ANTI-VEGFR2 HUMAN ANTIBODY FOR ANTI-ANGIOGENIC AND TARGETED CANCER THERAPY

FIELD OF THE INVENTION

The present invention relates generally to antibodies with anti-cancer activities, and more specifically to anti-VEGFR2 antibodies.

BACKGROUND OF THE INVENTION

Angiogenesis rarely occurs in adult healthy tissues. Vascular endothelial growth factor receptor 2 (VEGFR2) is expressed infrequently and at low levels in normal endothelial cells, as compared to tumor-associated endothelial cells. VEGFR2 expression is 3- to 5- folds higher in tumor vessels than that in normal vessels. Immunohistochemistry in biopsies of cancer patients further confirmed that VEGFR2 expression is significantly elevated in tumor vessels when compared with the vascular endothelium in normal tissues adjacent to the tumor region. Notably, expression of *VEGFR2* is greater in high-metastatic tumor vessels than in low-metastatic tumor vessels.

VEGFR2 expression was originally shown to be restricted to the vessels of tumor tissues. However, recent studies have provided evidence that VEGFR2 is also present in malignant tumor cells. Circulating tumor epithelial cells in the blood of breast cancer patients were found to express VEGFR2, and thus such expression is associated with tumor metastasis and prognosis. Therefore, blocking VEGFR2-mediated signaling transduction to concomitantly inhibit tumor endothelial and malignant cells is considered an excellent strategy for the development of anticancer therapeutics.

The results from clinical studies indicate that fully human therapeutic antibodies against VEGFR2 are safe and well-tolerated. They show promise as an emerging therapy for cancer by blocking tumor angiogenesis. Therefore, development of a novel anti-VEGFR2 human antibody with enhanced therapeutic efficacy will benefit cancer patients.

SUMMARY OF THE INVENTION

In one aspect, the invention relates to an isolated antibody or an antigen-binding fragment thereof that has a specific binding affinity to an epitope located within the domain 1 or domain 3 of human vascular endothelial growth factor receptor 2 (VEGFR2; SEQ ID NO: 74), wherein the epitope within the domain 3 of the VEGFR2 is located between amino acid residues 250 and 270 of SEQ ID NO: 74.

In one embodiment of the invention, the epitope comprises the amino acid sequence of NWEYPS (SEQ ID NO: 66). The epitope does not comprise the amino acid sequence of GID, KH, or GLMTK (SEQ ID NO: 75).

In another embodiment of the invention, the antibody or antigen-binding fragment thereof of the invention exhibits a specific binding affinity to tumor vascular endothelial cells.

The antibody or antigen-binding fragment thereof of the invention comprises a heavy chain variable region (V_H) and a light chain variable region (V_L), the V_H comprising V_H CDR1, V_H CDR2, and V_H CDR3, and the V_L comprising V_L CDR1, V_L CDR2, and V_L CDR3,

(i) wherein:

the V_H CDR1, V_H CDR2, and V_H CDR3 comprise the amino acid sequence of SEQ ID NO: 1, SEQ ID NO: 2, and SEQ ID NO: 3, respectively; and the V_L CDR1, V_L CDR2, and V_L CDR3 comprise the amino acid sequence of SEQ ID NO: 4, Ala Ala Ser, and SEQ ID NO: 5, respectively;

or (ii) wherein:

the V_H CDR1, V_H CDR2, V_H CDR3 comprise the amino acid sequence of SEQ ID NO: 6, SEQ ID NO: 7, and SEQ ID NO: 8, respectively; and the V_L CDR1, V_L CDR2, and V_L CDR3 comprise the amino acid sequence of SEQ ID NO: 9, Asp Ala Ser, and SEQ ID NO: 10 or 73, respectively;

or (iii) wherein:

the V_H CDR1, V_H CDR2, and V_H CDR3 comprise the amino acid sequence of SEQ ID NO: 11, SEQ ID NO: 12, and SEQ ID NO: 13, respectively; and the V_L CDR1, V_L CDR2, and V_L CDR3 comprise the amino acid sequence of SEQ ID NO: 14, Asp Ala Ser, and SEQ ID NO: 15, respectively;

or (iv) wherein:

the V_H CDR1, V_H CDR2, and V_H CDR3 comprise the amino acid sequence of SEQ ID NO: 16, SEQ ID NO: 17, and SEQ ID NO: 18, respectively; and the V_L CDR1, V_L CDR2, and V_L CDR3 comprise the amino acid sequence of SEQ ID NO: 19, Gly Ala Ser, and SEQ ID NO: 20, respectively;

or (v) wherein:

the V_H CDR1, V_H CDR2, and V_H CDR3 comprise the amino acid sequence of SEQ ID NO: 21, SEQ ID NO: 22, and SEQ ID NO: 23, respectively; and the V_L CDR1, V_L CDR2, and V_L CDR3 comprise the amino acid sequence of SEQ ID NO: 24, SEQ ID NO: 25, and SEQ ID NO: 26, respectively.

In another embodiment of the invention, the V_H CDR1, V_H CDR2, and V_H CDR3 comprise the amino acid sequence of SEQ ID NO: 6, SEQ ID NO: 7, and SEQ ID NO: 8, respectively; and the V_L CDR1, V_L CDR2, and V_L CDR3 comprise the amino acid sequence of SEQ ID NO: 9, Asp Ala Ser, and SEQ ID NO: 10 or 73, respectively.

In another embodiment of the invention, the antibody or antigen-binding fragment thereof comprises: (a) a heavy chain variable region (V_H) comprising the amino acid sequence of SEQ ID NO: 76; and (b) a light chain variable region (V_L) comprising the amino acid sequence of SEQ ID NO: 77 or 78.

In another embodiment of the invention, the antibody or antigen-binding fragment thereof is a single-chain variable fragment, a Fab fragment, or a Fv fragment.

In another embodiment of the invention, the antibody or antigen-binding fragment thereof is a fully human antibody.

In another embodiment of the invention, the antibody or antigen-binding fragment thereof is labeled with a detectable compound or an enzyme.

In another aspect, the invention relates to a composition comprising a therapeutically effective amount of an antibody or antigen-binding fragment thereof of the invention and a pharmaceutically acceptable vehicle or carrier.

In one embodiment of the invention, the composition further comprises a chemotherapeutic agent. In one embodiment of the invention, the chemotherapeutic agent is docetaxel.

Further in another aspect, the invention relates to use of an antibody or antigen-binding fragment thereof or a composition of the invention in the manufacture of a medicament for inhibiting tumor growth, tumor angiogenesis, and/or inducing cancer cell cytotoxicity in a subject in need thereof.

In one embodiment of the invention, the tumor and/or cancer cell express VEGFR2. The use of the antibody or antigen-binding fragment thereof may further comprise use of an additional chemotherapeutic agent such as docetaxel in the manufacture of a medicament for inhibiting tumor growth, tumor angiogenesis, and/or inducing cancer cell cytotoxicity in the subject in need thereof.

Alternatively, the invention relates to an antibody or antigen-binding fragment thereof or a composition of the invention for use in inhibiting tumor growth, tumor angiogenesis, and/or inducing cancer cell cytotoxicity in a subject in need thereof.

The invention also relates to a method for inhibiting tumor growth, tumor angiogenesis, and/or inducing cancer cell cytotoxicity, comprising:

administering to a subject in need thereof a composition comprising a therapeutically effective amount of an isolated antibody or antigen-binding fragment thereof of the invention and a pharmaceutically acceptable carrier, and thereby inhibiting tumor growth, tumor angiogenesis, and/or inducing cancer cell cytotoxicity in the subject in need thereof.

The method of the invention may further comprise administering to the subject in need thereof a composition comprising a therapeutically effective amount of a chemotherapeutic agent such as

docetaxel. In one embodiment of the invention, the chemotherapeutic agent is simultaneously administered to the subject in need thereof.

In one embodiment of the invention, the tumor or cancer is at least one selected from the group consisting of pancreatic, breast, lung, leukemia, prostate and ovary cancer.

Yet in another aspect, the invention relates to a method of detecting the presence of VEGFR2 on tumor vascular endothelial cells or cancer cells in a biological sample, comprising:

(i) admixing the antibody or antigen-binding fragment thereof of the invention with the biological sample;

(ii) allowing the antibody or antigen-binding fragment thereof and the VEGFR2 on the tumor vascular endothelial cells or cancer cells in the biological sample to interact and form a complex; and

(iii) detecting the presence of the VEGFR2 on the tumor vascular endothelial cells or cancer cells in the complex.

In one embodiment of the invention, the biological sample is a tissue specimen from a patient.

In another embodiment of the invention, the presence of the VEGFR2 on the tumor vascular endothelial cells or cancer cells in the complex is detected by immunoassay.

These and other aspects will become apparent from the following description of the preferred embodiment taken in conjunction with the following drawings, although variations and modifications therein may be affected without departing from the spirit and scope of the novel concepts of the disclosure.

The accompanying drawings illustrate one or more embodiments of the invention and, together with the written description, serve to explain the principles of the invention. Wherever possible, the same reference numbers are used throughout the drawings to refer to the same or like elements of an embodiment.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGs. 1A-E show the results of selection and identification of phage-displayed scFvs against VEGFR2. Phage display biopanning for VEGFR2-Fc recombinant protein. After four rounds of biopanning, the recovery rate of the phages was increased by 3,455-fold over that of the first round. cfu, colony-forming units. (B) Comparison of the binding of selected phage clones to VEGFR2-Fc protein by ELISA with a 1×10^{9} cfu phage titer. (C) Cellular VEGFR2 binding affinity of phage clones were evaluated on HUVECs by flow cytometry with 1×10^{10} cfu. (D) Soluble anti-VEGFR2 scFvs were purified and analyzed by SDS-PAGE with Coomassie blue staining. (E) Immunofluorescent staining for human tumor vasculature. Frozen sections of surgical specimens of lung cancer patients were probed with anti-VEGFR2 scFvs, followed by anti-E tag antibody and rhodamine-conjugated

secondary antibody staining. Vascular endothelium was stained with anti-human CD31 antibody, and then incubated with FITC-conjugated secondary antibody. Nuclei were stained with DAPI; Con-scFv, control scFv.

FIGs. 2A-F show that anti-VEGFR2 scFv suppresses VEGF-A binding and activation of VEGFR2 in HUVECs. (A) Analysis of the competition ability of anti-VEGFR2 scFv with VEGF-A by ELISA. The amount of VEGF-A binding to immobilized VEGFR2 in the absence of competitors was considered to be 100%. (B) Phosphorylated VEGFR2 (Pho. VEGFR2) expression in HUVECs treated with VEGF-A and scFv competitors was detected by Westernblot. Quantification of phosphorylated VEGFR2 was based on luminescence intensity, and normalized to total VEGFR2. (C to F) Epitope mapping of R2S12. (C) Sequence alignment of VEGFR2 domain 3 (VEGFR2-D3) of human (from 221 a.a. to 320 a.a. of SEQ ID NO: 74) and mouse (from 223 a.a. to 322 a.a. of SEQ ID NO: 80). Residues that differ between the two species are boxed. The residues involved in mutants M1 and M2 are underlined. The filled and open circles are used to indicate human VEGFR2-D3 residues in contact with 1121B and 6.64 antibodies, respectively. (D) Graphic depicting theVEGFR2-D3 backbone; NWEYPS (SEQ ID NO: 66) residues (M1) responsible for R2S12 binding are highlighted. (E) Model of the surface of VEGFR2-D3. NWEYPS (SEQ ID NO: 66) residues, i.e., the M1 area, is delineated by the black line. (F) The residues that make contact with 1121B and 6.64 on the surface of VEGFR2-D3 are indicated. The contacting residues of 1121B, which are localized in the M1 area, are also indicated. (N, N terminus; C, C terminus.)

FIGs. 3A-D show affinity maturation of anti-VEGFR2 hAb, and analysis of anti-VEGFR2-AF hAb activity. (A) Amino acids of the light chain variable domain of CDR3 (V_L-CDR3) of R2S12 (SEQ ID NO: 10) and R2S12AF (SEQ ID NO: 73). Residues that differ between R2S12 and R2S12AF are boxed. (B) Kinetic constants of anti-VEGFR2 and anti-VEGFR2-AF hAb, as determined using purified IgG and a BIACORE T100™. The K_d value was calculated using BIACORE T100™ evaluation software. (C) Competitive ELISA was performed to examine dose-dependent inhibition of VEGF-A binding to VEGFR2 by human antibody. A value of 100% was attributed to the binding of 4 nM VEGF-A to immobilized VEGFR2 in the absence of competitors. Error bar, SD; n=4. (D) Determination of the binding activity of anti-VEGFR2 antibody to HUVEC by flow cytometry analysis. Antibody concentration: 0.1 μg/ml.

FIGs. 4A-B show that anti-VEGFR2-AF hAb inhibits the VEGFR2 signaling pathway and disrupts capillary structure formation in HUVECs. (A) Capillary structure formation assays were performed using MATRIGEL®-coated μ-Slides. HUVECs (4×10^4 cells per well) were incubated with 0.2% FBS and treated with 40 ng/ml VEGF-A, or 40 ng/ml VEGF-A together with NHIgG, IMC-1121B, or anti-VEGFR2-AF antibody for 5 hours at 37°C. Tubular structures were observed

under phase contrast, and the relative sprout length (lower panel) and branching points (upper panel) were quantitatively measured with ImageJ software. All data were obtained from three independent experiments. (B) HUVECs were treated with 50 ng VEGF-A or 100 nM anti-VEGFR2-AF or IMC-1121B for 10 min at 37°C.Total protein was prepared from treated HUVECs and examined by Western blot analysis. α- tubulin was used as a loading control.

FIGs. 5A-F show characterization of VEGFR2 activity in human prostate cancer cells. (A) Analysis of *VEGFR2* expression in the indicated cell lines by quantitative RT-PCR. 293T cells were used as a negative control. Expression of *VEGFR2* was normalized to that of *GAPDH*. (B) PC-3 cells treated with VEGF-A were subjected to colony formation, MTT, and invasion assays. n=6 in each group. (C) PC-3 cells were treated with VEGFR2-targeted shRNA (shVEGFR2), and *VEGFR2* expression was analyzed by quantitative RT-PCR. Luciferase shRNA (shLuc) was used as a negative control. (D) MTT, colony formation, and Transwell invasion assays were performed to analyze shVEGFR2-PC-3 cells treated with VEGF-A. (E) Box plots showing relative *VEGFR2* expression in metastatic prostate tumors as compared with benign and primary tumors, as determined using a public microarray database. (F) Immunohistochemical staining of a human prostate cancer tissue array (PRC481, Pantomics) using anti-VEGFR2 antibody (55B11, Cell Signaling) to analyze VEGFR2 protein expression in human normal and tumor prostate tissues.

FIGs. 6A-E show analysis of the therapeutic efficacy of anti-VEGFR2-AF hAb in a PC-3 mouse xenograft model. (A) The treatment schedule. (B) The tumor growth profiles of mice of each group. (C) Body weight of each group. (D) At the end of the treatment period, tumor mass was dissected from each mouse.(E) Tumor weight was measured at the end of the treatment period. All data are shown as the mean of nine mice per group; bars, SE; *, $P< 0.05$.

FIGs. 7A-E show that anti-VEGFR2-AF hAb exhibits greater antitumor activity than IMC-1121B in a HL60 mouse xenograft model. (A) Kaplan-Meier survival analysis of mice of each group. Survival was significantly prolonged in the anti-VEGFR2-AF hAb group compared with the IMC-1121B group based on log-rank test (P =0.0284). (B) Body weight of mice of each group. (C) Ovaries were dissected from each mouse after death. The ovaries from a NSG mouse without leukemia were used as a normal control. (D) Ovary volume in mice treated with IMC-1121B or anti-VEGFR2-AF. (E) Morphometric analysis of lymph node (LN) changes in leukemia-tumor bearing mice. Leukemia cells that had metastasized to lymph nodes were dissected from mice of the indicated groups (n=9 for each group).

FIG. 8 shows construction of VEGFR2-expressing vectors. Schematic presentation of constructs expressing deletion or substitution mutants of human VEGFR2 domains. There are seven

immunoglobulin-like domains in the extracellular region of VEGFR2, which are labeled I to VII. TM: transmembrane domain.

FIGs. 9A-B show the results of selection and identification of scFvs against VEGFR2 using a phage-displayed synthetic scFv library. (A) Affinity maturation for R2S12. The phage-displayed R2S12-V_L-CDR3 mutagenic scFv library was incubated for 1 hour at 4°C with 0.1 μg VEGFR2-Fc immobilized on Protein G DYNABEADS®. Subsequently, the beads were washed four times with PBS containing 1% TWEEN™ 20. After four rounds of biopanning, the recovery rate of the phages was increased by 929-fold over that of the first round. (B) Comparison of the binding activity of the indicated concentrations of R2S12 and R2S12-AF scFv to VEGFR2-Fc protein, as assayed by ELISA. *Error bar*, SE.

FIGs. 10A-C show that anti-VEGFR2-AF hAb antagonized VEGF-A mediated cellular activity in PC-3 cells. (A) Transwell assays were carried out to examine the invasion capacity of PC-3 cells subjected to the indicated treatments. Upper panel: Giemsa staining of invasive cells. 100× magnification; n=3 in each group; scale bar, 150 μm. (B) A total of 1×10^3 PC-3 cells were seeded in a six-well plate, and treated with or without 100 ng/ml VEGF-A and 10 μg/ml of NHIgG, IMC-1121B, or anti-VEGFR2-AF antibody. The plate was incubated for 7 days to allow colony formation. Cell colonies were visualized by crystal violet staining. The relative number of colonies was calculated in each well after elution of crystal violet solution. n=3 in each group. (C) Wound healing assay. PC-3 cells were incubated in RPMI with 2% FBS, and stimulated by treatment with 100 ng/ml VEGF-A in the presence or absence of 10 μg/ml NHIgG, IMC-1121B, or anti-VEGFR2-AF, individually. Images were taken after 0, 16, and 36 hours of incubation. Scale bar, 150μm. n=3 in each group. *Error bar*, SE.

FIGs. 11A-B show investigation of vascular endothelium and apoptotic cells in tumor tissue after drug treatment. Frozen tumor sections were prepared from mice of each group at the end of the treatment period. (A) Sections were stained with anti-CD31 antibody to visualize tumor blood vessels. CD31-positive endothelium was quantitatively measured using ImageJ software. (B) Apoptotic cells in frozen tumor sections were analyzed using TUNEL assay. The apoptotic cells were quantified using ImageJ software. The sections were stained with DAPI for indication of all cells. n=5; Scale bar, 100 μm; 200× magnification; *Error bar*, SE; **, $P < 0.01$; ***, $P < 0.001$.

FIGs. 12A-B show histopathological phenotype of ovaries of human leukemia xenograft mice following antibody treatment. Ovaries were harvested from HL-60 tumor-bearing mice after treatment with saline, NHIgG, IMC-1121B, or anti-VEGFR2-AF hAb. The tissues were sliced and stained with hematoxylin and eosin (H&E), revealing infiltration with leukemia cells. The group

treated with Anti-VEGFR2-AF showed fewer blood vessels, and retained some primary oocytes. n=9 in each group. (A) Ovary at 200× magnification. Scale bar, 500 μm. (B) Scale bar, 150 μm.

DETAILED DESCRIPTION OF THE INVENTION

Various embodiments of the invention are now described in detail. Referring to the drawings, like numbers indicate like components throughout the views. As used in the description herein and throughout the claims that follow, the meaning of "a", "an", and "the" includes plural reference unless the context clearly dictates otherwise. Also, as used in the description herein and throughout the claims that follow, the meaning of "in" includes "in" and "on" unless the context clearly dictates otherwise. Additionally, some terms used in this specification are more specifically defined below.

DEFINITIONS

The terms used in this specification generally have their ordinary meanings in the art, within the context of the invention, and in the specific context where each term is used. Certain terms that are used to describe the invention are discussed below, or elsewhere in the specification, to provide additional guidance to the practitioner regarding the description of the invention. For convenience, certain terms may be highlighted, for example using italics and/or quotation marks. The use of highlighting has no influence on the scope and meaning of a term; the scope and meaning of a term is the same, in the same context, whether or not it is highlighted. It will be appreciated that same thing can be said in more than one way. Consequently, alternative language and synonyms may be used for any one or more of the terms discussed herein, nor is any special significance to be placed upon whether or not a term is elaborated or discussed herein. Synonyms for certain terms are provided. A recital of one or more synonyms does not exclude the use of other synonyms. The use of examples anywhere in this specification including examples of any terms discussed herein is illustrative only, and in no way limits the scope and meaning of the invention or of any exemplified term. Likewise, the invention is not limited to various embodiments given in this specification.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. In the case of conflict, the present document, including definitions will control.

The term "treating" or "treatment" refers to administration of an effective amount of the compound to a subject in need thereof, who has cancer, or a symptom or predisposition toward such a disease, with the purpose of cure, alleviate, relieve, remedy, ameliorate, or prevent the disease, the symptoms of it, or the predisposition towards it. Such a subject can be identified by a health care professional based on results from any suitable diagnostic method.

"An effective amount" refers to the amount of an active compound that is required to confer a therapeutic effect on the treated subject. Effective doses will vary, as recognized by those skilled in

the art, depending on rout of administration, excipient usage, and the possibility of co-usage with other therapeutic treatment.

The term "chemotherapeutic agent" refers to a pharmacological agent that is known to be of use in the treatment of cancer.

The "Guidance for Industry and Reviewers Estimating the Safe Starting Dose in Clinical Trials for Therapeutics in Adult Healthy Volunteers" published by the U.S. Department of Health and Human Services Food and Drug Administration discloses a "therapeutically effective amount" may be obtained by calculations from the following formula:

HED = animal dose in mg/kg × (animal weight in kg/human weight in kg) $^{0.33}$.

Ramucirumab (i.e., IMC-1121B) trade name is CYRAMZA®.

Human vascular endothelial growth factor receptor 2 (VEGFR2) domain 1 region is from 45 a.a. to 110 a.a., and domain 3 is from 224 a.a. to 320 a.a.

Sequence identifiers:

QQLDDIPIT (R2S12AF variable light chain CDR3; SEQ ID NO: 73); VGFR2 (HUMAN Vascular endothelial growth factor receptor 2; SEQ ID NO: 74); GLMTKK (SEQ ID NO: 75);

The V_H region of R2S12 and R2S12-AF are the same (SEQ ID NO: 76):
QVNLRESGGGLVKPGGSLRLSCAASGFTFGSYTMNWVRQAPGKGLEWVASITSGSSYIFYT DSVKGRFTISRDNSRSSLFLQMNSLRAEDTAIYYCARGSASAFDIWGQGTMVTVSS;

The V_L region of R2S12 (SEQ ID NO: 77):
DIQMTQSPSSLSASVGDRVTITCKASDDIINYLNWYQQKPGEAPKLLIYDASILETGVPSRFSG SGSGTDFTFTISSLQPEDIATYYC**QQ<u>Y</u>D<u>IL</u>P<u>L</u>T**FGGGTKLEIK;

The V_L region of R2S12AF (SEQ ID NO: 78):
DIQMTQSPSSLSASVGDRVTITCKASDDIINYLNWYQQKPGEAPKLLIYDASILETGVPSR FSGSGSGTDFTFTISSLQPEDIATYYC**QQ<u>L</u>D<u>DI</u>P<u>I</u>T**FGGGTKLEIK;

The amino acid residues that are different between R2S12 V_L and R2S12AF V_L are underlined above.

Human IgG1 constant region (SEQ ID NO: 79); Mouse VEGFR2 amino acid sequence (SEQ ID NO: 80).

Abbreviation: complementarity determining regions (CDRs); Fab (fragment, antigen-binding region); F_V region (variable domain).

EXAMPLES

Without intent to limit the scope of the invention, exemplary instruments, apparatus, methods and their related results according to the embodiments of the present invention are given below. Note that titles or subtitles may be used in the examples for convenience of a reader, which in no

way should limit the scope of the invention. Moreover, certain theories are proposed and disclosed herein; however, in no way they, whether they are right or wrong, should limit the scope of the invention so long as the invention is practiced according to the invention without regard for any particular theory or scheme of action.

Materials and Methods

Isolation of phages binding to VEGFR2 from a phage-displayed scFv library

A human naïve phage-displayed scFv library with 6×10^{10} complexity previously established in our laboratory was used for selection. The scFv library was subtracted non-specific binding with protein G DYNABEADS® (Invitrogen), and subsequently incubated with VEGFR2-Fc recombinant protein (R&D Systems)-immobilized DYNABEADS®. After washing with PBS containing 0.1% TWEEN™ 20 (PBST0.1), phages bound to VEGFR2-Fc were recovered by infection with *E. coli* TG1 cells. After determination of phage titer, the next round of biopanning was performed.

Competitive VEGF binding assay

Various concentrations of anti-VEGFR2 scFvs were mixed with 3 nM human VEGF-A (Peprotech), and added to 96-well plates coated with 1 μg/ml of VEGFR2-Fc and pre-blocked in 1% BSA. After incubation for 1 hr at RT and washes with PBST, the bound VEGF molecules were detected using anti-VEGF mAb (GeneTex) and HRP-labeled goat anti-mouse IgG. The reaction was developed with a mixture of OPD and H_2O_2, and subsequently terminated with 3 N HCl. The absorbance was determined using a microplate reader at 490 nm.

Human tumor vasculature staining with anti-VEGFR2 scFvs

Human lung cancer surgical specimens were obtained from the Department of Pathology, National Taiwan University Hospital. Frozen section slides were washed with PBS and then fixed with paraformaldehyde. After washing with PBS, slides were blocked with normal horse serum (Vector), and incubated with the scFv. After washing with PBST, a mixture of rabbit anti-E tag antibody (Bethyl Laboratories) and mouse anti-human CD31 mAb (BD) was added, and the slides were incubated for 1 hr. The coverslips were stained for 1 hour with FITC-labeled anti-mouse IgG, rhodamine-labeled goat anti-rabbit IgG, and DAPI, and captured using an Inverted Fluorescence Microscope (Zeiss, Axiovert 200M).

Tube formation assay

MATRIGEL® (BD Biosciences) was thawed at 4°C overnight, and 10 μl MATRIGEL® was added to each well of a pre-chilled μ-Slide Angiogenesis (Ibidi); the slide was then incubated at 37°C for 15 minutes. Starved HUVECs (4×10^4 cells) were added to EBM-2 containing 0.2% serum with or without 40 ng/ml VEGF-A and anti-VEGFR2 antibodies. After 24 hours of incubation, endothelial cell tube formation was assessed with an OLYMPUS inverted microscope and digital camera

(OLYMPUS, DP-12). Tubular lengths and branching points were quantitatively evaluated with ImageJ software. Inhibition percentage by antibodies was expressed as a percentage of that in VEGF-A-treated wells without competitor.

Clinical data set analysis

Raw microarray data were downloaded from the Gene Expression Omnibus at the the National Center for Biotechnology Information (NCBI) website. Raw data were normalized. GEO profile GDS2545 / 1954_at / KDR was used for metastatic prostate cancer analysis.

Construction and expression of anti-VEGFR2 human antibody

The V_H region of R2S12, R2S12-AF, and IMC-1121B (Lu et al., (2003) "Tailoring in vitro selection for a picomolar affinity human antibody directed against vascular endothelial growth factor receptor 2 for enhanced neutralizing activity" J Biol Chem *278*, 43496-43507) were cloned separately into modified expression vector pcDNA5-FRT-Gamma1 with a signal peptide and human IgG1 constant region, using *Age*I and *Nhe*I sites. In addition, the V_L region of R2S12, R2S12-AF, and IMC-1121B were separately cloned into modified expression vector p-Kappa-HuGs, using *Age*I and *Eco*RV sites. Both heavy and light chain gene-containing plasmids were combined into a biscistronic vector to generate a single vector system. The plasmids were transfected into FLPIN™-CHO cells (Invitrogen). The transfected cells were selected using hygromycin B after 2-3 weeks to establish stable clones; these clones were cultured in SFM4CHO media (Thermo Scientific) to produce human antibodies. After 2 weeks of incubation, cultured media of stable clones was collected, centrifuged, and filtered through a 0.45 μm membrane. The supernatant was then subjected to protein G column chromatography (GE healthcare) for purification of anti-VEGFR2 human IgG. After dialysis of eluents with PBS, the concentration of antibody was assessed using Bradford reagent (Thermo Scientific) and spectrophotometry.

Affinity maturation of anti-VEGFR2 human IgG

Affinity maturation was performed as previously described. Briefly, we constructed a synthetic phage-displayed scFv library comprised of the V_H and V_L gene repertoire of R2S12, with random mutations introduced at seven amino acid residues of V_L-CDR3. This synthetic library was used to perform biopanning for VEGFR2-Fc-immobilized DYNABEADS®. After four to five rounds of stringent *in vitro* biopanning, positive clones were screened and identified by ELISA. Superior VEGFR2-binding clones were identified through comparison to the respective parental clone.

Measurement of binding kinetics

The affinity and kinetics of anti-VEGFR2 antibodies were measured by surface plasmon resonance in a BIACORE T100™ (GE healthcare). VEGFR2-Fc protein was coupled to an EDC- and NHS-activated CM5 sensor chip in a BIACORE flow cell, and then blocked with ethanolamine

according to the manufacturer's directions. Associated and dissociated phases were monitored under continuous flow of 30 µl/min, using antibody concentrations ranging from 0.1 to 100 nM for 5 min. Regeneration was performed by injection of regenerate buffer (0.2 M NaCl, 10 mM glycine, pH 2.7). To determine binding constants, the sensorgrams were fit globally to a sample 1:1 interaction model using BIAevaluation software (GE healthcare).

Animal models

Procedures involving animals and their care were conducted according to the guidelines of the Academia Sinica Institutional Animal Care and Utilization Committee in compliance with national and international laws and policies. Non-obese diabetic-severe combined immunodeficiency (NOD/SCID) mice were purchased from the National Laboratory Animal Center (Taiwan). The human prostate cancer xenograft tumor model was developed by subcutaneously injecting 2×10^6 PC-3 cells into the dorsal flank of a six-week old male mouse. Animals were monitored daily for general health, and body weights were measured twice weekly. Tumor size was measured with slide calipers and calculated as length × width^2 × 0.52. Mice with size-matched tumors (50 mm^3) were randomly assigned to different treatment groups (n=9) and intravenously injected with normal human IgG (NHIgG; Jackson ImmunoResearch), IMC-1121B, anti-VEGFR2-AF antibodies, or an equivalent volume of PBS through the tail vein. An antibody dose of 20 mg/kg was injected twice a week for four weeks. For combination therapy, docetaxel (ScinoPharm Taiwan) was also intravenously administered at a dose of 5 mg/kg once a week for three weeks. At the end of the experiment, tumor tissue and visceral organs were removed and fixed for histological analysis.

For systemic leukemia engraftment studies, NOD/SCID/IL2Rγ$^{-/-}$ (NSG) mice were obtained from the Animal Center of the Institute of Cellular and Organismic Biology, Academia Sinica. Six-week old females were intravenously injected with 5×10^6 HL-60 cells through the tail vein. Animals were monitored daily for general health, and body weights were measured twice weekly. At 3 days after tumor inoculation, mice were randomly selected (n=9) for intravenous injection with 20 mg/kg NHIgG, IMC-1121B, anti-VEGFR2-AF antibodies, or an equivalent volume of PBS, twice weekly. Mice were observed daily for signs of toxicity, and the survival times were recorded. At the end-point of treatment, the visceral organs of each mouse were removed and fixed for further histological examination.

Cell culture

HUVEC (human umbilical vascular endothelial cells) were purchased from LONZA. HL-60 (human promyelocytic leukemia), PC-3 (human prostate cancer), EA.hy926 (human umbilical vein cell line), and 293T (human embryonic kidney cell) cell lines were obtained from the American Type Culture Collection (ATCC®).The hESC-H9 (human embryonic stem cell) line was purchased from

WiCell, and the FLP-IN™-CHO cell line was obtained from Invitrogen. HUVECs were cultured in endothelial growth medium (EBM-2, LONZA). HL-60 and PC-3 cells were cultured in RPMI 1640 medium (GIBCO™). *EA.hy926* and 293T cells were cultured in DMEM (GIBCO™). FLP-IN™-CHO cells were maintained in Ham's F12 medium. The hESC-H9 line was cultured as previously described. All cell lines were maintained in conditioned media supplemented with 10% fetal bovine serum (FBS; GIBCO™) and 100 μg/ml Penicillin/Streptomycin (P/S; GIBCO™) in a humidified incubator with 5% CO_2 at 37°C

Screening of anti-VEGFR2 phage clones by ELISA

The selected phages were further examined by ELISA screening. The 96-well plates were coated with 1μg/ml of VEGFR2-Fc, Met-Fc (R&D), or BSA (Sigma) protein in 0.1 M sodium bicarbonate overnight at 4°C. After blocking with 1% BSA in PBS (w/v) for 2 hr at room temperature, 70-randomly selected phage clones were added to the plates at a 1:2 dilution in 1% BSA, and incubated for 1 hr at room temperature. Following washes with PBST, the plates were incubated with a 1:2000 dilution of horseradish peroxidase (HRP)-conjugated mouse anti-M13 phage antibody (GE) for 1 hr. After washing with PBST, the colorimetric reaction was developed with the peroxidase substrate ortho-phenylenediamine (OPD; Sigma) plus H_2O_2 for 15 min, and then terminated by the addition of 3 N HCl. The absorbance at 490 nm was determined using a microplate reader (SpectraMax, Molecular Devices). Plasmid DNA of positive clones were isolated and sequenced using the pCANTAB5 sequencing primer set.

Plasmid construction

Human cDNA clone encoding the full-length VEGFR2 sequence (NM_002253.2) was purchased from Thermo Scientifics, and used as a PCR template for the following constructs. Various lengths of theVEGFR2 extracellular region with signal peptide, transmembrane domain, and truncated cytoplasmic domain were constructed, as follows (see also FIG. 8): VEGFR2(1-7), full-length extracellular region of VEGFR2, comprised of domains 1-7 from residues Met^{1} to Leu^{813}; VEGFR2(2-7), containing domains 2-7 from residues Ala^{111} to Leu^{813}; VEGFR2(3-7), containing domains 3-7 from residues Ser^{208} to Leu^{813}; VEGFR2(4-7), containing domains 4-7 from residues Phe^{321} to Leu^{813}; VEGFR2(del2-3), in which domains 2 and 3 of VEGFR2 were deleted by ligation of two fragments encoding domain 1 (Met^{1} to Glu^{140}) and domains 4-7 (Phe^{321} to Leu^{813}); VEGFR2(del3), in which domain 3 of VEGFR2 was deleted by ligation of two fragments encoding domains 1-2 (Met^{1} to Arg^{222}) and 4-7 (Phe^{321} to Leu^{813}); VEGFR2(M1), a mutagenic construct containing all seven domains of the full-length extracellular region of VEGFR2 (Met^{1} to Leu^{813}), in which ^{259}NWEYPS^{264} in domain 3 is replaced with ^{261}TWHSPP^{266} of the mouse homolog; and VEGFR2(M2), a mutagenic construct containing all seven domains of the full-length extracellular

region of VEGFR2 (Met^{1} to Leu^{813}), in which ^{279}TQSGSEM^{285} in domain 3 is replaced by ^{281}PFPGTVA^{287} of the mouse homolog.

Expression and purification of soluble scFv

E. coli strain HB2151 was infected with anti-VEGFR2 scFv phage clone PC-8, 12, 28, 29, or 45, and periplasmic extracts of bacteria were prepared. Soluble scFv was purified in periplasmic extracts using protein L agarose columns (Thermo Scientific) according to the manufacturer's instructions. Purified scFvs were completely dialyzed with PBS, and analyzed by reducing SDS-PAGE followed by Coomassie blue staining.

Proliferation assay

A total of 1×10^{4} HUVECs were seeded onto 96-well plates overnight. The cells were then starved in serum-free EBM-2 overnight. Subsequently, 8μg/ml of the selected scFv, together with 40 ng/ml VEGF in low-serum EBM-2 (0.2%), was added to the wells, and then incubated for 48 hours. Cell proliferation was assessed using MTT reagent (Invitrogen), according to the manufacturer's instructions.

Flow cytometry analysis

About 1×10^{4} HUVEC were incubated with the selected anti-VEGFR2 phage clones at 4°C for 1 hour in FACS buffer (PBS containing 1% fetal bovine serum). After the cells were washed with FACS buffer, they were first incubated with mouse anti-M13 phage Ab for 1 hr at 4°C, and then with R-phycoerythrin-conjugated goat anti-mouse IgG (Jackson Immuno Research) for 30 min at 4°C. Flow cytometry was performed with a FACSCantoII (BD), and emission fluorescence intensity was measured with FACS Diva software (BD) to quantitatively compare binding affinities.

Lentivirus-mediated short hairpin RNA (shRNA) knock-down

The lentiviral vector pLKO_TRCN0000199129, which encodes shRNA sequence, ccggcgctgacatgtacggtc tat gctcgagca tag accgta cat gtcagcgttttttg (SEQ ID NO: 70), and targets human *VEGFR2*, was obtained from the National RNAi Core Facility (Academia Sinica, Taiwan). The pLKO_TRCN0000072249 vector encoding shRNA against firefly *luciferase* was used as a negative control. For virus production, pLKO vector, the envelope plasmid pMD.G, and the packing plasmid pCMV-ΔR8.91 were co-transfected at a ratio of 10:1:9 into 293T cells using LIPOFECTAMINE® 2000 (Invitrogen). At 18 hours post-transfection, culture media were re-placed with fresh DMEM plus 10% FBS and 1% BSA. The supernatant containing virus particles was harvested after incubation for 24 and 48 hours.

PC-3 cells were seeded at a density of 1×10^{6}cells in a 60-mm dish one day before lentivirus transduction. Virus-containing media supplemented with 8 μg/ml polybrene (Sigma-Aldrich) was

added to PC-3cells,and incubated for 24 hours. Subsequently, the transduced cells were selected by incubation in growth media containing 2μg/ml puromycin for 3 days.

Immunohistochemical staining

Immunohistochemical staining was carried out as previously described. Briefly, sections were deparaffinized and rehydrated, and antigen retrieval was performed concomitantly using Trilogy buffer (Cell Marque). Endogenous peroxidase activity was then blocked by incubation in 3% H_2O_2 in methanol for 30 minutes. After washes with PBS, sections were incubated with 1% BSA for 30 min to block non-specific binding. Sections were then incubated with anti-VEGFR2 antibody (55B11, Cell Signaling) for 1hr at room temperature. After washing with PBST0.1, sections were treated with the polymer-based Super Sensitive IHC detection system (Biogenex, San Ramon) according to the manufacturer's instructions. Horseradish peroxidase activity was detected by the development of color with chromogenic subtract diaminobenzidine hydrochloride (DAB) (0.02%). The slides were lightly counterstained with hematoxylin (Sigma-Aldrich), mounted with Permount (Fisher Scientific), and examined by light microscopy.

Western blot analysis

Western blots were performed using standard protocols, as previously described. The primary antibodies were purchased from Cell Signaling Technology and used at a 1,000-fold dilution for protein detection; the antibodies used are as follows: anti-VEGFR2 (clone 55B11), anti-phospho-VEGFR2 (Tyr1175; clone 19A10), anti-FAK, anti-phospho-FAK (Tyr397; cloneD20B1), anti-p42/44 MAPK, anti-phospho-p42/44 anti-MAPK (Thr202/Tyr204; cloneD13.14.4E), anti-Akt, and anti-phospho-Akt (Ser473).

Immunofluorescence assay of frozen tissue sections

For tumor blood vessel studies, samples were fixed in OCT. Frozen blocks were cut into 50 μm sections, and frozen tumor tissue sections were fixed with 1% paraformaldehyde, permeabilized with 0.1% TRITON™-X 100, blocked with normal horse serum (Vector), and then incubated for 1 hour at room temperature (RT) with a 1:100 dilution of the primary antibody (rat anti-mouse CD31 (PECAM-1); BD Bioscience). Subsequently, the tissue sections were incubated with Alexa 549-conjugated goat anti-rat antibody (Invitrogen) at room temperature for 1 hour. Nuclei were stained with DAPI, and sections were then mounted with fluorescent mounting solution. Immunofluorescent images were acquired using a Zeiss Axiovert 200M microscope. Positive areas of CD31 endothelial cells were quantified by pixel area counting and normalized with DAPI staining using ImageJ software under low power magnification.

Terminal deoxynucleotidyltransferase-mediateddUTP nick end labeling (TUNEL)

The frozen tumor tissue sections were fixed with 1% paraformaldehyde and permeabilized with 0.1% TRITON™-X 100, before being incubated with terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling reaction mixture (Roche Diagnostics) at 37°C for 1 hour. After washing three times with PBS, the slides were incubated with FITC-anti-DIG antibody (1: 2000) and DAPI (1: 500). Slides were mounted with mounting solution and visualized under a fluorescent microscope. Slides were independently examined by three individuals. Areas with TUNEL-positive cells were quantified by pixel area counting, and normalized to DAPI staining using ImageJ software.

Hematoxylin and Eosin (H&E) staining

Tumors and indicated organs were dissected from mice and fixed in 4% paraformaldehyde overnight. Fixation and processing of specimens were performed in accordance with standard procedures. The specimens were embedded in paraffin and cut into 50 μm sections. Rehydrated paraffin-embedded tissue sections were stained with Mayer's hematoxylin solution (Wako) for 5 minutes and washed with water for 1-2 minutes. The slides were then stained with eosin solution (Wako) for 10 minutes. Tissues were visualized with Tissue Gnostics microscopes.

Quantitative RT-PCR

Total RNA extractions were performed using TrizolRNA isolation reagent (Invitrogen). Subsequently, cDNA was synthesized using oligo(dT) primers (Fermentas) and Super Script III reverse transcriptase (Invitrogen), according to the manufacturer's instructions. The forward and reverse primers used to amplify VEGFR2 cDNA through PCR are as follows: VEGFR2-F: gaacatttgggaaatctcttgc (SEQE ID NO: 71); VEGFR2-R: cggaagaacaatgtagtctttgc (SEQ ID NO: 72). Quantitative PCR was performed using the LightCycler480 System (Roche Applied Science). The transcript levels of VEGFR2 were normalized to those of GAPDH in the same sample. The ratio values were calculated accordingly for each sample. The reactions were performed in triplicate.

Results

Identification of phage-displayed scFv that binds to VEGFR2

A phage-displayed human naïve scFv library was used to isolate phages that bind to VEGFR2 recombinant protein. After four rounds of affinity selection (biopanning), the titer of bound phage increased by as much as 3,455-fold (FIG. 1A). Through ELISA screening and DNA sequencing, we identified five distinct phage clones (R2PC8, R2PC12, R2PC28, R2PC29, R2PC45; Table 1) that bind highly to VEGFR2-Fc, but not to c-Met-Fc control protein (FIG. 1B). We then used FACS assay of human umbilical vein endothelial cells (HUVEC) to confirm that all five clones have the ability to bind to VEGFR2 on the cell surface; of the five clones, R2PC12 exhibited the greatest

reactivity (FIG. 1C). Table 1 shows the amino acid sequence of V_H and V_L domains of anti-VEGFR2 scFvs.

Table 1

V_H domains

	FR1 (SEQ ID NO:)	**CDR1** (SEQ ID NO:)	**FR2** (SEQ ID NO:)	**CDR2** (SEQ ID NO:)
R2PC8	QVQLVQSGGGLVKPGGSL RLSCAAS (26)	**GFTFSSYS** (1)	MSWVRQAPGK GLEWVSS (27)	**ISSSSSYI** (2)
R2PC12	QVNLRESGGGLVKPGGSL RLSCAAS (34)	**GFTFGSYT** (6)	MNWVRQAPGK GLEWVAS (35)	**ITSGSSYI** (7)
R2PC28	EVQLVESGGALVQPGGSL RLSCVGS (42)	**EFTFSHYN** (11)	LHWVRQAPGK GLEWLAV (43)	**ISDDGRNK** (12)
R2PC29	QVQLQQSGAEMKKSGSSV KVSCKAS (50)	**GGNFISKG** (16)	ISWVRQAPGQG LEWMGG (51)	**IIPLFGTG** (17)
R2PC45	QVNLRESGGGVVQPGRSL RLSCAAS (58)	**GFTFSSYA** (21)	MHWVRQAPGK GLEWVAV (59)	**ISYDGSNK** (22)

	FR3	**CDR3**	**FR4**	**Family**
R2PC8	YYADSVKGRFTISRDNAK NSLYLQMNSLRAEDTAVY YC (28)	**ARSTDAFDI** (3)	WGQGTMVTVSS (29)	**V_H3**
R2PC12	FYTDSVKGRFTISRDNSRSS LFLQMNSLRAEDTAIYYC (36)	**ARGSASAFDI** (8)	WGQGTMVTVS S (37)	**V_H3**
R2PC28	YYGDSVKGRFTISRDNSKN TLYLQMNGLRAEDTAVYY C (44)	**ARVPTVWRG GVYDI** (13)	WGQGTMVTVS S (45)	**V_H3**
R2PC29	NYAQKFQGRVTITADESTT TVYLQLTSLTPEDTAMYFC (52)	**ATADVDYSDS LEAFDM** (18)	WGQGTMVTVS S (53)	**V_H1**
R2PC45	YYADSVKGRFTISRDNSKN TLYLQMNSLRAEDTAVYY C (60)	**AREQDYGSSS GDAFDI** (23)	WGQGTMVTVS S (61)	**V_H3**

V_L domains

	FR1	CDR1	FR2	CDR2
R2PC8	DIVMTQSPSSLSASVGDRVTI TCRAS (30)	**QRISNY** (4)	LNWYQHKSGE DPKLLIY (31)	**AAS** (Ala Ala Ser)
R2PC12	DIQMTQSPSSLSASVGDRVTI TCKAS (38)	**DDIINY** (9)	LNWYQQKPGE APKLLIY (39)	**DAS** (Asp Ala Ser)
R2PC28	EIVLTQSPATLSLSPGERATL SCRAS (46)	**QSVGSY** (14)	LAWYQQRPGQP PRLLIY (47)	**DAS** (Asp Ala Ser)
R2PC29	DIVMTQSPSSLSASVGDRVTI TCRAS (54)	**QSINNY** (19)	LNWYQQKPGK APNLLIY (55)	**GAS** (Gly Ala Ser)
R2PC45	DIQMTQSPSSLSASVGDRVTI TCRAS (62)	**QRISSY** (24)	LNWYQQKPGK APKLLIY (63)	**DAS** (Asp Ala Ser)
	FR3	**CDR3**	**FR4**	**Family**
R2PC8	SLQSGVPSRFSGSGSGTDFTL TISSLQPEDFATYYC (32)	**QQYDRYPPT** (5)	FGQGTKLEIK (33)	**V_κ1**
R2PC12	ILETGVPSRFSGSGSGTDFTF TISSLQPEDIATYYC (40)	**QQYDILPLT** (10)	FGGGTKLEIK (41)	**V_κ1**
R2PC28	NRATGVAARFSGSGSGTDFT LTIDSLEAEDAATYYC (48)	**HQSSSLPRT** (15)	FGQGTKLEIK (49)	**V_κ3**
R2PC29	SLQSGVPSRFRGSGSGTDFTL TISSLQPEDFATYYC (56)	**QQSYSTPL** (20)	FGQGTKLEIK (57)	**V_κ1**
R2PC45	NLQSGVPSRFSGSGSGTDFTL TINGLQPDDFAIYFC (64)	**HQSYSAPPT** (25)	FGQGTKVEIK (65)	**V_κ1**

Complementarity-determining regions 1-3 (CDR1-3) and framework regions 1-4 (FW1-4) for both the V_H and V_L domains are shown. The V domain families were aligned by IMGT database

Subsequently, we generated soluble scFv proteins from the five VEGFR2-binding phage clones, which were designated as R2S8, R2S12, R2S28, R2S29, and R2S45 (FIG. 1D). The binding ability of the anti-VEGFR2 scFvs to tumor vascular endothelium in human lung cancer surgical specimens was investigated through the use of immunofluorescence staining assays. We observed that the fluorescent signals of anti-VEGFR2 scFvs apparently colocalize with endothelial cell marker CD31 (FIG. 1E), suggesting that these scFvs are able to specifically recognize tumor vasculature.

Anti-VEGFR2 scFvs antagonized the VEGF-A/VEGFR2 interaction and VEGF-A-induced VEGFR2 phosphorylation

To determine whether the anti-VEGFR2 scFvs can block VEGF-A binding to VEGFR2, we performed a plate-based competition binding assay in which increasing concentrations of scFvs competed with VEGF-A for binding to the immobilized VEGFR2. The interaction of VEGF-A with VEGFR2 was strongly suppressed by R2S8 and R2S12, with half-maximal inhibitory concentrations (IC_{50}) of 7.03 and 3.26 nM, respectively, whereas R2S28 and R2S29 exhibited comparatively weak competitive ability (FIG. 2A). We next investigated whether the scFvs could antagonize VEGF-A-mediated activation of VEGFR2 in HUVECs; R2S8 and R2S12 apparently inhibited tyrosine phosphorylation of VEGFR2 by VEGF-A, and R2S12 exhibited the strongest inhibition activity (FIG. 2B).

Identification of binding epitopes of anti-VEGFR2 scFv

To map the binding domain responsible for anti-VEGFR2 scFv, we generated a series of VEGFR2 deletion mutants, which consist of signal peptide and transmembrane domain (FIG. 8). These protein mutants were ectopically expressed in 293T cells, and examined by immunofluorescent staining with R2S8, R2S12, and R2S28 (Table 2). We found that R2S28 bound to cells expressing VEGFR2(1-7), but not to cells expressing VEGFR2(2-7) or VEGFR2(4-7), suggesting that domain 1 of VEGFR2 is necessary for R2S28 binding. R2S8 and R2S12 bound to 293T cells expressing VEGFR2(1-7) and VEGFR2(2-7), but not to cells expressing constructs lacking domain 3, e.g., VEGFR2(4-7), VEGFR2(del2-3), or VEGFR2(del3), further indicating that their binding epitopes are located within domain 3. Table 2 shows epitope mapping of anti-VEGFR2 scFv.

Table 2

	R2S8	R2S12	R2S28
VEGFR2(1-7)	+	+	+
VEGFR2(2-7)	+	+	-
VEGFR2(3-7)	nd*	nd*	nd*
VEGFR2(4-7)	-	-	-
VEGFR2(del2-3)	-	-	+
VEGFR2(del3)	-	-	+
VEGFR2(M1)	-	-	+
VEGFR2(M2)	+	+	+

*nd, binding not determined because the construct was not expressed.

Furthermore, we found that both neutralizing scFvs, R2S8 and R2S12, did not cross-react with murine VEGFR2 protein. Amino acid sequence alignment between human and murine VEGFR2

revealed that domain 3, which has only 67% identity, is the most diverse of the seven domains of the extracellular region of VEGFR2. Thus, we speculated that the distinct epitopes of R2S8 and R2S12 in domain 3 of human VEGFR2 are not displayed in murine VEGFR2. Comparing domain 3 of human and mouse revealed that thirty residues are different, and that these residues are grouped into clusters. To identify the amino acid residues in domain 3 critical for R2S8 and R2S12 binding, we selected two major clusters (M1 and M2) for mutagenesis, as follows: the human NWEYPS (SEQ ID NO: 66) and TQSGSEM (SEQ ID NO: 67) residues were substituted with mouse TWHSPP (SEQ ID NO: 68) and PFPGTVA (SEQ ID NO: 69) residues, respectively (FIG. 2C). The results of immunofluorescent staining show that R2S8 and R2S12 do not recognize 293T cells expressing VEGFR2-M1 mutant protein. In contrast, mutations in the M2 region of VEGFR2 had no effect on binding to R2S8 and R2S12 (Table 2).

We built a molecular model of VEGFR2 domain 3 from previously reported crystal structural information and our mutagenesis data. The ribbon and surface models show that the NWEYPS (SEQ ID NO: 66) residues (M1 region) localize to a β-strand and middle surface of VEGFR2 domain 3 (FIGs. 2D and 2E). The contacting residues and binding surface of the neutralizing anti-VEGFR2 antibodies, IMC-1121B and 6.64, are located on VEGFR2 domain 3 (FIGs. 2C and 2F). We observed that the M1 region is close to the binding epitopes of the IMC-1121B and 6.64 antibodies. Two binding residues, Asn259 and Glu261, of IMC-1121B antibody were found to be situated in the M1 region. Therefore, our results suggest that the binding epitopes of R2S8 and R2S12 are most likely different from those of IMC-1121B and 6.64 antibodies.

Affinity maturation of R2S12 was performed to generate an anti-VEGFR2-AF human antibody with higher binding activity

Neutralizing antibodies with high affinity are important for therapeutic efficacy. As R2S12 was demonstrated to exhibit the greatest binding and antagonizing activity of the examined scFvs, we further improved its binding activity using phage display-based affinity maturation. After four rounds of stringent *in vitro* selection, a clone (R2S12AF) with superior binding activity was identified (FIGs. 9A-B). Four residues within V_L-CDR3 of R2S12-AF are different from its parental clone, R2S12 (FIG. 3A). The scFv format has been shown to be of limited clinical use on account of their short serum half-life (approximately 3.5 hours) and inability to trigger human effector functions. To overcome these challenges, we molecularly engineered the coding sequences of R2S12 and R2S12-AF scFv into a human IgG1 backbone to create anti-VEGFR2 and anti-VEGFR2-AF fully human antibody (hAb), respectively.

We analyzed the affinity of both human antibodies to VEGFR2 using a BIACORE T100™. Measurement of the kinetic parameters of antibody-antigen showed that anti-VEGFR2-AF hAb

possesses a sub-nmol/L affinity constant (K_d = 0.264 nM) and an 8-fold increase in binding affinity to VEGFR2 over its parental clone anti-VEGFR2 hAb (K_d = 2.1 nM; FIG. 3B). We performed solid-phase competitive assays to quantitatively evaluate the disruption of VEGF-A/VEGFR2 binding by individual antibodies. FIG. 3C shows that the IC_{50} value of VEGF-A binding to VEGFR2 was 0.88 nM for anti-VEGFR2-AF hAb, 1.87 nM for anti-VEGFR2 hAb, and 1.42 nM for IMC-1121B. These data indicate that anti-VEGFR2-AF hAb is superior to IMC-1121B at blocking the VEGF/VEGFR2 interaction. The results of FACS analysis of HUVECs further demonstrate that anti-VEGFR2-AF hAb exhibits stronger binding than IMC-1121B to cell-surface VEGFR2 (FIG. 3D).

Anti-VEGFR2-AF hAb inhibits activation of the VEGFR2-mediated signaling pathway and disrupts capillary structure formation in HUVECs

To elucidate the anti-angiogenic potential of anti-VEGFR2-AF hAb, we analyzed the impact of anti-VEGFR2-AF hAb on HUVEC growth, migration, and tube formation. We found that anti-VEGFR2-AF hAb suppressed VEGF-A-induced HUVEC proliferation and migration using MTT and wound-healing assays, respectively. To investigate the effect of anti-VEGFR2-AF hAb on endothelial cell tube formation (a critical step in angiogenesis), we examined HUVECs on MATRIGEL® layers in the absence or presence of VEGF-A with anti-VEGFR2-AF hAb (FIG. 4A). The ability of endothelial cells to migrate and organize into capillary-like structures was assessed and quantified using an inverted photomicroscope. We verified that anti-VEGFR2-AF hAb is more effective than IMC-1121B in suppressing VEGF-A-triggered capillary-like structures, based on measurements of tubule length and branch point number (FIG. 4A).

To investigate the molecular mechanism underlying the anti-angiogenic properties of anti-VEGFR2-AF hAb, we examined the signaling molecules and pathways by Western blotting (FIG. 4B). We found that anti-VEGFR2-AF hAb efficiently diminished VEGF-A-induced phosphorylation of VEGFR-2 and their downstream signaling molecules, such as Akt, MAPK, and FAK. In particular, the levels of phosphorylated VEGFR2, Akt, and MAPK in the anti-VEGFR2-AF-treated group were lower than those in IMC-1121B-treated group, whereas FAK phosphorylation was only marginally reduced by treatment with either antibody (FIG. 4B). The results indicate that anti-VEGFR2-AF hAb significantly inhibits several essential steps of vascular endothelial cell angiogenesis, suggesting that this antibody may have anti-angiogenic potential *in vivo*.

Anti-VEGFR2-AF hAb inhibits VEGF-A-induced cellular function in VEGFR2-expressing human prostate cancer cells

We chose the human prostate cancer cell line PC-3 as a model to study the therapeutic efficacy of anti-VEGFR2-AF hAb in inhibiting tumor growth. We performed quantitative reverse transcription-PCR (qRT-PCR) analysis to investigate the endogenous expression of *VEGFR2* in PC-3 cells (FIG.

5A). HUVECs, EA.hy926, and hESC-H9 cells are known to exhibit high expression of VEGFR2 mRNA, and were thus used as positive controls. We found that VEGFR2 mRNA was readily detectable in PC-3 cells, but was barely detectable in negative control 293T cells.

To characterize the functional significance of VEGF-A/VEGFR2 on PC-3 cells, we analyzed PC-3 cell activity upon VEGF-A treatment. We found that treatment with VEGF-A was able to enhance various cellular activities of PC-3 cells, including proliferation, colony formation, and invasive ability (FIG. 5B). We established VEGFR2-knock down PC-3 cells with lentivirus-mediated shRNA (shVEGFR2; FIG. 5C), and found that knockdown of VEGFR2 reduced proliferation, colony formation, and invasive ability of PC-3 cells as compared to PC-3 cells infected with control shRNA (shLuc; FIG. 5D). We treated PC-3 cells with anti-VEGFR2-AF hAb and demonstrated that VEGF-A-induced cellular activities can be suppressed by anti-VEGFR2-AF hAb (FIGs. 10A-C). Hence, these data show that the VEGF/VEGFR2 axis is crucial for clonogenic and tumorigenic capabilities of PC-3 cells.

We investigated *VEGFR2* expression patterns in relevant microarray data sets which are publicly available. We found that the amount of *VEGFR2* transcripts in metastatic prostate tumor was higher than that in primary tumor (FIG. 5E). We used a commercial anti-VEGFR2 antibody to stain a human prostate cancer tissue array, and revealed that VEGFR2 is detectable in tumor cells, and that its expression level is elevated in Grade III prostate adenocarcinoma as compared to Grade I (FIG. 5F). In normal prostate tissue specimens, VEGFR2 is present in vascular endothelium, but not in normal prostate cells.

Therapeutic efficacy of anti-VEGFR2-AF in human prostate cancer xenografts

We used the PC-3 xenograft prostate tumor model to elucidate the *in vivo* antitumor activity of anti-VEGFR2-AF hAb versus IMC-1121B. Docetaxel is a first-line chemotherapeutic agent for patients with metastatic castration-resistant prostate cancer.Thus, we also investigated the therapeutic effects of combining docetaxel and anti-VEGFR2-AF hAb or IMC-1121B. NOD/SCID mice bearing PC-3 xenografts were administrated with IMC-1121B, anti-VEGFR2-AF hAb, docetaxel, anti-VEGFR2-AF hAb plus docetaxel, or IMC-1121B plus docetaxel (FIG. 6A). By day 38, tumor growth reduction reached 90% for mice treated with the combination of anti-VEGFR2-AF hAb plus docetaxel, 82% for mice treated with the combination of IMC-1121B plus docetaxel, 70% for mice treated with docetaxel, 52% for mice treated with anti-VEGFR2-AF hAb, and 45% for mice treated with IMC-1121B (FIG. 6B). Body weight was used as a surrogate indicator of the health status of the mice (FIG. 6C). The anti-VEGFR2-AF hAb and IMC-1121B groups exhibited no significant changes in body weight during the treatment period as compared to the NHIgG group. Treatment with docetaxel alone caused a marked loss of body weight (about 20%). Mice treated with docetaxel in

combination with other antibodies lost a similar amount of body weight to mice treated with docetaxel alone, which indicates that anti-VEGFR2-AF hAb and IMC-1121B do not enhance docetaxel-induced toxicity.

Tumor weights were measured and found to be consistent with tumor volume (FIGs. 6D and 6E). We further examined tumor tissues in each group by using anti-CD31 antibody to detect tumor blood vessels and TUNEL assay to identify apoptotic cells. We found that anti-VEGFR2-AF hAb is superior to IMC-1121B at reducing tumor vascular density and enhancing cancer cell apoptosis (FIGs. 11A and 11B). These results show that anti-VEGFR2-AF hAb is more effective than IMC-1121B at attenuating tumor growth, and that anti-VEGFR2-AF hAb significantly enhances the effectiveness of docetaxel in the treatment of human prostate tumors in mice.

Anti-VEGFR2-AF hAb prolonged the survival of mice bearing HL-60 leukemia xenografts

The VEGF-A/VEGFR2 pathway has crucial functions not only in solid tumors, but also in liquid tumors, such as leukemia or lymphoma. Previous studies provided evidence that IMC-1121B inhibits HL-60 leukemia growth, and prolongs survive in a mouse model. To compare the anti-leukemia effect of IMC-1121B with that of anti-VEGFR2-AF hAb, we developed a HL-60 leukemia xenograft model in NSG mice. Mice received intravenous injections of 5×10^6 HL-60 cells, and were treated 3 days later with IMC-1121B, anti-VEGFR2-AF, NHIgG, or PBS. As shown in FIG. 7A, all PBS- or NHIgG-treated mice died within 36 days; however, leukemia-bearing mice treated with antibodies against VEGFR2 exhibited a marked extension in survival time. Mice treated with anti-VEGFR2-AF hAb survived longer (70 days; median = 53 days) than those treated with IMC-1121B (56 days; median = 43 days). No significant changes in body weight were observed between groups (FIG. 7B).

Post-mortem histopathological examinations of all mice showed that no obvious pathological changes were found in the livers, spleens, heart, or kidneys of mice in each group. The ovaries of leukemia-bearing mice were swollen as compared to normal ovaries (FIG. 7C). H&E staining revealed that the ovaries had been infiltrated by metastatic leukemia cells (FIGs. 12A and 12B). The average ovarian volume in the anti-VEGFR2-AF hAb-treated group was significantly smaller than that in the IMC-1121B-treated group (FIG. 7D).

Furthermore, lymph nodes with leukemia infiltration were identified in one of nine mice treated with anti-VEGFR2-AF hAb, whereas lymph nodes with leukemia infiltration were present in five of nine mice treated with IMC-1121B (FIG. 7E).

Anti-VEGFR2-AF hAb-mediated targeting of VEGFR2 on tumor endothelium not only disrupts VEGF-A-induced signaling, but also triggers antibody-dependent cell-mediated cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC) to directly kill the targeted cells, which may enhance current anti-angiogenesis therapy.

Anti-VEGFR2-AF hAb may be able to exert dual targeting and inhibition effects on both tumor vascular and malignant cells, as tumor cells also express VEGFR2. The dual targeting ability may have synergistic effects on cancer therapy. We produced a fully human antibody, anti-VEGFR2-AF, which exhibited superior binding to VEGFR2, antagonizing the activity of this receptor. Similar to IMC-1121B, anti-VEGFR2-AF hAb specifically bound to human VEGFR2, but not to murine VEGFR2. Compared to IMC-1121B, anti-VEGFR2-AF hAb presented with greater antitumor efficacy *in vitro* and *in vivo*, by interrupting VEGF-A/VEGFR2 axis-mediated signaling. We are the first to demonstrate that anti-VEGFR2 antibody can enhance the therapeutic efficacy of docetaxel in the treatment of prostate cancer. The findings suggest that anti-VEGFR2-AF hAb may be potentially used as a therapeutic antibody for cancer treatment by simultaneously and directly inhibiting angiogenesis and VEGFR2-expressing tumor cells.

In summary, compared to FDA-approved anti-VEGFR2 human antibody IMC-1121B (Ramucirumab), anti-VEGFR2-AF hAb possessed significantly superior activity, and suppressed VEGF-A-mediated capillary structure formation *in vitro*. We observed VEGFR2 expression in human prostate cancer cell line (PC-3) and leukemia cell line (HL-60), and demonstrated that VEGFR2 expression is associated with malignancy and metastasis of human prostate cancer. In PC-3-derived xenograft mouse models, treatment with anti-VEGFR2-AF hAb (monotherapy or combined with docetaxel) suppressed tumor growth and angiogenesis more effectively than treatment with IMC-1121B. In mice with HL-60-derived leukemia, anti-VEGFR2-AF hAb exhibited more significant efficacy than IMC-1121B in prolonging survival and reducing metastasis of leukemia cells to ovaries and lymph nodes.

The foregoing description of the exemplary embodiments of the invention has been presented only for the purposes of illustration and description and is not intended to be exhaustive or to limit the invention to the precise forms disclosed. Many modifications and variations are possible in light of the above teaching. All references cited and discussed in this specification are incorporated herein by reference in their entireties and to the same extent as if each reference was individually incorporated by reference.

What is claimed is:

1. An isolated antibody or an antigen-binding fragment thereof, which comprises a heavy chain variable region (V_H) and a light chain variable region (V_L), the V_H comprising V_H CDR1, V_H CDR2, and V_H CDR3, and the V_L comprising V_L CDR1, V_L CDR2, and V_L CDR3, wherein: the V_H CDR1, V_H CDR2, V_H CDR3 comprise the amino acid sequence of SEQ NO: 6, SEQ ID NO: 7, and SEQ ID NO: 8, respectively; and the V_L CDR1, V_L CDR2, and V_L CDR3 comprise the amino acid sequence of SEQ ID NO: 9, Asp Ala Ser, and SEQ ID NO: 10 or 73, respectively.

2. The antibody or antigen-binding fragment thereof of claim 1, wherein: the V_L CDR3 comprises the amino acid sequence of SEQ ID NO: 73.

3. The antibody or antigen-binding fragment thereof of claim 1, wherein the V_H comprises the amino acid sequence of SEQ ID NO: 76; and the V_L comprises the amino acid sequence of SEQ ID NO: 77 or 78.

4. The antibody or antigen-binding fragment thereof of claim 1, which is a single-chain variable fragment, a Fab fragment or a Fv fragment.

5. The antibody or antigen-binding fragment thereof of claim 1, wherein the antibody is a fully human antibody.

6. A composition comprising a therapeutically effective amount of the antibody or antigen-binding fragment thereof of claim 5 and a pharmaceutically acceptable vehicle or carrier.

7. The composition of claim 6, further comprising a therapeutically effective amount of a chemotherapeutic agent.

8. The antibody or antigen-binding fragment thereof of claim 3, wherein the antibody is a fully human antibody.

9. The antibody or antigen-binding fragment thereof of claim 4, wherein the antibody is a fully human antibody.

10. The antibody or antigen-binding fragment thereof of claim 2, wherein the antibody is a fully human antibody.

11. An isolated antibody or an antigen-binding fragment thereof, or a single-chain variable fragment, which comprises:

(a) a heavy chain variable region (V_H) comprising the amino acid sequence of SEQ ID NO: 76; and

(b) a light chain variable region (V_L) comprising the amino acid sequence of SEQ ID NO: 77 or 78.

12. A method of detecting the presence of VEGFR2 on tumor vascular endothelial cells or cancer cells in a biological sample, comprising:

(i) admixing the antibody or antigen-binding fragment thereof of claim 1 with the biological sample;

(ii) allowing the antibody or antigen-binding fragment thereof and the VEGFR2 on the tumor vascular endothelial cells or cancer cells in the biological sample to interact and form a complex; and

(iii) detecting the presence of the VEGFR2 on the tumor vascular endothelial cells or cancer cells in the complex.

13. A method of detecting the presence of VEGFR2 on tumor vascular endothelial cells or cancer cells in a biological sample, comprising:

(i) admixing the antibody or antigen-binding fragment thereof of claim 2 with the biological sample;

(ii) allowing the antibody or antigen-binding fragment thereof and the VEGFR2 on the tumor vascular endothelia cells or cancer cells in the biological sample to interact and from a complex; and

(iii) detecting the presence of the VEGFR2 on the tumor vascular endothelial cells or cancer cells in the complex.