

Original Article

# Function characterization of a glyco-engineered anti-EGFR monoclonal antibody cetuximab *in vitro*

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**Aim:** To evaluate the biochemical features and activities of a glyco-engineered form of the anti-human epidermal growth factor receptor monoclonal antibody (EGFR mAb) cetuximab *in vitro*.

**Methods:** The genes encoding the Chinese hamster bisecting glycosylation enzyme (GnTIII) and anti-human EGFR mAb were cloned and coexpressed in CHO DG44 cells. The bisecting-glycosylated recombinant EGFR mAb (bisec-EGFR mAb) produced by these cells was characterized with regard to its glycan profile, antiproliferative activity, Fc receptor binding affinity and cell lysis capability. The content of galactose- $\alpha$ -1,3-galactose ( $\alpha$ -Gal) in the bisec-EGFR mAb was measured using HPAEC-PAD.

**Results:** The bisec-EGFR mAb had a higher content of bisecting N-acetylglucosamine residues. Compared to the wild type EGFR mAb, the bisec-EGFR mAb exhibited 3-fold higher cell lysis capability in the antibody-dependent cellular cytotoxicity assay, and 1.36-fold higher antiproliferative activity against the human epidermoid carcinoma line A431. Furthermore, the bisec-EGFR mAb had a higher binding affinity for human Fc $\gamma$ RIa and Fc $\gamma$ RIIIa-158F than the wild type EGFR mAb. Moreover,  $\alpha$ -Gal, which was responsible for cetuximab-induced hypersensitivity reactions, was not detected in the bisec-EGFR mAb.

**Conclusion:** The glyco-engineered EGFR mAb with more bisecting modifications and lower  $\alpha$ -Gal content than the approved therapeutic antibody Erbitux shows improved functionality *in vitro*, and requires *in vivo* validations.

**Keywords:** EGFR mAb; Erbitux; glyco-engineering; antibody-dependent cellular cytotoxicity; antiproliferation; Fc receptor binding; galactose- $\alpha$ -1,3-galactose

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## Introduction

More than 300 monoclonal antibodies are in clinical trials for the treatment of human malignancies and inflammation<sup>[1, 2]</sup>. The clinical success of this drug class is demonstrated by the large number of therapeutic antibodies that have been brought to market and the increasing number of therapeutic antibodies in development. However, improving the efficacy of therapeutic antibodies and decreasing their side effects remain challenges<sup>[1]</sup>.

Glycosylation is one of the most important post-translational protein modifications and has essential roles in therapeutic antibody function, including effector function, immunogenicity, half-life in serum and other aspects of bioactivity<sup>[3, 4]</sup>. Some glycoforms of human antibodies exhibit stronger therapeutic

effects than other glycoforms, and some glycoforms possess undesired properties. For example, de-fucosylated Herceptin is at least 50-fold more active in Fc $\gamma$  receptor IIIa-mediated antibody-dependent cellular cytotoxicity (ADCC) assays than the form with alpha-1,6-linked fucose residues<sup>[5]</sup>. The anti-CD20 antibody with a higher content of bisecting acetylglucosamine (GlcNAc) residues has 10–20 times higher ADCC than its wild type counterpart<sup>[6]</sup>.

The therapeutic anti-human epidermal growth factor receptor monoclonal antibody (EGFR mAb) cetuximab (marketed under the name Erbitux) is expressed in the murine cell line Sp2/0 and approved for the treatment of colorectal cancer. This therapeutic antibody can bind EGFR and lead to EGFR autophosphorylation, as well as subsequent activation of signal transduction pathways that are involved in regulating cellular proliferation, differentiation, and survival<sup>[7]</sup>. Furthermore, this therapeutic EGFR mAb exhibits ADCC against cancer cells<sup>[8]</sup>.

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A high prevalence of hypersensitivity reactions to cetuximab have been reported in some areas of the United States<sup>[9]</sup>. In most subjects who had a hypersensitivity reaction, IgE antibodies that recognize the EGFR mAb were present in the circulation before treatment. The IgE antibodies were specific for galactose- $\alpha$ -1,3-galactose ( $\alpha$ -Gal), which is present on the Fab portion of the EGFR mAb heavy chain. Those patients who have such IgE antibodies before treatment tend to develop a hypersensitivity reaction after intravenous injection of monoclonal antibodies containing  $\alpha$ -Gal.

In this study, we first established a stable Chinese hamster ovary (CHO) cell line that can express the wild-type EGFR mAb and subsequently transfected these cells with cDNA encoding GnTIII (a Golgi-localized enzyme that catalyzes the addition of a bisecting N-acetylglucosamine residue on N-linked oligosaccharide chains). Following coexpression of GnTIII and wild type EGFR mAb in this cell line, we characterized the N-glycan profile of the GnTIII-modified EGFR mAb (bisec-EGFR mAb) using DNA sequencer-assisted-fluorophore-assisted capillary electrophoresis (DSA-FACE)<sup>[10,11]</sup> and quantified the  $\alpha$ -Gal content. The effects of the EGFR mAb on cell growth, ADCC and Fc $\gamma$ R binding capacity were investigated *in vitro* to assess the functions of the bisec-EGFR mAb.

## Materials and methods

### Cell lines

A431 (ATCC CRL-1555) is a cell line expressing EGFR. Daudi (ATCC CCL-213<sup>TM</sup>) is a B lymphoblast cell line expressing the inhibitory Fc $\gamma$  receptor (Fc $\gamma$ RIIb). HEK293 cells expressing human Fc $\gamma$ RIa, Fc $\gamma$ RIIa, Fc $\gamma$ RIIIa-158V, or Fc $\gamma$ RIIIa-158F were generated as previously described<sup>[12]</sup>.

### Expression of wild-type EGFR mAb and bisec-EGFR mAb

A GnTIII(-) CHO DG44 cell line was used in expression studies of the wild type EGFR mAb and the bisec-EGFR mAb. GnTIII and the heavy chain and light chain of the EGFR mAb (cDNA synthesized by Invitrogen and subcloned into an expression vector in our lab) were coexpressed in CHO DG44 cells using the OptiCHO<sup>TM</sup> Antibody Express System (Invitrogen). A cell line that stably expressed the wild type EGFR mAb was screened with 1  $\mu$ mol/L MTX and validated by whole cell ELISA (A431)<sup>[6]</sup>. The synthesized Chinese hamster GnTIII gene (NM\_001244074.1) was cloned into the pCDNA 3.1 Hygro(-) vector and the wild type EGFR mAb-expressing cell line was transfected with this vector. The bisec-EGFR mAb (GnTIII gene stably transfected) cell line was selected on 500  $\mu$ g/mL hygromycin.

### Glycosylation analysis of wild type EGFR mAb, bisec-EGFR mAb and the corresponding Fab and Fc fragments

The wild type EGFR mAb and bisec-EGFR mAb from cell supernatants were captured with Protein G-agarose and protein A-agarose, respectively. The corresponding Fc and Fab fragments were isolated using immobilized papain (Thermo Scientific, USA) following the manufacturer's instructions.

The digested supernatant was then loaded onto a Protein A column. The Fab fragments were collected as the flow-through fraction. The bound Fc fragments were eluted with 0.01 mol/L glycine, pH 3.0. N-glycan profiling of the wild type EGFR mAb, and bisec-EGFR mAb, and the corresponding Fab and Fc fragments was conducted by DSA-FACE.

### ADCC activity assay of wild type EGFR mAb and bisec-EGFR mAb

Peripheral blood mononuclear cells (PBMCs) were separated from heparinized fresh healthy human blood by standard centrifugation procedures using Ficoll/Hypaque (Sigma). The PBMCs used as effector cells were activated in RPMI with 10% FBS and 10 U/mL interleukin-2 (Roche) overnight. The ADCC activity assay was performed according to the manufacturer's instructions (CytoTox 96<sup>®</sup> Non-Radioactive cytotoxicity Assay, Promega, USA). Briefly, A431 cells were grown to the log phase and resuspended at  $4 \times 10^5$  cells/mL after washing in assay medium (DMEM). The target cells (A431) were added at 50  $\mu$ L/well into a 96-well flat-bottomed cell culture plate. Antibodies were serially diluted in assay medium and then added at 50  $\mu$ L/well in triplicate well in the plates. The plates were incubated at room temperature for 10 min prior to the addition of 100  $\mu$ L of serially diluted effector cells (PBMCs)<sup>[6]</sup>. The cell mixtures with antibodies were incubated at 37°C for 4 h in a humidified CO<sub>2</sub> incubator. One hundred microliters of supernatant was removed from each well and analyzed by measuring lactate dehydrogenase (LDH) activity released from damaged target cells using a CytoTox 96<sup>®</sup> Non-Radioactive Cytotoxicity Assay (Promega, USA). The effector and/or target cells were also included as controls. Specific lysis was calculated relative to a total lysis control generated by incubating the target cells with 100  $\mu$ L of 2% Triton X-100.

### Antiproliferative effects of wild type EGFR mAb and bisec-EGFR mAb

The A431 cell line was employed to test the Fab binding-mediated antiproliferative activity of the antibodies. In brief, A431 cells were incubated with the wild type EGFR mAb and bisec-EGFR mAb diluted in FBS-free medium for 72 h at 37°C with 5% CO<sub>2</sub>. After MTS solution (G5340, Promega) was added, the cells were incubated for another 3 h. Colorimetric evaluation was performed at 492 nm using a spectrophotometer. The inhibition of proliferation is reported as the IC<sub>50</sub> induced by the wild type EGFR mAb or bisec-EGFR mAb in comparison with that induced by a positive control (Erbitux).

### FcR binding affinity of wild type EGFR mAb and bisec-EGFR mAb

HEK293 cells expressing human Fc $\gamma$ RIa, Fc $\gamma$ RIIa, Fc $\gamma$ RIIIa-158V, or Fc $\gamma$ RIIIa-158F ( $1 \times 10^6$  cells) were incubated with the wild type EGFR mAb, bisec-EGFR mAb or Erbitux (10  $\mu$ g/mL) or 1% BSA in PBS at 4°C for 1 h and then washed and stained with FITC-labeled anti-human IgG (Sigma, USA). Cells were analyzed using light-scatter parameters on a MACS QUANT flow cytometer (Miltenyi Biotec, Germany). Blank controls were used, setting the cutoff at no more than 0.5% cells binding with FITC labeled anti-human IgG.

### $\alpha$ -Gal quantification of Erbitux and bisec-EGFR mAb

The standards for the calibration curve were created through serial dilution of a 100 mmol/L *D*-galactose stock solution (Sigma). Each sample was mixed with 2  $\mu$ L of (1-3,4,6)-galactosidase (Prozyme) and diluted to a final concentration of 1 mg/mL in the reaction buffer and incubated overnight at 37°C. High-performance anion exchange chromatographic separation of the analyte was performed using an ICS-3000 chromatography system (HPAEC, Dionex Corp). The solution was diluted 2 $\times$  to an antibody concentration of 0.5 mg/mL and detected using a pulsed amperometric detector (PAD) after elution through a CarboPac PA1 column.

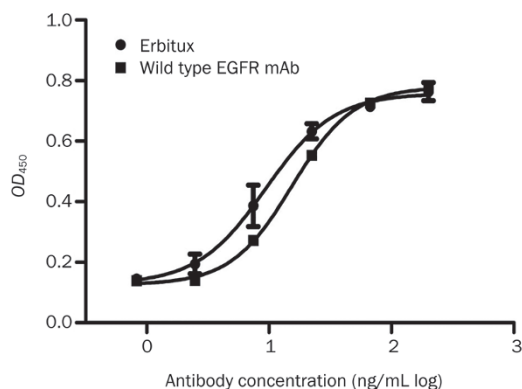
### Statistical analysis

All statistical analyses were performed with SPSS 16.0 for Windows software (SPSS, Chicago, IL, USA). Quantitative variables were compared using Student's *t* test and ANOVA. All reported *P*-values were two-tailed, and *P* values less than 0.05 were considered to be statistically significant.

## Results

### (Bisec-)EGFR mAb expression and N-Glycan analysis

The EGFR mAb was captured from the cell supernatant by using Protein A. Whole cell ELISA showed that the recombinant wild-type EGFR mAb exhibited dose-dependent binding to A431 cells, comparable to Erbitux (Figure 1).



**Figure 1.** Whole cell ELISA. The wild type EGFR mAb exhibited dose-dependent binding to A431 cells, comparable to Erbitux.

N-glycan analysis revealed 7 dominant N-glycan structures (peaks) in the N-glycan profile, with only peak 2 and peak 7 containing bisecting GlcNAc residues<sup>[10, 11]</sup>. Thus, the glycan-modified EGFR mAb with elevated abundances of peak 2 and peak 7 (bisec-EGFR mAb) was selected for further study. Typical N-glycan profiles of the wild type EGFR mAb and bisec-EGFR mAb are shown in Figure 2A-2B. For the wild type EGFR mAb, no bisecting GlcNAc glycoform was found in either the corresponding Fab or Fc fragments (Figure 2A). As expected, the bisecting GlcNAc content (peak 2 and peak 7) was elevated dramatically in both the Fab and Fc fragments of

bisec-EGFR mAb (Figure 2B) compared to the wide type EGFR mAb. Differences in N-glycan abundances between wild type EGFR mAb and bisec-EGFR mAb are shown in Figure 2C-2E. Notably, only galactosylated bisecting glycan (peak 2, 24%) was detected in Fc fragment of bisec-EGFR mAb (Figure 2E), while both agalactosylated and bigalactosylated bisecting GlcNAc (peak 2, 21%; peak 7, 21%) glycoforms were detected in the intact antibody (Figure 2C) and Fab fragment (Figure 2D).

### Bisec-EGFR mAb had increased ADCC

Both the wild type EGFR mAb and bisec-EGFR mAb effectively induced A431 cell lysis at effector:target cell (E:T) ratios of 5:1 and higher (Figure 3). The bisec-EGFR mAb showed a stronger lysis effect even at lower E:T ratios (*P*<0.05). At an E:T ratio of 40:1, the bisec-EGFR mAb yielded effective specific lysis with an approximately three-fold higher effect than that of the wild type EGFR mAb (89% vs 30%, *P*<0.001). The results indicated that the EGFR mAb that contained a larger amount of bisecting GlcNAc had a higher ADCC activity.

### Bisec-EGFR mAb had higher antiproliferative efficacy than wild type EGFR mAb

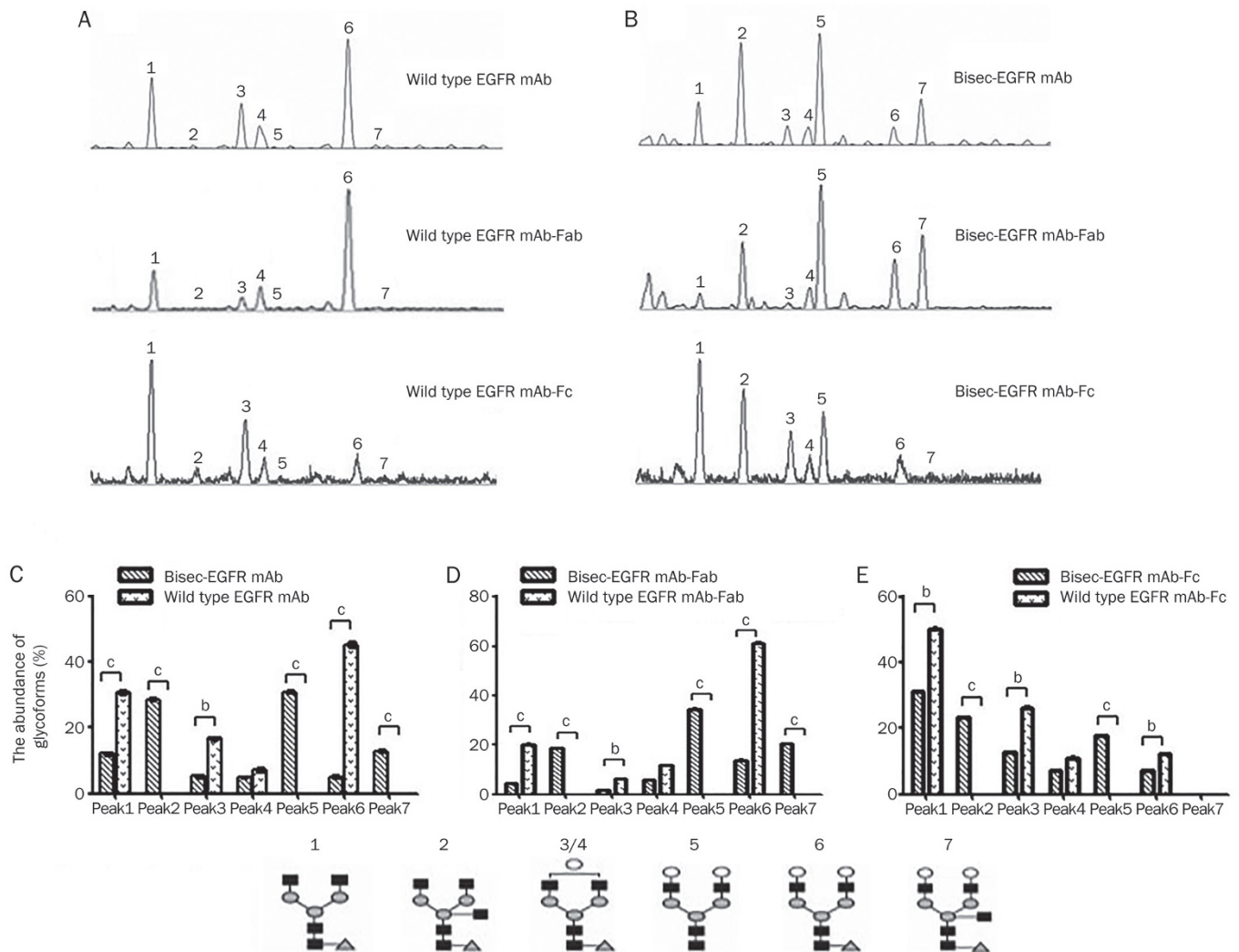
The antiproliferative activity of the EGFR mAb is mediated by the binding of the Fab fragment to EGFR. As indicated in Figure 4, both the wild type EGFR mAb and bisec-EGFR mAb showed significant inhibition of tumor cell growth. The 50% inhibitory concentration (IC<sub>50</sub>) values of Erbitux, the wild type EGFR mAb and the bisec-EGFR mAb were 0.162 (95% CI: 0.143-0.184), 0.178 (95% CI: 0.159-0.201) and 0.119 (95% CI: 0.095-0.150) mg/mL, respectively. Thus, the bisec-EGFR mAb was approximately 1.36-fold more potent than Erbitux, and the potency of the wild type EGFR mAb was comparable to that of the positive control (Erbitux).

### Bisec-EGFR mAb had improved binding affinity for Fcγ receptors

The binding activities of the wild type EGFR mAb, bisec-EGFR mAb, and Erbitux to Fcγ receptors (FcγRs, both inhibitory and activating FcγRs) were tested by flow cytometry. All of the EGFR mAbs bound to human FcγRs with the following rank order of affinity: FcγRIa>FcγRIIIa-158V>FcγRIIIa-158F>FcγRIIa>FcγRIIb (Figure 5). Compared to the wild type EGFR mAb, the bisec-EGFR mAb showed a higher binding affinity for human FcγRIa (*P*=0.001, Figure 5A) and FcγRIIIa-158F (*P*=0.013, Figure 5B). The binding affinities for 158V (Figure 5C), FcγRIIa (Figure 5D), and FcγRIIb (Figure 5E) of bisec-EGFR mAb and wild-type EGFR mAb were similar.

### $\alpha$ -Gal quantification of Erbitux and bisec-EGFR mAb

The digested Erbitux and bisec-EGFR mAb were analyzed in triplicate, and the results were measured against a calibration curve (Figure 6). The  $\alpha$ -Gal peak was observed at a retention time of approximately 12 min after injection of the digested Erbitux (Figure 7A). The molar ratio of  $\alpha$ -Gal per Erbitux molecule was determined to be 2.36 $\pm$ 0.09:1. For the bisec-EGFR mAb, no detectable peak was observed at a retention time of



**Figure 2.** Desialylated N-glycan profiling of wild type EGFR mAb and bisect-EGFR mAb. For the wild type EGFR mAb (A), the bisecting GlcNAc glycoforms: an agalacto core- $\alpha$ -1,6-fucosylated bisecting biantennary glycan (NGA2FB, peak 2) and a bigalacto core- $\alpha$ -1,6-fucosylated bisecting biantennary glycan (NA2FB, peak 7) were not found. However, the bisected GlcNAc content was elevated dramatically in both the Fab and Fc fragments of the bisect-EGFR mAb (B). Differences in N-glycan abundance between the wild type EGFR mAb and bisect-EGFR mAb are shown in C–E ( $^bP < 0.05$ ,  $^cP < 0.01$ ). Notably, only agalactosylated bisecting GlcNAc (peak 2, E) was detected for the Fc of bisect-EGFR mAb, whereas both agalactosylated and bigalactosylated GlcNAc (peak 2 and peak 7) were detected in the consensus intact antibody (C) and Fab fragment (D).

approximately 12 min after injection (Figure 7B), and a similar result was found for the wild type EGFR mAb (data not shown).

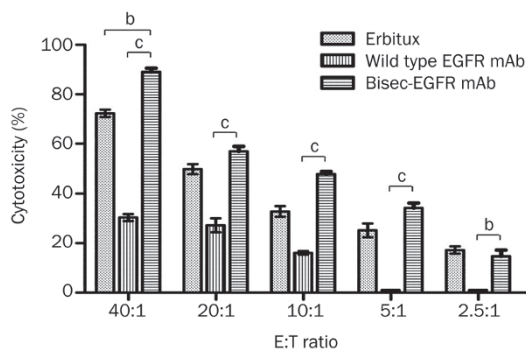
The retention time of the  $\alpha$ -Gal peak variant, at approximately 12 min, is highly sensitive to small changes in the sodium hydroxide concentration and the amount of dissolved carbon dioxide from the air, but the area of the peak is relatively consistent between different runs.

## Discussion

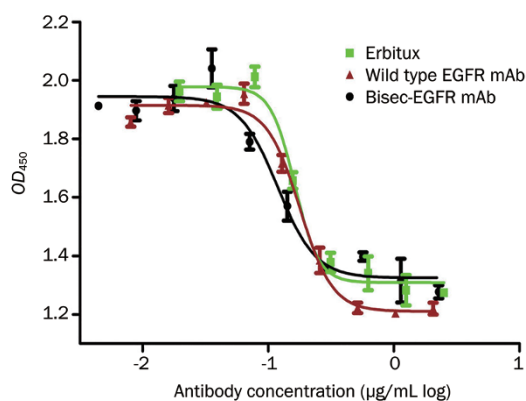
The effector functions elicited by therapeutic antibodies strongly depend on the carbohydrate moiety linked to the antibody protein. Therefore, several approaches have been developed to rationally manipulate these glycans and improve the biological functions of antibodies<sup>[13]</sup>.

GnT-III is a key enzyme in N-glycan biosynthesis and catalyzes the transfer of GlcNAc from UDP-GlcNAc, a glycosyl donor, to a core  $\beta$ -mannose residue in N-linked oligosaccharides via a  $\beta$ 1 $\rightarrow$ 4 linkage, resulting in the formation of a bisected sugar chain<sup>[14]</sup>. The GlcNAc residue added is referred to as a bisecting GlcNAc. The addition of this unique structure inhibits the action of other N-acetylglucosaminyl transferases, such as GnT-IV and GnT-V, both of which are involved in the formation of multi-antennary sugar chains<sup>[15]</sup>. Thus, GnT-III is a critical enzyme that has been used to improve the function of therapeutic antibodies.

Increasing evidence shows that N-linked oligosaccharides can affect the solubilities, clearance rates and effector functions of antibody molecules<sup>[16]</sup>. GnT-III is an ideal enzyme for manipulating the N-glycosylation of expressed proteins, as it



**Figure 3.** Comparisons of wild type EGFR mAb and bisec-EGFR mAb in ADCC. ADCC was evaluated in EGFR-positive A431 cells at different effector:target cell (E:T) ratios. Compared to the wild type EGFR mAb, the bisec-EGFR mAb resulted in comparable effective specific lysis at a lower E:T ratio ( $P < 0.05$ ). At the same E:T ratio (40:1), the bisec-EGFR mAb resulted in effective specific lysis, with approximately three-fold greater lysis than that mediated by the wild type EGFR mAb (88% vs 30%,  $P < 0.001$ ). <sup>b</sup> $P < 0.05$ , <sup>c</sup> $P < 0.01$ .



**Figure 4.** Antiproliferative efficacies of wild type EGFR mAb and bisec-EGFR mAb. Both the wild type EGFR mAb and bisec-EGFR mAb showed significant inhibition of tumor cell growth. The potency of the wild type EGFR mAb was comparable to that of Erbitux. The bisec-EGFR mAb exhibited a potency that was approximately 1.36-fold of that of Erbitux.

exerts a large degree of control over the glycosylation process by blocking the action of 1,6-FucT, ManII, and GnT-II<sup>[17,18]</sup>.

Previous studies have shown that over-expression of GnTIII in CHO DG44 cells leads to ADCC enhancement of an anti-neuroblastoma IgG1<sup>[18]</sup>, an anti-CD20 IgG1<sup>[19]</sup>, and an anti-human interleukin 5 receptor IgG1<sup>[20]</sup>. To further identify the roles of bisecting GlcNAc in EGFR mAb function, we over-

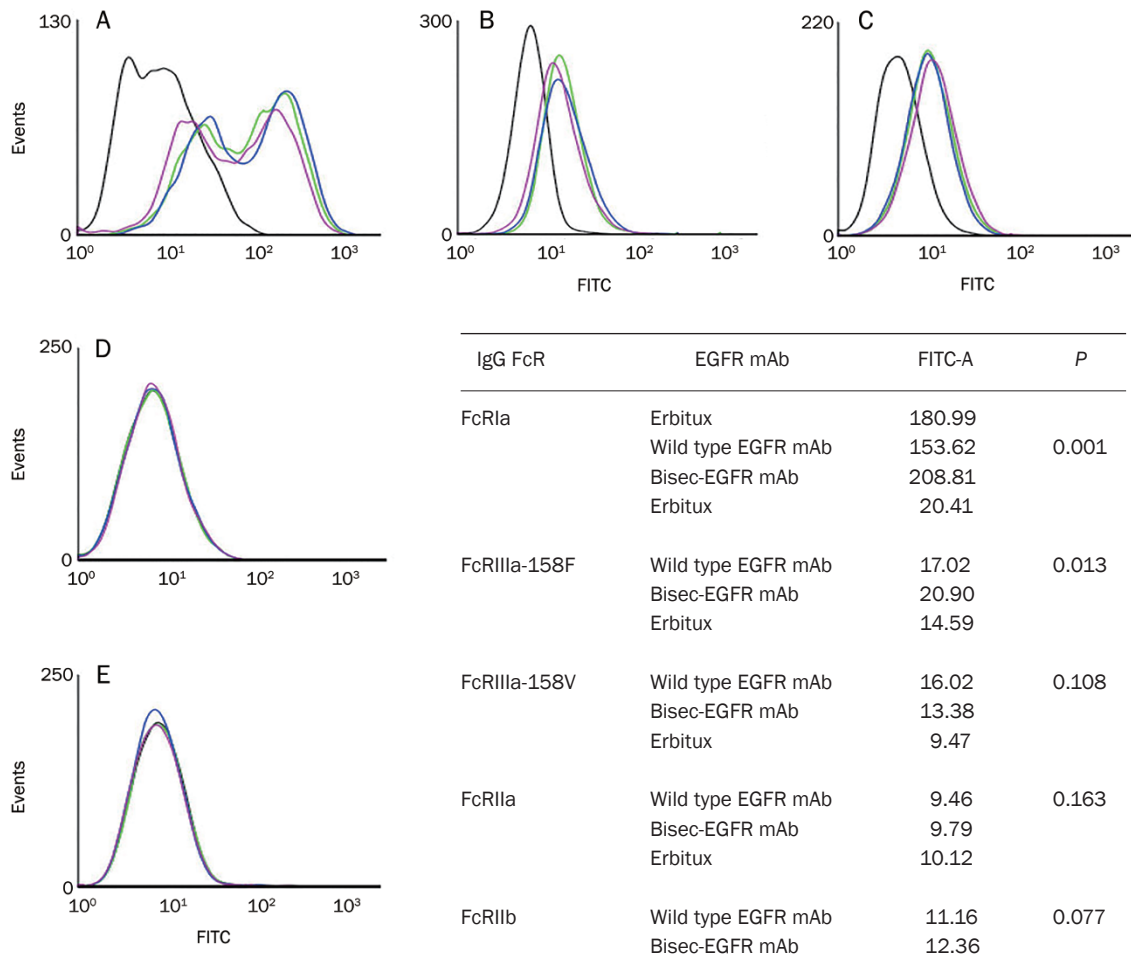
expressed GnTIII in an EGFR mAb producing cell line (CHO DG44) that lacks endogenous GnTIII expression<sup>[21]</sup>, and we confirmed that an antibody with bisecting GlcNAc (the bisec-EGFR mAb) enhanced ADCC compared to an antibody without bisecting GlcNAc (the wild type EGFR mAb).

However, all of the previous studies have only focused on the glycosylation of intact IgG. Considering the different oligosaccharide profiles of IgG Fab and Fc fragments revealed in some case-control studies<sup>[22,23]</sup>, we also analyzed the N-glycan profiles of antibody Fab and Fc fragments. Both the Fab and Fc fragments of the bisec-EGFR mAb showed an increased bisecting GlcNAc content compared to the wild type EGFR mAb, but the Fab and Fc fragments had different N-glycan profiles from each other. This finding demonstrated site specificity for the elongation of the oligosaccharide chains on the glycoprotein<sup>[22]</sup> and may explain why the antibodies with a higher bisecting GlcNAc content had higher ADCC activity. First, the enhanced effect may be due to an increased Fc affinity for Fcγ receptors<sup>[24]</sup>. Second, the improved ADCC activity may also result from a better specific binding of Fab to antigen-positive cells, which initiates the process of ADCC and target cell lysis<sup>[25]</sup>.

In the Fab-mediated cell antiproliferation assay, we found that the potency of the bisec-EGFR mAb was 50% higher than that of the wild type EGFR mAb. Meanwhile, a comparative study on Fc receptor binding activity revealed that the bisec-EGFR mAb had a higher binding affinity for activated human Fcγ receptors (mainly FcRIa and 158F-FcRIIIa) than did the wild type EGFR mAb. Taken together, these data suggest that the bisecting GlcNAc modified antibody has higher affinities for both activated FcR and EGFR-expressing cells.

Most of the recently marketed therapeutic antibodies are manufactured in CHO cells, in part because of the ability of these cells to produce proteins with desirable properties, including 'human-like' glycosylation profiles. Specific glycan structures may adversely affect an antibody's safety profile. For example, the terminal galactose-α-1,3-galactose (α-Gal) antigen can react with circulating anti α-Gal antibodies, which are present in most individuals, and induce an anaphylactic response<sup>[26]</sup>. It is now understood that murine cell lines, such as NS0 or SP2/0, contain the necessary biosynthetic machinery to produce proteins containing α-Gal epitopes<sup>[27-29]</sup>. However, it is generally accepted that CHO cells lack the biosynthetic machinery to synthesize glycoproteins with α-Gal antigens<sup>[30]</sup>. Some reports have revealed that CHO cells do not produce α-1,3-galactosyltransferase<sup>[31]</sup> and have a pattern of glycosylation that differs from that of the Erbitux host cell line Sp2/0. However, data from other groups have identified the presence of the CHO ortholog of N-acetylglucosaminyl 3-α-galactosyltransferase-1<sup>[32]</sup>, which is responsible for the synthesis of the α-Gal epitope. These groups also found that the amounts of terminal α-Gal in CHO clones ranged from 0 to 404 picomol per mg of protein; therefore, selection of a cell line without α-Gal expression is important for CHO-derived therapeutic antibody production.

In this study, we developed and quantified the concentra-



**Figure 5.** Binding affinities of wild type EGFR mAb and bisec-EGFR mAb to Fc $\gamma$ R $\alpha$ -positive and Fc $\gamma$ R1b-positive cells. The binding affinity of the wild type EGFR mAb (purple line), bisec-EGFR mAb (blue line), and Erbitux (green line) for Fc $\gamma$ R $\alpha$ s was tested by flow cytometry. The yellow line represents the blank control. The EGFR mAbs bound to Fc $\gamma$ R $\alpha$ s with the following rank order: Fc $\gamma$ R1a>Fc $\gamma$ R1a-158V>Fc $\gamma$ R1a-158F>Fc $\gamma$ R1a>Fc $\gamma$ R1b. Compared to the wild type EGFR mAb, the bisec-EGFR mAb showed significantly enhanced binding affinity for Fc $\gamma$ R1a (A,  $P=0.001$ ) and Fc $\gamma$ R1a-158F (B,  $P=0.013$ ). The binding affinities for human Fc $\gamma$ R1a-158V (C), Fc $\gamma$ R1a (D), and Fc $\gamma$ R1b (E) were comparable between the bisec-EGFR mAb and the wild type EGFR mAb.

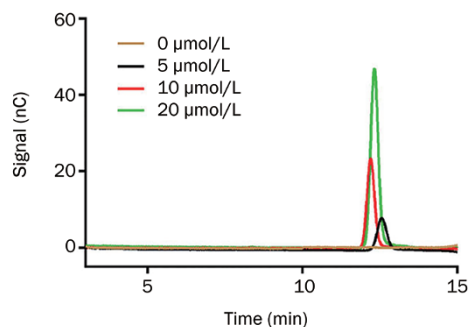
tion of  $\alpha$ -Gal in the bisec-EGFR mAb using HPAEC-PAD (high-performance anion exchange chromatography-pulsed amperometric detection). Consistent with a previous report<sup>[33]</sup>, we found that Erbitux contains an  $\alpha$ -Gal epitope ( $2.36\pm 0.09$   $\mu\text{mol}$  per  $\mu\text{mol}$  Erbitux), whereas the bisec-EGFR mAb we investigated did not show an obvious  $\alpha$ -Gal peak. Although the specific levels of  $\alpha$ -Gal required to trigger anaphylactic reactions are not yet clear, the fact that patients have high levels of circulating anti- $\alpha$ -Gal antibodies<sup>[9]</sup> suggests that controlling the levels of the  $\alpha$ -Gal epitope during EGFR mAb development may have a positive impact on drug safety. More evidence for avoiding the hypersensitivity reaction to  $\alpha$ -Gal epitope could be provided by clinical trials.

In conclusion, we modified the glyco-structure of the EGFR mAb by increasing the bisecting glycan abundance. This bisec-EGFR mAb showed higher ADCC and lower  $\alpha$ -Gal lev-

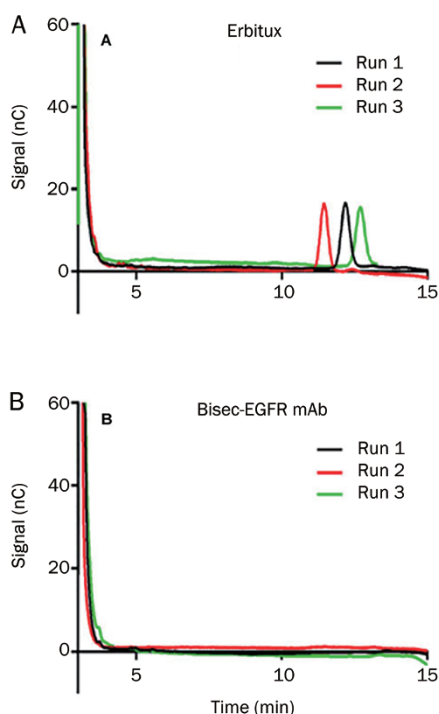
els than the approved therapeutic antibody (Erbitux). Additionally, the glycan-modified antibody showed higher binding to Fc $\gamma$ R1a and Fc $\gamma$ R1a-158F. These results indicate that the glyco-engineering of anti-EGFR antibodies could optimize their function and minimize their side effects (such as hypersensitivity). Validation *in vivo* will be required to confirm the improved efficacy of the glyco-engineered antibody. This research might provide a new, alternative, strategy for the production of engineered therapeutic monoclonal antibodies.

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**Figure 6.**  $\alpha$ -Galactose standard curve building. By high-performance anion exchange chromatography-pulsed amperometric detection (HPAEC-PAD), an  $\alpha$ -Galactose standard curve was created by the serial dilution of a 100 mmol/L D-galactose stock solution (Sigma). The  $\alpha$ -Gal peak was observed at a retention time of approximately 12 min after the injection of D-galactose solution.



**Figure 7.**  $\alpha$ -Galactose analysis of Erbitux and bisec-EGFR mAb. Digested Erbitux and bisec-EGFR mAb were analyzed in triplicate, and the results were measured against the standard curve. The  $\alpha$ -Gal peak was observed in digested Erbitux (A). For the bisec-EGFR mAb, no detectable  $\alpha$ -Gal peak was observed (B).

#### Author contribution

Chang-hong YI, Can-ping RUAN, and Chun-fang GAO designed the research; Chang-hong YI, Xin-yun XU, Yun-peng ZHAO, and Meng FANG performed research; Jun JI, Hao WANG, and Xing GU analyzed data; Chang-hong YI and Chun-fang GAO wrote the paper.

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