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(54) **Titre : UTILISATION D'ANTICORPS ANTI-EGFR DANS LE TRAITEMENT DE MALADIE MEDIEE PAR DES MUTANTS DE RECEPTEUR DU FACTEUR DE CROISSANCE EPIDERMIQUE (EGFR)**
(54) **Title: USE OF ANTI-EGFR ANTIBODIES IN TREATMENT OF EGFR MUTANT MEDIATED DISEASE**

(57) **Abrégé/Abstract:**
The present invention relates to the treatment of EGFR-mediated disease, particularly cancer, which is resistant to tyrosine kinase inhibitor therapies. Methods for treatment of cancer and reduction of tumor growth in individuals with secondary EGFR mutations, particularly tyrosine kinase domain mutations, resistant to standard therapy are provided. The invention provides methods for the treatment of tyrosine kinase inhibitor resistant cancers with anti-EGFR antibodies. Methods for treatment of recurrent lung cancer, including non-small cell lung carcinoma which is resistant to tyrosine kinase inhibitors, with the antibody anti-EGFR mAb806 are described.

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(54) Title: USE OF ANTI-EGFR ANTIBODIES IN TREATMENT OF EGFR MUTANT MEDIATED DISEASE

(57) Abstract: The present invention relates to the treatment of EGFR-mediated disease, particularly cancer, which is resistant to tyrosine kinase inhibitor therapies. Methods for treatment of cancer and reduction of tumor growth in individuals with secondary EGFR mutations, particularly tyrosine kinase domain mutations, resistant to standard therapy are provided. The invention provides methods for the treatment of tyrosine kinase inhibitor resistant cancers with anti-EGFR antibodies. Methods for treatment of recurrent lung cancer, including non-small cell lung carcinoma which is resistant to tyrosine kinase inhibitors, with the antibody anti-EGFR mAb806 are described.

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USE OF ANTI-EGFR ANTIBODIES IN TREATMENT OF EGFR MUTANT MEDIATED DISEASE

FIELD OF THE INVENTION

[0001] The present invention relates to the treatment of EGFR-mediated disease, particularly cancer, which is resistant to tyrosine kinase inhibitor therapies. Methods for treatment of cancer and reduction of tumor growth in individuals with secondary EGFR mutations, particularly tyrosine kinase domain mutations, resistant to standard therapy are provided.

BACKGROUND OF THE INVENTION

[0002] Targeted cancer therapy is designed to disrupt the function of specific molecules needed for carcinogenesis and tumor growth and thus either kills or prevents the growth of cancer cells (Ji H et al (2006) Cell Cycle 5(18):2072-2076 Epub 2006 Sep15). In contrast to conventional cytotoxic chemotherapy, such targeted cancer therapies may be more effective and less harmful to normal cells. A main effort in the targeted cancer therapy field has been the development of agents that target the epidermal growth factor receptor (EGFR). EGFR is a member of the ErbB family of closely related receptors including EGFR (ErbB-1), Her2/neu (ErbB-2), Her3 (ErbB-3) and Her4 (ErbB-4). Activation of EGFR leads to receptor tyrosine kinase activation and a series of downstream signaling events that mediate cellular proliferation, motility, adhesion, invasion, and resistance to chemotherapy as well as inhibition of apoptosis (2-4), processes that are crucial to the continual proliferation and survival of cancer cells.

[0003] To date, two major types of anti-EGFR agents have entered the clinical setting: anti-EGFR antibodies and small molecule EGFR tyrosine kinase inhibitors(TKIs) (5, 6). Anti-EGFR antibodies such as cetuximab were designed to bind to the extra-cellular domain of the EGFR and block activation of EGFR downstream signaling (7). Cetuximab (also known as antibody 225, U.S. Patent 4,943,533) was raised against A431 cells, which expresss high levels of wild type EGFR. In contrast, small molecule TKIs such as gefitinib (compound ZD1839, Iressa) or erlotinib (compound OSI-774, Tarceva) compete with ATP for binding to the intracellular catalytic domain of the EGFR tyrosine kinase and, thus, prevent EGFR autophosphorylation and downstream signaling(4).

[0004] Both of these anti-EGFR drug groups have shown some clinical efficacy in a subset of patients with a variety of different types of cancers. Treatment with gefitinib or erlotinib in patients with lung cancer having EGFR kinase domain mutations often generate dramatic clinical responses (5, 8). However, the effectiveness of gefitinib or erlotinib in lung adenocarcinoma with wild type EGFR or in other histological subtype, such as squamous cell carcinoma is limited (9, 10). Furthermore, it has been shown in pre-clinical and clinical trials that gefitinib or erlotinib are largely ineffective in inhibiting the function of the EGFRvIII mutant (11), a distinct activating EGFR mutation in which there is an in-frame deletion of exon II to VII (also denoted EGFR de2-7). EGFRvIII is commonly found in glioblastomas and recently found to be present in a subset of human lung squamous cell carcinomas (12) and a large fraction of head and neck cancers (13).

[0005] Cetuximab is shown to be effective in a small subset of non-small cell lung cancer (NSCLC) patients, and patients with head and neck cancers, as well as colorectal cancer patients. However, the response to cetuximab does not seem to correlate with expression levels of EGFR. Thus, it is unclear why these patients respond while other cancer patients whose tumors have high EGFR expression are refractory to cetuximab treatment (14).

[0006] As expression of the EGFR vIII mutant receptor is restricted to tumor cells, it represents a highly specific target for antibody therapy. Accordingly, both polyclonal and monoclonal antibodies specific to the unique peptide of de2-7 EGFR have been

generated. A series of mouse mAbs, isolated following immunization with the unique de2-7 peptide, all showed selectivity and specificity for the truncated receptor and targeted de2-7 EGFR positive xenografts grown in nude mice (Wikstrand CJ et al (1995) Cancer Res 55:3140-3148; Okamoto, S et al (1996) Br J Cancer 73:1366-1372; Hills D et al (1995) Int J Cancer 63:537-543; Reist CJ et al (1997) Cancer Res 57:1510-1515; Reist CJ et al (1995) Cancer Res 55:4375-4382; U.S. Patent 5,401,828). Examples of anti-EGFR vIII antibodies include ABX-EGF (panitumumab), DH8.3, L8A.4, and Y10.

[0007] MAb806 is a novel murine antibody, originally raised to recognize the unique truncation mutant, EGFRvIII using whole cells expressing EGFR vIII mutant as immunogen (15-17). Importantly, the epitope recognized by mAb806 is not accessible in inactive wild-type (*wt*) EGFR, but is exposed in a transitional form of wt EGFR in cells with overexpression of EGFR, and expression of EGFRvIII (18) . The epitope studies are supported by immunohistochemical studies demonstrating that the 806 antibody binds to epitopes present in gliomas, as well as a broad range of epithelial cancers, but not to normal human tissues (16, 19). These and other preclinical data suggest that mAb806 might have a different spectrum of clinical activity and side effect profile distinct from cetuximab and other anti-EGFR antibodies. In xenograft models, mAb806 has exhibited a potent anti-tumor activity with no targeting of normal tissues. Thus, the unique targeting capabilities of mAb806 represent a new paradigm for cancer-specific molecularly targeted therapy.

[0008] When overexpressed or activated by mutations, tyrosine kinases including EGFR contribute to the development of cancer and these mutated tyrosine kinase (TK) enzymes often provide a target or sensitivity for selective and specific cancer therapy. Somatic mutations in the tyrosine kinase domains of the EGFR gene are associated with sensitivity of lung cancers to certain tyrosine kinase inhibitors (TKIs) including gefitinib and erlotinib. In frame EGFR deletions in exon 19 (del L747-S752) and frequent point mutations in codon 858 (exon 21) (L858R) have been identified in non-small cell lung cancers and adenocarcinomas and associated with sensitivity to the TKIs gefitinib and erlotinib (Lynch TJ et al (2004) N Engl J Med 350:2129-2139; Paez JG et al (2004)

Science 304:1497-1500; Pao W et al (2004) PNAS 101(36):13306-13311). Recent studies have shown that 10-30% of NSCLC patients have EGFR kinase domain mutations while 5% of lung squamous cell carcinoma (SCC) patients have the extracellular domain *EGFRvIII* mutation (12, 20). Methods to determine the responsiveness of cancer to EGFR targeting treatments, based on assessment of mutations in EGFR, particularly in the kinase domain, and predicted inhibitor sensitivity in patients are described in Bell et al (WO 2005/094357 and US20060147959).

[0009] Acquired resistance to chemotherapy or targeted cancer therapy, mediated by secondary resistance or compensatory mutations is an ongoing challenge. Tumors that are sensitive to TKIs, including either gefitinib or erlotinib, eventually progress despite continued treatment with the TKIs. A secondary mutation at position 790 of EGFR (T790M) has been identified in tumor biopsy of relapsed and resistant patients (Kobayashi S et al (2005) N Engl J Med 352(8):786-792). This mutation is predicted to lead to steric hindrance of inhibitor binding in the ATP-kinase-binding pocket.

[0010] In view of the existence and prevalence of acquired resistance to TKIs in EGFR mediated disease and the significant cancer relapse rate, there is a clinical need for more broadly effective treatment protocols, employing EGFR targeted agents which are effective against, target, or avoid acquired resistance in EGFR mutants and EGFR mediated disease.

[0011] The citation of references herein shall not be construed as an admission that such is prior art to the present invention.

SUMMARY OF THE INVENTION

[0012] Activating epidermal growth factor receptor (EGFR) mutations have now been identified in a number of EGFR- mediated cancers, including lung cancer. EGFR mutations have been identified in human non-small cell lung cancer (NSCLC), with 5% of human lung squamous cell carcinomas having *EGFRvIII* mutations and 10~30% of

lung adenocarcinomas having *EGFR* kinase domain mutations. The EGFR targeting monoclonal antibody, mAb806, recognizes a conformational epitope of wild type (wt) EGFR as well as the truncated EGFRvIII mutant. In an effort to further characterize the application of mAb806 to EGFR-mediated cancer therapy, mAb806 was used to treat genetically engineered mice with lung tumors that were driven by either *EGFRvIII* or *EGFR* kinase domain mutations. The present invention confirms that anti-EGFR vIII antibody, particularly mAb806, is remarkably effective in blocking EGFRvIII signaling and inducing tumor cell apoptosis, resulting in dramatic tumor regression in *EGFRvIII* driven murine lung cancers. A distinct EGFR-targeting antibody, raised to cells expressing high levels of wild type EGFR, cetuximab, failed to show activity in these genetically defined lung tumors. In addition, treatment of murine lung tumors driven by a recognized and clinically relevant *EGFR* kinase domain mutation (L858R) with mAb806 induced a significant tumor regression. This kinase domain mutation has been shown to be sensitive to TKI therapy, particularly gefitinib or erlotinib.

[0013] Acquired resistance to TKIs, including either gefitinib or erlotinib, is an ongoing challenge and tumors that are sensitive to TKIs eventually progress despite continued therapy. This acquired resistance can be mediated by secondary resistance or compensatory mutations, particularly including a secondary mutation at position 790 of EGFR (T790M). The investigators now show that anti-EGFR antibody, particularly mAb806, is effective against the T790M mutation, resulting in dramatic tumor regression in EGFR T790M/L858R driven murine lung cancers. Taken together, these data demonstrate that anti-EGFR antibody, particularly mAb806, provides an effective alternative or adjunct in the treatment of patients with EGFR kinase domain mutations, including cancer patients, particularly lung cancer patients.

[0014] The invention provides a method of treating tyrosine kinase inhibitor resistant EGFR-mediated disease in a mammal, wherein said resistant EGFR-mediated disease is a result of a secondary mutation in EGFR to generate a mutant EGFR and wherein said mutation is distinct from the EGFR vIII mutation, comprising administering to said mammal an effective amount of an anti-EGFR antibody capable of binding to and

inhibiting the mutant EGFR. In a particular aspect the secondary EGFR mutation is an EGFR tyrosine kinase domain mutation. In a further aspect the tyrosine kinase domain mutation is T790M.

[0015] In a particular embodiment of the method the anti-EGFR antibody is mAb806 antibody or an active fragment thereof. MAb806 includes murine antibody, recombinant antibody or a humanized antibody.

[0016] Additional anti-EGFR antibodies, including those targeting the EGFRvIII mutant may be utilized in the therapeutic methods of the invention. Exemplary and known anti-EGFR antibodies may be selected from ABX-EGF (panitumumab), DH8.3, L8A4, and or active fragments thereof.

[0017] EGFR-mediated disease for treatment in the methods includes cancer. EGFR-mediated cancers include glioblastoma, head and neck cancer, pancreatic cancer, lung cancer, cancer of the nervous system, gastrointestinal cancer, prostate cancer, ovarian cancer, breast cancer, kidney cancer, retina cancer, skin cancer, liver cancer, genital-urinary cancer, and bladder cancer. In a particular aspect, the EGFR-mediated cancer is lung adenocarcinoma, lung squamous cell carcinoma or non-small cell lung cancer.

[0018] The invention provides a method for reducing EGFR-mediated tumor growth in a cancer patient, wherein said cancer patient has been previously treated with one or more tyrosine kinase inhibitor and has developed recurrent disease and tumor growth, comprising administering to said patient an effective amount of an anti-EGFR antibody such that the recurrent disease and tumor growth is inhibited and reduced.

[0019] In a particular embodiment of the method for reducing tumor growth the anti-EGFR antibody is mAb806 antibody or an active fragment thereof. MAb806 includes murine antibody, recombinant antibody or a humanized antibody.

[0020] Additional anti-EGFR antibodies, including those targeting the EGFRvIII mutant may be utilized. Exemplary and known anti-EGFR antibodies may be selected from ABX-EGF (panitumumab), DH8.3, L8A4, and or active fragments thereof.

[0021] In a particular clinical aspect, recurrent disease and tumor growth in the cancer patient is the result of a secondary EGFR mutation which is an EGFR tyrosine kinase domain mutation. A particular secondary EGFR mutation is the tyrosine kinase domain mutation T790M.

[0022] The invention further provides a method of treating EGFR-mediated cancer in a mammal comprising administering to said mammal a tyrosine kinase inhibitor and anti-EGFR antibody, wherein said anti-EGFR antibody is administered after treatment with the tyrosine kinase inhibitor as a second line of therapy to inhibit potential secondary mutant EGFRs resistant to tyrosine kinase inhibitors.

[0023] The EGFR-mediated cancer may be selected from glioblastoma, head and neck cancer, pancreatic cancer, lung cancer, cancer of the nervous system, gastrointestinal cencer, prostate cancer, ovarian cancer, breast cancer, kidney cancer, retina cancer, skin cancer, liver cancer, genital-urinary cancer, and bladder cancer. In particular the cancer is lung adenocarcinoma, lung squamous cell carcinoma or non-small cell lung cancer.

[0024] In one aspect of this method, the tyrosine kinase inhibitor is a reversible tyrosine kinase inhibitor. The reversible tyrosine kinase inhibitor may be an aniliniquinazoline and is selected from gefitinib, erlotinib, AG1478, ST1571 and SU-6668.

[0025] In a further aspect of this method, the tyrosine kinase inhibitor is an irreversible tyrosine kinase inhibitor. Exemplary irreversible tyrosine kinase inhibitor are known in the art and include, but are not limited to EKB-569, EKI-569, HKI-272, HKI-357 and BIBW 2992.

[0026] Other objects and advantages will become apparent to those skilled in the art from a review of the following description which proceeds with reference to the following illustrative drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

[0027] FIGURE 1 depicts murine lung tumors driven by EGFRvIII expression are sensitive to mAb806 and ch806 antibody treatment but resistant to cetuximab treatment. *Tet-op-EGFRvIII/CCSP-rtTA*, *Ink4A/Arf-/-* mice were treated with either mAb806 or ch806 at 0.5 mg per dose or cetuximab at 1 mg per dose through daily I.P. injection. Antibodies were given every two days at the same dose after the first week of treatment. Serial MRI were performed at indicated time points, corresponding sections of representative mice in each treatment group are shown. Bar diagram expressed as mean±standard deviation (SD) illustrating the tumor regression measured by MRI, and statistical analyses were performed using Student's exact t test. All mice were kept on a doxycycline diet throughout the experiment. H: indicates the area of the heart.

[0028] FIGURE 2A and 2B depicts histolopathological features of lung adenocarcinomas in EGFRvIII-driven mice treated with mAb806. **(A)** Lung adenocarcinoma driven by EGFRvIII expression for more than 8 weeks (upper panel). After 1 week of treatment with mAb806, tumors became smaller and had increased fibrosis (middle panel). Lung specimens were grossly normal when mAb806 treatment ended at 5 weeks (lower panel). Arrows show a fibrotic nodule, consisting of fibroblasts and macrophages. No tumor cells were found in this particular fibrosis area. Left panel: 100X, right panel: 800X. **(B)** Similar patterns and intensities of immunohistological staining of total EGFR can be observed in control mice and mice treated with mAb806 for 1 week (left upper and lower panel, respectively); intensity of phospho-EGFR staining of tumor cells decreased after 1 week of treatment (right lower panel) when compared with untreated tumors (right upper panel). Representative photos are taken under 200X magnification. **(C)** TUNEL staining shows increased apoptotic nuclei (red

arrows) in EGFRvIII driven lung tumors after 1 week of treatment with mAb806 (left lower panel) when compared with untreated tumors (left upper panel). Representative photos are taken under 200X magnification. Bar diagrams expressed as mean±SD illustrating the apoptotic indices in lung tumors before and after 1 week of mAb806 treatment were determined from at least 200 high-power fields (HPF). Statistical analyses were performed using Student's exact t test (right panel).

[0029] FIGURE 3 shows Western blot analysis of whole lung lysate from mAb806 treated *Tet-op-EGFRvIII/CCSP-rtTA*, *Ink4A/Arf-/-* mice. Whole lung lysates from tumors taken from mice at different time points of mAb806 treatment were analyzed. Inhibition of EGFR phosphorylation can be observed as soon as after 1 week of treatment, while total EGFR level decreases only after 5 weeks of treatment. Erk1,2 phosphorylation was inhibited by the antibody throughout the mAb806 administration, but AKT phosphorylation remained at a level comparable to untreated controls at both treatment time points. β-actin serves as a loading control.

[0030] FIGURE 4A and 4B. EGFR kinase domain mutation L858R-driven mouse lung adenocarcinoma responds to ch806 treatment. **(A)** *Tet-op-EGFR L858R-IRES-Luciferase/CCSP-rtTA* mice were treated with ch806 at 0.5 mg per dose by daily I.P. injection for 4 weeks. MRI showed decreased tumor volume after 2 and 4 weeks of treatment. Bar diagram expressed as mean±SD illustrating the tumor regression measured by MRI, and statistical analyses were performed using Student's exact t test. H: indicates the area of the heart. **(B)** Histopathological analysis shows shrinkage of tumors and marked macrophage infiltration in *Tet-op-EGFR L858R-IRES-Luciferase/CCSP-rtTA* mice (right two panels), when compared with the no treatment controls (left two panels). Arrows show foci of residual tumors. Photos from both 100X and 800X magnifications are shown as indicated by footnotes in the figure.

[0031] FIGURE 5A and 5B depicts the results of treatment of EGFR T790M-L858R lung tumors with mAb806 versus cetuximab. Mice on continuous doxycycline diets for more than 8 weeks underwent MRI to document the tumor burden. Mab806 was

delivered into mice bearing lung tumors through I.P. injection daily at 0.5 mg doses for 4 weeks. Cetuximab was administered to mice by I.P. injection at 1 mg per dose daily for 4 weeks. Littermates were used as controls for all the treatment studies (no treatment). **(A)** Mice were imaged with MRI at 0, 2, and 4 or 5 weeks to determine reduction in tumor volume. **(B)** After completion of treatment and MRI imaging, mice were sacrificed for further histological and biochemical studies.

DETAILED DESCRIPTION

[0032] In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook et al, "Molecular Cloning: A Laboratory Manual" (1989); "Current Protocols in Molecular Biology" Volumes I-III [Ausubel, R. M., ed. (1994)]; "Cell Biology: A Laboratory Handbook" Volumes I-III [J. E. Celis, ed. (1994))]; "Current Protocols in Immunology" Volumes I-III [Coligan, J. E., ed. (1994)]; "Oligonucleotide Synthesis" (M.J. Gait ed. 1984); "Nucleic Acid Hybridization" [B.D. Hames & S.J. Higgins eds. (1985)]; "Transcription And Translation" [B.D. Hames & S.J. Higgins, eds. (1984)]; "Animal Cell Culture" [R.I. Freshney, ed. (1986)]; "Immobilized Cells And Enzymes" [IRL Press, (1986)]; B. Perbal, "A Practical Guide To Molecular Cloning" (1984).

[0033] Therefore, if appearing herein, the following terms shall have the definitions set out below.

[0034] The term “antibody “ describes an immunoglobulin whether natural or partly or wholly synthetically produced. Antibody includes any immunoglobulin, including antibodies and fragments thereof, that binds a specific epitope. The term encompasses polyclonal, monoclonal, recombinant, humanized, and chimeric antibodies. The term also covers any polypeptide or protein having a binding domain which is, or is homologous to, an antibody binding domain. CDR grafted antibodies are also contemplated by this term.

[0035] As antibodies can be modified in a number of ways, the term "antibody" should be construed as covering any specific binding member or substance having a binding domain with the required specificity. Thus, this term covers antibody fragments, derivatives, functional equivalents and homologues of antibodies, including any polypeptide comprising an immunoglobulin binding domain, whether natural or wholly

or partially synthetic. Chimeric molecules comprising an immunoglobulin binding domain, or equivalent, fused to another polypeptide are therefore included. Cloning and expression of chimeric antibodies are described in EP-A-0120694 and EP-A-0125023 and U.S. Patent Nos. 4,816,397 and 4,816,567.

[0036] It has been shown that fragments of a whole antibody can perform the function of binding antigens. Examples of binding fragments are (i) the Fab fragment consisting of VL, VH, CL and CH1 domains; (ii) the Fd fragment consisting of the VH and CH1 domains; (iii) the Fv fragment consisting of the VL and VH domains of a single antibody; (iv) the dAb fragment (Ward, E.S. et al., Nature 341, 544-546 (1989)) which consists of a VH domain; (v) isolated CDR regions; (vi) F(ab')2 fragments, a bivalent fragment comprising two linked Fab fragments (vii) single chain Fv molecules (scFv), wherein a VH domain and a VL domain are linked by a peptide linker which allows the two domains to associate to form an antigen binding site (Bird et al, Science, 242, 423-426, 1988; Huston et al, PNAS USA, 85, 5879-5883, 1988); (viii) multivalent antibody fragments (scFv dimers, trimers and/or tetramers (Power and Hudson, J Immunol. Methods 242: 193-204 9 (2000))(ix) bispecific single chain Fv dimers (PCT/US92/09965) and (x) "diabodies", multivalent or multispecific fragments constructed by gene fusion (WO94/13804; P. Holliger et al Proc. Natl. Acad. Sci. USA 90 6444-6448, (1993)).

[0037] An "antibody combining site" is that structural portion of an antibody molecule comprised of light chain or heavy and light chain variable and hypervariable regions that specifically binds antigen.

[0038] The phrase "antibody molecule" in its various grammatical forms as used herein contemplates both an intact immunoglobulin molecule and an immunologically active portion of an immunoglobulin molecule.

[0039] Exemplary antibody molecules are intact immunoglobulin molecules, substantially intact immunoglobulin molecules and those portions of an immunoglobulin

molecule that contains the paratope, including those portions known in the art as Fab, Fab', $F(ab')_2$ and F(v), which portions are preferred for use in the therapeutic methods described herein.

[0040] Antibodies may also be bispecific, wherein one binding domain of the antibody is a specific binding member of the invention, and the other binding domain has a different specificity, *e.g.* to recruit an effector function or the like. Bispecific antibodies of the present invention include wherein one binding domain of the antibody is a specific binding member of the present invention, including a fragment thereof, and the other binding domain is a distinct antibody or fragment thereof, including that of a distinct anti-EGFR antibody, for instance antibody 528 (U.S. Patent No. 4,943,533), the chimeric and humanized 225 antibody (U.S. Patent No. 4,943,533 and WO/9640210), an anti-de2-7 antibody such as DH8.3 (Hills, D. et al (1995) Int. J. Cancer 63(4):537-543), antibody L8A4 and Y10 (Reist, CJ et al (1995) Cancer Res. 55(19):4375-4382; Foulon CF et al. (2000) Cancer Res. 60(16):4453-4460), ICR62 (Modjtahedi H et al (1993) Cell Biophys. Jan-Jun;22(1-3):129-46; Modjtahedi et al (2002) P.A.A.C.R. 55(14):3140-3148, or the antibody of Wikstrand et al (Wikstrand C. et al (1995) Cancer Res. 55(14):3140-3148). The other binding domain may be an antibody that recognizes or targets a particular cell type, as in a neural or glial cell-specific antibody. In the bispecific antibodies of the present invention the one binding domain of the antibody of the invention may be combined with other binding domains or molecules which recognize particular cell receptors and/or modulate cells in a particular fashion, as for instance an immune modulator (e.g., interleukin(s)), a growth modulator or cytokine (*e.g.* tumor necrosis factor (TNF), and particularly, the TNF bispecific modality demonstrated in U.S.S.N. 60/355,838 filed February 13, 2002 or a toxin (e.g., ricin) or anti-mitotic or apoptotic agent or factor.

[0041] Fab and $F(ab')_2$ portions of antibody molecules may be prepared by the proteolytic reaction of papain and pepsin, respectively, on substantially intact antibody molecules by methods that are well-known. See for example, U.S. Patent No. 4,342,566 to Theofilopolous et al. Fab' antibody molecule portions are also well-known and are

produced from $F(ab')_2$ portions followed by reduction of the disulfide bonds linking the two heavy chain portions as with mercaptoethanol, and followed by alkylation of the resulting protein mercaptan with a reagent such as iodoacetamide. An antibody containing intact antibody molecules is preferred herein.

[0042] The phrase "monoclonal antibody" in its various grammatical forms refers to an antibody having only one species of antibody combining site capable of immunoreacting with a particular antigen. A monoclonal antibody thus typically displays a single binding affinity for any antigen with which it immunoreacts. A monoclonal antibody may also contain an antibody molecule having a plurality of antibody combining sites, each immunospecific for a different antigen; *e.g.,* a bispecific (chimeric) monoclonal antibody.

[0043] The term "antigen binding domain" describes the part of an antibody which comprises the area which specifically binds to and is complementary to part or all of an antigen. Where an antigen is large, an antibody may bind to a particular part of the antigen only, which part is termed an epitope. An antigen binding domain may be provided by one or more antibody variable domains. Preferably, an antigen binding domain comprises an antibody light chain variable region (VL) and an antibody heavy chain variable region (VH).

[0044] The terms "mAb806", "806 antibody", "monoclonal antibody 806", "ch806", "humanized 806" and any variants not specifically listed, may be used herein interchangeably, and as used throughout the present application and claims refer to Accordingly, antibodies, including recombinant, chimeric, genetically modified, or alternative antibodies, displaying substantially equivalent or altered activity are likewise contemplated. These modifications may be deliberate, for example, such as modifications obtained through site-directed mutagenesis, or may be accidental, such as those obtained through mutations in hosts that are producers of the antibody or its fragments. Also, the terms "mAb806", "806 antibody", "monoclonal antibody 806", "ch806", "humanized 806" are intended to include within their scope proteins and immunoglobulins specifically recited herein and known to the skilled artisan, publicly

disclosed, as well as all substantially homologous analogs and allelic variations. The mAb806 antibody, including its generation, particular activities, amino acid and nucleic acid sequence, antigen binding domains, variable region sequences, are disclosed and known to the skilled artisan, including as provided in WO 02/092771; Luwor RB et al (2001) Cancer Res 61:5355-5361; Mishima K et al (2001) Cancer Res 61:5349-5354; Johns TG et al (2002) Int J Cancer 98:398-408; Jungbluth AA et al (2003) Proc Natl Acad Sci 100(2):639-644.

[0045] The amino acid residues described herein are preferred to be in the "L" isomeric form. However, residues in the "D" isomeric form can be substituted for any L-amino acid residue, as long as the desired fuctional property of immunoglobulin-binding is retained by the polypeptide. NH_2 refers to the free amino group present at the amino terminus of a polypeptide. COOH refers to the free carboxy group present at the carboxy terminus of a polypeptide. In keeping with standard polypeptide nomenclature, *J. Biol. Chem.*, **243**:3552-59 (1969), abbreviations for amino acid residues are shown in the following Table of Correspondence:

TABLE OF CORRESPONDENCE

SYMBOL		AMINO ACID
1-Letter	3-Letter	
Y	Tyr	tyrosine
G	Gly	glycine
F	Phe	phenylalanine
M	Met	methionine
A	Ala	alanine
S	Ser	serine
I	Ile	isoleucine
L	Leu	leucine
T	Thr	threonine

V	Val		valine
P	Pro		proline
K	Lys		lysine
H		His	histidine
Q	Gln		glutamine
E	Glu		glutamic acid
W	Trp		tryptophan
R	Arg		arginine
D	Asp		aspartic acid
N	Asn		asparagine
C	Cys		cysteine

[0046] It should be noted that all amino-acid residue sequences are represented herein by formulae whose left and right orientation is in the conventional direction of amino-terminus to carboxy-terminus. Furthermore, it should be noted that a dash at the beginning or end of an amino acid residue sequence indicates a peptide bond to a further sequence of one or more amino-acid residues. The above Table is presented to correlate the three-letter and one-letter notations which may appear alternately herein.

[0047] It should be appreciated that also within the scope of compositions for use in the methods of the present invention are DNA sequences encoding and/or expressing effective anti-EGFR antibodies, particularly incluing mAb806 and ch806, which code for anti-EGFR antibodies, antigen binding domains thereof, or active fragments thereof having the same amino acid sequence as the mAb806 antibody as publicly disclosed and known to the skilled artisan, but which are degenerate to the known mAb806 sequence(s). By "degenerate to" is meant that a different three-letter codon is used to specify a particular amino acid. It is well known in the art that the following codons can be used interchangeably to code for each specific amino acid:

Phenylalanine (Phe or F) UUU or UUC

Leucine (Leu or L)	UUA or UUG or CUU or CUC or CUA or CUG
Isoleucine (Ile or I)	AUU or AUC or AUA
Methionine (Met or M)	AUG
Valine (Val or V)	GUU or GUC of GUA or GUG
Serine (Ser or S)	UCU or UCC or UCA or UCG or AGU or AGC
Proline (Pro or P)	CCU or CCC or CCA or CCG
Threonine (Thr or T)	ACU or ACC or ACA or ACG
Alanine (Ala or A)	GCU or GCG or GCA or GCG
Tyrosine (Tyr or Y)	UAU or UAC
Histidine (His or H)	CAU or CAC
Glutamine (Gln or Q)	CAA or CAG
Asparagine (Asn or N)	AAU or AAC
Lysine (Lys or K)	AAA or AAG
Aspartic Acid (Asp or D)	GAU or GAC
Glutamic Acid (Glu or E)	GAA or GAG
Cysteine (Cys or C)	UGU or UGC
Arginine (Arg or R)	CGU or CGC or CGA or CGG or AGA or AGG
Glycine (Gly or G)	GGU or GGC or GGA or GGG
Tryptophan (Trp or W)	UGG
Termination codon	UAA (ochre) or UAG (amber) or UGA (opal)

[0048] It should be understood that the codons specified above are for RNA sequences. The corresponding codons for DNA have a T substituted for U.

[0049] Mutations can be made in anti-EGFR antibody sequence, including in mAb806 antibody sequence, such that a particular codon is changed to a codon which codes for a different amino acid. Such a mutation is generally made by making the fewest nucleotide changes possible. A substitution mutation of this sort can be made to change an amino acid in the resulting protein in a non-conservative manner (i.e., by changing the codon from an amino acid belonging to a grouping of amino acids having a

particular size or characteristic to an amino acid belonging to another grouping) or in a conservative manner (i.e., by changing the codon from an amino acid belonging to a grouping of amino acids having a particular size or characteristic to an amino acid belonging to the same grouping). Such a conservative change generally leads to less change in the structure and function of the resulting protein. A non-conservative change is more likely to alter the structure, activity or function of the resulting protein. The present invention should be considered to include sequences containing conservative changes which do not significantly alter the activity or binding characteristics of the resulting immunoglobulin and antibody.

[0050] Similarly, it is anticipated that certain EGFR mutations, which may effect or alter, even significantly, the activity of EGFR, for instance the EGFR kinase domain mutations described and utilized herein, may not affect the recognition, binding or inhibition of EGFR by anti-EGFR antibodies, particularly including anti-EGFR vIII mutant antibodies, particularly including the mAb806 antibody. Thus, it is anticipated that mAb806 may be similarly effective against other, as yet unrecognized or as yet unknown, EGFR mutations, particularly secondary mutations which arise during anti-cancer therapy. These mutations may arise as a result of TKI inhibition therapy or as a result of other therapies against EGFR-mediated disease which may target kinase or other activities of EGFR.

[0051] Amino acids may be grouped as similar or different, conserved or non-conserved. The grouping of amino acids may be based on their R groups (for instance nonpolar, uncharged polar, charged polar, those with phenyl groups), based on their molecular weight or the size of their R groups, and based on molecular weight. Particularly preferred substitutions are: Lys for Arg and vice versa such that a positive charge may be maintained; Glu for Asp and vice versa such that a negative charge may be maintained; Ser for Thr such that a free -OH can be maintained; and Gln for Asn such that a free NH_2 can be maintained.

[0052] Amino acid substitutions may also be introduced to substitute an amino acid with a particularly preferable property. For example, a Cys may be introduced a potential site for disulfide bridges with another Cys. A His may be introduced as a particularly "catalytic" site (i.e., His can act as an acid or base and is the most common amino acid in biochemical catalysis). Pro may be introduced because of its particularly planar structure, which induces β-turns in the protein's structure.

[0053] Two amino acid sequences are "substantially homologous" when at least about 70% of the amino acid residues (preferably at least about 80%, and most preferably at least about 90 or 95%) are identical, or represent conservative substitutions.

[0054] The phrase "pharmaceutically acceptable" refers to molecular entities and compositions that are physiologically tolerable and do not typically produce an allergic or similar untoward reaction, such as gastric upset, dizziness and the like, when administered to a human.

[0055] The phrase "therapeutically effective amount" is used herein to mean an amount sufficient to prevent, and preferably reduce by at least about 20 percent, more preferably by at least 30 percent, still more preferably by at least 50 percent, more preferably by at least 70 percent, more preferably by at least 90 percent, a clinically significant change in the S phase activity of a target cellular mass, or a significant change in the size or dimensions of a target cellular mass or tumor, or other feature of pathology as may attend its presence and activity.

[0056] The antibody or active fragment can be formulated into the therapeutic composition as neutralized pharmaceutically acceptable salt forms. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the polypeptide or antibody molecule) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed from the free carboxyl groups can

also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

[0057] The therapeutic antibody or active fragment-containing compositions are conventionally administered intravenously, as by injection of a unit dose, for example. The term "unit dose" when used in reference to a therapeutic composition of the present invention refers to physically discrete units suitable as unitary dosage for humans, each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required diluent; i.e., carrier, or vehicle.

The compositions are administered in a manner compatible with the dosage formulation, and in a therapeutically effective amount. The quantity to be administered depends on the subject to be treated, capacity of the subject's immune system to utilize the active ingredient, and degree of inhibition desired or extent of tumor mass being targeted. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner and are peculiar to each individual. However, suitable dosages may range from about 0.1 to 20, preferably about 0.5 to about 10, and more preferably one to several, milligrams of active ingredient per kilogram body weight of individual per day and depend on the route of administration. Suitable regimes for initial administration and booster shots are also variable, but are typified by an initial administration followed by repeated doses at one or more hour intervals by a subsequent injection or other administration. Alternatively, continuous intravenous infusion sufficient to maintain concentrations of ten nanomolar to ten micromolar in the blood are contemplated.

[0058] As used herein, "pg" means picogram, "ng" means nanogram, "ug" or "μg" mean microgram, "mg" means milligram, "ul" or "μl" mean microliter, "ml" means milliliter, "l" means liter.

[0059] In an effort to further characterize the application of mAb806 to EGFR-mediated cancer therapy, the present invention describes the use of mAb806 to treat genetically engineered mice with lung tumors that were driven by either *EGFRvIII* or *EGFR* kinase domain mutations. Each of these mutations are clinically relevant and significant for EGFR-mediated disease, particularly cancers, including lung cancer, pancreatic cancer, colorectal cancer, head and neck cancer, and glioblastoma. The present invention confirms that anti-EGFR vIII antibody, particularly mAb806, is remarkably effective in blocking EGFRvIII signaling and inducing tumor cell apoptosis, resulting in dramatic tumor regression in *EGFRvIII* driven murine lung cancers. A distinct EGFR-targeting antibody, raised to cells expressing high levels of wild type EGFR, cetuximab, failed to show activity in these genetically defined lung tumors. In addition, treatment of murine lung tumors driven by a recognized and clinically relevant *EGFR* kinase domain mutation (L858R) with mAb806 induced a significant tumor regression. This kinase domain mutation has been shown to be sensitive to TKI therapy, particularly gefitinib or erlotinib. Acquired resistance to TKIs, , including either gefitinib or erlotinib, is an ongoing challenge and tumors that are sensitive to TKIs eventually progress despite continued. This acquired resistance can be mediated by secondary resistance or compensatory mutations, particularly a secondary mutation at position 790 of EGFR (T790M). The investigators now show that anti-EGFR antibody, particularly mAb806 is effective against the T790M mutation, resulting in dramatic tumor regression in EGFR T790M/L858R driven murine lung cancers. Taken together, these data demonstrate that anti-EGFR antibody, particularly mAb806, provides an effective alternative or adjunct in the treatment of patients with EGFR kinase domain mutations, including cancer patients, particularly lung cancer patients.

[0060] Thus, both therapeutic and diagnostic applications and methods are provided and raised by the demonstration of the anti tumor activity of anti-EGFR antibody, particularly of mAb806. As suggested earlier and elaborated further on herein, the present invention contemplates pharmaceutical intervention in the cascade of reactions and signaling in which EGFR is implicated, to modulate the tumorigenic capacity

associated with EGFR mutations, including kinase domain mutations, both primary and secondary resistant mutations.

[0061] The data provided herein demonstrate mAb806 activity against EGFR kinase domain mutations, including L858R and TKI resistant T790M. It is anticipated that further kinase domain mutations or EGFR secondary mutations may exist or will arise with continued and advancing directed anti-EGFR therapy. The irreversible inhibitor HKI272, which binds to EGFR at cysteine 797 (corresponding to cysteine 530 in EGFR vIII deletion mutants), is advancing in preclinical protocols. Resistant secondary mutations with substitutions at the cysteine are likely if not anticipated. These additional EGFR secondary mutants would be candidates for anti-EGFR antibody therapy.

[0062] The invention thus provides a method of treating tyrosine kinase inhibitor resistant EGFR-mediated disease in a mammal, wherein said resistant EGFR-mediated disease is a result of a secondary mutation in EGFR to generate a mutant EGFR and wherein said mutation is distinct from the EGFR vIII mutation, comprising administering to said mammal an effective amount of an anti-EGFR antibody capable of binding to and inhibiting the mutant EGFR. In a particular aspect the secondary EGFR mutation is an EGFR tyrosine kinase domain mutation. In a further aspect the tyrosine kinase domain mutation is T790M. In a particular embodiment of the method the anti-EGFR antibody is mAb806 antibody or an active fragment thereof. MAb806 includes murine antibody, recombinant antibody or a humanized antibody. Additional anti-EGFR antibodies, including those targeting the EGFRvIII mutant may be utilized in the therapeutic methods of the invention. Exemplary and known anti-EGFR antibodies may be selected from ABX-EGF (panitumumab), DH8.3, L8A4, and or active fragments thereof.

[0063] EGFR-mediated disease for treatment is particularly cancer and may be selected from glioblastoma, head and neck cancer, pancreatic cancer, lung cancer, cancer of the nervous system, gastrointestinal cancer, prostate cancer, ovarian cancer, breast cancer, kidney cancer, retina cancer, skin cancer, liver cancer, genital-urinary cancer, and bladder

cancer. In a particular aspect, the EGFR-mediated cancer is lung adenocarcinoma, lung squamous cell carcinoma or non-small cell lung cancer.

[0064] The invention includes a method for reducing EGFR-mediated tumor growth in a cancer patient, wherein said cancer patient has been previously treated with one or more tyrosine kinase inhibitor and has developed recurrent disease and tumor growth, comprising administering to said patient an effective amount of an anti-EGFR antibody such that the recurrent disease and tumor growth is inhibited and reduced. In a particular embodiment of the method for reducing tumor growth the anti-EGFR antibody is mAb806 antibody or an active fragment thereof. MAb806 includes murine antibody, recombinant antibody or a humanized antibody. Additional anti-EGFR antibodies, including those targeting the EGFRvIII mutant may be utilized. Exemplary and known anti-EGFR antibodies may be selected from ABX-EGF (panitumumab), DH8.3, L8A4, and or active fragments thereof.

[0065] In a particular clinical aspect, recurrent disease and tumor growth in the cancer patient is the result of a secondary EGFR mutation which is an EGFR tyrosine kinase domain mutation. A particular secondary EGFR mutation is the tyrosine kinase domain mutation T790M.

[0066] The invention further provides a method of treating EGFR-mediated cancer in a mammal comprising administering to said mammal a tyrosine kinase inhibitor and anti-EGFR antibody. In one aspect, the tyrosine kinase inhibitor and anti-EGFR antibody are administered simultaneously. In one aspect, the tyrosine kinase inhibitor and anti-EGFR antibody are administered simultaneously or serially and repeatedly, before or after traditional chemotherapy.

[0067] The invention further provides a method of treating EGFR-mediated cancer in a mammal comprising administering to said mammal a tyrosine kinase inhibitor and anti-EGFR antibody, wherein said anti-EGFR antibody is administered after treatment with

the tyrosine kinase inhibitor as a second line of therapy to inhibit potential secondary mutant EGFRs resistant to tyrosine kinase inhibitors.

[0068] The tyrosine kinase inhibitor may be a reversible tyrosine kinase inhibitor or an irreversible tyrosine kinase inhibitor. The reversible tyrosine kinase inhibitor may be an aniliniquinazoline and selected from gefitinib, erlotinib, AG1478, ST1571 and SU-6668. Exemplary irreversible tyrosine kinase inhibitor are known in the art and include, but are not limited to EKB-569, EKI-569, HKI-272, HKI-357 and BIBW 2992 (Kwak EL et al (2005) Proc Natl Acad Sci U S A 102(21):7665-70).

[0069] EGFR-mediated cancers may be selected from glioblastoma, head and neck cancer, pancreatic cancer, lung cancer, cancer of the nervous system, gastrointestinal cencer, prostate cancer, ovarian cancer, breast cancer, kidney cancer, retina cancer, skin cancer, liver cancer, genital-urinary cancer, and bladder cancer. In particular the cancer is lung adanocarcinoma, lung squamous cell carcinoma or non-small cell lung cancer.

[0070] The anti-EGFR antibody, particularly mAb806 may be administered in the methods alone or in combination with other anti-EGFR antibodies. Thus, Mab806 may be administered serially or in combination with cetuximab. MAb806 may also be administered serially or in combination with other anti-EGFR vIII antibodies, including ABX-EGF (panitumumab), DH8.3, L8A4, and or active fragments thereof.

[0071] The anti-EGFR antibody(ies) may be prepared in pharmaceutical compositions, with a suitable carrier and at a strength effective for administration by various means to a patient. A variety of administrative techniques may be utilized, among them parenteral techniques such as subcutaneous, intravenous and intraperitoneal injections, catheterizations and the like. Quantities of the antibody or their active fragments may vary and in particular should be based upon the recommendations and prescription of a qualified physician or veterinarian, including upon consideration of the results and data provided herein.

[0072] The anti-EGFR antibodies of use in the invention, including mAb806, may provide useful diagnostic applications, including imaging applications or diagnostic biopsy applications, for diagnosing and/or monitoring cancer patients, including after or upon conclusion of TKI therapy.

[0073] The labels commonly employed for these studies are radioactive elements, enzymes, chemicals which fluoresce when exposed to ultraviolet light, and others. A number of fluorescent materials are known and can be utilized as labels. These include, for example, fluorescein, rhodamine, auramine, Texas Red, AMCA blue and Lucifer Yellow.

[0074] Antibodies of the invention may be labeled with a detectable or functional label. Detectable labels include, but are not limited to, radiolabels such as the isotopes ^{3}H, ^{14}C, ^{32}P, ^{35}S, ^{36}Cl, ^{51}Cr, ^{57}Co, ^{58}Co, ^{59}Fe, ^{90}Y, ^{121}I, ^{124}I, ^{125}I, ^{131}I, ^{111}In, ^{211}At, ^{198}Au, ^{67}Cu, ^{225}Ac, ^{213}Bi, ^{99}Tc and ^{186}Re, which may be attached to antibodies of the invention using conventional chemistry known in the art of antibody imaging. Labels also include fluorescent labels and labels used conventionally in the art for MRI-CT imagine. They also include enzyme labels such as horseradish peroxidase. Labels further include chemical moieties such as biotin which may be detected via binding to a specific cognate detectable moiety, *e.g.* labeled avidin.

[0075] Functional labels include substances which are designed to be targeted to the site of a tumor to cause destruction of tumor tissue. Such functional labels include cytotoxic drugs such as 5-fluorouracil or ricin and enzymes such as bacterial carboxypeptidase or nitroreductase, which are capable of converting prodrugs into active drugs at the site of a tumor.

[0076] The radiolabeled anti-EGFR antibodies and fragments thereof, are useful in *in vitro* diagnostics techniques and in *in vivo* radioimaging techniques and in radioimmunotherapy. In the instance of *in vivo* imaging, the specific binding members of the present invention may be conjugated to an imaging agent rather than a

radioisotope(s), including but not limited to a magnetic resonance image enhancing agent, wherein for instance an antibody molecule is loaded with a large number of paramagnetic ions through chelating groups. Examples of chelating groups include EDTA, porphyrins, polyamines crown ethers and polyoximes. Examples of paramagnetic ions include gadolinium, iron, manganese, rhenium, europium, lanthanium, holmium and ferbium. In a further aspect of the invention, radiolabelled specific binding members, particularly antibodies and fragments thereof, particularly radioimmunoconjugates, are useful in radioimmunotherapy, particularly as radiolabelled antibodies for cancer therapy. In a still further aspect, the radiolabelled specific binding members, particularly antibodies and fragments thereof, are useful in radioimmuno-guided surgery techniques, wherein they can identify and indicate the presence and/or location of cancer cells, precancerous cells, tumor cells, and hyperproliferative cells, prior to, during or following surgery to remove such cells.

[0077] Immunoconjugates or antibody fusion proteins of the present invention, wherein the specific binding members, particularly antibodies and fragments thereof, of the present invention are conjugated or attached to other molecules or agents further include, but are not limited to binding members conjugated to a chemical ablation agent, toxin, immunomodulator, cytokine, cytotoxic agent, chemotherapeutic agent or drug.

[0078] Radioimmunotherapy (RAIT) has entered the clinic and demonstrated efficacy using various antibody immunoconjugates. ^{131}I labeled humanized anti-carcinoembryonic antigen (anti-CEA) antibody hMN-14 has been evaluated in colorectal cancer (Behr TM et al (2002) Cancer 94(4Suppl):1373-81) and the same antibody with ^{90}Y label has been assessed in medullary thyroid carcinoma (Stein R et al (2002) Cancer 94(1):51-61). Radioimmunotherapy using monoclonal antibodies has also been assessed and reported for non-Hodgkin's lymphoma and pancreatic cancer (Goldenberg DM (2001) Crit Rev Oncol Hematol 39(1-2):195-201; Gold DV et al (2001) Crit Rev Oncol Hematol 39 (1-2) 147-54). Radioimmunotherapy methods with particular antibodies are also described in U.S. Patent 6,306,393 and 6,331,175. Radioimmunoguided surgery (RIGS) has also entered the clinic and demonstrated efficacy and usefulness, including

using anti-CEA antibodies and antibodies directed against tumor-associated antigens (Kim JC et al (2002) Int J Cancer 97(4):542-7; Schneebaum S et al (2001) World J Surg 25(12):1495-8; Avital S et al (2000) Cancer 89(8):1692-8; McIntosh DG et al (1997) Cancer Biother Radiopharm 12 (4):287-94).

[0079] Antibodies of the present invention may be administered to a patient in need of treatment via any suitable route, usually by injection into the bloodstream or CSF, or directly into the site of the tumor. The precise dose will depend upon a number of factors, including whether the antibody is for diagnosis or for treatment, the size and location of the tumor, the precise nature of the antibody (whether whole antibody, fragment, diabody, etc), and the nature of the detectable or functional label attached to the antibody. Where a radionuclide is used for therapy, a suitable maximum single dose is about 45 mCi/m^2, to a maximum of about 250 mCi/m^2. Preferable dosage is in the range of 15 to 40 mCi, with a further preferred dosage range of 20 to 30 mCi, or 10 to 30 mCi.. Such therapy may require bone marrow or stem cell replacement. A typical antibody dose for either tumor imaging or tumor treatment will be in the range of from 0.5 to 40 mg, preferably from 1 to 4 mg of antibody in F(ab')2 form. Naked antibodies are preferable administered in doses of 20 to 1000 mg protein per dose, or 20 to 500 mg protein per dose, or 20 to 100 mg protein per dose. This is a dose for a single treatment of an adult patient, which may be proportionally adjusted for children and infants, and also adjusted for other antibody formats in proportion to molecular weight. Treatments may be repeated at daily, twice-weekly, weekly or monthly intervals, at the discretion of the physician.

[0080] The invention may be better understood by reference to the following non-limiting Examples, which are provided as exemplary of the invention. The following examples are presented in order to more fully illustrate the preferred embodiments of the invention and should in no way be construed, however, as limiting the broad scope of the invention.

EXAMPLE 1

Therapeutic anti-EGFR antibody 806 generates responses in murine *de novo* EGFR mutant-dependant lung carcinomas

[0081] Activating epidermal growth factor receptor (EGFR) mutations occur in human non-small cell lung cancer (NSCLC), with 5% of human lung squamous cell carcinomas having *EGFRvIII* mutations and 10~30% of lung adenocarcinomas having *EGFR* kinase domain mutations. An EGFR targeting monoclonal antibody, mAb806, recognizes a conformational epitope of wild type (wt) EGFR as well as the truncated EGFRvIII mutant. To explore the anticancer spectrum of this antibody for EGFR targeted cancer therapy, mAb806 was used to treat genetically engineered mice with lung tumors that were driven by either *EGFRvIII* or *EGFR* kinase domain mutations. Our results demonstrate that mAb806 is remarkably effective in blocking EGFRvIII signaling and inducing tumor cell apoptosis and, thus resulting in dramatic tumor regression in the *EGFRvIII* driven murine lung cancers. Another EGFR-targeting antibody, cetuximab, failed to show activity in these genetically defined lung tumors. Furthermore, treatment of murine lung tumors driven by *EGFR* kinase domain mutation with the mAb806 induced a significant tumor regression, albeit to a less degree than that observed in EGFRvIII driven tumors. Taken together, these data support the hypothesis that mAb806 may provide significant activity in the treatment of the population of NSCLC patients with these two classes of EGFR mutations.

Introduction

[0082] Targeted cancer therapy is designed to disrupt the function of specific molecules needed for carcinogenesis and tumor growth and thus either kills or prevents the growth of cancer cells (1). In contrast to conventional cytotoxic chemotherapy, such targeted cancer therapies may be more effective and less harmful to normal cells. A main effort in the targeted cancer therapy field has been the development of agents that target the epidermal growth factor receptor (EGFR). EGFR is a member of the ErbB family of closely related receptors including EGFR (ErbB-1), Her2/neu (ErbB-2), Her3 (ErbB-3) and Her4 (ErbB-4). Activation of EGFR leads to receptor tyrosine kinase activation and a series of downstream signaling events that mediate cellular proliferation, motility, adhesion, invasion, and resistance to chemotherapy as well as inhibition of apoptosis (2-4), processes that are crucial to the continual proliferation and survival of cancer cells.

[0083] To date, two major types of anti-EGFR agents have entered the clinical setting: anti-EGFR antibodies and small molecule EGFR tyrosine kinase inhibitors (TKIs) (5, 6). Anti-EGFR antibodies such as cetuximab were designed to bind to the extra-cellular domain of the EGFR and block activation of EGFR downstream signaling (7). In contrast, small molecule TKIs such as gefitinib or erlotinib compete with ATP for binding to the intracellular catalytic domain of the EGFR tyrosine kinase and, thus, prevent EGFR autophosphorylation and downstream signaling (4).

[0084] Both of these anti-EGFR drug groups have shown some clinical efficacy in a subset of patients with a variety of different types of cancers. Treatment with gefitinib or erlotinib in patients with lung cancer having EGFR kinase domain mutations often generate dramatic clinical responses (5, 8). However, the effectiveness of gefitinib or erlotinib in lung adenocarcinoma with wild type EGFR or in other histological subtype, such as squamous cell carcinoma is limited (9, 10). Furthermore, it has been shown in pre-clinical and clinical trials that gefitinib or erlotinib are largely ineffective in inhibiting the function of the EGFRvIII mutant (11), a distinct activating EGFR mutation in which there is an in-frame deletion of exon II to VII. EGFRvIII is commonly found in

glioblastomas and recently found to be present in a subset of human lung squamous cell carcinomas (12) and a large fraction of head and neck cancers (13). Cetuximab is shown to be effective in a small subset of non-small cell lung cancer (NSCLC) patients, and patients with head and neck cancers, as well as colorectal cancer patients. However, the response to cetuximab does not seem to correlate with expression levels of EGFR. Thus, it is unclear why these patients respond while other cancer patients whose tumors have high EGFR expression are refractory to cetuximab treatment (14).

[0085] MAb806 is a novel murine antibody, originally raised to recognize the unique truncation mutant, EGFRvIII (15-17) . Importantly, the epitope recognized by mAb806 is not accessible in inactive wild-type (*wt*) EGFR, but is exposed in a transitional form of wt EGFR in cells with overexpression of EGFR, and expression of EGFRvIII (18) . The epitope studies are supported by immunohistochemical studies demonstrating that the 806 antibody binds to epitopes present in gliomas, as well as a broad range of epithelial cancers, but not to normal human tissues (16, 19). These and other preclinical data suggest that mAb806 might have a different spectrum of clinical activity and side effect profile distinct from cetuximab and other anti-EGFR antibodies. In xenograft models, mAb806 has exhibited a potent anti-tumor activity with no targeting of normal tissues. Thus, the unique targeting capabilities of mAb806 represent a new paradigm for cancer-specific molecularly targeted therapy.

[0086] Recent studies have shown that 10-30% of NSCLC patients have *EGFR* kinase domain mutations while 5% of lung squamous cell carcinoma (SCC) patients have the extracellular domain *EGFRvIII* mutation (12, 20). To investigate the clinical potential of mAb806 in cancer-specific targeted therapy in NSCLC patients harboring *EGFR* mutations, we utilized two established mouse lung cancer models that are dependent on EGFRvIII or EGFR kinase domain mutants. Our data show that mAb806 is very effective in the treatment of murine NSCLC driven by expression of either *EGFRvIII* or *EGFR* kinase domain mutation and suggest that this antibody is likely to have clinical activity in patients whose tumors have similar mutation.

Results

Treatment with mAb806, but not cetuximab, induces tumor regression in mice bearing lung tumors with *EGFRvIII* mutation

[0087] Previous studies have established the essential role of EGFRvIII mutation in tumor maintenance of murine lung tumors driven by the mutation. Blocking EGFRvIII activation results in dramatic tumor regression associated with apoptosis in the de novo murine lung cancer model(12). *Tet-op-EGFRvIII/CCSP-rtTA*, *Ink4A/Arf-/-* mice developed lung adenocarcinomas with bronchiolalvealor carcinoma (BAC) features after 8~10 weeks of doxycycline administration, (Figure 1, left panel; Figure 2A, upper panel). After tumor bearing mice were identified by MRI, 0.5 mg per dose of mAb806 was given by intraperitoneally (I.P.) injection daily for the first week and then every two days for the next 4 weeks. Serial MRI was performed at the end of 1, 3 and 5 weeks of treatment to determine changes in tumor volume and/or density. Tumor reduction was notable by MRI after 1 week of mAb806 treatment (average reduction of 60%±5% among 6 mice, Figure 1, upper panel). Tumor burden continued to decrease after 3 weeks of treatment (average reduction of 95% ±8%), and all 6 mice had complete tumor regression after 5 weeks of treatment. In contrast, treatment of mice with cetuximab was unable to induce tumor regression in 4 *Tet-op-EGFRvIII/CCSP-rtTA*, *Ink4A/Arf-/-* mice even after 5 weeks of treatment at 1mg per mouse with the same dosing schedule. We also observed that the mice treated with cetuximab became progressively frailer and that some even succumbed because of significant tumor burden during the treatment period (data not shown).

[0088] Pathologic examination of lungs from these mice correlated with the MRI findings: a decrease in tumor cellularity was present in adenocarcinomas after one week of treatment with mAb806 (Figure 2A, middle panel). After 5 weeks, lungs had focal fibrosis and scaring, with sparse monocytic cell infiltrates; potentially representing areas of continuing remodeling from regressed tumors (Figure 2A, lower panel). Although alive cancer cells could still be rarely observed in several foci of these fibrotic nodules, most of fibrosis and scarring area did not contain any tumor cells. In contrast, the tumors from mice treated with cetuximab appeared to be unaffected, with no visible histological

difference when compared with untreated tumors (data not shown). Thus, treatment with mAb806 antibody led to rapid and dramatic tumor regression in the *EGFRvIII* driven mouse lung cancer model while cetuximab treatment was largely ineffective.

MAb806 inhibits EGFRvIII phosphorylation and induces apoptosis of tumor cells in *Tet-op-EGFRvIII/CCSP-rtTA, Ink4A/Arf-/-* mice

[0089] To determine whether the mAb806 that was administered intraperitoneally recognized its target in the lung tumors, we performed immunohistochemical staining in lung tumors of mice treated with or without mAb806 using antibodies against total EGFR and phospho-EGFR. As expected, mAb806 treatment had no impact on the total EGFRvIII expression in tumor cells, (Figure 2B). However, the expression of phospho-EGFRvIII diminished after 1-week of mAb806 treatment (Figure 2B). We next confirmed these findings by immunoblotting analysis using lung lysates collected at different time points during treatment with mAb806. The level of phospho-EGFRvIII decreased dramatically after 1-week mAb806 treatment while the total EGFRvIII level remained similar to that of untreated controls (Figure 3), indicating a strong inhibitory effect of mAb806 on EGFRvIII phosphorylation. Interestingly, the total EGFRvIII level did finally decrease after 5 weeks of mAb806 administration. One explanation for this could be the dramatic decrease of the number of viable tumor cells. Consistent with this interpretation, greatly increased TUNEL staining was observed in lung tumors after 1-week of mAb806 treatment compared to untreated tumors (Fig 2C). Besides the changes on phospho-EGFR level, 1 week of mAb806 treatment also decreased phospho-AKt and phospho-Erk1,2 expression, these EGFR downstream signaling molecules are functionally associated with anti-apoptosis and proliferation pathways. Surprisingly, we observed a weak but reproducible increase of phopho-AKt level after 5 weeks of mAb806 treatment when compared to 1 week treatment. This phosphorylation of Akt is unlikely initiated by EGFRvIII, as phospho-EGFRvIII is low at this time point. Possibly, Akt could be activated by other signaling events that were involved in lung remodeling process. These data suggest that mAb806 induced tumor regression in *EGFRvIII* mice by blocking EGFR activation and increasing tumor cell apoptosis.

Ch806 treatment leads to a dramatic tumor regression in murine lung tumors with *EGFRvIII* mutation

[0090] Ch806 is a humanized form of mAb806 (22). To determine whether the humanized antibody could be as efficient as the murine mAb806 in treatment of lung adenocarcinoma in vivo, we treated tumor-bearing *Tet-op-EGFRvIII/CCSP-rtTA, Ink4A/Arf-/-* mice with one dose of 0.5 mg of ch806 by I.P. injection daily for the first week and then one dose every two days for another 7 weeks. These mice underwent re-imaging at 1.5, 5 and 8 weeks of the treatment and were then sacrificed for histological analysis. We observed a dramatic reduction in tumor volume by MRI scanning starting from 1.5 weeks of the treatment (43% ±3%), and near complete tumor regression (83% ± 7%) was achieved at 8 weeks of treatment in each of the 4 mice being treated with ch806 (Figure 1, lower panel). The histology of mice treated with ch806 (data not shown) was similar to that of tumors after mAb806 treatment and was consistent with the MRI data.

Ch806 is effective in treatment of murine lung tumors with *EGFR L858R* mutation

[0091] To address whether ch806 could be effective against *EGFR* kinase domain mutation driven lung cancer, *EGFR L858R-IRES-Luciferase/CCSP-rtTA* mice were employed. Ch806 was administered at 0.5 mg/mouse every day for 4 weeks and serial MRI scanning of all treated mice was performed at the end of 1, 2, and 4 weeks of treatment. Tumor regression was observed after 2 weeks of ch806 treatment (21%±2%) and was 41%±2 % at 4 weeks of ch806 treatment (Figure 4A). Microscopically, the lungs of ch806-treated mice showed an increased diffuse cellular infiltrate with macrophages, especially in areas surrounding the remaining viable tumors. Furthermore, macrophages were present in multiple areas of the tumors, suggesting that macrophage-mediated cytotoxicity might be one of the underlying mechanisms of antibody-induced tumor regression (Figure 4B). It should also be noted that the presence of consolidation within the lung due to increased accumulation of macrophages associated with tumor cells could overestimate tumor volume by MR imaging.

Discussion

[0092] EGFR mutations and activation events are common in human malignancies, including NSCLC. Activation of EGFR signaling can occur through receptor over expression as well as by constitutive signaling due to gain-of-function mutant forms of EGFR. Approximately 10-30% of NSCLC patients have *EGFR* kinase domain mutations in their lung tumors and about 5% of patients with squamous cell lung cancer have the specific *EGFRvIII* extracellular domain mutation (12, 20). Here we show that mAb806 and its humanized form, ch806, are effective in treating murine lung cancers with both types of *EGFR* mutations. The dramatic tumor regression observed was associated with blockage of EGFRvIII signaling and, consequently, increased apoptosis. The response to ch806 was not as impressive as that reported for erlotinib and cetuximab, in mice with lung tumors having *EGFR* kinase domain mutations, although they did have an objective response (41%±2%) radiographically and histologically(21, 23). In contrast, nearly complete tumor regression was achieved in mice with *EGFRvIII* driven lung tumors after treatment with mAb806, while cetuximab was without effect. This latter result is perhaps not surprising since cetuximab is designed to interfere with the interaction between ligand and the EGFR extracellular domain (24). It has been established that the EGFRvIII mutation leads to conformational changes and exhibits constitutive kinase activity independent of ligand stimulation which contributes to the tumor formation(25). Although cetuximab has been approved by the FDA for cancer patients, there is no clear biomarker to predict the efficacy of treatment with this antibody in individual patients, since response rates and overall survival are not correlated with EGFR protein expression by immunohistochemistry (14).

[0093] Although small molecule TKIs are effective in the treatment of many NSCLC patients with *EGFR* kinase domain mutations, all patients eventually develop resistance associated with a secondary mutation, T790M (10, 26). Consistently, in vitro studies have shown that tumor cells with T790M mutations are resistant to treatment with erlotinib (27, 28). Evidence from the crystal structure of the EGFR kinase domain with a secondary T790M mutation indicates that there should be little effect of T790M mutation

on the receptor function. It may be that the T790M mutation interferes with erlotinib for its binding to the ATPase pocket (27). Nonetheless, the extracellular domain of the T790M mutant potentially provides a good target for antibody-based cancer therapy including cetuximab and mAb806. This could mean that NSCLC tumors with secondary T790M point mutations, which are resistant to small TKI treatment, might respond to mAb806 treatment. Efforts to generate mice harboring the compound mutant *EGFR* alleles containing both the activating kinase domain mutations and the T790M mutation are ongoing in order to test this hypothesis.

[0094] Recently released data from a Phase I clinical trial has shown that the ch806 antibody, unlike cetuximab, selectively binds to tumor cells of lung cancers, including squamous cell lung carcinoma, but not to normal tissues (Scott, ASCO 2006). No significant toxicities of the ch806 antibody were observed in this trial. In comparison with other EGFR targeted cancer therapies, including cetuximab and TKI treatments, ch806 appears to have a much greater specificity, by targeting a conformationally dependent epitope of the EGFR on cancer cells while sparing wt EGFR on most, if not all, normal cells. Our results clearly indicate the effectiveness of mAb 806 on blocking EGFR signaling. Thus, the unique targeting capabilities of ch806 represent a new and exciting paradigm for cancer-specific molecularly targeted therapy, which may benefit patients whose cancers are dependent upon uncontrolled EGFR signaling due to overexpression or to gain-of-function mutations including *EGFRvIII* or *EGFR* kinase domain mutations.

Methods

Mouse cohorts.

[0095] The generation of *Tet-op-EGFRvIII/CCSP-rtTA, Ink4A/Arf-/-* mice and *Tet-op-EGFR L858R-IRES-Luciferase/CCSP-rtTA* mice were described previously (12, 21) . All mice were housed in the pathogen-free environment at Harvard School of Public Health and all mouse experiments performed were approved by the Institutional Animal Care and Use Committee (IACUC). Littermates are used as controls in all experiments. To induce *EGFRvIII* and *EGFR L858R* expression, mice were fed with a doxycycline diet (Research Diets, Inc.). Doxycycline withdraw experiment in previous studies clearly identified that lung tumors from both alleles are soley dependent on doxycycline.

Targeted therapies using either mAb806 or ch806 or cetuximab in vivo.

[0096] Mice on continuous doxycycline diets for more than 8 weeks underwent MRI to document the lung tumor burden. MAb806 or ch806 (generated by the Ludwig Institute for Cancer Research, Melbourne, Australia) was delivered into mice bearing lung tumors through I.P. injection at daily 0.5 mg per doses. After a 1-week treatment, antibodies were administered every two days at the same dose for the additional indicated weeks. Cetuximab (obtained commercially from BMS pharmaceuticals) was administered to mice by I. P. injection at 1 mg per dose using the same dosing schedule. Mice were imaged with MRI at the indicated time points to determine reduction in tumor volume and then sacrificed for further histological and biochemical studies after the completion of treatment. All the mice were kept on the doxycycline diet throughout the experiments. Littermates were used as controls for all the drug treatment studies.

Pathologic assessment of lung tumors.

[0097] Mice were euthanized at the indicated times and their left lungs were dissected and snap frozen for biochemical analysis. Their right lungs were then inflated under pressure (25 cm) with neutral buffered 10% formalin for 10 minutes and fixed overnight. Hematoxylin and eosin (H&E) stains were performed on 5 μm-thick sections from

formalin-fixed, paraffin-embedded tumor samples in the Department of Pathology at Brigham and Women's Hospital.

[0098] Immunohistochemical analysis was performed on formalin-fixed paraffin sections. Slides were deparaffinized in xylene and rehydrated sequentially in ethanol. For antibodies requiring antigen retrieval, antigen-unmasking solution (Vector Laboratories) was used according to the manufacturer's instructions. Slides were quenched in hydrogen peroxide (0.3%–3%) to block endogenous peroxidase activity and then washed in automation buffer (Fisher Scientific). Slides were blocked in 5% normal serum for 1 hr at room temperature and then incubated overnight at 4°C with primary antibody diluted in blocking buffer. The avidin biotin peroxidase complex method (Vector) was used, and slides were counterstained with hematoxylin. Slides were dehydrated sequentially in ethanol, cleared with xylene, and mounted with Permount (Fisher). Biotinylated DBA lectin (Vector) was used at 1:100. The antibodies used were total EGFR and phospho-EGFR Y1068 (1:50, Cell Signaling Technology). Apoptosis was measured by counting positive cells using the TUNEL assay (ApopTag kit; Intergen, Inc.).

Western blot analysis.

[0099] Snap frozen lung tissue samples were homogenized in RIPA buffer (Boston Bioproducts) containing the Complete Protease Inhibitors Cocktail and Phosphatase Inhibitors Cocktail Set I and II (EMD Biosciences). Lung lysates were cleared by centrifugation and boiled in 1x final sodium dodecyl sulfate (SDS) sample buffer (50mM Tris (pH6.8), 10% glycerol, 0.715M β-mercaptoethanol, 2% SDS and 0.01% bromophenol blue) for 5 minutes. Lysates were then separated by SDS- polyacrylamide-gel electrophoresis (PAGE), transferred to nitrocellulose membranes and detected by immunoblotting with antibodies using SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology). The antibodies used in this study were directed against total EGFR, phospho-EGFR (pY1068), total Akt, phospho-AKT (pS473), total Erk1/2 and phospho-ERK 1/2 (pT202/pY204) (all from Cell Signaling); and β-actin (Santa Cruz Biotechnology, Inc.). Antibodies were used according to the conditions recommended by the manufacturer.

MRI and tumor volume measurement.

[0100] Animals were anesthetized with 1.5-2% isoflurane (IsoFlo®, Abbot Laboratories) mixed in 100% oxygen via a nose cone. In order to eliminate motion issues, both cardiac and respiratory gating was applied to all MRI studies. Since the acquisition of the MR signal is synchronized with the cardiac and respiratory cycles, the MR signal was acquired at each cardiac phase and at end-expiratory phase allowing motion artifacts to be significantly reduced.

[0101] MRI protocols optimized for assessing pulmonary parenchyma and vessels in normal mice (29) were adapted for operation at 4.7 Tesla (Biospec 47/40, Bruker BioSpin, Karlsruhe, Germany). The system is equipped with shielded gradient systems with a maximum power gradient of 30 G/cm, and a cardiac-respiratory triggering system (BioTrig, Bruker BioSpin, Karlsruhe, Germany). Then, the animals were placed prone with the electrodes (both fore pads and left rear pad) for cardiac gating and a respiratory sensor on their bodies, head first into the system, with the thorax centered with respect to the center of the radio frequency birdcage coil (inner diameter 3 cm). For the purpose of reproducible positioning of the imaging region, a low-resolution multi-slice image, serving as the end-expiratory phase localizer, was firstly acquired for the entire lung in both transverse and coronal planes using a fast spin echo sequence (RARE: rapid acquisition with relaxation enhancement, TR/effective TE =1000/28 msec, bandwidth = 50 kHz, field of view = 30 mm, matrix = 128×128, slice thickness = 1 mm, number of excitation = 1). Further, two-dimensional (2D) multi-slice gradient echo imaging was performed in multi-slice transverse and coronal planes encompassing the entire lung with cardiac-respiratory gating. A pulse repetition time (TR) was selected less than the duration of one cardiac cycle (ranging 150 to 200 msec, average 178 msec), where one k-space line was filled for each image per single heartbeat. The minimum echo time (TE: 1.8 msec) was used to reduce the susceptibility effect arising from the interface between air/bone and tissue which would otherwise reduce the MR signal. Other scan parameters were: flip angle = 22°, matrix size = 256 × 256, field of view (FOV) = 2.56 cm^2, slice thickness = 1 mm, and number of excitation (NEX) = 4, affording a 100 μm^2 in-plane

resolution. Total scan time was approximately 6-7 minutes in each plane, depending on the individual animal's cardiac/respiratory rates. On each MR image, the areas indicating the pulmonary tumor were manually segmented and measured to calculate tumor volumes using ImageJ (ver. 1.33, National Institute of Health).

REFERENCES

1. Ji, H., Sharpless, N.E., and Wong, K.K. 2006. EGFR Target Therapy: View From Biological Standpoint. *Cell Cycle.* **5(18)**:2072-2076. Epub 2006 Sep15.
2. Hynes, N.E., and Lane, H.A. 2005. ERBB receptors and cancer: the complexity of targeted inhibitors. *Nat. Rev. Cancer.* **5**:341-354.
3. Arteaga, C.L. 2003. ErbB-targeted therapeutic approaches in human cancer. *Exp. Cell. Res.* **284**:122-130.
4. Mendelsohn, J., and Baselga, J. 2000. The EGF receptor family as targets for cancer therapy. *Oncogene.* **19**:6550-6565.
5. Snyder, L.C., Astsaturov, I., and Weiner, L.M. 2005. Overview of monoclonal antibodies and small molecules targeting the epidermal growth factor receptor pathway in colorectal cancer. *Clin. Colorectal Cancer.* **5** Suppl 2:S71-80.
6. Herbst, R.S. 2002. Targeted therapy in non-small-cell lung cancer. *Oncology (Williston Park).* **16**:19-24.
7. Groner, B., Hartmann, C., and Wels, W. 2004. Therapeutic antibodies. *Curr. Mol. Med.* **4**:539-547
8. Haber, D.A., et al. 2005. Molecular targeted therapy of lung cancer: EGFR mutations and response to EGFR inhibitors. *Cold Spring Harb Symp. Quant. Biol.* **70**:419-426.
9. Park, K., and Goto, K. 2006. A review of the benefit-risk profile of gefitinib in Asian patients with advanced non-small-cell lung cancer. *Curr. Med. Res. Opin.* **22**:561-573.
10. Sakurada, A., Shepherd, F.A., and Tsao, M.S. 2006. Epidermal growth factor receptor tyrosine kinase inhibitors in lung cancer: impact of primary or secondary mutations. *Clin. Lung Cancer.* **7** Suppl 4:S138-144.

11. Halatsch, M.E., Schmidt, U., Behnke-Mursch, J., Unterberg, A., and Wirtz, C.R. 2006. Epidermal growth factor receptor inhibition for the treatment of glioblastoma multiforme and other malignant brain tumours. *Cancer Treat. Rev.* **32**:74-89.

12. Ji, H., et al. 2006. Epidermal growth factor receptor variant III mutations in lung tumorigenesis and sensitivity to tyrosine kinase inhibitors. *Proc. Natl. Acad. Sci. U S A.* **103**:7817-7822.

13. Sok, J.C., et al. 2006. Mutant epidermal growth factor receptor (EGFRvIII) contributes to head and neck cancer growth and resistance to EGFR targeting. *Clin. Cancer. Res.* **12**:5064-5073.

14. Italiano, A. 2006. Targeting the epidermal growth factor receptor in colorectal cancer: advances and controversies. *Oncology.* **70**:161-167.

15. Jungbluth, A.A., et al. 2003. A monoclonal antibody recognizing human cancers with amplification/overexpression of the human epidermal growth factor receptor. *Proc. Natl. Acad. Sci. U S A.* **100**:639-644.

16. Luwor, R.B., et al. 2001. Monoclonal antibody 806 inhibits the growth of tumor xenografts expressing either the de2-7 or amplified epidermal growth. factor receptor (EGFR) but not wild-type EGFR. *Cancer Res.* **61**:5355-5361.

17. Mishima, K., et al. 2001. Growth suppression of intracranial xenografted glioblastomas overexpressing mutant epidermal growth factor receptors by systemic administration of monoclonal antibody (mAb) 806, a novel monoclonal antibody directed to the receptor. *Cancer Res.* **61**:5349-5354.

18. Johns, T.G., et al. 2004. Identification of the epitope for the epidermal growth factor receptor-specific monoclonal antibody 806 reveals that it preferentially recognizes an untethered form of the receptor. *J. Biol. Chem.* **279**:30375-30384.

19. Johns, T.G., et al. 2002. Novel monoclonal antibody specific for the de2-7 epidermal growth factor receptor (EGFR) that also recognizes the EGFR expressed in cells containing amplification of the EGFR gene. *Int. J. Cancer.* **98**:398-408.

20. Shigematsu, H., and Gazdar, A.F. 2006. Somatic mutations of epidermal growth factor receptor signaling pathway in lung cancers. *Int. J. Cancer.* **118**:257-262.

21. Ji, H., et al. 2006. The impact of human EGFR kinase domain mutations on lung tumorigenesis and in vivo sensitivity to EGFR-targeted therapies. *Cancer Cell.* **9**:485-495.

22. Panousis, C., et al. 2005. Engineering and characterisation of chimeric monoclonal antibody 806 (ch806) for targeted immunotherapy of tumours expressing de2-7 EGFR or amplified EGFR. *Br. J. Cancer.* **92**:1069-1077.

23. Politi, K., et al. 2006. Lung adenocarcinomas induced in mice by mutant EGF receptors found in human lung cancers respond to a tyrosine kinase inhibitor or to down-regulation of the receptors. *Genes. Dev.* **20**:1496-1510.

24. Li, S., etal. 2005. Structural basis for inhibition of the epidermal growth factor receptor by cetuximab. *Cancer Cell.* **7**:301-311.

25. Pedersen, M.W., and Poulsen, H.S. 2006. [Mutations in the epidermal growth factor receptor: structure and biological function in human tumors]. *Ugeskr. Laeger.* **168**:2354-2361.

26. Janne, P.A., Engelman, J.A., and Johnson, B.E. 2005. Epidermal growth factor receptor mutations in non-small-cell lung cancer: implications for treatment and tumor biology. *J Clin. Oncol.* **23**:3227-3234.

27. Kobayashi, S., et al. 2005. EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. *N. Engl. J. Med.* **352**:786-792.

28. Kobayashi, S.,et al. 2005. An alternative inhibitor overcomes resistance caused by a mutation of the epidermal growth factor receptor. *Cancer Res.* **65**:7096-7101.

29. Kubo, S., et al. 2006. Three-dimensional magnetic resonance microscopy of pulmonary solitary tumors in transgenic mice. *Magn. Reson. Med.* **56**:698-703.

EXAMPLE 2

806 ANTIBODY LEADS TO TUMOR REGRESSION IN LUNG TUMORS WITH EGFR T790M MUTATION

[0102] Mice expressing the human EGFR secondary mutation T790M were generated. Mab806 was delivered into mice bearing lung tumors through I.P. injection at daily 0.5 mg per dose per mouse for 4 weeks. Serial MRI scanning of treated mice was performed at the end of 2 and 4 weeks of treatment as described below.

Generation of the Tet-op-hEGFR T790M- L858R/CCSP-rtTA mouse cohort

[0103] To generate mice with inducible expression of human EGFR T790M-L858R mutant, we constructed a 4.7-kb DNA segment consisting of seven direct repeats of the tetracycline (tet)-operator sequence, followed by EGFR T790M-L858R cDNA and ?-globin polyA. The construct was injected into FVB/N blastocysts and progeny were screened using PCR strategy. Fifteen Tet-op-hEGFR T790M-L858R founders were identified and then crossed to CCSP-rtTA mice (an allele been shown specifically targeting the expression of the reverse tetracycline trans-activator protein (rtTA) in type II alveolar epithelial cells (Fisher GH et al (2001) Genes Dev 15(24):3249-62) to generate inducible bitransgenic mouse cohorts harboring both the activator and the responder transgenes (Fisher GH et al (2001) Genes Dev 15(24):3249-62; Perl AK, Tichelaar JW, and Whitsett JA. (2002) Transgenic Res 11(1):21-9). Four tightly regulated hEGFR T790M-L858R (#17, #19, #24 and #29) founders were identified by RT-PCR analysis and the copy numbers from individual founders were determined by quantitative real-time PCR (Ji H et al (2006) Cancer Cell 9(6):485-95).

Tightly regulated expression of EGFR T790M-L858R in lung tissue at RNA level

[0104] The inducibility of EGFR mutant transgene expression in the lung compartment was evaluated at the RNA level by RT-PCR with human EGFR specific primers. The lungs of the bitransgenic mouse Tet-op-hEGFR T790M-L858R/CCSP-rtTA cohort for each potential founder were collected before and after 8 weeks of doxycycline administration and after 3 days of doxycycline withdrawal following doxycycline administration of an 8-week period. The EGFR mutant transcript was undetectable from either non-transgenic mice or the bitransgenic mice without doxycycline treatment, while it became readily detectable after 8-week doxycycline administration; transcription of mutant EGFR was completely abolished by 3 days of doxycycline withdrawal in all of the lines. To further confirm that the mutant EGFR transcripts is inducible and tightly regulated by doxycycline, RT-PCR using the same primers as described above and quantitative real time PCR was performed for lung samples collected at serial time points of doxycycline administration and withdrawal from founder #19. EGFR expression was observed after 1 week of doxycycline administration and was kept at a comparable level throughout the 8-week period of administration; doxycycline withdrawal is sufficient to block the expression of mutant EGFR, and no expression of the transgene was observed after 12 weeks of doxycycline withdrawal.

[0105] Over-expression of the EGFR T790M-L858R mutant drives the development of lung adenocarcinomas with bronchioloalveolar features in parenchyma and papillary adenocarcinoma in airways

To determine if over-expression of the hEGFR mutants drive lung tumorigenesis, bitransgenic hEGFR T790M-L858R/CCSP-rtTA mice on continuous doxycycline administration underwent serial magnetic resonance imaging (MRI) and were sacrificed at various time points for histological examination of the lungs. Tumors could only be observed by MRI after 5~6 weeks of doxycycline administration and tumor volume, as defined by MRI, increased following prolonged doxycycline treatment. In contrast to untreated mice, early lesions started to develop in parenchyma of the lungs after 2-3 weeks of doxycycline treatment. After 4-5 weeks, typical BAC appeared.

[0106] Invasive adenocarcinoma with bronchioloalveolar features appeared after 7-9 weeks and become the dominant histological pattern after 12 weeks of doxycycline treatment. The lung parenchymal adenocarcinomas observed in our mouse model is histologically similar to that of EGFR L858R mouse model described previously (Ji, H., et al. (2006) Cancer Cell 9:485-495; Politi, K., et al. (2006) Genes Dev. 20:1496-1510) and also similar to that seen in a subset of NSCLC patients who originally responded to erlotinib.

[0107] In addition to parenchymal adenocarcinomas, hEGFR T790M-L858R/CCSP-rtTA mice also developed bronchial papillary adenocarcinomas. Early papillary neoplasia in the bronchioles was observed after 2-3 weeks of continuous doxycycline administration, and then developed into adenocarcinoma within additional 6 to 8 weeks. All of the four founders showed similar morphologic features and a similar latency of tumorigenesis. Bronchial tumors were found in all of the 4 founders of hEGFR T790M-L858R/CCSP-rtTA mice identified in the current study, but were absent in all of our EGFR L858R mice. Occasionally, metastatic foci of adenocarcinoma could be observed in lymph nodes of the mice that develop EGFR T790M-L858R driven lung tumors but not of the mice that has EGFR L858R driven tumors. IHC staining for both bronchial and parenchymal tumors with specific cell markers shows different patterns of differentiation. Prosurfactant protein C (SPC) is a unique biomarker for type II pneumocytes in the alveoli, while Clara cell secretory protein (CCSP) is specific to Clara cell in bronchiolar epithelium. The majority of parenchymal tumors show intensive SPC staining, implying a type II pneumocyte origin, as expected. In contrast, the bronchial tumors were negative for SPC. Interestingly, only a small subset of bronchial tumor cells is positive for CCSP. This could be possibly explained by Clara cell origin followed by poor differentiation which led to loss of the CCSP expression marker.

<u>Expression of the hEGFR T790M-L858R mutants is essential for tumor maintenance of both parenchymal and bronchial adenocarcinomas</u>

[0108] Both bronchial and parenchymal lung adenocarcinomas from hEGFR T790M-L858R/CCSP-rtTA mice were positively stained by total and phospho-EGFR antibodies, indicating that the expressed EGFR mutant is functionally active. After 3 days of doxycycline withdrawal, no positive signals from either of the antibodies were observed, implying that both types of tumors are driven by and dependent on EGFR T790M-L858R for their survival. We also observed an increase in positive staining of terminal deoxynucleotidyltransferase- mediated dUTP-biotin nick end labeling (TUNEL) assay after doxycycline withdrawal, indicating the apoptotic process had been triggered.

[0109] Consistent with the apoptosis suggested by TUNEL staining, MRI results demonstrate that EGFR T790M-L858R driven lung tumor completely regressed after 10 days of doxycycline withdrawal. Microscopic analysis of the lungs from the same mouse that examined by MRI shows grossly normal lung histology. No tumor lesions were found in either airways or parenchyma after 12 weeks of doxycycline withdrawal in other tumor bearing mice.

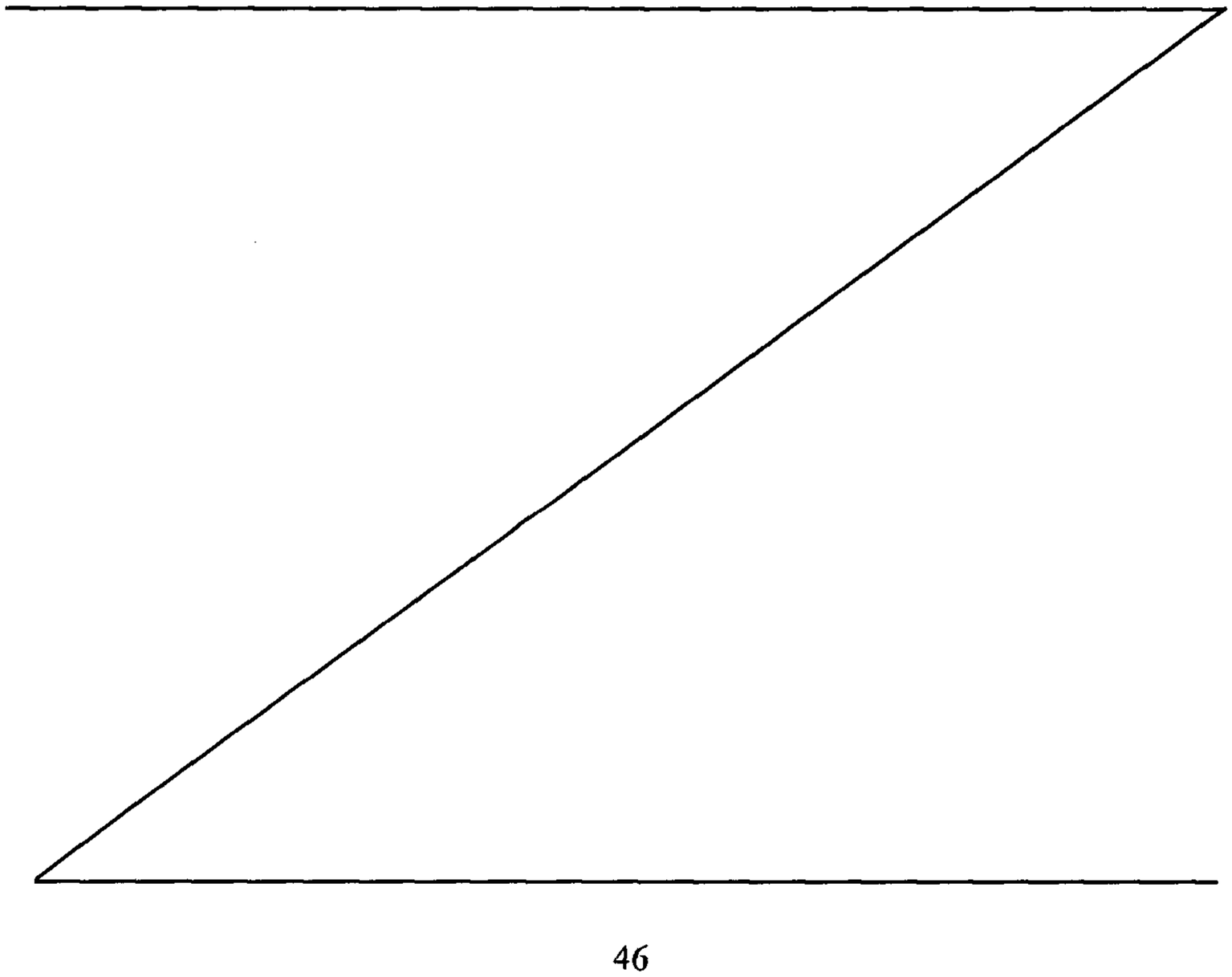
[0110] To better quantify mutant EGFR expression in tumors at the protein level, we performed western blotting using whole lung lysates from bitransgenic mice after different times of doxycycline administration. Although individual differences exist, EGFR phosphorylation was tightly regulated by doxycycline and was synchronized with the presence of tumors, confirming the essential role of mutant EGFR signaling in tumor maintenance as observed in IHC staining and MRI. Therefore, EGFR remains an attractive therapeutic target for our novel mouse lung cancer model.

[0111] Treatment of EGFR T790M-L858R driven lung tumors with mAb806

The results of treatment of EGFR T790M-L858R lung tumors with mAb806 versus cetuximab is depicted in FIGURE 5. Mice on continuous doxycycline diets for more than 8 weeks underwent MRI to document the tumor burden. Mab806 was delivered into mice bearing lung tumors through I.P. injection daily at 0.5 mg doses for 4 weeks. Cetuximab was administered to mice by I.P. injection at 1 mg per dose daily

for 4 weeks. Mice were imaged with MRI at 0, 2, and 4 or 5 weeks to determine reduction in tumor volume. Tumor volume was reduced at 2 weeks (over 20%) and more significantly at 4 weeks (over 30%) by treatment with mAb806. While tumor volume was initially reduced at 2 weeks by treatment with cetuximab, tumor volume grew significantly by 5 weeks of treatment with cetuximab (tumor volume observed at 5 weeks of cetuximab treatment was greater than original volume at 0 weeks). After completion of treatment and MRI imaging, mice were sacrificed for further histological and biochemical studies. Littermates were used as controls for all the treatment studies (no treatment).

[0112] This invention may be embodied in other forms or carried out in other ways without departing from the spirit or essential characteristics thereof. The present disclosure is therefore to be considered as in all aspects illustrate and not restrictive, the scope of the invention being indicated by the appended Claims, and all changes which come within the meaning and range of equivalency are intended to be embraced therein.



P1282CA00

WHAT IS CLAIMED IS :

1. The use of a mAb806 antibody, or an active fragment thereof, for treating tyrosine kinase inhibitor resistant EGFR-mediated lung cancer in a mammal, wherein said resistant EGFR-mediated lung cancer is a result of a secondary mutation at position 790 of EGFR (T790M) to generate a mutant EGFR, and wherein said mAb806 antibody, or an active fragment thereof, is capable of binding to and inhibiting the mutant EGFR.

2. The use of claim 1 wherein mAb806 is a humanized antibody.

3. The use of claim 1 wherein the lung cancer is lung adenocarcinoma.

4. The use of a mAb806 antibody, or an active fragment thereof, for reducing EGFR-mediated lung tumor growth in a cancer patient, wherein said cancer patient has been previously treated with one or more tyrosine kinase inhibitor and has developed recurrent disease and lung tumor growth, wherein said cancer patient has developed a secondary EGFR mutation which is T790M.

5. The use of claim 4 wherein mAb806 is a humanized antibody.

6. The use of claim 4 wherein the lung tumor is lung adenocarcinoma.

7. The use of claim 1, wherein mAb806 is labeled with a detectable or functional label.

8. The use of claim 7 wherein the functional label is a cytotoxic drug.

9. The use of claim 7, wherein the detectable or functional label is a radiolabel.

10. The use of claim 4, wherein mAb806 is labeled with a detectable or functional label.

11. The use of claim 10, wherein said detectable or functional label is a radiolabel.

12. The use of claim 11, wherein said detectable or functional label is a cytotoxic drug.

13. The use of claim 1, wherein mAb806 is a recombinant antibody.

14. The use of claim 1, wherein the lung cancer is lung squamous cell carcinoma.

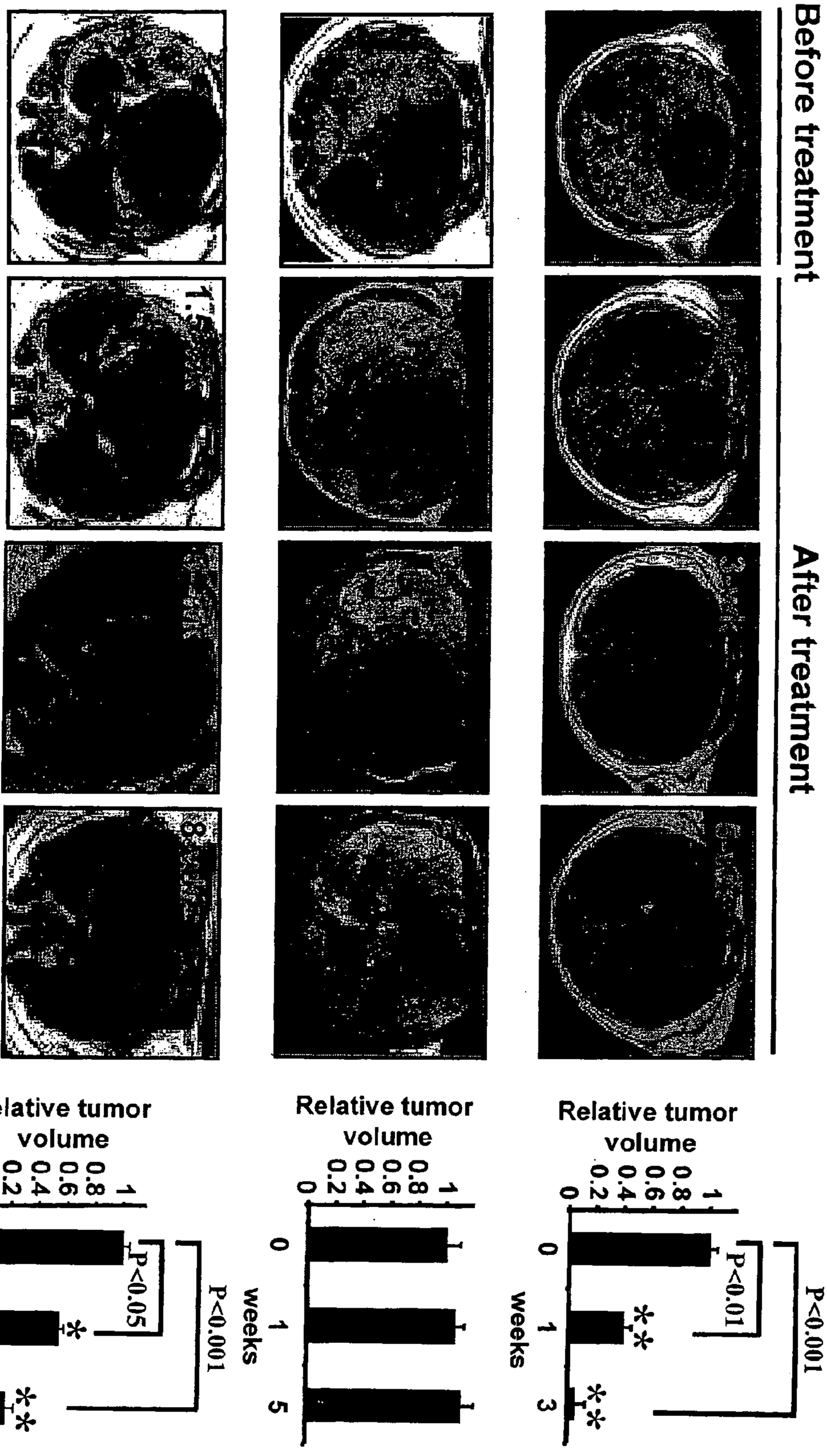
15. The use of claim 1, wherein the lung cancer is non-small cell lung cancer.

16. The use of claim 4, wherein mAb806 is a recombinant antibody.

17. The use of claim 4, wherein the lung tumor is lung squamous cell carcinoma.

18. The use of claim 4, wherein the lung tumor is non-small cell lung cancer.

Figure 1


ch806
cetuximab
mAb806
Before treatment
After treatment
1.5
8
Relative tumor volume
0
0.2
0.4
0.6
0.8
1
0
1.5
8
weeks
P<0.05
P<0.001
*
**
Relative tumor volume
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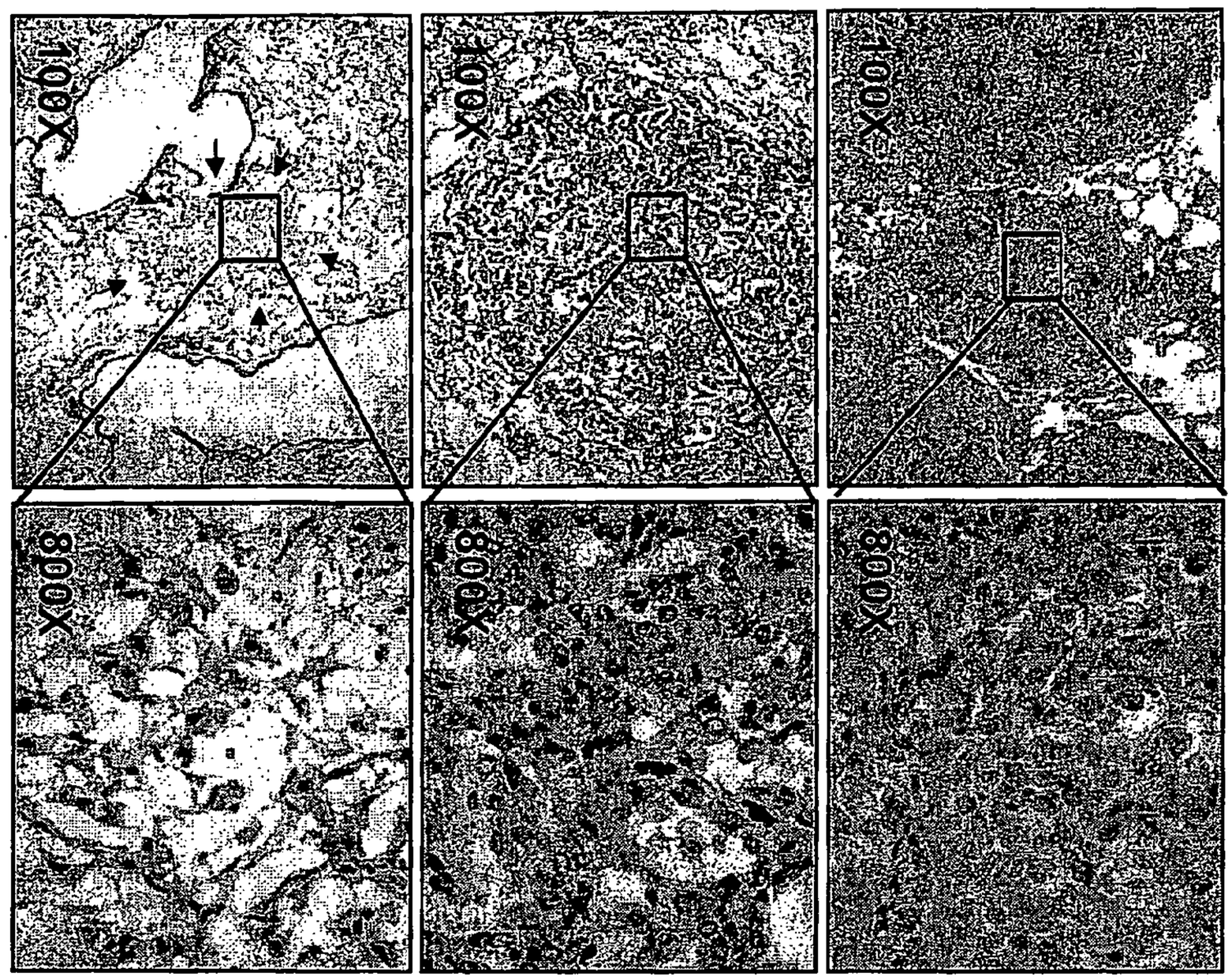
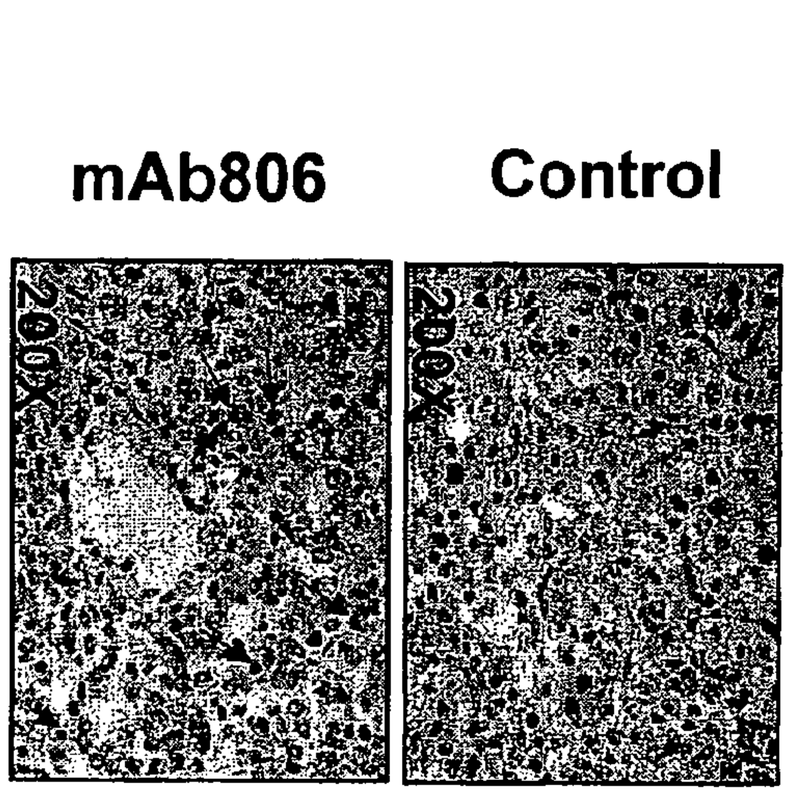
5
weeks
Relative tumor volume
0
1
3
weeks
P<0.01
P<0.001
**

Figure 2

A


5-wk mAb806
1-wk mAb806
Control
100X
100X
100X
X008
X008
X008

B


mAb806
Control
200X
200X
Number of apoptotic nuclei
0 1 2 3 4 5 6 7
control
1-wk Mab806
P<0.001
**

C

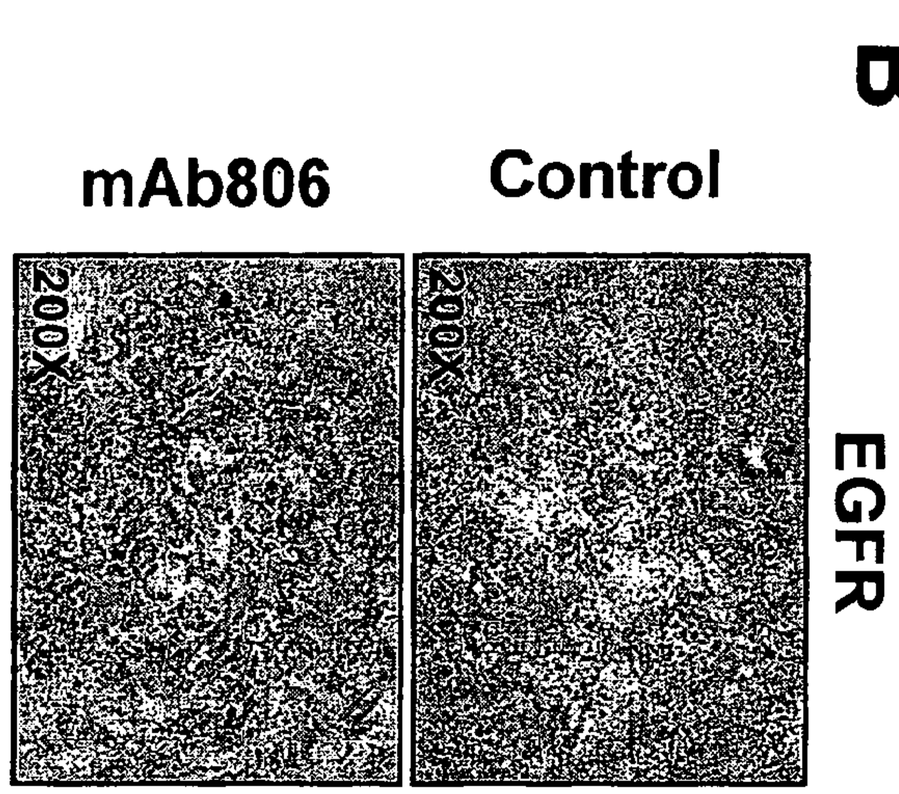

mAb806
Control
EGFR
p-EGFR
200X
200X
200X
200X

Figure 3

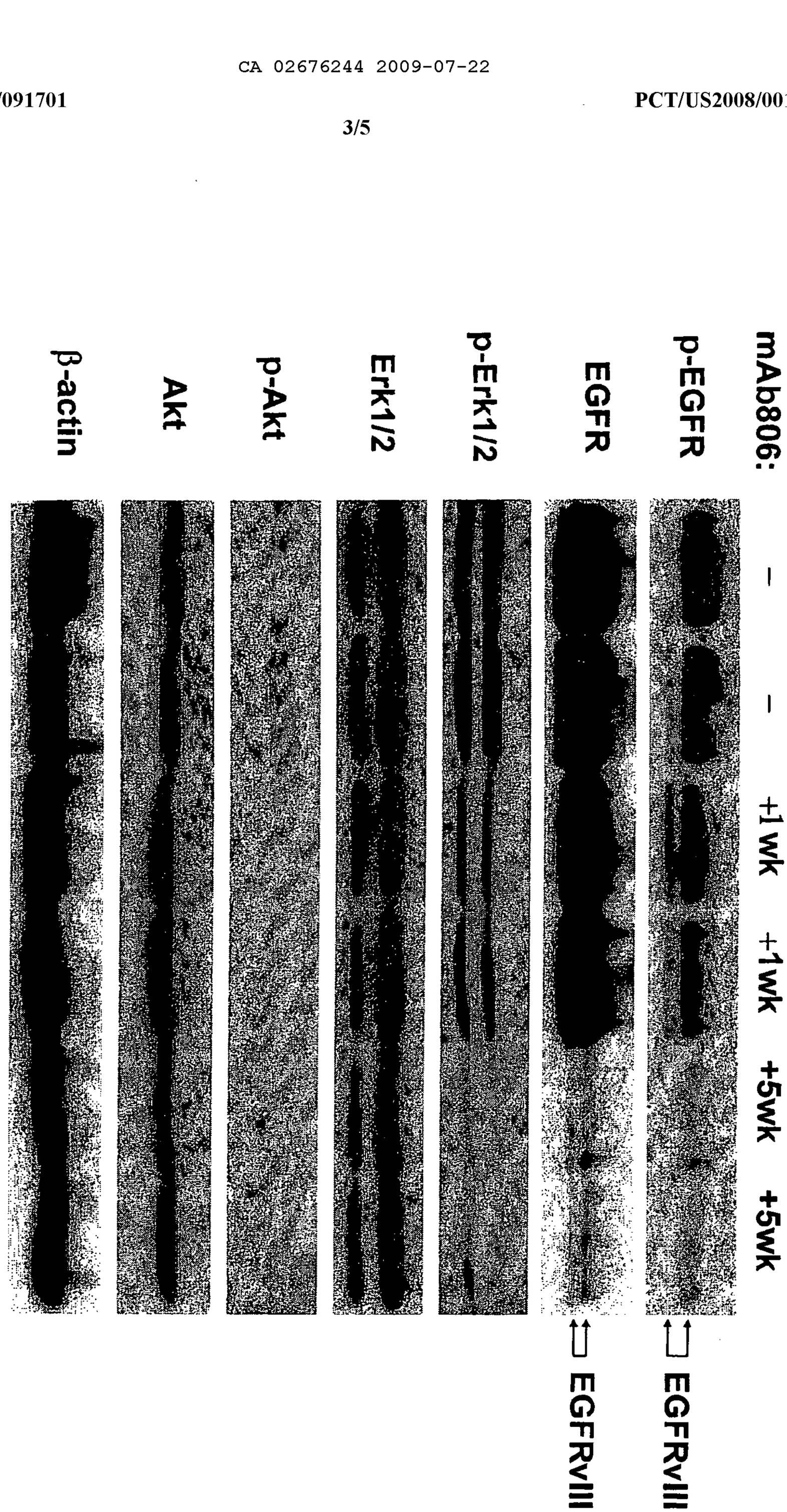

mAb806
p-EGFR
EGFR
p-Erk1/2
Erk1/2
p-Akt
Akt
β-actin
-
-
+1 wk
+1wk
+5wk
+5wk
EGFRvIII
EGFRvIII

Figure 4

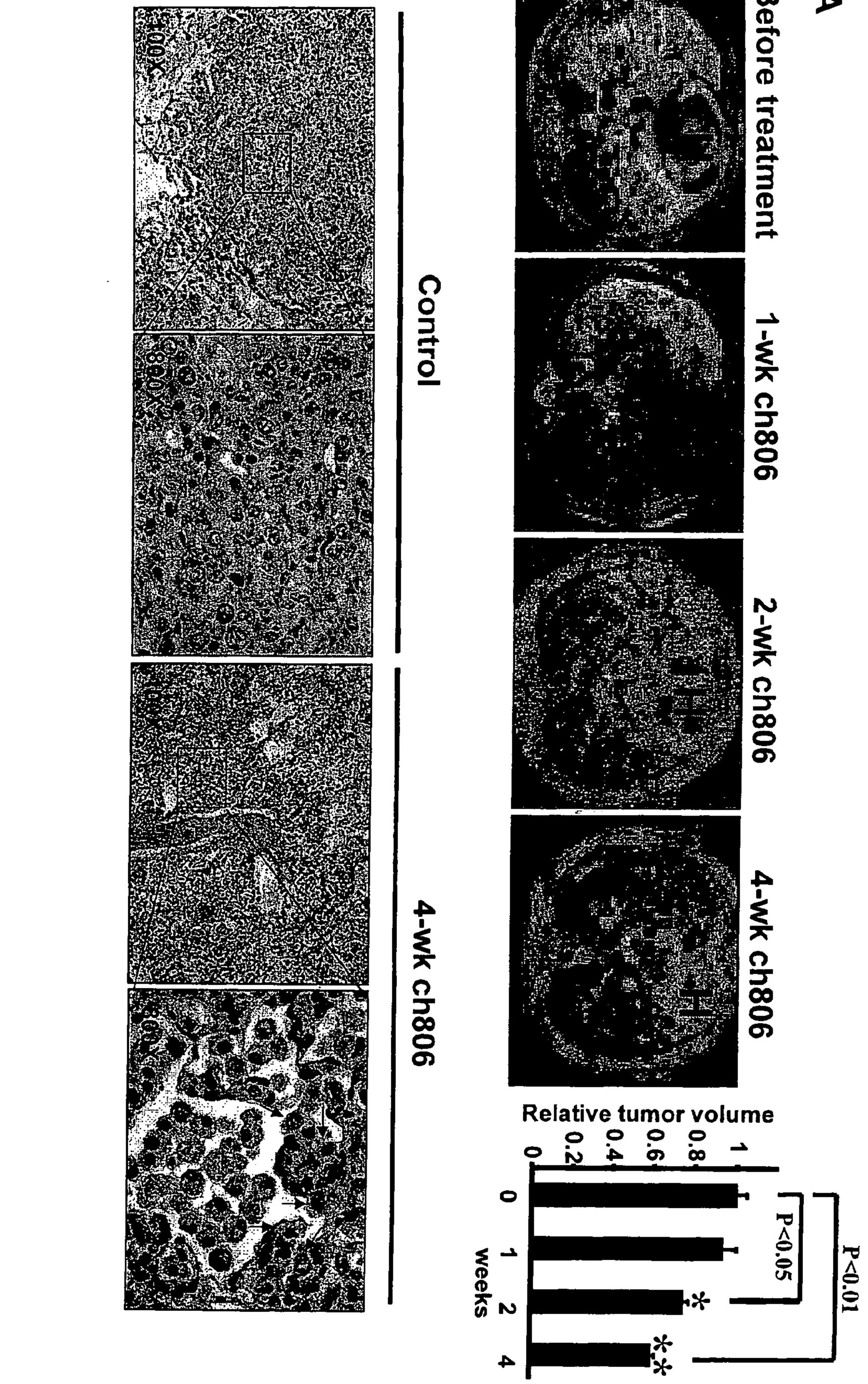
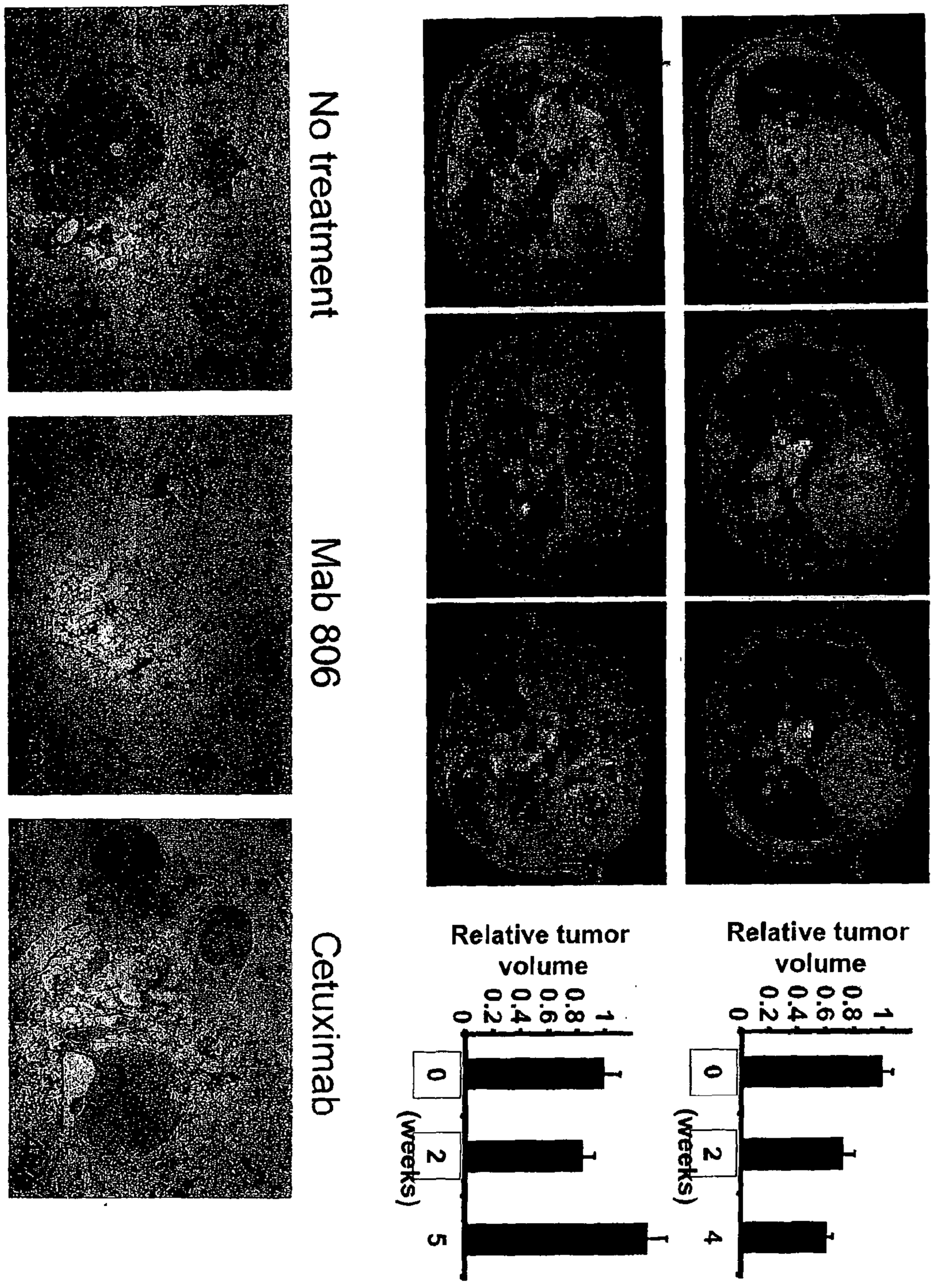

A
Before treatment
1-wk ch806
2-wk ch806
4-wk ch806
B
Control
4-wk ch806
Relative tumor volume
0
0.2
0.4
0.6
0.8
1
0
1
2
4
weeks
P<0.05
P<0.01
*
**

Figure 5


A
Cetuximab
Mab 806
B
No treatment
Mab 806
Cetuximab
Relative tumor volume
0
0.2
0.4
0.6
0.8
1
0
2
(weeks)
5
4