

ANTI-CEACAM6 ANTIBODIES AND METHODS OF USE

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of priority of Singapore application No. 10201608481W, filed 10 October 2016, the contents of it being hereby incorporated by reference in its entirety for all purposes.

FIELD OF THE INVENTION

[0001] The present invention relates generally to antibodies. Specifically, the invention relates to anti-CEACAM6 monoclonal antibodies and uses thereof.

BACKGROUND OF THE INVENTION

[0002] Carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) belongs to the carcinoembryonic antigen (CEA) family. CEACAM6 is a glycosyl phosphatidyl inositol (GPI) anchored cell surface glycoprotein that has been observed to be overexpressed in a variety of cancers including breast, pancreatic, colonic and non-small cell lung carcinoma. CEACAM6 acts as an oncogene in tumors and promotes cancer invasion, metastasis, anoikis resistance and chemoresistance, and inhibits differentiation.

[0003] To date, there are a very limited number of anti-CEACAM6 monoclonal antibodies that could be used for antibody therapy or as antibody-drug conjugates for cancer treatment. There is therefore a need to develop novel antibodies against CEACAM6 that can be used for antibody therapy or as antibody-drug conjugates for cancer treatment.

SUMMARY

[0004] In one aspect, there is provided an antigen-binding protein, or an antigen-binding fragment thereof, comprising (i) a heavy chain variable domain comprising a VHCDR1 having the amino acid sequence GNTFTSYVMH (SEQ ID NO: 3); a VHCDR2 having the amino acid sequence YINPYNDGTTYNEKFKG (SEQ ID NO: 4) and a VHCDR3 having the amino acid sequence STARATPYFYAMDY (SEQ ID NO: 5); and (ii) a light chain variable domain comprising a VLCDR1 having the amino acid sequence KSSQSLLWSVNQNSYLS (SEQ ID NO: 6), a VLCDR2 having the amino acid sequence GASIRES (SEQ ID NO: 7), and a VLCDR3 having the amino acid sequence QHNHGSFLPYT (SEQ ID NO: 8).

[0005] In another aspect, there is provided a composition comprising a physiologically acceptable carrier and a therapeutically effective amount of the antigen-binding protein, or an antigen-binding fragment thereof as disclosed herein.

5 [0006] In another aspect, there is provided a use of an antigen-binding protein, or an antigen-binding fragment thereof as disclosed herein, or composition as disclosed herein in the manufacture of a medicament for treating or preventing cancer.

[0007] In another aspect, there is provided an antigen-binding protein, or an antigen-binding fragment thereof as disclosed herein, or composition as disclosed herein for use in treating or preventing cancer.

10 [0008] In another aspect, there is provided a method of treating or preventing cancer comprising administering an antigen-binding protein, or an antigen-binding fragment thereof as disclosed herein, or composition as disclosed herein to a subject in need thereof.

[0009] In another aspect, there is provided a method for detecting cancer in a subject, the method comprising: contacting a sample obtained from the subject with an antigen-binding
15 protein, or an antigen-binding fragment thereof as disclosed herein *in vitro*; detecting the binding of the antigen-binding protein, or an antigen-binding fragment thereof in the sample; correlating the binding with a level of binding in a control sample to determine the level of binding in the sample, wherein an increase in the level of binding in the sample relative to the control sample is indicative of cancer.

20 [0010] In another aspect, there is provided a method for identifying a subject susceptible to cancer the method comprising: contacting a sample obtained from the subject with an antigen-binding protein, or an antigen-binding fragment thereof as disclosed herein *in vitro*; detecting the binding of the antigen-binding protein, or an antigen-binding fragment thereof in the sample; correlating the binding with a level of binding in a control sample to determine
25 the level of binding in the sample, wherein an increase in the level of binding in the sample relative to the control sample indicates that the subject is susceptible to cancer.

[0011] In one aspect, there is provided a kit when used in the method as disclosed herein, comprising an antigen-binding protein, or antigen-binding fragment thereof as disclosed herein, together with instructions for use.

DEFINITIONS

[0012] The following are some definitions that may be helpful in understanding the description of the present invention. These are intended as general definitions and should in no way limit the scope of the present invention to those terms alone, but are put forth for a better understanding of the following description.

[0013] The term “antigen binding protein” as used herein refers to antibodies, antibody fragments and other protein constructs, such as domains, which are capable of binding to CEACAM6.

[0014] The term “antibody” is used herein in the broadest sense to refer to molecules with an immunoglobulin-like domain and includes monoclonal, recombinant, polyclonal, chimeric, humanised, bispecific and heteroconjugate antibodies; a chimeric antigen receptor (CAR), a single variable domain, a domain antibody, antigen binding fragments, immunologically effective fragments, single chain Fv, diabodies, Tandabs™, etc (for a summary of alternative “antibody” formats see Holliger and Hudson, Nature Biotechnology, 2005, Vol 23, No. 9, 1126-1136).

[0015] The phrase “single variable domain” refers to an antigen binding protein variable domain (for example, V_H , V_{HH} , V_L) that specifically binds an antigen or epitope independently of a different variable region or domain.

[0016] A “domain antibody” or “dAb” may be considered the same as a “single variable domain” which is capable of binding to an antigen. A single variable domain may be a human antibody variable domain, but also includes single antibody variable domains from other species such as rodent (for example, as disclosed in WO 00/29004), nurse shark and Camelid V_{HH} dAbs. Camelid V_{HH} are immunoglobulin single variable domain polypeptides that are derived from species including camel, llama, alpaca, dromedary, and guanaco, which produce heavy chain antibodies naturally devoid of light chains. Such V_{HH} domains may be humanised according to standard techniques available in the art, and such domains are considered to be “domain antibodies”. As used herein V_H includes camelid V_{HH} domains.

[0017] As used herein the term “domain” refers to a folded protein structure which has tertiary structure independent of the rest of the protein. Generally, domains are responsible for discrete functional properties of proteins, and in many cases may be added, removed or transferred to other proteins without loss of function of the remainder of the protein and/or of

the domain. A “single variable domain” is a folded polypeptide domain comprising sequences characteristic of antibody variable domains. It therefore includes complete antibody variable domains and modified variable domains, for example, in which one or more loops have been replaced by sequences which are not characteristic of antibody variable domains, or antibody variable domains which have been truncated or comprise N- or C-terminal extensions, as well as folded fragments of variable domains which retain at least the binding activity and specificity of the full-length domain. A domain can bind an antigen or epitope independently of a different variable region or domain.

[0018] An antigen binding fragment may be provided by means of arrangement of one or more CDRs on non-antibody protein scaffolds such as a domain. The domain may be a domain antibody or may be a domain which is a derivative of a scaffold selected from the group consisting of CTLA-4, lipocalin, SpA, an Affibody, an avimer, GroEl, transferrin, GroES and fibronectin/adnectin, which has been subjected to protein engineering in order to obtain binding to an antigen, such as CEACAM6, other than the natural ligand.

[0019] An antigen binding fragment or an immunologically effective fragment may comprise partial heavy or light chain variable sequences. Fragments are at least 5, 6, 8 or 10 amino acids in length. Alternatively the fragments are at least 15, at least 20, at least 50, at least 75, or at least 100 amino acids in length.

[0020] The term “specifically binds” as used throughout the present specification in relation to antigen binding proteins means that the antigen binding protein binds to CEACAM6 with no or insignificant binding to other (for example, unrelated) proteins. However, the term does not exclude the fact that the antigen binding proteins may also be cross-reactive with closely related molecules. The antigen binding proteins described herein may bind to CEACAM6 with at least 2, 5, 10, 50, 100, or 1000 fold greater affinity than they bind to closely related molecules.

[0021] The term “neutralises” as used throughout the present specification means that the biological activity of CEACAM6 is reduced in the presence of an antigen binding protein as described herein in comparison to the activity of CEACAM6 in the absence of the antigen binding protein, *in vitro* or *in vivo*. Neutralisation may be due to one or more of blocking CEACAM6 binding to its receptor, preventing CEACAM6 from activating its receptor, down regulating CEACAM6 or its receptor, or affecting effector functionality. The reduction or

inhibition in biological activity may be partial or total. A neutralising antigen binding protein may neutralise the activity of CEACAM6 by at least 20%, 30%, 40%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 82%, 84%, 86%, 88%, 90%, 92%, 94%, 95%, 96%, 97%, 98%, 99% or 100% relative to CEACAM6 activity in the absence of the antigen binding protein.

- 5 Neutralisation may be determined or measured using one or more assays known to the skilled person or as described herein. For example, antigen binding protein binding to CEACAM6 can be assessed in a sandwich ELISA, by BIAcoreTM, FMAT, FORTEbio, or similar *in vitro* assays.

- 10 [0022] "CDRs" are defined as the complementarity determining region amino acid sequences of an antigen binding protein. These are the hypervariable regions of immunoglobulin heavy and light chains. There are three heavy chain and three light chain CDRs (or CDR regions) in the variable portion of an immunoglobulin. Thus, "CDRs" as used herein refers to all three heavy chain CDRs, all three light chain CDRs, all heavy and light chain CDRs, or at least two CDRs.

- 15 [0023] As used herein, the term "promoter" is intended to refer to a region of DNA that initiates transcription of a particular gene.

[0024] As used herein, the term "cancerous" relates to being affected by or showing abnormalities characteristic of cancer.

- 20 [0025] As used herein, the term "biological sample" or "sample" is meant a sample of tissue or cells from a patient that has been obtained from, removed or isolated from the patient.

[0026] The term "obtained or derived from" as used herein is meant to be used inclusively. That is, it is intended to encompass any nucleotide sequence directly isolated from a biological sample or any nucleotide sequence derived from the sample.

- 25 [0027] The method as described herein is suitable for use in a sample of fresh tissue, frozen tissue, paraffin- preserved tissue and/or ethanol preserved tissue. The sample may be a biological sample. Non-limiting examples of biological samples include whole blood or a component thereof (e.g. plasma, serum), urine, saliva lymph, bile fluid, sputum, tears, cerebrospinal fluid, bronchioalveolar lavage fluid, synovial fluid, semen, ascitic tumour fluid, 30 breast milk and pus. In one embodiment, the sample of nucleic acid is obtained from blood, amniotic fluid or a buccal smear. In a preferred embodiment, the sample is a whole blood sample.

[0028] A biological sample as contemplated herein includes cultured biological materials, including a sample derived from cultured cells, such as culture medium collected from cultured cells or a cell pellet. Accordingly, a biological sample may refer to a lysate, homogenate or extract prepared from a whole organism or a subset of its tissues, cells or component parts, or a fraction or portion thereof. A biological sample may also be modified prior to use, for example, by purification of one or more components, dilution, and/or centrifugation.

[0029] As used herein, the term “detectable label” or “reporter” refers to a detectable marker or reporter molecules, which can be attached to nucleic acids. Typical labels include fluorophores, radioactive isotopes, ligands, chemiluminescent agents, metal sols and colloids, and enzymes. Methods for labeling and guidance in the choice of labels useful for various purposes are discussed, e.g., in Sambrook et al., in *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press (1989) and Ausubel et al., in *Current Protocols in Molecular Biology*, Greene Publishing Associates and Wiley-Intersciences (1987).

[0030] As used herein, the term “susceptible to cancer” or “susceptibility to cancer” refers to the likelihood of a subject developing cancer. The term does not indicate that a subject will develop cancer with 100% certainty. Rather, the term “susceptible to cancer” refers to an increased probability that a subject will develop cancer when compared to an individual who is not “susceptible to cancer”.

[0031] As used herein, the term “about”, in the context of concentrations of components of the formulations, typically means $\pm 5\%$ of the stated value, more typically $\pm 4\%$ of the stated value, more typically $\pm 3\%$ of the stated value, more typically, $\pm 2\%$ of the stated value, even more typically $\pm 1\%$ of the stated value, and even more typically $\pm 0.5\%$ of the stated value.

[0032] Throughout this disclosure, certain embodiments may be disclosed in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the disclosed ranges. Accordingly, the description of a range should be considered to have specifically disclosed all the possible sub-ranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed sub-ranges such as from 1 to 3, from 1 to 4, from 1

to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 3, 4, 5, and 6. This applies regardless of the breadth of the range.

[0033] Certain embodiments may also be described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also
5 form part of the disclosure. This includes the generic description of the embodiments with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

[0034] Unless the context requires otherwise or specifically stated to the contrary, integers, steps, or elements of the invention recited herein as singular integers, steps or
10 elements clearly encompass both singular and plural forms of the recited integers, steps or elements.

[0035] The word “substantially” does not exclude “completely” e.g. a composition which is “substantially free” from Y may be completely free from Y. Where necessary, the word “substantially” may be omitted from the definition of the invention.

[0036] The invention illustratively described herein may suitably be practiced in the absence of any element or elements, limitation or limitations, not specifically disclosed herein. Thus, for example, the terms "comprising", "including", "containing", etc. shall be read expansively and without limitation. Additionally, the terms and expressions employed herein have been used as terms of description and not of limitation, and there is no intention
20 in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the inventions embodied therein herein disclosed may
25 be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention.

[0037] The invention has been described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form part of the invention. This includes the generic description of the invention with a proviso or
30 negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

[0038] Other embodiments are within the following claims and non-limiting examples. In addition, where features or aspects of the invention are described in terms of Markush groups, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group.

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BRIEF DESCRIPTION OF THE DRAWINGS

[0039] The invention will be better understood with reference to the detailed description when considered in conjunction with the non-limiting examples and the accompanying drawings, in which:

10 [0040] **Fig. 1** is a flow diagram depicting the methodology for generating Gefitinib resistant clones from PC-9 and cell lines used for immunization and hybridoma fusion of generation of monoclonal antibody panel.

[0041] **Fig. 2** shows flow cytometry binding of GR6A04 on A) immunizing GR lines, CL75, CL86 and CL131, and B) parental PC-9 and another GR PC-9 line. Gating was
15 performed at M=2% of negative control for each cell line. Absence of propidium iodide staining (FL-3) shows no inherent cytotoxicity from mAb GR 6A04 binding.

[0042] **Fig. 3** shows flow cytometry screening with A) PC-9 and derived gefitinib-resistant clones, B) NSCLC lines, C) HCC827 and derived gefitinib-resistant clones, D) breast cancer lines, E) colorectal cancer lines, and F) normal cell lines.

20 [0043] **Fig. 4** shows the characterization of GR 6A04 monoclonal antibody and its derivatives. A) GR 6A04 is of mouse IgG1, κ isotype. B) Variable heavy and light chain translated sequences with the CDR underlined. C) Direct conjugation of GR6A04 and an IgG1 isotype control (MG1.45) to Monomethyl auristatin E (MMAE) to obtain GR6A04-MMAE and MG1.45 respectively (DAR:Drug-antibody ratio). Flow cytometry binding is not
25 affected after conjugation. D) Human chimeric monoclonal antibody for GR 6A04 (Hu-GR6A04). V_H and V_L sequences were cloned into expression vector with human constant region backbone. Hu-GR6A04 cloned into CHO cells for expression. Coomassie staining showed expected protein size and flow cytometry binding was comparable to GR6A04.

[0044] **Fig. 5** shows the characterization of GR 6A04 antigen. A) Western blot performed
30 showed the antigen as a smear from 55-90kDa. Antigen smearing reduced after treatment with β -mercaptoethanol (reducing agent). B) GR 6A04 binding is PNGase sensitive. GR 6A04's binding to the antigen is abolished with PNGase treatment (lane 3), accompanied by a

drop in molecular weight indicating successful removal of N-glycans from the antigen. Anti-actin binding is not affected by the PNGase treatment. In other words, GR 6A04 binding is dependent on N-glycosylation of antigen.

[0045] **Fig. 6** shows A) immunoprecipitation with GR 6A04. Boxed area was excised for mass spectrometry. B) Mass spectrometry analysis of excised region identified the top 5 putative antigens after removal of non-specific hits found in the same region in the Column Control lane. The results showed that putative antigens are arranged based on the overall score, which is a function of the total protein coverage, and number of unique peptides found. The antigen identity was then validated by a pull-down with commercial antibodies against the target, and a cross-probe of the immunoprecipitated products with GR 6A04, and vice-versa. GRP78 was tested by cross-IP and ruled out as possible antigen for GR 6A04.

[0046] **Fig. 7** shows the validation of CEACAM6 as the putative antigen for GR 6A04. A) Cross-immunoprecipitation was performed with commercial anti-CEACAM6. Both the commercial anti-CEACAM6, and GR 6A04 recognised the antigen pulled-down by its counterpart. B) siRNA knock down of CEACAM6 was performed. The results showed that knockdown of the CEACAM6 expression in PC-9 cells led to a decrease in GR 6A04 binding observed in Western blot and flow cytometry.

[0047] **Fig. 8** shows immunohistology of GR 6A04 in cancer cell lines. A) FFPE on PC-9 cell pellets show both membrane bound and cytosolic localisation of GR 6A04. B) FFPE cell line array screening scored by ImmunoMembrane across duplicate cores.

[0048] **Fig. 9** shows immunohistology of GR 6A04 on tumour tissue. FFPE on tissue samples (Pantomics TMAs) using a mix of normal and tumour tissues, multiple organs (MNT241) and scored by ImmunoMembrane.

[0049] **Fig. 10** shows immunohistology of GR 6A04 on tumour tissue. FFPE on tumour tissues, multiple organs (MTU481).

[0050] **Fig. 11** shows immunohistology of GR 6A04 on tumour tissue. A) FFPE on tumour tissue, multiple organs (MTU951) and a summary of the cores stained. B) 2+ staining on two normal cores; esophageal and lung (false positive score). C) Representative positive scoring on cancers in multiple organs.

[0051] **Fig. 12** shows immunohistology of GR 6A04 on NSCLC tissue samples. Staining on lung tumour TMA with adjacent normal (LC 1001 2a) shows positive staining for 4/27 squamous and 9/18 adenocarcinoma, negative staining for paired adjacent normal tissue.

[0052] **Fig. 13** shows core images from the array LC1001 2a.

[0053] **Fig. 14** shows negative staining for 93/96 of normal tissue tested (MNO961). Non-specific staining was generally found on ductal linings (edge of cores) and/or necrotic tissue.

[0054] **Fig. 15** shows the comparison of GR 6A04 with a commercially available anti-CEACAM6 antibody. A) GR6A04 affected by Tunicamycin (N-glycosylation inhibitor) and PNGase digestion, but not commercial anti-CEACAM6 (Clone 9A6). B) Differences in role of glycosylation in mAb binding also leads to differences in specificity in immunohistochemistry. Cell line array with 1 additional core scored positive (boxed).

[0055] **Fig. 16** shows differences in binding profiles were also observed for MNO961 (multi-normal TMA) where commercial anti-CEACAM6 has more non-specific binding, with higher staining intensity. In particular, all 3 normal lung and 2 normal spleen cores were scored positive.

[0056] **Fig. 17** shows sorting of A549 cells. A) A549 lung adenocarcinoma cell line showed heterogeneous binding of GR6A04 and a positive binding tail of 15 – 30%. B) Sorting A549 cells with GR6A04 using Dynabeads Pan Ms-IgG beads followed by DNase bead release showed enrichment of GR6A04+ percentages at P0, but loss of binding was observed with subsequent passages (Round 1 of sorting from A549 parental cells (Positive fraction)). C) Multiple rounds of sorting established stable differential lines. D) A549-GR6A04+ single-cell (SC) clones that were generated showed improved homogeneity of GR6A04 binding. E) Differences in morphology between A549-GR6A04+ single-cell (SC) clones.

[0057] **Fig. 18** shows characterization of sorted populations. EGFR was down-regulated in A549-GR6A04+ cells and CEACAM1 was up-regulated in A549-GR6A04+ cells.

[0058] **Fig. 19** shows the *in vivo* functionality of GR6A04, using xenograft models of A549 lung adenocarcinoma; A549-GR6A04+ and A549 parental, A) A549-GR6A04+ cells have increased tumorigenicity. B) A549 parental, and Retention of GR6A04 binding at end-point where A549 parental was selected for GR6A04+ cells in xenograft (20% to >70%).

[0059] **Fig. 20** shows that the antigen of GR 6A04 is leached into conditioned media. Media was conditioned for 5 days with cultured cells, known volumes were blotted onto membranes in duplicates and probed with Hu-GR6A04. The results showed that higher antigen levels could be found in the conditioned media from GR 6A04(+) cells than from A549 parental cells and GR6A04 (-) cells.

[0060] **Fig. 21** shows characterization of the antibody drug conjugate of GR 6A04. A) The Mab-ZAP Antibody Internalization Kit as was used as proof of concept. GR 6A04 was indirectly conjugated with saporin using an anti-mouse secondary antibody linked with the toxin. B) GR 6A04 was internalized in PC-9 cells. Pre-incubation of GR 6A04, 10µg/mL at 4°C for 20 min was performed. Cells were fixed at time points and probed with anti-Ms AlexaFluor 488. Images were taken at 40x. The results showed that GR 6A04 localised as a ring (solid arrows) on the cell periphery/membrane at T = 0 min and as a ring (solid arrows) and also intracellularly (dashed arrows) at T = 120 min.

[0061] **Fig. 22** shows *in vitro* functionality of GR6A04-MMAE (direct conjugation of toxin), with dose-response curves estimating EC50 @ 19.58nM against PC-9; EC50 @ 3.33nM against A549-GR6A04+ cells.

[0062] **Fig. 23** shows antibody dependent cell cytotoxicity of GR 6A04. Promea ADCC kit was used with the Luciferase reporter for FcγR and Cetuximab as a positive ADCC control. EC50 for PC-9: 39.6nM; EC50 for A549-GR6A04+: 1.33nM.

[0063] **Fig. 24** shows GR 6A04 proof of concept as ADC in a xenograft model. Conditions used were GR6A04-MMAE; MG1.45-MMAE isotype Ctrl; Buffer Ctrl; GR6A04 (unconjugated); Hu-GR6A04 (unconjugated). Treatment regime was 10mg/kg (~200ug/mouse) and 3 doses, on D0, D4 and D8 (starting at tumor size >150mm³). The results indicated that xenografts treated with GR6A04-MMAE showed little tumour growth which demonstrated excellent *in vivo* functionality as an ADC. Xenografts treated with unconjugated GR6A04 and Hu-GR6A04 showed slower growth compared to the buffer control, which indicated some *in vivo* tumour growth inhibition via ADCC mechanisms.

DETAILED DESCRIPTION OF THE PRESENT INVENTION

[0064] In a first aspect, there is provided an antigen-binding protein, or an antigen-binding fragment thereof, comprising (i) a heavy chain variable domain comprising a VHCDR1 having the amino acid sequence GNTFTSYVMH (SEQ ID NO: 3); a VHCDR2 having the amino acid sequence YINPYNDGTTYNEKFKG (SEQ ID NO: 4) and a VHCDR3 having the amino acid sequence STARATPYFYAMDY (SEQ ID NO: 5); and (ii) a light chain variable domain comprising a VLCDR1 having the amino acid sequence KSSQSLLWSVNQNSYLS (SEQ ID NO: 6), a VLCDR2 having the amino acid sequence GASIRES (SEQ ID NO: 7), and a VLCDR3 having the amino acid sequence QHNHGSFLPYT (SEQ ID NO: 8).

[0065] The antigen-binding protein, or antigen-binding fragment thereof, may comprise heavy and light chain CDR regions that are about 80%, about 85%, about 90%, about 95%, about 96%, about 97%, about 98% or about 99% identical to the heavy and light chain CDR regions of (i) and (ii).

- 5 [0066] In one embodiment, the heavy chain variable region comprises the amino acid sequence

SGPELVKPGASVKMSCKASGNTFTSYVMHWVKQKPGQGLEWIGYINPYNDGTKYN
EKFKGKATLTSDKSSSTAYMELSSLTSEDSAVYYCARSTARATPYFYAMDYWGQGT
SVTVSS as set forth in SEQ ID NO:1. Alternatively, the heavy chain variable region may
10 comprise an amino acid sequence having about 80%, about 85%, about 90%, about 95%,
about 96%, about 97%, about 98% or about 99% identity to the amino acid sequence set
forth in SEQ ID NO:1.

[0067] In one embodiment, the light chain variable region comprises the amino acid sequence

- 15 DILMTQSPSSLAVTAGEKVTMRCKSSQSLLWSVNQNSYLSWYQLKQGQPPKLLLYG
ASIRESWVPDRFTGSGSGTDFTLTISNVHVEDLAVYYCQHNHGSFLPYTFGGGKLEI
K as set forth in SEQ ID NO:2. Alternatively, the antigen-binding protein, or antigen-binding
fragment thereof, may comprise a light chain variable region which comprises an amino acid
sequence having about 80%, about 85%, about 90%, about 95%, about 96%, about 97%,
20 about 98% or about 99% identity to the amino acid sequence set forth in SEQ ID NO:2.

- [0068] In one embodiment, the antigen-binding protein, or antigen-binding fragment
thereof, as claimed in any one of claims 1 to 6, wherein the antigen binding protein is
selected from the group consisting of monoclonal, recombinant, polyclonal, chimeric,
humanised, bispecific and heteroconjugate antibodies; a chimeric antigen receptor (CAR), a
25 single variable domain, a domain antibody, antigen binding fragments, immunologically
effective fragments, single chain Fv, a single chain antibody, a univalent antibody lacking a
hinge region, a minibody, diabodies, and Tandabs™

- [0002] In one embodiment, the antigen-binding protein, or antigen-binding fragment
thereof may be a monoclonal antibody. The monoclonal antibody may be GR 6A04. In one
30 embodiment, the monoclonal antibody may be humanised. Alternatively, the monoclonal
antibody may be chimeric.

[0069] In one embodiment, the antigen-binding protein, or antigen-binding fragment thereof may bind to CEACAM6. In one embodiment, the antigen-binding protein, or antigen-binding fragment thereof may bind to a glycan on CEACAM6. The antigen-binding protein, or antigen-binding fragment thereof as described herein may bind to an N-linked glycan on
5 CEACAM6.

[0070] In another embodiment, the antigen-binding protein, or antigen-binding fragment thereof as described herein may comprise a radioisotope or a cytotoxin conjugated thereto. The antigen-binding protein, or antigen-binding fragment thereof may be conjugated with a cytotoxin selected from the group consisting of monomethyl auristatin E (MMAE),
10 mertansine (DM-1), saporin, gemcitabine, irinotecan, etoposide, vinblastine, pemetrexed, docetaxel, paclitaxel, platinum agents (for example, cisplatin, oxaliplatin and carboplatin), vinorelbine, capecitabine, mitoxantrone, ixabepilone, eribulin, 5-fluorouracil, trifluridine and tipiracil.

[0071] In one embodiment, the antigen-binding protein, or an antigen-binding fragment as described herein may be internalized into a cell upon binding to CEACAM6.
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[0072] In another embodiment, the antigen-binding protein, or an antigen-binding fragment as described herein may not be internalized into a cell upon binding to CEACAM6.

[0073] In one embodiment, the antigen-binding protein, or an antigen-binding fragment as described herein may selectively bind to a gefitinib resistant lung cancer cell, a non-small cell
20 lung cancer cell, a breast cancer cell and/or a colorectal cancer cell.

[0074] In another aspect, there is provided a composition comprising a physiologically acceptable carrier and a therapeutically effective amount of the antigen-binding protein, or an antigen-binding fragment thereof as described herein. In one embodiment, the composition as disclosed herein may comprise one or more further therapeutic compounds.

[0075] The percentage of the the antigen-binding protein, or an antigen-binding fragment thereof, as described herein, in pharmaceutical compositions and preparations may, of course, be varied and, for example, may conveniently range from about 2% to about 90%, about 5% to about 80%, about 10% to about 75%, about 15% to about 65%; about 20% to about 60%, about 25% to about 50%, about 30% to about 45%, or about 35% to about 45%, of the weight
25 of the dosage unit. The amount of compound in therapeutically useful compositions is such that a suitable dosage will be obtained.
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[0076] The language "physiologically acceptable carrier" is intended to include solvents, dispersion media, coatings, anti-bacterial and anti-fungal agents, isotonic and absorption delaying agents, and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the compound, use thereof in the therapeutic compositions and methods of treatment and prophylaxis is contemplated. Supplementary active compounds may also be incorporated into the compositions according to the present invention. It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage.

5 [0077] "Dosage unit form" as used herein refers to physically discrete units suited as unitary dosages for the individual to be treated; each unit containing a predetermined quantity of compound(s) is calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The compound(s) may be formulated for convenient and effective administration in effective amounts with a suitable pharmaceutically acceptable carrier in an acceptable dosage unit. In the case of compositions containing supplementary active ingredients, the dosages are determined by reference to the usual dose and manner of administration of the said ingredients.

[0078] The composition may be conveniently administered by injection, for example, subcutaneous, intravenous, and the like. The composition may also be administered parenterally or intraperitoneally. In one embodiment, the compound may be administered by injection. In the case of injectable solutions, the carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by including various anti-bacterial and/or anti-fungal agents. Suitable agents are well known to those skilled in the art and include, for example, parabens, chlorobutanol, phenol, benzyl alcohol, ascorbic acid, thimerosal, and the like. In many cases, it may be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, and sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about

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by including in the composition an agent which delays absorption, for example, aluminium monostearate and gelatin.

[0079] Sterile injectable solutions can be prepared by incorporating the analogue in the required amount in an appropriate solvent with one or a combination of ingredients
5 enumerated above, as required, followed by filtered sterilisation. Generally, dispersions are prepared by incorporating the analogue into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above.

[0080] Under ordinary conditions of storage and use, pharmaceutical preparations may contain a preservative to prevent the growth of microorganisms. Preferably, the
10 pharmaceutical composition may further include a suitable buffer to minimise acid hydrolysis. Suitable buffer agent agents are well known to those skilled in the art and include, but are not limited to, phosphates, citrates, carbonates and mixtures thereof.

[0081] Single or multiple administrations of the pharmaceutical compositions according to the invention may be carried out. One skilled in the art would be able, by routine
15 experimentation, to determine effective, non-toxic dosage levels of the compound and/or composition of the invention and an administration pattern which would be suitable for treating the diseases and/or infections to which the compounds and compositions are applicable.

[0082] Further, it will be apparent to one of ordinary skill in the art that the optimal course
20 of treatment, such as the number of doses of the compound or composition of the invention given per day for a defined number of days, can be ascertained using convention course of treatment determination tests.

[0083] Generally, an effective dosage per 24 hours may be in the range of about 0.0001 mg to about 1000 mg per kg body weight; suitably, about 0.001 mg to about 750 mg per kg
25 body weight; about 0.01 mg to about 500 mg per kg body weight; about 0.1 mg to about 500 mg per kg body weight; about 0.1 mg to about 250 mg per kg body weight; or about 1.0 mg to about 250 mg per kg body weight. More suitably, an effective dosage per 24 hours may be in the range of about 1.0 mg to about 200 mg per kg body weight; about 1.0 mg to about 100 mg per kg body weight; about 1.0 mg to about 50 mg per kg body weight; about 1.0 mg to
30 about 25 mg per kg body weight; about 5.0 mg to about 50 mg per kg body weight; about 5.0 mg to about 20 mg per kg body weight; or about 5.0 mg to about 15 mg per kg body weight.

[0084] Alternatively, an effective dosage may be up to about 500mg/m². For example, generally, an effective dosage is expected to be in the range of about 25 to about 500mg/m², about 25 to about 350mg/m², about 25 to about 300mg/m², about 25 to about 250mg/m², about 50 to about 250mg/m², and about 75 to about 150mg/m².

5 [0085] In another aspect, there is provided use of an antigen-binding protein, or an antigen-binding fragment thereof as disclosed herein, or the composition as disclosed herein in the manufacture of a medicament for treating or preventing cancer.

[0086] In one embodiment, the cancer may be selected from the group consisting of gefitinib resistant lung cancer, non-small cell lung cancer, breast cancer, stomach cancer,
10 small intestine cancer, esophageal cancer and colorectal cancer.

[0087] In some embodiments, the medicament may be administered with one or more further active pharmaceutical ingredients. Alternatively, the medicament may be administered with chemotherapy. The further active pharmaceutical ingredients or chemotherapy may be administered separately, simultaneously or sequentially.

15 [0088] In another aspect, there is provided a method for detecting cancer in a subject, the method comprising: contacting a sample obtained from the subject with an antigen-binding protein, or an antigen-binding fragment thereof as disclosed herein *in vitro*; detecting the binding of the antigen-binding protein, or an antigen-binding fragment thereof in the sample; correlating the binding with a level of binding in a control sample to determine the level of
20 binding in the sample, wherein an increase in the level of binding in the sample relative to the control sample is indicative of cancer.

[0089] In another aspect, there is provided a method for identifying a subject susceptible to cancer the method comprising: contacting a sample obtained from the subject with an antigen-binding protein, or an antigen-binding fragment thereof as disclosed herein *in vitro*;
25 detecting the binding of the antigen-binding protein, or an antigen-binding fragment thereof in the sample; correlating the binding with a level of binding in a control sample to determine the level of binding in the sample, wherein an increase in the level of binding in the sample relative to the control sample indicates that the subject is susceptible to cancer.

[0090] In one embodiment, the control sample is from the same subject. Alternatively, the
30 control sample may be from a different subject.

[0091] In one embodiment, the antigen-binding protein, or antigen-binding fragment thereof as described herein may comprise a detectable label. The detectable label may be

selected from the group consisting of a fluorescent label, a chemiluminescent label, an enzymatic label and a radionuclide label.

[0092] In one embodiment, the detectable label may be selected from the group consisting of biotin, alkaline phosphatase, horseradish peroxidase, FITC, PE and Cy Dyes. The
5 detectable label may be detected in an assay selected from flow cytometry, tissue section, immunofluorescence, immunocytochemistry or immunohistochemistry.

[0093] In one aspect, there is provided a kit when used in the method as described herein, comprising an antigen-binding protein, or antigen-binding fragment thereof as described herein, together with instructions for use.

10 [0094] The invention illustratively described herein may suitably be practiced in the absence of any element or elements, limitation or limitations, not specifically disclosed herein. Thus, for example, the terms "comprising", "including", "containing", etc. shall be read expansively and without limitation. Additionally, the terms and expressions employed herein have been used as terms of description and not of limitation, and there is no intention
15 in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the inventions embodied therein herein disclosed may
20 be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention.

[0095] The invention has been described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form part of the invention. This includes the generic description of the invention with a proviso or
25 negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

[0096] Other embodiments are within the following claims and non-limiting examples. In addition, where features or aspects of the invention are described in terms of Markush groups, those skilled in the art will recognize that the invention is also thereby described in terms of
30 any individual member or subgroup of members of the Markush group.

EXAMPLES

[0097] Non-limiting examples of the invention and comparative examples will be further described in greater detail by reference to specific Examples, which should not be construed as in any way limiting the scope of the invention.

5 [0098] **Materials and Methods**

[0099] *Culture and generation of Gefitinib resistant cell lines*

[00100] PC-9 were cultured in RPMI (Invitrogen, USA) supplemented with 10% foetal bovine serum (HyClone GE Healthscience, South America). To obtain PC-9 clones with acquired gefitinib resistance, PC-9 cultures were exposed to increasing concentration of
10 gefitinib (Selleckchem, USA), starting from 2nM and gradually increased with each subsequent passage to a final concentration of 6.4µM. GR clones, CL75, CL86 and CL131, were maintained in 6.4µM gefitinib thereafter.

[00101] A549 were cultured in DMEM (Invitrogen, USA) supplemented with 10% FBS and 20mM L-glutamine (Invitrogen, USA). A549 has primary resistance to gefitinib, with
15 IC50 of >10µM.

[00102] *Generation of GR mAb panel*

[00103] Immunisation of PC-9 GR lines, CL75, CL86 and CL131, was done with 5E6 cells resuspended 1:1 with Freund's complete adjuvant. Immunisation was done once per week for the first immunisation in week 1-3 with only a single line each, and a mixed suspension of all
20 three lines for the subsequent immunisations in week 4-5. After 5 weeks, mice were sacrificed, and the B-cells collected for fusion with Sp2/0 mouse myeloma lines using STEMCELL Technologies ClonaCell™-HY kit as per manufacturer's instructions. Single hybridoma clones were picked into 96-wells, and the culture supernatant collected for screening by flow cytometry.

25 [00104] *Flow cytometry*

[00105] Cells were harvested as single cell suspensions using trypsin. 1E5 cells were used per sample, and incubated with 100µl of mAb culture supernatant for 30 min. Cells were then washed with 1% bovine serum albumin in PBS, and further incubated with 100µl of goat anti-mouse antibody fluorescein isothiocyanate (FITC)-conjugated (1:500, DAKO, Denmark)
30 for 15 min at 4°C in the dark. Cells were again washed and resuspended in 200µL of 1% BSA/PBS for analysis on Guava® easyCyte 8HT Benchtop Flow Cytometer (Merck Millipore, USA). For interrogation of intercellular binding, cells were fixed with 4%

PFA/PBS (Affymetrix, USA) at room temperature for 10 min, washed in PBS, and permeabilised with 0.1% triton/PBS for 5 min at room temperature, before proceeding with incubation with mAb supernatant/primary antibody. For staining with propidium iodide, PI was added to a final concentration of 5ug/mL for 5 min just prior to analysis by the flow
5 cytometer.

[00106] *Western blot and immunoprecipitation*

[00107] Membrane proteins were extracted from PC-9 cell pellets using the Membrane Protein Extraction Kit (BioVision, USA). Briefly, cell pellets of 5E7 cells were resuspended in 1mL of Homogenize Buffer and cell membranes broken in a dounce homogenizer. This
10 was transferred into an Eppendorf tube and centrifuged at 700 x g for 10 min at 4°C to remove cell debris. The supernatant was transferred to a new Eppendorf tube and centrifuged at 12,000 x g for 30 min at 4°C to pellet the membrane. The membrane was finally resuspended in 500µl of 1x Cell Lysis Buffer (Cell Signaling Technology, USA) containing protease inhibitors (Pierce ThermoScientific, USA). The membrane protein solution was
15 clarified with by centrifugation at 15,000 x g for 5 min at 4°C, to remove any insoluble proteins. Protein was quantified using the Pierce 660nM Protein Assay Reagent.

[00108] Immunoprecipitation (IP) was conducted using the automated Phynexus MEA system (Phynexus Inc., USA). GR6A04 was captured onto Protein G PhyTip columns containing 5ul of resin bed. The column was then washed with PBS to remove unbound
20 proteins, and PC-9 membrane protein extract was introduced to bind to GR6A04 that has been captured on the column. The column was then washed with Wash Buffer II (140mM NaCl, pH7.4) before elution at low pH with Elution Buffer (200 mM NaH₂PO₄/140 mM NaCl pH 2.5) and neutralized immediately with 1 M Tris-Cl pH 9.0. The IP product is then subjected to analysis by Western blot.

[00109] PC-9 membrane protein extracts, or IP products were denatured by in protein loading dye containing SDS at a final concentration of 1%, and heated at 95°C for 5 min. The sample was then loaded into pre-cast gradient gel (NuPAGE 4-12% gradient gel, Invitrogen), and separated by SDS-PAGE running MOPS Running Buffer (NuPAGE Invitrogen, USA). After gel electrophoresis, the resolved proteins were transferred onto a polyvinylidene
30 fluoride (PVDF) membrane (BioRad, USA) in a transfer buffer containing 20% methanol, 10% Tris-Glycine in DI water at constant voltage of 110V for 90 min.

[00110] The membrane was then blocked with 5% milk prepared in PBS/0.1% Tween-20 (PBS-T) for 30 min at room temperature. The membrane was then washed in PBS-T, followed by overnight incubation of GR6A04 at 2 μ g/mL in 2.5% milk at 4°C. Subsequently, the membrane was washed in PBS-T, before incubation with goat anti-mouse secondary antibodies horseradish peroxidase-conjugated (1:10000, Dako) for 1 hour at room temperature. After a final wash with PBS-T, the binding of HRP-conjugated secondary antibodies were visualized by ECL detection (GE Healthcare, Sweden).

[00111] *Coomassie blue staining and mass-spectrometry*

[00112] A parallel gel was run for the IP products, which was stained with Coomassie blue staining solution containing 0.1% Coomassie Blue R250 in 10% acetic acid, 50% methanol and 40% water for 1 hour at room temperature. The staining solution is then removed, and replaced by the de-staining solution containing 10% acetic acid, 50% methanol and 40% water. The de-staining solution is replaced with fresh solution, until the background of the gel is almost clear. The de-stained gel is then re-hydrated in water. The gel is then compared with the Western blot of the IP products to determine the position of the antigen band on the gel. This region is then excised for LC-MS analysis.

[00113] *Glycosylation studies*

[00114] PNGase digestion was carried out according to manufacturer's protocol (New England Biolabs). Briefly, 20 μ g of PC-9 membrane protein extract was first denatured in 1x glycoprotein Denaturing Buffer at 95°C for 10 minutes. Subsequently, 1x G7 Reaction Buffer and 10% NP-40 were added and incubated with PNGase F at 37°C for 1 hour. Digested proteins were subsequently analysed by Western blotting as described above.

[00115] Inhibition of N-glycosylation of proteins during cell culture was also achieved by addition of 1 μ M Tunicamycin (Sigma Aldrich, USA) in the culture media. PC-9 cells were seeded at 1E5 cells in a 6-well tissue culture place, and grown in culture media spiked with Tunicamycin, or DMSO for 3 days until confluent. The cells were then harvested for analysis by flow cytometry and Western blotting.

[00116] *Immunohistochemistry staining*

[00117] TMA slides containing FFPE tissues were first heated in an oven at 60°C for 30 min to remove any solvents. The slides were then dewaxed and re-hydrated through sequential immersion in HistoClear (2x), 100% ethanol (2x), 95% ethanol, 70% ethanol, and finally in DI water.

[00118] Heat-induced epitope retrieval was done in a solution containing 10mM Tris Base, 1mM EDTA, 0.05% Tween 20 at pH 9.0, and heated at 95°C for 20 min. The container with the antigen retrieval solution and slides was then removed and allowed to cool to room temperature for an additional 20 min. The slides were then washed in DI water. Endogenous
5 peroxidase activity was then blocked by incubation of the slides with 3% H₂O₂ in PBS for 30 min at room temperature. The slides were washed in DI water, followed by a blocking step with 10% normal goat serum in PBS for 30 min.

[00119] The slides were then incubated with GR6A04 at 5ug/mL in blocking solution overnight at 4°C. The slides were then washed and incubated with a polymer-based anti-mouse
10 secondary antibody conjugated with HRP (DAKO, USA) for 30 min at room temperature, and developed with the recommended DAB chromogen substrate solution for 2 min, and counterstained with Gill's Hematoxylin solution.

[00120] The stained slides were subsequently dehydrated through immersion in 50% ethanol, 70% ethanol, 90% ethanol, 100% ethanol (2x) and Histoclear (2x), before mounting
15 with a glass cover slip. The slides were then imaged with the Zeiss AxioScan Digital Slide Scanner.

[00121] *Enrichment of GR6A04 binding population from A549 cell line*

[00122] The CELLection™ Pan Mouse IgG Kit (ThermoScientific, USA) was used for the enrichment of GR6A04- and GR6A04+ sub-populations from the A549 parental lung
20 adenocarcinoma line, according to manufacturer's instructions. Briefly, A549 cells were harvested with trypsin to obtain a single-cell solution. Cells were incubated with GR6A04 at 10µg/1E7 cells for 30 min at 4°C. The cells were then centrifuged at 300 x g for 3 min to remove unbound GR6A04, before incubating it with the Dynabeads following kit recommendations. The cell-bead suspension was then applied on the magnetic rack, and
25 allowed to separate. The supernatant was collected as the GR6A04- population, while the bound beads and cells were collected as the GR6A04+ population. Both fractions were washed and subjected to the magnetic rack 3 times. The bound beads and cells were finally incubated with DNase I to release the cells from the Dynabeads. Cells were seeded into T75 tissue culture flasks at a density of 2.5E6 cells per flask.

30 [00123] *Antibody-dependent cell-mediated cytotoxicity (ADCC) assay*

[00124] ADCC activity was measured using a reporter bioassay (Promega; ADCC Reporter Bioassay, #G7010). The ADCC bioassay was carried out according to the manufacturer's

protocol Briefly, PC-9 and A549 cells were seeded at 5,000 cells per well in a 96-well clear bottom black tissue culture plates (Corning; #3904) in low 4% IgG-serum (Promega; #G711A) media and allowed to attach and spread overnight. Serial dilutions of Hu-GR6A04 were incubated in triplicate wells for approximately 15 min at 37°C, 5% CO₂. Following incubation, engineered effector cells were added to the wells at approximately 150,000 cells per well. After 6 hours, Bio-Glo™ Luciferase Assay Substrate (Promega; #G719A and #G720A) was added to the wells and luminescence was measured using the Infinite® 200 microplate reader (Tecan).

[00125] *Proliferation- CellTiter-Glo Luminescent Cell Viability (CTG) Assay*

10 [00126] Cells were seeded into a black coated 96 well plate (Grenier Bio-one, UK) at a range of density from 1000-5000 cells per well (depending on the cell type used) and 90uL per well. The plate was then incubated for 24 hours at 37°C in humidified air with 5% CO₂. After 24 hours, 10µL of mAb or buffer was added to each well and the plate was again placed at 37°C in humidified air with 5% CO₂. 4 days after addition of mAb or buffer, 100uL of CTG substrate (Promega, Wisconsin, USA) was added to each well. The plate was then left in the dark for 10 minutes, with vigorous shaking. The cell viability of the samples was then quantified using Tecan I-control (Tecan, Switzerland).

[00127] *Antibody Drug Conjugates (ADCs)*

[00128] GR6A04 and a commercial mouse IgG1 isotype antibody (Clone MG1.45 from BioLegend) were directly conjugated with MMAE toxin (Moradec, San Diego, USA) at a DAR of 3.0 and 3.3 respectively. Dose-response curve for the conjugated mAbs were established with the CTG assay with a serial dilution range of 0 to 4.5µg/ml. The cell viability of the cells was measure 4 days post treatment as described previously.

[00129] *In vivo Xenograft Model*

25 [00130] Xenograft models were established using A549-GR6A04+ cells in NCr Nude mice. 5E6 cells in DMEM basal media were mixed at a 1:1 ratio with Matrigel, and injected subcutaneously at a volume of 200µl. Tumours were allowed to form and reach a size of >150mm³, before they were randomised into 5 groups of 5 mice each. The groups were treated with mAbs as follows: (1) Buffer control; (2) GR6A04-MMAE; (3) Hu-GR6A04; (4) GR6A04; (5) MG1.45-MMAE isotype control. mAbs were injected via tail vein injection at a total volume of 100µl. Each dose, were applicable, is at 200µg (equivalent to 10mg/kg), and

treatment was done every 4 days, for a total of 3 doses (Day 0, 4 and 8). Tumour size was monitored over 50 days.

[00131] **Results and Discussion**

[00132] *Generation of GR resistant mAb panel*

5 [00133] GR6A04 was identified as part of a monoclonal antibody (mAb) panel generated against PC-9 lung adenocarcinoma with acquired resistance against Gefitinib, a small molecule inhibitor of epidermal growth factor (EGFR). Gefitinib resistant (GR) PC-9 clones were generated through culture in increasing concentration of Gefitinib until a stable line was obtained at a Gefitinib concentration of 6.4 μ M. This was used as a surrogate for acquired
10 resistance against Gefitinib observed in lung cancer patients undergoing treatment with the compound.

[00134] Three Gefitinib resistant PC-9 clones (CL75, CL86 and CL131) were used for immunisation in Balb/c mice in a semi-cyclic protocol (Fig. 1). The mice were sacrificed in Week 6, and the B-cells fused with Sp2/0 mouse myeloma cells using STEMCELL
15 Technologies ClonaCell™-HY kit, and mAb-producing hybridoma clones were obtained. The mAbs from this GR panel was screened for binding to the immunising lines by flow cytometry, of which GR6A04 was identified as one of the binding lead candidates from this panel.

[00135] *GR6A04 binding on cell lines by flow cytometry*

20 [00136] GR6A04 demonstrated strong reactivity (>50%) towards the three PC-9 Gefitinib resistant clones used for immunisation, with percentage positive-binding determined from FL-1 channel gated at 2% on the secondary-only control (Fig. 2). Propidium iodide staining on the FL-3 channel also showed no apparent cell cytotoxicity when cells were incubated with the mAb alone. GR6A04 binding was also tested on the Gefitinib sensitive parental PC-
25 9 cell line, and on another GR PC-9 clone not used in the immunisation, with similarly high binding for both lines. This flow cytometry binding data is summarised in Fig. 3A.

[00137] Flow cytometry binding of GR6A04 was also tested in other NSCLC lines, with partial binding on 2 of 4 lines tested (A549 and Calu-3). GR clones from another lung adenocarcinoma line, HCC827, were also obtained by culturing in increasing Gefitinib
30 concentrations. GR6A04 binding was observed for 2 of 6 of these GR HCC827 clones. In addition, binding on other cancer indications also showed GR6A04 reactivity in 3 of 13

breast cancer lines and 1 of 2 colorectal cancer lines tested. GR6A04 binding on the different cancer indications is summarised in Fig. 3B-E.

[00138] Importantly, using the same staining method, binding of GR6A04 on the cell surface of normal cells was found to be negligible (<8%) when tested with various normal cell lines including fibroblasts, endothelial and epithelial cells, and primary peripheral blood mononuclear cells (PBMCs), as summarised in Fig. 3F.

[00139] In summary, the flow cytometry binding characteristics of GR6A04 are a) GR6A04 binds to the cell surface of PC-9 and their derived gefitinib-resistant clones based on flow cytometry, b) GR6A04 demonstrates reactivity to other NSCLC, breast and colorectal cancer lines and c) there is no cross-reactivity to normal cell lines tested.

[00140] *Characterisation of GR6A04 mAb and derivatives*

[00141] The isotype of GR6A04 mAb was determined to be of a mouse IgG1 subtype, as determined by Pierce™ Rapid Antibody Isotyping Kit in Fig. 4A. The variable heavy chain (V_H) and light chain (V_L) amino acid sequences were determined as Fig. 4B by use of degenerate primers for mouse immunoglobulins. GR6A04 was purified from hybridoma culture supernatant using CaptureSelect™ IgG-Fc (ms) Affinity Matrix with the ÄKTA avant system.

[00142] Two derivatives of GR6A04 was made and characterised: an antibody drug conjugate with monomethyl auristatin E (MMAE) (GR6A04-MMAE), and a human chimeric mAb with the V_H and V_L cloned into a human Ig constant region backbone (Hu-GR6A04).

[00143] GR6A04-MMAE was conjugated with MMAE at a drug-to-antibody ratio (DAR) of 3.0, and at the same time, a commercial mouse IgG1 antibody (Clone MG1-45 from Biolegend) at a DAR of 3.3 using the same chemistry (Fig. 4C). Binding of GR6A04-MMAE on PC-9 and A549 cells was tested by flow cytometry, and found to be comparable to unconjugated GR6A04, while MG1-45-MMAE was determined to have negligible binding on these two lines.

[00144] Hu-GR6A04 was expressed in CHO cells, and the antibody purified from the culture supernatant using the same system as GR6A04. Purified Hu-GR6A04 was checked for correct protein size from the Coomassie blue stain from SDS-PAGE, with the heavy chain and light chain at the expected size of 50kDa and 25kDa respectively in the reducing lane, and the intact IgG at 150kDa in the non-reducing lane. Similarly, binding of Hu-GR6A04 on PC-9 and A549 was comparable to GR6A04 on flow cytometry, shown in Fig. 4E.

[00145] *GR6A04 binds to N-glycosylated CEACAM6*

[00146] Western blotting of PC-9 cell lysates was immunoblotted with GR6A04, and found to recognise a smear of between 55 – 90kDa on the non-reducing lane, shown in Fig. 5. Antigen binding intensity was also weaker under reducing conditions. Additionally, treatment of the cell lysate by PNGase to remove N-linked glycans also abolished binding of GR6A04 to the antigen band, demonstrating the importance of N-glycosylation in the antibody recognition site.

[00147] Immunoprecipitation (IP) with GR6A04 against PC-9 cell lysate enriched for the antigen, which was excised and sent for identification by mass spectrometry (Fig. 6A). The protein list was arranged by the overall score based on protein coverage and number of unique peptides found, and compared to the protein list from the column control. Top 5 putative antigens are listed in the table in Fig. 6B and validated by cross-IP with a commercial anti-CEACAM6 antibody (Clone 9A6 from Santa Cruz/Abcam) (Fig. 7A), and transient siRNA knock-down of CEACAM6 in PC-9 cells (Fig. 7B).

[00148] It can be concluded that GR6A04's antigen target is CEACAM6, whereby the N-glycan is important for antibody-antigen recognition.

[00149] *GR6A04 binding on patient FFPE tissue samples*

[00150] Having established that GR6A04 is specific to various cancer indications on flow cytometry, and does not bind to normal cells, we proceeded with determining GR6A04's binding on cancer patient tissue samples on formalin fixed paraffin embedded (FFPE) tissue microarrays (TMAs). Commercial FFPE TMAs were obtained from Pantomics.

[00151] Binding condition of GR6A04 for FFPE samples was optimised with FFPE cell line pellets, and determined to be at 5ug/mL with a pH 9 antigen retrieval step. GR6A04 was observed to be localised to both the membrane and cytosol of PC-9 cells. To extend the binding profile of GR6A04 in other cancer cell lines, immunohistochemistry (IHC) staining was conducted on a FFPE cell line arrays covering larger range of cancer indications. GR6A04 was found to be reactive in gastric, lung, colorectal and pancreatic cancer cell lines (Fig. 8).

[00152] IHC staining on various FFPE TMAs covering cancer tissue samples from a wide array of organ origins were also done. GR6A04 staining was scored with the open source software: ImmunoMembrane. In a vast majority of tissue cores that was stained positive (2+ and 3+), binding was localised to the cell membrane. Some non-specific staining was also

observed in necrotic regions. IHC staining on multi-tumour TMAs supported what was observed in cell line screening, whereby we observed that in addition to lung cancer, GR6A04 is also highly reactive to, but not limited to, gastro-intestinal (GI) and breast cancer (Fig. 9 to 11). In a focused array for NSCLC, GR6A04 stained positive for 15% of squamous cell cancer, and 50% of lung adenocarcinoma cores represented in the TMA. Importantly, the adjacent normal tissue did not have any positive staining, demonstrating GR6A04 specificity towards cancer tissue (Fig. 12 & 13).

[00153] In addition, GR6A04 was tested against FFPE normal tissues using the same staining protocol and scoring system. An FDA recommended TMA was used (MNO961), containing 35 different anatomical sites (Fig. 14). No staining of GR6A04 was observed in 93 of the 96 cores present, but 2 of 3 colon cores, and 1 of 3 prostate cores were weakly positive (2+). It should be noted that from the magnified images, the staining in these cases was mainly intracellular or in necrotic regions.

[00154] Hence, GR6A04 has demonstrated specificity towards a wide range of different cancer indications in patient tissue samples, and has negligible staining on normal tissue types, supporting our earlier binding profiles from flow cytometry and cell based screening.

[00155] *Increased specificity of GR6A04 due to glycan recognition site*

[00156] The commercial anti-CEACAM6 antibody used for the earlier validation is one that is not sensitive to changes in the glycosylation of CEACAM6 (i.e. Recognition site is not glycan dependent). This is demonstrated in Fig. 15A, where the binding intensities remained unchanged after PNGase and Tunicamycin treatment, unlike GR6A04.

[00157] When the two anti-CEACAM6 (GR6A04 and commercial) were compared in an FFPE cell line microarray, while staining profiles were largely similar, the commercial antibody had one additional staining core in HCC827, while BxPC3 (pancreatic cancer line) also showed more intense and intracellular staining (Fig. 15B).

[00158] This apparent non-specificity of the commercial anti-CEACAM6 was also observed in the TMA with normal tissues (MNO961), whereby 11 of 95 cores stained positive, as opposed to only 3 cores with GR6A04. Importantly, all three lung normal tissues, and 2 spleen normal tissues, showed staining on the cell membrane (Fig. 16).

[00159] The differences in staining profiles could be attributed to the differences in epitope binding sites on CEACAM6, especially that arising from the N-glycans on CEACAM6. This allowed for an extra degree of specificity in addition to binding on CEACAM6 protein alone,

which leads to reduced non-specific binding on normal tissues. This is important as it provides an added level of safety if GR6A04 is to be developed for therapy.

[00160] *Characteristics of GR6A04⁺ A549 subpopulations*

[00161] The lung adenocarcinoma cell line, A549, is a good biological model to investigate the role of glycosylated CEACAM6 expression in cancer due to heterogeneous binding for GR6A04, with only a small 15 – 30% GR6A04⁺ population (Fig. 17A). Through the use of magnetic sorting with CELlection™ Pan Mouse IgG Kit, both the GR6A04⁻ and GR6A04⁺ could be enriched for. Although it must be noted that reduction of GR6A04 binding was observed with increasing passages in culture. Multiple rounds of consecutive sorting mitigates this, and after 7 rounds of isolation for GR6A04⁺ and 5 rounds for GR6A04⁻ cells, stable differential lines were obtained, and subsequently referred to as A549-GR6A04+ and A549-GR6A04- respectively (Fig. 17B & C). Additionally, single cell clones from A549-GR6A04+ cultures were isolated that demonstrated strong, homogeneous binding of GR6A04 (D5, D7 and S4 clones). These were observed to have different cell morphology between clones (Fig. 17D & E). In particular, D5 clones showed dense clusters with round morphology, D7 showed spindle-shaped cells and S4 showed SCLC-like morphology with attachment independent growth.

[00162] Expression of EGFR and CEACAM1 was measured by flow cytometry on these sub-populations, and also the parental line. While expression of these two markers in A549-GR6A04- cells and the parental A549 were similar, A549-GR6A04+ cells had a much lower expression of EGFR (25% decrease in MFI), and an increase in CEACAM1 binding was also observed (Fig. 18).

[00163] GR6A04 expression was also found to affect tumorigenicity *in vivo*. In an A549 xenograft model in Nude mice, whereby the same initial cell numbers were injected subcutaneously, A549-GR6A04+ cells formed larger xenografts than the A549 parental cells (Fig. 19A). When the xenograft was dissociated at the end of the 50 day study, it was also found that percentage of GR6A04 in the xenografts obtained from A549 parental cells increased from around 20% at the point of injection, to >70% by Day 50 (Fig. 19B).

[00164] To establish if GR6A04 could have potential application as a serum biomarker for lung cancer, conditioned media from A549 subpopulations were spotted on a membrane and immunoblotted with GR6A04. The antigen is detectable in the conditioned media from the A549-GR6A04+ and A549 parental cell cultures, but not in the A549-GR6A04- cultures, and

intensity is proportional to the volume of media spotted (Fig. 20). The results show that GR6A04 may be used for antigen quantification in patient serum samples.

[00165] *In vitro and in vivo functional assays*

[00166] Having established GR6A04's specificity and its possible roles in cancer biology in the previous sections, this section focuses primarily on the functionality of the mAb as a cancer therapeutic. As an early test for function as an antibody-drug conjugate (ADC), GR6A04 was indirectly conjugated with saporin (ribosome inactivating protein) using an anti-mouse antibody conjugated with the toxin. Cell growth was inhibited in both the PC-9 sensitive parental line, and CL75 GR clone by 43% and 28% respectively (Fig. 21A). GR6A04 was also observed to be able to internalise within 120 min at 37°C, with the mAb being localised to the cell periphery after binding at 0 min, and subsequently found distributed within the cell cytoplasm (dashed arrows) at 120 min. A proportion of cells, however, has not internalised the mAb at 120 min, and the mAb was still localised on the surface (solid arrows) (Fig. 21B).

[00167] GR6A04 was subsequently directly conjugated to MMAE (Fig. 4C) for a more robust assay. A dose response curve was conducted on PC-9 and A549 binding cells, and H1299 non-binding cells (Fig 22). EC50 was established to be 19.8nM for PC-9 cells and 3.33nM for A549-GR6A04+ cells. GR6A04-MMAE did not have an effect on cell growth for non-binding cells, H1299 and A549-GR6A04- populations, demonstrating specificity to GR6A04 binding cells only.

[00168] GR6A04 was also developed as a chimeric mAb with a human IgG constant backbone (Fig. 4D). Hu-GR6A04 was tested for antibody-dependent cell cytotoxicity (ADCC) using a Promega ADCC kit, which as a Luciferase reporter for FcγR binding (Fig. 23). A dose-response curve on PC-9 and A549-GR6A04+ was done, and the EC50 established to be 39.6nM and 1.33nM respectively.

[00169] Hence, GR6A04 has demonstrated *in vitro* functionality on two lung cancer cell lines, PC-9 (gefitinib sensitive) and A549 (gefitinib-resistant), as a naked human chimeric mAb with ADCC activity, and as an ADC conjugated with MMAE.

[00170] Finally, *in vivo* functionality was tested using A549-GR6A04+ cells in a subcutaneous xenograft model (Fig. 24). Treatment with GR6A04 and their derivatives was initiated when the tumour reaches 150mm³, with I.V. mAb injection at 10mg/kg, every 4 days for 3 doses. Both the naked mAbs, Gr6A04 and Hu-GR6A04 showed repression of tumour

growth for the buffer control, attributed to ADCC mechanisms. Importantly, GR6A04-MMAE was extremely effective in controlling tumour size, with little tumour growth (<2-fold) throughout the 45 days of study. Hence, GR6A04 has demonstrated good *in vivo* functionality with a gefitinib-resistant A549 xenograft model, congruent with the *in vitro* results.

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