Research article

Characterization of monoclonal antibodies generated to the 287–302 amino acid loop of the human epidermal growth factor receptor

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Abstract

Background: The dysregulation of epidermal growth factor receptor (EGFR) has been implicated in the oncogenesis of various malignancies including glioblastoma and some epithelial cancers. Oncogenesis occurs from the overexpression of EGFR, often linked to gene amplification or receptor mutagenesis. The 287–302 loop in the extracellular domain is exposed completely on EGFR variant III (EGFRvIII), partially exposed on some cancers but cryptic on normal cells. We report on the generation of antibodies to this loop.

Methods: The 286–303 peptide was coupled chemically to keyhole limpet hemocyanin. After immunizations, sera were assayed for reactivity to the peptide. Mice with high titers were used for hybridoma production. Purified antibodies were isolated from hybridoma supernatants, while V regions were cloned and sequenced. Receptor binding was characterized using enzyme-linked immunosorbent assay and flow cytometry. A recombinant immunotoxin was generated from the 40H3 antibody and its cytotoxic activity characterized on relevant cancer cell lines.

Results: Seven monoclonal antibodies were generated to the 287–302 loop and characterized further. Each one reacted with EGFRvIII but not wild-type EGFR. Based on reactivity with the immunizing peptide, antibodies were mapped to one of three subgroups. One antibody, 40H3, also exhibited binding to MDA-MB-468 and A431 cells but not to non-cancerous WI-38 cells. Because of its unusual binding characteristics, a recombinant immunotoxin was generated from 40H3 antibody and its cytotoxic activity characterized on relevant cancer cell lines. Conclusions: Immunization with a peptide corresponding to a cryptic epitope from EGFR can produce tumor cell-binding antibodies. The 40H3 antibody was engineered as a cytotoxic recombinant immunotoxin and could be further developed as a therapeutic agent.

Statement of Significance: EGFRvIII (deletion of exons 2–7) is expressed only on cancer cells. The generation of antibodies against the 287–302 amino acid loop, exposed completely on EGFRvIII and partially on EGFR expressed by some epithelial cancers, may aid in the development of antibody-based therapeutics to this cancer-associated receptor.

KEYWORDS: Breast cancer; EGFR; immunotoxin; EGFRvIII; glioblastoma; immunotherapy; targeted therapy

INTRODUCTION

The epidermal growth factor receptor (EGFR) is a member of the receptor tyrosine kinase family and was the first receptor shown to be associated with human cancer [1]. In particular, EGFR frequently contributes to the oncogenic progression of human epithelial cancers [2]. Alterations in receptor expression include both gene amplifications and activating mutations. EGFR has an extracellular domain (ECD) of 621 amino acids, a single pass transmembrane domain (TM) of 23 amino acids and an enzymatically active intracellular domain (ICD) of 542 amino acids. Ligand
Figure 1. A schematic of wild type EGFR, EGFRvIII and amino acids 286–303 loop from the ECD. (A) Compared to wild type EGFR, EGFRvIII has an in-frame deletion (exon 2–7) resulting in the loss of 267 amino acids from the ECD. A disulfide-limited loop in the ECD (aa287 to 302) is constitutively exposed in EGFRvIII and partially exposed when wtEGFR is overexpressed on cancer cells. The arrows indicates the binding site location for the ma528 antibody. (B) Amino acids 286–303 were conjugated with KLH and injected repeatedly into mice as an immunogen.

binding leads to receptor dimer formation and the activation of the kinase domain. This produces signaling via one of several pathways that promote the growth, survival and spread of mammalian cells [3]. Activating mutations can occur in either the ECD or the ICD; there are also gene amplifications and large deletions exemplified by the loss of exons 2–7 to produce EGFR variant III (EGFRvIII) or the loss of exon 19 to generate constitutively active enzyme [2, 3]. The expression of EGFRvIII or the loss of exon 19 is reported to occur exclusively in cancer cells [4]. Current cancer treatments that focus on EGFR are either antibody-based agents directed to the ECD to prevent ligand binding [5] or small molecular weight drugs targeting the ICD to inhibit kinase activity [6]. Agents in the development pipeline include vaccines for targeting mutant versions of the receptor [7] and antibodies that react preferably with mutant receptors over the wild-type receptor [8].

Activation of wild-type EGFR (wtEGFR) leads to dimerization, which involves both ligand binding and a monomer to dimer transition with accompanying changes in receptor conformation [2]. There are several structures reported for the ECD of EGFR both in monomer and dimer conformations [9]. Analyses of these structures indicate the presence of residues that are not exposed in wild-type EGFR expressed under normal conditions. However, under oncogenic conditions, where receptors are highly expressed and may not be folded correctly, or where mutant versions of the receptor are expressed, cryptic structures may become exposed [4]. One structural element that is sterically unavailable under normal conditions is the 287–302 (numbering of mature receptor—which is equivalent to 301–326 for the full-length receptor) disulfide-limited loop (Fig. 1A) [10]. This loop is fully exposed in EGFRvIII and may become exposed when EGFR expression is very high or when ECD mutations alter the wild-type structure [4]. The development of dual targeting antibodies reactive for both EGFRvIII and oncogenic overexpressed EGFR (but not wtEGFR) can improve treatment outcomes while lowering toxicity for patients [11].

To produce monoclonal antibodies that could be useful in detecting and treating EGFR-driven cancers, we synthesized the 287–302 loop as a disulfide-limited peptide, coupled it with a carrier protein and immunized Balb/c mice. Sera from mice with high titers were identified for hybridoma production. Candidate hybridoma supernatants were then assayed for reactivity with wtEGFR and EGFRvIII. Seven antibodies with high reactivity for EGFRvIII and low reactivity for wtEGFR were isolated and characterized.

MATERIALS AND METHODS

Peptide synthesis

An 18-amino acid peptide, ‘ACGADSYEMEEDGVR KCK,’ which encompasses the 287–302 loop, including also an extra flanking amino acid at the amino and carboxyl termini (resulting in a 286–303 peptide), was synthesized at GenScript (Princeton, NJ, USA). A disulfide bond was generated between the cysteines at position 2 and 16 (corresponding to residues 287–302) by oxidation. For mouse immunizations, the peptide was conjugated to keyhole limpet hemocyanin (KLH) using glutaraldehyde at a final concentration of 0.125%. Several His-tag variants of the peptide were also synthetized for mapping the binding site of each antibody.
Cell culture

Mouse hybridomas were cultured in Dulbecco’s modified Eagle medium (DMEM), supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, NY, USA), 2 mM GlutaMAX (Thermo Fisher Scientific), Minimum Essential Media (MEM) non-essential amino acid (NEAA), which contains glycine, L-alanine, L-asparagine, L-aspartic acid, L-glutamic Acid, L-proline and L-serine at 100 μM each (Thermo Fisher Scientific), 100 U/ml Penicillin and 100 μg/ml streptomycin (Thermo Fisher Scientific). MDA-MB-468, A431 and WI-38 (purchased from ATCC, Manassas, VA, USA) were cultured in the above-mentioned medium without antibiotics. Further, F98EGFR and F98npEGFRvIII cells (purchased from ATCC, Manassas, VA, USA) were grown at 37°C with 5% CO2.

Flow cytometry

Antibodies were incubated with suspended cells (2.5 × 10^6 cells per well) in a 96-well plate at 4°C for 1 h in flow cytometry buffer consisting of phosphate-buffered saline (K D Medical, MD, USA), 2 mM ethylenediaminetetraacetic acid (K D Medical, MD, USA), 1% bovine serum albumin (BSA; Sigma-Aldrich, MO, USA) and 0.1% sodium azide (Sigma-Aldrich). Bound antibodies were detected with R-phycoerythrin-conjugated F(ab')2 goat anti-mouse IgG Fcγ (Cat # 115-116-071; Jackson ImmunoResearch, PA, USA) at 1: 250 dilution for 45–60 min at 4°C. Mouse IgG1 kappa and mouse IgG2b kappa (Cat# 14-4714-82 and 14-4732-82 respectively; Invitrogen, NY, USA) antibodies were used as isotype controls. Antibody binding was characterized with the SA3800 Spectral Analyzer (Sony Biotechnology, San Jose, CA, USA), the data were analyzed with FlowJo (Tree Star, Inc., Ashland, OR, USA) and displayed in histogram format with the median fluorescence intensity plotted. All flow cytometry experiments were repeated at least once.

ELISA

Human wtEGFR and EGFRvIII ECD C-terminal His-tagged proteins (Cat# EGR-H5222 and EGF1-H52H4, respectively) were purchased from ACROBiosystems (Newark, DE, USA). His-tagged proteins were bound to clear nickel-coated 96-well plates (Thermo Fisher Scientific) at 0.5 μg/ml for 1 h at room temperature. The plates were washed three times with PBS-Tween 20 (PBST) at 0.5% Tween (K D Medical and Sigma-Aldrich). Primary antibody or supernatant was incubated with rocking at room temperature for 1 h. Hybridoma supernatants were diluted to 1:100 or 1:10 in PBS with 5% BSA (Sigma-Aldrich). Purified antibodies were diluted in PBS with 5% BSA (Sigma-Aldrich) to the desired concentration. After the binding step, plates were washed three times with PBST. Peroxidase-conjugated donkey anti-mouse IgG (H + L) (Cat# 715-035-150; Jackson ImmunoResearch, ME, USA) was diluted at 1:20,000 and incubated with rocking at room temperature for 1 h. Plates were washed three times with PBST and the horseradish peroxidase (HRP)-labeled secondary antibody was developed with a 3,3′,5,5′-tetramethylbenzidine substrate solution (Thermo Fisher Scientific). The reaction was stopped with sulfuric acid 1 M (Sigma-Aldrich). The horseradish peroxidase reaction data were analyzed with a VersaMax plate reader using SoftMax Pro software (Molecular Devices, CA, USA).

Mouse immunization and hybridoma production

Mouse immunizations and hybridoma production were performed at GenScript. Briefly, five female 8-week old Balb/c mice were immunized intraperitoneally with KLH-conjugated peptide. The initial injection, on day 0, included 50 μg of immunogen per mouse with complete Freund’s adjuvant, then on days 14 and 28, 25 μg immunogen/mouse with incomplete Freund’s adjuvant were administered. After 35 days, sera were tested by enzyme-linked immunosorbent assay (ELISA) for antibody titers to the immunized peptide. High-titer mice were given a final boost at around day 56. When titers to the injected peptide were above background at a 1:128,000 dilution, the spleens were harvested and candidate hybridomas were produced through fusion with the myeloma cell line Sp2/0. The reactivity of supernatants from successful hybridoma clones was confirmed by ELISA against the peptide immunogen. Selected candidate hybridomas were then subcloned by limiting dilution and expanded for cryopreservation.

Antibody purification

Mouse hybridomas (cultured under conditions described above) were seeded at 11 × 10^6 cells per T175 flask in DMEM supplemented with 5% FBS, 2 mM GlutaMAX (Thermo Fisher Scientific), MEM NEAA, 100 U/ml penicillin and 100 μg/ml streptomycin (Thermo Fisher Scientific). Supernatants were harvested every 2 days and cells were resuspended in fresh media. Antibodies were purified with Protein A/G chromatography cartridges (Thermo Fisher Scientific) according to the manufacturer’s instructions. Fractions were eluted with 0.1 M sodium citrate pH 5 (Sigma-Aldrich) followed by 0.1 M glycine pH 2 (Sigma-Aldrich). All collected fractions were neutralized with 1 M Tris pH 8. Neutralized fractions eluted with 0.1 M glycine pH 2 were pooled and exchanged into PBS using Amicon Ultra-15 centrifugal filter units (Millipore, Ireland). The protein concentration of antibody preparations was determined with the bicinchoninic acid (BCA) assay using bovine serum albumin (BSA) fraction V as a standard (Thermo Fisher Scientific).

Cloning of heavy and light chain variable domains

For each hybridoma clone, total RNA was extracted with TRIzol Reagent (Cat # 15596-026; Ambion, TX, USA), following the manufacturer’s protocol. Total RNA was then reversed transcribed into complementary DNA (cDNA) using either isotype-specific antisense primers or universal primers with PrimeScript First Strand cDNA Synthesis Kit.
The binding affinity constant, \( K_d \), of the monoclonal antibody 40H3 against the EGFR peptide loop (GenScript), wtEGFR ECD or EGFRvIII ECD (Acrobiosystems) was measured using the Octet Red96 analyzer (Pall Life Sciences, New York, USA). All ligands contained a C-terminal His-tag and were captured on Ni-NTA biosensors and used as the ‘antigen’. Briefly, all ligand and antibodies were diluted in buffer composed of \( 1 \times \) PBS, 1% BSA and 0.05% Tween. Monoclonal 40H3 was diluted to 125 nM, 62.5 nM, 31.25 nM, 15.625 nM, 7.813 nM, 3.91 nM and 1.95 nM. 100 nM of each ligand was used as ‘antigen’. The Octet Red96 program was as follows: 10 min presoak, 60 s baseline establishment, 120 s antigen loading, 120 s baseline re-establishment after antigen loading, 120 s for ma40H3 association, and finally 20 min for dissociation. Binding kinetics were analyzed using the Octet RED96 software.

RESULTS

Selection of hybridomas reactive for EGFRvIII

EGFRvIII is expressed on the surface of a high percentage of malignant gliomas and some epithelial cancers [3, 16]. To generate cancer-reactive antibodies, mice were immunized with the disulfide-limited loop corresponding to amino acid 287–302 (numbering is for mature human EGFR which is represented in Fig. 1A) of the ECD of EGFR. Five Balb/c mice were immunized repeatedly with a KLH-conjugated peptide that included residues 286–303 (Fig. 1B). Hybridomas were established from two mice that exhibited particularly high titers to the immunogen (Supplementary Table 1). Initially, approximately 40 hybridoma supernatants were generated from each fusion and evaluated in an ELISA format for binding to the ECD of EGFRvIII-His and for cells expressing EGFRvIII (data not shown). From these, seven antibodies were chosen for further characterization.

Each of the supernatants of the seven selected hybridomas displayed a strong preference for binding to EGFRvIII-His over wtEGFR-His when screened by ELISA on nickel-coated 96-well plates (Fig. 2A). This result confirmed that the 287–302 loop is displayed on EGFRvIII but is poorly accessible on wtEGFR. Next, we assayed the same hybridoma supernatants (diluted 1:10) for binding to rat glioma F98 cells that had been transfected with either human EGFRvIII, F98pEGFRvIII (np = non phosphorylated), or human wtEGFR, F98EGFR [17]. Again, binding reflected a strong interaction of supernatants for the ECD of EGFRvIII (Fig. 2B) and poor reactivity for wtEGFR (Fig. 2C). The monoclonal antibody ma528 that reacts with domain III of EGFR and is expressed in both wild-type and EGFRvIII (Fig. 1A) [18, 19] was used as a positive control and was shown to bind equally well to the surface of either transfected cell line (Fig. 2B and C).

Characterization of purified antibodies

Using immobilized protein A/G, the seven antibodies from cloned hybridoma supernatants were purified for further characterization. (Fig. 3A). When compared with the original hybridoma supernatants, purified antibodies reacted in ELISA assays with qualitatively similar results. In fact, relatively low concentrations of antibodies (at 5 ng/ml) produced a robust signal with EGFRvIII-His and showed little or no binding to wtEGFR-His (Fig. 3B). When characterizing antibody binding to F98EGFR cells,
Figure 2. Binding of hybridoma supernatants to EGFR or EGFRvIII. (A) An ELISA was performed with diluted hybridoma supernatants (1:100) to detect binding to either His-tagged EGFRvIII ECD or His-tagged wtEGFR ECD. The ma528 antibody was used as a positive control at 5 ng/ml. Error bars represent standard deviation of triplicate. (B) and (C) Flow cytometry was used to detect binding of diluted hybridoma supernatants (1:10) to cells expressing either wtEGFR or EGFRvIII (F98EGFR or F98npEGFRvIII). The geometric mean of PE-Area (PE-A) plotted on the Y-axis represent the mean fluorescence intensity of antibody binding to cells. The ma528 antibody was used at 2 μg/ml as a positive control.

Histograms indicated strong binding by ma528 (median fluorescence intensity, from now on referred to only as the ‘median’, of 4041 units), very weak binding by ma40H3 (median of 187) and no binding by the remaining six monoclonal antibodies or the isotype controls (Fig. 3C). When binding was assessed on F98npEGFRvIII, the ma528 antibody showed the highest reactivity but was followed closely by substantial binding from all seven monoclonal antibodies with (median ranging from ~1000 to 1400) (Fig. 3C). The gene encoding EGFR is amplified and/or overexpressed in certain epithelial cancers. To assay binding to cells that overexpress EGFR, antibodies were added to either the triple-negative breast cancer cell line, MDA-MB-468 [20], or the epidermoid cancer line, A431 [21], or the non-cancerous normal lung fibroblast line, WI-38. Although neither MDA-MB-468 nor A431 expresses detectable amount of EGFRvIII, both lines overexpress EGFR with more than 10^6 receptors per cell [22]. On MDA-MB-468 cells, both ma528 and ma40H3 showed substantial binding, while the other six antibodies showed minimal reactivity over the isotype controls (Fig. 4—left panel). On A431 cells, a substantially similar result was achieved (Fig. 4—middle panel). However, on WI-38, which express wtEGFR at physiological levels, only ma528 showed substantial binding with a median of 2723 while all other antibodies had a median of less than 100 (Fig. 4—right panel). Thus, we conclude that functionally only antibody ma40H3 exhibited strong binding on EGFR overexpressing cancer cells. Of interest, when antibody binding to EGFRvIII-transfected F98 cells was assessed, all seven antibodies showed similar and substantial reactivity. In addition, no binding was observed with mouse mammary carcinoma cell line, 4 T1, or untransfected F98 cells (Supplementary Fig. 1) indicating
that all seven antibodies are specific for human EGFR. To characterize ma40H3 further, the binding affinity (described by the equilibrium dissociation constant, $K_D$) of ma40H3 against the 18 amino acids peptide loop (286–302), EGFRvIII ECD or wtEGFR ECD was examined. The $K_D$ for the 18 amino acids peptide loop was $\sim 1.1$ nM (Supplementary Fig. 2), while the $K_D$ for EGFRvIII ECD was $\sim 270$ pM (Supplementary Fig. 2). Given that there was little-to-no binding of ma40H3 to wtEGFR ECD, no $K_D$ value was obtained (Supplementary Fig. 2). The curve-fitting model employed for $K_D$ determination, using 1:1 binding model, provided a better fit relative to the 1:2 bivalent analyte model (data not shown). This suggested that there was minimal avidity artifact influencing the calculation of the $K_D$ value. The $K_D$ values indicate that ma40H3 exhibits a higher binding affinity toward EGFRvIII ECD relative to the 18 amino acid exposed peptide. The reasons for this are not yet clear.

**Sequence analysis of the antibodies, grouping by sequence**

The variable regions of both the heavy chains and lights of the seven antibodies were sequenced and the deduced amino acids determined (Fig. 5A and B). Based on sequence comparison and hierarchical clustering using a neighbor-joining algorithm of both $V_H$ and $V_L$ regions, the seven antibodies can be divided into four groups (Fig. 5C). There were small numbers of amino acid differences within each group. Specifically, in group 1, both 3D10 and 9G11 shared similar sequences with only two amino acids differing in the $V_H$ chain (Supplementary Fig. 2). Based on sequence comparison and hierarchical clustering using a neighbor-joining algorithm of both $V_H$ and $V_L$ regions, the seven antibodies can be divided into four groups (Fig. 5C). There were small numbers of amino acid differences within each group. Specifically, in group 1, both 3D10 and 9G11 shared similar sequences with only two amino acids differing in the $V_H$ chain (Supplementary Fig. 2). Given that there was little-to-no binding of ma40H3 to wtEGFR ECD, no $K_D$ value was obtained (Supplementary Fig. 2). The curve-fitting model employed for $K_D$ determination, using 1:1 binding model, provided a better fit relative to the 1:2 bivalent analyte model (data not shown). This suggested that there was minimal avidity artifact influencing the calculation of the $K_D$ value. The $K_D$ values indicate that ma40H3 exhibits a higher binding affinity toward EGFRvIII ECD relative to the 18 amino acid exposed peptide. The reasons for this are not yet clear.

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Figure 4. The 40H3 antibody reacts with EGFR expressed on cancer cell lines. Antibodies (2 μg/ml) were incubated with MDA-MB-468, A431 or WI-38 cell lines. The flow cytometry data are represented in a histogram where the number of cells (Y-axis) is plotted against fluorescent intensity (X-axis). The ma40H3 antibody, but not six others, reacted with MDA-MB-468 and A431 cells but not WI-38 cells. The ma528 was used as a positive control. The tables underneath the histograms indicate the median fluorescence intensity for each antibody in terms of median fluorescent intensity as well as the total number of cells (count) that were analyzed.

Locating key amino acids within the EGFR\textsubscript{286–303}, grouping by binding location

To aid in mapping the binding site of each antibody, three charged residues within the EGFR\textsubscript{287–302} loop, D290, E293 and R300 were each changed to alanine and produced as His-tagged peptides (Fig. 6). The wild-type sequence of the 287–302 loop was produced similarly. Because the charge residues were spread out across the loop, relative antibody binding activity could provide information about the location of critical residues involved in the interaction of each antibody. The results indicated that antibodies fell into one of three groups: 1) binding was lost when residues D290 and E293 were changed to alanine, 2) binding was lost only with a change of E293 to alanine; and finally, 3) reactivity was lost when R300 was changed to alanine. In summary, the seven EGFRvIII-reactive antibodies segregated into at least three groups based on binding characteristics toward variants of the 287–302 loop peptide. Group 1 contained five of the seven antibodies: 1D9, 3D10, 4A4, 9G11 and 11G3. Group 2 had 11E11 while group 3 included 40H3 (Supplementary Table 3).

Cytotoxicity of 40H3-PE38 to EGFR overexpressing cancer cell lines

Antibodies, with a few exceptions, are rarely cytotoxic for tumor cells, even when they bind with high affinities. Often, this prompts the addition of a toxic payload to the antibody. One such strategy is to produce therapeutics by engineering recombinant immunotoxins. Recombinant immunotoxins are fusion proteins that consist of an enzymatic domain of a protein toxin (the payload) fused genetically to a single-chain variable antibody fragment (scFv). To generate a 40H3-PE38 immunotoxin, the scFv region of 40H3 was produced synthetically and then fused to a 38 kDa C-terminal fragment of \textit{Pseudomonas aeruginosa} exotoxin A (PE) \cite{27, 28}. The cytotoxic potential of 40H3-PE38 was evaluated against cells that expressed either EGFRvIII or EGFR (F98\textsubscript{npEGFRvIII} and F98\textsubscript{EGFR} respectively) as well as cancer cell lines that are known for EGFR overexpression (i.e. MDA-MB-468 and A431). 40H3-PE38 exhibited cytotoxic activity against F98\textsubscript{npEGFRvIII} cells with an IC\textsubscript{50} of less than 1 nM (\textasciitilde0.4 nM) and was tenfold more potent relative to the same cells expressing wild-type EGFR, F98\textsubscript{EGFR}, which had an IC\textsubscript{50} of \textasciitilde4 nM (Supplementary Fig. 3). This result confirmed the antibody’s preferred binding specificity for EGFRvIII over wild-type EGFR, established by flow cytometry analysis (Fig. 3C). WI-38 cells which are derived from...
lungs fibroblasts and have normal EGFR expression did not show any loss of viability when incubated with 40H3-PE38 (IC50 > 10 nM) (Supplementary Fig. 3). MDA-MB-468 and A431 cells were treated with either 40H3-PE38 or with the parent antibody, ma40H3, for 72 h. 40H3-PE38 had an IC50 of less than 1 nM for A431 and an IC50 of less than 10 nM for MDA-MB-468 (Fig. 7). Although the immunotoxin was toxic in the low nanomolar range for both cancer cell lines, the parent antibody was not cytotoxic up to a concentration of 66 nM (Fig. 7). These data confirm both the selectivity and utility of a 40H3-derived scFv as a potential agent for antibody-directed cancer therapy.

**DISCUSSION**

EGFR is frequently mutated and/or overexpressed in various types of cancer and is a target of several kinds of therapies [3]. One approach is to target EGFR with monoclonal antibodies, such as cetuximab, which has been approved for metastatic colorectal cancer, head and neck cancer, non–small-cell lung cancer and squamous cell skin cancer [29]. Another approach uses tyrosine kinase inhibitors that inhibit the phosphorylation of EGFR substrates [30]. However, a major issue with current EGFR-targeted therapies are side effects stemming from the interactions with EGFR expressed by non-target normal tissues [31] (see below). The main purpose of this study was to identify novel antibodies that could differentiate mutated versions of EGFR from the wild-type receptor. This is especially relevant for glioblastoma where 25–33% of patients express the EGFRvIII variant [16]. EGFRvIII (Fig. 1A), is a tumor-specific variant, with an extracellular deletion of amino acids 6 to 273 causing structural changes to the remaining ECD and exposing a normally cryptic loop (amino acids 287–302) [4, 32]. This loop is hidden in both the wild-type receptor’s monomeric state as well as the fully active, ligand bound state [32]. Because the loop is exposed only under oncogenic conditions, it may be possible to differentially target oncogenic EGFR over wtEGFR. In addition, current tyrosine kinase inhibitors as well as monoclonal antibodies (i.e. cetuximab), have not proven efficacious in patients with glioblastoma [33]. The identification of novel antibodies that target the EGFRvIII could allow for the development of targeted treatments for glioblastomas and other cancers that express mutant EGFR.

By focusing on the conformational abnormalities that potentially exist in oncogenic versions of EGFR, we were able to raise seven antibodies that were all reactive for EGFRvIII. This set of antibodies, which can be divided...
Figure 6. Antibody reactivity for alanine replacement variants of the 286–303 peptide. Purified antibodies were tested for binding to each of three alanine-substituted peptides as well as a non-substituted peptide (i.e. wild type). Squared residues in the insert image indicate the location of residues that were replaced by alanine.

Figure 7. The 40H3-PE38 immunotoxin was cytotoxic for cancer cells expressing high levels of EGFR. An immunotoxin engineered with the 40H3 scFv killed A431 and MDA-MB-468 cells. The parent antibody at the same concentrations was not cytotoxic. The black line denotes the IC$_{50}$ of the immunotoxins.

into three distinct groups based on their epitope binding profiles, showed minimal binding to either the ECD of wild-type EGFR or cell lines expressing wild-type EGFR. In addition to reactivity for EGFRvIII, one of the anti-EGFR antibodies, 40H3, exhibited a relatively high affinity for epidermoid and breast cancer lines that overexpress EGFR but do not express EGFRvIII. Perhaps related to this, of the seven antibodies, only 40H3 lost reactivity with an alanine replacement for arginine at position 300, suggesting that this antibody binds near the C-terminus of the 287–302 loop. Because the other six antibodies, all reactive with the N-terminus of the loop, did not bind cells with ‘overexpressed EGFR’, we speculate that the N-terminus of this loop remains cryptic while the C-terminus can be exposed. The exposure of the C-terminal portion of the 287–302 loop could be the basis of future structural analysis and lead to the selection of additional antibodies focused more narrowly to this substructure.

The unique properties of the 40H3 antibody prompted us to develop a cytotoxic antibody-based therapeutic. We confirmed that antibody binding led to cell death when a payload was attached to a scFv variant of the parent antibody. The unmodified 40H3 was not toxic, establishing the need for toxic payload. Further, the 40H3-PE38 immunotoxin effectively killed A431 and MDA-MB-468 cells but not the WI-38 cell line which expressed wt EGFR at physiological levels. This result will likely spur the development of additional antibody-based therapeutics.

Although we have performed an initial characterization of our antibodies and conducted several proof-of-principle experiments, additional experiments and further development are necessary to bring these antibodies to the clinic. We need to characterize in vivo antitumor activity using mouse xenograft models of human cancer. Although antibodies alone did not have a cytotoxic effect in vitro, assess-
ing their effect in vivo might provide a different outcome given the complex role that the tumor microenvironment plays [34, 35], the nature of intercellular interactions [36] and the ligand-dependent nature of EGFR in certain cancer cell types [37]. Combinations of antibody-based therapeutics and small molecular weight EGFR inhibitors such as tyrosine kinase inhibitor could be examined also [38]. Other antibody-based treatments, such as antibody–drug conjugates and chimeric antigen receptor (CAR) T cell therapy, will be also likely be pursued.

Approved antibodies to the ECD of EGFR include cetuximab and panitumumab, both targeting domain III of EGFR (one of the ligand binding domains). Antibodies were developed to block ligand binding sites on EGFR and reduce signaling through this receptor. However, these antibodies do not readily distinguish EGFR expressed on cancer cells from EGFR expressed on normal cells. Causing problems clinically where patients experience difficult side effects from damage to normal tissues. This has led to the development of therapeutics with selectivity for cancer over normal tissues. In fact, a series of publications spanning the past 12 years has reported on the biology of the monoclonal antibody, 806 (and its variants), suggesting that it may exhibit fewer side effects than either of the two approved antibodies. Because of the improved selectivity for cancer cells, the 806 antibody was modified to carry a cytotoxic payload which is now termed ABT-414 [10, 12, 28–30]. ABT-414 is currently in advanced clinical trials, producing encouraging results in the treatment of patients with glioblastomas [39, 40]. The smaller size of immunotoxins (60 kDa) relative to the full-length antibody–drug conjugates (~155 kDa) would offer a better option for penetrating solid tumor (e.g. glioblastoma). Pre-screening patients for EGFR and/or EGFRvIII positive tumors will also increase the efficacy of treatment. Finally, the approach of producing cancer-selective therapeutics now includes the development of small molecular weight agents with a binding preference for mutant forms of EGFR such as the kinase inhibitors osimertinib and rociletinib which bind with high affinity to the T790 M mutant of EGFR.

Here we report on the development of antibodies reactive for EGFR with a preference for forms that are expressed on cancer cells. Of the seven antibodies we isolated, six bound EGFRvIII but not full-length EGFR variants even those that are over expressed on cancer cells. However, one of the seven reacted with EGFRvIII but also bound to cancer-expressed EGFR such as those expressed from amplified EGFR genes in A431 and MDA-MD-468 cells. When the location of antibody binding was mapped within the 287–302 peptide, six of the seven antibodies reacted near the N-terminus, while the one antibody (40H3) which reacted with overexpressed EGFR was mapped to the C-terminus of the 287–302 peptide. These results may direct the future development of therapeutic antibodies to locally misfolded epitopes on receptors that are overexpressed on cancer cells.

Supplementary Data

Supplementary Data are available at ABT Online.

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