 1, 2, 3, or 4) conservative amino acid substitutions per CDR.

[0107] Polypeptides can comprise one of the individual variable light chains or variable heavy chains described herein. Antibodies and polypeptides can also comprise both a variable light chain and a variable heavy chain. The variable light chain and variable heavy chain sequences of murine and humanized EGFR-7 and EGFR-12 antibodies are provided in Tables 3 and 4 below.

Table 3: Variable heavy chain amino acid sequences

Antibody	VH Amino Acid Sequence
muEGFR-7 V _H	QVQLQQSGAELARPGASVKLSCKASGYTFTSYWMQWVKQRPQGQLECIIGTIYPG DGDTTYTQKFKGKATLTADKSSSTAYMQLSSLASEDSAVYYCARYDAPGYAMD YWGQGTSTVTVSS (SEQ ID NO:19)
muEGFR-12 V _H	QVQLQQSGTELARPGASVKLSCKASGYTFTSYWMQWVKQRPQGQLECIIGTIYPG DGDTRYIQKFKGKATLTADKSSSTAYMQLSSLASEDSAVYYCARYDAPGYAMD YWGQGTSTVTVSS

Antibody	VH Amino Acid Sequence
	(SEQ ID NO:20)
huEGFR-7 V _H	QVQLVQSGAEVAKPGASVKLSCKASGYTFTSYWMQWVKQRPQGQGLECIGTIYP GDGDDTTYTQKFQGKATLTADKSSSTAYMQLSSLRSEDSAVYYCARYDAPGYAM DYWGQGTLLTVSS (SEQ ID NO:21)
huEGFR-7 V _H _CDR grafted	QVQLVQSGAEVKKPGASVKVSKASGYTFTSYWMQWVRQAPQGQGLEWMGIIY PGDGDTTYTQKFQKGRVTMTRDTSTSTVYMELSSLRSEDTAVYYCARYDAPGYA MDYWGQGTLLTVSS (SEQ ID NO:22)
huEGFR-12 V _H	QVQLVQSGAEVAKPGASVKLSCKASGYTFTSYWMQWVKQRPQGQGLECIGTIYP GDGDTRYIQKFQGKATLTADKSSSTAYMQLSSLRSEDSAVYYCARYDAPGYAM DYWGQGTLLTVSS (SEQ ID NO:23)
muEGFR-6 V _H	QVQLQQSGAELARPGASVKLSCKASGYTFTSYWMQWVKQRPQGQGLECIGALYP GDGDARYTQKFQKATLTADRSSSTAYMQLSSLASEDSAVYYCARYDAPGYAM DYWGQGTSTVTVAS (SEQ ID NO:69)
huEGFR-6 V _H v1.0	QVQLVQSGAEVAKPGASVKLSCKASGYTFTSYWMQWVKQRPQGQGLECIGALYP GDGDARYTQKFQGKATLTADTSSSTAYMQLSSLRSEDSAVYYCARYDAPGYAM DYWGQGTLLTVSS (SEQ ID NO:71)
huEGFR-6 V _H v1.11	QVQLVQSGAEVAKPGASVKLSCKASGYTFTSYWMQWVKQRPQGQGLEWIGALY PGDGDARYTQKFQGKATLTADTSSSTAYMQLSSLRSEDSAVYYCARYDAPGYA MDYWGQGTLLTVSS (SEQ ID NO:72)
huEGFR-7 V _H v1.11	QVQLVQSGAEVAKPGASVKLSCKASGYTFTSYWMQWVKQRPQGQGLEWIGTIYP GDGDDTTYTQKFQGKATLTADKSSSTAYMQLSSLRSEDSAVYYCARYDAPGYAM DYWGQGTLLTVSS (SEQ ID NO:73)

Table 4: Variable light chain amino acid sequences

Antibody	VL Amino Acid Sequence
muEGFR-7 V _L	DIQMTQSPSSLSASLGKVTITCKASQDINNYLAWYQHKGKGPRLLIHYTSTLHP GIPSRFSGSGSGRDYSFSSISNLEPEDIATYYCLQYDNLLYTFGGGKLEIKR (SEQ ID NO:24)
muEGFR-12 V _L	DVQITQSPSYLAASPGETITINCRASKSISKYLAWYQEKPGKTNKLLIYSGSTLQSGI PSRFSGSGSGTDFTLTSSLEPEDFAMYYCQHQHNEYPWTFGGGKLEIKR (SEQ ID NO:25)
huEGFR-7 V _L v1.0	DIQMTQSPSSLSASVGDRVITITCRASQDINNYLAWYQHKGKGPRLLIHYTSTLHP GIPSRFSGSGSGRDYSFSSISLEPEDIATYYCLQYDNLLYTFGQGTLEIKR (SEQ ID NO:26)
huEGFR-7 V _L v1.01	DIQMTQSPSSLSASVGDRVITITCKASQDINNYLAWYQHKGKGPRLLIHYTSTLHP GIPSRFSGSGSGRDYSFSSISLEPEDIATYYCLQYDNLLYTFGQGTLEIKR (SEQ ID NO:27)
huEGFR-7 V _L _CDR grafted	DIQMTQSPSSLSASVGDRVITITCRASQDINNYLAWYQQKPGKAPKLLIYYTSTLHP GVPSRFSGSGSGTDFTFTISLQPEDATYYCLQYDNLLYTFGQGTKEIKR (SEQ ID NO:28)
huEGFR-12 V _L v1.0	DVQITQSPSSLAASVGERITINCRASQISIRYLAWYQEKPGKTNKLLIYSGSTLQSG IPSRFSGSGSGTDFTLTSSLEPEDFAMYYCQHQHNEYPWTFGQGTLEIKR (SEQ ID NO:29)
huEGFR-12 V _L	DVQITQSPSSLAASVGERITINCRASKSISKYLAWYQEKPGKTNKLLIYSGSTLQSG IPSRFSGSGSGTDFTLTSSLEPEDFAMYYCQHQHNEYPWTFGQGTLEIKR

Antibody	VL Amino Acid Sequence
v1.01	(SEQ ID NO:30)
muEGFR6 V _L	DIQMTQSPSSLSASLGGKVTITCKASQDINNYIAWYQHKGKGPRLLIHYTSTLHP GIPSRFSGSGSGRDYSFISISNLEPEDIATYYCLQYDNLLYTFGGGKLEIKR (SEQ ID NO:70)

[0108] Also provided are polypeptides that comprise: (a) a polypeptide having at least about 90% sequence identity to SEQ ID NOs:19-23, 69, and 71-76; and/or (b) a polypeptide having at least about 90% sequence identity to SEQ ID NOs:24-30 and 70. In certain embodiments, the polypeptide comprises a polypeptide having at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% sequence identity to SEQ ID NOs:19-30 and 69-76. Thus, in certain embodiments, the polypeptide comprises (a) a polypeptide having at least about 95% sequence identity to SEQ ID NOs: 19-23, 69, and 71-76, and/or (b) a polypeptide having at least about 95% sequence identity to SEQ ID NOs:24-30 and 70. In certain embodiments, the polypeptide comprises (a) a polypeptide having the amino acid sequence of SEQ ID NOs: 19-23, 69, and 71-76; and/or (b) a polypeptide having the amino acid sequence of SEQ ID NOs:24-30 and 70. In certain embodiments, the polypeptide is an antibody and/or the polypeptide specifically binds EGFR. In certain embodiments, the polypeptide is a murine, chimeric, or humanized antibody that specifically binds EGFR. In certain embodiments, the polypeptide having a certain percentage of sequence identity to SEQ ID NOs: 19-30 and 69-76 differs from SEQ ID NOs: 19-30 and 69-76 by conservative amino acid substitutions only.

Table 5: Full-length heavy chain and light chain amino acid sequences

Antibody	VH Amino Acid Sequence
huEGFR-7 HC	QVQLVQSGAEVAKPGASVKLSCKASGYTFTSYWMQWVKQRPGQGLECIGTIYPGD GDTTYTQKFQGKATLTADKSSSTAYMQLSSLRSEDSAVYYCARYDAPGYAMDYW GQGTLTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPTVSWNSGALT SGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCD KTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIE KTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENN YKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG (SEQ ID NO:31)
huEGFR-7 HC_CDR grafted	QVQLVQSGAEVKKPGASVKVSKASGYTFTSYWMQWVRQAPGQGLEWMGTIYPG DGDTTYTQKFGRVTMTRDTSTSTVYMELSSLRSEDTAVYYCARYDAPGYAMDYW GQGTLTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPTVSWNSGALT SGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDK THTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVD GVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTIS KAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTT PPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG (SEQ ID NO:32)
huEGFR-7	DIQMTQSPSSLSASVGDRVITITCRASQDINNYLAWYQHKGKGPRLLIHYTSTLHPGI

Antibody	VH Amino Acid Sequence
LCv1.0	PSRFSGSGSGRDYSFSISSLEPEDIATYYCLQYDNLLYTFGQGTKLEIKRTVAAPSVFIF PPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDYSL SSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC (SEQ ID NO:33)
huEGFR-7 LCv1.01	DIQMTQSPSSLSASVGDRVTITCKASQDINNYLAWYQHKGPKGPKLLIHYTSTLHPGI PSRFSGSGSGRDYSFSISSLEPEDIATYYCLQYDNLLYTFGQGTKLEIKRTVAAPSVFIF PPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDYSL LSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC (SEQ ID NO:34)
huEGFR-7 LC CDR grafted	DIQMTQSPSSLSASVGDRVTITCRASQDINNYLAWYQQKPGKAPKLLIYYTSTLHPG VPSRFSGSGSGTDFTFTISSLPEDIATYYCLQYDNLLYTFGQGTKVEIKRTVAAPSV FIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDS TYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC (SEQ ID NO:35)
huEGFR-12 HC	QVQLVQSGAEVAKPGASVKLSCKASGYTFTSYWMQWVKQRPGQGLECIGTIYPGD GDTRYIQKFQGKATLTADKSSSTAYMQLSSLRSEDSAVYYCARYDAPGYAMDY WGQGLTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGAL TSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSC DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNW YVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAP IEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPE NNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFCSCVMHEALHNHYTQKSLSL SPG (SEQ ID NO:36)
huEGFR-12 LCv1.0	DVQITQSPSSLAASVGERITINCRASQISRYLAWYQEKPQKTNKLLIYSGSTLQSGI PSRFSGSGSGTDFTLTISLEPEDFAMYQCQHNEYPWTFGQGTKLEIKRTVAAPSV FIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDS TYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC (SEQ ID NO:37)
huEGFR-12 LCv1.01	DVQITQSPSSLAASVGERITINCRASKSISKYLAWYQEKPQKTNKLLIYSGSTLQSGI PSRFSGSGSGTDFTLTISLEPEDFAMYQCQHNEYPWTFGQGTKLEIKRTVAAPSV FIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKD STYLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC (SEQ ID NO:38)
huEGFR-6 HCv1.0	QVQLVQSGAEVAKPGASVKLSCKASGYTFTSYWMQWVKQRPGQGLECIGALYPG DGDARYTQKFQGKATLTADTSSSTAYMQLSSLRSEDSAVYYCARYDAPGYAMDY WGQGLTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGA LTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKS CDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFN WYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQ PENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFCSCVMHEALHNHYTQKSL SLSPG (SEQ ID NO:74)
huEGFR-6 HCv1.11	QVQLVQSGAEVAKPGASVKLSCKASGYTFTSYWMQWVKQRPGQGLEWIGALYP DGDARYTQKFQGKATLTADTSSSTAYMQLSSLRSEDSAVYYCARYDAPGYAMDY YWGQGLTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSG ALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPK SCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKF NWWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKAL PAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQ PENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFCSCVMHEALHNHYTQKSL SLSPG (SEQ ID NO:75)
huEGFR-7 HCv1.11	QVQLVQSGAEVAKPGASVKLSCKASGYTFTSYWMQWVKQRPGQGLEWIGTIYPG DGDTTYTQKFQGKATLTADKSSSTAYMQLSSLRSEDSAVYYCARYDAPGYAMDY WGQGLTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGA LTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKS CDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFN WYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP

Antibody	VH Amino Acid Sequence
	APIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQ PENNYKTTTPVLDSGDFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSL SLSPG (SEQ ID NO:76)

[0109] Also provided are polypeptides that comprise: (a) a polypeptide having at least about 90% sequence identity to SEQ ID NOs:31, 32, and 36; and/or (b) a polypeptide having at least about 90% sequence identity to SEQ ID NOs:33-35, 37, and 38. In certain embodiments, the polypeptide comprises a polypeptide having at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% sequence identity to SEQ ID NOs:31-38. Thus, in certain embodiments, the polypeptide comprises (a) a polypeptide having at least about 95% sequence identity to SEQ ID NOs: 31, 32, and 36, and/or (b) a polypeptide having at least about 95% sequence identity to SEQ ID NOs: 33-35, 37, and 38. In certain embodiments, the polypeptide comprises (a) a polypeptide having the amino acid sequence of SEQ ID NOs: 31, 32, and 36; and/or (b) a polypeptide having the amino acid sequence of SEQ ID NOs: 33-35, 37, and 38. In certain embodiments, the polypeptide is an antibody and/or the polypeptide specifically binds EGFR. In certain embodiments, the polypeptide is a humanized antibody that specifically binds EGFR. In certain embodiments, the polypeptide having a certain percentage of sequence identity to SEQ ID NOs:31-38 differs from SEQ ID NOs:31-38 by conservative amino acid substitutions only.

[0110] In certain embodiments, the EGFR antibody is the antibody produced from a hybridoma selected from the group consisting of ATCC Deposit Designation PTA-11331 (EGFR-6), deposited with the ATCC on October 6, 2010, ATCC Deposit Designation PTA-11332 (EGFR-7), deposited with the ATCC on October 6, 2010, and ATCC Deposit Designation PTA-11333 (EGFR-12), deposited with the ATCC on October 6, 2010. In certain embodiments, the antibody comprises the VH-CDRs and the VL-CDRS of the antibody produced from a hybridoma selected from the group consisting of PTA-11331, PTA-11332, and PTA-11333.

[0111] Monoclonal antibodies can be prepared using hybridoma methods, such as those described by Kohler and Milstein (1975) Nature 256:495. Using the hybridoma method, a mouse, hamster, or other appropriate host animal, is immunized as described above to elicit the production by lymphocytes of antibodies that will specifically bind to an immunizing antigen. Lymphocytes can also be immunized *in vitro*. Following immunization, the lymphocytes are isolated and fused with a suitable myeloma cell line using, for example, polyethylene glycol, to form hybridoma cells that can then be selected away from unfused lymphocytes and myeloma

cells. Hybridomas that produce monoclonal antibodies directed specifically against a chosen antigen as determined by immunoprecipitation, immunoblotting, or by an *in vitro* binding assay (e.g. radioimmunoassay (RIA); enzyme-linked immunosorbent assay (ELISA)) can then be propagated either *in vitro* culture using standard methods (Goding, Monoclonal Antibodies: Principles and Practice, Academic Press, 1986) or *in vivo* as ascites tumors in an animal. The monoclonal antibodies can then be purified from the culture medium or ascites fluid as described for polyclonal antibodies above.

[0112] Alternatively monoclonal antibodies can also be made using recombinant DNA methods as described in U.S. Patent 4,816,567. The polynucleotides encoding a monoclonal antibody are isolated from mature B-cells or hybridoma cell, such as by RT-PCR using oligonucleotide primers that specifically amplify the genes encoding the heavy and light chains of the antibody, and their sequence is determined using conventional procedures. The isolated polynucleotides encoding the heavy and light chains are then cloned into suitable expression vectors, which when transfected into host cells such as E. coli cells, simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, monoclonal antibodies are generated by the host cells. Also, recombinant monoclonal antibodies or fragments thereof of the desired species can be isolated from phage display libraries expressing CDRs of the desired species as described (McCafferty et al., 1990, Nature, 348:552-554; Clackson et al., 1991, Nature, 352:624-628; and Marks et al., 1991, J. Mol. Biol., 222:581-597).

[0113] The polynucleotide(s) encoding a monoclonal antibody can further be modified in a number of different manners using recombinant DNA technology to generate alternative antibodies. In some embodiments, the constant domains of the light and heavy chains of, for example, a mouse monoclonal antibody can be substituted 1) for those regions of, for example, a human antibody to generate a chimeric antibody or 2) for a non-immunoglobulin polypeptide to generate a fusion antibody. In some embodiments, the constant regions are truncated or removed to generate the desired antibody fragment of a monoclonal antibody. Site-directed or high-density mutagenesis of the variable region can be used to optimize specificity, affinity, etc. of a monoclonal antibody.

[0114] In some embodiments, the monoclonal antibody against the human EGFR is a humanized antibody. In certain embodiments, such antibodies are used therapeutically to reduce antigenicity and HAMA (human anti-mouse antibody) responses when administered to a human subject.

Humanized antibodies can be produced using various techniques known in the art. In certain alternative embodiments, the antibody to EGFR is a human antibody.

[0115] Human antibodies can be directly prepared using various techniques known in the art. Immortalized human B lymphocytes immunized *in vitro* or isolated from an immunized individual that produce an antibody directed against a target antigen can be generated (See, e.g., Cole et al., *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, p. 77 (1985); Boemer et al., 1991, *J. Immunol.*, 147 (1):86-95; and U.S. Patent 5,750,373). Also, the human antibody can be selected from a phage library, where that phage library expresses human antibodies, as described, for example, in Vaughan et al., 1996, *Nat. Biotech.*, 14:309-314, Sheets et al., 1998, *Proc. Nat'l. Acad. Sci.*, 95:6157-6162, Hoogenboom and Winter, 1991, *J. Mol. Biol.*, 227:381, and Marks et al., 1991, *J. Mol. Biol.*, 222:581). Techniques for the generation and use of antibody phage libraries are also described in U.S. Patent Nos. 5,969,108, 6,172,197, 5,885,793, 6,521,404; 6,544,731; 6,555,313; 6,582,915; 6,593,081; 6,300,064; 6,653,068; 6,706,484; and 7,264,963; and Rothe et al., 2007, *J. Mol. Bio.*, doi:10.1016/j.jmb.2007.12.018 (each of which is incorporated by reference in its entirety). Affinity maturation strategies and chain shuffling strategies (Marks et al., 1992, *Bio/Technology* 10:779-783, incorporated by reference in its entirety) are known in the art and can be employed to generate high affinity human antibodies.

[0116] Humanized antibodies can also be made in transgenic mice containing human immunoglobulin loci that are capable upon immunization of producing the full repertoire of human antibodies in the absence of endogenous immunoglobulin production. This approach is described in U.S. Patents 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; and 5,661,016.

[0117] This invention also encompasses bispecific antibodies that specifically recognize a EGFR. Bispecific antibodies are antibodies that are capable of specifically recognizing and binding at least two different epitopes. The different epitopes can either be within the same molecule (e.g. the same EGFR) or on different molecules such that both, for example, the antibodies can specifically recognize and bind a EGFR as well as, for example, 1) an effector molecule on a leukocyte such as a T-cell receptor (e.g. CD3) or Fc receptor (e.g. CD64, CD32, or CD16) or 2) a cytotoxic agent as described in detail below.

[0118] Exemplary bispecific antibodies can bind to two different epitopes, at least one of which originates in a polypeptide of the invention. Alternatively, an anti-antigenic arm of an immunoglobulin molecule can be combined with an arm which binds to a triggering molecule on a leukocyte such as a T cell receptor molecule (e.g. CD2, CD3, CD28, or B7), or Fc receptors for

IgG so as to focus cellular defense mechanisms to the cell expressing the particular antigen. Bispecific antibodies can also be used to direct cytotoxic agents to cells which express a particular antigen. These antibodies possess an antigen-binding arm and an arm which binds a cytotoxic agent or a radionuclide chelator, such as EOTUBE, DPTA, DOTA, or TETA. Techniques for making bispecific antibodies are common in the art (Millstein et al., 1983, *Nature* 305:537-539; Brennan et al., 1985, *Science* 229:81; Suresh et al., 1986, *Methods in Enzymol.* 121:120; Traunecker et al., 1991, *EMBO J.* 10:3655-3659; Shalaby et al., 1992, *J. Exp. Med.* 175:217-225; Kostelny et al., 1992, *J. Immunol.* 148:1547-1553; Gruber et al., 1994, *J. Immunol.* 152:5368; and U.S. Patent 5,731,168). Antibodies with more than two valencies are also contemplated. For example, trispecific antibodies can be prepared (Tutt et al., *J. Immunol.* 147:60 (1991)). Thus, in certain embodiments the antibodies to EGFR are multispecific.

[0119] In certain embodiments are provided an antibody fragment to, for example, increase tumor penetration. Various techniques are known for the production of antibody fragments. Traditionally, these fragments are derived via proteolytic digestion of intact antibodies (for example Morimoto et al., 1993, *Journal of Biochemical and Biophysical Methods* 24:107-117; Brennan et al., 1985, *Science*, 229:81). In certain embodiments, antibody fragments are produced recombinantly. Fab, Fv, and scFv antibody fragments can all be expressed in and secreted from *E. coli* or other host cells, thus allowing the production of large amounts of these fragments. Such antibody fragments can also be isolated from the antibody phage libraries discussed above. The antibody fragment can also be linear antibodies as described in U.S. Patent 5,641,870, for example, and can be monospecific or bispecific. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner.

[0120] According to the present invention, techniques can be adapted for the production of single-chain antibodies specific to EGFR (see U.S. Pat. No. 4,946,778). In addition, methods can be adapted for the construction of Fab expression libraries (Huse, et al., *Science* 246:1275-1281 (1989)) to allow rapid and effective identification of monoclonal Fab fragments with the desired specificity for EGFR, or derivatives, fragments, analogs or homologs thereof. Antibody fragments can be produced by techniques in the art including, but not limited to: (a) a F(ab')₂ fragment produced by pepsin digestion of an antibody molecule; (b) a Fab fragment generated by reducing the disulfide bridges of an F(ab')₂ fragment, (c) a Fab fragment generated by the treatment of the antibody molecule with papain and a reducing agent, and (d) Fv fragments.

[0121] It can further be desirable, especially in the case of antibody fragments, to modify an antibody in order to increase its serum half-life. This can be achieved, for example, by incorporation of a salvage receptor binding epitope into the antibody fragment by mutation of the appropriate region in the antibody fragment or by incorporating the epitope into a peptide tag that is then fused to the antibody fragment at either end or in the middle (e.g., by DNA or peptide synthesis).

[0122] Heteroconjugate antibodies are also within the scope of the present invention. Heteroconjugate antibodies are composed of two covalently joined antibodies. Such antibodies have, for example, been proposed to target immune cells to unwanted cells (U.S. Pat. No. 4,676,980). It is contemplated that the antibodies can be prepared *in vitro* using known methods in synthetic protein chemistry, including those involving crosslinking agents. For example, immunotoxins can be constructed using a disulfide exchange reaction or by forming a thioether bond. Examples of suitable reagents for this purpose include iminothiolate and methyl-4-mercaptobutyrimidate.

[0123] For the purposes of the present invention, it should be appreciated that modified antibodies can comprise any type of variable region that provides for the association of the antibody with the polypeptides of a human EGFR. In this regard, the variable region can comprise or be derived from any type of mammal that can be induced to mount a humoral response and generate immunoglobulins against the desired tumor associated antigen. As such, the variable region of the modified antibodies can be, for example, of human, murine, non-human primate (e.g. cynomolgus monkeys, macaques, etc.) or lupine origin. In some embodiments both the variable and constant regions of the modified immunoglobulins are human. In other embodiments the variable regions of compatible antibodies (usually derived from a non-human source) can be engineered or specifically tailored to improve the binding properties or reduce the immunogenicity of the molecule. In this respect, variable regions useful in the present invention can be humanized or otherwise altered through the inclusion of imported amino acid sequences.

[0124] In certain embodiments, the variable domains in both the heavy and light chains are altered by at least partial replacement of one or more CDRs and, if necessary, by partial framework region replacement and sequence changing. Although the CDRs can be derived from an antibody of the same class or even subclass as the antibody from which the framework regions are derived, it is envisaged that the CDRs will be derived from an antibody of different class and possibly from an antibody from a different species. It is not always necessary to replace all of the

CDRs with the complete CDRs from the donor variable region to transfer the antigen binding capacity of one variable domain to another. Rather, in some cases it is only necessary to transfer those residues that are necessary to maintain the activity of the antigen binding site. Given the explanations set forth in U.S. Pat. Nos. 5,585,089, 5,693,761 and 5,693,762, it will be well within the competence of those skilled in the art, either by carrying out routine experimentation or by trial and error testing to obtain a functional antibody with reduced immunogenicity.

[0125] Alterations to the variable region notwithstanding, those skilled in the art will appreciate that the modified antibodies of this invention will comprise antibodies (e.g., full-length antibodies or immunoreactive fragments thereof) in which at least a fraction of one or more of the constant region domains has been deleted or otherwise altered so as to provide desired biochemical characteristics such as increased tumor localization or reduced serum half-life when compared with an antibody of approximately the same immunogenicity comprising a native or unaltered constant region. In some embodiments, the constant region of the modified antibodies will comprise a human constant region. Modifications to the constant region compatible with this invention comprise additions, deletions or substitutions of one or more amino acids in one or more domains. That is, the modified antibodies disclosed herein can comprise alterations or modifications to one or more of the three heavy chain constant domains (CH1, CH2 or CH3) and/or to the light chain constant domain (CL). In some embodiments, modified constant regions wherein one or more domains are partially or entirely deleted are contemplated. In some embodiments, the modified antibodies will comprise domain deleted constructs or variants wherein the entire CH2 domain has been removed (Δ CH2 constructs). In some embodiments, the omitted constant region domain will be replaced by a short amino acid spacer (e.g. 10 residues) that provides some of the molecular flexibility typically imparted by the absent constant region.

[0126] Besides their configuration, it is known in the art that the constant region mediates several effector functions. For example, binding of the C1 component of complement to antibodies activates the complement system. Activation of complement is important in the opsonisation and lysis of cell pathogens. The activation of complement also stimulates the inflammatory response and can also be involved in autoimmune hypersensitivity. Further, antibodies bind to cells via the Fc region, with a Fc receptor site on the antibody Fc region binding to a Fc receptor (FcR) on a cell. There are a number of Fc receptors which are specific for different classes of antibody, including IgG (gamma receptors), IgE (eta receptors), IgA (alpha receptors) and IgM (mu

receptors). Binding of antibody to Fc receptors on cell surfaces triggers a number of important and diverse biological responses including engulfment and destruction of antibody-coated particles, clearance of immune complexes, lysis of antibody-coated target cells by killer cells (called antibody-dependent cell-mediated cytotoxicity, or ADCC), release of inflammatory mediators, placental transfer and control of immunoglobulin production.

[0127] In certain embodiments, the EGFR-binding antibodies provide for altered effector functions that, in turn, affect the biological profile of the administered antibody. For example, the deletion or inactivation (through point mutations or other means) of a constant region domain can reduce Fc receptor binding of the circulating modified antibody thereby increasing tumor localization. In other cases, it can be that constant region modifications, consistent with this invention, moderate complement binding and thus reduce the serum half-life and nonspecific association of a conjugated cytotoxin. Yet other modifications of the constant region can be used to eliminate disulfide linkages or oligosaccharide moieties that allow for enhanced localization due to increased antigen specificity or antibody flexibility. Similarly, modifications to the constant region in accordance with this invention can easily be made using well known biochemical or molecular engineering techniques well within the purview of the skilled artisan.

[0128] In certain embodiments, a EGFR-binding agent that is an antibody does not have one or more effector functions. For instance, in some embodiments, the antibody has no antibody-dependent cellular cytotoxicity (ADCC) activity and/or no complement-dependent cytotoxicity (CDC) activity. In certain embodiments, the antibody does not bind to an Fc receptor and/or complement factors. In certain embodiments, the antibody has no effector function.

[0129] It will be noted that in certain embodiments, the modified antibodies can be engineered to fuse the CH3 domain directly to the hinge region of the respective modified antibodies. In other constructs it can be desirable to provide a peptide spacer between the hinge region and the modified CH2 and/or CH3 domains. For example, compatible constructs could be expressed wherein the CH2 domain has been deleted and the remaining CH3 domain (modified or unmodified) is joined to the hinge region with a 5-20 amino acid spacer. Such a spacer can be added, for instance, to ensure that the regulatory elements of the constant domain remain free and accessible or that the hinge region remains flexible. However, it should be noted that amino acid spacers can, in some cases, prove to be immunogenic and elicit an unwanted immune response against the construct. Accordingly, in certain embodiments, any spacer added to the construct

will be relatively non-immunogenic, or even omitted altogether, so as to maintain the desired biochemical qualities of the modified antibodies.

[0130] Besides the deletion of whole constant region domains, it will be appreciated that the antibodies of the present invention can be provided by the partial deletion or substitution of a few or even a single amino acid. For example, the mutation of a single amino acid in selected areas of the CH2 domain can be enough to substantially reduce Fc binding and thereby increase tumor localization. Similarly, it can be desirable to simply delete that part of one or more constant region domains that control the effector function (e.g. complement CLQ binding) to be modulated. Such partial deletions of the constant regions can improve selected characteristics of the antibody (serum half-life) while leaving other desirable functions associated with the subject constant region domain intact. Moreover, as alluded to above, the constant regions of the disclosed antibodies can be modified through the mutation or substitution of one or more amino acids that enhances the profile of the resulting construct. In this respect it can be possible to disrupt the activity provided by a conserved binding site (e.g. Fc binding) while substantially maintaining the configuration and immunogenic profile of the modified antibody. Certain embodiments can comprise the addition of one or more amino acids to the constant region to enhance desirable characteristics such as decreasing or increasing effector function or provide for more cytotoxin or carbohydrate attachment. In such embodiments it can be desirable to insert or replicate specific sequences derived from selected constant region domains.

[0131] The present invention further embraces variants and equivalents which are substantially homologous to the chimeric, humanized and human antibodies, or antibody fragments thereof, set forth herein. These can contain, for example, conservative substitution mutations, i.e. the substitution of one or more amino acids by similar amino acids. For example, conservative substitution refers to the substitution of an amino acid with another within the same general class such as, for example, one acidic amino acid with another acidic amino acid, one basic amino acid with another basic amino acid or one neutral amino acid by another neutral amino acid. What is intended by a conservative amino acid substitution is well known in the art.

[0132] The polypeptides of the present invention can be recombinant polypeptides, natural polypeptides, or synthetic polypeptides comprising an antibody, or fragment thereof, against a human EGFR. It will be recognized in the art that some amino acid sequences of the invention can be varied without significant effect of the structure or function of the protein. Thus, the invention further includes variations of the polypeptides which show substantial activity or which

include regions of an antibody, or fragment thereof, against EGFR protein. Such mutants include deletions, insertions, inversions, repeats, and type substitutions.

[0133] The polypeptides and analogs can be further modified to contain additional chemical moieties not normally part of the protein. Those derivatized moieties can improve the solubility, the biological half-life or absorption of the protein. The moieties can also reduce or eliminate any desirable side effects of the proteins and the like. An overview for those moieties can be found in REMINGTON'S PHARMACEUTICAL SCIENCES, 20th ed., Mack Publishing Co., Easton, PA (2000).

[0134] The isolated polypeptides described herein can be produced by any suitable method known in the art. Such methods range from direct protein synthetic methods to constructing a DNA sequence encoding isolated polypeptide sequences and expressing those sequences in a suitable transformed host. In some embodiments, a DNA sequence is constructed using recombinant technology by isolating or synthesizing a DNA sequence encoding a wild-type protein of interest. Optionally, the sequence can be mutagenized by site-specific mutagenesis to provide functional analogs thereof. See, e.g. Zoeller et al., Proc. Nat'l. Acad. Sci. USA 81:5662-5666 (1984) and U.S. Pat. No. 4,588,585.

[0135] In some embodiments a DNA sequence encoding a polypeptide of interest would be constructed by chemical synthesis using an oligonucleotide synthesizer. Such oligonucleotides can be designed based on the amino acid sequence of the desired polypeptide and selecting those codons that are favored in the host cell in which the recombinant polypeptide of interest will be produced. Standard methods can be applied to synthesize an isolated polynucleotide sequence encoding an isolated polypeptide of interest. For example, a complete amino acid sequence can be used to construct a back-translated gene. Further, a DNA oligomer containing a nucleotide sequence coding for the particular isolated polypeptide can be synthesized. For example, several small oligonucleotides coding for portions of the desired polypeptide can be synthesized and then ligated. The individual oligonucleotides typically contain 5' or 3' overhangs for complementary assembly.

[0136] Once assembled (by synthesis, site-directed mutagenesis or another method), the polynucleotide sequences encoding a particular isolated polypeptide of interest will be inserted into an expression vector and operatively linked to an expression control sequence appropriate for expression of the protein in a desired host. Proper assembly can be confirmed by nucleotide sequencing, restriction mapping, and expression of a biologically active polypeptide in a suitable

host. As is well known in the art, in order to obtain high expression levels of a transfected gene in a host, the gene must be operatively linked to transcriptional and translational expression control sequences that are functional in the chosen expression host.

[0137] In certain embodiments, recombinant expression vectors are used to amplify and express DNA encoding antibodies, or fragments thereof, against human EGFR. Recombinant expression vectors are replicable DNA constructs which have synthetic or cDNA-derived DNA fragments encoding a polypeptide chain of an anti-EGFR antibody, or fragment thereof, operatively linked to suitable transcriptional or translational regulatory elements derived from mammalian, microbial, viral or insect genes. A transcriptional unit generally comprises an assembly of (1) a genetic element or elements having a regulatory role in gene expression, for example, transcriptional promoters or enhancers, (2) a structural or coding sequence which is transcribed into mRNA and translated into protein, and (3) appropriate transcription and translation initiation and termination sequences, as described in detail below. Such regulatory elements can include an operator sequence to control transcription. The ability to replicate in a host, usually conferred by an origin of replication, and a selection gene to facilitate recognition of transformants can additionally be incorporated. DNA regions are operatively linked when they are functionally related to each other. For example, DNA for a signal peptide (secretory leader) is operatively linked to DNA for a polypeptide if it is expressed as a precursor which participates in the secretion of the polypeptide; a promoter is operatively linked to a coding sequence if it controls the transcription of the sequence; or a ribosome binding site is operatively linked to a coding sequence if it is positioned so as to permit translation. Structural elements intended for use in yeast expression systems include a leader sequence enabling extracellular secretion of translated protein by a host cell. Alternatively, where recombinant protein is expressed without a leader or transport sequence, it can include an N-terminal methionine residue. This residue can optionally be subsequently cleaved from the expressed recombinant protein to provide a final product.

[0138] The choice of expression control sequence and expression vector will depend upon the choice of host. A wide variety of expression host/vector combinations can be employed. Useful expression vectors for eukaryotic hosts, include, for example, vectors comprising expression control sequences from SV40, bovine papilloma virus, adenovirus and cytomegalovirus. Useful expression vectors for bacterial hosts include known bacterial plasmids, such as plasmids from *Escherichia coli*, including pCR 1, pBR322, pMB9 and their derivatives, wider host range plasmids, such as M13 and filamentous single-stranded DNA phages.

[0139] Suitable host cells for expression of a EGFR-binding polypeptide or antibody (or a EGFR protein to use as an antigen) include prokaryotes, yeast, insect or higher eukaryotic cells under the control of appropriate promoters. Prokaryotes include gram negative or gram positive organisms, for example *E. coli* or bacilli. Higher eukaryotic cells include established cell lines of mammalian origin as described below. Cell-free translation systems could also be employed. Appropriate cloning and expression vectors for use with bacterial, fungal, yeast, and mammalian cellular hosts are described by Pouwels et al. (Cloning Vectors: A Laboratory Manual, Elsevier, N.Y., 1985), the relevant disclosure of which is hereby incorporated by reference. Additional information regarding methods of protein production, including antibody production, can be found, e.g., in U.S. Patent Publication No. 2008/0187954, U.S. Patent Nos. 6,413,746 and 6,660,501, and International Patent Publication No. WO 04009823, each of which is hereby incorporated by reference herein in its entirety.

[0140] Various mammalian or insect cell culture systems are also advantageously employed to express recombinant protein. Expression of recombinant proteins in mammalian cells can be performed because such proteins are generally correctly folded, appropriately modified and completely functional. Examples of suitable mammalian host cell lines include the COS-7 lines of monkey kidney cells, described by Gluzman (Cell 23:175, 1981), and other cell lines capable of expressing an appropriate vector including, for example, L cells, C127, 3T3, Chinese hamster ovary (CHO), HeLa and BHK cell lines. Mammalian expression vectors can comprise nontranscribed elements such as an origin of replication, a suitable promoter and enhancer linked to the gene to be expressed, and other 5' or 3' flanking nontranscribed sequences, and 5' or 3' nontranslated sequences, such as necessary ribosome binding sites, a polyadenylation site, splice donor and acceptor sites, and transcriptional termination sequences. Baculovirus systems for production of heterologous proteins in insect cells are reviewed by Luckow and Summers, Bio/Technology 6:47 (1988).

[0141] The proteins produced by a transformed host can be purified according to any suitable method. Such standard methods include chromatography (e.g., ion exchange, affinity and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for protein purification. Affinity tags such as hexahistidine, maltose binding domain, influenza coat sequence and glutathione-S-transferase can be attached to the protein to allow easy purification by passage over an appropriate affinity column. Isolated proteins can also be

physically characterized using such techniques as proteolysis, nuclear magnetic resonance and x-ray crystallography.

[0142] For example, supernatants from systems which secrete recombinant protein into culture media can be first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. Following the concentration step, the concentrate can be applied to a suitable purification matrix. Alternatively, an anion exchange resin can be employed, for example, a matrix or substrate having pendant diethylaminoethyl (DEAE) groups. The matrices can be acrylamide, agarose, dextran, cellulose or other types commonly employed in protein purification. Alternatively, a cation exchange step can be employed. Suitable cation exchangers include various insoluble matrices comprising sulfopropyl or carboxymethyl groups. Finally, one or more reversed-phase high performance liquid chromatography (RP-HPLC) steps employing hydrophobic RP-HPLC media, e.g., silica gel having pendant methyl or other aliphatic groups, can be employed to further purify a EGFR-binding agent. Some or all of the foregoing purification steps, in various combinations, can also be employed to provide a homogeneous recombinant protein.

[0143] Recombinant protein produced in bacterial culture can be isolated, for example, by initial extraction from cell pellets, followed by one or more concentration, salting-out, aqueous ion exchange or size exclusion chromatography steps. High performance liquid chromatography (HPLC) can be employed for final purification steps. Microbial cells employed in expression of a recombinant protein can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents.

[0144] Methods known in the art for purifying antibodies and other proteins also include, for example, those described in U.S. Patent Publication No. 2008/0312425, 2008/0177048, and 2009/0187005, each of which is hereby incorporated by reference herein in its entirety.

[0145] In certain embodiments, the EGFR-binding agent is a polypeptide that is not an antibody. A variety of methods for identifying and producing non-antibody polypeptides that bind with high affinity to a protein target are known in the art. See, e.g., Skerra, *Curr. Opin. Biotechnol.*, 18:295-304 (2007), Hosse et al., *Protein Science*, 15:14-27 (2006), Gill et al., *Curr. Opin. Biotechnol.*, 17:653-658 (2006), Nygren, *FEBS J.*, 275:2668-76 (2008), and Skerra, *FEBS J.*, 275:2677-83 (2008), each of which is incorporated by reference herein in its entirety. In certain embodiments, phage display technology has been used to identify/produce the EGFR-binding polypeptide. In certain embodiments, the polypeptide comprises a protein scaffold of a type

selected from the group consisting of protein A, a lipocalin, a fibronectin domain, an ankyrin consensus repeat domain, and thioredoxin.

[0146] In some embodiments, the agent is a non-protein molecule. In certain embodiments, the agent is a small molecule. Combinatorial chemistry libraries and techniques useful in the identification of non-protein EGFR-binding agents are known to those skilled in the art. See, e.g., Kennedy et al., *J. Comb. Chem.*, 10:345-354 (2008), Dolle et al, *J. Comb. Chem.*, 9:855-902 (2007), and Bhattacharyya, *Curr. Med. Chem.*, 8:1383-404 (2001), each of which is incorporated by reference herein in its entirety. In certain further embodiments, the agent is a carbohydrate, a glycosaminoglycan, a glycoprotein, or a proteoglycan.

[0147] In certain embodiments, the agent is a nucleic acid aptamer. Aptamers are polynucleotide molecules that have been selected (e.g., from random or mutagenized pools) on the basis of their ability to bind to another molecule. In some embodiments, the aptamer comprises a DNA polynucleotide. In certain alternative embodiments, the aptamer comprises an RNA polynucleotide. In certain embodiments, the aptamer comprises one or more modified nucleic acid residues. Methods of generating and screening nucleic acid aptamers for binding to proteins are well known in the art. See, e.g., U.S. Patent No. 5,270,163, U.S. Patent No. 5,683,867, U.S. Patent No. 5,763,595, U.S. Patent No. 6,344,321, U.S. Patent No. 7,368,236, U.S. Patent No. 5,582,981, U.S. Patent No. 5,756,291, U.S. Patent No. 5,840,867, U.S. Patent No. 7,312,325, U.S. Patent No. 7,329,742, International Patent Publication No. WO 02/077262, International Patent Publication No. WO 03/070984, U.S. Patent Application Publication No. 2005/0239134, U.S. Patent Application Publication No. 2005/0124565, and U.S. Patent Application Publication No. 2008/0227735, each of which is incorporated by reference herein in its entirety.

III. Immunoconjugates

[0148] The present invention is also directed to conjugates (also referred to herein as immunoconjugates), comprising the anti-EGFR antibodies, antibody fragments, and their functional equivalents as disclosed herein, linked or conjugated to a drug or prodrug. Suitable drugs or prodrugs are known in the art. The drugs or prodrugs can be cytotoxic agents. The cytotoxic agent used in the cytotoxic conjugate of the present invention can be any compound that results in the death of a cell, or induces cell death, or in some manner decreases cell viability, and includes, for example, maytansinoids and maytansinoid analogs. Other suitable cytotoxic agents are for example benzodiazepines, taxoids, CC-1065 and CC-1065 analogs, duocarmycins and duocarmycin analogs, enediynes, such as calicheamicins, dolastatin and dolastatin analogs

including auristatins, tomaymycin derivatives, leptomycin derivatives, methotrexate, cisplatin, carboplatin, daunorubicin, doxorubicin, vincristine, vinblastine, melphalan, mitomycin C, chlorambucil and morpholino doxorubicin.

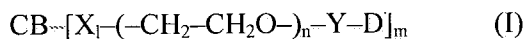
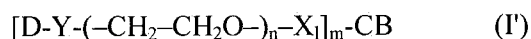
[0149] Such conjugates can be prepared by using a linking group in order to link a drug or prodrug to the antibody or functional equivalent. Suitable linking groups are well known in the art and include, for example, disulfide groups, thioether groups, acid labile groups, photolabile groups, peptidase labile groups and esterase labile groups.

[0150] The drug or prodrug can, for example, be linked to the anti-EGFR antibody or fragment thereof through a disulfide bond. The linker molecule or crosslinking agent comprises a reactive chemical group that can react with the anti-EGFR antibody or fragment thereof. The reactive chemical groups for reaction with the cell-binding agent can be *N*-succinimidyl esters and *N*-sulfosuccinimidyl esters. Additionally the linker molecule comprises a reactive chemical group, which can be a dithiopyridyl group that can react with the drug to form a disulfide bond. Linker molecules include, for example, *N*-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) (see, e.g., Carlsson et al., *Biochem. J.*, 173: 723-737 (1978)), *N*-succinimidyl 4-(2-pyridyldithio)butanoate (SPDB) (see, e.g., U.S. Patent No. 4,563,304), *N*-succinimidyl 4-(2-pyridyldithio)2-sulfobutanoate (sulfo-SPDB) (see US Publication No. 20090274713), *N*-succinimidyl 4-(2-pyridyldithio) pentanoate (SPP) (see, e.g., CAS Registry number 341498-08-6), 2-iminothiolane, or acetylsuccinic anhydride. For example, the antibody or cell binding agent can be modified with crosslinking reagents and the antibody or cell binding agent containing free or protected thiol groups thus derived is then reacted with a disulfide- or thiol-containing maytansinoid to produce conjugates. The conjugates can be purified by chromatography, including but not limited to HPLC, size-exclusion, adsorption, ion exchange and affinity capture, dialysis or tangential flow filtration.

[0151] In another aspect of the present invention, the anti-EGFR antibody is linked to cytotoxic drugs via disulfide bonds and a polyethylene glycol spacer in enhancing the potency, solubility or the efficacy of the immunoconjugate. Such cleavable hydrophilic linkers are described in WO2009/0134976. The additional benefit of this linker design is the desired high monomer ratio and the minimal aggregation of the antibody-drug conjugate. Specifically contemplated in this aspect are conjugates of cell-binding agents and drugs linked via disulfide group (-S-S-) bearing polyethylene glycol spacers ((CH₂CH₂O)_{n=1-14}) with a narrow range of drug load of 2-8 are described that show relatively high potent biological activity toward cancer cells and have the

desired biochemical properties of high conjugation yield and high monomer ratio with minimal protein aggregation.

[0152] Specifically contemplated in this aspect is an anti-EGFR antibody drug conjugate of formula (I) or a conjugate of formula (I'):



wherein:

[0153] CB represents an anti-EGFR antibody or fragment;

[0154] D represents a drug;

[0155] X represents an aliphatic, an aromatic or a heterocyclic unit attached to the cell-binding agent via a thioether bond, an amide bond, a carbamate bond, or an ether bond;

[0156] Y represents an aliphatic, an aromatic or a heterocyclic unit attached to the drug via a disulfide bond;

[0157] l is 0 or 1;

[0158] m is an integer from 2 to 8; and

[0159] n is an integer from 1 to 24.

[0160] In some embodiments, m is an integer from 2 to 6.

[0161] In some embodiments, m is an integer from 3 to 5.

[0162] In some embodiments, n is an integer from 2 to 8. Alternatively, as disclosed in, for example, U.S. Patent No. 6,441,163 and 7,368,565, the drug can be first modified to introduce a reactive ester suitable to react with a cell-binding agent. Reaction of these drugs containing an activated linker moiety with a cell-binding agent provides another method of producing a cell-binding agent drug conjugate. Maytansinoids can also be linked to anti-EGFR antibody or fragment using PEG linking groups, as set forth for example in U.S. Patent 6,716,821. These PEG non-cleavable linking groups are soluble both in water and in non-aqueous solvents, and can be used to join one or more cytotoxic agents to a cell binding agent. Exemplary PEG linking groups include heterobifunctional PEG linkers that react with cytotoxic agents and cell binding agents at opposite ends of the linkers through a functional sulfhydryl or disulfide group at one end, and an active ester at the other end. As a general example of the synthesis of a cytotoxic conjugate using a PEG linking group, reference is again made to U.S. Patent 6,716,821 which is incorporated entirely by reference herein. Synthesis begins with the reaction of one or more cytotoxic agents bearing a reactive PEG moiety with a cell-binding agent, resulting in

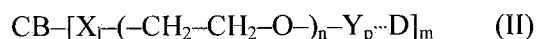
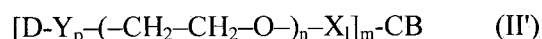
displacement of the terminal active ester of each reactive PEG moiety by an amino acid residue of the cell binding agent, to yield a cytotoxic conjugate comprising one or more cytotoxic agents covalently bonded to a cell binding agent through a PEG linking group. Alternatively, the cell binding can be modified with the bifunctional PEG crosslinker to introduce a reactive disulfide moiety (such as a pyridyldisulfide), which can then be treated with a thiol-containing maytansinoid to provide a conjugate. In another method, the cell binding can be modified with the bifunctional PEG crosslinker to introduce a thiol moiety which can then be treated with a reactive disulfide-containing maytansinoid (such as a pyridyldisulfide), to provide a conjugate.

[0163] Antibody-maytansinoid conjugates with non-cleavable links can also be prepared. Such crosslinkers are described in the art (see US Publication No. 20050169933) and include but are not limited to, *N*-succinimidyl 4-(maleimidomethyl) cyclohexanecarboxylate (SMCC). In some embodiments, the antibody is modified with crosslinking reagents such as succinimidyl 4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate (SMCC), sulfo-SMCC, maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS), sulfo-MBS or succinimidyl-iodoacetate, as described in the literature, to introduce 1-10 reactive groups (Yoshitake et al, Eur. J. Biochem., 101:395-399 (1979); Hashida et al, J. Applied Biochem., 56-63 (1984); and Liu et al, Biochem., 18:690-697 (1979)). The modified antibody is then reacted with the thiol-containing maytansinoid derivative to produce a conjugate. The conjugate can be purified by gel filtration through a Sephadex G25 column or by dialysis or tangential flow filtration. The modified antibodies are treated with the thiol-containing maytansinoid (1 to 2 molar equivalent/maleimido group) and antibody-maytansinoid conjugates are purified by gel filtration through a Sephadex G-25 column, chromatography on a ceramic hydroxyapatite column, dialysis or tangential flow filtration or a combination of methods thereof. Typically, an average of 1-10 maytansinoids per antibody are linked. One method is to modify antibodies with succinimidyl 4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate (SMCC) to introduce maleimido groups followed by reaction of the modified antibody with a thiol-containing maytansinoid to give a thioether-linked conjugate. Again conjugates with 1 to 10 drug molecules per antibody molecule result. Maytansinoid conjugates of antibodies, antibody fragments, and other proteins are made in the same way.

[0164] In another aspect of the invention, the EGFR antibody is linked to the drug via a non-cleavable bond through the intermediacy of a PEG spacer. Suitable crosslinking reagents comprising hydrophilic PEG chains that form linkers between a drug and the anti-EGFR antibody or fragment are also well known in the art, or are commercially available (for example

from Quanta Biodesign, Powell, Ohio). Suitable PEG-containing crosslinkers can also be synthesized from commercially available PEGs themselves using standard synthetic chemistry techniques known to one skilled in the art. The drugs can be reacted with bifunctional PEG-containing cross linkers to give compounds of the following formula, $Z-X_l-(CH_2-CH_2-O)_n-Y_p-D$, by methods described in detail in US Patent Publication 20090274713 and in WO2009/0134976, which can then react with the cell binding agent to provide a conjugate. Alternatively, the cell binding can be modified with the bifunctional PEG crosslinker to introduce a thiol-reactive group (such as a maleimide or haloacetamide) which can then be treated with a thiol-containing maytansinoid to provide a conjugate. In another method, the cell binding can be modified with the bifunctional PEG crosslinker to introduce a thiol moiety which can then be treated with a thiol-reactive maytansinoid (such as a maytansinoid bearing a maleimide or haloacetamide), to provide a conjugate.

[0165] Accordingly, another aspect of the present invention is an anti-EGFR antibody drug conjugate of formula (II) or of formula (II'):



wherein, CB represents an anti-EGFR antibody or fragment;

[0166] D represents a drug;

[0167] X represents an aliphatic, an aromatic or a heterocyclic unit bonded to the cell-binding agent via a thioether bond, an amide bond, a carbamate bond, or an ether bond;

[0168] Y represents an aliphatic, an aromatic, or a heterocyclic unit bonded to the drug via a covalent bond selected from the group consisting of a thioether bond, an amide bond, a carbamate bond, an ether bond, an amine bond, a carbon-carbon bond and a hydrazone bond;

[0169] l is 0 or 1;

[0170] p is 0 or 1;

[0171] m is an integer from 2 to 15; and

[0172] n is an integer from 1 to 2000.

[0173] In some embodiments, m is an integer from 2 to 8; and

[0174] In some embodiments, n is an integer from 1 to 24.

[0175] In some embodiments, m is an integer from 2 to 6.

[0176] In some embodiments, m is an integer from 3 to 5.

[0177] In some embodiments, *n* is an integer from 2 to 8. Examples of suitable PEG-containing linkers include linkers having an *N*-succinimidyl ester or *N*-sulfosuccinimidyl ester moiety for reaction with the anti-EGFR antibody or fragment thereof, as well as a maleimido- or haloacetyl-based moiety for reaction with the compound. A PEG spacer can be incorporated into any crosslinker known in the art by the methods described herein.

[0178] Many of the linkers disclosed herein are described in detail in U.S. Patent Publication Nos. 20050169933 and 20090274713, and in WO2009/0134976; the contents of which are entirely incorporated herein by reference.

[0179] The present invention includes aspects wherein about 2 to about 8 drug molecules ("drug load"), for example, maytansinoid, are linked to an anti-EGFR antibody or fragment thereof, the anti-tumor effect of the conjugate is much more efficacious as compared to a drug load of a lesser or higher number of drugs linked to the same cell binding agent. "Drug load", as used herein, refers to the number of drug molecules (e.g., a maytansinoid) that can be attached to a cell binding agent (e.g., an anti-EGFR antibody or fragment thereof). In one aspect the number of drug molecules that can be attached to a cell binding agent can average from about 2 to about 8 (e.g., 1.9, 2.0, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3.0, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4.0, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5.0, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, 6.0, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, 7.0, 7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9, 8.0, 8.1). *N*^{2'}-deacetyl-*N*^{2'}-(3-mercapto-1-oxopropyl)-maytansine (DM1) and *N*^{2'}-deacetyl-*N*^{2'}-(4-mercapto-4-methyl-1-oxopentyl) maytansine (DM4) can be used.

[0180] The anti-EGFR antibody or fragment thereof can be modified by reacting a bifunctional crosslinking reagent with the anti-EGFR antibody or fragment thereof, thereby resulting in the covalent attachment of a linker molecule to the anti-EGFR antibody or fragment thereof. As used herein, a "bifunctional crosslinking reagent" is any chemical moiety that covalently links a cell-binding agent to a drug, such as the drugs described herein. In another method, a portion of the linking moiety is provided by the drug. In this respect, the drug comprises a linking moiety that is part of a larger linker molecule that is used to join the cell-binding agent to the drug. For example, to form the maytansinoid DM1, the side chain at the C-3 hydroxyl group of maytansine is modified to have a free sulfhydryl group (SH). This thiolated form of maytansine can react with a modified cell-binding agent to form a conjugate. Therefore, the final linker is assembled from two components, one of which is provided by the crosslinking reagent, while the other is provided by the side chain from DM1.

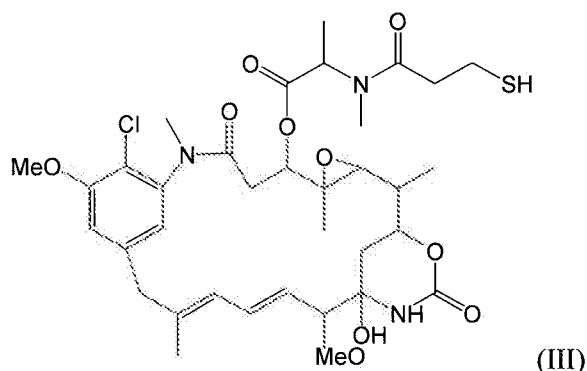
[0181] The drug molecules can also be linked to the antibody molecules through an intermediary carrier molecule such as serum albumin.

[0182] As used herein, the expression "linked to a cell-binding agent" or "linked to an anti-EGFR antibody or fragment" refers to the conjugate molecule comprising at least one drug derivative bound to a cell-binding agent anti-EGFR antibody or fragment via a suitable linking group, or a precursor thereof. One linking group is SMCC.

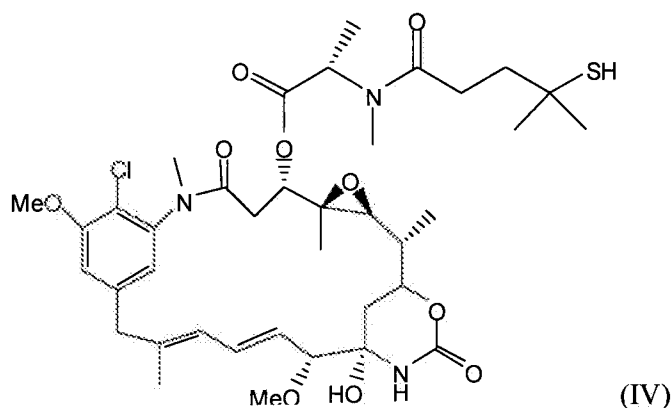
[0183] In certain embodiments, cytotoxic agents useful in the present invention are maytansinoids and maytansinoid analogs. Examples of suitable maytansinoids include esters of maytansinol and maytansinol analogs. Included are any drugs that inhibit microtubule formation and that are highly toxic to mammalian cells, as are maytansinol and maytansinol analogs.

[0184] Examples of suitable maytansinol esters include those having a modified aromatic ring and those having modifications at other positions. Such suitable maytansinoids are disclosed in U.S. Patent Nos. 4,424,219; 4,256,746; 4,294,757; 4,307,016; 4,313,946; 4,315,929; 4,331,598; 4,361,650; 4,362,663; 4,364,866; 4,450,254; 4,322,348; 4,371,533; 5,208,020; 5,416,064; 5,475,092; 5,585,499; 5,846,545; 6,333,410; 7,276,497 and 7,473,796.

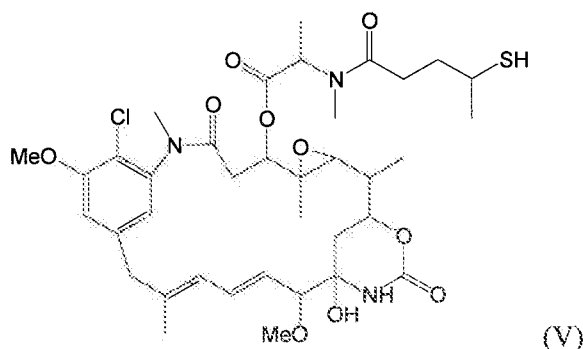
[0185] In a certain embodiment, the immunoconjugates of the invention utilize the thiol-containing maytansinoid (DM1), formally termed *N*^{2'}-deacetyl-*N*^{2'}-(3-mercapto-1-oxopropyl)-maytansine, as the cytotoxic agent. DM1 is represented by the following structural formula (III):



[0186] In another embodiment, the conjugates of the present invention utilize the thiol-containing maytansinoid *N*^{2'}-deacetyl-*N*^{2'}-(4-methyl-4-mercapto-1-oxopentyl)-maytansine (e.g., DM4) as the cytotoxic agent. DM4 is represented by the following structural formula (IV):



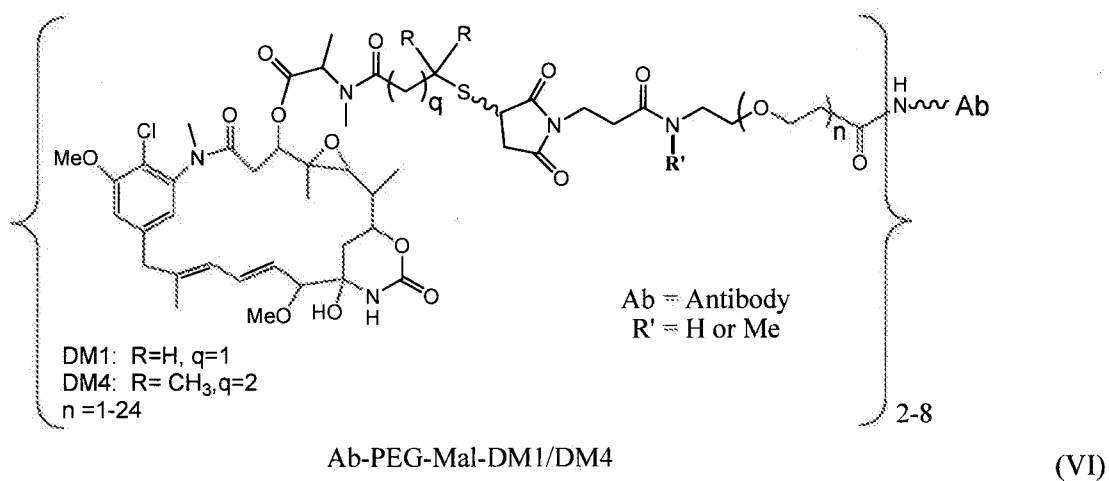
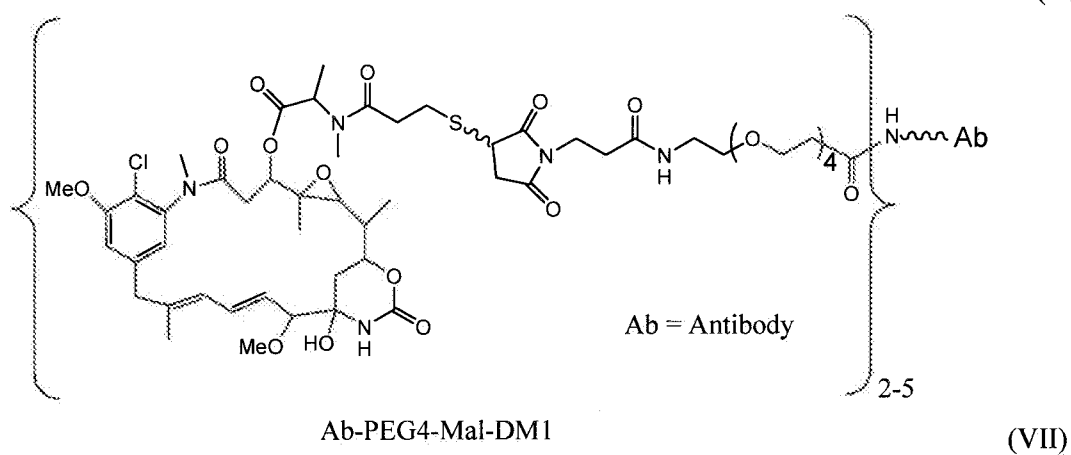
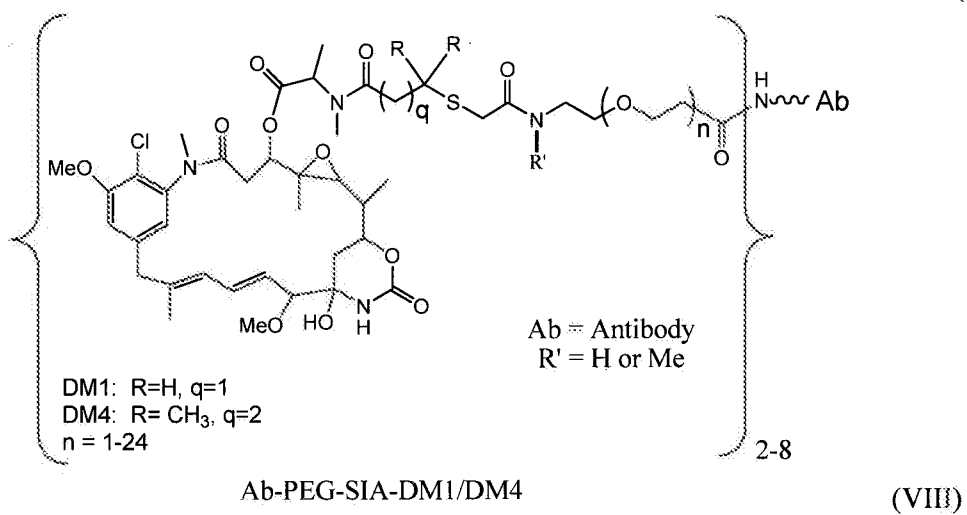
[0187] Another maytansinoid comprising a side chain that contains a sterically hindered thiol bond is *N*^{2'}-deacetyl-*N*-2'(4-mercapto-1-oxopentyl)-maytansine (termed DM3), represented by the following structural formula (V):

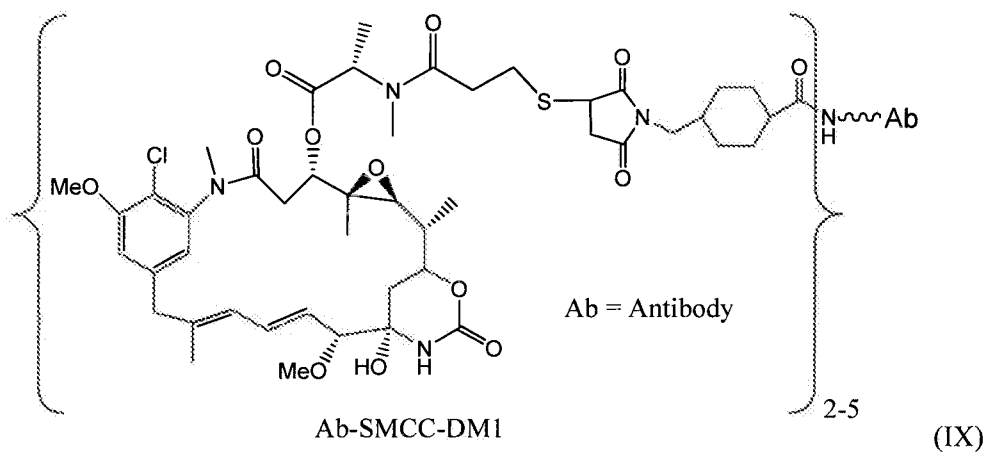
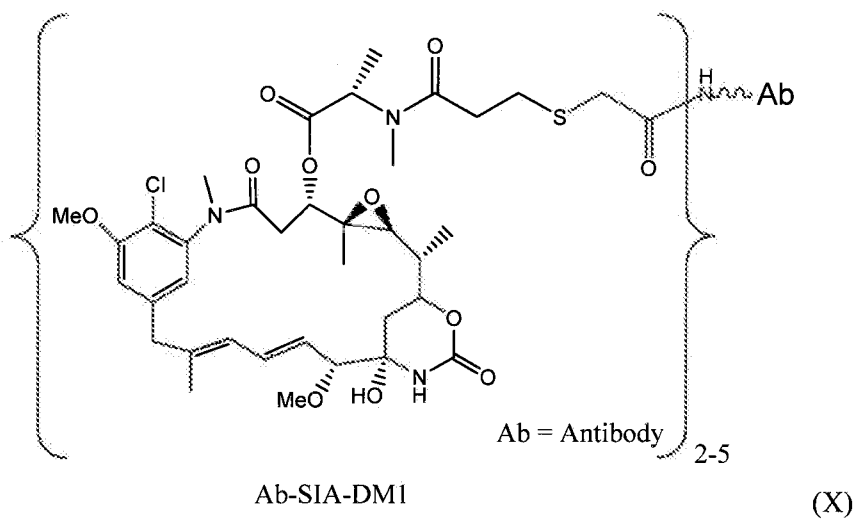
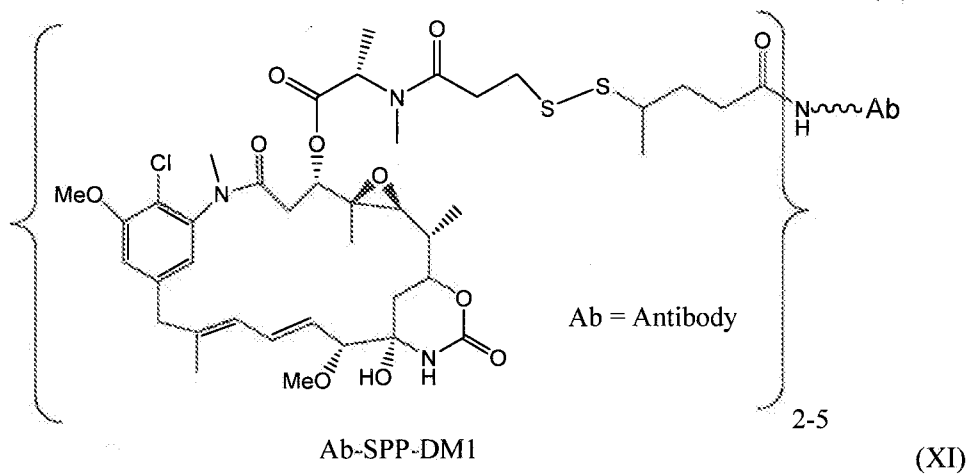


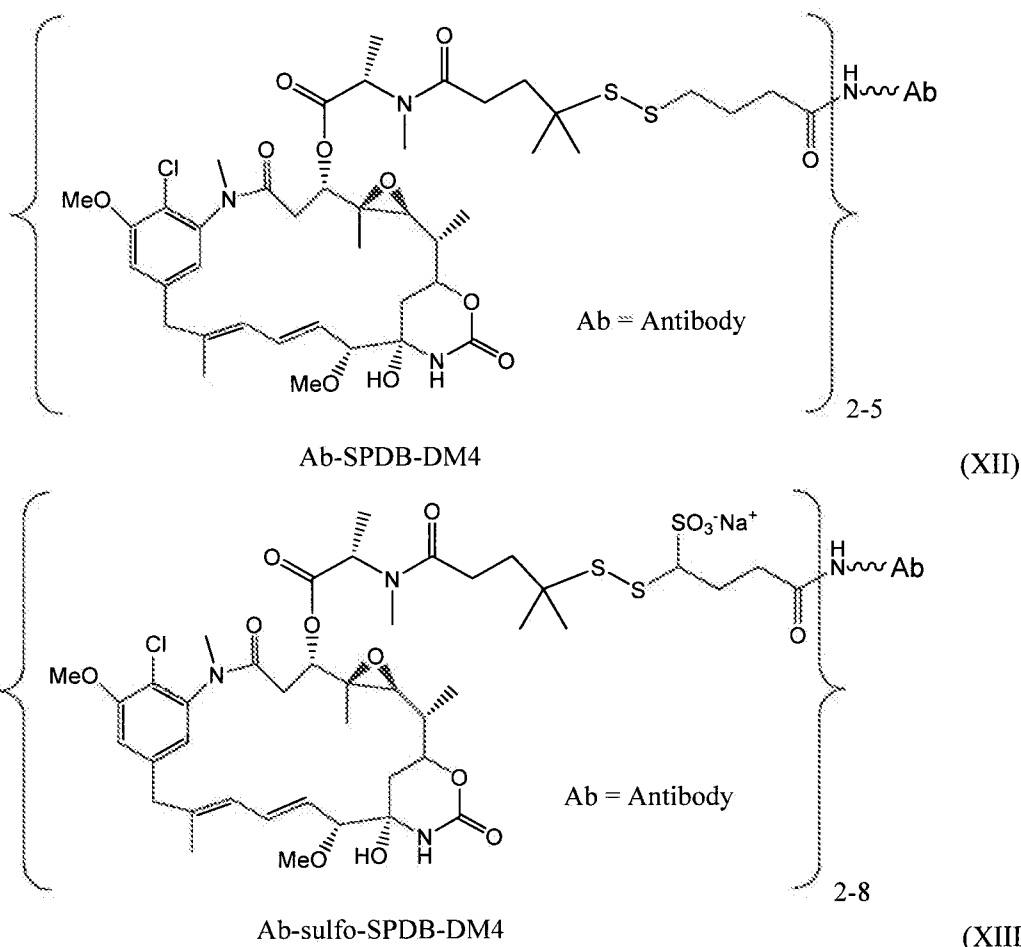
[0188] Each of the maytansinoids taught in US Patent No. 5,208,020 and 7,276,497, can also be used in the conjugate of the present invention. In this regard, the entire disclosure of 5,208,020 and 7,276,697 is incorporated herein by reference.

[0189] Many positions on maytansinoids can serve as the position to chemically link the linking moiety. For example, the C-3 position having a hydroxyl group, the C-14 position modified with hydroxymethyl, the C-15 position modified with hydroxy and the C-20 position having a hydroxy group are all expected to be useful. In some embodiments, the C-3 position serves as the position to chemically link the linking moiety, and in some particular embodiments, the C-3 position of maytansinol serves as the position to chemically link the linking moiety.

[0190] Structural representations of some conjugates are shown below:







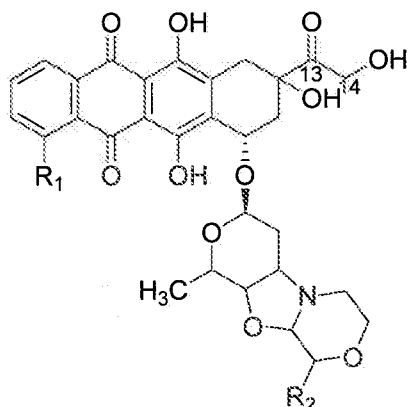
[0191] Several descriptions for producing such antibody-maytansinoid conjugates are provided in U.S. Patent Nos. 6,333,410, 6,441,163, 6,716,821, and 7,368,565, each of which is incorporated herein in its entirety.

[0192] In general, a solution of an antibody in aqueous buffer can be incubated with a molar excess of maytansinoids having a disulfide moiety that bears a reactive group. The reaction mixture can be quenched by addition of excess amine (such as ethanolamine, taurine, etc.). The maytansinoid-antibody conjugate can then be purified by gel filtration.

[0193] The number of maytansinoid molecules bound per antibody molecule can be determined by measuring spectrophotometrically the ratio of the absorbance at 252 nm and 280 nm. The average number of maytansinoid molecules/antibody can be, for example, 1-10 or 2-5.

[0194] Anthracycline compounds, as well as derivatives, intermediates and modified versions thereof, can also be used to prepare anti-EGFR immunoconjugates. For example, doxorubicin, doxorubicin derivatives, doxorubicin intermediates, and modified doxorubicins can be used in anti-EGFR conjugates. Exemplary compounds are described in WO 2010/009124, which is

herein incorporated by reference in its entirety. Such compounds include, for example, compounds of the following formula:



[0195] wherein R_1 is a hydrogen atom, hydroxy or methoxy group and R_2 is a C_1 - C_5 alkoxy group, or a pharmaceutically acceptable salt thereof.

[0196] Conjugates of antibodies with maytansinoid or other drugs can be evaluated for their ability to suppress proliferation of various unwanted cell lines *in vitro*. For example, cell lines such NCI-H226, NCI-H292, and NCI-H322M, can easily be used for the assessment of cytotoxicity of these compounds. Cells to be evaluated can be exposed to the compounds for 4 to 5 days and the surviving fractions of cells measured in direct assays by known methods. IC_{50} values can then be calculated from the results of the assays.

[0197] The immunoconjugates can, according to some embodiments described herein, be internalized into cells. The immunoconjugate, therefore, can exert a therapeutic effect when it is taken up by, or internalized, by a EGFR-expressing cell. In some particular embodiments, the immunoconjugate comprises an antibody, antibody fragment, or polypeptide, linked to a cytotoxic agent by a cleavable linker, and the cytotoxic agent is cleaved from the antibody, antibody fragment, or polypeptide, wherein it is internalized by a EGFR-expressing cell.

[00121] In some embodiments, the immunoconjugates are capable of reducing tumor volume. For example, in some embodiments, treatment with an immunoconjugate results in a %T/C value that is less than about 50%, less than about 45%, less than about 40%, less than about 35%, less than about 30%, less than about 25%, less than about 20%, less than about 15%, less than about 10%, or less than about 5%.

[0198] In another aspect of the invention siRNA molecules can be linked to the antibodies of the present invention instead of a drug. siRNAs can be linked to the antibodies of the present invention by methods commonly used for the modification of oligonucleotides (see, for example, US Patent Publications 20050107325 and 20070213292). Thus the siRNA in its 3' or 5'-

phosphoromidite form can be reacted with one end of the crosslinker bearing a hydroxyl functionality to give an ester bond between the siRNA and the crosslinker. Similarly reaction of the siRNA phosphoramidite with a crosslinker bearing a terminal amino group results in linkage of the crosslinker to the siRNA through an amine. Alternatively, the siRNA can be derivatized by standard chemical methods to introduce a thiol group. This thiol-containing siRNA can be reacted with an antibody that has been modified to introduce an active disulfide or maleimide moiety, to produce a cleavable or non-cleavable conjugate. Between 1 - 20 siRNA molecules can be linked to an antibody by this method.

III. Polynucleotides

[0199] In certain embodiments, the invention encompasses polynucleotides comprising polynucleotides that encode a polypeptide that specifically binds EGFR or a fragment of such a polypeptide. For example, the invention provides a polynucleotide comprising a nucleic acid sequence that encodes an antibody to a human EGFR or encodes a fragment of such an antibody. The polynucleotides of the invention can be in the form of RNA or in the form of DNA. DNA includes cDNA, genomic DNA, and synthetic DNA; and can be double-stranded or single-stranded, and if single stranded can be the coding strand or non-coding (anti-sense) strand.

[0200] In certain embodiments, the polynucleotides are isolated. In certain embodiments, the polynucleotides are substantially pure.

[0201] The invention provides a polynucleotide comprising a polynucleotide encoding a polypeptide comprising a sequence selected from the group consisting of SEQ ID NOs:1-38 and 69-76.

[0202] The invention further provides a polynucleotide comprising a sequence selected from those shown in Tables 7-9 below.

Table 7: Variable heavy chain polynucleotide sequences

Antibody	VH Polynucleotide Sequence
muEGFR-7 V _H	caggttcagctccagcagctctggggctgagctggcaagacctggggcttcagtgaaagttgtcctgcaagg cttctggctacacctttactagctactggatgcagtggtgaaacagaggcctggacagggctctggaatg tattgggactatttatcctggagatggtgatactacgtacactcagaagttcaagggaaggccacattg actgcagataaatcctccagcacagcctacatgcaactcagcagcttggcatctgaggactctcggtct attactgtgcaagatatgatgccccggctatgctatggactactggggctcaaggaaacctcagtcaccgt ctcctca (SEQ ID NO:39)
muEGFR-12 V _H	caggttcagctccagcagctctgggactgagctggcaagacctggggcttcagtgaaagttgtcctgcaagg cttctggctacacctttactagctactggatgcagtggtgaaacagaggcctggacagggctctggaatg tattgggactatctatcctggagatggtgatactaggacattcagaagttcaagggaaggccacattg

Antibody	VH Polynucleotide Sequence
	actgcagataaatcctccagcacagcctacatgcaactcagcagcttggcatctgaggactctgcggctct attactgtgcaagatatgatgccccggctatgctatggactactgggggtcaaggaacctcagtcaccgt ctctca (SEQ ID NO:40)
huEGFR- 7 V _H	aagcttggccaccatgggctggctcatgtatcattctgttcttgggtggccaccgcaaccgggtgccattccca gggtgcagctcgtgcagagcgggggtgaagtggccaagccaggtgcttctgtcaaattgtcttgaaggcc agtgggtacaccttcacaagctactggatgcagtgggttaagcaacgccaggccagggactggagtgc atcggcaccatttatccagggtatggagataccactatacacaagtttcaaggcaagccaccctgac cggcgacaaatccagcagcacagcatacatgcagctttctagcctcaggtctgaagactccggctgtact attgtgcccgtacgacgccccggctatgcaatggattactggggccagggtactctggtcacagtgtcct ccgcctctacaaagggccc (SEQ ID NO:41)
huEGFR- 7 V _H CDR grafted	aagcttggccaccatggggtggctctgtataactgtttctgggtggccactgccacaggagtccacagccaag tgcagctgggtgcagagtgggcgtgagggtcaagaagcctggggcatccgtcaagtttcttgaaggcatctgg atataccttcacttctattggatgcagtgggtgagacaggcaccaggacagggactggagtggatgggact atttatccaggtgacgggtgacactacttatactcagaattcaagggcgagtgacctgactcgtgatactagc actagtagcgtgtatggagcttagttctctccgggtccaggacacagcagcttactactgtgctagatatgacg caccggatagccatggactattgggggcaggggcacctgtgaccgtgagttccggcagcactaagggccc (SEQ ID NO:42)
huEGFR- 12 V _H	aagcttggccaccatgggctggctctgtattatcctcttttgggtggccactgctaccggcgtacacagtcaggtgc agctgggtgcagtcgggggtgaagtggcaagcccggggcctccgtaaagctcttgaaggcatccggct acacttttacttctactggatgcagtggtgcaaacagcggccaggacagggggtggaatgtataggtacaatct atccggcgatgggtgacacacgatataccagaagttccagggaaggtaccctgactccgacaaatcttcta gcaccgcttatatgcagctgtcatctcttgaagtgaagactctgcagtgtattactgcgcccgatagacgcaccc ggttacgccatggattactgggggtcaggggaccttgtaaccgtatcaagcgcagtagccaagggccc (SEQ ID NO:43)
muEGFR- 6 V _H	cagggttcagctccagcagcttggggctgagctggcaagacctggggcttcagtgaa ttgtcctgcaaggcttctggctacaccttactagctactggatgcagtggtgaaaacagaggcctggac agggtctggaatgtattggggctcttactcctggagatgggtgctaggtacactcagaaattcaaggg caaggccacattgactgcagatagatcctccagcacagcctacatgcaactcagcagcttggcatctgag gactctgcggctattactgtgcaagatatgatgccccggctatgctatggactactgggggtcaaggaa cctcagtcaccgtcgctca (SEQ ID NO:77)
huEGFR-6 V _H v1.0	aagcttggccaccatggggtggagttgtatcctcttcttctgtcgtaccggcactggagtgcatccaggtg cagttggtgcaatctggcgccgaggtggccaagcccgggtgcctccgtaaaattgagttgaaagcctctggcta tacattacatcttattggatgcagtggtgcaagcagcgccctgggtcaaggcctggagtgcatcggagctctgta tcttggcgacggggacggccgttactcagaaattcagggcaagctaccctaccgcagatacatccagc agcactgcttatatgcaacttagtagcctccgcagcgaggatagtgccgtgtactactgtgccagatatgacgcc caggttatgctatggactactgggggtcaaggaaccctgggtgacagtgcaagcgtagcacaagggccc (SEQ ID NO:78)
huEGFR-6 V _H v1.11	aagcttggccaccatggggtggagttgtatcctcttcttctgtcgtaccggcactggagtgcatccaggtgca gttgggtgcaatctggcgccgaggtggccaagcccgggtgcctccgtaaaattgagttgaaagcctctggctataca tttacatcttattggatgcagtggtgcaagcagcgccctgggtcaaggcctggagtggtatcggagctctgtatcctgg cgacggggacggccgttactcagaaattcagggcaagctaccctaccgcagatacatccagcagcactg cttatatgcaacttagtagcctccgcagcgaggatagtgccgtgtactactgtgccagatatgacgccccaggttat gctatggactactgggggtcaaggaaccctgggtgacagtgcaagcgttagcacaagggccc (SEQ ID NO:79)
huEGFR-7 V _H v1.11	aagcttggccaccatgggctggctcatgtatcattctgttcttgggtggccaccgcaaccgggtgccattccaggtgcag ctcgtgcagagcgggggtgaagtggccaagccaggtgcttctgtcaaattgtcttgaaggccagtggggtacactt cacaagctactggatgcagtggttaagcaacgccaggccagggtgagtggtatcggcaccatttatccagg ggatggagataccactatacacaagtttcaaggcaagccaccctgaccggcgacaaatccagcagcagc atacatgcaagcttctagcctcaggtctgaagactccggctgtactattgtgcccgtacgacgccccggctatgc

Antibody	VH Polynucleotide Sequence
	aatggaattactggggccagggtactctgtcacagtctctccgcctctacaaaggccc (SEQ ID NO:80)

Table 8: Variable light chain polynucleotide sequences

Antibody	VL Polynucleotide Sequence
muEGFR-7 V _L	gacatccagatgacacagtctccatctcactgtctgcatctctgggaggcaaaagtcaccatcacittgca aggcaagccaagacattaacaactatttggcttggtaccaacacaagcctggaaaaggctcctaggctgct catacattacacatctacattacatccaggcatcccatcaaggttcagtggagtggtctgggagagat tattccttcagcatcagcaacctggagcctgaagatattgcaacttatttctacagtatgataatc ttctgtacacgttcggaggggggaccgaagctggaataaaacgg (SEQ ID NO:44)
muEGFR-12 V _L	gatgtccagataaccagctctccatcttattctgtgcatctcctggagaaaccattactattaattgca gggcaagtaagagcattagcaaatatttagcctgggtatcaagagaaacctgggaaaactaataagcttct tatctactctggatccactttgcaatctggaattccatcaaggttcagtggcagtggtctgtgacagat ttcactctccatcagtagcctggagcctgaagattttgcaatgtattactgtcaacagcataatgaat acctgtggagcttcggtggaggcaccgaagctggaatacaaacgg (SEQ ID NO:45)
huEGFR-7 V _L v1.0	gaattcgccaccatgggctggagctgcatcatcttgttcttggtcgccactgccacaggagtgcatagcgata ttcagatgaccagctctccagctctctgagcgttagcgtgggcgcatcgggtgactattacttgcctgcatc ccaggatatcaacaactacttggcctggtaccagcacaagccggcaaggcccaagctgctgacccact ataccagtacactgcacctggtatccctctagattcagcggctccggtagtggctgggattactcattctcta tctcttccctggagcccaggatatagtacataattattgtctccagtagcatalaattcttctacacatttggacag gggacaaaagctggagatcaaacgtacg (SEQ ID NO:46)
huEGFR-7 V _L v1.01	gaattcgccaccatgggatggctctgcattatcttcttcttggtcgccaccgccacaggcgtccactctgacata caaatgaccagtcctcttctcactgagcgcctcgttggggatagagtacaatcacttgtaaagctagccag gacatcaacaactatctgcttgggtatcagcacaacctgggaagggaaccaagctcttgattcattacacctcta ccttgcaccaggcataccaagccgtttagcggtagtggcagtgccgcgattactcattctccatcagttcctt gggaaccagaagatagccacctattattgtctccagtagataatttgcctctacacttttggccagggcaccaaac ttgagatcaagcgtacg (SEQ ID NO:47)
huEGFR-7 V _L CDR grafted	gaattcgccaccatgggatggctctgcattatcttcttcttggttagctaccgctacaggcgttcacagcattca gatgacacagagcccctcttctgtccgctcctcgtgggcgatagagtacaaatcacctgccgcgcaagccag gatatacaacaactaccttgcatggtaccagcagaagcctggaaaagccccaaagctgctcatatactacacctc cacccttcaccagggagttccatccaggttctctgggtctggaagtggaaacagatttaccttcacaatcagctcat tgcaaccggaggacatagctacataattactgctgcagtagacaatctgctgtacacatttggacagggaacca aaattgaaatcaaacgtacg (SEQ ID NO:48)
huEGFR-12 V _L v1.0	gaattcgccaccatgggctggagtgcatcatcctgttcttgggtgctaccgcaaccggagtacactccgacgt gcagatcacccaatctccatcatcctcgcgcaggtggtggagaacgaattactatcaactgccgagcaagc cagagtatcagccgttatctggcatggtaccaggagaaaccggtaagactaacaactgttgatttactcagg cagtacactgcaatctggatcccttagccgctttagcggctccggcagtggcaccgatttaccctgacaatttc ctccctggagccagaggatttcgcaatgtattattgtcagcaacacaacgagtacccatggacatttggccagg gcacaaaagctggagattaagcgtacg (SEQ ID NO:49)
huEGFR-12 V _L v1.01	gaattcgccaccatgggatggctctgcattatcttctcctcgtggcaacagctacaggggtgcatagcgatgtg cagatcacccagtcaccaagctcccttgagcgttccgttggtgagcgcattaccatcaactgtcgagctagtaag tctatttcaagtacctggcttgggtatcaagagaagccaggaaagacaaacagctgctcattacagtggtctcta cccttcagtcgggtattccctctagatttagtggcagtggttagtgaaccgattttacccttacaatiagctctctgg aaccagaagacttcgcaatgtactactccagcaacacaatgagtacccatggacttttggccagggaacaaaag ctggaaattaaacgtacg (SEQ ID NO:50)
muEGFR-6 V _L	gacatccagatgacacagtctccatctcactgtctgcatctctgggaggcaaaagtcaccatc acttgaaggccaagccaagacattaacaactatataagcttgggtaccaacacaagcctggaaaaggctcta ggctgctcattcattacatctacattacatccaggcatcccatcaaggttcagtggagtggtctgg gagagattatcttcagcatcagcaacctggagcctgaagatattgcaacttatttctacagtat gataatcttctacacgttcggaggggggaccgaagctggaataaaacgg (SEQ ID NO:81)

Table 9: Full-length heavy and light chain polynucleotide sequences

Antibody	Polynucleotide Sequence
huEGFR-7 HC	<p>aagcttgccaccatgggctggtcatgtatctgttctgttgccaccgcaaccgggtgccattcccaggtg cagctcgtgcagagcggggtgaagtggccaagccaggtgcttctgtcaaattgtctgtaaggccagtggtg acaccttcacaagctactggatgcagtggttaagcaacgcccaggccaggactggagtgcatcggcacca tttatccaggggatggagataccacttatacacaagtttaaggcaaagccacctgaccgcccagacaaatcc agcagcacagcatatgcagcttctagcctcaggtctgaagactccgctgtactattgtgcccgtacga cgccccgggctatgcaatggattactggggccagggtactctgtgcacagtgctctccgctctacaaagggc ccatcagtttcccttggtccaagtctaaatccacaagcgggtggaacagctgcactgggatgctcgtttaa gattatttccctgagcctgtgacagtgagctggaatagcggagcattgacttcaggtgtgcacactttccgctg tgttgagctctccggtctgtactactgtccagtgctgaaccgtccctctagcagctgggaaccagaccta catctgaacgtcaaccataaaccatccaacacaaagggtgataagaaggtgaacaaagagctgtgataag acacatacatgcccctctgtctgcaccagagctcctcgaggtccatctgtgttctgtttcccccaaacca aggacactcttatgatctctgtactccagaggtcacctgtgtgtgtgcagctgagccatgaagatcccagg ttaaattcaactggtacgtggatggagtcgaggttcacaatccaagaccaagcccaggaggagcaataaa ttctacatatcgggtagtgcagctgtgaccgtgctccaccaagattggctcaatggaaaagagtacaagtgc gggtgccacaaggctcttcccgtccattgagaaaactatccaaagccaaggggcagccacgggaaccc caggtgtatacattgccccatctagagacgagctgaccaagaaccaggtgagctcactgtctgttgcagg gtttacccttctgacattgcttagagtggtgagctaacggacagccagaaaacaactacaagacaactcccc cagtgctggacagcgacgggagcttcttctactccaagttgactgtagacaagtctagatggcagcaaggaa acgttttctcctgctcagtaatgcaggtctgtgcacaatcactataccagaaatcactgtcccttagccagg tgactcag (SEQ ID NO:51)</p>
huEGFR-7 LC v1.0	<p>gaattgccaccatgggctggagctgcatcattgttctgttgccactgccacaggagtgcatagcgatattc agaagaccagcttcccagctctctgagcgttagcgtggcgatcgggtgactattacttgcctgcatccag gatatacaaaactacttggcctgtaccagcacaagcccggcaaggcccaagctgctgatccactataccag tactgcaccctggtatccctctagattcagcggctccggtagtgggtcgggattactcattctctatcttccctg gagcccagggatatagtacataattattgtctccagtagcagataatcttctgtacacatttgacaggggacaaagc tgagatcaagcgtacggtggctgcaccatctgtcttcatcttcccgcctctgatgagcagttgaaatctggaac tgctctgtgtgtgctgtgaataacttctatccagagaggccaaagtacagtggaaggtggataacgcctc caatcgggtaactccaggagagtgacacagagcaggacagcaaggacagcacctacagcctcagcagcacc ctgacgtgagcaaaagcagactacgagaaacaaaagtctacgcctgcgaagtcacccatcagggcctgagct cgcccgtcacaagagcttcaacaggggagagtgtag (SEQ ID NO:52)</p>
huEGFR-7 LC v1.01	<p>gaattgccaccatggggtggtcctgcatattatcttctgttgccaccgcccacaggcgtccacttgacatac aatgaccagctccccttctactgagcgcctcgttggggatagagttaaatcactgttaaagctagccagga catacaaaactatctggcttggatcagcataaacctgggaagggaaccaagctctgattcattacacctctact tgcaccaggcagatacaagccgcttagcggtagtgagcagtgccgcgattactcattctccatcagttccttgg aaccagaagatagccacctattattgtctccagtagataattgtctctacatttggccagggcaccaaactg agatcaagcgtacggtggctgcaccatctgtcttcatcttcccgcctctgatgagcagttgaaatctggaactgc ctctgtgtgtgctgtgaataacttctatccagagaggccaaagtacagtggaaggtggataacgcctcca atcgggtaactccaggagagtgacacagagcaggacagcaaggacagcacctacagcctcagcagcacc ctgacgtgagcaaaagcagactacgagaaacaaaagtctacgcctgcgaagtcacccatcagggcctgagct cgcccgtcacaagagcttcaacaggggagagtgtag (SEQ ID NO:53)</p>
huEGFR-7 HC_CDR grafted	<p>aagcttgccaccatggggtggtcctgtataatactgttctgttgccactgccacaggagtgccacagccaag tgcagctggtgcagagtggtcgtgaggtcaagaagcctggggcatccgcaagggttcttgaaggcatctg gatataccttcacttctattggatgcagtggtgagacaggcaccaggacaggactggagtgatgggc actatttatccaggtgacggtgacactactatactcagaaattcaagggcgagtgaccatgactctgtgatac tagcactagtaccgtgtatatggagcttagttcttccggtccgaggacacagcagctactactgtgctagata tgacgcaaccggatagccatggactattggggcagggcaccctggtcaccgtgagttccggcagcactaa gggcccacagtttcccttggtccaagttctaaatccacaagcgggtggaacagctgcactgggatgctcgt ttaaagattatttccctgagcctgtgacagtgagctggaatagcggagcattgacttcaggtgtgcacactttcc cgctgtgtgagctctccggtctgtactactgtccagtgctgaaccgtccctctagcagcttgggaaccca gacctacatctgaacgtcaaccataaaccatccaacacaaagggtgataagaaggtgaacaaaagagctg tgataagacacatacatgcccctctgtctgcaccagagctcctcgagggtccatctgtgttctgtttcccc</p>

Antibody	Polynucleotide Sequence
	aaaccaaggacactcttatgatctctgactccagaggtcacctgtgtgtgtcgcacgtgagccatgaagat cccgaggttaaattcaactggtagtgagtcgaggtcacaatgccaagaccaagcccaggaggga gcaatataattctacatatcggtagtgagcgttctgaccgtgctccaccaagattggctcaatggaaaagag tacaagtgaaggtgtccaacaaggctcttcccgtcccatgagagacgagctgaccaagaaccaggtgagctc gccacgggaaccccaggtgtatattgccccatctagagacgagctgaccaagaaccaggtgagctc actgtctgtgcaaggggtttacccttctgacattgctgtagagtgaggagctaacggacagccagaaaac aactacaagacaactccccagtgctggacagcgacgggagcttctcctactccaagttgactgtaga caagtctagatggcagcaaggaaacgtttctcctgctcagtaatgcatgaggtctgcacaatcactatacc cagaatcactgtcccttagcccaagggtgactcag (SEQ ID NO:54)
huEGFR- 7 LC_CDR grafted	gaattcgcaccatgggatggagttgcattattttgttctgtagctaccgctacaggcgttcacagcatt cagatgacacagagccccctcttctgtcgcctcctggggcgatagagtcacaatcacctgccgcgaag ccagatatacaactaccttgcattggtaccagcagaagcctggaaaagcccaagctgctcatatact acacctccacccttccccaggttccatccaggttctctgggtctggaagtggacagattttaccctcac aatcagctcattgcaacccgaggacatagctacatattactgcctgcagtatgacaatctgctgtacacattg gacagggaaacaaagtgaatcaagegtacgggtgctgaccatctgtcttcatcttcccgccatctgatga gcagttgaaatctggaactgcctctgtgtgtgctgctgaataacttctatccagagaggccaaagtacag tggaggtggataacgccctccaatcgggtaactccaggagagtgctcacagagcaggacagcaaggac agcacctacagcctcagcagcacctgacgtgagcaaagcagactacgagaacacaaagtctacgcct gcgaagtcacccatcaggcctgagctcgcctgcacaaagagcttcaacaggggagagtgtag (SEQ ID NO:55)
huEGFR- 12 HC	aagcttgcaccatgggctggtcctgtattatcctcttttgggtggccactgctaccggcgacacagtcagg tgcagctgggtgcagtcgggggctgaagtggcaaacgggggctccgtaagctctcttgaaggcat ccggctacacttttacttctactggatgcagtggtgcaaacagcggccaggacaggggttggaatgtata ggtacaatctatccggcgatggtgacacacgatataccagaagttccaggggcaagggtaccctgactg ccgacaaatctttagcaccgcttatatgcagctgtcatctctcgaagtgaagactctgcagtgattactg cggcgatagacgcacccgggtacgccatggattactggggtcaggggaccttgtaaccgtatcaagc gccagtaccaagggcccatcagtttccccttggctccaagtictaaatccacaagcgggtggaacagctgca ctgggatgctcgttaaagattattccctgagcctgtgacagtgagctggaatagcggagcattgacttcag gtgtgcacacttttccgctgtgttgacgtctcgggtctgtactcactgtccagtgctgtaaccgtcccttcta gcagcttgggaaccagacctacatctgtaacgtcaaccataaaccatccaacacaaaggtggataagaa ggttgaaacaaagagctgtgataagacacatacatgccctcctgtcctgcaccagagctcctcggaggtcc atctgtgttctgtttcccccaaaccaaggacactcttatgatctctgactccagaggtcacctgtgtgtt gtcgacgtgagccatgaagatcccgaggttaaattcaactggtagctggatggagtcgaggttcacaatgcc aagaccaagcccaggaggagcaatataattctacatatcgggtagtgagcgttctgaccgtgtccacca agattggctcaatggaaaagagtaaaagtgcaaggtgtccaacaaggctcttcccgtcccattgagaaaa ctatctccaagccaaggggcagccacgggaaccccaggtgtatacattgccccatctagagacgagc tgaccaagaaccaggtgagctcacttgtctgtgcaaggggtttacccttctgacattgctgtagagtgagg agtctaacggacagccagaaaacaactacaagacaactccccagtgctggacagcgacgggagcttct tctctactccaagttgactgtagacaagctagatggcagcaaggaaacgttttctcctgctcagtaatgcat gaggtctgcacaatcactataccagaaatcactgtcccttagcccaagggtgactcgag (SEQ ID NO:56)
huEGFR- 12 LC v1.0	gaattcgcaccatgggctggagttgcattatcctgttcttgggtgctaccgcaaccggagtacactccgacg tgcagatcacccaatciccatcctcctcggccagtggtgggagaacgaattactatcaactgccgagcaa gccagagtatcagccgttatctggcatggtaccaggagaaacccgtaagactaacaactgttgattactc aggcagtacactgcaatctggtatccctagccgttttagcggctccggcagtggaaccgatttcacctgac aatttctcctcggagccagaggatttcgcaatgtattatgtcagcaacacaacagtagcccatggacattgg ccagggcacaaagctggagattaagcgtacgggtgctgaccatctgtcttcatcttcccgccatctgatgag cagttgaaatctggaactgcctctgtgtgtgctgctgaataacttctatccagagaggccaaagtacagtg aaggtggataacgccctccaatcgggtaactccaggagagtgctcacagagcaggacagcaaggacagcac ctacagcctcagcagcacctgacgtgagcaaagcagactacgagaacacaaagtctacgcctgcgaag tcaccatcagggcctgagctcggcgtcacaagagcttcaacaggggagagtgtag (SEQ ID NO:57)

Antibody	Polynucleotide Sequence
huEGFR-12 LC v1.01	<p>gaattgccaccatgggatggctcgtcattatcctgttccctgtggcaacagctacaggggtgcatagc gatgt gcagatcacccagtcaccaagctcccttgacgttccgttggtgagcgcattaccatcaactgtcagagctagta agtctatttccaagtacctggcttggatcaagagaagccaggaaagacaaaagctgctcatttacagtggc tctacccttcagtcgggtattccctctagatttagtgaggcagtggttagtggaaaccgattttacccttacaattagctct ctggaaccagaagacttcgcaatgtactactgccagcaacacaatgagtacccatggacttttggccaggga caaagctggaaattaaacgtacgggtggctgcaccatctgtcttcatcttcccgccatctgatgagcaggtgaaat ctggaactgcctctgttgtgtgcctgctgaataactctatcccagagaggccaaagtacagtggaaaggtggata acgccctccaatcggttaactcccaggagagtgctcacagagcaggacagcaaggacagcacctacagcctc agcagcacctgacgtgagcaaacgagactacgagaacacaaagtctacgctgcgaagtcacccatca gggctgagctcggcgtcacaaagagcttcaacaggggagagtgtag (SEQ ID NO:58)</p>
huEGFR-6 HC v1.0	<p>aagcttgccaccatgggggtggagttgtatcatcctcttctgtcgtaccgccactggagtgcatccaggtg cagttgggtgaatctggcgccgaggtggccaagcccgggtgcctccgtaaaattgagttgtaaagcctctggcta tacatttacatcttattggatgcagtggggtcaagcagcgccctggtaaggcctggagtgcatcggagctctgta tcttggcgacggggacgcccgttacactcagaaattcaggggcaaaagctaccctaccgcagatacatccagc agcactgcttatatgcaacttagtagcctccgcagcgaggatagtgccgtgtactactgtgccagatalgacgcc ccaggttatgctatggactactggggtcaaggaaccctgggtgacagtgtaagcgctagcacaaagggcccatc agtttccccctggctccaagttctaaatccacaagcggtggaacagctgcactgggatgcctcgttaaagattatt tccctgagcctgtgacagtgagctggaatagcggagcattgacttcaggtgtgcacacttttccgctgtgttgca gtctccgggtgtactcactgtccagtgctgaaccgtccctttagcagcttgggaaccagacctacatctgta acgtcaaccataaaccatccaacacaaaggtggataagaaggtgaaccaaagagctgtgataagacacatac atgccctccttgcctgcaccagagctcctcggaggtccatctgtgtcctgtttcccccaaaccaaggacact cttatgatctctcgtactccagaggtcacctgtgtgtgtgcagctgagccatgaagatcccagggttaaaattcaa ctggtagctggatggagtcgaggttcacaatgccaagaccaagcccaggaggagcaatataattctacatc gggtagtgagcgttctgaccgtgtccaccaagattgggtcaatggaaaagagtacaagtgcaaggtgtccaac aaggtcttcccgtcccattgagaaaactatctccaaagccaaggggcagccacgggaaccccagggtgtatac attgcccccatctagagacgagctgaccaagaaccaggtgagctcacttctgtctggtcaaggggttttacccttctg acattgtgtagagtgaggagctaacggacagccagaaaacaactacaagacaactccccagtgctggacag cgacgggagcttctccttactccaagttgactgtagacaagcttagatggcagcaaggaaacgttttctcctgctc agtaatgcatgaggtctgcacaatcactataccagaaatcactgtcccttagcccagggtgactcgag (SEQ ID NO:82)</p>
huEGFR-6 HC v1.11	<p>aagcttgccaccatgggggtggagttgtatcatcctcttctgtcgtaccgccactggagtgcatccaggtgca gttgggtgaatctggcgccgaggtggccaagcccgggtgcctccgtaaaattgagttgtaaagcctctggctatacat ttacatcttattggatgcagtggggtcaagcagcgccctggtaaggcctggagtggtatcgtatcctggc gacggggacgcccgttacactcagaaattcaggggcaaaagctaccctaccgcagatacatccagcagcactgt tatatgcaacttagtagcctccgcagcgaggatagtgccgtgtactactgtgccagatatgacgcccaggttatgc tatggactactggggtcaaggaaccctgggtgacagtgtaagcgctagcacaaagggcccatcagtttcccccttg gtccaagttctaaatccacaagcggtggaacagctgcactgggatgcctcgttaaagattatttccctgagcctgtg acagtgagctggaatagcggagcattgacttcaggtgtgcacacttttccgctgtgttgacgtcctccggtgtgac tcactgtccagtgctgaaccgtccctttagcagcttgggaaccagacctacatctgtaacgtcaaccataaacca tccaacacaaaggtggataagaaggtgaaccaaagagctgtgataagacacatacatgccctccttgcctgcacc agagctcctcggaggtccatctgtgtcctgttcccccaaaccaaggacactcttatgatctctcgtactccagagg tcacctgtgtgtgtgcagctgagccatgaagatcccagggttaaaattcaactgtgtacgtggatggagtcgaggttca caatgccaagaccaagcccaggaggagcaatataattctacatcgggtagtgagcgttctgaccgtgtccacca agattggctcaatggaaaagagtacaagtgcaaggtgtccaacaaggctcttcccgtcccattgagaaaactatctc caaagccaaggggcagccacgggaaccccagggtgtatacattgccccatctagagacgagctgaccaagaacc aggtgagtgctcactgtgtgtgcaaggggttttacccttctgacattgtgtagagtgaggagctaacggacagccaga aaacaactacaagacaactccccagtgctggacagcgacgggagcttctccttactccaagttgactgtagacaa gtctagatggcagcaaggaaacgttttctcctgctcagtaatgcatgaggtctgcacaatcactataccagaaatca ctgtcccttagcccagggtgactcgag (SEQ ID NO:83)</p>
huEGFR-7 HC	<p>aagcttgccaccatgggctgtgcatgtatcattctgttccctgggtggccaccgcaaccgggtgtccattccaggtgcagct cgtgcagagcggggctgaagtgcccaagccaggtgtctgtcaaatgtctgtgaaggccagtggttacaccttca</p>

Antibody	Polynucleotide Sequence
v1.11	agctactggatgcagtgggtaagcaacgccaggccaggactggagtggatcggcaccatttatccaggggatgg agataccacttatacaciaaagttcaaggcaaaagccacctgaccgccgacaaatccagcagcacagcatacatgcag ctttctagcctcaggtctgaagactccgcgtgtactattgtgcccgtacgacgccccggctatgcaatggattactggg gccagggtactctggtcacagtgtcctccgccttacaaagggcccatcagttttcccttggtccaagtctaaatccaca agcgggtggaacagctgcactgggatgcctcgtaaagattttccctgagcctgtgacagtgcagtggaatagcggagca ttgacttcaggtgtgcacactttcccgctgtgttcagtcctccggctgtactcactgtccagtgtcgtaaccgtcccttctag cagcttgggaaccagacctacatctgtaacgtcaaccataaacatccaacacaaagggtgataagaagggtgaaccaa agagctgtgataagacacatacatgccctcctgtcctgcaccagagctcctcgagggtccatctgtgtcctgtttcccc caaaccaaggaactcttatgatctctgtactccagaggtcacctgtgtgtgtgcagctgagccatgaagatcccgga ggttaaattcaactggtacgtggatggagtcaggttcacaatgccaaagaccaagcccaggaggagcaataataattcta catatcgggtagtgagcgttctgaccgtgtccaccaagattggctcaatggaaaagagtacaagtgaaggtgtccaac aaggtcttcccgctccattgagaaaactatctccaaagccaaggggcagccacgggaaccccggtgtataattgcc cccatctagagacgagctgaccaagaaccaggtgagtctcactgtgtgtcaagggttttacccttctgacattgtgtga gagtgggagtgtaacggacagccagaaaacaactacaagacaactccccagtgctggacagcgacgggagcttcttc ctctactccaagtgactgtagacaagctagatggcagcaaggaaacgttttctcctgctcagtaatgcatgaggctctgc acaactactataccagaaatcactgtcccttagcccagggtgactcgag (SEQ ID NO:84)

[0203] Also provided is a polynucleotide having at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% sequence identity to SEQ ID NOs:39-58, 77-84. Thus, in certain embodiments, the polypeptide comprises (a) a polypeptide having at least about 95% sequence identity to SEQ ID NOs:39-43, 51, 54, 56, 77-80 or 82-84 and/or (b) a polypeptide having at least about 95% sequence identity to SEQ ID NOs:44-50, 52, 53, 55, 57, 58, 81. In certain embodiments, the polypeptide comprises (a) a polypeptide having the amino acid sequence of SEQ ID NOs: 39-43, 51, 54, 56, 77-80 or 82-84; and/or (b) a polypeptide having the amino acid sequence of SEQ ID NOs: 44-50, 52, 53, 55, 57, 58, 81.

[0204] In certain embodiments the polynucleotides comprise the coding sequence for the mature polypeptide fused in the same reading frame to a polynucleotide which aids, for example, in expression and secretion of a polypeptide from a host cell (e.g. a leader sequence which functions as a secretory sequence for controlling transport of a polypeptide from the cell). The polypeptide having a leader sequence is a preprotein and can have the leader sequence cleaved by the host cell to form the mature form of the polypeptide. The polynucleotides can also encode for a proprotein which is the mature protein plus additional 5' amino acid residues. A mature protein having a prosequence is a proprotein and is an inactive form of the protein. Once the prosequence is cleaved an active mature protein remains.

[0205] In certain embodiments the polynucleotides comprise the coding sequence for the mature polypeptide fused in the same reading frame to a marker sequence that allows, for example, for purification of the encoded polypeptide. For example, the marker sequence can be a hexahistidine tag supplied by a pQE-9 vector to provide for purification of the mature polypeptide

fused to the marker in the case of a bacterial host, or the marker sequence can be a hemagglutinin (HA) tag derived from the influenza hemagglutinin protein when a mammalian host (e.g. COS-7 cells) is used.

[0206] The present invention further relates to variants of the hereinabove described polynucleotides encoding, for example, fragments, analogs, and derivatives.

[0207] The polynucleotide variants can contain alterations in the coding regions, non-coding regions, or both. In some embodiments the polynucleotide variants contain alterations which produce silent substitutions, additions, or deletions, but do not alter the properties or activities of the encoded polypeptide. In some embodiments, nucleotide variants are produced by silent substitutions due to the degeneracy of the genetic code. Polynucleotide variants can be produced for a variety of reasons, e.g., to optimize codon expression for a particular host (change codons in the human mRNA to those preferred by a bacterial host such as *E. coli*).

[0208] Vectors and cells comprising the polynucleotides described herein are also provided.

IV. Methods of use and pharmaceutical compositions

[0209] The EGFR-binding agents (including antibodies, immunoconjugates, and polypeptides) of the invention are useful in a variety of applications including, but not limited to, therapeutic treatment methods, such as the treatment of cancer. In certain embodiments, the agents are useful for inhibiting tumor growth, inducing differentiation, reducing tumor volume, and/or reducing the tumorigenicity of a tumor. The methods of use can be *in vitro*, *ex vivo*, or *in vivo* methods. In certain embodiments, the EGFR-binding agent or antibody or immunoconjugate, or polypeptide is not antagonistic of the human EGFR to which it binds.

[0210] In one aspect, anti-EGFR antibodies and immunoconjugates of the invention are useful for detecting the presence of EGFR in a biological sample. The term "detecting" as used herein encompasses quantitative or qualitative detection. In certain embodiments, a biological sample comprises a cell or tissue.

[0211] In one aspect, the invention provides a method of detecting the presence of EGFR in a biological sample. In certain embodiments, the method comprises contacting the biological sample with an anti-EGFR antibody under conditions permissive for binding of the anti-EGFR antibody to EGFR, and detecting whether a complex is formed between the anti-EGFR antibody and EGFR.

[0212] In one aspect, the invention provides a method of diagnosing a disorder associated with increased expression of EGFR. In certain embodiments, the method comprises contacting a test

cell with an anti-EGFR antibody; determining the level of expression (either quantitatively or qualitatively) of EGFR by the test cell by detecting binding of the anti-EGFR antibody to EGFR; and comparing the level of expression of EGFR by the test cell with the level of expression of EGFR by a control cell (e.g., a normal cell of the same tissue origin as the test cell or a cell that expresses EGFR at levels comparable to such a normal cell), wherein a higher level of expression of EGFR by the test cell as compared to the control cell indicates the presence of a disorder associated with increased expression of EGFR. In certain embodiments, the test cell is obtained from an individual suspected of having a disorder associated with increased expression of EGFR. In certain embodiments, the disorder is a cell proliferative disorder, such as a cancer or a tumor.

[0213] In certain embodiments, a method of diagnosis or detection, such as those described above, comprises detecting binding of an anti-EGFR antibody to EGFR expressed on the surface of a cell or in a membrane preparation obtained from a cell expressing EGFR on its surface. In certain embodiments, the method comprises contacting a cell with an anti-EGFR antibody under conditions permissive for binding of the anti-EGFR antibody to EGFR, and detecting whether a complex is formed between the anti-EGFR antibody and EGFR on the cell surface. An exemplary assay for detecting binding of an anti-EGFR antibody to EGFR expressed on the surface of a cell is a "FACS" assay.

[0214] Certain other methods can be used to detect binding of anti-EGFR antibodies to EGFR. Such methods include, but are not limited to, antigen-binding assays that are well known in the art, such as western blots, radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoprecipitation assays, fluorescent immunoassays, protein A immunoassays, and immunohistochemistry (IHC).

[0215] In certain embodiments, anti-EGFR antibodies are labeled. Labels include, but are not limited to, labels or moieties that are detected directly (such as fluorescent, chromophoric, electron-dense, chemiluminescent, and radioactive labels), as well as moieties, such as enzymes or ligands, that are detected indirectly, e.g., through an enzymatic reaction or molecular interaction.

[0216] In certain embodiments, anti-EGFR antibodies are immobilized on an insoluble matrix. Immobilization entails separating the anti-EGFR antibody from any EGFR that remains free in solution. This conventionally is accomplished by either insolubilizing the anti-EGFR antibody before the assay procedure, as by adsorption to a water-insoluble matrix or surface (Bennich et al., U.S. Pat. No. 3,720,760), or by covalent coupling (for example, using glutaraldehyde cross-

linking), or by insolubilizing the anti-EGFR antibody after formation of a complex between the anti-EGFR antibody and EGFR, e.g., by immunoprecipitation.

[0217] Any of the above embodiments of diagnosis or detection can be carried out using an immunoconjugate of the invention in place of or in addition to an anti-EGFR antibody.

[0218] In certain embodiments, the disease treated with the EGFR-binding agent or antagonist (e.g., an anti-EGFR antibody) is a cancer. In certain embodiments, the cancer is characterized by EGFR expressing cells to which the EGFR-binding agent (e.g., antibody) binds.

[0219] In a further aspect, the invention is directed to an improved method for treating cell proliferation disorders wherein EGFR is expressed, particularly wherein EGFR is abnormally expressed (e.g. overexpressed), including cancers of the bladder, brain, head and neck, pancreas, lung, breast, ovary, colon, prostate, skin, and kidney, comprising administering a therapeutically effective amount of an anti-EGFR binding agent of the present invention to a human subject in need thereof. In another embodiment the antibody is humanized. Examples of cell proliferation disorders that can be treated by an anti-EGFR binding agent of the present invention include, but are not limited to neoplasms located in the: abdomen, bone, breast, digestive system, liver, pancreas, peritoneum, endocrine glands (adrenal, parathyroid, pituitary, testicles, ovary, thymus, thyroid), eye, head and neck, nervous system (central and peripheral), lymphatic system, pelvic, skin, soft tissue, spleen, thoracic region, and urogenital system.

[0220] Similarly, other cell proliferation disorders can also be treated by the anti-EGFR binding agents of the present invention. Examples of such cell proliferation disorders include, but are not limited to: adrenal cortex hyperplasia (Cushing's disease), congenital adrenal hyperplasia, endometrial hyperplasia, benign prostatic hyperplasia, breast hyperplasia, intimal hyperplasia, focal epithelial hyperplasia (Heck's disease), sebaceous hyperplasia, compensatory liver hyperplasia, and any other cell proliferation disease, besides neoplasia, located in an organ system listed above.

[0221] The present invention further provides methods for inhibiting tumor growth using the antibodies or other agents described herein. In certain embodiments, the method of inhibiting the tumor growth comprises contacting the cell with an EGFR-binding agent (e.g., antibody) *in vitro*. For example, an immortalized cell line or a cancer cell line that expresses EGFR is cultured in medium to which is added the antibody or other agent to inhibit tumor growth. In some embodiments, tumor cells are isolated from a patient sample such as, for example, a tissue

biopsy, pleural effusion, or blood sample and cultured in medium to which is added an EGFR-binding agent to inhibit tumor growth.

[0222] In some embodiments, the method of inhibiting tumor growth comprises contacting the tumor or tumor cells with the EGFR-binding agent (e.g., antibody) *in vivo*. In certain embodiments, contacting a tumor or tumor cell with a EGFR-binding agent is undertaken in an animal model. For example, EGFR-binding agents can be administered to xenografts expressing one or more EGFRs that have been grown in immunocompromised mice (e.g. NOD/SCID mice) to inhibit tumor growth. In some embodiments, cancer stem cells are isolated from a patient sample such as, for example, a tissue biopsy, pleural effusion, or blood sample and injected into immunocompromised mice that are then administered a EGFR-binding agent to inhibit tumor cell growth. In some embodiments, the EGFR-binding agent is administered at the same time or shortly after introduction of tumorigenic cells into the animal to prevent tumor growth. In some embodiments, the EGFR-binding agent is administered as a therapeutic after the tumorigenic cells have grown to a specified size.

[0223] In certain embodiments, the method of inhibiting tumor growth comprises administering to a subject a therapeutically effective amount of a EGFR-binding agent. In certain embodiments, the subject is a human. In certain embodiments, the subject has a tumor or has had a tumor removed.

[0224] In certain embodiments, the tumor expresses the EGFR to which the EGFR-binding agent or antibody binds.

[0225] In addition, the invention provides a method of reducing the tumorigenicity of a tumor in a subject, comprising administering a therapeutically effective amount of a EGFR-binding agent to the subject. In certain embodiments, the tumor comprises cancer stem cells. In certain embodiments, the frequency of cancer stem cells in the tumor is reduced by administration of the agent.

[0226] The invention further provides methods of differentiating tumorigenic cells into non-tumorigenic cells comprising contacting the tumorigenic cells with a EGFR-binding agent (for example, by administering the EGFR-binding agent to a subject that has a tumor comprising the tumorigenic cells or that has had such a tumor removed).

[0227] The use of the EGFR-binding agents, polypeptides, or antibodies described herein to induce the differentiation of cells, including, but not limited to tumor cells, is also provided. For example, methods of inducing cells to differentiate comprising contacting the cells with an

effective amount of a EGFR-binding agent (e.g., an anti-EGFR antibody) described herein are envisioned. Methods of inducing cells in a tumor in a subject to differentiate comprising administering a therapeutically effective amount of a EGFR-binding agent, polypeptide, or antibody to the subject are also provided. In certain embodiments, the tumor is a pancreatic tumor. In certain other embodiments, the tumor is a colon tumor. In some embodiments, the treatment methods comprise administering a therapeutically effective amount of the EGFR-binding agent, polypeptide, or antibody to the subject.

[0228] The present invention further provides pharmaceutical compositions comprising one or more of the EGFR-binding agents described herein. In certain embodiments, the pharmaceutical compositions further comprise a pharmaceutically acceptable vehicle. These pharmaceutical compositions find use in inhibiting tumor growth and treating cancer in human patients.

[0229] In certain embodiments, formulations are prepared for storage and use by combining a purified antibody or agent of the present invention with a pharmaceutically acceptable vehicle (e.g. carrier, excipient) (Remington, The Science and Practice of Pharmacy 20th Edition Mack Publishing, 2000). Suitable pharmaceutically acceptable vehicles include, but are not limited to, nontoxic buffers such as phosphate, citrate, and other organic acids; salts such as sodium chloride; antioxidants including ascorbic acid and methionine; preservatives (e.g. octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride; benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens, such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight polypeptides (e.g. less than about 10 amino acid residues); proteins such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; carbohydrates such as monosaccharides, disaccharides, glucose, mannose, or dextrans; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g. Zn-protein complexes); and non-ionic surfactants such as TWEEN or polyethylene glycol (PEG).

[0230] The pharmaceutical compositions of the present invention can be administered in any number of ways for either local or systemic treatment. Administration can be topical (such as to mucous membranes including vaginal and rectal delivery) such as transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders; pulmonary (e.g., by inhalation or insufflation of powders or aerosols, including by nebulizer; intratracheal,

intranasal, epidermal and transdermal); oral; or parenteral including intravenous, intraarterial, subcutaneous, intraperitoneal or intramuscular injection or infusion; or intracranial (e.g., intrathecal or intraventricular) administration.

[0231] An antibody or immunoconjugate of the invention can be combined in a pharmaceutical combination formulation, or dosing regimen as combination therapy, with a second compound having anti-cancer properties. The second compound of the pharmaceutical combination formulation or dosing regimen can have complementary activities to the ADC of the combination such that they do not adversely affect each other. Pharmaceutical compositions comprising the EGFR-binding agent and the second anti-cancer agent are also provided.

[0232] For the treatment of the disease, the appropriate dosage of an antibody or agent of the present invention depends on the type of disease to be treated, the severity and course of the disease, the responsiveness of the disease, whether the antibody or agent is administered for therapeutic or preventative purposes, previous therapy, patient's clinical history, and so on all at the discretion of the treating physician. The antibody or agent can be administered one time or over a series of treatments lasting from several days to several months, or until a cure is effected or a diminution of the disease state is achieved (e.g. reduction in tumor size). Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the patient and will vary depending on the relative potency of an individual antibody or agent. The administering physician can easily determine optimum dosages, dosing methodologies and repetition rates. In certain embodiments, dosage is from 0.01 μ g to 100 mg per kg of body weight, and can be given once or more daily, weekly, monthly or yearly. In certain embodiments, the antibody or other EGFR-binding agent is given once every two weeks or once every three weeks. In certain embodiments, the dosage of the antibody or other EGFR-binding agent is from about 0.1 mg to about 20 mg per kg of body weight. The treating physician can estimate repetition rates for dosing based on measured residence times and concentrations of the drug in bodily fluids or tissues.

[0233] The combination therapy can provide "synergy" and prove "synergistic", i.e. the effect achieved when the active ingredients used together is greater than the sum of the effects that results from using the compounds separately. A synergistic effect can be attained when the active ingredients are: (1) co-formulated and administered or delivered simultaneously in a combined, unit dosage formulation; (2) delivered by alternation or in parallel as separate formulations; or (3) by some other regimen. When delivered in alternation therapy, a synergistic effect can be

attained when the compounds are administered or delivered sequentially, e.g. by different injections in separate syringes. In general, during alternation therapy, an effective dosage of each active ingredient is administered sequentially, i.e. serially, whereas in combination therapy, effective dosages of two or more active ingredients are administered together.

VI. Kits comprising EGFR binding agents

[0234] The present invention provides kits that comprise the antibodies, immunoconjugates or other agents described herein and that can be used to perform the methods described herein. In certain embodiments, a kit comprises at least one purified antibody against EGFR in one or more containers. In some embodiments, the kits contain all of the components necessary and/or sufficient to perform a detection assay, including all controls, directions for performing assays, and any necessary software for analysis and presentation of results. One skilled in the art will readily recognize that the disclosed antibodies, immunoconjugates or other agents of the present invention can be readily incorporated into one of the established kit formats which are well known in the art.

[0235] Further provided are kits comprising a EGFR-binding agent (e.g., a EGFR-binding antibody), as well as a second anti-cancer agent. In certain embodiments, the second anti-cancer agent is a chemotherapeutic agent (e.g., rituximab).

Examples

[0236] It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application.

Example 1

Production of murine EGFR antibodies

[0237] To produce murine anti-EGFR antibodies, head and neck squamous carcinoma cell lines such as CA922 (Japanese Collection of Research Bioresources (JCRB), Shinjuku, Japan) and HSC4 (JCRB, Shinjuku, Japan) were injected subcutaneously into Balb/c female mice (Charles River Laboratory, Wilmington, MA) at the dose of 5×10^6 cells per mouse every 2 weeks for 5 times. Three days before being sacrificed for hybridoma generation, the immunized mice

received intraperitoneal injection of another dose of antigen. The spleen from the mouse was collected according to standard animal protocols and was ground between two sterile, frosted microscopic slides to obtain a single cell suspension in RPMI-1640 medium. After the red blood cells were lysed with ACK lysing buffer, the spleen cells were then mixed with murine myeloma P3X63Ag8.653 cells (P3 cells) (J. F. Kearney et al. 1979, *J Immunol*, 123: 1548-1550) at ratio of 1 P3 cells: 3 spleen cells. The mixture of spleen cells and P3 cells was washed and treated with pronase in fusion media (0.3M mannitol/D-sorbitol, 0.1mM CaCl₂, 0.5mM MgCl₂ and 1mg/ml BSA) at room temperature for 3 min. The reaction was stopped by addition of FBS (Fetal Bovine Serum, Invitrogen); cells were then washed, resuspended in 2 ml cold fusion media and fused with BTX ECM 2001 electrofusion machine (Harvard Apparatus). The fused cells were added gently to RPMI-1640 selection medium containing hypoxanthine-aminopterin-thymidine (HAT) (Sigma Aldrich), incubated for 20 min at 37°C, and then seeded into flat bottom 96-well plates at 200 µl/well. The plates were then incubated in 5% CO₂ incubator at 37°C until hybridoma clones were ready for antibody screening. Other techniques of immunization and hybridoma production can also be used, including those described in J. Langone and H. Vunakis (Eds., *Methods in Enzymology*, Vol. 121, "Immunochemical Techniques, Part I"; Academic Press, Florida) and E. Harlow and D. Lane ("*Antibodies: A Laboratory Manual*"; 1988; Cold Spring Harbor Laboratory Press, New York, NY).

Murine hybridoma screening and selection

[0238] Hybridoma screening was done using flow cytometric binding assay with immunizing cells that are either untreated or treated with EGF (R&D Systems). Because incubation with EGF downregulates EGFR level on the cell surface, EGFR specific hybridoma supernatant would only react to the untreated cells and not to the EGF-treated cells. Cells for screening were first cultured in serum free media for overnight and separated into two parts. One part of the cells was left untreated and the other part was treated with EGF for 3 hours at 37°C. The EGF treated cells were labeled with CellTrace™ far red DDAO-SE (Invitrogen), mixed with untreated cells at 1:1 ratio and incubated with the hybridoma supernatant for 2 hours on ice. Cells were then washed, incubated with FITC-labeled anti-mouse IgG (Jackson Immunoresearch), washed, fixed with formalin and analyzed using FACScalibur (BD Bioscience). The EGFR specific hybridomas were expanded and the supernatants were rescreened by ELISA using soluble recombinant human EGFR extracellular domain (RELIAtech) as antigen. The positive hybridomas were rescreened using flow cytometric binding assay with human EGFR-expressing A431 epidermal

carcinoma cell line (ATCC) and monkey EGFR-expressing Vero cell line (an African green monkey kidney epithelial cell line) (ATCC). In brief, the hybridoma supernatant was incubated with A431 cells and DDAO-labeled Vero cells on ice for 1 hour. The cells were washed twice and incubated with PE-conjugated goat anti-mouse IgG antibody (Jackson ImmunoResearch) for 1 hour on ice. The cells were then washed with FACS buffer, fixed in formalin and analyzed using a FACSCalibur flow cytometer (BD Biosciences).

[0239] The positive hybridoma clones that reacted to both human and monkey antigens were tested for the capacity to inhibit basal proliferation of EGFR-overexpressing HCC827 cells (ATCC). In brief, exponentially growing HCC827 cells were plated at 2000 cells/well in 96 well plates in 100 μ l complete media containing 10% FBS. 100 μ l of hybridoma supernatant was added to the cells and the mixture was incubated at 37°C in a humidified 5% CO₂ incubator for 5 days. Level of cell proliferation was determined using colorimetric WST-8 assay (Dojindo Molecular Technologies, Rockville, MD). WST-8 is reduced by dehydrogenases in the living cells to an orange formazan product that is soluble in tissue culture medium, and the amount of formazan produced is directly proportional to the number of living cells. 10% of the final volume of WST-8 was added to each well and plates were incubated at 37°C for an additional 2-4h. Plates were then analyzed by measuring the absorbance at 450 nm (A450) in the Spectra Max M2 plate reader (Molecular Devices, Sunnyvale, CA). Background A450 absorbance of wells with media and WST-8 only was subtracted from all values. The surviving fraction was calculated by dividing each treated sample value by the average value of wells with untreated cell (surviving fraction = (A450 treated sample – A450 background)/ (A450 untreated sample – A450 background)). The results were normalized so that surviving fraction 0 indicated the value of wells without cells and 1 indicated the level of cell proliferation in the serum containing media without any EGFR antibody. Hybridoma clones that inhibited at least 50% HCC827 cell growth were subcloned by limiting dilution. One subclone from each hybridoma, which showed the same reactivity against EGFR as the parental cells by flow cytometry, was chosen for subsequent analysis. Stable subclones were cultured and the antibody was isotyped using commercial isotyping reagents (Roche).

Antibody purification

[0240] Antibodies were purified from hybridoma subclone supernatants using standard methods, such as, for example Protein A or G chromatography (HiTrap Protein A or G HP, 1 mL, Amersham Biosciences). Briefly, supernatant was prepared for chromatography by the addition

of 1/10 volume of 1M Tris/HCl buffer, pH 8.0. The pH-adjusted supernatant was filtered through a 0.22µm filter membrane and loaded onto column equilibrated with binding buffer (PBS, pH 7.3). The column was washed with binding buffer until a stable baseline was obtained with no absorbance at 280nm. Antibody was eluted with 0.1M acetic acid buffer containing 0.15M NaCl, pH 2.8, using a flow rate of 0.5mL/min. Fractions of approximately 0.25mL were collected and neutralized by the addition of 1/10 volume of 1M Tris/HCl, pH 8.0. The peak fraction(s) was dialyzed overnight twice against 1x PBS and sterilized by filtering through a 0.2 µm filter membrane. Purified antibody was quantified by absorbance at A280.

[0241] Protein A purified fractions were further polished using ion exchange chromatography (IEX) with quaternary ammonium (Q) chromatography for murine antibodies. Briefly, samples from protein A purification were buffer exchanged into binding buffer (10mM Tris, 10mM sodium chloride, pH 8.0) and filtered through 0.22 µm filter. The prepared sample was then loaded onto a Q fast flow resin (GE Lifesciences) that was equilibrated with binding buffer at a flow rate of 120cm/hr. Column size was chosen to have sufficient capacity to bind all the MAb in the sample. The column was then washed with binding buffer until a stable baseline was obtained with no absorbance at 280nm. Antibody was eluted by initiating a gradient from 10mM to 500mM sodium chloride in 20 column volume (CV). Peak fractions were collected based on absorbance measurement at 280nm (A280). The percentage of monomer was assessed with size exclusion chromatography (SEC) on a TSK gel G3000SWXL, 7.8 × 300 mm with a SWXL guard column, 6.0 × 40 mm (Tosoh Bioscience, Montgomeryville, PA) using an Agilent HPLC 1100 system (Agilent, Santa Clara, CA). Fractions with monomer content above 95% were pooled, buffer exchanged to PBS (pH 7.4) using a TFF system, and sterilized by filtering through a 0.2µm filter membrane. The IgG concentration of purified antibody was determined by A280 using an extinction coefficient of 1.47. Alternative methods such as ceramic hydroxyapatite (CHT) were also used to polish antibodies with good selectivity. Type II CHT resin with 40µm particle size (Bio-Rad Laboratories) were used with a similar protocol as described for IEX chromatography. The binding buffer for CHT corresponds to 20mM sodium phosphate, pH 7.0 and antibody was eluted with a gradient of 20-160mM sodium phosphate over 20 CV.

Example 2

Binding affinity to human and monkey EGFR antigen

[0242] To determine the binding affinity of the EGFR antibodies to the human and monkey antigens, EGFR expressing human tumor cell lines such as MDA-MB468 (ATCC) and A431 (ATCC), and Vero monkey kidney cell line (ATCC) were used in a flow cytometric binding assay. In brief, the cells were incubated with various concentration of EGFR antibody for 1h at 4°C. The cells were washed and incubated with PE-conjugated secondary antibody (Jackson ImmunoResearch) for 1h at 4°C. The cells were then washed, fixed in formalin and analyzed in FACSarray (BD Bioscience). To determine the binding affinity of these antibodies, geometric mean fluorescence intensity was plotted against the antibody concentration in a semi-log plot. A dose-response curve was generated by non-linear regression and the EC50 value of the curve, which corresponds to the apparent dissociation constant (Kd) of each antibody, was calculated using GraphPad Prism v4 (GraphPad software). The EGFR antibodies of the invention as well as positive control antibodies, cetuximab and panitumumab, showed strong specific binding to both human tumor cells and monkey Vero cells. A table shown in Figure 1 lists the Kd of each antibody to the human and monkey EGFR. The EGFR antibodies of the invention exhibited similarly strong binding affinity to both human and monkey antigens.

Example 3

Inhibition of ligand-induced EGFR activation

[0243] EGFR ligand binding induces EGFR phosphorylation followed by activation of downstream signaling pathways. To investigate the effect of anti-EGFR antibodies in ligand-induced EGFR activation, Western blot analysis was performed using MDA-MB468 tumor cell line and human primary adult keratinocytes. In brief, cells were seeded at 1e6 cells/well in a 6 well plate and cultured in normal media for overnight. Cells were washed and starved in serum free media containing 0.1% BSA for 2 hours at 37°C. 10µg/ml antibody was added to the cells and the mixture was incubated for 3 hours at 37°C. 50 ng/ml EGF (R&D Systems) was added to the mixture and incubated for 15 minutes at 37°C. Cells were then washed with ice-cold PBS and lysed in RIPA buffer containing protease and phosphatase inhibitors. The protein lysates were separated in SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked with 5% BSA and incubated with anti-phosphotyrosine antibody (clone 4G10, Millipore)

for overnight at 4°C. The membrane was washed, incubated with HRP conjugated anti-mouse antibody (Jackson ImmunoResearch) for 1 hour at room temperature, and washed again. The signal was detected using an ECL (enhanced chemiluminescence) system (GE Healthcare). To ensure equal amount of proteins loaded into each lane, the membrane was stripped and reprobed with anti- β -tubulin antibody (Sigma Aldrich).

[0244] As shown in Figure 2, EGF stimulation led to strong EGFR phosphorylation in both MDA-MB468 cells and human primary keratinocytes. Treatment of cells with cetuximab and panitumumab strongly inhibited the EGF-induced EGFR phosphorylation while the EGFR antibodies of the invention did not completely inhibit EGFR activation. Anti-KTI (Kunitz Trypsin inhibitor) antibody (produced from hybridoma obtained from ATCC) was used as negative control.

Example 4

Agonistic activity of EGFR antibodies

[0245] To investigate the effect of the EGFR antibodies of the invention on EGFR signaling in absence of EGFR ligands, MDA-MB468 tumor cells were starved in serum free media as described in Example 5. The cells were then incubated with 10 μ g/ml EGFR antibodies for 3 hours at 37°C. As positive control, untreated cells were incubated with 50 ng/ml EGF for 15 minutes at 37°C. The protein lysates were prepared and analyzed using Western blot as described in Example 5. The representative result is shown in Figure 3. EGF treatment clearly induced a strong EGFR phosphorylation in MDA-MB468 cells while the EGFR antibodies of the invention did not affect EGFR signaling in absence of the ligand.

Example 5

Ligand binding competition

[0246] One mechanism of EGFR signaling inhibition is blockade of ligand binding. To examine if the EGFR antibodies inhibit the ligand binding to the EGFR, the binding of biotinylated EGFR ligand to the A431 cells was measured by flow cytometry in the presence of anti-EGFR antibodies. Biotinylation of TGF α was done using EZ-link Micro Sulfo-NHS-LC-biotinylation kit (Pierce, Rockland, IL) according to the manufacturer's instruction. Biotinylated EGF was obtained from Invitrogen. Prior to competition assay, the binding curve of the biotinylated ligands was established. Varying concentrations of anti-EGFR antibodies were pre-mixed with

biotinylated ligands at EC50 concentration (1.8 nM and 10 nM for EGF and TGF α , respectively), and the mixture was incubated with the cells for 30 min on ice. Cells were then washed twice with FACS buffer and incubated with streptavidin-APC conjugate (Jackson ImmunoResearch) for 15 min on ice. Cells were washed twice with FACS buffer and analyzed in FACS Calibur (BD Bioscience) using FlowJo program (Tree Star). The geometric mean fluorescence intensities were plotted against the antibody concentration in a semi-log plot. As shown in Figure 4, the negative control antibody, anti-KTI antibody does not affect the ligand binding while all anti-EGFR antibodies compete the ligand binding with the following EC50 (Table 10).

Table 10

	EC50 of TGF α binding competition (nM)	EC50 of EGF binding competition (nM)
Ligand	1.336	2.097
Cetuximab	0.769	1.226
EGFR-6	1.181	2.552
EGFR-12	1.341	2.927
EGFR13	1.321	1.957

[0247] The EGFR antibodies of the invention completely blocked the TGF α and EGF binding with similar EC50 as cetuximab (Figure 4 and Table 10). This result cannot explain the differential effect of cetuximab and the EGFR antibodies of the invention on ligand induced EGFR signaling (Example 3) and growth of normal epithelial cell lines including human primary keratinocytes (Example 6).

Example 6

Growth inhibition assay of human primary keratinocytes and normal epithelial cell line

[0248] Human normal basal epithelial cells in skin, gastrointestinal tract and other organs physiologically express EGFR. The EGFR signaling in these tissues is critical for the epithelial cell growth. Disruption of EGFR signaling pathway by cetuximab and panitumumab as well as small tyrosine kinase inhibitors such as erlotinib and gefitinib causes significant skin toxicity. To mimic the potential toxicity in skin and other epithelial cells, proliferation assays using human primary keratinocytes (Invitrogen) and a non-tumorigenic breast epithelial cell line, MCF10A (ATCC), were established. In this assay, cells were plated at 1,500-2,000 cells per well in EGFR ligand-containing media suggested by the manufacturers and incubated with anti-EGFR antibodies at 37°C for 5 days. In the keratinocytes assay (Figure 5), cells were grown in presence

of 1nM EGF with varying concentration of antibodies. While in MCF10A cell assay (Figure 6), cells were grown in presence of 10nM EGF with a fixed concentration (10 μ g/ml) of antibodies. Level of cell proliferation was determined using colorimetric WST-8 assay (Dojindo Molecular Technologies, Rockville, MD) as described in example 1. The surviving fraction was calculated by dividing each treated sample value by the average value of wells with untreated cell (surviving fraction = (A450 treated sample – A450 background)/ (A450 untreated sample – A450 background)). The results were normalized so that 0 indicated the level of cell proliferation in absence of EGF and 1 indicated the level of cell proliferation in presence of EGF without any anti-EGFR antibody treatment.

[0249] The binding of the anti-EGFR antibodies to human primary keratinocytes as well as MCF10A cells was confirmed before the proliferation assays were performed. A representative result of the keratinocytes proliferation assay is shown in Figure 5. As expected from the toxicity profile, cetuximab and panitumumab strongly inhibited the keratinocytes proliferation in dose dependent manner with the maximal inhibition of 40% for cetuximab and 78% for panitumumab. Surprisingly, the EGFR antibody of the invention, similar as the negative control chimeric KTI antibody, had little or no effect on the keratinocytes. This result was confirmed in the MCF10A proliferation assay (Figure 6). The cetuximab and panitumumab strongly inhibited MCF10A cell proliferation while the EGFR antibodies of the invention had little or no effect on MCF10A cell growth. These data suggest that the EGFR antibodies of the invention are less toxic than cetuximab and panitumumab on normal epithelial cells that express EGFR.

Example 7

Inhibition of basal proliferation of HCC827 and NCI-H292 cell lines

[0250] To determine the capacity of anti-EGFR antibodies in inhibiting the basal proliferation of tumor cells, proliferation assays with EGFR-expressing HCC827 (ATCC) and NCI-H292 (ATCC) lung tumor cell lines were established. Cells were plated at 2,000 cells per well in normal growth media containing 10% FBS and grown at 37°C for 5 days in presence of varying concentration of anti-EGFR antibodies. Level of cell proliferation was determined using colorimetric WST-8 assay. The OD results were normalized so that surviving fraction 1 represents cells grown in normal growth media without anti-EGFR antibodies, and 0 represents the value of wells without cells.

[0251] Figures 7A and 7B show the representative proliferation assay results with HCC827 and NCI-H292 cells, respectively. In HCC827 cell line (Figure 7A), cetuximab strongly inhibited the tumor cell growth in dose dependent manner. Despite being harmless to EGFR expressing normal epithelial cells, anti-EGFR antibodies of the invention showed similar or better inhibitory activity than cetuximab. Table 11 describes the EC50 and % maximal inhibition of each antibody.

Table 11. Anti-proliferative activity of anti-EGFR antibodies in HCC827 cells

Antibody	EC50 (nM)	Maximal inhibition (%)
Cetuximab	0.13	65
2	0.32	70
5	1.3	72
6	0.3	72
7	0.03	72
12	0.7	63
13	0.03	69
15	0.7	67
17	0.06	64

[0252] In NCI-H292 cell line (Figure 7B), cetuximab and panitumumab strongly inhibited the basal cell proliferation in dose dependent manner. The EGFR antibodies of the invention were also significantly active in this cell line with the maximal proliferation inhibition was similar to cetuximab and panitumumab. Table 12 describes the EC50 and % maximal inhibition of each antibody.

Table 12. Anti-proliferative activity of anti-EGFR antibodies in NCI-H292 cells

Antibody	EC50 (nM)	Maximal inhibition (%)
Panitumumab	0.039	76
Cetuximab	0.035	74
2	1.3	75
5	4.7	60
6	5.6	65
7	0.4	77
13	0.4	78
15	4.7	75
17	1.5	67

[0253] These data strongly argue that the EGFR antibodies of the invention are as active as cetuximab and panitumumab in EGFR overexpressing cells while they do not affect the normal epithelial cell growth.

Example 8

Anti-EGFR antibody binding competition

[0254] To distinguish binding epitopes of anti-EGFR antibodies, antibody binding competition assays were done using flow cytometry. In this experiment, binding of 0.3 nM biotinylated 528 (Figure 8A), 0.2 nM biotinylated cetuximab (Figure 8B) and 0.2 nM biotinylated EGFR-7 antibody (Figure 8C) to the MDA-MB468 cells was measured in presence of varying concentration of 'competing' antibodies. In brief, the biotinylated antibody was pre-mixed with varying concentration of 'competing' antibodies. The antibody mixture was then incubated with cells on ice for 2h. The cells were washed and incubated with streptavidin-alexa 488 conjugate on ice for 1h. After washing, the cells were fixed and analyzed in FACScalibur. The geometric mean fluorescence intensity was plotted against antibody concentration in semi-log plot and normalized so that 100% represents the maximal binding of the biotinylated antibody in absence of other antibody and 0% represents the background staining in absence of the biotinylated antibody. As shown in Figure 8, all the EGFR antibodies compete with each other. Cetuximab and panitumumab competed the 528 antibody binding as strong as the naked 528 antibody while the EGFR antibodies of the invention had slightly less capacity to compete with 528 antibody (Figure 8A). All EGFR antibodies competed the binding of cetuximab and EGFR-7 biotinylated antibodies in similar manner (Figure 8B and 8C).

Example 9

Cloning and sequencing of the VL and VH regions of the EGFR antibodies

[0255] Total cellular RNA was prepared from 5×10^6 cells of the EGFR hybridomas using an RNeasy kit (QIAGEN) according to the manufacturer's protocol. cDNA was subsequently synthesized from total RNA using the SuperScript II cDNA synthesis kit (Invitrogen).

[0256] The procedure for the first round degenerate PCR reaction on the cDNA derived from hybridoma cells was based on methods described in Wang et al. ((2000) *J Immunol Methods*. 233:167-77) and Co et al. ((1992) *J Immunol*. 148:1149-54). VH sequences were amplified by PCR using the following degenerate primers: EcoMH1 CTTCCGGAATTCSARGTNMAGCTGSAGSAGTC (SEQ ID NO:59), EcoMH2 CTTCCGGAATTCSARGTNMAGCTGSAGSAGTCWGG (SEQ ID NO:60) and BamIlg1 GGAGGATCCATAGACAGATGGGGGTGTCGTTTTGGC (SEQ ID NO:61). VL sequences were amplified by PCR using the following degenerate primers: SacIMK

GGAGCTCGAYATTGTGMTSACMCARWCTMCA (SEQ ID NO:62) and HindIII
TATAGAGCTCAAGCTTGGATGGTGGGAAGATGGATACAGTTGGTGC (SEQ ID NO:85).
(Mixed bases are defined as follows: N=G+A+T+C, S=G+C, Y=C+T, M=A+C, R=A+G,
W=A+T). The PCR reaction mixtures were then run on a 1% low melt agarose gel, the 300 to
400 bp bands were excised, purified using Zymo DNA mini columns, and sent to Agencourt
Biosciences for sequencing. The respective 5' and 3' PCR primers were used as sequencing
primers to generate the variable region cDNAs from both directions. The amino acid sequences
of VH and VL regions were predicted from the DNA sequencing results.

[0257] Since the degenerate primers used to clone the VL and VH cDNA sequences alters the 5'
end sequences, additional sequencing efforts were needed to verify the complete sequences. The
preliminary cDNA sequences were used to search the NCBI IgBlast site
(<http://www.ncbi.nlm.nih.gov/igblast/>) for the murine germline sequences from which the
antibody sequences are derived. PCR primers were then designed to anneal to the germline
linked leader sequence of the murine antibody so that this new PCR reaction would yield a
complete variable region cDNA sequence, unaltered by the PCR primers. The PCR reactions,
band purifications, and sequencing were performed as described above.

Mass determination for sequence confirmation

[0258] The cDNA sequence information for the variable region was combined with the germline
constant region sequence to obtain full length antibody cDNA sequences. The molecular weights
of the heavy chain and light chain were then calculated and compared with the molecular weights
obtained by LC/MS analyses of the murine EGFR antibodies. The molecular weight
measurements are consistent with the cDNA sequences for both the EGFR-7 and EGFR-12 light
and heavy chains.

Composite CDR sequences for the EGFR-7 variants

[0259] A number of murine anti-EGFR hybridomas were sequenced and found to express
antibodies with light and heavy chain variable region sequences nearly identical to EGFR-7, but
with some CDR amino acid substitutions, particularly in heavy chain CDR2 (Figure 9). Since
these CDR variants of murine EGFR-7 were found to be functionally identical, they provide
some structural insight into the sequence flexibility of the EGFR-7 CDR's. Light chain CDR's 2
and 3 as well as heavy chain CDR 1 were identical in each of the variants while a single residue
substitution was found at a low frequency in light chain CDR1 and heavy chain CDR3. As

opposed to the CDR's with an apparently tight sequence conservation, the variants of EGFR-7 frequently contained as many as 4 amino acid substitutions in heavy chain CDR2. These sequence variants of EGFR-7 suggest that the 5 tightly conserved CDR's may provide the structural basis for EGFR binding, while heavy chain CDR2 has some sequence flexibility resulting in a somatic mutation hotspot during affinity maturation. Table 4 provides a composite CDR sequence listing based on EGFR-7 variants together with the CDR variants described above for humanization. Humanized antibodies derived from these composite CDR's would be expected to preserve the functional attributes of EGFR-7.

Table13

EGFR-7 variant composite CDR's
Light Chain
CDR1: [KorR]ASQDINNY[LorI]A (SEQ ID NO:14)
CDR2: YTSTLHP (SEQ ID NO:11)
CDR3: LQYDNLLYT (SEQ ID NO:12)
Heavy Chain
CDR1: TSYWMQ (SEQ ID NO:1)
CDR2: [TorA][lorL]YPGDGD[TorA][T,R,orS] (SEQ ID NO:4)
Kabat CDR2: [TorA][lorL]YPGDGD[TorA][T,R,orS][YTorI]QKF[QorK]G (SEQ ID NO:6)
CDR3: YDAPGY[AorT]MDY (SEQ ID NO:5)

Example 10

Antibody humanization

[0260] The EGFR-7 and EGFR-12 antibodies were humanized following resurfacing methods previously described, such as, for example in Roguska et al., Proc. Natl. Acad. Sci., USA, 91(3):969-973 (1994) and Roguska et al., Protein Eng. 9(10):895-904 (1996), which are incorporated in their entirety herein by reference. Resurfacing generally involves identification of the variable region framework surface residues in both the light and heavy chains and replacing them with human equivalents. The murine CDR's are preserved in the resurfaced antibody. Exemplary CDRs of EGFR-7 and EGFR-12 antibodies are defined as indicated in Table 13. To minimize concerns about the impact of conjugating lysines that fall in CDR's, lysine 24 in murine EGFR-7 antibody light chain CDR1 was replaced with arginine for

humanized version 1.0 (shown in *italic*), so both versions of the LC CDR1 are given. Similarly, lysine 27 and lysine 31 in murine EGFR-12 antibody light chain CDR1 were replaced with glutamine and arginine, respectively, for humanized version 1.0 (shown in *italic*). The replacement of lysine 27 with glutamine instead of arginine was because glutamine is reserved in human germline sequences at this position. In addition to the AbM heavy chain CDR2 definition employed for resurfacing, the table provides exemplary Kabat defined heavy chain CDR2's for both the murine and human versions of EGFR-7 and EGFR-12 antibodies. The underlined sequence marks the portion of the Kabat heavy chain CDR2 that was not considered a CDR for resurfacing.

[0261] Surface residue positions were defined as any position with a relative accessibility of 30% or greater (Pedersen J.T. et. Al, J. Mol. Biol. 1994; 235: 959-973). The calculated surface residues were then aligned with human germline surface sequences to identify the most homologous human surface sequence. The human germline sequence used as the replacement surface for the light chain variable domains of EGFR-7 antibody was IGKV1-12*01 while IGKV1-16*01 was used as the replacement surface for EGFR-12 antibody VL. The human germline sequences used as the replacement surfaces for the heavy chain variable domains of EGFR-7 and EGFR-12 antibodies were IGHV1-69*02 and IGHV1-69*08, respectively. The specific framework surface residue changes for EGFR-7 and EGFR-12 antibodies are given in Figures 10 and 11, respectively. Since the resurfaced light chain of both antibodies included the CDR1 lysine substitution(s) for preferred version, a resurfaced version (v1.01) was also generated with murine lysine(s) retained in CDR-L1. Finally, heavy chain framework 2 for murine EGFR-7 and its variants, contained a somatic W47C mutation resulting in an unpaired cysteine residue. For this reason, EGFR-7 heavy chain W47 germline revertant versions (v1.11) were also tested. Figure 12 shows the alignment of the resurfaced sequences for EGFR-7 and EGFR-12 antibodies variable domain of both light and heavy chain with their murine counterparts.

Table 14

EGFR-7 CDR's (Resurfacing)	EGFR-12 CDR's
Light Chain	Light Chain
Murine and resurfaced v1.01 CDR1: KASQDINNYLA (SEQ ID NO:10)	Murine and resurfaced v1.01 CDR1: RASKSISKYLA (SEQ ID NO:15)
Resurfaced v1.0 CDR1: RASQDINNYLA (SEQ ID NO:13)	Resurfaced v1.0 CDR1: RASQSISRYLA (SEQ ID NO:16)

CDR2: YTSTLHP (SEQ ID NO:11)	CDR2: SGSTLQS (SEQ ID NO:17)
CDR3: LQYDNLLYT (SEQ ID NO:12)	CDR3: QQHNEYPWT (SEQ ID NO:18)
Heavy Chain	Heavy Chain
CDR1: TSYWMQ (SEQ ID NO:1)	CDR1: TSYWMQ (SEQ ID NO:1)
CDR2: TIYPGDGDTT (SEQ ID NO:2)	CDR2: TIYPGDGDTR (SEQ ID NO:7)
CDR3: YDAPGYAMDY (SEQ ID NO:3)	CDR3: YDAPGYAMDY (SEQ ID NO:3)
Kabat EGFR-7 HC CDR2	Kabat EGFR-12 HC CDR2
Murine HC CDR2: TIYPGDGDTTYTQKFKG (SEQ ID NO:63)	Murine HC CDR2: TIYPGDGDTRYIQKFKG (SEQ ID NO:8)
Humanized HC CDR2: TIYPGDGDTTYTQKFQG (SEQ ID NO:64)	Humanized HC CDR2: TIYPGDGDTRYIQKFQG (SEQ ID NO:9)

[0262] In addition to humanization by variable domain resurfacing, EGFR-7 antibody was also humanized following complementary determining region (CDR) grafting technology described, such as for example in Jones et al., Nature 321: 604-608 (1986) and Verhoeven et al., Science 239: 1534-1536 (1988). CDR grafting method consists of grafting the CDRs from a naturally evolved murine antibody onto the Fv framework regions (FRs) of a human antibody. The main step of the process was to choose the appropriate human acceptor frameworks. Kabat numbering scheme and Kabat CDR definition were used for CDR grafting of EGFR-7 antibody. Exemplary CDRs of EGFR-7 antibody for CDR grafting are defined as indicated in Table 15. Human immunoglobulin germline sequence with the highest homology with the murine EGFR-7 antibody was identified through the interactive tool, V-QUEST, of the International ImMunoGeneTics information system® (IMGT (<http://imgt.cines.fr/>)) as described in Lefranc, Nucleic Acids Res. 29: 207-209 (2001). The human germline sequences used as the acceptor frameworks for the VL and VH domains of EGFR-7 antibody were IGKV1-33*01 and IGHV1-46*03, respectively. To minimize concerns about the impact of conjugating lysines that fall in CDR's, lysine 24 in murine EGFR-7 antibody light chain CDR1 was replaced with arginine in CDR grafting (Table 6). The specific framework residue changes as well as the substitution in CDR-L1 in CDR-grafting of EGFR-7 antibody are given in Figure 13, and the alignments of the CDR-grafted sequences for EGFR-7 antibody variable domains with its murine counterparts is illustrated in Figure 14.

Table 15

EGFR-7 CDR's (CDR grafting)
Light Chain

Murine CDR1: KASQDINNYLA (SEQ ID NO:10)
CDR grafted CDR1: RASQDINNYLA (SEQ ID NO:13)
CDR2: YTSTLHP (SEQ ID NO:11)
CDR3: LQYDNLLYT (SEQ ID NO:12)
Heavy Chain
CDR1: TSYWMQ (SEQ ID NO:1)
CDR2: TIYPGDGDTTYTQKFKG (SEQ ID NO:63)
CDR3: YDAPGYAMDY (SEQ ID NO:3)

Recombinant expression of the humanized EGFR antibodies

[0263] The variable region sequences for huEGFR-7 and huEGFR-12 were codon-optimized and synthesized by Blue Heron Biotechnology. The sequences were flanked by restriction enzyme sites for cloning in-frame with the respective constant sequences in single chain mammalian expression plasmids. The light chain variable region was cloned into EcoRI and BsiWI sites in the pAbKZeo plasmid. The heavy chain variable region was cloned into the HindIII and ApaI sites in the pAbG1Neo plasmid. These plasmids were used to express the recombinant antibodies in either transient or stable mammalian cell transfections. Transient transfections to express recombinant antibodies in HEK 293T cells were performed using a modified PEI procedure (Durocher, Y. et al., *Nucleic Acids Res.* 30:E9 (2002)). Supernatant was purified by Protein A and polishing chromatography steps using standard procedures as described above for murine antibodies.

Example 11

Binding competition between murine and humanized anti-EGFR antibodies

To examine the binding of humanized anti-EGFR antibodies, antibody competition assays were performed as described in Example 8. Because muEGFR-6 and muEGFR-7 antibodies cross-compete with one another (Figure 8C), this experiment examines the ability of murine or humanized antibodies of EGFR-6 (Figure 15A) or EGFR-7 (Figure 15B) to compete with the binding of biotinylated muEGFR-7 antibody to the MDA-MB468 cells. In brief, 1 nM of biotinylated muEGFR-7 antibody was pre-mixed with various concentration of 'competing' antibody. The antibody mixture was then incubated with target cells on ice for 1 hour, washed, incubated with streptavidin-APC conjugate on ice for another hour, washed, fixed and analyzed using flow cytometer. The geometric mean fluorescence intensity was plotted against antibody concentration in semi-log plot and normalized so that 100% represents the maximal binding of the biotinylated antibody in absence of other antibody and 0% represents the background

staining in absence of the biotinylated antibody. As shown in Figure 15A, both muEGFR-6 and huEGFR-6 antibodies compete the binding of muEGFR7 at similar EC50 (8.13 nM for muEGFR-6 and 11.09 nM for huEGFR-6). Figure 15B shows that muEGFR-7, hu-EGFR-7 and huEGFR-7R antibodies also compete the muEGFR-7 antibody binding at similar EC50 (3.54 nM for muEGFR7, 3.35 nM for huEGFR-7R and 4.12 nM for huEGFR-7). These results indicate that humanization does not affect the binding of antibodies of the invention.

Anti-tumor activity of humanized anti-EGFR antibodies

[0264] To examine the anti-tumor activity of the humanized antibodies of the invention, H292 tumor cell growth inhibition assays were performed as described in Example 7. As shown in Figure 16, murine and humanized antibodies of EGFR-6 and EGFR-7 were potent in inhibiting H292 tumor cell growth with EC50 included in Table 16. It is apparent that all humanized antibodies maintain the anti-tumor activity of the murine counterparts and humanization does not affect the biological activity of these antibodies.

Table 16. Anti-proliferative activity of anti-EGFR antibodies in NCI-H292 cells

Antibody	EC50 (nM)
muEGFR-6	2.59
huEGFR-6	1.61
muEGFR-7	0.21
huEGFR-7	0.17
huEGFR-7R	0.11

Example 12

Antibody-dependent-cellular-cytotoxicity (ADCC) activity of huEGFR antibodies

[0265] A lactate dehydrogenase (LDH) release assay was used to measure antibody-dependent cell mediated cytotoxicity (ADCC) of tumor cells lines using freshly isolated human natural killer (NK) cells as effector cells (Shields RL, *J Biol Chem.* 2001 276(9):6591-604). The NK cells were first isolated from human peripheral blood from a normal donor (Research Blood Components, Inc., Brighton, MA) using a modified protocol for the NK cell Isolation Kit II (#130-091-152; Miltenyi Biotec, Auburn, CA). Peripheral blood was diluted 2-fold with 1x PBS. 25 mL of diluted blood was carefully layered over 25 mL of Ficoll Paque in a 50 mL conical tube and centrifuged at 400 g for 45 min at RT. The peripheral blood mononuclear cells (PBMC) were collected from the interface, transferred into a new conical 50 mL tube, and washed once with 1x

PBS. The PBMC were counted and resuspended at concentration of 2.5×10^7 cells/100 μ L with MACS buffer (1x PBS, 0.5% BSA, 2mM EDTA), and then 1/4x volume of NK cell Biotin-Antibody Cocktail were added to the cell suspension. The NK cell Biotin-Antibody Cocktail contains biotinylated antibodies that bind to the lymphocytes, except for NK cells, resulting in a negative selection of NK cells. The mixture was incubated at 4°C for 10 min, and then 3/5x volume of MACS buffer and 2/5x volume of NK cell MicroBead cocktail that would bind to the biotinylated antibodies were added. The cell-antibody mixture was incubated for another 15 min at 4°C. Next, cells were washed once with 50 mL of MACS buffer and resuspended in 3 mL of MACS buffer. NK cells were separated as negative fraction using autoMACS separator (Miltenyi Biotec). The resulting NK cells were plated into 30 mL of complete RPMI media (RPMI-1640 supplemented with 5% fetal bovine serum, 1% penicillin-streptomycin, 1 mM HEPES, 1 mM Sodium Pyruvate, 1% 100X MEM non-essential Amino Acid Solution) overnight. The subsequent assay and all dilutions were carried out in RHBP medium (RPMI-1640 medium supplemented with 20 mM HEPES, pH 7.4, 0.1% BSA and 1% penicillin-streptomycin).

[0266] Various concentrations of antibodies in RHBP medium were aliquoted in duplicate at 50 μ L/well into a round bottom 96-well plate. The target cells (in this experiment A431 cell line) were resuspended at 10^6 cells/mL in RHBP medium and added at 100 μ L/well to each well containing antibody dilutions. The plate containing target cells and antibody dilutions was incubated for 30 min at RT. NK cells were then added to the wells containing the target cells at 50 μ L/well. The typical ratio was 1 target cell to 3-4 NK cells. The following controls were set up for each experiment: NK cells alone, target cells alone (spontaneous LDH release), target cells with NK cells (antibody independent LDH release), target cells with 10% Triton X-100 (maximum LDH release). The mixtures were incubated at 37°C for 4 h to allow for cell lysis. Plates were centrifuged for 10 min at 1200 rpm, and 100 μ L of the supernatant was carefully transferred to a new flat-bottom 96-well plate. LDH reaction mixture (100 μ L/well) from the Cytotoxicity Detection Kit (Roche 1 644 793) was added to each well and incubated at room temperature for 5 to 30 min. The optical density (OD) of samples was measured at 490 nm (OD₄₉₀). The percent specific lysis of each sample was determined using the following formula: percent specific lysis = (sample value - spontaneous release) / (maximum release - spontaneous release) * 100.

[0267] Figure 17 shows a representative ADCC activity of huEGFR-6 and huEGFR-7R antibodies in comparison to that of chKTI antibody. The huEGFR-6 and huEGFR-7R antibodies

induced NK cell mediated killing of target cells in dose dependent manner with maximal specific killing reached around 20% and EC50 of 22 ng/ml and 17 ng/ml for huEGFR6 and huEGFR-7R, respectively. In contrast, chKTI antibody that did not bind to target cells failed to mediate ADCC.

Example 13

Preparation of huEGFR-7R-SMCC-DM1

[0268] The (Succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC, Pierce Biotechnology, Inc) linker was dissolved in dimethylacetamide (DMA). The huEGFR antibody was modified with SMCC to introduce maleimides into the antibody by incubating the antibody at 5 mg/mL in 50mM potassium phosphate, 50mM NaCl, 2mM EDTA, pH 6.5 with a 10 molar excess of SMCC. After approximately 100 minutes at ambient temperature, the reaction mixture was purified using a SEPHADEX™ G25 column equilibrated with the same potassium phosphate buffer. Antibody containing fractions were pooled and used for subsequent steps.

[0269] The SMCC-modified antibody was reacted with a 10 mM solution of DM1 at a 1.7 molar excess relative to the maleimide linker. The reaction was stirred at ambient temperature under for approximately 18 hours. The conjugation reaction mixture was filtered through a SEPHADEX™ G25 gel filtration column equilibrated with 1×PBS at pH 6.5. The huEGFR antibody-SMCC-DM1 conjugate was then dialyzed into buffer containing 10 mM histidine, 250 mM glycine, 1% sucrose pH 5.5. The number of DM1 molecules linked per antibody molecule was determined using the previously reported extinction coefficients for antibody and DM1 (Liu et al., *Proc. Natl. Acad. Sci. USA*, 93, 8618-8623 (1996)). The percentage of free maytansinoid present after the conjugation reaction was determined by injecting 20-50 µg conjugate onto a HiSep column equilibrated in 25% acetonitrile in 100 mM ammonium acetate buffer, pH 7.0, and eluting in acetonitrile. The peak area of total free maytansinoid species (eluted in the gradient and identified by comparison of elution time with known standards) was measured using an absorbance detector set to a wavelength of 252 nm and compared with the peak area related to bound maytansinoid (eluted in the conjugate peak in the column flow-through fractions) to calculate the percentage of total free maytansinoid species. Conjugates with 3.5-4 DM1 molecules per huEGFR antibody were obtained with <1% present as unconjugated maytansinoid.

Preparation of huEGFR-7R-SPDB-DM4

[0270] The exemplary N-succinimidyl 4-(2-pyridyldithio) butanoate (SPDB) linker was dissolved in ethanol. The huEGFR antibody was incubated at 8 mg/mL with a 5.5-5 fold molar excess of SPDB linker for approximately 2 hours at room temperature in 50 mM potassium phosphate buffer (pH 6.5) containing 50 mM NaCl, 2 mM EDTA, and 3% ethanol. The SPDB modified antibody was diluted 2-fold in PBS, pH 6.5 and modified with a 1.5 fold molar excess of the maytansinoid DM4 by the addition of a concentrated solution (15-30 mM) of DM4 in dimethylacetamide (DMA). After overnight incubation at room temperature, the conjugated antibody was purified by chromatography on SEPHADEX™ G25F equilibrated with 10 mM histidine, 250 mM glycine, 1% sucrose pH 5.5. The number of DM4 molecules linked per antibody molecule was determined using the previously reported extinction coefficients for antibody and maytansinoid (Widdison WC et al. J Med Chem, 49:4392-4408 (2006)). The percentage of total free maytansinoid species were determined as described above. Conjugates with 3.5-4 DM4 molecules per huEGFR antibody were obtained with <1% present as unconjugated maytansinoid.

Example 14

Binding affinity of maytansinoid conjugates

[0271] Binding affinity of the huEGFR-6 and huEGFR-7R antibody maytansinoid conjugates was compared with that of the naked antibodies using MDA-MB468 cells as described in the Example 2. The Kds calculated from binding curves of the huEGFR-6 antibody and conjugates (Figure 18 A) were 0.81 nM for naked huEGFR-6 antibody and 1.18 nM for huEGFR-6-SMCC-DM1 conjugate. The Kds calculated from binding curves of the huEGFR-7R antibody and conjugates (Figure 18 B) were 0.67 nM for naked huEGFR-7R antibody and 0.83 nM for huEGFR-7R-SMCC-DM1 conjugate. This data demonstrates that DM conjugation does not notably alter the binding affinity of the huEGFR-6 and huEGFR-7R antibody to the huEGFR antigen.

Example 15

In vitro cytotoxic assay on tumor cells

[0272] The ability of EGFR antibody maytansinoid conjugates to inhibit the tumor cell growth was measured using *in vitro* cytotoxicity assays. Briefly, target cells were plated at 1,500 to 3,000 cells per well in 100 μ L complete RPMI media containing 10% FBS. Conjugates were diluted into complete RPMI media using 5-fold dilution series and 100 μ L were added per well. The final concentration typically ranged from 3×10^{-8} M to 8×10^{-14} M. Cells were incubated at 37°C in a humidified 5% CO₂ incubator for 5 days. Viability of the remaining cells was determined by colorimetric WST-8 assay and the absorbance at 450 nm (A₄₅₀) was measured in a multiwell plate reader. The surviving fraction was calculated by dividing each treated sample value by the average value of untreated controls. The surviving fraction value was plotted against the antibody-conjugate concentration in a semi-log plot for each treatment.

[0273] The *in vitro* cytotoxicity of naked antibodies and antibody-maytansinoid conjugates of the invention was compared to the activity of a non-specific antibody and its corresponding maytansinoid conjugate such as chKTI and chKTI-SMCC-DM1. The results from a typical cytotoxicity assay are shown in Figures 19 and 20.

[0274] In Figure 19A, the activity of naked antibodies and maytansinoid conjugates of the invention was tested in FaDu cell line. The huEGFR-6 and huEGFR-7R naked antibodies inhibited 40% and 55% H292 cell growth, respectively while the chKTI antibody had no activity. Maytansinoid conjugation further enhances the activity of the EGFR antibody of the invention. Both huEGFR-6 and huEGFR-7R-SMCC-DM1 conjugates completely abolished the target cells with EC₅₀ of 0.22 nM and 0.06 nM, respectively. The control chKTI-SMCC-DM1 conjugate also killed the target cells but with much lower EC₅₀ (0.59 μ M).

[0275] In Figure 19B, the activity of naked antibodies and maytansinoid conjugates of the invention was tested in H292 cell line. The huEGFR-6 and huEGFR-7R naked antibodies inhibited 60-70% H292 cell growth while the chKTI antibody had no activity. Maytansinoid conjugation further enhances the activity of the EGFR antibody of the invention. Both huEGFR-6 and huEGFR-7R-SMCC-DM1 conjugates completely abolished the target cells with EC₅₀ of 0.16 nM and 0.03 nM, respectively. The control chKTI-SMCC-DM1 conjugate also killed the target cells but with much lower EC₅₀ (38.51 nM).

[0276] In Figure 20A, the activity of naked antibodies and maytansinoid conjugates of the invention was compared with cetuximab in H226 cell line. The cetuximab, huEGFR-6, huEGFR-

7R naked antibodies as well as the chKTI control antibody had no anti-proliferative activity. Maytansinoid conjugates of both huEGFR-6 and huEGFR-7R antibodies completely eliminated the target cells with EC₅₀ of 0.68 nM and 0.14 nM, respectively while the control chKTI-SMCC-DM1 conjugate failed to kill the target cells.

[0277] In Figure 20B, the activity of naked antibodies and maytansinoid conjugates of the invention was compared with cetuximab in the SCC-4 cell line. The cetuximab, huEGFR-6 and huEGFR-7R naked antibodies showed a dose dependent growth inhibition with maximal inhibition of around 30%, while the chKTI antibody had no activity. Maytansinoid conjugation further potentiates the activity of the EGFR antibody of the invention. Both huEGFR-6 and huEGFR-7R-SMCC-DM1 conjugates completely eliminated the target cells with EC₅₀ of 0.07 nM and 0.03 nM, respectively. The control chKTI-SMCC-DM1 conjugate also killed the target cells but with a much lower EC₅₀ (17.62 nM). Altogether, these results show that the maytansinoid conjugation dramatically enhances the anti-tumor activity of the EGFR antibodies of the invention.

Example 16

In vivo efficacy study comparing huEGFR-6 and huEGFR-7R antibodies and maytansinoid conjugates

The activity of naked antibody and antibody-maytansinoid conjugates of the huEGFR-6 and huEGFR-7R antibodies was tested in EGFR expressing H292 NSCLC (non-small cell lung cancer) (Figure 21) and FaDu SCCHN (squamous cell carcinoma of head and neck) (Figure 22) tumor xenograft models. 1×10^7 tumor cells were injected subcutaneously into SCID mice. Animals were randomized by tumor volume into treatment groups when tumors reached a mean tumor volume of approximately 100 mm³ and injected once with the indicated dosage of naked antibodies or antibody-maytansinoid conjugates. Median tumor volume of each treatment groups is plotted against time post tumor cell inoculation (Figures 21 and 22). Tables 17 and 18 show the number of mice with complete response (CR) (no palpable tumor) and percent of tumor growth inhibition (% T/C) which corresponds to the median of tumor volume of each treated group divided by the median tumor volume of control group when the tumor volume of the control group reaches a predetermined size. A treatment with a % T/C value of below 42% is considered active, while a treatment with a % T/C value of below 12% is considered highly active.

[0278] Both the naked antibodies and antibody maytansinoid conjugates of the invention were very active and they significantly delayed the growth of both H292 and FaDu tumor xenografts

(Figures 21 and 22). In H292 tumor xenograft study (Figure 21 and Table 17), all mice treated with the huEGFR-6-SMCC-DM1 and huEGFR-7R-SMCC-DM1 conjugates as low as 3 mg/kg exhibited a complete response. Even the huEGFR-6 and huEGFR-7R antibodies at 10 mg/kg were highly active. In FaDu tumor xenograft study (Figure 22 and Table 18), the huEGFR-6 and the huEGFR-7R antibodies at 10 mg/kg were active and the antibody-maytansinoid conjugates were even more active with complete response in some mice.

Table 17. Activity of EGFR Ab and maytansinoid conjugates in H292 tumor xenograft

Ab and conjugate	% T/C	CR
huEGFR-6 Ab 10 mg/kg	3.1	1/6
huEGFR-6-SMCC-DM1 3 mg/kg	0.0	6/6
huEGFR-6-SMCC-DM1 10 mg/kg	0.0	6/6
huEGFR-7R Ab 10 mg/kg	2.2	4/6
huEGFR-7R-SMCC-DM1 3 mg/kg	1.2	6/6
huEGFR-7R-SMCC-DM1 10 mg/kg	0.6	6/6

Table 18. Activity of EGFR Ab and maytansinoid conjugates in FaDu tumor xenograft

Ab and conjugate	% T/C	CR
huEGFR-6 Ab 10mg/kg	17.5	0/6
huEGFR-6-SMCC-DM1 10mg/kg	5.8	2/6
huEGFR-7R Ab 10mg/kg	13.4	0/6
huEGFR-7R-SMCC-DM1 10mg/kg	5.5	1/6

In vivo efficacy study comparing huEGFR-7R-SMCC-DM1 and huEGFR-7R-PEG-MAL-DM1

[0279] The activity of naked antibody, the huEGFR-7R-SMCC-DM1 and the huEGFR-7R-PEG-MAL-DM1 conjugates was compared in EGFR expressing H292 NSCLC (non-small cell lung cancer) (Figure 25), HSC2 SCCHN (squamous cell carcinoma of head and neck) (Figure 26) and FaDu SCCHN (Figure 27) tumor xenograft models. The experiment and data analysis were done as described above.

[0280] In H292 NSCLC tumor xenograft study (Figure 25 and Table 19), all of the test articles were highly active at 3 mg/kg single dose. All mice treated with both the huEGFR-7R-SMCC-DM1 and the huEGFR-7R-PEG-MAL-DM1 conjugates showed a complete response, while none of the mice treated with the huEGFR-7R antibody had a complete response. In HSC2 SCCHN tumor xenograft study (Figure 26 and Table 20), both the huEGFR-7R-SMCC-DM1 and the huEGFR-7R-PEG-MAL-DM1 conjugates were highly active with T/C of 8%, while the huEGFR-7R antibody was barely active at 5 mg/kg single dose. In FaDu SCCHN tumor xenograft study (Figure 27 and Table 21), both the huEGFR-7R-SMCC-DM1 and the huEGFR-7R-PEG-MAL-DM1 conjugates were active with T/C of 15% and 28%, respectively. The huEGFR-7R antibody treatment showed some tumor growth inhibition but it was not significantly active. In conclusion, these results show that the naked antibodies of the invention are potent in inhibiting the growth of NSCLC and SCCHN tumors, and the conjugation with maytansinoid further enhances the anti-tumor activity.

Table 19. Activity of huEGFR-7R Ab and maytansinoid conjugates in H292 tumor xenograft

Ab and conjugate	% T/C	CR
huEGFR-7R Ab 3 mg/kg	10	0/6
huEGFR-7R-SMCC-DM1 3 mg/kg	< 1	6/6
huEGFR-7R-PEG-MAL-DM1 3 mg/kg	< 1	6/6

Table 20. Activity of huEGFR-7R Ab and maytansinoid conjugates in HSC2 tumor xenograft

Ab and conjugate	% T/C	CR
huEGFR-7R Ab 5 mg/kg	41	0/6
huEGFR-7R-SMCC-DM1 5 mg/kg	8	0/6
huEGFR-7R-PEG-MAL-DM1 5 mg/kg	8	0/6

Table 21. Activity of huEGFR-7R Ab and maytansinoid conjugates in FaDu tumor xenograft

Ab and conjugate	% T/C	CR
huEGFR-7R Ab 5 mg/kg	45	0/6
huEGFR-7R-SMCC-DM1 5 mg/kg	15	0/6
huEGFR-7R-PEG-MAL-DM1 5 mg/kg	28	0/6

Example 17

In vitro cytotoxicity assay on human primary keratinocytes

[0281] EGFR signaling plays a key role in human primary keratinocyte proliferation. Inhibition of EGFR signaling by small molecule tyrosine kinase inhibitors or antagonistic anti-EGFR antibodies such as cetuximab leads to growth arrest and apoptosis in keratinocyte culture (Stoll et

al., Oncogene 16, 1493-1499 (1998)). Keratinocyte apoptosis is thought to be one of the mechanism underlying dermatologic toxicities caused by the anti-EGFR therapies in the clinic. To examine the potential of skin toxicity, the EGFR antibodies and antibody-maytansinoid conjugates of the invention was tested in an *in vitro* cytotoxicity assay using human primary keratinocytes. Briefly, human primary keratinocytes (Invitrogen) were plated at 1,500 to 3,000 cells per well in 100 μ L EGF containing media suggested by the manufacturer. Test articles were diluted in EGF containing media using 5-fold dilution series and 100 μ L were added per well. The final concentration typically ranged from 3×10^{-8} M to 8×10^{-14} M. Cells were incubated at 37°C in a humidified 5% CO₂ incubator for 5 days. Viability of the remaining cells was determined by colorimetric WST-8 assay and the absorbance at 450 nm (A₄₅₀) was measured in a multiwell plate reader. The surviving fraction was calculated by dividing each treated sample value by the average value of untreated controls. The surviving fraction value was plotted against the antibody-conjugate concentration in a semi-log plot for each treatment.

[0282] The *in vitro* cytotoxicity of huEGFR-7R naked antibodies and huEGFR-7R-SMCC-DM1 conjugate was compared to the activity of a non-specific antibody (chKTI), chKTI-SMCC-DM1 conjugate, a non antagonistic antibody (huML66) and its corresponding maytansinoid conjugate on the human primary keratinocytes (Figure 23A) as well as H292 tumor cells (Figure 23B) in the same experiment. In H292 cell line (Figure 23B), huML66 antibody had no activity, while the huEGFR-7R antibody inhibited cell growth up to 55%. The maytansinoid conjugates of huML66 and huEGFR-7R had the best activity; they were able to completely inhibit tumor cell growth with EC₅₀ between 0.05 and 0.07 nM. In human primary keratinocytes (Figure 23A), chKTI and huML66 antibodies had no effect on keratinocyte proliferation. However, the huML66-SMCC-DM1 was very potent in killing the keratinocytes with 0.55 nM EC₅₀. The huEGFR-7R naked antibody had very little effect on keratinocytes. Surprisingly, the huEGFR-7R-SMCC-DM1 conjugate was much less toxic to the keratinocytes as compared to the huML66-SMCC-DM1 conjugate. At the concentration of 3.3 nM, the huEGFR-7R naked antibody and its corresponding conjugate only inhibited less than 40% of the keratinocyte growth. In summary, antibody and antibody-maytansinoid conjugate of the invention has little effect on the human primary keratinocyte cell growth while they are very potent in eliminating tumor cells.

Example 18

Chemokine/cytokine production by human primary keratinocytes

[0283] The skin epithelium, which is composed mainly of keratinocytes interspersed with dendritic cells, melanocytes, and rare T lymphocytes and monocytes, is highly committed to host defense. Physical, chemical, or immune-specific insults rapidly evoke an epidermal response characterized by the increase expression of chemokine and cytokines, which attract and activate distinct leukocyte subpopulations to induce inflammatory response. $\text{TNF}\alpha$ induces human keratinocytes to express numerous chemokines and cytokines including CCL5/RANTES, CXCL10/IFN γ -inducible-protein 10 and CXCL8/IL-8. CCL5 attracts T cells, monocytes as well as neutrophils. CXCL10 induces migration of type 1 T cells. CXCL8 is a chemoattractant active in neutrophil recruitment as well as in epithelial and endothelial cell proliferation.

[0284] EGFR signaling governs the homeostatic maintenance and repair of epithelial tissue. EGFR activation leads to keratinocyte proliferation, migration and controlled differentiation. In response to $\text{TNF}\alpha$, keratinocytes produce EGFR ligands which activate EGFR signaling. The enhanced EGFR activation in keratinocytes increases CXCL8 expression and reduces CCL5 and CXCL10 expression. In contrast, impairment of EGFR signaling led to an opposite pattern. Skin application of a selective EGFR tyrosine kinase inhibitor led to more severe contact hypersensitivity responses, with increased epidermal levels of CCL5 and CXCL10, and a higher number of monocytes/macrophages and T cells in the skin. These findings suggested that EGFR signaling modulates skin inflammation by affecting chemokine expression in keratinocytes (Pastore, *J. Immunol.*, 174: 5047-5056 (2005)). It is now believed that skin toxicity manifested in the clinic upon EGFR therapies are caused by apoptosis and sustained inflammation in the skin.

[0285] The effect of antibodies and antibody-maytansinoid conjugates of the invention in modulating chemokine/cytokine production by human primary keratinocytes was tested in the following in vitro assay. 1×10^5 human primary keratinocytes/well (Invitrogen) were first seeded in 6 well plate. The cells were starved overnight and then cultured with 100 ng/ml $\text{TNF}\alpha$ and 10 $\mu\text{g/ml}$ test antibodies in the EGF containing media for 14 hours. The amount of CCL5, CXCL10 and CXCL8 in the culture supernatant was measured using ELISA kits from R&D systems according to the manufacturer's protocol. As shown in Figure 24, cetuximab reduced the expression of CXCL8 and increased the production of CXCL10 and CCL5. In contrast, the naked antibodies of the invention had no or little effect on the expression of these chemokines/cytokines when compared to the chKTI control. Surprisingly, the maytansinoid conjugates of the EGFR

antibodies of the invention also had no or little effect on the keratinocytes. Altogether, the results shown in Examples 17 and 18 strongly suggest that both the antibodies and antibody maytansinoid conjugates of the invention had minimal effect on the human primary keratinocytes in vitro, therefore are likely to be less toxic to the skin in humans. In contrast to the effect on keratinocytes, the antibodies and antibody maytansinoid conjugates of the invention are very potent in killing the EGFR positive tumor cells in vitro and in vivo as shown in Examples 7, 12, 15 and 16. In summary, the antibodies and antibody cytotoxic agent conjugates of the invention are unique class of anti-EGFR molecules that have distinct effect on normal vs. tumor cells.

Example 19

Epitope mapping

[0286] The human EGFR is a large (1186 residues), monomeric glycoprotein with an extracellular ligand binding region, a single transmembrane region and a cytoplasmic tyrosine kinase domain flanked by noncatalytic regulatory regions. The extracellular domain (ECD) of human EGFR (residues 1-618) contains four subdomains (Figure 28), here termed domain I (amino acids 1-165), domain II (amino acids 166-309), domain III (amino acids 310-481), and domain IV (amino acids 482-618). These domains are also referred to as L1, CR1, L2, and CR2, where L and CR are acronyms for large and Cys-rich, respectively. The epitope of the huEGFR-7R of the invention were mapped mainly to the defined region containing amino acids 460-480 in the human EGFR ECD domain III by engineering truncated and chimeric human/murine EGFR molecules.

EGFR variants cloning and expression

[0287] The entire human EGFR ECD (amino acids 1-618) was expressed as an Fc fusion protein (huEGFR-Fc). The protein sequence was codon optimized, synthesized, and cloned in frame with a murine IgG2A hinge, CH2, and CH3 region in the pmuFc2ANL mammalian expression vector by Blue Heron Biotechnologies. As antibodies of the invention compete with cetuximab binding to EGFR (Figure 8B), and cetuximab binds exclusively to domain III, the epitope of the antibodies of the invention may also be located in domain III and might overlap with that of cetuximab. To further identify the epitope, an Fc fusion of the truncated human EGFR (huEGFRdIII-Fc), containing entire domain III (amino acids 310-481) plus 20 extra residues from domain IV (amino acids 482-501), which was suggested to be required for binding of

cetuximab, was similarly constructed as huEGFR-Fc. Further, a truncated murine EGFR containing amino acids 310-501 (muEGFRdIII-Fc), and chEGFRdIII-Fc, a chimeric version containing murine EGFR amino acids 310-501 with nucleotide sequence coding for amino acids 460-481 being replaced by the corresponding sequence from human EGFR, were also similarly constructed to be expressed as Fc fusion proteins (Figure 29). The chEGFRdIII-Fc constructs consists of 10 amino acids mutations to their human counterparts, including residues 460, 461, 467, 468, 471, 473, 474, and 478-480. All forms of EGFR ECD Fc tagged proteins were expressed via transient transfection of HEK 293T cells and purified from the supernatant of the transfected cells using protein A affinity chromatography.

Antibody binding to various EGFR ECD-Fc constructs

[0288] The huEGFR-7R was tested in ELISA format for binding to the various EGFR-Fc constructs described above. As shown in Figure 30, huEGFR-7R antibody binds to both human EGFR (huEGFR-Fc) and human EGFR domain III (huEGFRdIII-Fc) with similar affinity. Figure 30 also demonstrates that huEGFR-7R antibody practically does not recognize the murine EGFR domain III (muEGFRdIII-Fc), despite the high sequence homology with the human receptor (88% sequence identity in domain III). Additionally, the huEGFR-7R antibody binds to the human/murine EGFR chimera (chEGFRdIII-Fc), containing mainly murine EGFR domain III sequence with ten amino acids at positions 460, 461, 467, 468, 471, 473, 474, and 478-480 mutated to their human counterparts (Figure 30). These data indicate that huEGFR-7R antibody binds exclusively to the domain III of human EGFR and the binding epitope is largely confined within amino acid positions 460-480. When the binding affinities to different truncated formats of huEGFR are compared, it is apparent that there was an approximately two fold decrease in the binding affinity of the huEGFR-7R antibody to chEGFRdIII-Fc as compared to huEGFR and huEGFRdIII, suggesting that the epitope of huEGFR-7R antibody consists of additional amino acid residues besides those in the positions 460-480. This data was confirmed with the muEGFR-7 antibody binding results (Figure 32).

[0289] In parallel, other anti-EGFR antibodies of the invention, such as huEGFR-6 (Figure 31), muEGFR-6 (Figure 33), muEGFR-12 (Figure 34) and muEGFR-13 (Figure 35) antibodies that share unique biological activities with the EGFR-7 antibody, exhibit similar binding properties as the EGFR-7 antibody. They bind to human EGFR domain III (huEGFRdIII-Fc) but not murine EGFR domain III (muEGFRdIII-Fc), and importantly, they all bind to the chEGFRdIII-Fc at a

lower affinity than to the huEGFRdIII-Fc. These data suggest that the epitope recognized by the antibodies of the invention constitutes other residues in domain III in addition to the amino acid residues in positions 460-480. In contrast, cetuximab binds to chEGFRdIII as well as wild type human EGFR and huEGFRdIII at similar affinity (Figure 36), suggesting that the cetuximab binding epitope is confined to amino acid residues in positions 460-480. In summary, the huEGFR-7R antibody along with other anti-EGFR antibodies of the invention binds exclusively to domain III of the huEGFR extracellular domain. Moreover, through constructing chimeric EGFR, it has been confirmed that the epitope recognized by the antibodies of the invention is displaced toward the C-terminus of huEGFR domain III and largely overlaps with, but is not identical, to the cetuximab binding site and very likely consists of additional critical amino acids

[0290] The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying knowledge within the skill of the art, readily modify and/or adapt for various applications such specific embodiments, without undue experimentation, without departing from the general concept of the present invention. Therefore, such adaptations and modifications are intended to be within the meaning and range of equivalents of the disclosed embodiments, based on the teaching and guidance presented herein. It is to be understood that the phraseology or terminology herein is for the purpose of description and not of limitation, such that the terminology or phraseology of the present specification is to be interpreted by the skilled artisan in light of the teachings and guidance.

[0291] The breadth and scope of the present invention should not be limited by any of the above-described exemplary embodiments, but should be defined only in accordance with the following claims and their equivalents.

WHAT IS CLAIMED IS:

1. An antibody or antigen binding fragment thereof that specifically binds to human EGFR, wherein said antibody has at least one characteristic selected from the group consisting of: (a) inhibits at least 80% of epidermal growth factor (EGF) and transforming growth factor alpha (TGF α) binding to A431 cells at a concentration of 10 nM or higher, (b) causes at least 50% inhibition of H292 and HCC827 tumor cell proliferation at 30 nM or higher, and (c) does not inhibit more than 20% proliferation of keratinocytes and MCF-10A epithelial cells at 60 nM or lower.
2. The antibody or antigen binding fragment thereof of claim 1, wherein said antibody has at least two characteristics selected from the group consisting of: (a) inhibits at least 80% of EGF and TGF α binding to A431 cells at a concentration of 10 nM or higher, (b) causes at least 50% inhibition of H292 and HCC827 tumor cell proliferation at 30 nM or higher, and (c) does not inhibit more than 20% proliferation of keratinocytes and MCF-10A epithelial cells at 60 nM or lower.
3. An antibody or antigen binding fragment thereof that specifically binds to human EGFR, wherein said antibody (a) inhibits at least 80% of EGF and TGF α binding to A431 cells at a concentration of 10 nM or higher, (b) causes at least 50% inhibition of H292 and HCC827 tumor cell proliferation at 30 nM or higher, and (c) does not inhibit more than 20% proliferation of keratinocytes and MCF-10A epithelial cells at 60 nM or lower.
4. The antibody or antigen binding fragment thereof of any of claims 1-3, wherein the antibody comprises (a) a VH sequence at least 90% identical to a reference VH sequence selected from the group consisting of SEQ ID NOs:19-23, 69, and 71-73; and (b) a VL sequence at least 90% identical to a reference VL sequence selected from the group consisting of SEQ ID NOs:24-30 and 70.
5. The antibody or antigen binding fragment thereof of claim 4, wherein the VH and VL sequences are at least 95% identical to the reference VH and VL sequences.
6. The antibody or antigen binding fragment thereof of claim 5, wherein the VH and VL sequences are at least 99% identical to the reference VH and VL sequences.
7. The antibody or antigen binding fragment thereof of claim 6, which comprises (a) a VH sequence selected from the group consisting of SEQ ID NOs:19-23, 69, and 71-73; and (b) a VL sequence selected from the group consisting of SEQ ID NOs:24-30 and 70.
8. The antibody or antigen binding fragment thereof of claim 7, which comprises SEQ ID NO:19 and SEQ ID NO:24.

9. The antibody or antigen binding fragment thereof of claim 7, which comprises SEQ ID NO:20 and SEQ ID NO:25.
10. The antibody or antigen binding fragment thereof of claim 7, which comprises SEQ ID NO:21 and SEQ ID NO:26.
11. The antibody or antigen binding fragment thereof of claim 7, which comprises SEQ ID NO:21 and SEQ ID NO:27.
12. The antibody or antigen binding fragment thereof of claim 7, which comprises SEQ ID NO:22 and SEQ ID NO:28.
13. The antibody or antigen binding fragment thereof of claim 7, which comprises SEQ ID NO:23 and SEQ ID NO:29.
14. The antibody or antigen binding fragment thereof of claim 7, which comprises SEQ ID NO:23 and SEQ ID NO:30.
15. The antibody or antigen binding fragment thereof of claim 7, which comprises SEQ ID NO:69 and SEQ ID NO:70.
16. The antibody or antigen binding fragment thereof of claim 7, which comprises SEQ ID NO:71 and SEQ ID NO:26.
17. The antibody or antigen binding fragment thereof of claim 7, which comprises SEQ ID NO:71 and SEQ ID NO:27.
18. The antibody or antigen binding fragment thereof of claim 7, which comprises SEQ ID NO:72 and SEQ ID NO:26.
19. The antibody or antigen binding fragment thereof of claim 7, which comprises SEQ ID NO:72 and SEQ ID NO:27.
20. The antibody or antigen binding fragment thereof of claim 7, which comprises SEQ ID NO:73 and SEQ ID NO:26.
21. The antibody or antigen binding fragment thereof of claim 7, which comprises SEQ ID NO:73 and SEQ ID NO:27.

22. An antibody or antigen binding fragment thereof that competitively inhibits binding of a second antibody to human EGFR, wherein said second antibody comprises the heavy chain variable region of SEQ ID NO:21 and the light chain variable region of SEQ ID NOs: 26 or 27, and wherein the first antibody or antigen binding fragment: (a) inhibits at least 80% of epidermal growth factor (EGF) and transforming growth factor alpha (TGF α) binding to A431 cells at a concentration of 10 nM or higher, (b) causes at least 50% inhibition of H292 and HCC827 tumor cell proliferation at 30 nM or higher, or (c) does not inhibit more than 20% proliferation of keratinocytes and MCF-10A epithelial cells at 60 nM or lower.

23. An antibody or antigen binding fragment thereof produced by hybridoma selected from the group consisting of ATCC Deposit Designation PTA-11331, deposited with the ATCC on October 6, 2010, ATCC Deposit Designation PTA-11332, deposited with the ATCC on October 6, 2010, and ATCC Deposit Designation PTA-11333, deposited with the ATCC on October 6, 2010.

24. An antibody or antigen binding fragment thereof that specifically binds to the same EGFR epitope as an antibody selected from the group consisting of ATCC Deposit Designation PTA-11331, deposited with the ATCC on October 6, 2010, ATCC Deposit Designation PTA-11332, deposited with the ATCC on October 6, 2010, and ATCC Deposit Designation PTA-11333, deposited with the ATCC on October 6, 2010.

25. An antibody or antigen binding fragment thereof that competitively inhibits binding of a reference antibody to human EGFR, wherein said reference antibody is selected from the group consisting of ATCC Deposit Designation PTA-11331, deposited with the ATCC on October 6, 2010, ATCC Deposit Designation PTA-11332, deposited with the ATCC on October 6, 2010, and ATCC Deposit Designation PTA-11333, deposited with the ATCC on October 6, 2010, and wherein the antibody or antigen binding fragment: (a) inhibits at least 80% of epidermal growth factor (EGF) and transforming growth factor alpha (TGF α) binding to A431 cells at a concentration of 10 nM or higher, (b) causes at least 50% inhibition of H292 and HCC827 tumor cell proliferation at 30 nM or higher, or (c) does not inhibit more than 20% proliferation of keratinocytes and MCF-10A epithelial cells at 60 nM or lower.

26. An antibody or antigen binding fragment thereof that specifically binds to the same EGFR epitope as an antibody selected from the group consisting of:

- a) an antibody comprising the VH polypeptide of SEQ ID NO:19 and the VL polypeptide of SEQ ID NO:24;
- b) an antibody comprising the VH polypeptide of SEQ ID NO:20 and the VL polypeptide of SEQ ID NO:25;

- c) an antibody comprising the VH polypeptide of SEQ ID NO:21 and the VL polypeptide of SEQ ID NO:26;
- d) an antibody comprising the VH polypeptide of SEQ ID NO:21 and the VL polypeptide of SEQ ID NO:27;
- e) an antibody comprising the VH polypeptide of SEQ ID NO:22 and the VL polypeptide of SEQ ID NO:28;
- f) an antibody comprising the VH polypeptide of SEQ ID NO:23 and the VL polypeptide of SEQ ID NO:29;
- g) an antibody comprising the VH polypeptide of SEQ ID NO:23 and the VL polypeptide of SEQ ID NO:30;
- h) an antibody comprising the VH polypeptide of SEQ ID NO:69 and the VL polypeptide of SEQ ID NO:70;
- i) an antibody comprising the VH polypeptide of SEQ ID NO:71 and the VL polypeptide of SEQ ID NO:26;
- j) an antibody comprising the VH polypeptide of SEQ ID NO:71 and the VL polypeptide of SEQ ID NO:27;
- k) an antibody comprising the VH polypeptide of SEQ ID NO:72 and the VL polypeptide of SEQ ID NO:26;
- l) an antibody comprising the VH polypeptide of SEQ ID NO:72 and the VL polypeptide of SEQ ID NO:27;
- m) an antibody comprising the VH polypeptide of SEQ ID NO:73 and the VL polypeptide of SEQ ID NO:26; and
- n) an antibody comprising the VH polypeptide of SEQ ID NO:73 and the VL polypeptide of SEQ ID NO:27.

27. An antibody or antigen binding fragment thereof that competitively inhibits binding of a reference antibody to human EGFR, wherein said reference antibody is selected from the group consisting of:

- a) an antibody comprising the VH polypeptide of SEQ ID NO:19 and the VL polypeptide of SEQ ID NO:24;
- b) an antibody comprising the VH polypeptide of SEQ ID NO:20 and the VL polypeptide of SEQ ID NO:25;
- c) an antibody comprising the VH polypeptide of SEQ ID NO:21 and the VL polypeptide of SEQ ID NO:26;
- d) an antibody comprising the VH polypeptide of SEQ ID NO:21 and the VL polypeptide of SEQ ID NO:27;
- e) an antibody comprising the VH polypeptide of SEQ ID NO:22 and the VL polypeptide of SEQ ID NO:28;
- f) an antibody comprising the VH polypeptide of SEQ ID NO:23 and the VL polypeptide of SEQ ID NO:29;
- g) an antibody comprising the VH polypeptide of SEQ ID NO:23 and the VL polypeptide of SEQ ID NO:30;
- h) an antibody comprising the VH polypeptide of SEQ ID NO:69 and the VL polypeptide of SEQ ID NO:70;
- i) an antibody comprising the VH polypeptide of SEQ ID NO:71 and the VL polypeptide of SEQ ID NO:26;
- j) an antibody comprising the VH polypeptide of SEQ ID NO:71 and the VL polypeptide of SEQ ID NO:27;
- k) an antibody comprising the VH polypeptide of SEQ ID NO:72 and the VL polypeptide of SEQ ID NO:26;
- l) an antibody comprising the VH polypeptide of SEQ ID NO:72 and the VL polypeptide of SEQ ID NO:27;
- m) an antibody comprising the VH polypeptide of SEQ ID NO:73 and the VL polypeptide of SEQ ID NO:26; and

- n) an antibody comprising the VH polypeptide of SEQ ID NO:73 and the VL polypeptide of SEQ ID NO:27.
28. An antibody or antigen binding fragment thereof that specifically binds to human EGFR, wherein the antibody or antigen binding fragment thereof comprises:
- a) an immunoglobulin heavy chain variable region comprising CDR1, CDR2, and CDR3, which, with the exception of 1, 2, or 3 conservative amino acid substitutions, are respectively identical to the reference heavy chain CDR1 sequence of SEQ ID NO:1, the reference heavy chain CDR2 sequence of SEQ ID NO: 2, 4, 6, 63, or 64, and the reference heavy chain CDR3 sequence of SEQ ID NO: 3 or 5; and
 - b) an immunoglobulin a light chain variable region comprising CDR1, CDR2, and CDR3, which, with the exception of 1, 2, or 3 conservative amino acid substitutions, are respectively identical to the reference light chain CDR1 sequence of SEQ ID NO: 10, 13, or 14, the reference light chain sequence CDR2 of SEQ ID NO:11, and the reference light chain CDR3 sequence of SEQ ID NO: 12.
29. The antibody or antigen binding fragment thereof of claim 27, which comprises:
- a) (a) an immunoglobulin heavy chain variable region comprising CDR1, CDR2, and CDR3, which are respectively identical to the reference heavy chain CDR1 sequence of SEQ ID NO:1, the reference heavy chain CDR2 sequence of SEQ ID NO: 2, 4, 6, 63, or 64, and the reference heavy chain CDR3 sequence of SEQ ID NO: 3 or 5; and
 - b) an immunoglobulin a light chain variable region comprising CDR1, CDR2, and CDR3, which are respectively identical to the reference light chain CDR1 sequence of SEQ ID NO: 10, 13, or 14, the reference light chain sequence CDR2 of SEQ ID NO:11, and the reference light chain CDR3 sequence of SEQ ID NO: 12.
30. An antibody or antigen binding fragment thereof that specifically binds to human EGFR, wherein the antibody or antigen binding fragment thereof comprises:
- a) an immunoglobulin heavy chain variable region comprising CDR1, CDR2, and CDR3, which, with the exception of 1, 2, or 3 conservative amino acid substitutions, are respectively identical to the reference heavy chain CDR1 sequence of SEQ ID NO:1, the reference heavy chain CDR2 sequence of SEQ ID NO: 7, 8, or 9, and the reference heavy chain CDR3 sequence of SEQ ID NO: 3; and

- b) an immunoglobulin a light chain variable region comprising CDR1, CDR2, and CDR3, which, with the exception of 1, 2, or 3 conservative amino acid substitutions, are respectively identical to the reference light chain CDR1 sequence of SEQ ID NO: 15 or 16, the reference light chain CDR2 sequence of SEQ ID NO:17, and the reference light chain CDR3 sequence of SEQ ID NO: 18.
31. The antibody or antigen binding fragment thereof of claim 30, which comprises:
- a) an immunoglobulin heavy chain variable region comprising CDR1, CDR2, and CDR3, which are respectively identical to the reference heavy chain CDR1 sequence of SEQ ID NO:1, the reference heavy chain CDR2 sequence of SEQ ID NO: 7, 8, or 9, and the reference heavy chain CDR3 sequence of SEQ ID NO: 3; and
 - b) an immunoglobulin a light chain variable region comprising CDR1, CDR2, and CDR3, which are respectively identical to the reference light chain CDR1 sequence of SEQ ID NO: 15 or 16, the reference light chain CDR2 sequence of SEQ ID NO:17, and the reference light chain CDR3 sequence of SEQ ID NO: 18.
32. An antibody or antigen binding fragment thereof that specifically binds to human EGFR, wherein the antibody or antigen binding fragment thereof comprises:
- a) an immunoglobulin heavy chain variable region comprising CDR1, CDR2, and CDR3, which, with the exception of 1, 2, or 3 conservative amino acid substitutions, are respectively identical to the reference heavy chain CDR1 sequence of SEQ ID NO:1, the reference heavy chain CDR2 sequence of SEQ ID NO: 65, 66, or 67, and the reference heavy chain CDR3 sequence of SEQ ID NO: 3; and
 - b) an immunoglobulin a light chain variable region comprising CDR1, CDR2, and CDR3, which, with the exception of 1, 2, or 3 conservative amino acid substitutions, are respectively identical to the reference light chain CDR1 sequence of SEQ ID NO: 68 or 13, the reference light chain sequence CDR2 of SEQ ID NO:11, and the reference light chain CDR3 sequence of SEQ ID NO: 12.
33. The antibody or antigen binding fragment thereof of claim 32, which comprises:
- a) an immunoglobulin heavy chain variable region comprising CDR1, CDR2, and CDR3, which are respectively identical to the reference heavy chain CDR1 sequence of SEQ ID

NO:1, the reference heavy chain CDR2 sequence of SEQ ID NO: 65, 66, or 67, and the reference heavy chain CDR3 sequence of SEQ ID NO: 3; and

- b) an immunoglobulin a light chain variable region comprising CDR1, CDR2, and CDR3, which are respectively identical to the reference light chain CDR1 sequence of SEQ ID NO: 68 or 13, the reference light chain sequence CDR2 of SEQ ID NO:11, and the reference light chain CDR3 sequence of SEQ ID NO: 12.

34. The antibody or antigen binding fragment thereof of any one of claims 1-33, wherein said antibody or antigen binding fragment thereof is murine, non-human, humanized, chimeric, resurfaced, or human.

35. The antibody or antigen binding fragment thereof of any one of claims 1-34, which is a full length antibody.

36. The antibody or antigen binding fragment thereof of any one of claims 1-33, which is an antigen binding fragment.

37. The antibody or antigen binding fragment thereof of any one of claims 1-36, wherein said antibody or antigen binding fragment thereof comprises a Fab, Fab', F(ab')₂, Fd, single chain Fv or scFv, disulfide linked Fv, V-NAR domain, IgNar, intrabody, IgGACH2, minibody, F(ab')₃, tetrabody, triabody, diabody, single-domain antibody, DVD-Ig, Fcab, mAb2, (scFv)₂, or scFv-Fc.

38. A polypeptide comprising the VH and VL sequences of any of claims 1-37.

39. The antibody or polypeptide of any of claims 1-38, wherein the antibody or antigen binding fragment thereof bind both human and macaque EGFR with a substantially similar binding affinity.

40. The antibody or polypeptide of claim 39, which binds to human and macaque EGFR with a K_d of about 1.0 to about 10 nM.

41. The antibody or polypeptide of claim 40, which binds to human and macaque EGFR with a K_d of about 1.0 nM or better.

42. The antibody or polypeptide of any of claims 39-41, wherein the binding affinity is measured by flow cytometry, Biacore, or radioimmunoassay.

43. An isolated cell producing the antibody or antigen binding fragment thereof, or polypeptide of any one of claims 1-42.

44. A method of making the antibody or antigen-binding fragment thereof, or polypeptide of any one of claims 1-42, comprising (a) culturing the cell of claim 43; and (b) isolating said antibody, antigen-binding fragment thereof, or polypeptide from said cultured cell.
45. The method of claim 44, wherein said cell is a eukaryotic cell.
46. An immunoconjugate having the formula (A) - (L) - (C), wherein:
- (A) is an antibody or antigen binding fragment thereof, or polypeptide of any one of claims 1-42;
- (L) is a linker; and
- (C) is a cytotoxic agent; and
- wherein said linker (L) links (A) to (C).
47. The immunoconjugate of claim 46, wherein said linker is selected from the group consisting of a cleavable linker, a non-cleavable linker, a hydrophilic linker, and a dicarboxylic acid based linker.
48. The immunoconjugate of claim 47, wherein said linker is a non-cleavable linker.
49. The immunoconjugate of claim 48, wherein said linker is selected from the group consisting: N-succinimidyl 4-(2-pyridyldithio)pentanoate (SPP); N-succinimidyl 4-(2-pyridyldithio)butanoate (SPDB) or N-succinimidyl 4-(2-pyridyldithio)-2-sulfobutanoate (sulfo-SPDB); N-succinimidyl 4-(maleimidomethyl) cyclohexanecarboxylate (SMCC); N-sulfosuccinimidyl 4-(maleimidomethyl) cyclohexanecarboxylate (sulfoSMCC); N-succinimidyl-4-(iodoacetyl)-aminobenzoate (SIAB); and N-succinimidyl-[(N-maleimidopropionamido)-tetraethyleneglycol] ester (NHS-PEG4-maleimide).
50. The immunoconjugate of claim 49, wherein said linker is N-succinimidyl-[(N-maleimidopropionamido)-tetraethyleneglycol] ester (NHS-PEG4-maleimide).
51. The immunoconjugate of any one of claims 46-50, wherein said cytotoxic agent is selected from the group consisting of a maytansinoid, maytansinoid analog, doxorubicin, a modified doxorubicin, benzodiazepine, taxoid, CC-1065, CC-1065 analog, duocarmycin, duocarmycin analog, calicheamicin, dolastatin, dolastatin analog, aristatin, tomaymycin derivative, and leptomycin derivative or a prodrug of the agent.
52. The immunoconjugate of claim 51, wherein said cytotoxic agent is a maytansinoid.

53. The immunoconjugate of claim 52, wherein said cytotoxic agent is N(2')-deacetyl-N(2')-(3-mercapto-1-oxopropyl)-maytansine (DM1) or N(2')-deacetyl-N2-(4-mercapto-4-methyl-1-oxopentyl)-maytansine (DM4).
54. A pharmaceutical composition comprising the antibody or antigen binding fragment thereof, or polypeptide of any one of claims 1-42, or the immunoconjugate of any one of claims 46-53 and a pharmaceutically acceptable carrier.
55. The pharmaceutical composition of claim 54, further comprising a second anti-cancer agent.
56. A diagnostic reagent comprising the antibody or antigen binding fragment thereof, or polypeptide of any one of claims 1-42, or the immunoconjugate of any one of claims 46-53 which is labeled.
57. The diagnostic reagent of claim 56, wherein said label is selected from the group consisting of a radiolabel, a fluorophore, a chromophore, an imaging agent and a metal ion.
58. A kit comprising the antibody or antigen binding fragment thereof, or polypeptide of any one of claims 1-42, or the immunoconjugate of any one of claims 46-53.
59. A method for inhibiting the growth of a cell expressing EGFR comprising contacting the cell with the antibody or antigen binding fragment thereof, or polypeptide of any one of claims 1-42, the immunoconjugate of any one of claims 46-53, or the pharmaceutical composition of claim 54 or 55 to the subject.
60. The method of claim 59, wherein said cell is a tumor cell.
61. A method for treating a patient having cancer comprising administering to said patient a therapeutically effective amount of the antibody or antigen binding fragment thereof, or polypeptide of any one of claims 1-42, the immunoconjugate of any one of claims 46-53, or the pharmaceutical composition of claim 54 or 55 to the subject.
62. The method of claim 61, further comprising administering a second anti-cancer agent to the subject
63. The method of claim 62, wherein said second anti-cancer agent is a chemotherapeutic agent.
64. A method for treating a cell proliferative disorder comprising administering to said patient a therapeutically effective amount of the antibody or antigen binding fragment thereof, or polypeptide of any one of claims 1-42, the immunoconjugate of any one of claims 46-53, or the pharmaceutical composition of claim 54 or 55 to the subject.

65. The method of claim 64, wherein said cell proliferative disorder is selected from the group consisting of: adrenal cortex hyperplasia (Cushing's disease), congenital adrenal hyperplasia, endometrial hyperplasia, benign prostatic hyperplasia, breast hyperplasia, intimal hyperplasia, focal epithelial hyperplasia (Heck's disease), sebaceous hyperplasia, compensatory liver hyperplasia, and any other cell proliferation disease, besides neoplasia.

66. An isolated polynucleotide comprising a sequence that encodes a polypeptide at least 90% identical to a sequence selected from the group consisting of SEQ ID NOs: 39-43, 77-80, and 82-84.

67. The isolated polynucleotide of claim 66, wherein said sequence is at least 95% identical a sequence selected from the group consisting of SEQ ID NOs: 39-43, 77-80, and 82-84.

68. The isolated polynucleotide of claim 67, wherein said sequence is at least 99% identical to a sequence selected from the group consisting of SEQ ID NOs: 39-43, 77-80, and 82-84.

69. The isolated polynucleotide which comprises a sequence that is at least 90% identical to SEQ ID NOs: 44-50 and 81.

70. The isolated polynucleotide of claim 69, wherein the polynucleotide comprises a sequence that is at least 95% identical to SEQ ID NOs: 44-50 and 81.

71. The isolated polynucleotide of claim 70, wherein the polynucleotide comprises a sequence that is at least 99% identical to SEQ ID NOs: 44-50 and 81.

72. An isolated polynucleotide comprising a sequence selected from the group consisting of SEQ ID NOs: 39-58 and 77-84.

73. A vector comprising the polynucleotide of any one of claims 66-72.

74. A host cell comprising the vector of claim 73.

75. The immunoconjugate of claim 46, wherein said immunoconjugate can eliminate EGFR positive tumor cells, but only inhibit 40% or less the growth of human primary keratinocytes at concentration of 3.3 nM.

76. An immunoconjugate having the formula (A) - (L) - (C), wherein:

(A) is an antibody or antigen binding fragment thereof comprising the heavy chain variable region of SEQ ID NO:21 and the light chain variable region of SEQ ID NOs: 26 or 27;

(L) is a N-succinimidyl 4-(maleimidomethyl) cyclohexanecarboxylate (SMCC) linker;
and

(C) is the cytotoxic agent N(2')-deacetyl-N(2')-(3-mercapto-1-oxopropyl)-maytansine (DM1).