

FGF-R4 receptor-specific antagonists

The subject of the present invention is antagonists specific for the FGF receptor 4 (FGF-R4) which make it possible to inhibit the activity of this receptor. These
5 antagonists are in particular antibodies directed specifically against the FGF receptor 4 (FGF-R4).

A subject of the present invention is also the therapeutic use of these antagonists, in particular in the angiogenesis field and in the treatment of certain types of cancer.

10 FGFs (Fibroblast Growth Factors) are among the first molecules described as being capable of stimulating vascular cell proliferation, migration and differentiation *in vitro* and *in vivo*. An abundant literature describes the induction of angiogenesis and the formation of blood capillaries *in vitro* and *in vivo* by FGFs. FGFs are also involved in tumour angiogenesis by promoting the formation of blood vessels recruited by the
15 tumour.

The human FGF family is composed of at least 23 members which all have a conserved central domain of 120 amino acids. They exert their biological activity by interacting with their high affinity receptors of tyrosine-kinase type (FGF-R) and
20 heparan sulphate proteoglycans, which are components present on most cell surfaces and extracellular matrices (low-affinity binding site), so as to form a ternary complex. Some FGFs have a high affinity for several FGF-Rs, whereas others activate specifically one receptor or one isoform of a receptor.

25 An FGF-R4-specific ligand has been identified by Xie and al (Cytokine, 1999, 11:729-35.) This ligand, called FGF19, is a ligand with high affinity for FGF-R4 exclusively, and the binding of which to the receptor is heparin-dependent or heparan sulphate-dependent. FGF19 has been identified in adult animals, only in the hepatocytes and the small intestine where it regulates the synthesis of bile acid by
30 the liver. It appears to be a growth factor involved during embryonic development, and appears to be involved in foetal brain development in the zebra fish and humans.

Other FGF-R4 ligands are described, such as FGF1 or FGF2. These ligands strongly

activate FGF-R4, but are not specific of FGF-R4: they also bind to other FGF-Rs (Ornitz and al., *J. Biol. Chem.*, 1996, 271:15292-7).

- The activation of the FGF-R4 receptor results in several types of cell signalling.
- 5 Among these, the most conventional form corresponds to the setting up of a phosphorylation cascade-mediated signalling pathway subsequent to stimulation of FGF-R4 by FGF. This induction results in the autophosphorylation of the tyrosine kinase domain of FGF-R4 and serves to initiate an intracellular signalling pathway dependent on the phosphorylation of other signalling proteins such as AKT, p44/42,
- 10 JNK etc. This phosphorylation-mediated signalling varies according to the cell type and according to the coreceptors or the adhesion molecules present at the surface of these cells (Cavallaro and al., *Nat. Cell Biol.* 3/7, 650-657 (2001); Stadler and al., *Cell. Signal.* 18/6, 783-794. (2006); Lin and al., *J Biol Chem.*, 2007, 14:27277-84. (2007)). Another method of signalling that is important for FGF-Rs, including FGF-
- 15 R4, is the internalization of the receptor after activation in combination with its ligand. This mechanism is not dependent on the tyrosine kinase activity of the receptor, but on a short C-terminal sequence of FGF-R4 (Klingenberg and al., *J. Cell Sci.*, 113/Pt10:1827-1838 (2000)).
- 20 Four distinct forms of FGF-R4 are described in the literature. A full-length form with 2 polymorphic variants at position 388, namely FGF-R4 Gly388, which is the normal form of the receptor, and the Arg388 form which is described in the context of several tumours (Bange and al., *Cancer Res.* 62/3, 840-847 (2002); Spinola and al., *J Clin Oncol* 23, 7307-7311(2005); Stadler and al., *Cell. Signal.* 18/6, 783-794.
- 25 (2006)). A soluble form, which is expressed in mammary tumour cells, has also been discovered (Takaishi and al., *Biochem Biophys Res Commun.*, 2000, 267:658-62). A fourth form, which is truncated in the extracellular portion, has been described in certain hypophyseal adenomas (Ezzat and al., *J Clin Invest.*, 2002, 109:69-78).
- FGF-R4 is mainly expressed in tissues derived from the endoderm, such as the
- 30 gastrointestinal tract, the pancreas, the liver, the muscles and the adrenal glands.

FGF-R4 is known in the literature as having several cellular roles, the principal three of which are described below:

Firstly, this receptor is involved in the control of various cell differentiation processes *in vitro* and *in vivo*, such as skeletal muscle differentiation and regeneration, mesenchymal tissue differentiation, or osteogenesis, or else in the formation of alveoli during post-natal hepatic development.

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Secondly, FGF-R4 is described in the control of bile acid and cholesterol homeostasis and is thought to be involved in the control of adiposity. Furthermore, the balance between bile acid production and cholesterol production is controlled by FGF-R4 *in vitro* and *in vivo*.

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Thirdly, FGF-R4 is involved in certain tumoral phenomena such as the development of hepatocellular carcinomas or colon cancers, or in the proliferation of mammary fibroadenoma cells or of mammary cancer epithelial cells, such as mammary or colorectal carcinoma cell motility. The tumoral involvement of FGF-R4 is predominantly associated with the appearance of the polymorphism (Gly388Arg) correlated with the acceleration of tumour progression in mammary and colorectal tumours (Bange and al., *Cancer Res.* 62/3, 840-847, 2002), prostate tumours (Wang and al., *Clin. Cancer Res.* 10/18, 6169-6178, 2004) or hepatic tumours (Nicholes and al., *Am. J. Pathol.* 160/6, 2295-307, 2002). This polymorphism is also associated with a poor prognosis in the case of sarcomas (Morimoto and al., *Cancer* 98/10, 2245-2250, 2003), of pulmonary adenocarcinomas (Spinola and al., *J Clin Oncol* 23, 7307-7311, 2005) or of squamous sarcomas (da Costa Andrade and al., *Exp Mol Pathol* 82, 53-7, 2007). The overexpression of FGF-R4 is also described in certain pancreatic cancer lines (Shah and al., *Oncogene* 21/54, 8251-61, 2002) and correlates with astrocytoma malignancy (Yamada and al., *Neurol. Res.* 24/3, 244-8, 2002). In addition, the use of an anti-FGF19 monoclonal antibody in *in vivo* models of colon tumour xenografts or in hepatocellular carcinoma models shows that the inactivation of FGF19 and therefore the blocking of FGF-R4 activation can be beneficial in the treatment of colon or liver cancer.

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It is accepted in the literature that the FGF/FGF-R1 and FGF/FGF-R2 pairs participate in the formation of new blood vessels in a normal or pathological context. However, the potential involvement of FGF-R4 in the control of this cell phenomenon has never been studied. It has, in fact, up until now been supposed that the

activation of angiogenesis is mediated by FGF-R1 and/or FGF-R2 (Presta and al, Cytokine Growth Factor Rev., 2005, 16:159-78).

5 Examples of FGF-R4 antagonists are described in the literature, in particular: small molecules, but they do not target FGF-R4 specifically, thus leading to adverse effects. Thus, tyrosine-kinase domain-inhibiting small chemical molecules which inhibit several FGF-Rs and also other receptor tyrosine kinases have been described by Thompson and al. (Thompson and al *J Med Chem.*, 2000, 43:4200-11.). Small chemical molecules which inhibit FGF-Rs by association with their extracellular
10 portion have also been described in application WO2007/080325.

Antibodies have also been studied, such as the anti-FGFR1 and/or anti-FGFR4 antibodies described in international applications WO2005/066211 and WO2008052796 or by Chen and al. (*Hybridoma* 24/3, 152-159, 2005). Application WO2005/037235 describes antibodies which are FGF-R antagonists, for the
15 treatment of obesity and diabetes. In addition, anti-FGF-R4 antibodies which are agonists are described in application WO03/063893.

The subject of the present invention is an FGF-R4 receptor antagonist, characterized in that it binds specifically to said FGF-R4 receptor.

20 Advantageously, said antagonist is an antibody specific for the FGF-R4 receptor.

In one embodiment, the antagonist which is the subject of the invention binds to the D2-D3 domain of the FGF-R4 receptor. In one advantageous embodiment, the antagonist which is the subject of the invention binds to the D2 domain of the FGF-
25 R4 receptor. In an even more advantageous embodiment, the antagonist binds to the sequence SEQ ID No. 70.

Advantageously, the FGF-R4 receptor-specific antagonist has a K_D with respect to the FGF-R4 receptor, determined by the Biacore technique, of less than 10^{-8} M, less
30 than 5×10^{-9} M, less than 2×10^{-9} M or less than 1×10^{-9} M.

In one advantageous embodiment, the FGF-R4 receptor-specific antagonist is active against both human FGF-R4 and murine FGF-R4.

In another advantageous embodiment, the FGF-R4 receptor-specific antagonist is

active at the same time against human FGF-R4, murine FGF-R4 and rat FGF-R4.

In one advantageous embodiment, the FGF-R4 receptor-specific antagonist antibody comprises at least one CDR having a sequence identical to SEQ ID No. 9, 10, 11,
5 12, 13, 14, 73, 74, 75, 78, 79, 80, 83, 84, 85, 88, 89, 90, 93, 94, 95, 98, 99, 100, 103, 104, 105, 108, 109 or 110 or at least one CDR of which the sequence differs by one or two amino acids compared with the sequences SEQ ID No. 9, 10, 11, 12, 13, 14, 73, 74, 75, 78, 79, 80, 83, 84, 85, 88, 89, 90, 93, 94, 95, 98, 99, 100, 103, 104, 105, 108, 109 or 110, provided that the antibody keeps its binding specificity.

10 In one particularly advantageous embodiment, the FGF-R4 receptor-specific antagonist antibody comprises the CDRs of sequence SEQ ID No. 9, 10, 11, 12, 13, 14, 73, 74, 75, 78, 79, 80, 83, 84, 85, 88, 89, 90, 93, 94, 95, 98, 99, 100, 103, 104, 105, 108, 109 or 110 or CDRs of which the sequences differ by one or two amino acids compared, respectively, with the abovementioned sequences, provided that
15 this does not modify the FGF-R4 receptor-binding specificity of the antibody.

In one advantageous embodiment, the antibodies of the invention comprise at least one heavy chain and at least one light chain, said heavy chain comprising three CDR sequences having amino acid sequences selected from the group constituted of SEQ ID No. 9, 10 and 11 or 73, 74 and 75, 83, 84 and 85, or 93, 94 and 95, or 103,
20 104 and 105, said light chain comprising three CDR sequences having amino acid sequences selected from the group constituted of SEQ ID No. 12, 13 and 14 or 78, 79 and 80, or 88, 89 and 90, or 98, 99 and 100, or 108, 109 and 110.

In one advantageous embodiment, the heavy chain variable regions of the FGF-R4
25 receptor-specific antagonist antibody comprise a sequence having at least 80%, 90%, 95% or 99% identity with the sequence SEQ ID No. 6, 77, 87, 97 or 107.

In one advantageous embodiment, the light chain variable regions of the FGF-R4 receptor-specific antagonist antibody comprise a sequence having at least 80%, 90%, 95% or 99% identity with the sequence SEQ ID No. 8, 72, 82, 92 or 102.

30 A subject of the present invention is also an FGF-R4 receptor-specific antagonist antibody comprising a heavy chain comprising a variable region encoded by a nucleotide sequence having at least 80%, 90%, 95% or 99% identity with sequence SEQ ID No. 5, 76, 86, 96 or 106.

A subject of the present invention is also an FGF-R4 receptor-specific antagonist

antibody comprising a light chain comprising a variable region encoded by a nucleotide sequence having at least 80%, 90%, 95% or 99% identity with the sequence SEQ ID No. 7, 71, 81, 91 or 101.

5 A subject of the present invention is also an FGF-R4 receptor-specific antagonist antibody comprising a heavy chain comprising a variable region of polypeptide sequence SEQ ID No. 6, 77, 87, 97 or 107.

A subject of the present invention is also an FGF-R4 receptor-specific antagonist antibody comprising a light chain comprising a variable region of polypeptide sequence SEQ ID No. 8, 72, 82, 92 or 102.

10 A subject of the present invention is also an FGF-R4 receptor-specific antagonist antibody comprising sequences encoded by the nucleotide sequences SEQ ID Nos. 5 and 7 or 71 and 76, or 81 and 86, or 91 and 96, or 101 and 106.

A subject of the present invention is also an FGF-R4 receptor-specific antagonist antibody comprising the polypeptide sequences SEQ ID Nos. 6 and 8, or 72 and 77,
15 or 82 and 87, or 92 and 97 or 102 and 107.

In one advantageous embodiment, the FGF-R4 receptor-specific antagonist antibody comprises sequences at least 80%, 90%, 95% or 99% identical to SEQ ID No. 2 and/or SEQ ID No. 4; or SEQ ID No. 72 and/or SEQ ID No. 77; or SEQ ID No. 82 and/or SEQ ID No. 87; or SEQ ID No. 92 and/or SEQ ID No. 97; or SEQ ID No. 102
20 and/or SEQ ID No. 107.

In one particularly advantageous embodiment, the FGF-R4 receptor-specific antagonist antibody comprises a heavy chain encoded by a nucleotide sequence at least 80%, 90%, 95% or 99% identical to the sequence SEQ ID No. 1.

25 A subject of the present invention is also an FGF-R4 receptor antagonist antibody comprising a heavy chain of polypeptide sequence at least 80%, 90%, 95% or 99% identical to the sequence SEQ ID No. 2.

A subject of the present invention is also an FGF-R4 receptor-specific antagonist antibody comprising a light chain encoded by a nucleotide sequence at least 80%,
30 90%, 95% or 99% identical to the sequence SEQ ID No. 3.

A subject of the present invention is also an FGF-R4 receptor-specific antagonist antibody comprising a light chain of polypeptide sequence at least 80%, 90%, 95% or 99% identical to the sequence SEQ ID No. 4.

A subject of the present invention is also an FGF-R4 receptor-specific antagonist

antibody comprising the sequences encoded by the nucleotide sequences SEQ ID No. 1 and 3.

- In one even more advantageous embodiment, the FGF-R4-specific antagonist antibody comprises a heavy chain comprising the sequence SEQ ID No. 2 and a
5 light chain comprising the sequence SEQ ID No. 4.

The antibody composed of a heavy chain of sequence SEQ ID No. 2 and of a light sequence SEQ ID No. 4 will be called 40-12 in the rest of the application.

- 10 In one embodiment of the invention, the FGF-R4-specific antibodies are active at the same time against human FGF-R4, murine FGF-R4 and rat FGF-R4.

In one advantageous embodiment, the FGF-R4 receptor-specific antagonist induces inhibition of AKT/p38 cell pathways.

- 15 In one advantageous embodiment, the FGF-R4 receptor-specific antagonist induces inhibition of Erk1/2 cell pathways.

In one advantageous embodiment, the FGF-R4 receptor-specific antagonist induces inhibition of FGF-R4-controlled cell signalling pathways.

- 20 In another advantageous embodiment, the FGF-R4 receptor-specific antagonist induces inhibition of tumour cell proliferation.

In yet another advantageous embodiment, the FGF-R4 receptor-specific antagonist induces inhibition of angiogenesis.

- 25 In another particularly advantageous embodiment, the FGF-R4 receptor-specific antagonist has an affinity for FGF-R4 which is 10 times greater than its affinity for the other FGF receptors.

- In one particularly advantageous embodiment, the antibody according to the
30 invention is an FGF-R4 receptor-specific humanized antagonist antibody.

In one embodiment, the FGF-R4 receptor-specific humanized antagonist antibody comprises a light chain of which the variable region is encoded by a nucleotide sequence at least 80%, 90%, 95% or 99% identical to the sequence SEQ ID No. 29 or the sequence SEQ ID No. 31.

In another embodiment, the FGF-R4 receptor-specific humanized antagonist antibody comprises a light chain of which the variable region is at least 80%, 90%, 95% or 99% identical to the sequence SEQ ID No. 30 or to the sequence SEQ ID No. 32.

- 5 In another embodiment, the FGF-R4 receptor-specific humanized antagonist antibody comprises a light chain in which the variable region is encoded by a sequence identical to the nucleotide sequence SEQ ID No. 29 or to the sequence SEQ ID No. 31.

- 10 A subject of the present invention is also an FGF-R4 receptor-specific humanized antagonist antibody comprising a heavy chain of which the variable region is encoded by a sequence at least 80%, 90%, 95% or 99% identical to the sequence SEQ ID No. 33, to the sequence SEQ ID No. 35 or to the sequence SEQ ID No. 37.

- A subject of the present invention is also an FGF-R4 receptor-specific humanized antagonist antibody comprising a heavy chain of which the variable region is at least
15 80%, 90%, 95% or 99% identical to the sequence SEQ ID No. 34, to the sequence SEQ ID No. 36 or to the sequence SEQ ID No. 38.

A subject of the present invention is also an FGF-R4 receptor-specific humanized antagonist antibody comprising a heavy chain encoded by a nucleotide sequence SEQ ID No. 33 and/or SEQ ID No. 35 and/or SEQ ID No. 37.

- 20 A subject of the present invention is also an FGF-R4 receptor-specific humanized antagonist antibody of which the humanized sequences of sequence SEQ ID No. 30 or 32 are used in combination with the humanized sequences of sequence SEQ ID No. 34, 36 or 38.

- In another embodiment, the FGF-R4 receptor-specific antagonist antibody comprises
25 the CDRs of sequence SEQ ID Nos. 73, 74, 75, 78, 79 and 80 or CDRs of which the sequences differ by one or two amino acids compared, respectively, with the abovementioned sequences, provided that this does not modify the FGF-R4 receptor-binding specificity of the antibody.

- In another embodiment, the FGF-R4 receptor-specific antagonist antibody comprises
30 the CDRs of sequence SEQ ID Nos. 83, 84, 85, 88, 89 and 90 or CDRs of which the sequences differ by one or two amino acids compared, respectively, with the abovementioned sequences, provided that this does not modify the FGF-R4 receptor-binding specificity of the antibody.

In another embodiment, the FGF-R4 receptor-specific antagonist antibody comprises

the CDRs of sequence SEQ ID Nos. 93, 94, 95, 98, 99 and 100 or CDRs of which the sequences differ by one or two amino acids compared, respectively, with the abovementioned sequences, provided that this does not modify the FGF-R4 receptor-binding specificity of the antibody.

- 5 In another embodiment, the FGF-R4 receptor-specific antagonist antibody comprises the CDRs of sequence SEQ ID Nos. 103, 104, 105, 108, 109 and 110 or CDRs of which the sequences differ by one or two amino acids compared, respectively, with the abovementioned sequences, provided that this does not modify the FGF-R4 receptor-binding specificity of the antibody.
- 10 In one preferred embodiment of the invention, the FGF-R4 receptor-specific antagonist antibody comprises the CDRs of sequence SEQ ID Nos. 83, 84, 85, 88, 89 and 90.

- In one advantageous embodiment, the FGF-R4 receptor-specific antagonist antibody
- 15 is a human antibody of which the heavy chain variable regions comprise a nucleotide sequence having at least 80%, 90%, 95% or 99% identity with the sequence SEQ ID No. 76, 86, 96 or 106.

- In one advantageous embodiment, the FGF-R4 receptor-specific antagonist antibody is a human antibody of which the light chain variable regions comprise a nucleotide
- 20 sequence having at least 80%, 90%, 95% or 99% identity with the sequence SEQ ID No. 71, 81, 91 or 101.

- A subject of the present invention is also an FGF-R4 receptor-specific human antagonist antibody comprising a heavy chain comprising a variable region encoded by a protein sequence having at least 80%, 90%, 95% or 99% identity with the
- 25 sequence SEQ ID No. 77, 87, 97 or 107.

- A subject of the present invention is also an FGF-R4 receptor-specific human antagonist antibody comprising a light chain comprising a variable region encoded by a protein sequence having at least 80%, 90%, 95% or 99% identity with the
- sequence SEQ ID No. 72, 82, 92 or 102.

- 30 A subject of the present invention is also an FGF-R4 receptor-specific human antagonist antibody comprising sequences encoded by the nucleotide sequences SEQ ID Nos. 71 and 76 or the nucleotide sequences SEQ ID Nos. 81 and 86 or the nucleotide sequences SEQ ID Nos. 91 and 96 or the nucleotide sequences SEQ ID

Nos. 101 and 106.

A subject of the present invention is also an FGF-R4 receptor-specific human antagonist antibody comprising the polypeptide sequences SEQ ID Nos. 72 and 77 or the polypeptide sequences SEQ ID Nos. 82 and 87 or the polypeptide sequences
5 SEQ ID Nos. 92 and 97 or the polypeptide sequences SEQ ID Nos 102 and 107.

In one advantageous embodiment, the FGF-R4 receptor-specific antagonist antibody comprises sequences at least 80%, 90%, 95% or 99% identical to SEQ ID No. 72 and/or SEQ ID No. 77.

10 In another advantageous embodiment, the FGF-R4 receptor-specific human antagonist antibody comprises sequences at least 80%, 90%, 95% or 99% identical to SEQ ID No. 82 and/or SEQ ID No. 87.

In another advantageous embodiment, the FGF-R4 receptor-specific human antagonist antibody comprises sequences at least 80%, 90%, 95% or 99% identical
15 to SEQ ID No. 92 and/or SEQ ID No. 97.

In another advantageous embodiment, the FGF-R4 receptor-specific human antagonist antibody comprises sequences at least 80%, 90%, 95% or 99% identical to SEQ ID No. 102 and/or SEQ ID No. 107.

20 In one preferred embodiment, a subject of the present invention is an FGF-R4 receptor-specific human antagonist antibody comprising a light chain encoded by a nucleotide sequence at least 80%, 90%, 95% or 99% identical to the sequence SEQ ID No. 82 and comprising a light chain of polypeptide sequence at least 80%, 90%, 95% or 99% identical to the sequence SEQ ID No. 87.

25 Even more preferably, a subject of the present invention is an FGF-R4 receptor-specific human antagonist antibody comprising sequences encoded by the nucleotide sequences SEQ ID Nos. 82 and 87.

The antibody composed of a heavy chain of sequence SEQ ID No. 77 and of a light
30 sequence SEQ ID No. 72 will be called clone 8 in the rest of the application.

The antibody composed of a heavy chain of sequence SEQ ID No. 87 and of a light sequence SEQ ID No. 82 will be called clone 31 in the rest of the application.

The antibody composed of a heavy chain of sequence SEQ ID No. 97 and of a light sequence SEQ ID No. 92 will be called clone 33 in the rest of the application.

The antibody composed of a heavy chain of sequence SEQ ID No. 107 and of a light sequence SEQ ID No. 102 will be called clone 36 in the rest of the application.

5 The field of the present invention is not limited to the antibodies comprising these sequences. In fact, all the antibodies that specifically bind to FGF-R4, having an antagonistic action on this receptor, are part of the field of the present invention.

A subject of the present invention is also an FGF-R4 receptor-specific antagonist antibody conjugated to a cytotoxic agent.

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A subject of the present invention is the use of an FGF-R4 receptor-specific antagonist in the treatment of diseases associated with angiogenesis.

A subject of the present invention is the use of an FGF-R4 receptor-specific antagonist in the treatment of a cancer.

15 A subject of the present invention is the use of an FGF-R4 receptor-specific antagonist in the treatment of hepatocarcinomas or of any other type of hepatic cancer.

A subject of the present invention is the use of an FGF-R4 receptor-specific antagonist in the treatment of pancreatic cancer.

20 A subject of the present invention is an FGF-R4 receptor-specific antibody which is of use both in the treatment of diseases associated with angiogenesis and in the treatment of hepatocarcinomas or of any other type of hepatic cancer.

A subject of the present invention is an FGF-R4 receptor-specific antibody which is of use at the same time in the treatment of diseases associated with angiogenesis, in
25 the treatment of hepatocarcinomas or of any other type of hepatic cancer, and in the treatment of pancreatic cancer, or cancer of the organs of the gastrointestinal tract or any other organ expressing FGF-R4.

A subject of the present invention is a pharmaceutical composition comprising an
30 FGF-R4 receptor-specific antagonist and excipients.

A subject of the present invention is a method of treating a cancer, comprising the administration, to the patient, of an FGF-R4 receptor-specific antagonist antibody.

A subject of the present invention is a method of treating a disease associated with a

pathological increase in angiogenesis, comprising the administration, to the patient, of an FGF-R4 receptor-specific antagonist antibody.

5 A subject of the present invention is a method of selecting an FGF-R4 receptor-specific antagonist monoclonal antibody, comprising the following steps:

- a. immunizing mice,
- b. taking lymph nodes from the mice, and
- c. screening the hybridoma supernatants.

10 A subject of the present invention is a cell line that produces FGF-R4 receptor-specific antagonist antibodies.

15 A subject of the present invention is a method of producing an FGF-R4 receptor-specific antagonist antibody, comprising culturing a cell line that produces FGF-R4 receptor antagonist antibodies.

A subject of the present invention is a drug comprising an FGF-R4 receptor-specific antagonist.

20 A subject of the present invention is also a polynucleotide encoding a polypeptide selected from the group constituted of SEQ ID Nos. 2, 4, 6, 8, 9, 10, 11, 12, 13, 14, 30, 32, 34, 36, 38, 72, 73, 74, 75, 77, 78, 79, 80, 82, 83, 84, 85, 87, 88, 89, 90, 92, 93, 94, 95, 97, 98, 99, 100, 102, 103, 104, 105, 107, 108, 109 and 110, and the sequences at least 80%, 90%, 95% or 99% identical to one of these sequences.

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A subject of the present invention is a recombinant vector comprising a polynucleotide as described above or encoding a polypeptide as described above.

30 In order to enable the expression of heavy chains and/or light chains of the FGF-R4 receptor antagonist antibody, which is a subject of the invention, the polynucleotides encoding said chains are inserted into expression vectors. These expression vectors may be plasmids, YACs, cosmids, retroviruses, EBV-derived episomes, and any of the vectors that those skilled in the art may deem appropriate for the expression of said chains.

A subject of the present invention is a host cell comprising a recombinant vector as described above.

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Detailed description of the invention

10 A subject of the present invention is the use of antibodies specifically directed against FGF-R4 (without cross-reaction with FGF-R1, R2 or R3) for inhibiting angiogenesis and tumour growth.

Unexpectedly, the inventors have shown that FGF-R4 plays an active and specific role in the control of angiogenesis.

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This function of FGF-R4 had never previously been shown or proposed. Consequently, this receptor may be used as a target for treating pathologies exhibiting an angiogenic dysfunction. The FGF-R4 ligands capable of modulating the activity of said receptor are therefore potential therapeutic agents for numerous
20 angiogenesis-related pathologies.

The present invention can therefore be used in the treatment of all pathologies involving a dysregulation of angiogenesis and requiring inhibition thereof. The pathologies covered may be cancer, with the use of the antagonists according to the
25 invention as tumour angiogenesis inhibitors, or pathologies for which a dysregulation of angiogenesis is described, such as: age-related macular degeneration or ARMD, inflammatory diseases such as rheumatoid arthritis, osteoarthritis, colitis, ulcers or any inflammatory disease of the intestines, atherosclerosis, or else in the treatment of obesity.

30 The use of these antibodies is also illustrated in the inhibition of tumour growth. The antagonists according to the present invention can therefore be used for the treatment of certain cancers involving a dysregulation of FGF-R4, and more particularly, liver cancer, colon cancer, breast cancer, lung cancer, prostate cancer, pancreatic cancer, skin cancer or oesophageal cancer.

One of the major advantages of the antagonists according to the present invention is to specifically target an FGF receptor, in the case in point FGF-R4. This specificity makes it possible to limit the adverse effects that small chemical molecules which inhibit the tyrosine kinase domain can have. In addition, since FGF-R4 is not expressed ubiquitously, but is in particular expressed on endothelial cells, for instance on hepatocyte cells, biliary cells, mammary cells, prostatic cells, ovarian cells, pancreatic cells or renal cells, this provides a method of treating diseases related to a dysregulation of FGF-R4 activity which limits the side effects.

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The term "antagonist" refers to any ligand capable of reducing or completely inhibiting the activity of FGF-R4. This antagonist compound is thus also referred to as FGF-R4 inhibitor.

15 This antagonist may be any FGF-R4 ligand, such as a chemical molecule, a recombinant protein, an oligosaccharide, a polysaccharide, an oligonucleotide or an antibody capable of specifically binding to the FGF-R4 receptor, with the exclusion of any other FGFR.

20 A subject of the invention is therefore an FGF-R4-specific antagonist. According to the invention, an antagonist that binds specifically to FGF-R4 refers to a ligand which does not bind to the other FGF receptors, namely FGF-R1, FGF-R2 or FGF-R3. In particular, an FGF-R4-specific antibody is an antibody which does not exhibit any cross-reaction with FGF-R1, FGF-R2 or FGF-R3.

25 "Specific binding" refers to a difference, by a factor of at least 10, between the intensity of the binding to one receptor compared with another, in this case between the binding to FGF-R4 and the possible bindings to FGF-R1, FGF-R2 or FGF-R3.

In one embodiment of the invention, the FGF-R4 ligand is an oligosaccharide or a polysaccharide.

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An "oligosaccharide" refers to any saccharide polymer containing from three to ten units of simple sugars. Natural oligosaccharides such as, for example, fructo-oligosaccharides (FOS), and synthetic oligosaccharides such as, for example, heparin-mimetic antithrombotics, exist.

The term "polysaccharide" refers to any polymer constituted of more than ten monosaccharides linked to one another by glycosidic linkages. Natural polysaccharides such as, for example, mucopolysaccharides, fucoids, carrageenans or bacterial exopolysaccharides exist, as do synthetic polysaccharides. Thus, low-molecular-weight fucoidans or highly sulphated exopolysaccharides have shown pro-angiogenic activities (Chabut and al., *Mol Pharmacol.*, 2003, 64:696-702; Matou and al., *Biochem Pharmacol.*, 2005, 69:751-9). Conversely, heparin-derived weakly sulphated oligosaccharides or phosphomannopentose sulphates can have anti-angiogenic characteristics (Parish and al., 1999, 15:3433-41; Casu and al., *J Med Chem.*, 2004, 12:838-48)

In one embodiment of the invention, the FGF-R4 ligand is an antibody.

The term "antibody" refers to antibodies or derived molecules of any type, such as polyclonal and monoclonal antibodies. Included among the molecules derived from monoclonal antibodies are humanized antibodies, human antibodies, multispecific antibodies, chimeric antibodies, antibody fragments, nanobodies, etc.

In one embodiment of the invention, the FGF-R4-specific antagonist is a polyclonal antibody.

A "polyclonal antibody" is an antibody which has been produced from a mixture of antibodies originating from several B lymphocyte clones and which recognize a series of different epitopes.

In one advantageous embodiment, the FGF-R4-specific antagonist is a monoclonal antibody.

A "monoclonal antibody" is an antibody obtained from a substantially homogeneous population of antibodies derived from a single type of B lymphocyte, clonally amplified. The antibodies making up this population are identical except for possible naturally occurring mutations that may be present in minor amounts. These antibodies are directed against a single epitope and are therefore highly specific.

The term "epitope" refers to the site of the antigen to which the antibody binds. If the antigen is a polymer, such as a protein or a polysaccharide, the epitope may be

made up of contiguous or noncontiguous residues.

In one advantageous embodiment of the invention, the anti-FGF-R4 antagonist antibody binds to an epitope belonging to the D2-D3 domain of the FGF-R4 receptor.

5 In an even more advantageous embodiment, the antibody binds to an epitope included in the domain comprising amino acids 144 to 365 of the FGF-R4 receptor.

In an even more advantageous embodiment, the antibody binds to an epitope included in the D2 domain of the FGF-R4 receptor, this epitope corresponding to amino acids 145 to 242 described in the sequence SEQ ID No. 70.

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An antibody, also known as an immunoglobulin, is composed of two identical heavy chains ("VH") and of two identical light chains ("VL") which are linked by a disulphide bridge. Each chain contains a constant region and a variable region. Each variable region comprises three segments called "complementarity determining regions" (CDRs) or "hypervariable regions", which are mainly responsible for the binding to the epitope of an antigen.

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The term "VH" refers to the variable regions of an immunoglobulin heavy chain of an antibody, including the heavy chains of an Fv, scFv, dsFv, Fab, Fab' or F(ab)' fragment.

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The term "VL" refers to the variable regions of an immunoglobulin light chain of an antibody, including the light chains of an Fv, scFv, dsFv, Fab, Fab' or F(ab)' fragment.

25

The term "antibody fragment" refers to any part of said antibody: Fab (fragment antigen binding), Fv, scFv (single chain Fv), Fc (fragment crystallizable). Preferably, these functional fragments will be fragments of the type Fv, scFv, Fab, F(ab')₂, Fab', scFv-Fc or diabodies, which generally have the same binding specificity as the chimeric or humanized, monoclonal antibody from which they are derived. According to the present invention, antibody fragments of the invention can be obtained from chimeric or humanized, monoclonal antibodies by methods such as digestion with enzymes, for instance pepsin or papain, and/or by cleavage of the disulphide bridges by chemical reduction.

30

The term "CDR regions or CDRs" is intended to denote the immunoglobulin heavy and light chain hypervariable regions as defined by Kabat and al. (Kabat and al., Sequences of proteins of immunological interest, 5th Ed., U.S. Department of Health and Human Services, NIH, 1991, and later editions). There are 3 heavy chain CDRs and 3 light chain CDRs. The term CDR or CDRs is used herein to denote, as appropriate, one or more of these regions or even all of these regions which contain the majority of the amino acid residues responsible for the affinity binding of the antibody for the antigen or the epitope that it recognizes. The most conserved regions of the variable domain are called FR (for "framework") regions or sequences.

In another embodiment of the invention, the FGF-R4-specific antagonist is a chimeric antibody.

The term "chimeric antibody" refers to an antibody in which the constant region, or a portion thereof, is altered, replaced or exchanged, such that the variable region is linked to a constant region of a different species, or belongs to another antibody class or subclass.

The term "chimeric antibody" also refers to an antibody in which the variable region, or a portion thereof, is altered, replaced or exchanged, such that the constant region is linked to a variable region of a different species, or belongs to another antibody class or subclass.

The methods of producing chimeric antibodies are known to those skilled in the art. See, for example, Morrison, 1985, *Science*, **229**:1202; Oi and al., 1986, *Bio Techniques*, 4:214; Gillies and al., 1989, *J. Immunol. Methods*, **125**:191-202; U.S. Pat. Nos. 5,807,715; 4,816,567; and 4,816,397.

These chimeric versions of the antibody may comprise the fusion of the VL and VH variable regions to the Ckappa and the CH (IgG1) constant domains of human origin in order to generate a chimeric monoclonal antibody.

The CH (IgG1) domain can also be modified by point mutations in order to increase the affinity of the Fc fragment for the FcγRIIIa receptor and thereby to increase the effector functions of the antibody (Lazar and al., 2006, *Proc. Natl. Acad. Sci. USA* 103: 4005-4010; Stavenhagen and al., 2007, *Cancer Res.* **67**: 8882-8890).

The present invention includes the humanized versions of the antibodies.

The term "humanized antibody" refers to an antibody which contains mainly human immunoglobulin sequences. This term generally refers to a non-human
5 immunoglobulin which has been modified by incorporation of human sequences or of residues found in human sequences.

In general, humanized antibodies comprise one or typically two variable domains in which all or part of the CDR regions correspond to parts derived from the non-human
10 parent sequence and in which all or part of the FR regions are those derived from a human immunoglobulin sequence. The humanized antibody can then comprise at least one portion of an immunoglobulin constant region (Fc), in particular that of the human immunoglobulin template chosen.

The goal is thus to have an antibody that is minimally immunogenic in a human.
15 Thus it is possible that one or two amino acids in one or more CDRs can be modified by one that is less immunogenic to a human host, without substantially reducing the specific binding function of the antibody to FGF-R4. Similarly, the residues of the framework regions may not be human, and it is possible for them not to be modified since they do not contribute to the immunogenic potential of the antibody.

20 Several methods of humanization known to those skilled in the art exist for modifying a non-human parent antibody so as to give an antibody that is less immunogenic to humans. An overall sequence identity with a human antibody is not necessarily required. This is because the overall sequence identity is not necessarily a predictive
25 indicator of reduced immunogenicity, and the modification of a limited number of residues can result in humanized antibodies having a greatly reduced immunogenic potential in humans (Molecular Immunology (2007) 44, 1986-1998).

Various methods are, for example, CDR grafting (EPO 0 239 400; WO 91/09967; and U.S. Pat. Nos. 5,530,101 and 5,585,089), resurfacing (EPO 0 592 106; EPO 0 519 596; Padlan, 1991, Molec Imm 28(4/5):489-498; Studnicka and al., 1994, Prot Eng 7(6):805-814; and Roguska and al., 1994, PNAS 91:969-973) or else chain
30 shuffling (U.S. Pat. No. 5,565,332).

The present invention relates in particular to humanized antibodies of which the variable portions are modified according to the following technology:

The light and heavy chains most similar to the corresponding chains of the anti-FGF-R4 murine antibody 40-12 are identified by comparison with the Protein Data Bank (H.M. Berman, J. Westbrook, Z. Feng, G. Gilliland, T.N. Bhat, H. Weissig, I.N. Shindyalov, P.E. Bourne. *Nucleic Acids Research*, 2000, 28:235-242). The sequence alignment uses the BLAST algorithm (*J Mol Biol.* 1990 Oct 215:403-410). These are tridimensional structures corresponding to the PDB codes 1NDM & 1ETZ respectively used to build up the homology models of the variable domain light and heavy chains. These tridimensional models are subsequently energy minimized using the standard procedure implemented in the MOE software (Molecular Operating Environment, Chemical Computing Group, Quebec, Canada). A molecular dynamic (MD) simulation of these minimized tridimensional models of the antibody is subsequently performed with the Amber software (D.A. Case, T.E. Cheatham, III, T. Darden, H. Gohlke, R. Luo, K.M. Merz, Jr., A. Onufriev, C. Simmerling, B. Wang and R. Woods. *J. Computat. Chem.* 2005, 26:1668-1688). This simulation is done with harmonic constraints applied to the protein backbone atoms at a temperature of 500 K for a period of 1.1 nanoseconds in a generalized Born implicit solvent (Gallicchio & Levy, *J Comput Chem* 2004, 25:479-499). Ten diverse conformations are thus extracted from this first simulation, one tridimensional conformation every one hundred picoseconds, during the last nanosecond of the simulation. These ten diverse conformations are then each subjected to a molecular dynamic simulation, without constraints on the protein backbone, at a temperature of 27°C for 2.3 nanoseconds in a generalized Born implicit solvent. The bonds involving a hydrogen atom are constrained using the SHAKE algorithm (Barth. and al., *J Comp Chem*, 1995, 16:1192-1209), the time step is 1 femtosecond, and the simulation was run based on the Langevin equation at constant volume and a constant temperature of 27°C. For each of the ten molecular dynamic simulations, the last two thousand conformations, extracted at a frequency of one every picosecond, are then used to quantify, for each amino acid of the antibody to be humanized, the deviation of the atomic positions with respect to an average, or medoid, conformation of the amino acid. The Scientific Vector Language (SVL) of the MOE software is used to code all of the analysis described below. The medoid conformation of the amino acid is the conformation derived from the molecular dynamic which is the closest to the average

conformation calculated from the position of the atoms of all the conformations of the amino acid. The distance used for detecting the medoid conformation is the route mean square (RMSD) of the scalar distances between the atoms of two conformations of the amino acid. Similarly, the deviation of the positions of the atoms of one conformation of an amino acid compared with the medoid conformation is quantified by calculating the RMSD of the scalar distances between the atoms of the amino acid of one conformation of the simulation and the same atoms of the medoid conformation. Subsequently, by comparing the RMSD of the positions of the atoms of a given amino acid (i), averaged over all the ten molecular dynamic simulations (Fi), with the RMSD of the positions of all the amino acids of the antibody, averaged over all the ten molecular dynamic simulations (Fm), it is decided whether the amino acid is flexible enough to be considered able to potentially interact with the T-cell receptors and trigger activation of the immune system. An amino acid i is considered flexible if its flexibility score Zi, defined as $Z_i = (F_i - F_m)/F_m$, is above 0.15. 45 amino acids are thus identified as flexible in the variable domain of the antibody, with the exclusion of the antigen complementarity determining region (CDR) and of its immediate vicinity. The immediate vicinity of the CDR is defined as any amino acid with an alpha carbon at a distance of 5 angströms (Å) or less to an alpha carbon of the CDR.

20

The motions of the 60 most flexible amino acids of the antibody, during the 20 nanoseconds (10x2ns) of simulation, are then compared to the motions of the corresponding amino acids of 49 homology models of human antibody germ lines, for each of which ten molecular dynamic simulations (10x2 ns) have been run using the same protocol. The 60 most flexible amino acids exclude the antigen complementarity determining region (CDR) and its immediate vicinity. The 49 human antibody germ line models were built by systematically combining the 7 most frequent human light chains (vk1, vk2, vk3, vk4, vlamba1, vlamba2, vlamba3) and the 7 most frequent human heavy chains (vh1a, vh1b, vh2, vh3, vh4, vh5, vh6) (Nucleic Acids Research, 2005, Vol. 33, Database issue D593-D597).

30

The similarity of the antibody to be humanized to the 49 human germ line models is quantified by sampling the positions of specific atoms of the 60 flexible amino acids of an antibody, over the course of the ten molecular dynamic simulations, by means

of a unique tridimensional cubic grid which has a 1Å resolution. This is referred to as quadridimensional similarity. The tridimensional grid used is made of 445 740 points and is initialized using the tridimensional structure of the human antibody corresponding to the PDB code 8FAB. The 8FAB structure is also used to position all the conformations of an antibody to be sampled in the tridimensional grid. For this purpose, the medoid conformation of the molecular dynamic of the antibody is superposed onto the 8FAB structure. This superposition consists of aligning the moments of inertia of the two conformations, followed by the optimization of the scalar distances between the alpha carbon atoms of both conformations. All the remaining conformations of the molecular dynamic of the antibody are superposed onto the medoid conformation using the same method.

Two types of sampling are performed, which result in two similarities (electrostatic similarity and lipophilic similarity), for a pair of antibodies being compared. These two similarities are then added to obtain the total similarity. The first sampling, the electrostatic sampling, considers all atoms of the amino acid side chain. The value in one point, x , of the grid is obtained by applying, to the atoms of the amino acid side chain, a tridimensional Gaussian function $f(x)$ weighted with the atomic partial charge as described in the Amber99 force field (Cieplak, J., and al.; J. Comp. Chem. 2001, 22:1048-1057). The $f(x)$ function is applied on the 3 Cartesian coordinate axes and

corresponds to the following formula: $f(x) = \left(s\sqrt{2\pi}\right)^{-3} \times \exp\left(\frac{-(x-u)^2}{2s^2}\right)$, with x and u

being, respectively, the Cartesian coordinates of a grid point x and of a sampled atom, and $s = r/1.6$ (r = covalence radius of the atom). The sampling is repeated for all conformations of the amino acid and the obtained results are averaged at all points x of the tridimensional grid. The second sampling, the lipophilic sampling, considers only the lipophilic atoms of the amino acid side chain. The value at one point, x , of the grid is calculated with the same Gaussian function $f(x)$ without weighting. As a result, the two ensembles of conformations from the molecular dynamic simulations, of the two antibodies being compared, are sampled by the same tridimensional grid. The electrostatic similarity (sim-elec) between antibody a and antibody b is measured with the following formula:

$$sim - elec = \frac{\sum_{i=1}^{445740} \left(\left| x_i^a + x_i^b \right| - \left| x_i^a - x_i^b \right| \right)}{\sum_{i=1}^{445740} \left(\left| x_i^a + x_i^b \right| \right)}$$

The lipophilic similarity is calculated with the

same formula applied to the data generated by the lipophilic sampling previously described.

The human germ line model vlamba2-vh2 thus displays the highest quadridimensional similarity (total similarity = 58%) of these 60 flexible amino acids with respect to the flexible amino acids of the murine antibody 40-12. The human germ line model vlamba2-vh2 has thus been used to humanize the antibody to be humanized, while focusing on the 45 flexible amino acids. In order to determine the mutations to be made, the tridimensional structure of the model of the murine antibody 40-12 is superposed on that of the model derived from the germ lines showing the highest similarity, with the positions of the alpha carbons of the amino acids being optimized. The amino acids identified as flexible are mutated with the corresponding amino acids in the sequence of the model showing the highest similarity.

The unwanted sequence motifs considered are the following: Aspartate-Proline (peptide bond labile in acidic medium), Asparagine-X-Serine/Threonine (glycosylation site, X=any amino acid except Proline), Aspartate-Glycine/Serine/Threonine (potential formation of succinimide/isoaspartate in the flexible zones), Asparagine-Glycine/Histidine/Serine/Alanine/Cysteine (exposed deamidation sites), Methionine (oxidation in the exposed zones). The humanized sequences thus obtained are finally compared, by means of the BLAST sequence comparison algorithm, with the sequences of the IEDB database (<http://www.immuneepitope.org/> The immune epitope database and analysis resource: from vision to blueprint. PLoS Biol. 2005 Mar;3(3):e91) so as to be sure that the sequences do not contain any epitopes known to be recognized by B and T lymphocytes. If the sequence contains residues which have unwanted sequences, they are then also modified. If the composite sequence contains a known epitope listed in the IEDB, another germ line structure template showing a high similarity is then used as model.

More advantageously, the antibody according to the invention comprises sequences having at least 80%, 90%, 95% or 99% identity with the sequence SEQ ID No. 30 or the sequence SEQ ID No. 32 are used, in combination with the sequences having at least 80%, 90%, 95% or 99% identity with the sequence SEQ ID No. 34, the sequence SEQ ID No. 36 or the sequence SEQ ID No. 38.

In one embodiment, the antibody according to the invention comprises variable light chains of sequence SEQ ID No. 30 and variable heavy chains of sequence SEQ ID No. 34.

- 5 In another embodiment, the antibody comprises variable light chains of sequence SEQ ID No. 32 and variable heavy chains of sequence SEQ ID No. 38.

In another embodiment, the antibody comprises variable light chains of sequence SEQ ID No. 30 and variable heavy chains of sequence SEQ ID No. 36.

- 10 In another embodiment, the antibody comprises variable light chains of sequence SEQ ID No. 32 and variable heavy chains of sequence SEQ ID No. 34.

In another embodiment, the antibody comprises variable light chains of sequence SEQ ID No. 32 and variable heavy chains of sequence SEQ ID No. 36.

- 15 In another embodiment, the antibody comprises variable light chains of sequence SEQ ID No. 30 and variable heavy chains of sequence SEQ ID No. 38.

- A subject of the present invention is also FGF-R4-specific human antagonist antibodies. Such antibodies can be obtained by phage display according to methods known to those skilled in the art (McCafferty J. and *al*, 1990; Hoogenboom, HR and
20 *al*, 2005). Other technologies are available for the preparation of human antibodies, such as the XenoMouse technology described in US patent 5,939,598.

- In one particular embodiment, the FGF-R4 receptor-specific antagonist antibody is a human antibody of which the heavy chain variable regions comprise a sequence
25 having at least 80%, 90%, 95% or 99% identity with the sequence SEQ ID No. 76, 86, 96 or 106.

- In another embodiment, the FGF-R4 receptor-specific antagonist antibody is a human antibody of which the light chain variable regions comprise a sequence having at least 80%, 90%, 95% or 99% identity with the sequence SEQ ID No. 71,
30 81, 91 or 101.

A subject of the present invention is also an FGF-R4 receptor-specific human antagonist antibody comprising a heavy chain comprising a variable region encoded by a nucleotide sequence having at least 80%, 90%, 95% or 99% identity with the sequence SEQ ID No. 77, 87, 97 or 107.

A subject of the present invention is also an FGF-R4 receptor-specific human antagonist antibody comprising a light chain comprising a variable region encoded by a nucleotide sequence having at least 80%, 90%, 95% or 99% identity with the sequence SEQ ID No. 72, 82, 92 or 102.

5

A subject of the present invention is also an FGF-R4 receptor-specific human antagonist antibody comprising sequences encoded by the nucleotide sequences SEQ ID Nos. 71 and 76 or the nucleotide sequences SEQ ID Nos. 81 and 86 or the nucleotide sequences SEQ ID Nos. 91 and 96 or the nucleotide sequences SEQ ID Nos. 101 and 106.

10

A subject of the present invention is also an FGF-R4 receptor-specific human antagonist antibody comprising the polypeptide sequences SEQ ID Nos. 72 and 77 or the polypeptide sequences SEQ ID Nos. 82 and 87 or the polypeptide sequences SEQ ID Nos. 92 and 97 or the polypeptide sequences SEQ ID Nos. 102 and 107.

15

Particularly preferably, an FGF-R4 receptor-specific human antagonist antibody comprises the polypeptide sequences SEQ ID Nos. 82 and 87.

The amino acid sequences thus modified can also be modified by means of post-translational modifications during the production in the mammalian cell. In particular, the use of stable lines deficient in fucose biosynthesis can make it possible to produce monoclonal antibodies in which the N-glycan of the Fc (position N297) partially or completely lacks fucose and makes it possible to increase the ADCC effector effect (Kanda and *al.*, 2006, *Biotechnol. Bioeng.* 94:680-688 and Ripka and *al.*, 1986 *Arch Biochem Bioph* 249: 533-545).

25

In another embodiment of the invention, the FGF-R4-specific antagonist is a conjugated antibody.

The antibodies may be conjugated to a cytotoxic agent. The term "cytotoxic agent" denotes herein a substance which reduces or blocks the function or the growth of the cells, or causes destruction of the cells.

30

In one embodiment, the antibody or a binding fragment thereof can be conjugated to a drug, such as a maytansinoid, so as to form a "prodrug" which has cytotoxicity with respect to the cells expressing the antigen.

The cytotoxic agent of the present invention may be any compound which results in the death of a cell, or induces the death of a cell, or decreases cell viability in various ways. The preferred cytotoxic agents include, for example, maytansinoids and maytansinoid analogues, taxoids, CC-1065 and CC-1065 analogues, dolastatin and
5 dolastatin analogues, defined above. These cytotoxic agents are conjugated to antibodies, antibody fragments, functional equivalents, improved antibodies and analogues thereof, as described in the present application.

The conjugated antibodies may be prepared by *in vitro* methods. A linker group is
10 used to link a drug or a prodrug to the antibody. Suitable linker groups are well known to those skilled in the art and include, in particular, disulphide groups, thioether groups, labile acid groups, photolabile groups, labile peptidase groups and labile esterase groups. Preferred linker groups are disulphide groups and thioether groups. For example, a conjugate can be constructed by using a disulphide
15 exchange reaction or by forming a thioether bridge between the antibody and the drug or prodrug.

Compounds such as: methotrexate, daunorubicin, vincristine, vinblastine, melphalan, mitomycin C, chlorambucil, calicheamicin, tubulysin and tubulysin analogues,
20 duocarmycin and duocarmycin analogues, dolastatin and dolastatin analogues, are also suitable for the preparation of conjugates of the present invention. The molecules may also be linked to the antibody molecules via an intermediate molecule such as serum albumin. Doxorubicin and doxorubicin compounds, as described, for example, in patent application US 09/740991, may also be useful cytotoxic agents.

25 The antibodies which are the subject of the present invention may be combined with a cytotoxic molecule or compound. They may also be combined with an anti-angiogenic compound that acts on other angiogenic pathways.

The expression "cells expressing FGF-R4" refers to any eukaryotic cell, especially
30 mammalian cell, and in particular human cell, which expresses an FGF-R4 receptor in its native form or in a mutated form. The FGF-R4 may also be in its whole form or in a truncated form comprising, for example, the extracellular domain of FGR-R4, and in particular the D2-D3 domains. The FGF-R4 may also be recombined in a chimeric form.

The compounds of the invention may be formulated in pharmaceutical compositions for the purpose of topical, oral, parenteral, intranasal, intravenous, intramuscular, subcutaneous, intraocular, etc., administration. Preferably, the pharmaceutical compositions contain carriers that are pharmaceutically acceptable for an injectable formulation. They may in particular be sterile, isotonic, saline solutions (monosodium phosphate, disodium phosphate, sodium chloride, potassium chloride, calcium chloride or magnesium chloride etc., or mixtures of such salts), or dry, in particular lyophilized, compositions which by means of the addition, as appropriate, of sterilized water or physiological saline, can form injectable solutes.

The pathologies targeted may be all diseases related to angiogenesis, whether said angiogenesis is tumoral or non-tumoral.

The pathologies targeted may be cancer (with the use of the invention as an inhibitor of tumour angiogenesis), in particular liver cancer, colon cancer, breast cancer, lung cancer, prostate cancer, pancreatic cancer or skin cancer, or else pathologies for which a dysregulation of angiogenesis is described, such as: age-related macular degeneration or ARMD, inflammatory diseases such as rheumatoid arthritis, osteoarthritis, colitis, ulcers or any inflammatory disease of the intestines, atherosclerosis or else in the treatment of obesity.

The anti-FGF-R4 antibodies may also be used for treating cancer, in particular hepatocarcinomas and other hepatic cancers, and pancreatic cancer as an inhibitor having an action directly on tumour growth.

The present invention is illustrated, without however being limited, by the examples which follow.

Description of the figures

Figures 1A and 1B: *In vitro* (Fig. 1A), human endothelial cells of HUVEC type are capable of forming a network of pseudotubules, known as angiogenesis. This network is stimulated by the addition of 1 ng/ml of FGF2. This induction can also be obtained by adding 10 ng/ml of FGF19, which is a ligand specific for FGF-R4,

whereas 10 ng/ml of FGF4 (ligand which does not activate FGF-R4) are not capable of stimulating angiogenesis. In the same way, *in vivo* (Fig. 1B) in a murine model of induction of angiogenesis in a sponge implant on the back of the mouse, FGF2 is capable of inducing the recruitment of functional neovessels to the sponge, which is
5 characterized by an increase in the haemoglobin content of these sponges in comparison with the control. FGF19 is also capable of inducing this angiogenesis in the sponge.

Figure 2A: Map of the plasmid pXL4614 enabling the expression of hFGFR4-Histag
10 (SEQ ID No. 40).

Figure 2B: Map of the plasmid pXL4613 enabling the expression of hFGFR4-Streptag (SEQ ID No. 69).

15 **Figure 3:** Map of the plasmid pXL4615 enabling the expression of hFGFR4(D2,D3)-Histag (SEQ ID No. 42).

Figure 4: Map of the plasmid pXL4621 enabling the expression of mFGFR4-Histag (SEQ ID No. 44).
20

Figure 5: Map of the plasmid pXL4328 enabling the expression of hFGFR1-Fc (of sequence SEQ ID No. 46).

Figure 6: Map of the plasmid pXL4327 enabling the expression of hFGFR2-Fc (of
25 sequence SEQ ID No. 48).

Figure 7: ELISA plates are coated with the 4 human FGF receptors. The ability of the anti-FGFR4 antibodies 40-12 and 64-12 to recognize these various FGF-Rs is measured by ELISA assay. Clone 40-12 (black histogram) is specific for FGF-R4.
30 Clone 64-12 (grey histogram) recognizes, in addition, FGF-R3 very weakly.

Figures 8A and 8B: The active antagonist antibody 40-12 and its inactive control 64-12 have no effect per se on basal angiogenesis. On the other hand, at the dose of 30 µg/ml (approximately 200 nM), the anti-FGFR4 monoclonal antibody 40-12 is

capable of inhibiting the FGF2-induced angiogenesis of HUVEC cells, whereas the control antibody 64-12 is not capable of doing so (Fig. 8A).

FGF2 labelled with an AlexaFluor® 488 nm is capable of binding to the FGF-R4 expressed by 300-19 cells (white histogram). This interaction can be dissociated by
5 unlabelled FGF2 (black histograms) or by the anti-FGFR4 antibody 40-12 (dark grey histograms), whereas the control antibody 64-12 is not capable of doing so (light grey histograms) (Fig. 8B).

Figure 8C and 8D: Effect of the anti-FGFR4 antibodies derived from clones 8, 31,
10 33 and 36, at 10 µg/ml, on the *in vitro* angiogenesis induced by 3 ng/ml of FGF-2. Clones 8 (Fig. 8C) and 31, 33 and 36 (Fig. 8D) inhibit the FGF-2-induced angiogenesis of HUVEC cells.

Figures 9A and 9B: Hep3b human hepatocarcinoma cells are stimulated with
15 FGF19 at 30 ng/ml. This stimulation induces FGF-R4-specific cell signalling, resulting in synthesis of the cFos and JunB proteins and in the phosphorylation of Erk1/2, observed by Western blotting (Fig. 9A). Each band is then quantified. This quantification is represented in the form of a histogram (Fig. 9B). The antibody 40-12 at 100 µg/ml completely inhibits the induction of the FGF-R4-specific cell signalling,
20 whereas the control antibody has no effect. Specifically, the antibody 40-12 completely blocks the synthesis of JunB and of cFos and also the phosphorylation of Erk1/2 induced by FGF19 after a stimulation of 3 h.

Figure 9C: The inhibitory effect of the anti-FGFR4 antibody 40-12 on the
25 phosphorylation of Erk1/2 induced in the Hep3b cells by FGF-19 (30 ng/ml) is confirmed by means of the anti-phosphoErk1/2 ELISA. The antibody 40-12 is also capable of inhibiting the phosphorylation of Erk1/2 in Hep3b cells stimulated with FGF-2 (1 ng/ml) or with serum (FCS) at 10%.

30 **Figure 9D:** Percentage inhibition of Erk1/2 phosphorylation induced by FGF-19 (30 ng/ml) obtained by ELISA on Hep3b cells using the antibodies derived from clones 8, 31, 33 and 36.

Figures 10A and 10B: The proliferation of Hep3b cells can be stimulated by adding
35 serum (Fig. 10A) or FGF19 (Fig. 10B). The induction with serum is partially inhibited

by the antibody 40-12 at 100 µg/ml, whereas the control antibody has no effect (Fig. 10A). The proliferation induced by FGF19 is completely blocked with 10 µg/ml of anti-FGFR4 antibody 40-12, whereas at the same dose, the control antibody is not capable of doing so (Fig. 10B).

5

Figures 11A to 11D: The anti-FGFR4 antibody 40-12 is capable of reducing the development of pancreatic tumors in a RipTag murine model by inhibiting tumour angiogenesis: The Rip1-Tag2 model is a murine model in which transgenic mice expressing the SV40 T antigen in the insulin-producing β cells of the pancreatic islets (Hanahan D. Nature, 1985, 9-15:115-22.). This T antigen is expressed during embryonic development of the pancreas up to 4 to 5 weeks of life, without apparent effect. Some of the pancreatic islets expressing the T antigen then progress, during the next 5 weeks, towards the formation of angiogenic islets associated with activation of the vasculature and then towards the development of small tumours of adenoma type. A few weeks later, some adenomas develop, so as to form invasive carcinomas (Fig.11A).

These Rip1-Tag2 mice are treated subcutaneously once a week, between weeks 10 and 13, at the dose of 25 mg/kg, with the antibody 40-12 or with the control antibody. After 13 weeks, the mice are sacrificed. The tumour volume and also the number of tumours per pancreas and the vascular density are determined. The treatment with the anti-FGFR4 antibody 40-12 makes it possible to significantly reduce the tumour volume by 55%, whereas the control antibody shows no effect (Fig. 11B). The antibody 40-12 also makes it possible to reduce by 34% the number of tumours per pancreas, compared with the control (Fig. 11C). This reduction in tumour volume is accompanied by a reduction in the vascular density, whether in terms of the number of small, medium or large vessels (Fig. 11D).

Figure 12: The ability of the antibodies 40-12 and 64-12 to recognize the complete extracellular domain of human or murine FGF-R4, and also the extracellular domain of FGF-R4 deleted of its D1 domain, is measured by ELISA. Clone 40-12 is capable of binding equally with the 3 FGF-R4 constructs, whereas clone 64-12 recognizes the murine form of FGF-R4 less well.

Figures 13A to 13C: The antagonistic effect of the anti-FGFR4 antibody clone 40-12

on FGF2/FGF-R4 binding is measured by means of competition binding experiments with FGF2 labelled with an AlexaFluor® 488 nm. Clone 40-12 is capable of blocking the binding of human (Fig. 13A), murine (Fig. 13B) and rat (Fig. 13C) FGF2 on murine 300-19 cells transfected with cDNA encoding the human, murine or rat forms of the FGF-R4 receptor, with the same effectiveness (3500, 4110 and 3940 ng/ml, i.e. 23, 27 and 26 nM for the human, murine or rat complexes, respectively).

Figure 14A: Map of the plasmid pXL4794 enabling the expression of hFGFR4_D1: Fc.

10

Figure 14B: Amino acid sequence of the hFGFR4_D1: Fc protein secreted in the HEK293 line transfected with the plasmid pXL4794.

Figure 15A: Map of the plasmid pXL4796 enabling the expression of hFGFR4_D2: Fc.

15

Figure 15B: Amino acid sequence of the hFGFR4_D2: Fc protein secreted in the HEK293 line transfected with the plasmid pXL4796.

Figure 16A: Map of the plasmid pXL4799 enabling the expression of hFGFR4_D3: Fc.

20

Figure 16B: Amino acid sequence of the hFGFR4_D3: Fc protein secreted in the HEK293 line transfected with the plasmid pXL4799.

25

EXAMPLES:

Example 1: Demonstration of the role of FGF-R4 in the control of angiogenesis

In order to demonstrate the role of FGF-R4 in the control of angiogenesis, *in vitro* angiogenesis experiments were carried out with human endothelial cells of HUVEC type stimulated with several FGFs: FGF2, a ligand which activates most of the FGF receptors; FGF19, a ligand which specifically activates FGF-R4; and FGF4, a ligand which does not activate FGF-R4.

30

To do this, gels were prepared by distributing, into each well of a chamberslide

(Biocoat Cellware collagen, type I, 8-well culture slides: Becton dickinson 354630), 160 µl of matrigel diluted to 1/6 (Growth factor reduced Matrigel: Becton dickinson 356230) in collagen (rat Tail collagen, type I: Becton dickinson 354236). The gels are maintained at 37°C for 1 hour so as to enable them to polymerize. Next, the human

5 vein endothelial cells (HUVEC ref: C-12200 – Promocell) were seeded at 15×10^3 cells/well in 400 µl of EBM medium (Clonetics C3121) + 2% FBS + 10 µg/ml of hEGF. This protocol can be adapted to 96-well plates: 60 µl per well of 96-well plates (Biocoat collagenI cellware, Becton Dickinson 354407). The matrix is prepared by mixing 1/3 of matrigel, 1 mg/ml final concentration of collagen, NaOH

10 (0.026x the volume of collagen in µl), 1x PBS, the volume then being adjusted with water. The endothelial cells are stimulated with 1 ng/ml of FGF2 (R&D, 133-FB-025) or 10 ng/ml of FGF4 (R&D, 235-F4-025) or of FGF19 (R&D, 969-FG-025) for 24 h at 37°C in the presence of 5% CO₂. After 24 hours, the length of the network of microtubules formed is measured using a computer-assisted image analysis system

15 (Imagenia Biocom, Courtaboeuf, France) and the total length of the pseudotubules in each well is determined. The mean of the total length of the microcapillary network is calculated in µm for each condition corresponding to the mean of 6 replicates.

The stimulation with FGF2 or FGF19 enables the induction of the formation of new tubules, whereas FGF4 has no effect (Fig. 1A). These results show that the specific

20 activation of FGF-R4 makes it possible to induce the formation of neovessels and therefore to conclude that FGF-R4 controls angiogenesis *in vitro*.

The *in vivo* correlation with the *in vitro* data was obtained using the model of angiogenesis induction *in vivo* in cellulose implants in mice. This model is an

25 adaptation of the model described by Andrade and *al.* (*Microvascular Research*, 1997, 54, 253-61).

The animals, inbred white BALB/c J mice, are anaesthetized with a xylazine (Rompun®, 10mg/kg)/ketamine (Imalgene 1000, 100 mg/kg) mixture given intraperitoneally. The back of the animal is shaved and disinfected with

30 Hexomedine®. A pocket of air is created subcutaneously on the back of the mouse by injection of 5 ml of sterile air. An incision of approximately 2 cm is made on the upper back of the animal in order to introduce a sterile cellulose implant (disc 1 cm in diameter, 2 mm thick, Cellspan® ref 0501) impregnated with 50 µl of sterile solution containing the protein or the product to be tested. The incision is then sutured and

cleaned with Hexomedine®. During the days following the implantation of the implant, the mice received, in the implant, the protein or the product by means of an injection through the skin (50 µl/implant/day) under gas anaesthesia (5% isoflurane (Aerrane®, Baxter)).

- 5 Seven days after the implantation of the sponge, the mice are sacrificed by means of a lethal dose of sodium pentobarbital (CEVA santé animale), administered intraperitoneally. The skin is then cut, approximately 1 cm around the sponge, avoiding the scar, in order to free the skin and the sponge. The sponge is then cut up into several pieces and placed in a Ribolyser® tube containing 1 ml of lysis buffer
10 (Cell Death Detection ELISA, Roche). The tubes are shaken 4 consecutive times, for 20 sec, force 4, in a cell homogenizer (FastPrep® FP 120). The tubes are then centrifuged for 10 min at 2000 g at 20°C and the supernatants are frozen at -20°C while awaiting the haemoglobin assay.

- On the day of the assay, the tubes are again centrifuged after thawing, and the
15 haemoglobin concentration is measured with the Drabkin reagent (Sigma, volume for volume) by reading on a spectrophotometer at 405 nm against a standard range of bovine haemoglobin (Sigma). The haemoglobin concentration in each sample is expressed in mg/ml according to the polynomial regression performed on the basis of the standard range. The results are expressed in mean value ($\pm$ sem) for each
20 group. The differences between the groups are tested with an ANOVA followed by a Dunnett's test on the square root of the values.

- In this model, FGF19 at 50 ng per site and 5 re-injections is capable of significantly inducing colonization of the sponge by newly formed mature vessels, with the same effectiveness as FGF2 at 5 ng per site. The presence of functional blood vessels in
25 the sponge is demonstrated by the presence of haemoglobin (Fig. 1B). These results show that the specific activation of FGF-R4 enables the recruitment of functional blood vessels, indicating that FGF-R4 also controls angiogenesis *in vivo*.

Example 2: Description of the FGFR proteins used as immunogen and antigen

- 30 FGFR growth factor receptors, and in particular the extracellular domains of FGF-R1, FGF-R2, FGF-R3, FGF-R4, are fused to a tag (Histag) or to the immunoglobulin Fc domain.

The cDNA encoding the extracellular domain of human FGF-R4 corresponds to the protein described in SwissProt FGF-R4_HUMAN position 1-365 with the L136P

mutation. It was cloned into the eukaryotic expression vector pXL4614 represented in Figure 2, in order to express a protein containing a Histag in the C-terminal position in the extracellular domain.

The proteins named hFGFR4-Histag, of sequence SEQ ID No. 40, were produced by
5 transient transfection in the HEK293 EBNA line (Invitrogen) using the plasmid pXL4614 and the helper plasmids pXL4544 and pXL4551 which enable the expression of two N-glycan glycosylation enzymes, i.e. α -2,3-sialyltransferase and β -1,4-galactosyltransferase, as described in application WO2008/065543.

10 The hFGFR4-Histag protein expressed in the HEK293 EBNA cell culture supernatant was purified by chromatography on an Ni-chelating sepharose column (Amersham Biosciences; ref. 17-0575-01), elution being carried out in an imidazole buffer, and then formulated in PBS buffer (Invitrogen; ref. 14190-094). The analysis of the monosaccharide composition and the quantification of the sialic acids of the
15 N-glycans, as described by Saddic and *al.* 2002. (Methods Mol. Biol. 194:23-36 and Anumula and *al.* 1998. Glycobiology 8:685-694), made it possible to demonstrate that the protein was very highly sialylated (91%). Consequently, the protein had all the characteristics for having sufficient pharmacokinetic properties.

In a comparable manner, the hFGFR4-Streptag protein (SEQ ID No. 69) was purified
20 by chromatography on a Strep-Tactin Superflow column (IBA; ref. 2-1206), elution being carried out in a desthiobiotin buffer, and then formulated in PBS buffer.

The hFGFR4(D2,D3)-Histag protein corresponds to the sequence SEQ ID No. 42. The cDNA was cloned into the eukaryotic expression plasmid pXL4615 represented
25 in Figure 3, and the protein was produced and purified under conditions comparable to the hFGFR4-Histag protein.

The cDNA encoding the mFGFR4-Histag protein (SEQ ID No. 43) was cloned into the eukaryotic expression plasmid pXL4621 represented in Figure 4, and the protein was produced and purified under conditions comparable to the hFGFR4-Histag
30 protein.

The hFGFR1-Fc protein (SEQ ID No. 46) contains the extracellular domain of human FGF-R1 IIIc fused, in the C-terminal position, to the Fc domain of human IgG1. The cDNA was cloned into the eukaryotic expression plasmid pXL4728 represented in

Figure 5, and the protein was produced under conditions comparable to the hFGFR4-Histag protein and then purified by chromatography on a protein G Sepharose affinity column (Amersham Biosciences), elution being carried out in 100 mM glycine/HCl buffer, pH 2.7, and then formulated in PBS buffer.

5 The hFGFR2-Fc protein, of sequence SEQ ID No. 48, contains the extracellular domain of human FGF-R2 IIIc fused, in the C-terminal position, to the Fc domain of human IgG1. The cDNA was cloned into the eukaryotic expression plasmid pXL4327 represented in Figure 6, and the protein was produced and then purified under conditions comparable to the hFGFR1-Fc protein.

10 The hFGFR3-Fc protein is a protein which fuses the extracellular domain of human FGF-R3 IIIc to the Fc domain of human IgG1, and was obtained from R&D Systems (ref: 760-FR).

The hFGFR4-Fc protein is a protein which fuses the extracellular domain of human FGF-R4 to the Fc domain of human IgG1, and was obtained from R&D Systems

15 (ref: 685-FR).

Various subdomains of the extracellular portion of the hFGFR4 protein were fused to the Fc domain of human IgG1. The D1 subdomain is contained in the construct SABVA4794 (SEQ ID No. 112 and Figs 14A and 14B). The D2 subdomain is
20 contained in the construct SABVA4796 (SEQ ID No. 114 and Figs 15A and 15B). The D3 subdomain is contained in the construct SABVA4799 (SEQ ID No. 116 and Figs 16A and 16B). These three subdomains extend respectively from positions 1 to 179 for SABVA4794, 1 to 32 plus 145 to 242 for SABVA4796, and 1 to 32 plus 228 to 360 for SABVA4799 (positions described in SwissProt FGF-R4_HUMAN). These
25 were produced using the plasmids pXL4794 (coding sequence SEQ ID No. 111), pXL4796 (coding sequence SEQ ID No. 113) and pXL4799 (coding sequence SEQ ID No. 115) under conditions comparable to the FGFR1-Fc protein.

Example 3: Generation and screening of anti-FGF-R4 monoclonal antibodies

30

A – Antibodies obtained by immunization

The monoclonal antibodies were obtained by immunization with the hFGFR4-Histag immunogen in five BALB/cJ mice (Charles River), 6 to 8 weeks old, each immunized with a total of 24 µg of hFGFR4-Histag by the RIMMS method described by Kilpatrick

and *al.* (1997. Hybridoma 16: 381389) and the fusion protocol described in ClonaCell™- HY Hybridoma Cloning Kit (StemCell Technologies; ref 03800).

- Two days after the final injection, the mice were sacrificed and the lymph nodes were
- 5 fused with P3X63-AG8.653 myeloma cells (ATCC, CRL-1580) in a 5:1 ratio in the presence of polyethylene glycol (ClonaCell™-HY ref. 03806). The cell suspension was distributed aseptically into Petri dishes incubated at 37°C in the presence of 5% CO₂. The colonies that had appeared after 12 days of incubation were isolated and cultured in medium E (ClonaCell™-HY; ref. 03805) in 96-well plates.
- 10 The primary screening of monoclonal antibodies obtained by immunization with hFGFR4-Histag was carried out by ELISA assay using hFGFR4–Streptag as capture antigen. The capture antigen was bound to Immulon-4 enzyme-linked plates (VWR Scientific Inc. Swedesboro, NJ). The hybridoma culture supernatants were subsequently added and then detection was carried out using the peroxidase-
- 15 conjugated anti-mouse IgG rabbit antibody (Sigma; ref. A9044-dilution to 1:50 000). The revealing was carried out with the TMB-H₂O₂ substrate (Interchim; ref UP664780) and the measurements were carried out with the plate reader at 450 nm. Among the 444 hybridomas tested, 129 were positive by ELISA assay with the hFGFR4-Streptag antigen, and 120 of these hybridomas were also positive with the
- 20 hFGFR4-Fc dimer protein.

- A secondary screen was carried out, in order to select only the FGF-R4-specific antibodies, by ELISA assay using as capture antigen the hFGFR4-Streptag protein, and then with the hFGFR1-Fc, hFGFR2-Fc and hFGFR3-Fc proteins described in
- 25 Example 2. The capture antigen was bound to Immulon-4 enzyme-linked plates (VWR Scientific Inc. Swedesboro, NJ). The hybridoma culture supernatants were subsequently added and then detection was carried out using the peroxidase-conjugated anti-mouse IgG rabbit antibody (Sigma; ref. A9044-dilution to 1:50 000). The revealing was carried out with the TMB-H₂O₂ substrate (Interchim; ref
- 30 UP664780) and the measurements were carried out with the plate reader at 450 nm. Among the 129 hybridomas that tested positive by ELISA assay with the antigen, 84 hybridomas were positive with hFGFR4-Streptag and had no affinity for either hFGFR1-Fc, hFGFR2-Fc or hFGFR3-Fc. 39 hybridomas were conserved, as a function of their growth and their morphology. Their isotype was determined using

the SEROTEC kit (ref. MMT1); 95% were IgG1s.

A tertiary screen was carried out on a test for FGF2-induced proliferation of Baf/3 modified cells, in order to characterize the inhibition by the anti-FGFR4 antibodies.

- 5 The murine hybridomas expressing the anti-FGFR4 antagonist antibodies were cloned by limiting dilution. Using the hybridoma cells cultured in the exponential phase, the coding sequence (cDNA) was determined after extraction of the mRNA using the Oligotex kit (Qiagen; ref 72022); production and amplification of the cDNA by the RACE-RT method with the Gene Racer kit, the SuperScript III reverse
10 transcriptase (Invitrogen; ref L1500) and the primers described in Table 2 below; amplification of the cDNA fragments using the Phusion polymerase (Finnzymes; ref. F-530S), the primers and the temperature conditions described in Table 2. The amplified fragments containing the coding regions for VH (variable region of the heavy chain HC) or VL (variable region of the light chain LC) were cloned into the
15 pGEM-T Easy vector from Promega; ref A137A, and the inserts of the plasmids obtained were sequenced, such that the coding sequence of each variable domain was analysed in the 5'-3' and 3'-5' direction on at least 6 plasmids corresponding to the anti-FGFR4 antibody 40-12 and the anti-FGFR4 antibody 64-12. The analyses of sequence, contigs and alignments were carried out using the software available on
20 Vector NTI (Invitrogen).

The plasmids containing the consensus sequences encoding the variable regions of the anti-FGFR4 antibodies were conserved. The plasmid pXL4691 contains the sequence encoding the VH of sequence SEQ ID No. 5 of the anti-FGFR4 antibody
25 40-12, and the plasmid pXL4693 contains the sequence encoding the VH of sequence SEQ ID No. 19 of the anti-FGFR4 antibody 64-12, as shown in Table 2 below.

The plasmid pXL4690 contains the nucleotide sequence SEQ ID No. 7 encoding the VL of sequence SEQ ID No. 8 of the anti-FGFR4 antibody 40-12, and the plasmid
30 pXL4692 contains the nucleotide sequence SEQ ID No. 21 encoding the VL of sequence SEQ ID No. 22 of the anti-FGFR4 antibody 64-12, as shown in Table 2 below.

Table 2 – Operating conditions and analysis of the reverse transcriptase and PCR

reactions – identification of the plasmids pXL4690 to pXL4693.

Anti-FGFR4	40-12	64-12
RT reaction Temperature Primers	55°C SEQ ID No. 64 (HC) SEQ ID No. 65 (LC)	55°C SEQ ID No. 64 (HC) SEQ ID No. 66 & SEQ ID No. 67 (LC)
5' Primer-GeneRacer (SEQ ID No. 63)	5'-CGACTGGAGCACGAGGACACTGA-3'	
3' Primer-internal to murine hinge (SEQ ID No. 64)	5'-TATGCAAGGCTTACAACCACA-3'	
3' Primer-internal to murine C κ (SEQ ID No. 65)	5'-CTCATTCTGTT GAAGCTCTT GAC-3'	
3' Primers-internal to murine C λ (SEQ ID No. 66 and SEQ ID No. 67)		5'- ACACTCAGCACGGG ACAAACTCTTCTC-3' 5'- ACACTCTGCAGGAG ACAGACTCTTTTC-3'
HC PCR: Temperature Primers	55°C SEQ ID No. 63 - SEQ ID No. 64	55°C SEQ ID No. 63 – SEQ ID No. 64
Sequence of the cloned PCR products (VH)	9 clones have the same VH sequence and a CH1 sequence identical to that of mCH1-	6 clones have the same VH sequence and a CH1 sequence identical to that of mCH1
Plasmid containing VH	pXL4691	pXL4693
LC PCR: Temperature Primers	55°C SEQ ID No. 63 – SEQ ID No. 65	55°C SEQ ID No. 63 – SEQ ID No. 66 & 67
Sequence of the cloned PCR products (VL)	11 clones have the same VL sequence and a C κ sequence identical to mC κ	6 clones have the same VL sequence and a C λ sequence identical to mC λ
Plasmid containing VL	pXL4690	pXL4692

The amino acid sequences of the light and heavy variable regions, respectively, of the anti-FGFR4 antibody 64-12 and the anti-FGFR4 antibody 40-12 are different. The numbers of the sequences used, obtained and deduced are indicated in Table 5 7.

The antibodies 40-12 and 64-12 were produced in T500 flasks. The culture supernatant was harvested after 7 days. The anti-FGFR4 antibodies were affinity-purified on protein G and then dialysed against PBS, filtered sterile, and stored at 10 4°C.

The purified antagonist antibodies have a K_D of 6.5×10^{-9} M (anti-FGFR4 40-12) and 5.75×10^{-8} M (anti-FGFR4 64-12).

B – Antibody selected using the phage display method

15 The primary screening of monoclonal antibodies obtained by phage display with hFGFR4-Histag was carried out by ELISA assay using hFGFR4-Histag as capture antigen. The capture antigen was bound to Immulon-2 enzyme-linked plates (VWR Scientific Inc. Swedesboro, NJ). The culture supernatants from *E. coli* infected with the phages were subsequently added and then detection was carried out using the 20 peroxidase-conjugated anti-M13 mouse antibody (GE Healthcare; ref. 27-9421-01, dilution to 1:5000). The revealing was carried out with the TMB-H₂O₂ substrate (Interchim; ref UP664780) and the optical density (O.D.) measurements were carried out at 450 nm.

A secondary screen was carried out in order to select only the FGF-R4-specific 25 antibodies, by ELISA assay using as capture antigen the hFGFR4-Histag protein, and then with the hFGFR1-Fc, hFGFR2-Fc and hFGFR3-Fc proteins described in Example 2. The capture antigen was bound to Immulon-2 enzyme-linked plates (VWR Scientific Inc. Swedesboro, NJ). The culture supernatants from *E. coli* infected with the phages were subsequently added and then detection was carried out using 30 the peroxidase-conjugated anti-M13 mouse antibody (GE Healthcare; ref. 27-9421-01, dilution to 1:5000). The revealing was carried out with the TMB-H₂O₂ substrate (Interchim; ref UP664780) and the optical density (O.D.) measurements were carried out at 450 nm.

The FGF-R4-specific clones selected were sequenced and recloned into an

expression vector for transient transfection of HEK293 cells.

For this, in a first step, the regions encoding Fab, i.e. the light chain of the antibody, a bacterial ribosome binding site, and the heavy chain variable region of the antibody, are extracted from the phagemid by restriction and inserted into an IgG
5 expression plasmid for mammalian cells, downstream of a eukaryotic antibody signal sequence and upstream of the constant region of a human IgG1 heavy chain. In a second step, the region containing the bacterial ribosome binding site and the bacterial signal peptide for the heavy chain is exchanged against an IRES sequence and a eukaryotic signal sequence. The IgGs are expressed by transient transfection
10 of HEK293 cells. This process is described in detail in *T. Jostock et al., Journal of Immunological Methods* 289 (2004) 65–80.

An example of a human IgG1 constant region sequence that can be used in the present invention is the sequence SEQ ID No. 117.

15 A tertiary screen was carried out on a test for FGF2-induced proliferation of modified Baf/3 cells, described in Example 4, in order to characterize the inhibition by the anti-FGFR4 antibodies, using the culture supernatants from HEK293 cells transiently transfected with the antibody expression vectors. This screen made it possible to identify the antibodies of clones 8, 31, 33 and 36. The corresponding sequences are
20 described in Table 7.

Example 4: Establishment of the BaF/3 FGF-R4-hMpl murine clonal line and cell proliferation protocol

25 The extracellular and transmembrane domain of FGF-R4 was cloned, as a translational fusion with the intracellular domain of hMpl, into a mutated pEF6/V5-His A vector in order to obtain the presence, in the 5' position, of the HA tag in front of the chimeric FGF-R4-hMpl receptor.

30 Construction of the mutated pEF6A

The pEF6/V5-His A vector (Invitrogen, reference V961-20) was improved in order to integrate the MCS (multi cloning site) associated with the HA tag placed under the signal peptide of IgGk of pDisplay (Invitrogen, reference V660-20). To do this, the MCS associated with the HA tag and with its signal peptide were amplified by PCR

between the sense primer of sequence SEQ ID No. 51 and the reverse primer of sequence SEQ ID No. 52, making it possible to insert the KpnI restriction site in the 5' position and the XbaI restriction site in the 3' position. The PCR fragment was digested with the KpnI and XbaI enzymes and then cloned into the pEF6/V5-His A vector opened with the same enzymes. Finally, the first BamHI site of the MCS was replaced with the BsrGI site by digesting the newly formed vector with the KpnI and SpeI enzymes and inserting, between these sites, the primers of sequences SEQ ID No. 53 and SEQ ID No. 54, hybridized to one another and containing the BsrGI enzyme site. The vector obtained was called: pEF6mut-HA.

Construction of the pEF6mut-HA-FGF-R4-hMpl vector

The intracellular domain of Mpl was amplified in a vector pEF6/V5-His TOPO (Invitrogen, reference K9610-20) between the sense primer of sequence SEQ ID No. 55 allowing the insertion of the SacI digestion site and the reverse primer of sequence SEQ ID No. 56, commonly called revBGH. The PCR fragments generated were then digested with the SacI and NotI enzymes.

The extracellular and transmembrane domain of FGF-R4 was amplified using the pair of primers (sense: sequence SEQ ID No. 57; reverse: sequence SEQ ID No. 58). These primers make it possible to insert the BamHI enzymatic site in the 5' position and the SacI enzymatic site in the 3' position. The PCR fragment obtained was then digested with the BamHI and SacI enzymes.

The amplifications of DNA encoding hMpl and encoding FGF-R4 were then cloned simultaneously into the pEF6mut-HA vector opened with BamHI-NotI. The resulting construct was called "pEF6mut-HA FGF-R4 α IIIc-hMpl2". This construct was then improved by site-directed mutagenesis in order to change the transmembrane domain of FGF-R4 (protein sequence SEQ ID No. 59) for the sequence SEQ ID No. 60. To do this, the "QuickChange® Site-directed mutagenesis kit" (Clontech, reference 200518) was used with the sense primer of sequence SEQ ID No. 61 and the reverse primer of sequence SEQ ID No. 62. The new chimeric construct obtained was called pEF6mut-HA FGF-R4 α IIIcmut-hMpl2.

Creation of a stable line by transfection of the BaF/3 murine line with the FGF-R4 α IIIcmut-hMpl2 construct:

The "pEF6mut-HA FGF-R4 α IIIcmut-hMpl2" construct was stably introduced, by electroporation, into the genome of the BaF/3 murine cells. The line obtained was selected in the presence of FGF2 at 20 ng/ml (R&D, reference 234-FSE-025) and of heparin at 100 ng/ml (Sigma, reference H3149). The transfected and selected line is then of clonal type.

Cell proliferation protocol with the BaF/3 FGFR4-hMpl cell line:

The BaF/3 FGFR4-hMpl cells were cultured and maintained in complete RPMI 1640 medium (Invitrogen; ref. 32404-014) (10% FCS (Hyclone; ref. SH30070.03), 2 mM glutamine, 1x MEM non essential amino acid (Gibco, ref 11140-035), 1x MEM sodium pyruvate (Gibco, ref 11360-039), supplemented with FGF2 at 20 ng/ml (R&D Systems, ref 234-FSE) and heparin at 3 ng/ml (Sigma, ref H3149). On day one, the cells are seeded at 0.4×10^6 cells/ml in complete RPMI 1640 medium supplemented with FGF2 at 20 ng/ml and with heparin at 3 ng/ml. The following day, 50 μ l of BaF/3 FGFR4-hMpl cell suspension in complete RPMI 1640 medium supplemented with FGF2 at 20 ng/ml and with heparin at 3 ng/ml, at 0.2×10^6 cells/ml, were dispensed into 96-well plates (Corning, ref 214006), followed by 50 μ l of hybridoma supernatant containing the antibody to be tested. The plates were then placed at 37°C, 5% CO₂ for 24 to 30 h. For reading the cell proliferation, the amount of ATP was quantified by adding 100 μ l of Cell Titer Glo Luminescent Cell Viability Assay (Promega, ref G7571) and the luminescence was read using a luminometer.

The clones exhibiting, in this test, a signal 50% weaker than that of the complete RPMI 1640 medium containing the additives FGF2 at 20 ng/ml and heparin at 3 ng/ml were selected.

When the anti-FGFR4 antibodies, specific for FGF-R4, were incubated with the BaF/3-FGFR4-hMpl cells and with the following additives FGF2 at 20 ng/ml and heparin at 3 ng/ml, an antagonist effect was observed on cell proliferation. Among the 39 hybridomas tested, 14 are capable of inhibiting FGF-induced cell proliferation of BaF/3-FGFR4-hMpl cells in the presence of FGF2.

It was shown by ELISA (Fig. 12) that the anti-FGFR4 antagonist antibodies 40-12 and 64-12 had affinity for the murine protein mFGFR4 and human protein hFGFR4 (D2, D3).

Finally, a last screen was carried out by Surface Plasmon Resonance (BIAcore 2000) in order to determine the affinity constants of the anti-FGFR4 antibodies. The interaction between the FGF-R4 protein and the anti-FGFR4 antibody present in the hybridoma culture supernatant was analysed after having bound the anti-FGFR4 antibody to an anti-Fc antibody, itself bound to the CM chip. The kinetics are measured according to the protocol of Canziani et al 2004. Anal. Biochem. 325: 301-307.

Two antibodies among those having affinity constants of 10^{-8} to 10^{-9} M and dissociation rates of 10^{-3} to 10^{-5} s $^{-1}$ were selected. The characteristics of these antibodies are described in Table 1 below.

The reference method used to determine the K_D is Surface Plasmon Resonance (BIAcore).

Table 1A: Affinity and association/dissociation constants of the purified anti-FGFR4 antagonist antibodies 40-12 and 64-12

Anti-FGFR4	k_{on} (M $^{-1}$.s $^{-1}$)	k_{off} (s $^{-1}$)	K_D (M)
40-12	1.94×10^5	1.26×10^{-3}	6.50×10^{-9}
64-12	1.05×10^4	6.02×10^{-4}	5.75×10^{-8}

Table 1B: Kinetic parameters for binding of the anti-FGFR4 antibodies 8, 31, 33 and 36, measured by Surface Plasmon Resonance (BIAcore 3000):

	on h-FGFR4-Histag			on m-FGFR4-Histag		
	K_{on} (M $^{-1}$ s $^{-1}$)	K_{off} (s $^{-1}$)	K_D (M)	K_{on} (M $^{-1}$ s $^{-1}$)	K_{off} (s $^{-1}$)	K_D (M)
Clone 8	8.93×10^5	2.92×10^{-4}	3.27×10^{-10}	1.20×10^6	1.36×10^{-4}	1.15×10^{-10}
Clone 31	6.48×10^5	9.80×10^{-4}	1.52×10^{-9}	9.14×10^5	1.80×10^{-3}	1.97×10^{-9}
Clone 33	8.05×10^5	6.17×10^{-4}	7.71×10^{-10}	1.01×10^6	7.04×10^{-4}	6.92×10^{-10}
Clone 36	2.44×10^5	6.31×10^{-4}	2.62×10^{-9}	3.30×10^5	7.52×10^{-4}	2.28×10^{-9}

Example 5: Specificity of the anti-FGFR4 antibodies

A- Specificity of the anti-FGFR4 antibody 40-12 for FGF-R4

The specificity of each antibody is established by ELISA according to the protocol described in Example 4. In this way, the ability of each antibody to bind to each

5 FGF-R is observed. This experiment clearly shows that the antibody 40-12 recognizes only FGF-R4 and is therefore specific for FGF-R4. The antibody 64-12 is capable of binding mainly to FGF-R4, but also weakly to FGF-R3 (Fig. 7).

B – Specificity of the anti-FGFR4 antibodies 8, 31, 33 and 36 for FGF-R4

- 10 The specificity of each antibody is established by ELISA according to the following protocol: suspensions of phages displaying, at their surface, the antibodies in the Fab format are generated by infection of *E. coli* bacteria. The capture antigens were bound to Immulon-2 enzyme-linked dishes (VWR Scientific Inc. Swedesboro, NJ). The phage suspensions were subsequently added and then
- 15 detection was carried out using the peroxidase-conjugated anti-M13 phage mouse antibody (GE Healthcare, ref. 27-9421-01, dilution to 1:5000). The revealing was carried out with the TMB-H₂O₂ substrate (Interchim; ref UP664780) and the optical density (OD) measurements were carried out at 450 nm. Table 2 summarizes the results obtained:
- 20 In this way, the ability of each antibody to bind to each FGF-R is observed. This experiment clearly shows that the antibodies 8, 31, 33, 36 recognize only FGF-R4 and are therefore specific for FGF-R4.

Table 2: Specificity of the anti-FGF-R4 antibodies derived from clones 8, 31, 33 and

25 36, established by ELISA (Signal = OD, 450 nm)

	h-FGFR4(D2,D3)-histag	h-FGFR1-Fc	h-FGFR2-Fc	h-FGFR3-Fc
Clone 8	2.55	0.05	0.00	0.00
Clone 31	2.52	0.00	0.00	0.01
Clone 33	2.73	0.00	0.00	-0.01
Clone 36	1.87	-0.01	-0.01	-0.02

Example 6: Antagonistic effect of the antibody 40-12 and of the antibodies

30 derived from clones 8, 31, 33, 36, on angiogenesis (*in vitro*)

In order to determine the biological activity of the anti-FGFR4 monoclonal antibody 40-12 over the course of human endothelial cell angiogenesis, *in vitro* angiogenesis experiments were carried out using HUVEC cells stimulated with FGF2 in the presence of the antibody 40-12 or of the control antibody at increasing doses of 1 to 30 µg/ml (Figs. 8A and 8B).

In this context, the active anti-FGFR4 antagonist monoclonal antibody 40-12 is capable of inhibiting the FGF2-induced angiogenesis of HUVEC cells, at the dose of 30 µg/ml or 200 nM, whereas the control antibody 64-12 has no effect. Furthermore, the antibody 40-12 has no effect per se on the basal angiogenesis.

These results indicate that an FGF-R4-specific antagonist antibody is capable of inhibiting angiogenesis.

As for the antibody 40-12, the anti-FGFR4 antibodies of clones 8, 31, 33 and 36, derived from the phage display, were evaluated with regard to their ability to inhibit the FGF-2-induced angiogenesis of human endothelial cells of HUVEC type. These 4 antibodies block the *in vitro* stimulation of angiogenesis obtained with FGF-2, said antibodies being at the dose of 10 µg/ml (Figs. 8C and 8D).

Example 7: Antagonistic effect of the antibody 40-12 and of the antibodies derived from clones 8, 31, 33 and 36, on human hepatocarcinoma cells (*in vitro*)

In order to determine the antitumour effect of the anti-FGFR4 antagonist monoclonal antibody 40-12, experiments were carried out on Hep3b human hepatocarcinoma cells in which the proliferation and the signalling pathways resulting therein are dependent on the ligand-receptor pair: FGF19/FGF-R4.

Firstly, the study of the FGF-R4-dependent signalling pathways resulting in the proliferation of Hep3b cells was undertaken by Western blotting. This cell signalling involves the neosynthesis of the cFos and JunB proteins and also the phosphorylation of Erk1/2 (Lin and al., *J Biol Chem.*, 2007, 14:27277-84). To do this, 5×10⁵ cells are seeded into dishes 35 mm in diameter, in 2 ml of complete medium (DMEM, 10% FCS, 2 mM glutamine). 24 h later, the cells are subjected to conditions of deficiency for 24 h in 1.8 ml of serum-free medium. The cells are then stimulated

for 3 h with 200 µl of 10-times concentrated FGF19, in the absence or in the presence of control anti-FGFR4 antibody or anti-FGFR4 antibody 40-12. The medium is then removed, and the cells are washed once with cold PBS and lysed on the dish for 30 min at 4°C with 75 µl of RIPA buffer supplemented with protease inhibitor. The total protein extract is then centrifuged for 10 min at 4°C at 13 000 rpm and the supernatant is analysed by the Western blotting technique. The membranes are then incubated for 2 h at ambient temperature in TBS, 0.05% tween, 5% milk and then the anti-cFos (Cell Signaling Technology, ref 2250), anti-JunB (Cell Signaling Technology, ref 3746) and anti-phosphoErk1/2 (Cell Signaling Technology, ref 4377) primary antibodies are added at 1/1000th and incubated overnight at 4°C with slow shaking. The membrane is rinsed three times with TBS, 0.05% tween, and the secondary antibody coupled to HRP is incubated for 4 h at 4°C, diluted to 1/2000th in TBS, 0.05% tween, 5% milk. The Western blotting results are then quantified using a Chemigenius machine (Syngene). The intensity of the bands obtained with the various antibodies are weighted with the intensity of the bands obtained with the anti-actin antibody directly coupled to HRP and used at 1/3000th (Santa Cruz Biotechnology, ref Sc-8432-HRP).

FGF19 at 30 ng/ml induces the synthesis of the JunB and cFos proteins and also the phosphorylation of Erk1/2 in Hep3b cells. This neosynthesis of protein and the phosphorylation of Erk are completely inhibited by the anti-FGFR4 antibody 40-12 at 100 µg/ml, whereas the control antibody has no inhibitory effect. These effects observed on the Western blotting membranes (Fig. 9A) are quantified and the intensities of the bands of each membrane are represented in the form of a graph (Fig. 9B).

Secondly, cell proliferation experiments per se were carried out. 5000 cells are seeded into a 96-well plate in 100 µl of DMEM medium, containing 10% FCS and 2 mM glutamine. 24 h later, the cells are serum deprived in a serum-free culture medium for 24 h. The Hep3b cells are then stimulated for 72 h with 100 µl of serum-free medium supplemented with 10 ng/ml of FGF19 (internal production at Sanofi-Aventis R&D) or with 10% of serum, in the absence or in the presence of control antibodies or of anti-FGFR4 antagonist monoclonal antibody 40-12. After 3 days, the cell proliferation is quantified using the CellTiter Glo kit (Promega, France).

It emerges from these experiments that serum and FGF19 are capable of stimulating Hep3b proliferation. The anti-FGFR4 antagonist antibody 40-12 partially inhibits this

serum-induced proliferation at 100 µg/ml, whereas the control antibody does not show any inhibitory activity (Fig. 10A). In addition, the antibody 40-12 at 10 µg/ml completely blocks the proliferation induced by FGF19 (Fig. 10B). The control antibody has no effect.

- 5 This demonstrates that the anti-FGFR4 antagonist antibody which is the subject of the invention can be used as an antitumour therapeutic agent in the context of FGF19-dependent or FGF-R4-dependent tumours, and that this antibody would be particularly effective in the treatment of hepatocarcinomas.
- 10 In order to simplify the study of the effect of the anti-FGFR4 antibodies on Hep3b cells, an ELISA assay was developed for detecting the phosphorylation of Erk1/2 following the stimulation of these cells with FGF-19 (in correlation with the experiments described in Example 8), FGF-2 or foetal calf serum.
- To do this, 50 000 Hep3b cells are seeded into 96-well black clear-bottom plates
- 15 (COSTAR, ref 3603) in 100 µl of DMEM medium containing 10% FCS and 2 mM glutamine. After 24 h, the cells are subjected to conditions of deficiency for 24 h in FCS-free DMEM medium containing 2 mM glutamine. The medium is then drawn off and replaced with 100 µl of deficiency medium preequilibrated at 37°C, containing FGF or FCS, and also the antibodies evaluated, at the various doses. The cells are
- 20 incubated for 3 h at 37°C, 5% CO₂. The stimulation medium is then drawn off, the wells are rinsed with PBS at 4°C and the cells are fixed by adding 200 µl of 4% PFA (paraformaldehyde) in PBS for 15 min at ambient temperature. The PFA is drawn off and the cells are washed three times with 200 µl of PBS. The antibody labelling for detecting phospho-Erk1/2 directly on the Hep3b cells begins by saturating the
- 25 nonspecific sites with 100 µl of saturation buffer (21.25 ml of PBS, 1.25 ml of 10% non-immune goat serum (Zymed, ref 50-062Z), 75 µl of triton X100) for 2 h. The saturation buffer is replaced with 50 µl of anti-phosphoErk1/2 primary antibody (Cell Signaling Technology, ref 4377) diluted to 1/100th in PBS buffer containing 1% BSA and 0.3% triton X100. The primary antibody is incubated with cells overnight at 4°C.
- 30 It is then rinsed off with 3 washes of 200 µl of PBS and revealed using an anti-rabbit secondary antibody coupled to AlexaFluor 488 (Molecular Probes, ref A11008) diluted to 1/5000th in PBS buffer containing 1% BSA and 0.3% triton X100 for 4 h. The secondary antibody is then rinsed off with 3 washes of 200 µl of PBS, and 100 µl of PBS is then added to each well. The fluorescence is read over with an EnVision

2103 Multilabel Reader (Perkin Elmer) using the FITC filter.

This technique makes it possible to confirm that FGF-19 at 30 ng/ml induces the phosphorylation of Erk1/2 in Hep3b cells and that 40-12 is capable of blocking this stimulation from doses of 3 µg/ml (Fig. 9C). FGF-2 and the serum are also capable
5 of inducing the system. In the latter 2 cases, the antibody 40-12 inhibits the effect of FGF-2 and of the serum at higher doses (30-100 µg/ml; Fig. 9C).

The detection of phospho-Erk1/2 by ELISA also made it possible to show that anti-FGFR4 antibodies that are active in the *in vitro* angiogenesis model are also capable of blocking between 70% and 95% of the phosphorylation of Erk1/2 induced
10 by FGF-19 at the dose of 3 µg/ml (Fig. 9D).

Advantageously, the antibodies of the present invention have an antagonistic effect both on the pathological angiogenesis associated with tumour development and on hepatic tumour growth per se, in particular on a model of hepatocarcinoma.

15

Example 8: Antagonistic effect of the antibody 40-12 in the murine model of pancreatic cancer

For this pharmacological model, female Rip1-Tag2 mice (Charles River Laboratory, France) with a C57Bl/6J genetic background are used. Starting from week 9 after
20 birth, the animals have drinking water supplemented with 5% sucrose. The mice are treated from week 10 to week 12.5 in an intervention treatment protocol, once a week with a subcutaneous injection of the anti-FGFR4 antibody 40-12 or the control antibody, at the dose of 25 mg/kg (Fig. 14A). This protocol is approved by the
25 "Comité expérimentation Animale (Animal Care and Use Committee)" of Sanofi-Aventis Recherche. Our zootechnical facilities, the attention given to the animals and also the treatment protocols are in accordance with the principles laid down by the European Convention on the protection of vertebrate animals, sacrificed after the period of treatment or when the tumour burden and/or the side effects render their
30 withdrawal from the study obligatory.

For measuring the tumour burden, the animals are sacrificed by euthanasia at the end of the experiment and the tumours are microdissected from freshly excised pancreases. The tumour volume in mm³ is measured using a calliper rule, applying the formula [volume = 0.52 x (width)² x (length)] in order to approach the volume of a

spheroid. The tumour burden per mouse is calculated by the cumulation of the volume of the tumours of each mouse.

For the histochemical analysis of the vascular density, the animals are anaesthetized, and the pancreases are collected, fixed overnight in accustain®
5 (Sigma) and then embedded in paraffin. Sections 5 µm thick are prepared for each sample. The endothelial cells are detected by incubating the sections with trypsin (Zymed, ref 00-3003) at 37°C for 10 min, and then with an anti-mouse CD31 antibody, produced in rats, diluted to 1/50th (BD Pharmingen). To reveal the regions labelled with the antibody, the sections are incubated with a biotin-coupled anti-rat
10 antibody for 30 min, and then with HRP-coupled streptavidin, also for 30 min (Vectastain® ABC kit, Vector) and, finally, with DAB for 5 min (Vector, ref SK4100). The sections are then stained with hematoxylin diluted to 1/10th (Dako, S-3309). The photographs are taken with a camera mounted on a microscope (Nikon, E-800) at a total magnification of x200. The images are analysed using software (Visiolab,
15 Biocom). The blood vessels in the tumour are counted and classified according to their surface area: small vessels between 5 and 20 µm², medium vessels between 21 and 100 µm² and large vessels starting from 101 µm². Two slides per pancreas are analysed in order to determine the vascular density corresponding to the total number of elements labelled per field.

20

In this model, the subcutaneous treatment using the anti-FGFR4 antibody 40-12 at 25 mg/kg once a week between the tenth and twelfth weeks makes it possible to significantly reduce the tumour burden by 55% (Fig11B) and has a tendency to reduce the number of tumours per pancreas by 34% (Fig. 11C), whereas the control
25 treatment has no effect. This inhibition of tumour development by virtue of the anti-FGFR4 antibody 40-12 is accompanied by a significant reduction of 31% in the total vascular density (Fig. 11D) corresponding to an observed reduction in the number of blood vessels in all the vessel-size groups, labelled with an anti-CD31 antibody (Fig. 11D).

30 These *in vivo* results clearly show that an anti-FGFR4 antagonist antibody is capable of inhibiting the recruitment and the formation of blood vessels in the tumour. This inhibition of tumour vascularization is accompanied by a reduction in the number of tumours per pancreas and in the total tumour volume.

Advantageously, the antibodies of the present invention have an antagonistic effect both on pathological angiogenesis and on hepatic tumour growth (on a model of hepatocarcinoma) and pancreatic tumour growth.

5 Example 9: Cross-reactivity of the antibody with human, murine and rat FGF-R4s

Firstly, competition binding experiments were carried out. To do this, FGF2 was labelled on the 2 free cysteines with an AlexaFluor® 488 nm C-5 maleimide
10 (Molecular Probes, A10254) according to the supplier's recommendations. This FGF2-AF488, at 10 ng/ml, is capable of binding to the human FGF-R4s expressed at the surface of transfected 300-19 cells (Fig. 8). This binding is specific since the addition of excess unlabelled FGF2 makes it possible to displace the FGF2/FGF-R4 interaction (Fig. 8A). The same experiment was carried out with increasing doses of
15 the antibody 40-12 or of the control antibody 64-12 being added. Only the antagonist antibody 40-12 is capable of displacing the FGF2-AF488/FGF-R4 binding, with an IC_{50} of 3500 ng/ml, i.e. 23 nM, showing that the antagonistic effect of this antibody is due to its ability to displace FGF/FGF-R4 binding.

Secondly, and for the purpose of determining the ability of clone 40-12 to block
20 FGF/FGF-R4 binding in species other than humans, a dissociation experiment was carried out using the abovementioned mouse, rat and human FGF2/FGF-R4 pairs. To do this, murine FGF2 (R&D, ref 3139-FB-025) or rat FGF2 (R&D, ref 3339-FB-025) was labelled in the same way as human FGF2, with AlexaFluor 488. The dissociation experiments were carried out using the 300-19 lines transfected with
25 mouse or rat FGF-R4 receptors. The results show that the anti-FGFR4 antagonist antibody 40-12 is capable of dissociating the FGF2/FGF-R4 binding in a mouse or rat system, with the same effectiveness as in the human system. In fact, the IC_{50} values are 3500, 4110 and 3940 ng/ml, i.e. 23, 27 and 26 nM, for the human, murine or rat FGF2/FGF-R4 complexes, respectively (Fig. 13A, 13B and 13C, respectively).
30 This ability to bind to rodent FGF-R4 was verified by ELISA. The antibody 40-12 binds both to human FGF-R4 and to murine FGF-R4 (Fig. 12).

These results indicate that the anti-FGFR4 antibody 40-12 may be used in pharmacological models on rodents (at least mice and rats) and that the results obtained should be predictive of the effectiveness in humans.

Similarly, studies carried out on the antibodies derived from clones 8, 31, 33 and 36 show that these antibodies recognize both human FGF-R4 and murine FGF-R4 (Table 4 below)

5

Table 4: Kinetic parameters for binding of the anti-FGFR4 antibodies 8, 31, 33 and 36, measured by Surface Plasmon Resonance (BIAcore 3000):

	on h-FGFR4-Histag			on m-FGFR4-Histag		
	Kon ($M^{-1}s^{-1}$)	Koff (s^{-1})	KD (M)	Kon ($M^{-1}s^{-1}$)	Koff (s^{-1})	KD (M)
Clone 8	8.93×10^5	2.92×10^{-4}	3.27×10^{-10}	1.20×10^6	1.36×10^{-4}	1.15×10^{-10}
Clone 31	6.48×10^5	9.80×10^{-4}	1.52×10^{-9}	9.14×10^5	1.80×10^{-3}	1.97×10^{-9}
Clone 33	8.05×10^5	6.17×10^{-4}	7.71×10^{-10}	1.01×10^6	7.04×10^{-4}	6.92×10^{-10}
Clone 36	2.44×10^5	6.31×10^{-4}	2.62×10^{-9}	3.30×10^5	7.52×10^{-4}	2.28×10^{-9}

10

Example 10: Determination of the epitope recognized by the anti-FGF-R4 antibodies

A- Determination of the epitope recognized by the anti-FGFR4 antibody 40-12

15

A screen was carried out in order to determine the specific domain of FGFR4 recognized by the antibody 40-12, by ELISA assay. Through the use of a deleted form of the D1 domain of FGF-R4, in ELISA, it was established that the antibody 40-12 interacted with the D2-D3 domains of FGF-R4 (Fig. 12).

20

A second screen was carried out by ELISA assay, using, as capture antigen, the constructs containing either the D1 domain (SABVA4794) or the D2 domain (SABVA4796) or the D3 domain (SABVA4799) of the hFGFR4 protein, as described in Example 2. The capture antigen was bound to Immulon-4 enzyme-linked plates (VWR Scientific Inc. Swedesboro, NJ). The hybridoma 40-12 was subsequently added and then detection was carried out using the peroxidase-conjugated anti-mouse IgG rabbit antibody (Sigma; ref. A9044-dilution to 1:50 000). The revealing

25

was carried out with the TMB-H₂O₂ substrate (Interchim; ref UP664780) and the optical density (OD) measurements were carried out at 450 nm. Table 5 summarizes the results obtained:

- 5 Table 5: Measurement of the binding of the antibody 40-12 to the various subdomains of FGF-R4

Domain studied	D1 SABVA4794	D2 SABVA4796	D3 SABVA4799	D1-D3 FGFR4 -Fc
Signal (O.D., 450 nm)	0.185	3.000	0.105	3.000

- 10 The anti-FGFR4 antibody therefore recognizes the D2 domain of the extracellular portion of the FGFR4 protein.

Furthermore, the FGFR4-Fc protein denatured with FCS is not recognized by the antibody 40-12 in Western blotting analysis, thereby indicating that the epitope targeted by 40-12 on the D2 domain of FGFR4 is of conformational type.

- 15 B- Determination of the epitope recognized by the anti-FGFR4 antibodies 8, 31, 33, 36.

- A screen was carried out in order to determine the specific domain of FGFR4 recognized by the antibodies 8, 31, 33 and 36, by ELISA assay using, as capture antigen, the constructs containing either the D2 and D3 domains (SEQ ID No. 42) or the hFGFR4-Histag protein (SABVA4614, SEQ ID No. 40). The capture antigen was bound to Immulon-4 enzyme-linked plates (VWR Scientific Inc. Swedesboro, NJ). Culture supernatants from HEK293 cells transiently transfected with plasmids which enable the secretion of the antibodies 8, 31, 33 and 36 were subsequently added and then detection was carried out using the peroxidase-conjugated anti-human IgG rabbit antibody (DakoCytomation, ref. P0214-dilution to 1:5000). The revealing was carried out with the TMB-H₂O₂ substrate (Interchim; ref UP664780) and the optical density (OD) measurements were carried out at 450 nm. Table 6 below summarizes the results obtained:

30

Table 6: Binding of the antibodies derived from clones 8, 31, 33 and 36 to whole FGF-R4 or to the D2-D3 subdomain (OD signal at 450nm)

	hFGFR4-Histag	hFGFR4(D2,D3)-Histag
Clone 8	3.898	3.860
Clone 31	3.859	3.741
Clone 33	3.752	3.621
Clone 36	3.751	3.616

The antibodies 8, 31, 33 and 36 therefore recognize the D2-D3 domain of the extracellular portion of the FGFR4 protein.

5

Table 7: Sequences used, obtained and deduced

	Nucleotide sequences	Protein sequences
Antibody 40-12		
VH + CH	SEQ ID No. 1	SEQ ID No. 2
VL + CL	SEQ ID No. 3	SEQ ID No. 4
VH	SEQ ID No. 5	SEQ ID No. 6
VL	SEQ ID No. 7	SEQ ID No. 8
CDR VH		SEQ ID No. 9; 10; 11
CDR VL		SEQ ID No. 12; 13; 14
Antibody 64-12		
VH + CH	SEQ ID No. 15	SEQ ID No. 16
VL + CL	SEQ ID No. 17	SEQ ID No. 18
VH	SEQ ID No. 19	SEQ ID No. 20
VL	SEQ ID No. 21	SEQ ID No. 22
CDR VH		SEQ ID No. 23; 24; 25
CDR VL		SEQ ID No. 26; 27; 28

Humanized sequences		
Light chain 1	SEQ ID No. 29	SEQ ID No. 30
Light chain 2	SEQ ID No. 31	SEQ ID No. 32
Heavy chain 1	SEQ ID No. 33	SEQ ID No. 34
Heavy chain 2	SEQ ID No. 35	SEQ ID No. 36
Heavy chain 3	SEQ ID No. 37	SEQ ID No. 38
Constructs		
hFGFR4-Histag	SEQ ID No. 39	SEQ ID No. 40
hFGFR4-Streptag	SEQ ID No. 68	SEQ ID No. 69
hFGFR4(D2D3)-Histag	SEQ ID No. 41	SEQ ID No. 42
mFGFR4-Histag	SEQ ID No. 43	SEQ ID No. 44
hFGFR1-Fc	SEQ ID No. 45	SEQ ID No. 46
hFGFR2-Fc	SEQ ID No. 47	SEQ ID No. 48
rFGFR4	SEQ ID No. 49	SEQ ID No. 50
Primer sequences for establishing clonal line (Example 4)	SEQ ID No. 51; 52; 53; 54; 55; 56; 58 SEQ ID No. 61; 62	
FGFR4 transmembrane domain		SEQ ID No. 59; 60
Primer oligonucleotides Table 1	SEQ ID No. 63; 64; 65; 66; 67	
Epitope in D2 domain		SEQ ID No. 70

Antibody clone 8		
Whole light chain	SEQ ID No. 71	SEQ ID No. 72
Light chain CDR		SEQ ID No. 73; 74; 75
Whole heavy chain	SEQ ID No. 76	SEQ ID No. 77

Heavy chain CDR		SEQ ID No. 78; 79; 80
Antibody clone 31		
Whole light chain	SEQ ID No. 81	SEQ ID No. 82
Light chain CDR		SEQ ID No. 83; 84; 85
Whole heavy chain	SEQ ID No. 86	SEQ ID No. 87
Heavy chain CDR		SEQ ID No. 88; 89; 90
Antibody clone 33		
Whole light chain	SEQ ID No. 91	SEQ ID No. 92
Light chain CDR		SEQ ID No. 93; 94; 95
Whole heavy chain	SEQ ID No. 96	SEQ ID No. 97
Heavy chain CDR		SEQ ID No. 98; 99; 100
Antibody clone 36		
Whole light chain	SEQ ID No. 101	SEQ ID No. 102
Light chain CDR		SEQ ID No. 103; 104; 105
Whole heavy chain	SEQ ID No. 106	SEQ ID No. 107
Heavy chain CDR		SEQ ID No. 108; 109; 110
Subdomains of the extracellular portion of hFGFR4, fused to the Fc domain of IgG1		
fGFR4_D1::Fc	SEQ ID No. 111	SEQ ID No. 112
fGFR4_D2::Fc	SEQ ID No. 113	SEQ ID No. 114
fGFR4_D3::Fc	SEQ ID No. 115	SEQ ID No. 116
Human IgG1 constant region sequence		SEQ ID No. 117

Claims

1. Use of a FGF-R4 antagonist antibody for the manufacture of a medicament for treatment of hepatocarcinoma or of any other type of hepatic cancer.
5
2. Use of a FGF-R4 antagonist antibody according to claim 1 , wherein the antibody can bind both human FGF-R4 and murine FGF-R4.
3. Use of a FGF-R4 antagonist antibody according to claim 1 or 2, wherein the
10 antibody comprises the CDRs of sequence SEQ ID Nos. 9, 10, 11, 12, 13 and 14; or 73, 74, 75, 78, 79 and 80; or 83, 84, 85, 88, 89 and 90; or 93, 94, 95, 98, 99 and 100; or 103, 104, 105, 108, 109 and 110, wherein one of the CDRs may differ by one or two amino acids compared with at least one of the sequences mentioned above, provided that the antibody keeps its binding specificity.
15
4. Use of a FGF-R4 antagonist antibody according to any one of the preceding claims wherein the variable region of the heavy chain of the antibody comprises a nucleotide sequence having at least 80% identity with the
20 sequence SEQ ID No. 5, 76, 86, 96 or 106 and wherein the variable region of the light chain of the antibody comprises a nucleotide sequence having at least 80% identity with the sequence SEQ ID No. 7, 71, 81, 91 or 101, provided that the antibody keeps its binding specificity.
5. Use of a FGF-R4 antagonist antibody according to any one of the preceding
25 claims wherein the sequence of the antibody comprises the polypeptide sequences SEQ ID Nos. 2 and 4; or 6 and 8; or 72 and 77; or 82 and 87; or 92 and 97; or 102 and 107 or sequences corresponding to these sequences, having at least 80% identity therewith, provided that the antibody keeps its binding specificity.
30

6. Use of a FGF-R4 antagonist antibody according to any one of the preceding claims, wherein the antibody affinity for FGF-R4 is 10 times greater than the affinity for the other FGF receptors.
- 5 7. Use of a FGF-R4 antagonist antibody according to any one of the preceding claims, wherein the antibody is a humanized antibody.
8. Use of a FGF-R4 antagonist antibody according to claim 7, wherein the antibody comprises a variable light chain having at least 80% identity with one of the polypeptide sequences SEQ ID No. 30 or 32 and a variable heavy chain having at least 80% identity with a sequence SEQ ID No. 34, 36 or 38.
- 10 9. Use of a FGF-R4 antagonist antibody according to any one of the preceding claims, wherein the antibody is conjugated to a cytotoxic agent.