

Anti-EphA4 Antibody

Technical Field

[0001]

The present invention relates to an antibody that binds to EphA4, a nucleic acid that encodes the antibody, a vector that comprises the nucleic acid, a cell comprising the vector, a method of producing the antibody, and a pharmaceutical composition comprising the antibody.

Background Art

[0002]

EphA4 is a member of the receptor tyrosine kinase family. Ephrin type A and type B are known as ligands for EphA4, and when EphA4 binds with ephrin which is a ligand thereof, de-adhesion signal is induced. So far, the involvement of EphA4 in the pathology of Alzheimer's disease (hereinafter also referred to as "AD") has been suggested (Non-Patent Literatures 1-4), and it has been reported that the inhibition of binding between EphA4 and ephrin rescues amyloid β ($A\beta$)₍₁₋₄₂₎ oligomer-mediated dysfunction of neurotransmission (Patent Literature 1). In AD, it is thought that aggregates (neurofibrillary tangles) formed by excessively phosphorylated tau are involved in nerve cell death (Non-Patent Literature 5), and it has also been reported that suppression of tau phosphorylation suppresses neurodegeneration of synapse disappearance (Non-Patent Literature 6 and Non-Patent Literature 7), as well as improves memory deficits or cognitive dysfunction (Non-Patent Literatures 8-11). There are reports suggesting that activation of CDK5 is a cause of tau phosphorylation (Non-Patent Literature 12 and Non-Patent Literature 13). A genetically modified mouse expressing a P301L mutation which has been found in familial frontotemporal dementia (rTg4510 mouse) is an AD model mouse, and similar to AD, hyperphosphorylation of tau and abnormal accumulation of tau in neuronal cells are found in the mouse. In rTg4510 mouse, neurofibrillary tangles, a pathological feature of AD, are formed and bring cognitive dysfunction by brain atrophy and loss of neuron

(Non-Patent Literature 14 and Non-Patent Literature 5).

[0003]

EphA4 is abundantly expressed in the hippocampus or the cerebral cortex, and is neural activity-dependently cleaved by matrix metalloprotease (MMP), ADAM (a disintegrin and metalloproteinase), and γ selectase. It is known that this cleavage reaction of EphA4 stabilizes the spine which is a key structure in neural function (Non-Patent Literature 15). It has been reported that the density of spine is decreased in AD (Non-Patent Literature 16), and since decrease of cleaved fragments of EphA4 is also confirmed in AD at NFT stages V and VI, it is thought that EphA4 cleavage reaction is involved in the pathology of AD (Non-Patent Literature 17).

[0004]

Although KYL peptide and compound 1 etc. are known as existing EphA4 inhibitory drugs (Patent Literature 2, Non-Patent Literature 18, and Non-Patent Literature 19), there has been no reports regarding an antibody having activity that enhances the cleavage of EphA4.

Citation List

[0005]

[Patent Literature 1] WO2016/019280A1

[Patent Literature 2] WO2012/156351A1

[0006]

[Non-Patent Literature 1] Vargas LM et al., PLoS One. 2014 Mar 21; 9 (3)

[Non-Patent Literature 2] Fu AK et al., Proc Natl Acad Sci U S A. 2014 Jul 8; 111 (27): 9959-64

[Non-Patent Literature 3] Rosenberger AF et al., Acta Neuropathol Commun. 2014 Jul 16; 2: 79

[Non-Patent Literature 4] Huang TY et al., J Exp Med. 2017 Dec 4; 214 (12): 3669-3685.

[Non-Patent Literature 5] Santa Cruz et al., Science. 2005 Jul 15; 309 (5733): 476-81

[Non-Patent Literature 6] Seo J et al., J Neurosci. 2017 Oct 11; 37 (41): 9917-9924

[Non-Patent Literature 7] Patrick GN et al., Nature. 1999 Dec 9; 402

(6762): 615-22.

[Non-Patent Literature 8] Onishi T et al., J Neurochem. 2011 Dec; 119 (6): 1330-40

[Non-Patent Literature 9] Belfiore R et al., Aging Cell. 2019 Feb; 18 (1): e12873.

[Non-Patent Literature 10] Webster SJ et al., Front Genet. 2014 Apr 23; 5: 88.

[Non-Patent Literature 11] Grayson B et al., Behav Brain Res. 2015 May 15; 285: 176-93.

[Non-Patent Literature 12] Cancino GI et al., Neurobiol Aging. 2011 Jul; 32 (7): 1249-61.

[Non-Patent Literature 13] Vargas LM et al., Biochim Biophys Acta Mol Basis Dis. 2018 Apr; 1864: 1148-1159.

[Non-Patent Literature 14] Ramsden M et al., J Neurosci. 2005 Nov 16; 25 (46): 10637-47.

[Non-Patent Literature 15] Inoue E et al., J Cell Biol. 2009 May 4; 185 (3): 551-64

[Non-Patent Literature 16] Boros et al., Ann Neurol. 2017 Oct; 82 (4): 602-614

[Non-Patent Literature 17] Matsui C et al., Brain Pathol. 2012 Nov; 22 (6): 776-87. doi: 10.1111/j.1750-3639

[Non-Patent Literature 18] Goldshmit et al., PLoS one. 2011; 6 (9): e24636

[Non-Patent Literature 19] Van Hoecke et al., Nature Medicine. 2012 Sep; 18 (9): 1418-22,2012

Summary of the Invention

Problems to be Solved by the Invention

[0007]

The object of the present disclosure is to provide an anti-EphA4 antibody that can bind to EphA4 and enhance the cleavage of EphA4, as well as a pharmaceutical composition comprising the antibody as the active ingredient.

Means for Solving the Problems

[0008]

As a result of extensive investigation to solve the above problems, the present inventors obtained a mouse anti-EphA4 monoclonal antibody that can bind to EphA4 and enhance the cleavage of EphA4, and produced a humanized antibody of the antibody to thereby come to complete the antibody of interest.

[0009]

The present disclosure encompasses the following characteristics.

(1) An anti-EphA4 antibody, wherein

the anti-EphA4 antibody comprises heavy and light chains, and comprises:

(a) a heavy chain CDR1 consisting of the amino acid sequence shown in SEQ ID NO. 44;

(b) a heavy chain CDR2 consisting of the amino acid sequence shown in SEQ ID NO. 27;

(c) a heavy chain CDR3 consisting of the amino acid sequence shown in SEQ ID NO. 28;

(d) a light chain CDR1 consisting of the amino acid sequence shown in SEQ ID NO. 29;

(e) a light chain CDR2 consisting of the amino acid sequence shown in SEQ ID NO. 30; and

(f) a light chain CDR3 consisting of the amino acid sequence shown in SEQ ID NO. 31.

[0010]

(2) The anti-EphA4 antibody according to (1), wherein the anti-EphA4 antibody is humanized.

[0011]

(3) The anti-EphA4 antibody according to (1) or (2), wherein the anti-EphA4 antibody specifically binds to EphA4 and enhances the cleavage of EphA4.

[0012]

(4) The anti-EphA4 antibody according to any of (1) - (3), wherein the anti-EphA4 antibody specifically binds to EphA4 and inhibits the binding between EphA4 and ephrin.

[0013]

(5) The anti-EphA4 antibody according to any of (1) - (4),

wherein

the variable region of the heavy chain consists of the amino acid sequence shown in SEQ ID NO. 45, and

the variable region of the light chain consists of the amino acid sequence shown in SEQ ID NO. 46.

[0014]

(6) The anti-EphA4 antibody according to any of (1) - (5), wherein

the constant region of the heavy chain and the constant region of the light chain comprise amino acid sequences derived from a human antibody.

[0015]

(7) The anti-EphA4 antibody according to (6), wherein the constant region of the heavy chain is the constant region of human IgG.

[0016]

(8) The anti-EphA4 antibody according to (7), wherein the constant region of human IgG is the constant region of human IgG₂.

[0017]

(9) The anti-EphA4 antibody according to (8), wherein the constant region of human IgG₂ comprises the amino acid sequence shown in SEQ ID NO. 47.

[0018]

(10) The anti-EphA4 antibody according to any of (6) - (9), wherein

the constant region of the light chain is the constant region of human Igκ.

[0019]

(11) The anti-EphA4 antibody according to (10), wherein the constant region of human Igκ comprises the amino acid sequence shown in SEQ ID NO. 48.

[0020]

(12) An anti-EphA4 antibody, wherein the anti-EphA4 antibody comprises heavy and light chains, the heavy chain comprises the amino acid sequence shown in SEQ

ID NO. 59, and

the light chain comprises the amino acid sequence shown in SEQ

ID NO. 60.

[0021]

(13) The anti-EphA4 antibody according to (12), wherein the C-terminal lysine of the heavy chain is deleted.

[0022]

(14) An anti-EphA4 antibody, wherein the anti-EphA4 antibody comprises heavy and light chains, the heavy chain comprises the amino acid sequence shown in SEQ

ID NO. 59,

the light chain comprises the amino acid sequence shown in SEQ

ID NO. 60, and

the C-terminal lysine of the heavy chain is deleted.

[0023]

(15) An isolated nucleic acid encoding the anti-EphA4 antibody according to any of (1) - (14).

[0024]

(16) A vector comprising the nucleic acid according to (15).

[0025]

(17) A host cell comprising the vector according to (16).

[0026]

(18) A method of producing an anti-EphA4 antibody, comprising a step of culturing the host cell according to (17).

[0027]

(19) A pharmaceutical composition comprising the anti-EphA4 antibody according to any of (1) - (14).

[0028]

(20) A pharmaceutical composition according to (19), wherein the pharmaceutical composition comprises at least one pharmaceutically acceptable carrier.

[0029]

(21) The pharmaceutical composition according to (19) or (20), for treating Alzheimer's disease.

[0030]

(22) The anti-EphA4 antibody according to any of (1) - (14)

for use in the treatment of Alzheimer's disease.

[0031]

(23) A method for treating Alzheimer's disease, comprising administering to a patient in need thereof a therapeutically effective amount of the anti-EphA4 antibody according to any of (1) - (14).

[0032]

(24) Use of the anti-EphA4 antibody according to any of (1) - (14) for manufacturing a pharmaceutical composition for treating Alzheimer's disease.

[0033]

(25) The pharmaceutical composition according to (19) or (20), for treating tauopathy.

[0034]

(26) The anti-EphA4 antibody according to any of (1) - (14) for use in the treatment of tauopathy.

[0035]

(27) A method for treating tauopathy, comprising administering to a patient in need thereof a therapeutically effective amount of the anti-EphA4 antibody according to any of (1) - (14).

[0036]

(28) Use of the anti-EphA4 antibody according to any of (1) - (14) for manufacturing a pharmaceutical composition for treating tauopathy.

[0037]

(29) The pharmaceutical composition, the anti-EphA4 antibody, the method for treating, or the use according to any of (25) - (28), wherein the tauopathy is Alzheimer's disease or frontotemporal lobar degeneration with tau pathology.

[0038]

(30) The pharmaceutical composition, the anti-EphA4 antibody, the method for treating, or the use according to (29), wherein the tauopathy is Alzheimer's disease.

[0039]

(31) The pharmaceutical composition, the anti-EphA4 antibody, the method for treating, or the use according to (29), wherein the

tauopathy is frontotemporal lobar degeneration with tau pathology.
[0040]

(32) The pharmaceutical composition, the anti-EphA4 antibody, the method for treating, or the use according to (31), wherein the frontotemporal lobar degeneration with tau pathology is progressive supranuclear palsy, corticobasal degeneration, argyrophilic grain dementia, senile dementia of the neurofibrillary tangle type, or Pick's disease.

[0041]

(33) The pharmaceutical composition, the anti-EphA4 antibody, the method for treating, or the use according to (32), wherein the frontotemporal lobar degeneration with tau pathology is progressive supranuclear palsy.

[0042]

(34) The pharmaceutical composition, the anti-EphA4 antibody, the method for treating, or the use according to (32), wherein the frontotemporal lobar degeneration with tau pathology is corticobasal degeneration.

[0043]

(35) The pharmaceutical composition, the anti-EphA4 antibody, the method for treating, or the use according to (32), wherein the frontotemporal lobar degeneration with tau pathology is argyrophilic grain dementia.

[0044]

(36) The pharmaceutical composition, the anti-EphA4 antibody, the method for treating, or the use according to (32), wherein the frontotemporal lobar degeneration with tau pathology is senile dementia of the neurofibrillary tangle type.

[0045]

(37) The pharmaceutical composition, the anti-EphA4 antibody, the method for treating, or the use according to (32), wherein the frontotemporal lobar degeneration with tau pathology is Pick's disease.

Effects of the Invention

[0046]

The present disclosure provides an anti-EphA4 antibody that can bind to EphA4 and enhance the cleavage of EphA4, a nucleic acid that encodes the antibody, a vector that comprises the nucleic acid, a cell comprising the vector, a method of producing the antibody, and a pharmaceutical composition comprising the antibody as the active ingredient.

Brief Description of the Drawings

[0047]

Figure 1 shows the binding affinity of the anti-EphA4 monoclonal antibody (antibody A) against mouse and human EphA4.

Figure 2 shows EphA4 cleavage enhancement activity of the anti-EphA4 monoclonal antibody (antibody A) employing hippocampus neurons.

Figure 3 shows mouse EphA4-mouse ligand binding inhibitory activity of the anti-EphA4 monoclonal antibody (antibody A).

Figure 4 shows human EphA4-human ligand binding inhibitory activity of the anti-EphA4 monoclonal antibody (antibody A).

Figure 5 shows the selectivity of the anti-EphA4 monoclonal antibody (antibody A) against each human Eph receptor.

Figure 6 shows the selectivity of the anti-EphA4 monoclonal antibody (antibody A) against each mouse Eph receptor.

Figure 7 shows the reactivity of the anti-EphA4 monoclonal antibody (antibody A) against mouse, rat, monkey, and human EphA4.

Figure 8 shows the reactivity of the anti-EphA4 monoclonal antibody (antibody A) against human EphA4 extracellular region (ECD), ligand binding domain (LBD), fibronectin type III domain 1 (FN1), and fibronectin type III domain 2 (FN2).

Figure 9 shows the effect of the anti-EphA4 monoclonal antibody (antibody A) on increasing the number of spines in the hippocampus neuron.

Figure 10 shows the the effect to suppress tau phosphorylation of the anti-EphA4 monoclonal antibody *in vivo*.

Figure 11A shows the amino acids of the EphA4-Ligand Binding Domain (EphA4-LBD) on the horizontal axis, and the structural region of antibody A-Fab on the vertical axis. Black bits show the

intersecting points of combinations where interaction is present.

Figure 11B shows the surface structure of EphA4-Ligand Binding Domain (EphA4-LBD). In Figure 11B, amino acid names and residue numbers contained in the binding region are shown at the corresponding positions, and the CDRs of H-chain and L-chain of the binding antibody A-Fab are shown in a ribbon model.

Figure 12 shows the affinity of the humanized anti-EphA4 monoclonal antibody (antibody B) against human EphA4.

Figure 13 shows EphA4 cleavage enhancement activity of the humanized anti-EphA4 monoclonal antibody (antibody B) in the hippocampus neuron.

Figure 14 shows human EphA4-human ligand binding inhibitory activity of the humanized anti-EphA4 monoclonal antibody (antibody B).

Figure 15 shows mouse EphA4-mouse ligand binding inhibitory activity of the humanized anti-EphA4 monoclonal antibody (antibody B).

Figure 16 shows the selectivity of the humanized anti-EphA4 monoclonal antibody (antibody B) against human Eph receptor.

Figure 17 shows the selectivity of the humanized anti-EphA4 monoclonal antibody (antibody B) against mouse Eph receptor.

Figure 18 shows the reactivity of the humanized anti-EphA4 monoclonal antibody (antibody B) against mouse, rat, monkey, and human EphA4.

Figure 19 shows the reactivity of the humanized anti-EphA4 monoclonal antibody (antibody B) against human EphA4 extracellular region (ECD), ligand binding domain (LBD), fibronectin type III domain 1 (FN1), and fibronectin type III domain 2 (FN2).

Figure 20 shows the effect of the humanized anti-EphA4 monoclonal antibody (antibody B) on increasing the number of spines in the hippocampus neuron.

Figure 21 shows human EphA4 cleavage enhancement activity of the humanized anti-EphA4 monoclonal antibody (antibody B) in the hippocampus neuron.

Figure 22 shows the effect of the humanized anti-EphA4 monoclonal antibody (antibody B) via MMP and ADAM on increasing the

number of spines in the hippocampus neuron.

Figure 23 shows the the effect to suppress tau phosphorylation of the humanized anti-EphA4 monoclonal antibody (antibody B) *in vivo*.

Description of Embodiments

[0048]

The regions specified or coded by the SEQ ID NOs. used herein are as follows:

Seq No.	Sequence Region	SEQ No	Sequence Region
1	Mouse EphA4 (amino acid sequence)	32	Monkey EphA4 (amino acid sequence)
2	Extracellular region of mouse EphA4 (amino acid sequence)	33	Extracellular region of monkey EphA4 (amino acid sequence)
3	Mouse EphA4 extracellular region-SEAP-His protein (amino acid sequence)	34	Signal sequence of human EphA4 (amino acid sequence)
4	Signal sequence of mouse EphA4 (amino acid sequence)	35	Signal sequence of preprotrypsin (amino acid sequence)
5	Human EphA4 (amino acid sequence)	36	Extracellular region of human EphA4 (amino acid sequence)
6	Extracellular region of human EphA4 (amino acid sequence)	37	Ligand binding domain of human EphA4 (amino acid sequence)
7	OligoDNA ad29S (nucleic acid sequence)	38	Fibronectin type III domain 1 of human EphA4 (amino acid sequence)
8	OligoDNA ad29AS (nucleic acid sequence)	39	Fibronectin type III domain 2 of human EphA4 (amino acid sequence)
9	5' Forward primer (nucleic acid sequence)	40	Light chain FR IGKV1-17*01 of human antibody (amino acid sequence)

10	3' Reverse primer for mouse IgG heavy chain (nucleic acid sequence)	41	Light chain FR JK4 of human antibody (amino acid sequence)
11	3' Reverse primer for mouse Igκ light chain (nucleic acid sequence)	42	Heavy chain FR IGHV3-33*03 of human antibody (amino acid sequence)
12	Heavy chain signal sequence of antibody A (amino acid sequence)	43	Heavy chain FR JH6 of human antibody (amino acid sequence)
13	Heavy chain variable region of antibody A (amino acid sequence)	44	Heavy chain CDR1 of antibody B (amino acid sequence)
14	Light chain signal sequence of antibody A (amino acid sequence)	45	Heavy chain variable region HK2-42 of antibody B (amino acid sequence)
15	Light chain variable region of antibody A (amino acid sequence)	46	Light chain variable region L1-8 of antibody B (amino acid sequence)
16	Heavy chain signal sequence of antibody A (nucleic acid sequence)	47	Human IgG ₂ heavy chain constant region of antibody B (amino acid sequence)
17	Heavy chain variable region of antibody A (nucleic acid sequence)	48	Human Igκ light chain constant region of antibody B (amino acid sequence)
18	Light chain signal sequence of antibody A (nucleic acid sequence)	49	Heavy chain CDR1 of antibody B (nucleic acid sequence)
19	Light chain variable region of antibody A (nucleic acid sequence)	50	Heavy chain CDR2 of antibody B (nucleic acid sequence)
20	5' Forward primer for antibody A heavy chain (nucleic acid sequence)	51	Heavy chain CDR3 of antibody B (nucleic acid sequence)
21	5' Forward primer for	52	Light chain CDR1 of

	antibody A light chain (nucleic acid sequence)		antibody B (nucleic acid sequence)
22	3' Reverse primer for antibody A heavy chain (nucleic acid sequence)	53	Light chain CDR2 of antibody B (nucleic acid sequence)
23	3' Reverse primer for antibody A light chain (nucleic acid sequence)	54	Light chain CDR3 of antibody B (nucleic acid sequence)
24	Heavy chain constant region of antibody A (amino acid sequence)	55	Heavy chain variable region HK2-42 of antibody B (nucleic acid sequence)
25	Light chain constant region of antibody A (amino acid sequence)	56	Light chain variable region L1-8 of antibody B (nucleic acid sequence)
26	Heavy chain CDR1 of antibody A (amino acid sequence)	57	Human IgG ₂ heavy chain constant region of antibody B (nucleic acid sequence)
27	Heavy chain CDR2 of antibody A (amino acid sequence)	58	Human Igκ light chain constant region of antibody B (nucleic acid sequence)
28	Heavy chain CDR3 of antibody A (amino acid sequence)	59	Heavy chain full length sequence of antibody B (amino acid sequence)
29	Light chain CDR1 of antibody A (amino acid sequence)	60	Light chain full length sequence of antibody B (amino acid sequence)
30	Light chain CDR2 of antibody A (amino acid sequence)	61	Heavy chain full length sequence of antibody B (nucleic acid sequence)
31	Light chain CDR3 of antibody A (amino acid sequence)	62	Light chain full length sequence of antibody B (nucleic acid sequence)

[0049]

The present disclosure relates to an anti-EphA4 antibody that

binds to EphA4.

The anti-EphA4 antibody according to the present disclosure is an antibody that can recognize and bind to EphA4, and as described below, the antibody may be an intact antibody, or may be a synthetic antibody (such as a recombinant antibody, a chimeric antibody, a humanized antibody, and the like), as long as it possesses binding affinity with EphA4. EphA4 herein can be understood as referring to human-, mouse-, rat-, and monkey-derived EphA4. Human-, mouse-, rat-, and monkey-derived EphA4 can be obtained from a public database where sequence information is registered, such as Genbank provided by the United States National Center for Biotechnology Information, or EphA4 gene sequence information can be obtained by designing primers based on the base sequence information of EphA4 of a closely related animal specie, and then cloning from RNA extracted from the desired animal specie. For example, the base sequence information of human, mouse, rat, and monkey EphA4 is registered in the database as Genbank Accession Nos. NM_004438.5, NM_007936.3, NM_001162411.1, and NM_001260870.1, respectively.

[0050]

In one aspect, the anti-EphA4 antibody is an antibody that specifically binds to EphA4. The term "specific binding" is a term well-known to those skilled in the pertaining technical field, and methods for determining the specific binding between an antibody or an antigen binding fragment thereof and an antigen or an epitope are also well-known. In one embodiment, "specific binding" is understood as that the anti-EphA4 antibody can bind to EphA4 by immunological reaction with higher binding affinity and binding activity, more rapidly, and/or for a longer period of time compared to when binding with other target molecules. This does not mean that the antibody that specifically binds to EphA4 does not bind to other target molecules. In another embodiment, "specific binding" may be shown by an antibody having a K_D against EphA4 of at least about 10^{-7} M, or at least about 10^{-8} M, or at least about 10^{-9} M, or lower. Moreover, in another further embodiment, "specific binding" is understood as binding to EphA4 by immunological reaction but does not substantially bind to other family molecules of the Eph receptor.

[0051]

In one aspect, the anti-EphA4 antibody is an antibody that binds to the extracellular region of EphA4. In one embodiment, the anti-EphA4 antibody is an antibody that binds to the ligand binding domain (LBD) among the extracellular regions of EphA4.

[0052]

In one embodiment, the anti-EphA4 antibody can specifically bind to EphA4 and enhance the cleavage of EphA4. In a particular embodiment, the anti-EphA4 antibody can specifically bind to EphA4 and enhance the cleavage of the EphA4 extracellular domain by matrix metalloprotease (MMP) or ADAM (a disintegrin and metalloproteinase).

[0053]

In one embodiment, the anti-EphA4 antibody can specifically bind to EphA4 and inhibit the binding between EphA4 and ephrin which is a ligand thereof.

[0054]

In another embodiment, the anti-EphA4 antibody can specifically bind to EphA4 and increase the number of spines in the hippocampus neuron or stabilize the spines in the hippocampus neuron.

[0055]

In one embodiment, the present disclosure encompasses an anti-EphA4 antibody that can specifically bind to at least one of human EphA4, mouse EphA4, rat EphA4, and monkey EphA4 and inhibit the binding with a ligand thereof. In another embodiment, the present disclosure encompasses an anti-EphA4 antibody that can specifically bind to two or more of human EphA4, mouse EphA4, rat EphA4, and monkey EphA4 and inhibit the binding with a ligand thereof. In another further embodiment, the present disclosure encompasses an anti-EphA4 antibody that can specifically bind to all of human EphA4, mouse EphA4, rat EphA4, and monkey EphA4 and inhibit the binding with a ligand thereof.

[0056]

For the method for measuring the antigen binding property (such as binding affinity and cross-species reactivity) of the anti-EphA4 antibody, methods well-known to those skilled in the art in the pertaining technical field may be employed. For example, binding

affinity may be measured using, but not limited to, Biacore™ biosensor, KinExA biosensor, scintillation proximity assay, ELISA, ORIGEN immunoassay (IGEN), flow cytometry, fluorescence quenching, fluorescence metastasis, yeast display, and/or immunostaining. The neutralizing activity of anti-EphA4 antibody against the binding between EphA4 and a ligand thereof may be measured using, but not limited to, Biacore™ biosensor, ELISA, and/or flow cytometry.

[0057]

The anti-EphA4 antibody according to the present disclosure may be a monoclonal antibody as long as it binds to EphA4.

[0058]

The anti-EphA4 antibody according to the present disclosure may be of any class such as IgG, IgA, or IgM (or subclasses thereof), and is not limited to a particular class. Immunoglobulins are classified into different classes depending on the antibody amino acid sequence of the constant region of the heavy chain (may be referred to as H-chain). There are five major immunoglobulin classes: IgA, IgD, IgE, IgG, and IgM, and some of these may be further subdivided into subclasses (isotypes) of e.g. IgG₁, IgG₂, IgG₃, IgG₄, IgA₁, and IgA₂. The constant regions of the heavy chain corresponding to the different classes of immunoglobulins are called α , δ , ϵ , γ , and μ , respectively. Moreover, the types of the light chain (may be referred to as L-chain) of the antibody are the λ chain and the κ chain. The anti-EphA4 antibody according to the present disclosure may be an IgG antibody, for example may be an IgG₁ antibody or an IgG₂ antibody, and the like. Moreover, the anti-EphA4 antibody according to the present disclosure may be in some instances in the form of a monomer, a dimer, or a multimer.

[0059]

The variable region of the antibody according to the present disclosure may mean the variable region of the antibody light chain and/or the variable region of the antibody heavy chain, and the constant region of the antibody may mean the constant region of the antibody light chain and/or the constant region of the antibody heavy chain. The variable regions of the heavy and light chains each consist of four framework regions (FRs) connected by three CDRs also

known as complementarity-determining regions. The CDRs in each chain are kept in proximity by FRs, and along with the CDRs in the other chain, contribute to the formation of the antigen binding site of the antibody. Technologies for determining CDRs include, but are not limited to, for example, (1) an approach based on cross-species sequence variability (e.g. Kabat et al, Sequences of Proteins of Immunological Interest, 5th ed., 1991, National Institutes of Health, Bethesda MD); and (2) an approach based on crystal structure research of antigen-antibody complexes (Al-lazikani et al., 1997 J. Molec. Biol. 273: 927-948). These and other approaches may be employed in combination.

[0060]

A monoclonal antibody herein may mean an antibody obtained from a population of essentially uniform antibodies. In other words, individual antibodies contained in the population is identical except for natural mutants that may possibly be present in small amounts. Monoclonal antibodies are against single antigenic sites, and are very specific. Further, in contrast to a typical polyclonal antibody which targets different antigens or different epitopes, each monoclonal antibody targets a single epitope of an antigen. The modifier "monoclonal" indicates the property of an antibody obtained from an essentially uniform antibody population, and is not to be understood in a limited way as requiring the production of the antibody by a particular method.

[0061]

The anti-EphA4 antibody according to the present disclosure may be a mouse antibody, a chimeric antibody, or a humanized antibody. A chimeric antibody is e.g. an antibody where the variable region of a non-human (such as a mouse or a rat) antibody is fused with the constant region of a human antibody, and for example may refer to an antibody where the variable region is derived from a non-human antibody and the constant region is derived from a human antibody. A humanized antibody is e.g. an antibody where a complementarity-determining region (CDR (may also be referred to as a hypervariable region)) of a non-human antibody is introduced into a human antibody, and for example may refer to an antibody where the

CDR is derived from a non-human antibody and the remaining regions of the antibody are derived from a human antibody. Note that the borderline between a chimeric antibody and a humanized antibody does not necessarily need to be clear, and an antibody may be in a state that may be called a chimeric antibody or a humanized antibody. Moreover, in a chimeric antibody or a humanized antibody, the antibody region derived from a human antibody (FR, constant region) does not necessarily need to be all composed of amino acids derived from a human antibody, and may comprise one or multiple amino acids derived from a non-human antibody, as long as it can be used normally in a human subject. One embodiment of a humanized antibody is an antibody where the CDR is derived from a rodent antibody and the remaining regions of the antibody are derived from a human antibody. A particular embodiment of a humanized antibody is an antibody where the CDR is derived from a mouse antibody and the remaining regions of the antibody are derived from a human antibody. In these embodiments, the CDR may comprise one or multiple amino acids derived from a non-rodent antibody or one or multiple amino acids derived from a non-mouse antibody, and the antibody regions other than the CDR may comprise one or multiple amino acids derived from a non-human antibody. Here, "multiple" is, but it not limited to, 2 - 20, or 2 - 15, such as 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, or 2, or within 10%, within 9%, within 8%, within 7%, within 6%, within 5%, within 4%, within 3%, within 2%, or within 1% of the number of amino acids in an amino acid sequence. Humanization can be performed with a CDR transplantation method (Kontermann and Dubel, Antibody Engineering, Springer Lab Manual (2001) and Tsurushita et al., Methods 36: 69-83 (2005)), and further may also be performed by substituting the CDR sequence with a corresponding sequence in the human antibody with methods well-known in the pertaining technical field (see e.g. Jones et al., Nature 321: 522-525 (1986); Riechmann et al., Nature 332: 323-327 (1988); and Verhoeyen et al., Science 239: 1534-1536 (1988)).

[0062]

In order to decrease antigenicity, it may be important to select the use of human variable regions in both light and heavy chains

upon preparation of the humanized antibody. According to the "best-fit" method, the variable region sequence of the rodent antibody is screened against the entire library of known human FR sequences. Following that, a human sequence that is the closest to the rodent sequence is accepted as the human FR of the humanized antibody. See e.g. Sims et al., J. Immunol. 151: 2296-2308 (1993) and Chothia et al., J. Mol. Biol. 196: 901-917 (1987). In another method, a particular framework derived from a sequence common in all human antibodies of a particular subgroup of the light or heavy chain is employed. The same framework may be employed for several different humanized antibodies. See e.g. Carter et al., Proc. Natl. Acad. Set USA 89: 4285-4289 (1992) and Presta et al., J. Immunol. 151: 2623-2632 (1993).

[0063]

Further, in general, it is desirable that the humanized antibody retains the high binding affinity towards the antigen and other preferred biological properties. To this end, according to one method, humanized antibodies are prepared by an analysis step of the parent sequence and various conceptual humanized products employing three-dimensional models of the parent and humanized sequences. In general, three-dimensional immunoglobulin models are available and known to those skilled in the art. Computer programs that illustrate and present promising three-dimensional conformations of selected candidate immunoglobulin sequences are available. Investigation of these presentations will allow analysis of possible roles of residues in the functions of candidate immunoglobulin sequences, i.e. analysis of residues that influence the ability of candidate immunoglobulins to bind to their antigens. With this method, FR residues can be selected from recipient and imported sequences and used in combination so that desirable antibody properties such as increase in the binding affinity to single or multiple target antigen(s) (such as EphA4 or a fragment thereof) are accomplished.

[0064]

Needless to say, an antibody with appropriate alteration (such as modification of the antibody, or partial substitution, addition

and/or deletion of the amino acid sequence of the antibody) in the chimeric or humanized antibody exemplified above while retaining the function of the antibody (or in order to add or improve the function of the antibody) is also encompassed in the anti-EphA4 antibody according to the present disclosure. More specifically, an antibody having alteration in the amino acid sequence of the constant region in order to modify the effector function of the antibody is also included in the scope of the present disclosure. For example, an antibody having valine (Val) at position 234 (Eu numbering) of human IgG₂ antibody substituted to alanine (Ala) and having glycine (Gly) at position 237 substituted to alanine (Ala) in order to reduce antibody-dependent cellular cytotoxicity (ADCC) activity and/or antibody-dependent cellular phagocytosis (ADCP) activity etc. is also included in the scope of the present disclosure. Further, a bispecific antibody that has an antibody binding site having the CDR sequence of the anti-EphA4 antibody according to the present disclosure together with an antigen binding site that binds to a different antigen (Kontermann (2012), mAbs 4, 182-97) is also included in the scope of the present disclosure.

[0065]

The anti-EphA4 antibody according to the present disclosure may be modified as desired. Modification of the anti-EphA4 antibody may be a modification that changes (a) the three-dimensional structure of the amino acid sequence in the region to be modified, such as sheet or helix conformation etc.; (b) the charge or hydrophobic state of the molecule at the target site; or (c) the effect of the modification on the maintenance of the side chain volume, or may be a modification in which these changes are not clearly observed.

[0066]

Modification of the anti-EphA4 antibody according to the present disclosure may be accomplished by e.g. substitution, deletion, addition, and the like of the constituent amino acid residues.

[0067]

An amino acid herein is employed in its broadest meaning, and includes not only natural amino acids, such as serine (Ser),

asparagine (Asn), valine (Val), leucine (Leu), isoleucine (Ile), alanine (Ala), tyrosine (Tyr), glycine (Gly), lysine (Lys), arginine (Arg), histidine (His), aspartic acid (Asp), glutamic acid (Glu), glutamine (Gln), threonine (Thr), cysteine (Cys), methionine (Met), phenylalanine (Phe), tryptophan (Trp), and proline (Pro), but also non-natural amino acids such as amino acid variants and derivatives. Those skilled in the art will naturally understand in light of this broad definition that e.g. L-amino acids; D-amino acids; chemical modified amino acids such as amino acid variants and amino acid derivatives; amino acids that will not be protein components in the body such as norleucine, β -alanine, and ornithine; and chemically synthesized compounds having amino acid properties well-known to those skilled in the art, and the like are included as the amino acids of the present specification. Examples of a non-natural amino acid can include, e.g., α -methylamino acids (such as α -methylalanine), D-amino acids (such as D-aspartic acid and D-glutamic acid), histidine-like amino acids (such as 2-amino-histidine, β -hydroxy-histidine, homohistidine, α -fluoromethyl-histidine, and α -methyl-histidine), amino acids having excess methylene on the side chain ("homo" amino acid), and amino acids where the carboxylate functional group amino acid in the side chain is substituted with a sulfonate group (such as cysteic acid).

[0068]

Naturally-occurring amino acid residues may be classified into e.g. groups below based on general side chain properties:

- (1) Hydrophobic: Met, Ala, Val, Leu, Ile;
- (2) Neutral hydrophilic: Asn, Gln, Cys, Ser, Thr;
- (3) Acidic: Asp, Glu;
- (4) Basic: His, Lys, Arg;
- (5) Residues that influence chain orientation: Gly, Pro; and
- (6) Aromatic: Trp, Tyr, Phe.

[0069]

Nonconservative substitution of the amino acid sequence constituting the antibody may be performed by exchanging an amino acid belonging to one of these groups with an amino acid belonging to another group. A more conservative substitution may be performed

by exchanging an amino acid belonging to one of these groups with another amino acid in the same group. Similarly, deletion or substitution of the amino acid sequence may be appropriately performed.

[0070]

Modification of amino acids configuring the antibody may be e.g. glycosylation by a carbohydrate or post-translational modifications such as acetylation or phosphorylation. The antibody may be glycosylated at a conserved position in its constant region. Glycosylation of an antibody is ordinarily either N-linked or O-linked. N-linked means the binding of a carbohydrate portion to the side chain of an asparagine residue. Tripeptide sequences asparagine-X-serine, asparagine-X-threonine, and asparagine-X-cysteine (wherein X is any amino acid other than proline) are recognition sequences for enzymatically adding the carbohydrate portion to the asparagine side chain. When one of these tripeptide sequences is present in the antibody, a potential glycosylation site is present. O-linked glycosylation may be the binding of either N-acetylgalactosamine, galactose, or xylose to a hydroxy amino acid (such as serine or threonine), and in some instances may be the binding to 5-hydroxyproline or 5-hydroxylysine. Those skilled in the art can appropriately select the glycosylation condition (such as the type of host cell or cell medium and pH etc., when glycosylation is performed with a biological means) according to the objective.

[0071]

The anti-EphA4 antibody according to the present disclosure may further be modified by other modification methods, alone or in combination, based on common technical knowledge well-known to those skilled in the art.

[0072]

The anti-EphA4 antibody according to the present disclosure can be produced by a method well-known to those skilled in the art. For example, the antibody may be produced by integrating the nucleic acid encoding the anti-EphA4 antibody according to the present disclosure into an expression vector, introducing the expression

vector into a host cell, and culturing the host cell. Accordingly, the present disclosure encompasses a nucleic acid encoding the anti-EphA4 antibody, a vector that comprises the nucleic acid, a host cell comprising the vector, and a method of producing the anti-EphA4 antibody comprising a step of culturing the host cell.

[0073]

The nucleic acid encoding the anti-EphA4 antibody according to the present disclosure may have DNA encoding a signal sequence, or may have DNA encoding a signal sequence at the 5' terminal of the DNA encoding the heavy chain variable region and the DNA encoding the light chain variable region. A signal sequence is amino acid residues present at the N-terminal of the protein that is necessary for a secretory protein or an integral membrane protein to pass through the lipid bilayer after being synthesized on a ribosome, and in the present disclosure, is not particularly limited as long as it is a sequence having this function. Signal sequences that may be contained in the anti-EphA4 antibody according to the present disclosure include signal sequences derived from human, mouse, rat, rabbit, donkey, goat, horse, bird, dog, cat, yeast, and the like. Specifically, in the present disclosure, a peptide comprising an amino acid sequence represented by SEQ ID NO. 12 or 16 can be included as a signal sequence related to the heavy chain, and a peptide comprising an amino acid sequence represented by SEQ ID NO. 14 or 18 can be included as a signal sequence related to the light chain. Moreover, as long as it is functionally equivalent, the signal sequence may have substitution, addition, and/or deletion of one or multiple (such as 2, 3, 4, or 5) amino acids in the amino acid sequence represented by SEQ ID NO. 12 or 16 and the amino acid sequence represented by SEQ ID NO. 14 or 18.

[0074]

The anti-EphA4 antibody according to the present disclosure may be that isolated or purified according to a method well-known to those skilled in the art.

[0075]

"Isolated" or "purified" herein means artificially isolated or purified from the natural state. If the molecule or composition

is naturally occurring, it is "isolated" or "purified" when it is changed or removed from the original environment, or both. Examples of an isolation or purification method include, but are not limited to, means by electrophoresis, molecular biology, immunology, or chromatography, and the like, specifically, ion exchange chromatography, hydrophobic chromatography, reverse phase HPLC chromatography, isoelectric focusing, or alkali extraction method, and the like.

[0076]

In one embodiment, the anti-EphA4 antibody comprises the following CDRs:

- (a) a heavy chain CDR1 consisting of the amino acid sequence shown in SEQ ID NO. 44;
- (b) a heavy chain CDR2 consisting of the amino acid sequence shown in SEQ ID NO. 27;
- (c) a heavy chain CDR3 consisting of the amino acid sequence shown in SEQ ID NO. 28;
- (d) a light chain CDR1 consisting of the amino acid sequence shown in SEQ ID NO. 29;
- (e) a light chain CDR2 consisting of the amino acid sequence shown in SEQ ID NO. 30; and
- (f) a light chain CDR3 consisting of the amino acid sequence shown in SEQ ID NO. 31.

[0077]

In one embodiment, the anti-EphA4 antibody is a humanized antibody or a chimeric antibody, and in a particular embodiment a humanized antibody.

[0078]

In another embodiment, the anti-EphA4 antibody comprises heavy and light chains, the variable region of the heavy chain comprises the amino acid sequence shown in SEQ ID NO. 45, and the variable region of the light chain comprises the amino acid sequence shown in SEQ ID NO. 46. Note that in the embodiment, the variable region of the heavy chain and/or the variable region of the light chain may comprise an amino acid sequence having one or multiple amino acids substituted, added, and/or deleted in the amino acid sequence shown in SEQ ID NO.

45 and/or the amino acid sequence shown in SEQ ID NO. 46. Here, "multiple" is not limited as long as it retains the binding affinity towards EphA4 and enhances the cleavage of EphA4, and is 2 - 15, or 2 - 10, such as 9, 8, 7, 6, 5, 4, 3, or 2, or within 10%, such as within 9%, within 8%, within 7%, within 6%, within 5%, within 4%, within 3%, within 2%, or within 1% of the number of amino acids in an amino acid sequence.

[0079]

In one embodiment, the heavy chain of the anti-EphA4 antibody comprises the constant region of human IgG₂.

[0080]

In a particular embodiment, the constant region of human IgG₂ comprises the amino acid sequence of SEQ ID NO. 47.

[0081]

In one embodiment, the light chain of the anti-EphA4 antibody comprises the constant region of human Igκ.

[0082]

In a particular embodiment, the constant region of human Igκ comprises the amino acid sequence of SEQ ID NO. 48.

[0083]

In one embodiment, the anti-EphA4 antibody comprises a heavy chain comprising the amino acid sequence shown in SEQ ID NO. 59 and a light chain comprising the amino acid sequence shown in SEQ ID NO. 60.

[0084]

In another embodiment, for example, for reasons such as decreasing the ununiformity of antibodies produced by antibody-producing cells (U.S. Patent Application Publication No. 2010/0297697 or Liu H et al., MAbs. 2014 Sep-Oct; 6 (5): 1145-1154), the anti-EphA4 antibody has lysine positioned at the C-terminal (carboxy terminal) of the heavy chain deleted. In the present disclosure, an anti-EphA4 antibody having the C-terminal lysine of the heavy chain deleted also includes an anti-EphA4 antibody having the C-terminal lysine of the heavy chain deleted by genetic modification or an anti-EphA4 antibody having the C-terminal lysine of the heavy chain cleaved post-translationally by carboxypeptidase

etc., and the like. Moreover, in the present disclosure, an anti-EphA4 antibody having the C-terminal lysine of the heavy chain deleted includes not only an anti-EphA4 antibody having the C-terminal lysine deleted in both heavy chains, but also an anti-EphA4 antibody having the C-terminal lysine deleted in only one heavy chain. [0085]

In one aspect, the present disclosure relates to an isolated nucleic acid encoding an anti-EphA4 antibody. An isolated nucleic acid encoding an anti-EphA4 antibody refers to one or more nucleic acid molecules encoding the heavy chain and/or light chain of an anti-EphA4 antibody. In one embodiment, the nucleic acid according to the present disclosure encodes the heavy chain of the anti-EphA4 antibody. In another embodiment, the nucleic acid according to the present disclosure encodes the light chain of the anti-EphA4 antibody. In another further embodiment, the nucleic acid according to the present disclosure encodes the heavy and light chains of the anti-EphA4 antibody. The nucleic acid according to the present disclosure also includes a first nucleic acid molecule encoding the heavy chain of the anti-EphA4 antibody and a second nucleic acid molecule encoding the light chain of the anti-EphA4 antibody. [0086]

In another aspect, the present disclosure relates to a vector comprising the isolated nucleic acid encoding the anti-EphA4 antibody. The vector according to the present disclosure refers to one or more vectors comprising the isolated nucleic acid encoding the anti-EphA4 antibody. In one embodiment, the vector according to the present disclosure is a vector comprising the nucleic acid encoding the heavy chain of the anti-EphA4 antibody and the nucleic acid encoding the light chain of the anti-EphA4 antibody. In another embodiment, the vector according to the present disclosure is a vector comprising the nucleic acid encoding the heavy and light chains of the anti-EphA4 antibody. In another further embodiment, the vector according to the present disclosure comprises a first vector comprising the nucleic acid encoding the heavy chain of the anti-EphA4 antibody and a second vector comprising the nucleic acid encoding the light chain of the anti-EphA4 antibody. The vector according

to the present disclosure may be, but not limited to, a plasmid, a cosmid, a virus, a phage, and the like. For example, as a viral vector, retroviral, lentiviral, adenoviral, adeno-associated viral, or herpes simplex viral vector, and the like are also included in the vector according to the present disclosure.

[0087]

In yet another aspect, a host cell comprising the vector according to the present disclosure, and a method of producing an anti-EphA4 antibody comprising a step of culturing the host cell are also included in the present disclosure. The host cell according to the present disclosure may be, but not limited to, E. coli cells, monkey COS cells, Chinese hamster ovary (CHO) cells, NS0 cells, and the like. In one embodiment, the method of producing an anti-EphA4 antibody comprises a step of culturing the host cell, and a step of recovering the anti-EphA4 antibody secreted from the host cell (or culture medium of host cell).

[0088]

In one aspect, the present disclosure relates to a pharmaceutical composition comprising an anti-EphA4 antibody. The pharmaceutical composition according to the present disclosure can be manufactured according to known methods such as methods described in the Pharmacopeia of Japan (JP), the United States Pharmacopeia (USP), or the European Pharmacopeia (EP), and the like.

[0089]

The anti-EphA4 antibody according to the present disclosure may be useful for treating Alzheimer's disease. In other words, in other aspects, the present disclosure encompasses a method for treating Alzheimer's disease comprising a step of administering a therapeutically effective amount of an anti-EphA4 antibody to a subject having Alzheimer's disease. Moreover, in other aspects, the present disclosure encompasses the use of an anti-EphA4 antibody for manufacturing a therapeutic drug for Alzheimer's disease. In other aspects, the present disclosure encompasses an anti-EphA4 antibody for use in the treatment of Alzheimer's disease.

[0090]

The anti-EphA4 antibody according to the present disclosure

may be useful for treating tauopathy. In other words, in other aspects, the present disclosure encompasses a method for treating tauopathy comprising a step of administering a therapeutically effective amount of an anti-EphA4 antibody to a subject having tauopathy. Moreover, in other aspects, the present disclosure encompasses the use of an anti-EphA4 antibody for manufacturing a therapeutic drug for tauopathy. In other aspects, the present disclosure encompasses an anti-EphA4 antibody for use in the treatment of tauopathy. Tauopathy of the present disclosure includes Alzheimer's disease or frontotemporal lobar degeneration with tau pathology (FTLD-tau). Moreover, frontotemporal lobar degeneration with tau pathology includes progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), argyrophilic grain dementia (AGD), senile dementia of the neurofibrillary tangle type (SD-NFT), Pick's disease (PiD), and the like.

[0091]

The anti-EphA4 antibody according to the present disclosure can be employed in the therapeutic method alone or in combination with other agents or compositions. For example, the anti-EphA4 antibody according to the present disclosure may be administered at the same or different times as another agent. Such combination therapy includes combination administration (two or more agents are included in the same or separate formulation) and separate administration (such as simultaneous or sequential). When two or more agents are separately administered, the administration of the anti-EphA4 antibody according to the present disclosure may be performed before or after the accompanying therapeutic method.

[0092]

The subject for administering the pharmaceutical composition according to the present disclosure is not limited, and can be employed for e.g. a human or non-human mammal (such as monkey, mouse, rat, rabbit, cow, horse, and goat).

[0093]

The method of administering the pharmaceutical composition according to the present disclosure to a subject (such as administration route, dosage, the number of administrations per day,

and administration timing) is not limited, and can be appropriately decided by those skilled in the art (such as a physician) according to the health state of the subject, the extent of the disease, the type of agents used in combination, and the like.

[0094]

Those skilled in the art shall recognize that as long as it is not technically contradicting, the present invention may be carried out in an appropriate combination of any one or more of any and all aspects described herein. Further, those skilled in the art shall recognize that as long as it is not technically contradicting, it is preferred that the present invention is carried out in an appropriate combination of any and all preferred or advantageous aspects described herein.

[0095]

All of the disclosures of the literatures cited herein shall be regarded as clearly cited herein by reference, and those skilled in the art can understand the related disclosure contents in these literatures by citing them as a part of the present specification according to the context herein without departing from the spirit and scope of the present invention.

[0096]

The literatures cited herein are provided with the sole purpose of disclosing the related technology preceding the filing date of the present application, and are not to be construed as an admission by the present inventors that the present invention does not hold the right to precede said disclosures due to prior inventions or for any other reason. All of the description of all these literatures is based on the information available to the present applicants, and does not configure in any way an acknowledgement that these description contents are accurate.

[0097]

The terms used herein are employed for describing particular embodiments, and do not intend to limit the invention.

[0098]

The term "comprise" as used herein, unless the content clearly indicates to be understood otherwise, intends the presence of the

described items (such as components, steps, elements, or numbers), and does not exclude the presence of other items (such as components, steps, elements, and numbers). The term "consist of" encompasses the aspects described by the terms "consist of" and/or "consist essentially of."

[0099]

The term "neutralizing activity" as used herein means the activity to inhibit the binding between EphA4 and a ligand thereof, and/or the activity to inhibit signal transduction or molecular expression response or functionality change of cells which are induced by EphA4 due to binding to a ligand thereof in the human body.

[0100]

Unless otherwise defined, all terms used herein (including technical and scientific terms) have the same meanings as those broadly recognized by those skilled in the art of the technology to which the present invention belongs. The terms used herein, unless explicitly defined otherwise, are to be construed as having meanings consistent with the meanings herein and in related technical fields, and shall not be construed as having idealized or excessively formal meanings.

[0101]

Terms such as first and second are employed to express various elements, and it should be recognized that these elements are not to be limited by these terms per se. These terms are employed solely for the purpose of discriminating one element from another, and it is for example possible to describe a first element as a second element, and similarly, to describe a second element as a first element without departing from the scope of the present invention.

[0102]

The numeric values employed herein for indicating component content or numeric value range and the like, unless explicitly indicated, are to be understood as being modified by the term "about." For example, "4°C," unless explicitly indicated, is understood to mean "about 4°C," and it is obvious that those skilled in the art can rationally understand the extent thereof in concordance with technical common sense and the meaning of the passages herein.

[0103]

It should be recognized that unless clearly indicated to mean otherwise in context, when used in the specification and the claims herein, each aspect represented in singular form may also be a plural form as long as it is not technically contradicting, and vice versa.

[0104]

The present invention will now be described in further detail with reference to Examples. However, the present invention can be embodied by various aspects, and shall not be construed as being limited to the Examples described herein. Those skilled in the art of related technical fields will be able to carry out the present invention with various modifications, additions, deletions, substitutions, and the like without altering the spirit or scope of the present invention.

Examples

[0105]

Reference Example 1: Preparation of anti-EphA4 monoclonal antibody (A) Preparation of mouse anti-EphA4 monoclonal antibody

In order to prepare a monoclonal antibody that binds to mouse EphA4 (Genbank Accession No. NP_031962.2, SEQ ID NO. 1), a protein having secretory alkaline phosphatase (SEAP) and histidine tag fused to the extracellular region of mouse EphA4 (positions 20-547) (SEQ ID NO. 2) (hereinafter referred to as "mouse EphA4 extracellular region-SEAP-His protein," SEQ ID NO. 3) was prepared by the following steps.

[0106]

First, the DNA sequences encoding the signal sequence (SEQ ID NO. 4) and the extracellular region (SEQ ID NO. 2) of mouse EphA4 were amplified by RT-PCR with total RNA derived from mouse brain, and cloned into the Sal I/Not I site of pENTR1A vector (Invitrogen/LifeTechnologies) having the DNA sequence encoding SEAP and histidine tag. Next, the DNA sequence encoding the signal sequence and extracellular region of mouse EphA4, SEAP, and histidine tag was transferred to pcDNA 3.1_rfcB vector by LR reaction of Gateway System (Invitrogen/LifeTechnologies) to construct pcDNA 3.1-mouse

EphA4 extracellular region-SEAP-His expression vector. The constructed pcDNA 3.1-mouse EphA4 extracellular region-SEAP-His expression vector was transfected into HEK293 EBNA cells (Invitrogen/LifeTechnologies) with TransIT-LT1 (TAKARA). After 6 days of incubation (5% CO₂, 37°C), the culture supernatant was recovered. From the recovered culture supernatant, mouse EphA4 extracellular region-SEAP-His protein (SEQ ID NO. 3) was purified with Protino column (MACHEREY-NAGEL).

[0107]

Twenty micrograms of mouse EphA4 extracellular region-SEAP-His protein was mixed with the same amount of TiterMax Gold adjuvant (TiterMax USA) or GERBU adjuvant (GERBU Biotechnik GmbH), and subcutaneously injected into the footpad of Balb/c mice. Mouse EphA4 extracellular region-SEAP-His protein was then similarly administered on Days 3, 7, and 10. Here, TiterMax Gold adjuvant (TiterMax USA) was used only on Day 10, and GERBU adjuvant (GERBU Biotechnik GmbH) was used on Days 3, 7, and 10. Mice were sacrificed on Day 13, and peripheral lymph nodes were recovered to prepare lymph node cells. The prepared lymph node cells and P3U1 myeloma cells (endowed from Kyoto University) were fused at a proportion of 5:1 in the presence of GenomeONE-CF (Ishihara Sangyo Kaisha, Ltd.). The fused cells were cultured in a 96-well plastic plate. After 7 days of incubation (5% CO₂, 37°C), the culture supernatant was recovered.

[0108]

Employing the culture supernatant obtained, wells having reactivity against mouse, rat, and human EphA4 were picked up.

[0109]

Reactivity against mouse, rat, and human EphA4 was evaluated with ELISA with proteins having the Fc region of human IgG₁ and histidine tag fused to the extracellular region of mouse EphA4, the extracellular region (positions 20-547) of rat EphA4 (Genbank Accession No. NP_001155883.1), or the extracellular region (positions 20-547) (SEQ ID NO. 6) of human EphA4 (Genbank Accession No. NP_004429.1, SEQ ID NO. 5) (hereinafter referred to "mouse EphA4 extracellular region-Fc-His protein," "rat EphA4 extracellular region-Fc-His protein," or "human EphA4 extracellular region-Fc-His

protein," respectively).

[0110]

Mouse, rat, or human EphA4 extracellular region-Fc-His proteins were prepared by the following steps. Initially, pcDNA 3.1-mouse, rat, or human EphA4 extracellular region-Fc-His expression vectors were constructed. First, the DNA sequences encoding the signal sequence and the extracellular region of mouse, rat, or human EphA4 were amplified by RT-PCR with total RNA derived from mouse, rat, or human brain, and cloned into the Sal I/Not I site of pENTR1A vector (Invitrogen/LifeTechnologies) having the DNA sequence encoding Fc and histidine tag. Next, the DNA sequences encoding the signal sequence and extracellular region of mouse, rat, or human EphA4, Fc, and histidine tag were transferred to pcDNA 3.1_rfcB vector by LR reaction of Gateway System (Invitrogen/LifeTechnologies) to construct pcDNA 3.1-mouse, rat, or human EphA4 extracellular region-Fc-His expression vectors. These expression vectors constructed were transfected into HEK293 EBNA cells (Invitrogen/LifeTechnologies) with TransIT-LT1 (TAKARA). After 6 days of incubation (5% CO₂, 37°C), the culture supernatant was recovered.

[0111]

ELISA employing mouse, rat, or human EphA4 extracellular region-Fc-His proteins was performed following the steps below. Anti-human IgG antibody (Jackson ImmunoResearch Laboratories) was coated onto the wells of a 96-well plate (Nunc). After incubating at 4°C overnight, wells were blocked at room temperature for one hour with 1 x Block ACE (Dainippon Seiyaku). After washing three times with 0.02% Tween 20/PBS (Nacalai Tesque), a culture supernatant comprising mouse, rat, or human EphA4 extracellular region-Fc-His protein was added to each well (final concentration 1 nM), and this was incubated at room temperature for one hour. After washing three times, the culture supernatant of the fused cells was added to each well. After incubating at room temperature for one hour and washing three times, horseradish peroxidase-labeled anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories) was added, and this was incubated at room temperature for one hour. After washing three times,

TMBZ (3,3',5,5'-tetramethylbenzidine, Sigma) solution was added to each well, and this was incubated at room temperature for 5-20 minutes. An equal amount of the stop solution (2 N H₂SO₄, Wako Pure Chemical) was added to each well, and absorbance at 450 nm was read with a microplate reader (PerkinElmer).

[0112]

Hybridomas were cloned from the wells picked up through the above steps by limiting dilution method, and hybridoma clones expressing mouse anti-EphA4 antibody having binding activity against mouse, rat, and human EphA4 was ultimately obtained.

[0113]

The hybridoma clones obtained were cultured, and mouse anti-EphA4 monoclonal antibody was purified from the culture supernatant with Protein A (GE Healthcare).

[0114]

(B) Evaluation of EphA4 cleavage enhancement activity

Preparation of rat hippocampus neurons was performed following the steps below. Fetuses were taken out of a rat at Day 18 of pregnancy (Charles River Laboratories Japan), and the head was cut open to take out the brain. The hippocampus region was cut out under a stereomicroscope, placed in the digestion solution (137 mM NaCl (Wako Pure Chemical), 5 mM KCl (Wako Pure Chemical), 7 mM Na₂HPO₄ (Wako Pure Chemical), 25 mM Hepes (DOJINDO), 0.5 mg/mL DNase (Sigma), and 0.25% trypsin (Life technologies)), and shaken at 37°C for 10 minutes. The solution was removed, and 20% Fetal bovine serum/Hanks buffer (Sigma) was added. After removing the solution and washing twice in Hanks buffer, the hippocampus tissue was pipetted in Hanks buffer to prepare a cell suspension. Cells were seeded in a 96-well dish (Falcon) coated with poly-L-lysine containing culture fluid (Neurobasal medium (Life technologies), 1 x B-27 supplement (Life technologies), and 0.5 mM L-glutamine (Life technologies)).

[0115]

Evaluation of EphA4 cleavage enhancement activity employing hippocampus neurons was performed following the steps below. Rat hippocampus neurons seeded in a 96-well dish (Falcon) were treated

with anti-EphA4 monoclonal antibody (67 nM) and γ selectase inhibitory drug Compound E (50 nM, Enzo Life Sciences). Sixteen hours later, this was washed with PBS (Wako Pure Chemical), SDS sample buffer (Laemmli sample buffer (Bio-Rad) and 5% 2-mercaptoethanol (Bio-Rad)) was added to recover the cells, and this was boiled for 5 minutes. SDS-PAGE was performed with this sample, western blotting with anti-EphA4 monoclonal antibody (Abnova) was performed, the band strength was quantified, and the value of EphA4 C-terminal fragment/full length EphA4 was calculated.

[0116]

Mouse anti-EphA4 monoclonal antibody having activity that enhances the cleavage of EphA4 (antibody A) was obtained. The isotype of antibody A was determined with monoclonal antibody isotyping kit (Serotec) to be IgG₁ for the heavy chain and κ for the light chain.

[0117]

(C) Sequence analysis of antibody A

The DNA sequence encoding the signal sequence and the variable region of heavy and light chains of antibody A was amplified by 5'-RACE (5'-rapid amplification of cDNA ends) method. Total RNA was prepared from the hybridoma with RNeasy (QIAGEN), and treated with DNase (QIAGEN, RNase free DNase set). Double-stranded cDNA was prepared from the total RNA with cDNA synthesis kit (TAKARA). 5' Adaptor obtained by annealing of oligoDNA ad29S (ACATCACTCCGT) (SEQ ID NO. 7) and oligoDNA ad29AS (ACGGAGTGATGTCCGTCGACGTATCTCTGCGTTGATACTTCAGCGTAGCT) (SEQ ID NO. 8) was added to the cDNA. The cDNA obtained was amplified with 5' forward primer (5'-PCR4 primer, AGCTACGCTGAAGTATCAACGCAGAG) (SEQ ID NO. 9) and 3' reverse primer (GCCAGTGGATAGACTGATGG (SEQ ID NO. 10) was employed for amplification of the mouse IgG heavy chain, and GATGGATACAGTTGGTGCAGC (SEQ ID NO. 11) was employed for amplification of the mouse Ig κ light chain). The amplified cDNA was inserted into pCR2.1 vector (Invitrogen/LifeTechnologies). The gene sequence of antibody A was analyzed with ABI 3130XL. As amino acid sequences coded by the gene sequence of antibody A identified by the present analysis, the heavy chain signal sequence is the sequence shown in

SEQ ID NO. 12, the heavy chain variable region is the sequence shown in SEQ ID NO. 13, the light chain signal sequence is the sequence shown in SEQ ID NO. 14, and the light chain variable region is the sequence shown in SEQ ID NO. 15. As nucleotide sequences coding the gene sequence of antibody A, heavy chain signal sequence is the sequence shown in SEQ ID NO. 16, the heavy chain variable region is the sequence shown in SEQ ID NO. 17, the light chain signal sequence is the sequence shown in SEQ ID NO. 18, and the light chain variable region is the sequence shown in SEQ ID NO. 19.

[0118]

The full length sequences of the heavy and light chains of antibody A were obtained with the following steps. Total RNA was prepared from the hybridoma with RNeasy (QIAGEN), and treated with DNase (QIAGEN, RNase free DNase set). Reverse transcription products were prepared from the total RNA with RNA PCR kit (TAKARA). Employing the reverse transcription products obtained as templates, the gene sequence encoding the heavy and light chains of antibody A was amplified with PCR with 5' forward primer (GCGAAGCTTGCCGCCACCATGGCTGTCTGGTGCTGCTCC (primer ID 7455) (SEQ ID NO. 20) was used for amplification of the heavy chain, and GCGAAGCTTGCCGCCACCATGGACATGAGGGTTCCTGCTCACG (primer ID 7453) (SEQ ID NO. 21) was used for amplification of the light chain) and 3' reverse primer (GCGGAATTCATCATTTACCAGGAGAGTGGGAGAGGC (primer ID 7257) (SEQ ID NO. 22) was used for amplification of the heavy chain, and CGCGAATTCATACTAACAATTCCTGTTGAAGCTCTTGAC (primer ID 7249) (SEQ ID NO. 23) was used for amplification of the light chain), and respectively cloned into pEE6.4 and pEE12.4 vectors (Lonza). The gene sequence was analyzed with ABI3130XL. As amino acid sequences coded by the gene sequence of antibody A identified by the present analysis, the heavy chain constant region is the sequence shown in SEQ ID NO. 24, and the light chain constant region is the sequence shown in SEQ ID NO. 25.

[0119]

The CDR of antibody A was determined with the following method. The amino acid sequence of antibody A was numbered according to the Kabat numbering system with Abysis software (UCL). Based on this

numbering, decision was made according to Kabat definition for CDR identification. The amino acid sequences of CDR of antibody A are shown in Table 1.

[Table 1]

Amino acid sequences of CDR of antibody A

Name	Sequence
Heavy chain CDR1	RYGVH (SEQ ID NO. 26)
Heavy chain CDR2	VIWRGGSTDYNAAFMS (SEQ ID NO. 27)
Heavy chain CDR3	ESLFGVYYDYGYYSDY (SEQ ID NO. 28)
Light chain CDR1	RASQEISGYLS (SEQ ID NO. 29)
Light chain CDR2	AASTLDS (SEQ ID NO. 30)
Light chain CDR3	LQYASYPLT (SEQ ID NO. 31)

[0120]

Reference Example 2: Binding affinity of anti-EphA4 monoclonal antibody against mouse and human EphA4

The binding affinity of antibody A against mouse and human EphA4 was determined by surface plasmon resonance (SPR method) employing Biacore T200 (GE Healthcare). First, anti-His antibody (GE Healthcare, 28-9950-56) was fixed onto a sensor chip CM5. Fixation was performed by amine coupling method employing N-hydroxysuccinimide (NHS) and N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), and ethanolamine was employed for blocking (sensor chip and fixation reagents were all from GE Healthcare). This was diluted to 3.5 µg/mL with the fixation buffer (10 mM sodium acetate, pH 4.5), and fixed on the sensor chip according to the protocol attached to Biacore T200. Mouse or human EphA4 extracellular region-SEAP-His10 was diluted with the running buffer HBS-EP (GE Healthcare, BR-1001-88), and the solution was sent onto a flow cell for 120 seconds for capture (the captured amount about 10 RU). Subsequently, antibody A serially diluted to the range of 100, 50, 25, 12.5, 6.3, 3.2, 1.6, and 0 nM with HBS-EP was added to the sensor chip for 120 seconds, and the binding reaction curve at the time of addition (binding phase, 120 seconds) and after addition had completed (dissociation phase, 600 seconds) was sequentially observed. After

the completion of each observation, 4 M MgCl₂ (60 seconds, Wako Pure Chemical) was added to regenerate the sensor chip. Fitting analysis by 1:1 binding model employing BIA evaluation software attached to the system was performed on the binding reaction curve obtained, and the binding affinity (KD = kd/ka) against mouse and human EphA4 was calculated.

[0121]

The binding affinity of antibody A against mouse and human EphA4 (KD value) was 1.32×10^{-9} M and 1.19×10^{-9} M, respectively (Figure 1). Other binding parameters against mouse and human EphA4 were almost to the same extent. Accordingly, it is thought that antibody A has the same extent of binding affinity towards mouse and human EphA4.

[0122]

Reference Example 3: EphA4 cleavage enhancement activity of anti-EphA4 monoclonal antibody in hippocampus neurons

For antibody A, evaluation of EphA4 cleavage enhancement activity employing hippocampus neurons was performed following the steps below. Rat hippocampus neurons seeded in a 96-well dish (Falcon) were treated with antibody A (2.0, 6.7, and 20 nM) and γ selectase inhibitory drug Compound E (50 nM, Enzo Life Sciences). Twenty-four hours later, this was washed with PBS (Wako Pure Chemical), SDS sample buffer (Laemmli sample buffer (Bio-Rad) and 5% 2-mercaptoethanol (Bio-Rad)) was added to recover the cells, and this was boiled for 5 minutes. SDS-PAGE was performed with this sample, western blotting with anti-EphA4 monoclonal antibody (Abnova) was performed, the band strength was quantified, and the value of EphA4 C-terminal fragment/full length EphA4 was calculated.

[0123]

Antibody A concentration-dependently enhanced EphA4 cleavage reaction in hippocampus neurons (Figure 2).

[0124]

Reference Example 4: Mouse EphA4-mouse ligand binding inhibitory activity of anti-EphA4 monoclonal antibody

For antibody A, the evaluation of the inhibitory activity of binding between mouse EphA4 and mouse ligand was performed following the steps below. Anti-alkaline phosphatase antibody (Thermo SCIENTIFIC) was coated onto the wells of a 96-well plate (Nunc). After incubating at 4°C overnight, wells were blocked at room temperature for one hour with 1% Block ACE (DS Pharma Biomedical). After washing three times with 0.02% Tween 20/PBS (Thermo SCIENTIFIC), mouse EphA4 extracellular region-SEAP-His protein was added to the wells (final concentration 10 nM), and this was incubated at room temperature for one hour. After washing three times, the ligand and antibody A (0, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30, 100, 300, 1000, and 3000 nM) were added to the wells. Note that biotinylated mouse Ephrin A1-Fc chimera (R&D Systems, final concentration 6 nM) and biotinylated mouse Ephrin B2-Fc chimera (R&D Systems, final concentration 2.5 nM) were employed as ligands. After incubating at room temperature for one hour and washing three times, horseradish peroxidase-labeled streptavidin (GE Healthcare) was added, and this was incubated at room temperature for one hour. After washing three times, TMBZ (3,3',5,5'-tetramethylbenzidine, Sigma) solution was added to the wells, and this was incubated at room temperature for 2 minutes. An equal amount of the stop solution (1N H₂SO₄, Wako Pure Chemical) was added to the wells, and absorbance at 450 nm was read with a microplate reader (PerkinElmer).

[0125]

Antibody A concentration-dependently suppressed the binding between mouse EphA4 and mouse ligand, and the IC₅₀ values against mouse Ephrin A1 and Ephrin B2 binding were about 5.9 nM and 3.1 nM, respectively (Figure 3). Accordingly, it was shown that antibody A strongly inhibits the binding between mouse EphA4 and mouse ligand.

[0126]

Reference Example 5: Human EphA4-human ligand binding inhibitory activity of anti-EphA4 monoclonal antibody

For antibody A, the evaluation of the inhibitory activity of binding between human EphA4 and human ligand was performed following the steps below. Anti-alkaline phosphatase antibody (Thermo

SCIENTIFIC) was coated onto the wells of a 96-well plate (Nunc). After incubating at 4°C overnight, wells were blocked at room temperature for one hour with 1% Block ACE (DS Pharma Biomedical). After washing three times with 0.05% Tween 20/PBS (Thermo SCIENTIFIC), human EphA4 extracellular region-SEAP-His protein was added to the wells (final concentration 10 nM), and this was incubated at room temperature for one hour. After washing three times, the ligand and serially diluted antibody A (0, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30, 100, 300, 1000, and 3000 nM) were added to the wells. Note that biotinylated human Ephrin A5-Fc chimera (R&D Systems, final concentration 0.7 nM) and biotinylated human Ephrin B3-Fc chimera (R&D Systems, final concentration 2.3 nM) were employed as ligands. After incubating at room temperature for one hour and washing three times, horseradish peroxidase-labeled streptavidin (GE Healthcare) was added, and this was incubated at room temperature for one hour. After washing three times, TMBZ (3,3',5,5'-tetramethylbenzidine, Sigma) solution was added to the wells, and this was incubated at room temperature for 2-5 minutes. An equal amount of the stop solution (1N H₂SO₄, Wako Pure Chemical) was added to the wells, and absorbance at 450 nm was read with a microplate reader (Molecular Devices or PerkinElmer).

[0127]

Antibody A concentration-dependently suppressed the binding between human EphA4 and human ligand, and the IC₅₀ values against human Ephrin A5 and Ephrin B3 binding were about 2.8 nM and 1.4 nM, respectively (Figure 4). Accordingly, it was shown that antibody A also strongly inhibits the binding between human EphA4 and human ligand.

[0128]

Reference Example 6: Selectivity of anti-EphA4 monoclonal antibody against human Eph receptor

Following the method for preparing mouse EphA4 extracellular region-SEAP-His protein described in Reference Example 1, the DNA sequences encoding the signal sequence and the extracellular region of each Eph receptor of human (EphA1, EphA2, EphA3, EphA4, EphA5,

EphA6, EphA7, EphA8, EphA10, EphB1, EphB2, EphB3, EphB4, and EphB6) were amplified by RT-PCR with total RNA derived from tissue, and cloned into pENTR1A vector (Invitrogen/LifeTechnologies) having the DNA sequence encoding SEAP and histidine tag. Next, the DNA sequences encoding the signal sequence and extracellular region of each Eph receptor of human, SEAP, and histidine tag were transferred to pcDNA 3.1_rfcB vector by LR reaction of Gateway System (Invitrogen/LifeTechnologies) to construct vectors (referred to as "Eph receptor extracellular region-SEAP-His protein expression vector") expressing a protein having SEAP and His tag fused to the extracellular region of each Eph receptor of human (referred to as "Eph receptor extracellular region-SEAP-His protein.")

[0129]

Next, each Eph receptor extracellular region of human-SEAP-His protein expression vector was introduced into Expi293F cells (Gibco/ThermoFisher) with Expi293 expression system (Gibco/ThermoFisher). After 5 days of culture (5% CO₂, 37°C, 120 rpm), the culture supernatant was recovered, and this was centrifuged at room temperature, at 1500 rpm, and for 5 minutes. The centrifuged supernatant was filtered with a 0.45 µm filter (Millipore).

[0130]

For antibody A, the evaluation of the binding activity of human Eph receptor was performed following the steps below.

Rabbit anti-6-His antibody (Bethyl Laboratories) was coated onto the wells of a 96-well plate (Nunc). After incubating at 4°C overnight, wells were blocked at room temperature for one hour with 1% Block ACE (DS Pharma Biomedical). After washing three times with 0.05% Tween 20/PBS (Thermo SCIENTIFIC), each Eph receptor extracellular region of human-SEAP-His protein (final concentration 1 nM) was seeded in each well, and this was incubated at room temperature for one hour. After washing three times, human IgG solution (100 µg/mL, Mitsubishi Pharma Corporation) and antibody A (10 µg/mL) were added to the wells, and this was incubated at room temperature for one hour. Horseradish peroxidase-labeled donkey anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories) was added, and this was incubated at room temperature for one hour. After

washing three times, TMBZ (3,3',5,5'-tetramethylbenzidine, Sigma) solution was added to the wells, and upon confirmation of a moderate amount of coloring, an equal amount of the stop solution (1N H₂SO₄, Wako Pure Chemical) was added to the wells, and absorbance at 450 nm was read with a microplate reader (PerkinElmer).

[0131]

Among the human Eph receptor family, antibody A had specific binding activity only towards human EphA4 (Figure 5).

[0132]

Reference Example 7: Selectivity of anti-EphA4 monoclonal antibody against mouse Eph receptor

Following the method for preparing EphA4 extracellular region-Fc-His protein according to Reference Example 1, the DNA sequences encoding the signal sequence and the extracellular region of each Eph receptor of mouse (EphA1, EphA3, EphA4, EphA5, EphA6, EphA7, EphA8, EphA10, EphB1, EphB2, EphB3, EphB4, and EphB6) were amplified by RT-PCR with total RNA derived from tissue, and cloned into pENTR1A vector (Invitrogen/LifeTechnologies) having the DNA sequence encoding the Fc region of human IgG₁ and histidine tag. Next, the DNA sequences encoding the signal sequence and extracellular region of each Eph receptor of mouse, Fc, and histidine tag (EphA1, EphA3, EphA4, EphA5, EphA6, EphA7, EphA8, EphA10, EphB1, EphB2, EphB3, EphB4, and EphB6) were transferred to pcDNA 3.1_rfcB vector by LR reaction of Gateway System (Invitrogen/LifeTechnologies) to construct the extracellular region of each Eph receptor of mouse-Fc-His protein expression vector. In the construction of the extracellular region of mouse EphA2-Fc-His protein expression vector, the DNA sequences encoding the signal sequence and the extracellular region of mouse EphA2 were amplified by RT-PCR with total RNA derived from tissue, and cloned into pcDNA 3.1 vector having the DNA sequence encoding Fc and histidine tag to construct a mouse EphA2 extracellular region-Fc-His protein expression vector.

[0133]

Next, each Eph receptor extracellular region of mouse-Fc-His protein expression vector was introduced into Expi293F cells

(Gibco/ThermoFisher) with Expi293 expression system (Gibco/ThermoFisher). After 5 days of culture (5% CO₂, 37°C, 120 rpm), the culture supernatant was recovered, and this was centrifuged at room temperature, at 1500 rpm, and for 5 minutes. The centrifuged supernatant was filtered with a 0.45 µm filter (Millipore).

[0134]

For antibody A, the evaluation of the binding activity of mouse Eph receptor was performed following the steps below.

Rabbit anti-6-His antibody (Bethyl Laboratories) was coated onto the wells of a 96-well plate (Nunc). After incubating at 4°C overnight, wells were blocked at room temperature for one hour with 1% Block ACE (DS Pharma Biomedical). After washing three times with 0.05% Tween 20/PBS (Thermo SCIENTIFIC), each Eph receptor extracellular region of mouse-Fc-His protein (final concentration 1 nM) was seeded in each well, and this was incubated at room temperature for one hour. After washing three times, human IgG solution (100 µg/mL, Sigma) and antibody A (10 µg/mL) were added to the wells, and this was incubated at room temperature for one hour. Horseradish peroxidase-labeled donkey anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories) was added, and this was incubated at room temperature for one hour. After washing three times, TMBZ (3,3',5,5'-tetramethylbenzidine, Sigma) solution was added to the wells, and upon confirmation of a moderate amount of coloring, an equal amount of the stop solution (1N H₂SO₄, Wako Pure Chemical) was added to the wells, and absorbance at 450 nm was read with a microplate reader (PerkinElmer).

[0135]

Among the mouse Eph receptor family, antibody A had specific binding activity only towards mouse EphA4 (Figure 6).

[0136]

Reference Example 8: Reactivity of anti-EphA4 monoclonal antibody against mouse, rat, monkey, and human EphA4

The preparation of mouse, rat, monkey, and human EphA4 extracellular regions-Fc-His proteins was performed following the steps below. First, following the method for preparing EphA4

extracellular region-Fc-His protein according to Reference Example 1, monkey EphA4 extracellular region-Fc-His protein expression vector was constructed. The amino acid sequence of monkey EphA4 utilized in vector construction is shown as SEQ ID NO. 32, and the extracellular region thereof is shown as SEQ ID NO. 33. Various EphA4 extracellular region-Fc-His proteins were prepared employing the monkey EphA4 extracellular region-Fc-His protein expression vector as well as the mouse, rat, and human EphA4 extracellular region-Fc-His protein expression vectors described in Reference Example 1.

[0137]

For antibody A, the evaluation of the binding activity with various EphA4 extracellular regions was performed following the steps below.

Donkey anti-human IgG antibody (Jackson ImmunoResearch Laboratories) was coated onto the wells of a 96-well plate (Nunc). After incubating at 4°C overnight, wells were blocked at room temperature for one hour with 1% Block ACE (DS Pharma Biomedical). After washing three times with 0.05% Tween 20/PBS (Thermo SCIENTIFIC), mouse, rat, monkey, and human EphA4 extracellular regions-Fc-His proteins (final concentration 1 nM) were seeded in the wells, and this was incubated at room temperature for one hour. After washing three times, human IgG solution (100 µg/mL, Mitsubishi Pharma Corporation) and antibody A (0, 0.00013, 0.00064, 0.0032, 0.016, 0.08, 0.4, 2, and 10 µg/mL) were added to the wells, and this was incubated at room temperature for one hour. Horseradish peroxidase-labeled donkey anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories) was added, and this was incubated at room temperature for one hour. After washing three times, TMBZ (3,3',5,5'-tetramethylbenzidine, Sigma) solution was added to the wells, and upon confirmation of a moderate amount of coloring, an equal amount of the stop solution (1N H₂SO₄, Wako Pure Chemical) was added to the wells, and absorbance at 450 nm was read with a microplate reader (PerkinElmer).

[0138]

Antibody A had equivalent binding activity in all of mouse, rat, monkey, and human EphA4 (Figure 7).

[0139]

Reference Example 9: Reactivity of anti-EphA4 monoclonal antibody against human EphA4 extracellular region, ligand binding domain, fibronectin type III domain 1, fibronectin type III domain 2

The preparation of proteins having the extracellular region (ECD), ligand binding domain (LBD), fibronectin type III domain 1 (FN1), or fibronectin type III domain 2 (FN2) of human EphA4 fused with maltose-binding protein (MBP) and histidine tag (hereinafter referred to as "human EphA4 extracellular region-MBP-His protein," "human EphA4 ligand binding domain-MBP-His protein," "human EphA4 fibronectin type III domain 1-MBP-His protein," and "human EphA4 fibronectin type III domain 2-MBP-His protein") was performed following the steps below. Initially, pcDNA 3.4-human EphA4 extracellular region, ligand binding domain, fibronectin type III domain 1, or fibronectin type III domain 2-MBP-His expression vectors were constructed. First, the signal sequence of human EphA4 (SEQ ID NO. 34) or the signal sequence of preprotrypsin (SEQ ID NO. 35) and the DNA sequences encoding each domain of human EphA4 were amplified by PCR, and cloned into pcDNA 3.4 vector having the DNA sequence encoding MBP and histidine tag (Invitrogen/LifeTechnologies) to construct human EphA4 extracellular region-MBP-His protein, human EphA4 ligand binding domain-MBP-His protein, human EphA4 fibronectin type III domain 1-MBP-His protein, and human EphA4 fibronectin type III domain 2-MBP-His protein expression vectors. The amino acid sequence of human EphA4 utilized in vector construction is shown as SEQ ID NO. 5, the extracellular region thereof as SEQ ID NO. 36, the ligand binding domain as SEQ ID NO. 37, the fibronectin type III domain 1 as SEQ ID NO. 38, and the fibronectin type III domain 2 as SEQ ID NO. 39. The above expression vectors were transfected into Expi293F cells (Thermo SCIENTIFIC) with Expi293 expression system (Thermo SCIENTIFIC). Four days later, the culture supernatant was recovered, and passed through a 0.45 μ m filter (Millipore). Crude purification was performed with Amylose resin (NEB), and the buffer was substituted to PBS (Wako Pure Chemical) with Zeba Spin Desalting column (Thermo

SCIENTIFIC). The monomer fraction was differentially purified with Superdex 200 10/300 (GE Healthcare).

[0140]

For antibody A, the evaluation of the binding activity with various domains in human EphA4 was performed following the steps below.

Rabbit anti-6-His antibody (Bethyl Laboratories) was coated onto the wells of a 96-well plate (Nunc). After incubating at 4°C overnight, wells were blocked at room temperature for one hour with 1% Block ACE (DS Pharma Biomedical). After washing twice with 0.02% Tween 20/PBS (Nacalai Tesque), human EphA4 extracellular region-MBP-His protein, human EphA4 ligand binding domain-MBP-His protein, human EphA4 fibronectin type III domain 1-MBP-His protein, and human EphA4 fibronectin type III domain 2-MBP-His protein (final concentration 10 nM) were seeded in the wells, and this was incubated at room temperature for one hour. After washing three times, antibody A (final concentration 10 nM) was added to the wells, and this was incubated at room temperature for one hour. Horseradish peroxidase-labeled goat anti-mouse IgG Fcγ fragment antibody (Jackson ImmunoResearch Laboratories) was added, and this was incubated at room temperature for one hour. After washing five times, TMB solution (KPL) was added to the wells, and upon confirmation of a moderate amount of coloring, an equal amount of the stop solution (2 N H₂SO₄, Wako Pure Chemical) was added to the wells. Absorbance at 450 nm and 650 nm were read by a microplate reader (PerkinElmer).

[0141]

Antibody A had binding activity for human EphA4 extracellular region (ECD) and ligand binding domain (LBD) (Figure 8). There was no reaction for fibronectin type III domain 1 (FN1) and fibronectin type III domain 2 (FN2). Accordingly, it was found that antibody A specifically binds to the ligand binding domain of the human EphA4 extracellular region.

[0142]

Reference Example 10: Effect of anti-EphA4 monoclonal antibody on increasing the number of spines in the hippocampus neuron

Preparation of rat hippocampus neurons was performed as described in the above Reference Example 1 (B). Rat hippocampus neurons were introduced with EGFP gene employing Nucleofector (Lonza), mixed with rat hippocampus neurons without gene introduction, and seeded in a 24-well plate (Falcon) containing a cover glass (Matsunami Glass Industries) coated with poly-L-lysine.

[0143]

The counting of spine employing hippocampus neurons was performed following the steps below. Rat hippocampus neurons introduced with EGFP at culture Day 13 seeded in a 24-well plate (Falcon) containing a cover glass (Matsunami Glass Industries) coated with poly-L-lysine were treated with control antibody (mouse IgG₁; BioLegend) or antibody A (6.7 and 20 nM) for 24 hours. The cover glass was then transferred to 2% PFA (Wako Pure Chemical)/4% sucrose (Wako Pure Chemical)/PBS, and this was left standing for 20 minutes to fix the cells. After removing the fixative solution and washing the cells three times with PBS, 0.25% Triton X-100 (Wako Pure Chemical)/PBS was added, and cell permeabilization was performed for 15 minutes. The solution was removed, the cover glass was transferred to 2% BSA (Sigma)/0.25% Triton X-100/Opti-MEM (GIBCO) and subjected to one hour of blocking, after which anti-GFP antibody (Nacalai Tesque) was allowed to react for 1.5 hours. After removing the primary antibody solution and washing three times with PBS, the secondary antibody was allowed to react for one hour. After removing the secondary antibody solution and washing three times with PBS, Prolong Gold antifade reagent (Molecular probes) was added for mounting, and observation was performed with LSM800 (ZEISS). The experiment described above was carried out three times, and for each experiment, neurons were extracted from two cover glasses, spines on each dendrite were counted with image analysis software Imaris® (Bitplane), and the number of spines per 10 μm for each neuron was calculated.

[0144]

Antibody A increased the number of spines in the hippocampus neuron (Figure 9). This result shows that antibody A has the activity to stabilize spines in hippocampus neurons.

[0145]

Reference Example 11: Effect of anti-EphA4 monoclonal antibody to suppress tau phosphorylation *in vivo*

The evaluation of the effect to suppress tau phosphorylation *in vivo* employing tau transgenic mouse (rTg4510) was performed following the steps below. Tau transgenic mice (rTg4510) were subcutaneously administered twice a week from 20 to 26 weeks-old with antibody A or control antibody prepares by conventional methods by immunizing a mouse with dinitrophenol (mouse anti-dinitrophenol antibody) at a dose of 100 mg/kg (10 mL/kg) each. On 3.5 days after the final administration, anesthesia was done with 2% isoflurane (Intervet) and a mix of three types of anesthetic drugs (4.0 mg/kg of Dormicum (Astellas Pharma), 0.3 mg/kg of Domitor (Nippon Zenyaku Kogyo), and 5.0 mg/kg of Vetorphale (Meiji Seika Pharma)), perfusion was performed under anesthesia with PBS (Wako Pure Chemical) containing 3 units/mL of heparin (Ajinomoto) and 1% phosphatase inhibitor cocktail (Nacalai Tesque), and the cerebral hemisphere was resected. The cerebral hemisphere collected was fixed at 4°C while shaking overnight in 2% paraformaldehyde (TAAB)/0.1 M phosphate buffer (Wako Pure Chemical). The cerebral hemisphere was substituted with 20% sucrose (Wako Pure Chemical)/0.1 M phosphate buffer (Wako Pure Chemical) and subsequently 25% sucrose/0.1 M phosphate buffer (Wako Pure Chemical), and then embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek Japan)/25% sucrose, and frozen with liquid nitrogen. Slices were created at a thickness of 7 μ m with cryostat CM1860 (Leica), adhered on a slide glass (Muto Pure Chemicals), air-dried with cold air, and then placed in a sealed bag and stored at -80°C. The slide glass used for immunostaining was thawed, air-dried with cold air, and then washed with PBS (Wako Pure Chemical), immersed in 1% BSA (Sigma)/10% normal donkey serum (Jackson ImmunoResearch Laboratories)/0.5% Triton X-100 (Wako Pure Chemical)/PBS solution, and subjected to one hour of blocking, after which anti-phosphorylated tau antibody AT8 (Fujirebio Europe N.V.) was allowed to react overnight at ordinary temperatures. After washing three times with PBS, the secondary antibody was allowed to

react for one hour. After washing three times with PBS, Prolong Gold antifade reagent (Molecular probes) was placed on the slice and mounted, and observation was performed with LSM700 (ZEISS). The AT8 signal-positive area in the hippocampus CA1 stratum radiatum was measured with image analysis software ImageJ, and the proportion of the AT8-positive signal area against the total area was calculated. [0146]

Antibody A decreased the signal of phosphorylated tau in the hippocampus CA1 region (Figure 10). This result shows that antibody A has the activity to suppress the progression of tau pathology in tau transgenic mouse (rTg4510).

[0147]

Reference Example 12: Epitope mapping of EphA4-ligand binding domain (EphA4-LBD) by X-Ray crystal structure analysis

The preparation of antibody A-Fab was performed following the steps below. Antibody A at 101.1 mg was dissolved in 0.1 M sodium phosphate buffer (pH 7.0) comprising 30 mM L-cysteine and 2 mM EDTA at a concentration of 15 mg/mL. To this antibody solution, papain (Sigma) was added at 1/200 amount to the antibody, and enzymatic digestion was performed at 37°C for 18 hours. The antibody A enzymatic digestion juice was dialyzed against PBS, and the precipitate was removed by centrifugation (the precipitate produced was redissolved in PBS and mixed with the centrifugation supernatant). Next, the following steps were performed with the purpose of removing impurities other than antibody A-Fab.

1) Purification by Protein A column

This enzymatic digestion solution was applied to 2 mL of ProSep vA High Capacity (Millipore) equilibrated with PBS, and the pass-through fraction and the PBS wash fraction were recovered.

2) Affinity purification employing anti-human IgG Fcγ antibody

An affinity column having the anti-human IgG Fcγ antibody (Jackson ImmunoResearch Laboratories) covalently bound to NHS-Activated Sepharose 4FF (GE Healthcare) was prepared according to the manual of this Sepharose. The solution recovered in the above 1) was charged to this affinity column, and the pass-through solution

and the PBS wash solution thereof were recovered.

3) Purification by gel filtration

The pass-through fraction obtained in the above 2) was concentrated with an ultrafiltration membrane. Superose 12 (GE Healthcare) was equilibrated with PBS, the concentrated sample was applied and separated and purified. A part of the separated and purified fraction was analyzed with SDS-PAGE, and the fraction comprising antibody A-Fab with high purity was recovered and pooled. The sample purified in this way was set as antibody A-Fab.

[0148]

EphA4-LBD was prepared in order to prepare a complex of antibody A-Fab and the antigen EphA4-LBD (Qin H. et al., J. Biol. Chem., 283: 29473-29484 (2008)). EphA4-LBD at 0.68 μmol (200 μM , 3.4 mL) and antibody A-Fab at 0.45 μmol (300 μM , 1.5 mL) were mixed so that EphA4-LBD will have a molar ratio of about 1.5 folds against antibody A-Fab. Next, the mixed solution was applied to HILOAD 26/60 Superdex 75 prep grade (GE Healthcare), and eluted with the buffer for chromatography (25 mM Tris/HCl (pH 7.5), 100 mM NaCl). The fraction comprising the complex was analyzed with SDS PAGE, the fractions having high purity were gathered and concentrated to about 40.8 mg/mL, and this was employed for crystallization.

[0149]

Crystallization of the complex was performed by sitting drop vapor diffusion method with an auto crystallization device Hydra II Plus One System (Matrix Technologies Corp., Ltd.). MRC-2 (Molecular Dimensions) was used as the plate. The composition of the reservoir solution was 100 mM HEPES (pH 7.5), 10% Polyethylene Glycol 8000, and 8% Ethylene Glycol, and this reservoir solution and the above complex solution were mixed so that the volume ratio was 1:1 to generate crystallization droplets. The crystallization plate generated was left standing at 20°C.

[0150]

Upon crystallization under the above condition, crystals having space group P212121, lattice constants $a = 71.0 \text{ \AA}$, $b = 84.5 \text{ \AA}$, and $c = 116.1 \text{ \AA}$ were obtained. Radiation light X-Ray ($1.0 \text{ \AA}$) was incidented to the crystals obtained to obtain diffraction data of

1.79 Å. The diffraction data was processed by HKL2000 (HKL Research Inc.), and phase determination was performed by molecular substitution method. The program PHASER (version 2.5.0, McCoy A. J. et al., J. Appl. Cryst. 40: 658-674 (2007)) included in CCP4 Software Suite (Collaborative computational project number 4, [CCP4] version 6.5.0, Acta Cryst. D 67: 235-242 (2011)) was employed for the molecular substitution method. The crystal structure of EphA4-LBD (PDBID:3CKH) and the crystal structure of the Fab region of IgG (PDBID:2VXT (L-chain) and 1FGN (H-chain)) were employed as the search model of the molecular substitution method. A molecular model was constructed with the program COOT (Emsley P. et al., Acta Cryst. D 60: 2126-2132n (2004)) so as to fit the electron density obtained from the phase determined, and structure refinement was performed with the program REFMAC (Murshudov G.N., Acta Cryst. D 53: 240-255 (1997)).

The complex crystal structure of 2.0 Å resolution was obtained by structural calculation (R = 0.212, Rfree = 0.258).

[0151]

The crystal structure of the antibody A-Fab/EphA4-LBD complex obtained was analyzed with the interaction detection tool equipped in the computational chemical system MOE 2018.0101 (Chemical Computing Group Inc.), and the amino acid residues on EphA4-LBD which are in direct contact with antibody A-Fab were identified (Figure 11A). The identified amino acid residues are Glu51, Gly52, Ile59, Gln71, Cys73, Asn74, Val75, Met76, Glu77, Thr104, Arg106, Leu111, Pro112, Met115, Arg162, Met164, Cys191, Ala193, and Val195. Figure 11B shows the surface structure of EphA4-LBD generated with Maestro (version 11.0, Schrodinger, LLC). As a result, the present inventors concluded that the region where these amino acid residues are present is the antibody A-Fab binding region in EphA4-LBD.

[0152]

Example 1: Preparation of humanized antibody of antibody A Preparation of humanized anti-EphA4 antibody

The variable region of the humanized antibody was designed. Based on the high homology against the framework region (FR) of

antibody A, among the FRs of human antibody, IGHV3-33*03 (SEQ ID NO. 42) and JH6 (SEQ ID NO. 43) for the heavy chain and IGKV1-17*01 (SEQ ID NO. 40) and JK4 (SEQ ID NO. 41) for the light chain were selected as the FRs for the humanized antibody. The amino acids in the FR that interact with the amino acids of the CDR were then predicted employing a 3D structure prediction model of mouse antibody A, these were transplanted together with the CDR of antibody A having Y32F mutation in the CDR1 of the heavy chain (SEQ ID NO. 44, 27, 28, and 29-31), and HK2-42 (SEQ ID NO. 45) was designed as the heavy chain variable region of the humanized antibody and L1-8 (SEQ ID NO. 46) was designed as the light chain variable region of the humanized antibody. The amino acid sequences of the transplanted CDR are shown in Table 2, and the nucleic acid sequences are shown in Table 3. [0153]

The constant region of human IgG₂ (SEQ ID NO. 47) was employed as the heavy chain constant region. Human IgK (SEQ ID NO. 48) was employed as the light chain constant region. An expression vector (pcDNA 3.4) comprising the gene sequence encoding the amino acid sequence of the humanized antibody was transfected into Expi293F cells (Gibco/ThermoFisher) with Expi293 expression system (Gibco/ThermoFisher). As the gene sequence encoding the amino acid sequence of the humanized antibody, the nucleic acid sequence shown in SEQ ID NO. 55 was employed as heavy chain variable region, the nucleic acid sequence shown in SEQ ID NO. 56 was employed as light chain variable region, the nucleic acid sequence shown in SEQ ID NO. 57 was employed as the heavy chain constant region, and the nucleic acid sequence shown in SEQ ID NO. 58 was employed as the light chain constant region, respectively. The amino acid sequence of the full length of the heavy chain (not including the signal sequence) of the humanized antibody is the amino acid sequence shown in SEQ ID NO. 59, the amino acid sequence of the full length of the light chain (not including the signal sequence) is the amino acid sequence shown in SEQ ID NO. 60. The nucleic acid sequence encoding the full length of the heavy chain of the humanized antibody is the nucleic acid sequence shown in SEQ ID NO. 61, and the nucleic acid sequence encoding the full length of the light chain is the nucleic acid sequence shown

in SEQ ID NO. 62. The supernatant was recovered, and the humanized antibody of antibody A (antibody B) was purified with MabSelectSuRe (GE Healthcare).

[0154]

[Table 2]

Amino acid sequences of CDR of antibody B

Name	Sequence
Heavy chain CDR1	RFGVH (SEQ ID NO. 44)
Heavy chain CDR2	VIWRGGSTDYNAAFMS (SEQ ID NO. 27)
Heavy chain CDR3	ESLFGVYYDYGYYSDY (SEQ ID NO. 28)
Light chain CDR1	RASQEISGYLS (SEQ ID NO. 29)
Light chain CDR2	AASTLDS (SEQ ID NO. 30)
Light chain CDR3	LQYASYPLT (SEQ ID NO. 31)

[Table 3]

Nucleic acid sequences of CDR of antibody B

Name	Sequence
Heavy chain CDR1	AGATTTGGAGTGCAT (SEQ ID NO. 49)
Heavy chain CDR2	GTGATCTGGAGGGGAGGATCCACCGACTACAACGCTGCTTTTATGAGC (SEQ ID NO. 50)
Heavy chain CDR3	GAGAGCCTGTTTCGGCGTGTACTATGACTACGGCTACTATTCTATGGAT TAT (SEQ ID NO. 51)
Light chain CDR1	CGCGCCTCCCAGGAGATCTCTGGCTACCTGTCC (SEQ ID NO. 52)
Light chain CDR2	GCTGCCTCCACCCTGGACTCT (SEQ ID NO. 53)
Light chain CDR3	CTGCAGTACGCTTCCTATCCACTGACC (SEQ ID NO. 54)

[0155]

Example 2: Affinity of humanized anti-EphA4 monoclonal antibody against human EphA4

The binding affinity of antibody B obtained in Example 1 against human EphA4 was determined by surface plasmon resonance (SPR method) employing Biacore T200 (GE Healthcare). First, anti-His antibody (GE Healthcare, 28-9950-56) was fixed onto a sensor chip CM5. Fixation was performed by amine coupling method employing N-hydroxysuccinimide (NHS) and N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide hydrochloride

(EDC), and ethanolamine was employed for blocking (sensor chip and fixation reagent were all from GE Healthcare). This was diluted to 3.5 µg/mL with the fixation buffer (10 mM sodium acetate, pH 4.5), and fixed on the sensor chip according to the protocol attached to Biacore T200. Human EphA4 extracellular region-SEAP-His10 was diluted with the running buffer HBS-EP (GE Healthcare, BR-1001-88), and the solution was sent onto a flow cell for 120 seconds for capture (the captured amount about 10 RU). Subsequently, antibody B serially diluted to the range of 100, 50, 25, 12.5, 6.3, 3.2, 1.6, and 0 nM with HBS-EP was added to the sensor chip for 120 seconds, and the binding reaction curve at the time of addition (binding phase, 120 seconds) and after addition had completed (dissociation phase, 600 seconds) was sequentially observed. After the completion of each observation, 4 M MgCl₂ (60 seconds, Wako Pure Chemical) was added to regenerate the sensor chip. Fitting analysis by 1:1 binding model employing BIA evaluation software attached to the system was performed on the binding reaction curve obtained, and the affinity (KD = kd/ka) against human EphA4 was calculated.

[0156]

The binding affinity of antibody B against human EphA4 (KD value) was 1.34×10^{-9} M (Figure 12). It was shown that antibody B shows an almost equivalent affinity as antibody A which is the antibody before humanization.

[0157]

Example 3: EphA4 cleavage enhancement activity of humanized anti-EphA4 monoclonal antibody in hippocampus neurons

For antibody B obtained in Example 1, evaluation of EphA4 cleavage enhancement activity employing hippocampus neurons was performed following the steps below.

Rat hippocampus neurons seeded in a 96-well dish (Falcon) were treated with antibody B (2.0, 6.7, and 20 nM) and γ selectase inhibitory drug Compound E (50 nM, Enzo Life Sciences). Twenty-four hours later, this was washed with PBS (Wako Pure Chemical), SDS sample buffer (Laemmli sample buffer (Bio-Rad) and 5% 2-mercaptoethanol (Bio-Rad)) was added to recover the cells, and this was boiled for

5 minutes. SDS-PAGE was performed with this sample, western blotting with anti-EphA4 monoclonal antibody (Abnova) was performed, the band strength was quantified, and the value of EphA4 C-terminal fragment/full length EphA4 was calculated.

[0158]

Antibody B concentration-dependently enhanced EphA4 cleavage reaction in hippocampus neurons (Figure 13)

[0159]

Example 4: Human EphA4-human ligand binding inhibitory activity of humanized anti-EphA4 monoclonal antibody

For antibody B obtained in Example 1, the evaluation of the inhibitory activity of binding between human EphA4 and human ligand was performed following the steps below. Anti-alkaline phosphatase antibody (Thermo SCIENTIFIC) was coated onto the wells of a 96-well plate (Nunc). After incubating at 4°C overnight, wells were blocked at room temperature for one hour with 1% Block ACE (DS Pharma Biomedical). After washing three times with 0.05% Tween 20/PBS (Thermo SCIENTIFIC), human EphA4 extracellular region-SEAP-His protein (final concentration 10 nM) was seeded in the wells, and this was incubated at room temperature for one hour. After washing three times, the ligand and serially diluted antibody B (0, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30, 100, 300, 1000, and 3000 nM) were added to the wells. Note that biotinylated human Ephrin A5-Fc chimera (R&D Systems, final concentration 0.7 nM) and biotinylated human Ephrin B3-Fc chimera (R&D Systems, final concentration 2.3 nM) were employed as ligands. After incubating at room temperature for one hour and washing three times, horseradish peroxidase-labeled streptavidin (GE Healthcare) was added, and this was incubated at room temperature for one hour. After washing three times, TMBZ (3,3',5,5'-tetramethylbenzidine, Sigma) solution was added to the wells, and this was incubated at room temperature for 2-5 minutes. An equal amount of the stop solution (1N H₂SO₄, Wako Pure Chemical) was added to the wells, and absorbance at 450 nm was read with a microplate reader (Molecular Devices or PerkinElmer).

[0160]

Antibody B concentration-dependently suppressed the binding between human EphA4 and human ligand, and the IC₅₀ values against human Ephrin A5 and Ephrin B3 binding were about 4.9 nM and 1.6 nM, respectively. Accordingly, it was found that antibody B strongly inhibits the binding between human EphA4 and human ligand, and shows an almost equivalent inhibitory activity as antibody A which is the antibody before humanization (Figure 14).

[0161]

Example 5: Mouse EphA4-mouse ligand binding inhibitory activity of humanized anti-EphA4 monoclonal antibody

For antibody B obtained in Example 1, the evaluation of the inhibitory activity of binding between mouse EphA4 and mouse ligand was performed following the steps below. Anti-alkaline phosphatase antibody (Thermo SCIENTIFIC) was coated onto the wells of a 96-well plate (Nunc). After incubating at 4°C overnight, wells were blocked at room temperature for one hour with 1% Block ACE (DS Pharma Biomedical). After washing three times with 0.02% Tween 20/PBS (Thermo SCIENTIFIC), mouse EphA4 extracellular region-SEAP-His protein was added to the wells (final concentration 10 nM), and this was incubated at room temperature for one hour. After washing three times, the ligand and serially diluted antibody B (0, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30, 100, 300, 1000, and 3000 nM) were added to the wells. Note that biotinylated mouse Ephrin A1-Fc chimera (R&D Systems, final concentration 6 nM) and biotinylated mouse Ephrin B2-Fc chimera (R&D Systems, final concentration 2.5 nM) were employed as ligands. After incubating at room temperature for one hour and washing three times, horseradish peroxidase-labeled streptavidin (GE Healthcare) was added, and this was incubated at room temperature for one hour. After washing three times, TMBZ (3,3',5,5'-tetramethylbenzidine, Sigma) solution was added to the wells, and this was incubated at room temperature for 2 minutes. An equal amount of the stop solution (1N H₂SO₄, Wako Pure Chemical) was added to the wells, and absorbance at 450 nm was read with a microplate reader (Molecular Devices or PerkinElmer).

[0162]

Antibody B concentration-dependently suppressed the binding between mouse EphA4 and mouse ligand, and the IC₅₀ values against mouse Ephrin A1 and Ephrin B2 binding were about 8.7 nM and 4.2 nM, respectively. Accordingly, it was found that antibody B strongly inhibits the binding between mouse EphA4 and mouse ligand, and shows an almost equivalent inhibitory activity as antibody A which is the antibody before humanization (Figure 15).

[0163]

Example 6: Selectivity of humanized anti-EphA4 monoclonal antibody against human Eph receptor

Similarly to the method for preparing mouse EphA4 extracellular region-SEAP-His protein described in Reference Example 1, the DNA sequences encoding the signal sequence and the extracellular region of each Eph receptor of human (EphA1, EphA2, EphA3, EphA4, EphA5, EphA6, EphA7, EphA8, EphA10, EphB1, EphB2, EphB3, EphB4, and EphB6) were amplified by RT-PCR with total RNA derived from tissue, and cloned into pENTR1A vector (Invitrogen/LifeTechnologies) having the DNA sequence encoding SEAP protein and histidine tag. Next, the DNA sequences encoding the signal sequence and extracellular region of each Eph receptor of human, SEAP protein and histidine tag were transferred to pcDNA 3.1_rfcB vector by LR reaction of Gateway System (Invitrogen/LifeTechnologies) to construct vectors (referred to as "Eph receptor extracellular region-SEAP-His protein expression vector") expressing a protein having SEAP protein and His tag fused to the extracellular region of each Eph receptor of human (referred to as "Eph receptor extracellular region-SEAP-His protein.")

[0164]

Next, each of human Eph receptor extracellular region-SEAP-His protein expression vectors was introduced into Expi293F cells (Gibco/ThermoFisher) with Expi293 expression system (Gibco/ThermoFisher). After five days of incubation (5% CO₂, 37°C), the culture supernatant was recovered, and this was centrifuged at room temperature, at 1500 rpm, and for 5 minutes. The centrifuged supernatant was filtered with a 0.45 µm filter (Millipore).

[0165]

For antibody B obtained in Example 1, the evaluation of the binding activity of human Eph receptor was performed following the steps below.

Rabbit anti-6-His antibody (Bethyl Laboratories) was coated onto the wells of a 96-well plate (Nunc). After incubating at 4°C overnight, wells were blocked at room temperature for one hour with 1% Block ACE (DS Pharma Biomedical). After washing three times with 0.05% Tween 20/PBS (Thermo SCIENTIFIC), each of human Eph receptor extracellular region-SEAP-His proteins (final concentration 1 nM) was seeded in each well, and this was incubated at room temperature for one hour. After washing three times, human IgG solution (100 µg/mL, Mitsubishi Pharma Corporation) and antibody B (10 µg/mL) were added to the wells, and this was incubated at room temperature for one hour. Horseradish peroxidase-labeled donkey anti-human IgG antibody (Jackson ImmunoResearch Laboratories) was added, and this was incubated at room temperature for one hour. After washing three times, TMBZ (3,3',5,5'-tetramethylbenzidine, Sigma) solution was added to the wells, and upon confirmation of a moderate amount of coloring, an equal amount of the stop solution (1N H₂SO₄, Wako Pure Chemical) was added to the wells, and absorbance at 450 nm was read with a microplate reader (PerkinElmer).

[0166]

It was found that antibody B, similarly to antibody A which is the antibody before humanization, specifically binds to human EphA4 among the human Eph receptor family (Figure 16).

[0167]

Example 7: Selectivity of humanized anti-EphA4 monoclonal antibody against mouse Eph receptor

Following the method for preparing EphA4 extracellular region-Fc-His protein according to Reference Example 1, the DNA sequences encoding the signal sequence and the extracellular region of each Eph receptor of mouse (EphA1, EphA3, EphA4, EphA5, EphA6, EphA7, EphA8, EphA10, EphB1, EphB2, EphB3, EphB4, and EphB6) were amplified by RT-PCR with total RNA derived from tissue, and cloned

into pENTR1A vector (Invitrogen/LifeTechnologies) having the DNA sequence encoding the Fc region of human IgG₁ and histidine tag. Next, the DNA sequences encoding the signal sequence and extracellular region of each Eph receptor (EphA1, EphA3, EphA4, EphA5, EphA6, EphA7, EphA8, EphA10, EphB1, EphB2, EphB3, EphB4, and EphB6) of mouse, Fc, and histidine tag were transferred to pcDNA 3.1_rfcB vector by LR reaction of Gateway System (Invitrogen/LifeTechnologies) to construct each of mouse Eph receptor extracellular region-Fc-His protein expression vectors. In the construction of mouse EphA2 extracellular region-Fc-His protein expression vector, the DNA sequences encoding the signal sequence and the extracellular region of mouse EphA2 were amplified by RT-PCR with total RNA derived from tissue, and cloned into pcDNA 3.1 vector having the DNA sequence encoding Fc and histidine tag to construct a mouse EphA2 extracellular region-Fc-His protein expression vector.

[0168]

Next, each of mouse Eph receptor extracellular region-Fc-His protein expression vectors was introduced into Expi293F cells (Gibco/ThermoFisher) with Expi293 expression system (Gibco/ThermoFisher). After 5 days of culture (5% CO₂, 37°C, 120 rpm), the culture supernatant was recovered, and this was centrifuged at room temperature, at 1500 rpm, and for 5 minutes. The centrifuged supernatant was filtered with a 0.45 µm filter (Millipore).

[0169]

For antibody B, the evaluation of the binding activity of mouse Eph receptor was performed following the steps below.

Rabbit anti-6-His antibody (Bethyl Laboratories) was coated onto the wells of a 96-well plate (Nunc). After incubating at 4°C overnight, wells were blocked at room temperature for one hour with 1% Block ACE (DS Pharma Biomedical). After washing three times with 0.05% Tween 20/PBS (Thermo SCIENTIFIC), each of mouse Eph receptor extracellular region-Fc-His proteins (final concentration 1 nM) was seeded in each well, and this was incubated at room temperature for one hour. After washing three times, human IgG solution (100 µg/mL, Sigma) and antibody B (10 µg/mL) were added to the wells, and this was incubated at room temperature for one hour. Horseradish

peroxidase-labeled goat anti-human Kappa Light Chain antibody (IBL) was added, and this was incubated at room temperature for one hour. After washing three times, TMBZ (3,3',5,5'-tetramethylbenzidine, Sigma) solution was added to the wells, and upon confirmation of a moderate amount of coloring, an equal amount of the stop solution (1N H₂SO₄, Wako Pure Chemical) was added to the wells, and absorbance at 450 nm was read with a microplate reader (PerkinElmer).

[0170]

Among the mouse Eph receptor family, antibody B had specific binding activity only towards mouse EphA4 (Figure 17).

[0171]

Example 8: Reactivity of humanized anti-EphA4 monoclonal antibody against mouse, rat, monkey, and human EphA4

[0172]

For antibody B, the evaluation of the binding activity with various EphA4 was performed following the steps below.

Anti-alkaline phosphatase antibody (Thermo SCIENTIFIC) was coated onto the wells of a 96-well plate (Nunc). After incubating at 4°C overnight, wells were blocked at room temperature for one hour with 1% Block ACE (DS Pharma Biomedical). After washing three times with 0.05% Tween 20/PBS (Thermo SCIENTIFIC), mouse, rat, monkey, and human EphA4 extracellular region-SEAP-His proteins (final concentration 1 nM) were seeded in the wells, and this was incubated at room temperature for one hour. After washing three times, human IgG solution (100 µg/mL, Mitsubishi Pharma Corporation) and antibody B (0, 0.00013, 0.00064, 0.0032, 0.016, 0.08, 0.4, 2, and 10 µg/mL) were added to the wells, and this was incubated at room temperature for one hour. Horseradish peroxidase-labeled donkey anti-human IgG antibody (Jackson ImmunoResearch Laboratories) was added, and this was incubated at room temperature for one hour. After washing three times, TMBZ (3,3',5,5'-tetramethylbenzidine, Sigma) solution was added to the wells, and upon confirmation of a moderate amount of coloring, an equal amount of the stop solution (1N H₂SO₄, Wako Pure Chemical) was added to the wells, and absorbance at 450 nm was read with a microplate reader (PerkinElmer).

[0173]

Antibody B had equivalent binding activity in all of mouse, rat, monkey, and human EphA4 (Figure 18).

[0174]

Example 9: Reactivity of humanized anti-EphA4 monoclonal antibody against human EphA4 extracellular region, ligand binding domain, fibronectin type III domain 1, fibronectin type III domain 2

For antibody B obtained in Example 1, the evaluation of the binding activity with various domains in human EphA4 was performed following the steps below.

Rabbit anti-6-His antibody (Bethyl Laboratories) was coated onto the wells of a 96-well plate (Nunc). After incubating at 4°C overnight, wells were blocked at room temperature for one hour with 1% Block ACE (DS Pharma Biomedical). After washing twice with 0.02% Tween 20/PBS (Nacalai Tesque), human EphA4 extracellular region-MBP-His protein, human EphA4 ligand binding domain-MBP-His protein, human EphA4 fibronectin type III domain 1-MBP-His protein, and human EphA4 fibronectin type III domain 2-MBP-His protein (final concentration 10 nM) were seeded in the wells, and this was incubated at room temperature for one hour. After washing three times, antibody B (final concentration 10 nM) was added to the wells, and this was incubated at room temperature for one hour. Horseradish peroxidase-labeled rabbit anti-human IgG Fcγ fragment antibody (Jackson ImmunoResearch Laboratories) was added, and this was incubated at room temperature for one hour. After washing five times, TMB (KPL) solution was added to the wells, and upon confirmation of a moderate amount of coloring, an equal amount of the stop solution (2 N H₂SO₄, Wako Pure Chemical) was added to the wells. Absorbance at 450 nm and 650 nm were read by a microplate reader (PerkinElmer).

[0175]

Antibody B had binding activity for human EphA4 extracellular region (ECD) and ligand binding domain (LBD) (Figure 19). There was no reaction for fibronectin type III domain 1 (FN1) and fibronectin type III domain 2 (FN2). Accordingly, it was found that antibody B specifically binds to the ligand binding domain of the human EphA4

extracellular region.

[0176]

Example 10: Effect of humanized anti-EphA4 monoclonal antibody on increasing the number of spines in the hippocampus neuron

Preparation of rat hippocampus neurons was performed as described in Reference Example 1 (B). Rat hippocampus neurons were introduced with EGFP gene employing Nucleofector (Lonza), and seeded in a 24-well plate (Falcon) containing a cover glass (Matsunami Glass Industries) coated with poly-L-lysine.

[0177]

The counting of spine employing hippocampus neurons was performed following the steps below. Rat hippocampus neurons introduced with EGFP at culture Day 13 seeded in a 24-well plate (Falcon) containing a cover glass (Matsunami Glass Industries) coated with poly-L-lysine were treated with control antibody (human IgG₂; Sigma) or antibody B (6.7 and 20 nM) for 24 hours. The cover glass was then transferred to 2% PFA (Wako Pure Chemical)/4% sucrose (Wako Pure Chemical)/PBS, and this was left standing for 20 minutes to fix the cells. After removing the fixative solution and washing the cells three times with PBS, 0.25% Triton X-100 (Wako Pure Chemical)/PBS was added, and cell permeabilization was performed for 15 minutes. The solution was removed, the cover glass was transferred to 2% BSA (Sigma)/0.25% Triton X-100/OPTI-MEM (GIBCO) and subjected to one hour of blocking, after which anti-GFP antibody (Nacalai Tesque) was allowed to react for 1.5 hours. After removing the primary antibody solution and washing three times with PBS, the secondary antibody was allowed to react for one hour. After removing the secondary antibody solution and washing three times with PBS, Prolong Gold antifade reagent (Molecular probes) was added for mounting, and observation was performed with LSM800 (ZEISS). The experiment described above was carried out three times, and for each experiment, neurons were extracted from two cover glasses, spines on each dendrite were counted with image analysis software Imaris® (Bitplane), and the number of spines per 10 μm for each neuron was calculated.

[0178]

Antibody B increased the number of spines in the hippocampus neuron (Figure 20). This result shows that antibody B has the activity to stabilize spines in hippocampus neurons.

[0179]

Example 11: Human EphA4 cleavage enhancement activity of humanized anti-EphA4 monoclonal antibody

For antibody B obtained in Example 1, the evaluation of cleavage enhancement activity on human EphA4 was performed following the steps below.

Preparation of rat hippocampus neurons was performed as described in Reference Example 1 (B). Rat hippocampus neurons were introduced with a human EphA4-HA protein expression vector employing Nucleofector (Lonza), and seeded in a 96-well dish (Falcon) coated with poly-L-lysine. The seeded rat hippocampus neurons were treated with antibody B (6.7, 20, and 67 nM) and γ selectase inhibitory drug Compound E (50 nM, Enzo Life Sciences). About twenty-four hours later, this was washed with PBS (Wako Pure Chemical), SDS sample buffer (Laemmli sample buffer (Bio-Rad) and 5% 2-mercaptoethanol (Bio-Rad)) was added to recover the cells, and this was boiled for 5 minutes. SDS-PAGE was performed with this sample, western blotting with rat anti-HA monoclonal antibody (Roche) was performed, the band strength was quantified, and the value of EphA4 C-terminal fragment/full length EphA4 was calculated.

[0180]

Antibody B enhanced human EphA4 cleavage reaction in hippocampus neurons (Figure 21).

[0181]

Example 12: Involvement of MMP and ADAM against the effect of humanized anti-EphA4 monoclonal antibody on increasing the number of spines in the hippocampus neuron

Preparation of rat hippocampus neurons was performed as described in Reference Example 1 (B). A part of rat hippocampus neurons were introduced with EGFP gene employing Nucleofector (Lonza), and seeded in a 24-well plate (Falcon) containing a cover glass

(Matsunami Glass Industries) coated with poly-L-lysine.

[0182]

The counting of spine employing hippocampus neurons was performed following the steps below. Rat hippocampus neurons introduced with EGFP at culture Day 13 seeded in a 24-well plate (Falcon) containing a cover glass (Matsunami Glass Industries) coated with poly-L-lysine were treated with control antibody (human IgG₂; Sigma) or antibody B (20 nM), as well as DMSO (Sigma) or MMP and ADAM inhibitor GM6001 (2.5 μ M, MedChemExpress) for 24 hours. The cover glass was then transferred to 2% PFA (Wako Pure Chemical)/4% sucrose (Wako Pure Chemical)/PBS, and this was left standing for 20 minutes to fix the cells. After removing the fixative solution and washing the cells three times with PBS, 0.25% Triton X-100 (Wako Pure Chemical)/PBS was added, and cell permeabilization was performed for 15 minutes. The 0.25% Triton X-100/PBS was removed, the cover glass was transferred to 2% BSA (Sigma)/0.25% Triton X-100/OPTI-MEM (GIBCO) and subjected to one hour of blocking, after which anti-GFP antibody (Nacalai Tesque) was allowed to react for 1.5 hours. After removing the primary antibody solution and washing three times with PBS, the secondary antibody was allowed to react for one hour. After removing the secondary antibody solution and washing three times with PBS, Prolong Gold antifade reagent (Molecular probes) was added for mounting, and observation was performed with LSM800 (ZEISS). The experiment described above was carried out three times, and for each experiment, neurons were extracted from two cover glasses, spines on each dendrite were counted with image analysis software Imaris® (Bitplane), and the number of spines per 10 μ m for each neuron was calculated.

[0183]

The increase in the number of spines in the hippocampus neuron by antibody B was inhibited by simultaneous treatment with GM6001 (Figure 22). This result shows that antibody B has spine stabilization activity via MMP and ADAM in hippocampus neurons.

[0184]

Example 13: Effect of humanized anti-EphA4 monoclonal antibody to

suppress tau phosphorylation *in vivo*

The evaluation of the effect to suppress tau phosphorylation *in vivo* employing tau transgenic mouse (rTg4510) was performed following the steps below. Tau transgenic mice (rTg4510) were subcutaneously administered twice a week from 20 to 26 weeks-old with antibody B at a dose of 100 mg/kg (10 mL/kg). PBS (Wako Pure Chemical) was subcutaneously administered at 10 mL/kg for the control group. On 3.5 days after the final administration, mice were anesthetized with 2-2.5% isoflurane inhalation anesthetic drug (Intervet) and a mix of three types of anesthetic drugs (4.0 mg/kg of Dormicum (Astellas Pharma), 0.3 mg/kg of Domitor (Nippon Zenyaku Kogyo), and 5.0 mg/kg of Vetorphale (Meiji Seika Pharma)), perfusion was performed under anesthesia with PBS (Wako Pure Chemical) containing 3 units/mL of heparin (Ajinomoto) and 1% phosphatase inhibitor cocktail (Nacalai Tesque), and the cerebral hemisphere of mice was resected. The cerebral hemisphere collected was fixed at 4°C while shaking overnight immersed in 2% paraformaldehyde (TAAB)/0.1 M phosphate buffer (Wako Pure Chemical). The cerebral hemisphere was substituted with 10% sucrose (Wako Pure Chemical)/0.1 M phosphate buffer (Wako Pure Chemical) and subsequently 20% sucrose/0.1 M phosphate buffer (Wako Pure Chemical), and then embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek Japan)/20% sucrose, and frozen employing an aluminum block cooled with liquid nitrogen. Slices of the cerebral hemisphere were created at a thickness of 7 μ m with cryostat CM1860 (Leica). The slices were adhered on a silane-coated slide glass (Muto Pure Chemicals), air-dried with cold air, and then placed in a sealed bag and stored at -80°C. The slide glass used for immunostaining was taken out from -80°C, air-dried with cold air, and then washed with PBS (Wako Pure Chemical), immersed in 1% BSA (Sigma)/10% normal donkey serum (Jackson ImmunoResearch Laboratories)/0.5% Triton X-100 (Wako Pure Chemical)/PBS solution, and subjected to one hour of blocking, after which anti-phosphorylated tau antibody AT8 (Fujirebio Europe N.V.) was allowed to react overnight at ordinary temperatures. After washing three times with PBS, the secondary antibody was allowed to react for one hour. After washing three times with PBS, Prolong Gold

antifade reagent (Molecular probes) was placed on the slice and mounted, and observation was performed with LSM700 (ZEISS). The AT8 signal-positive area in the hippocampus CA1 stratum radiatum was measured with image analysis software Metamorph, and the proportion of the AT8-positive signal area against the total area was calculated. [0185]

Antibody B decreased the signal of phosphorylated tau in the hippocampus CA1 region (Figure 23). This result shows that antibody B has the activity to suppress the progression of tau pathology in tau transgenic mouse (rTg4510).

CLAIMS

1. An anti-EphA4 antibody, wherein
the anti-EphA4 antibody comprises heavy and light chains, and
comprises:
 - (a) a heavy chain CDR1 consisting of the amino acid sequence
shown in SEQ ID NO. 44;
 - (b) a heavy chain CDR2 consisting of the amino acid sequence
shown in SEQ ID NO. 27;
 - (c) a heavy chain CDR3 consisting of the amino acid sequence
shown in SEQ ID NO. 28;
 - (d) a light chain CDR1 consisting of the amino acid sequence
shown in SEQ ID NO. 29;
 - (e) a light chain CDR2 consisting of the amino acid sequence
shown in SEQ ID NO. 30; and
 - (f) a light chain CDR3 consisting of the amino acid sequence
shown in SEQ ID NO. 31.
2. The anti-EphA4 antibody according to claim 1, wherein the
anti-EphA4 antibody is humanized.
3. The anti-EphA4 antibody according to claim 1 or 2, wherein the
anti-EphA4 antibody specifically binds to EphA4 and enhances the
cleavage of EphA4.
4. The anti-EphA4 antibody according to any one of claims 1 to
3, wherein the anti-EphA4 antibody specifically binds to EphA4 and
inhibits the binding between EphA4 and ephrin.
5. The anti-EphA4 antibody according to any one of claims 1 to
4, wherein
 - the variable region of the heavy chain consists of the amino
acid sequence shown in SEQ ID NO. 45, and
 - the variable region of the light chain consists of the amino
acid sequence shown in SEQ ID NO. 46.

6. The anti-EphA4 antibody according to any one of claims 1 to 5, wherein
the constant region of the heavy chain and the constant region of the light chain comprise amino acid sequences derived from a human antibody.
7. The anti-EphA4 antibody according to claim 6, wherein
the constant region of the heavy chain is the constant region of human IgG.
8. The anti-EphA4 antibody according to claim 7, wherein
the constant region of human IgG is the constant region of human IgG₂.
9. The anti-EphA4 antibody according to claim 8, wherein
the constant region of human IgG₂ comprises the amino acid sequence shown in SEQ ID NO. 47.
10. The anti-EphA4 antibody according to any one of claims 6 to 9, wherein
the constant region of the light chain is the constant region of human Igκ.
11. The anti-EphA4 antibody according to claim 10, wherein
the constant region of human Igκ comprises the amino acid sequence shown in SEQ ID NO. 48.
12. An anti-EphA4 antibody, wherein
the anti-EphA4 antibody comprises heavy and light chains,
the heavy chain comprises the amino acid sequence shown in SEQ ID NO. 59, and
the light chain comprises the amino acid sequence shown in SEQ ID NO. 60.
13. The anti-EphA4 antibody according to claim 12, wherein
the C-terminal lysine of the heavy chain is deleted.

14. An isolated nucleic acid encoding the anti-EphA4 antibody according to any one of claims 1 to 13.
15. A vector comprising the nucleic acid according to claim 14.
16. A host cell comprising the vector according to claim 15.
17. A method of producing an anti-EphA4 antibody, comprising a step of culturing the host cell according to claim 16.
18. A pharmaceutical composition comprising the anti-EphA4 antibody according to any one of claims 1 to 13.
19. The pharmaceutical composition according to claim 18 for treating Alzheimer's disease.