(54) Titre : MUTATIONS DANS LE DOMAINE EXTRACELLULAIRE III DU GENE DU RECEPTEUR DE FACTEUR DE CROISSANCE EPIDERMIQUE
(54) Title: MUTATIONS IN THE EXTRACELLULAR DOMAIN III OF EPIDERMAL GROWTH FACTOR RECEPTOR GENE

(57) Abrégé/Abstract:

The invention relates to new identified mutations in the epidermal growth factor receptor gene, leading to amino acidic changes which highly correlate with the resistance to a therapy regimen comprising cetuximab. The invention includes peptide sequences and primers to detect the mutations, as well as kits for predicting the response of a subject to a therapy regime comprising cetuximab. In particular, the invention is useful in the therapy regimen applicable to metastasic colorectal cancer.

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Mutations in the extracellular domain III of epidermal growth factor receptor gene

The present invention is directed to new mutations of the human epidermal
growth factor receptor gene, as a marker for determining response to
monoclonal antibody treatment.

BACKGROUND ART

Epidermal growth factor receptor gene (EGFR) is a transmembrane tyrosine-
kinase receptor that belongs to the epidermal growth factor family of
receptors (ErbB family), which includes four closely related receptor tyrosine
kinases: EGFR (ErbB-1), HER2/c-neu (ErbB-2), Her 3 (ErbB-3) and Her 4
(ErbB-4). Upon ligand binding, EGFR activates intracellular signalling
pathways, mainly the RAS-RAF-MEK-ERK cascade and the PI3K-AKT
pathway, that regulate key oncogenic events such as apoptosis, cell growth,
angiogenesis and metastasis. Aberrant activation or overexpression of EGFR
has been reported in several types of cancer (i.e. Mendelsohn J, Baselga J et
al., "Epidermal growth factor receptor targeting in cancer". Semin Oncol -
2006, Vol. 33, pp.: 369–38). Mutations in EGFR gene have been described in
lung cancer. Examples of such mutations are disclosed for instance in the
document of Lynch TJ et al., "Activating mutations in the epidermal growth
factor receptor underlying responsiveness of non-small-cell lung cancer to
gefitinib", N Engl J Med-2004, Vol. 350, pp:2129-2139.

Metastasic colorectal cancer (mCRC) is the second leading cause of death
from cancer in the Western Countries world.

A therapy based on monoclonal antibodies (moAbs), e.g. cetuximab and
panitumumab, which are directed against the extracellular domain III of EGFR,
provides significant survival benefit to patients with mCRC and are now
standard components of therapy regimens for these patients, i.e. either alone
or in combination with other antineoplastic drug(s).

The moAbs bind to foreign antigens expressed on cancer cells. Once bound,
the cancer cells are marked for destruction by the patient's immune system. In
addition to targeting cancer cells, moAbs can be designed to act on other cell

types and molecules necessary for tumor growth. For example, antibodies can neutralize growth factors and thereby inhibit tumor expansion. It is possible to create a moAb specific to almost any extracellular/cell surface target (such as cancer cells). In summary, moAbs can be used to destroy malignant tumor cells and prevent tumor growth by blocking specific cell receptors. Therapeutic moAbs cetuximab and panitumumab bind to EGFR and prevent the activation of intracellular signalling pathways driven by EGFR (i.e., the RAS-RAF-MEK-ERK cascade and PI3K-AKT pathway).

Not all patients with mCRC respond to a therapy regimen comprising moAbs. The lack of response of a patient with mCRC to such a treatment could be primary (i.e. since the beginning of anti-EGFR moAb treatment), known as primary resistance. Moreover, all mCRC patients that initially respond to anti-EGFR moAbs invariably develop secondary resistance, i.e. acquired resistance to anti-EGFR moAb. In both cases, the result is treatment failure. The mechanisms that contribute to the acquisition of such treatment resistance in mCRC patients is not fully known yet.

KRAS (also known as V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog) is an EGFR downstream effector, and a marker of primary resistance to anti-EGFR moAbs. KRAS has a significant impact on the optimization of treatment of mCRC patients. Forty per cent of colorectal tumors harbour a mutation in the KRAS gene and these patients do not benefit from anti-EGFR moAbs. In current clinical practice all mCRC patients who are being considered for anti-EGFR moAb therapy should undergo KRAS testing, and patients should be excluded from cetuximab or panitumumab therapy if a KRAS mutation is detected.

While the use of *KRAS* mutations and more recently NRAS (Neuroblastomas Ras viral oncogene homolog) mutations as markers of primary resistance to anti-EGFR moAbs has meant a significant step towards optimization of treatment of mCRC patients, the understanding of molecular changes underlying acquired resistance to anti-EGFR moAb is currently a crucial challenge to improve the clinical benefit of these drugs. Recently, mechanisms of secondary resistance (acquired resistance) have been elucidated in patients. The most common event is the emergence of *KRAS* mutations or gene amplification in approximately 50% of the cases, as deducible from

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Misale et al., "Emergence of KRAS mutations and acquired resistance to anti-EGFR therapy in colorectal cancer", Nature – 2012, Vol. No. 486, pp.: 532-536.

5   Other mechanisms of secondary resistance include acquisition of a mutation in the extracellular domain of EGFR abrogating binding of cetuximab to EGFR, as illustrated by Montagut et al., "Identification of a mutation in the extracellular domain of the Epidermal Growth Factor Receptor conferring cetuximab resistance in colorectal cancer", Nature Medicine - 2012, Vol. No.
10   18, pp.:221-223. The mutation is the polymorphism in the extracellular portion of the EGFR gene, resulting in the amino acid substitution S492R at domain III of the codified protein.

With the aim of studying monoclonal antibody interaction with EGFR epitopes,
15   several reports are directed to the mapping of critical epitopes. These reports provide data of mutations obtained by site-directed mutagenesis at domain III of EGFR. An example of these reports is the one of Voigt et al., "Functional Dissection of the Epidermal Growth Factor Receptor Epitopes Targeted by Panitumumab and Cetuximab", Neoplasia – 2012, Vol. No. 14(11), pp.: 1023-
20   1031. This document discloses mutations in which the wild-type amino acid has been mostly changed by an alanine, according to the protocols and tools of site-mutagenesis assays. Voigt concludes that in-vitro data from site-mutagenesis may not be meaningful in vivo because residues defined as critical for cetuximab or panitumumab binding by an alanine scanning
25   approach may well be mutated in vivo to other amino acids without functional consequences. Therefore, those key positions in a defined epitope identified by site-mutagenesis do not suggest the in vivo meaningful mutation (the particular amino acid exchange).

30   Drug resistance is then a major challenge in colorectal cancer patients treated with anti-EGFR drugs, namely cetuximab and panitumumab. Elucidation of the molecular mechanisms of resistance represents a great goal, but this implies detection of meaningful mutations or other gene alterations as markers for predicting a response and, at the same time, for determining if a particular
35   medical regimen has to be modified due to acquired resistance (secondary resistance). In summary, the state of the art provides useful tools for detecting primary and secondary resistance to anti-EGFR moAb therapies in patients

with mCRC, but it is necessary to identify additional and alternative predictive biomarkers of resistance in order to cover patients with different mutations, or with a different evolution of the resistance molecular mechanisms.

5   SUMMARY OF THE INVENTION

The inventors have identified new mutations in the extracellular domain of human EGFR (domain III) that correlate with resistance to the treatment with some moAbs used in the cancer therapy. The mutations lead to the amino
10   acid substitutions of an arginine by a cysteine at position 451 of the EGFR protein; of a serine by a leucine at position 464 of the EGFR; of a glycine by an arginine at position 465 of the EGFR protein; and of a lysine by a threonine at position 467 of the EGFR protein.

15   Wild type human EGFR protein has the amino acid sequence SEQ ID NO: 2, and the mutations are known herein as R451C, S464L, G465R and K467T. Mutations may be detected alone or in combination with each of the others in patients with mCRC after treatment with anti-EGFR moAbs.

20   All these mutations are located in a particular amino acid sequence fragment of the cetuximab binding epitope. Namely, they are located in a fragment from amino acid at position 450 to amino acid at position 470 of SEQ ID NO: 2, this SEQ ID NO: 2 corresponding to the consensus wild-type amino acid sequence of human EGFR, This amino acid sequence fragment of the cetuximab
25   binding epitope is herewith referred also as SEQ ID NO: 12 (LRSLKEISDGDVIISGNKNLC). Interestingly, inventors discovered that this fragment includes many of the particular amino acid exchanges (mutations) that lead to a real impairment (i.e not effectivity) of many anti-EGFR moAb treatments. As above exposed, many amino acid positions have been
30   determined as key positions by mutagenesis while mapping anti-EGFR-moAb binding sites, nonetheless, it is also known that mapping assays are not conclusive for determining resistance to treatments.

Therefore, the inventors provide for the first time a fragment of the
35   extracellular domain III of EGFR that contains or summarizes many mutation points with a real effect on therapy. Analysing or determining the sequence within this fragment (SEQ ID NO: 12) provides the advantage of detecting

many of the possibly resistant patients to treatments including anti-EGFR moAb. Examples of amino acids within this SEQ ID NO: 12 (LRSLKEISDGDVIISGNKNLC) that lead to resistance to the widely employed anti-EGFR moAb cetuximab are indicated in bold and underwritten.

All these mutations are located in exon 12 of the mRNA variant 1 of the human *EGFR* gene finally coding for EGFR protein of SEQ ID NO: 2. In addition, all of them relate to a change of wild-type amino acids to bulky amino acids (i.e. those with a side-chain consisting of branched or unbranched C1-C4 hydrocarbons, optionally with a terminal amino group) and/or polar or charged amino acids. In particular, most of the mutations relate to a change of a polar and/or charged amino acid with a side-chain comprising a terminal amino (-NH2). More in particular, two of the mutationsrelate to a change of an amino acid with a side-chain comprising a terminal amino (-NH2). In addition, mutations R451C and K467T imply the substitution of an amino acid with a side-chain comprising a terminal amino (-NH2) for a polar amino acid, whose carbohydrate side-chain comprises radicals with atoms from the oxygen group, namely –OH and –SH, and they have a similar chain size as depicted below:

$$H_2N-CHC(=O)-OH$$
$$|$$
$$CH_2$$
$$|$$
$$CH_2$$
$$|$$
$$CH_2$$
$$|$$
$$NH$$
$$|$$
$$C=NH$$
$$|$$
$$NH_2$$

Arg, R

$$H_2N-CHC(=O)-OH$$
$$|$$
$$CH_2$$
$$|$$
$$CH_2$$
$$|$$
$$CH_2$$
$$|$$
$$CH_2$$
$$|$$
$$NH_2$$

Lys, K

$$H_2N-CHC(=O)-OH$$
$$|$$
$$CHOH$$
$$|$$
$$CH_3$$

Thr, T

$$H_2N-CHC(=O)-OH$$
$$|$$
$$CH_2$$
$$|$$
$$SH$$
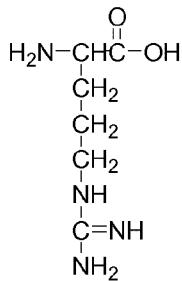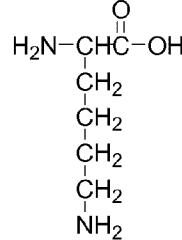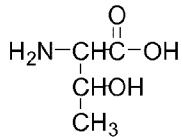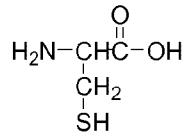
Cys, C

Thus, inventors provide for the first time the association of mutations in domain III of human EGFR changing a basic amino acid with a side-chain comprising a terminal amino (-NH2), with proved resistance to the treatment

with some moAbs used in the cancer therapy. More particularly, this association is seen when these basic amino acids change to certain polar amino acids selected from cysteine and threonine.

5   Besides, changes of amino acids within the above-mentioned SEQ ID NO: 12 being said amino acids polar or neutral and substituted by bulky amino acids, being charged or neutral, are also associated with proved resistance to the treatment with some moAbs used in the cancer therapy. This is the case, for example, of mutation S464L and of mutation G465R.

10

Particular mutations R451C and K467T are detected in a mutated protein comprising the peptide sequence defined in SEQ ID NO: 1.

Besides, the above-indicated mutations and mutations S464L and G465R are
15   detected in a mutated protein comprising the peptide sequence defined in SEQ ID NO: 13.

These markers may then be used to track the evolution of the acquired resistance mechanisms to anti-EGFR therapies. Detection of acquired
20   resistance may be a useful tool for proposing another therapeutic approach or medical regimen along the follow-up of the disease evolution.

Thus, in a first aspect the invention relates to a peptide sequence with a length from 17 to 100 amino acids and comprising the sequence SEQ ID NO:
25   13

X^1SLKEISDGDVII$X^4$$X^5NX^2$, wherein

X^1 is selected from R and C;
30   X^4 is selected from S and L;
X^5 is selected from G and R;
X^2 is selected from K and T; and wherein at least one of X^1, X^4, X^5 and X^2 is, respectively, C, L, R or T.

35   SEQ ID NO: 13 encompasses any of the above-defined mutations, but at least one of them: R451C, S464L, G465R or K467T.

In a particular embodiment, the invention relates to a peptide sequence with a length from 17 to 100 amino acids and comprising the sequence SEQ ID NO: 1

5   X^1SLKEISDGDVIISGNX^2, wherein:

X^1 is selected from R and C;
X^2 is selected from K and T; and

10   wherein if X^1 is C, then X^2 is selected independently from K and T, and if X^1 is R, then X^2 is T.

SEQ ID NO: 1 encompasses any of the above-defined mutations, but at least
15   one of them: R451C or K467T. In other words, X^1 and X^2 have the indicated meaning but with the proviso that at least one of X^1 or X^2 are, respectively, the mutated forms C or T; or both X^1 and X^2 are the mutated forms C and T.

This SEQ ID NO: 1 derives from human EGFR protein of SEQ ID NO: 2. Thus, it is a fragment of the human protein sequence, said fragment including at
20   least one of the indicated mutations. Therefore, the resting of the amino acids up to 100 are the ones located in the protein sequence of SEQ ID NO: 2, being either flanking said SEQ ID NO: 1 or a sequence linked to the C-terminal end of SEQ ID NO: 1 and defined by the amino acid X^2.

25   Advantageously, these mutations (R451C, S464L, G465R or K467T) represent alternatives that can be tested in a sample of a subject suspected of having acquired resistance or a primary resistance to the anti-EGFR moAb therapies. Thus, besides other mutations that may be present or not in the sample of a subject, the mutations proposed in SEQ ID NO: 1 (R451C or
30   K467T) or even in SEQ ID NO: 13 (R451C, S464L, G465R or K467T) serve to detect possible resistant subjects not detectable by other means. In particular, mutations R451C and K467T imply the additional advantage of indicating that some anti-EGFR moAb therapies are still permissive (or efficient). In particular, mutations R451C and K467T are permissive to panitumumab. This
35   means that, if at least one of these two mutations are detected, at least panitumumab treatment may be recommended.

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In a second aspect, the invention relates to an oligonucleotide comprising a sequence coding for SEQ ID NO: 1 or SEQ ID NO: 13.

5   The isolated peptide comprising SEQ ID NO: 1 or SEQ ID NO: 13 is the key product leading to the detection of mutated forms of EGFR protein of great interest in the field of cancer therapy. These mutated forms of the protein are also detectable in the form of an oligonucleotide comprising a sequence coding for SEQ ID NO: 1 or for SEQ ID NO: 13.

10   Oligonucleotides coding for SEQ ID NO: 1 or for SEQ ID NO: 13 are those including the nucleotide changes that lead to at least one of the above mentioned amino acid changes taking into account the codon degeneracy (redundancy of the genetic code) in these mutation positions. These oligonucleotides may be used then as hybridization probes for detecting the

15   particular mutations that lead to the amino acid changes.

In addition, all these oligonucleotides are suitable probes allowing detecting the presence or not of the nucleotide mutations leading to amino acid changes in a peptide sequence comprising SEQ ID NO: 1 or SEQ ID NO: 13.

20

In particular, the invention is based on the surprising identification of the amino acid substitutions of an arginine by a cysteine at position 451 of the EGFR protein; of a serine by a leucine at position 464 of the EGFR; of a glycine by an arginine at position 465; and of a lysine by a threonine at

25   position 467 of the EGFR protein.

The amino acid change K467T is the result of the nucleotide change A→C at nucleotide 1400 (also known herein as A1400C) of the mRNA variant 1 of the *EGFR* gene (Codon AAA is changed to ACA). The amino acid change R451C

30   is the result of the nucleotide change C→T at nucleotide 1351 (also known herein as C1351T) of the mRNA variant 1 of the *EGFR* gene (Codon CGC is changed to TGC). The amino acid change S464L is the result of the nucleotide change C→T at nucleotide 1391 (also known herein as C1391T) of the mRNA variant 1 of the *EGFR* gene (Codon TCA is changed to TTA). The

35   amino acid change G465R is the result of the nucleotide change G→A at nucleotide 1393 (also known herein as G1393A) of the mRNA variant 1 of the *EGFR* gene (Codon GGA is changed to AGA).

All these amino acid changes may be the result of other mutations in the codon coding for them. In particular, all those nucleotide changes leading to a cysteine at position 451 of SEQ ID NO: 2 (human EGFR protein), to a leucine
5   at position 464 of SEQ ID NO: 2, to an arginine at position 465 of SEQ ID NO: 2; and to a threonine at position 467 of SEQ ID NO: 2.

As already indicated above, each of the above nucleotide changes refers to the mRNA, transcript variant 1 sequence of the *EGFR* gene (also known as
10   ERBB1, PIG61, proto-oncogene c-ErbB-1, avian erythroblastic leukemia viral (v-erb-b) oncogene homolog receptor tyrosine-protein kinase erbB-1, or HER1). The sequence of the mRNA, transcript variant 1, of the *EGFR* gene is that corresponding to SEQ ID NO: 3 (or GenBank accession number NM_005228.3, version 3 of sequence and database release available on
15   18.05.2014) as well as any variant thereof, wherein said variant codes for the EGFR protein. The EGFR protein corresponds to SEQ ID NO: 2 (GenBank accession number NP_005219.2 version 2 of sequence and database release of 18.05.2014) or any variant thereof that maintains the basic structure of the EGFR protein. SEQ ID NOs: 2 and 3 are from human (*Homo sapiens*).
20   Nonetheless, EGFR is highly conserved in most of the mammals and the herewith mutation points comprise in the wild-type sequences the same amino acids in most of mammals. Therefore, the invention encompasses the same mutations but determined in a sequence of EGFR protein or gene of any mammal.
25
Another aspect of the invention is a set of primers consisting of SEQ ID Nos: 6 (CAAAGTTTTCAGGGATACATTGTTTTT) and 7 (TTAAATGGGAATAGCCCTTCAATATT).

30   This set of primers allows amplifying the genomic region comprising the portion of the *EGFR* coding region wherein the nucleotide changes resulting in the mutations of the present invention are located. They are thus related with the novel amino acidic mutations identified by the inventors, and they allow amplifying the EGFR coding region coding for the fragment herewith named
35   SEQ ID NO: 12 (LRSLKEISDGDVIISGNKNLC) that has been surprisingly found as a key region including many mutations leading to resistance (acquired or primary) to treatment with anti-EGFR moAbs. Particularly, the set

of primers consisting of SEQ ID NO: 6 and 7 allow amplification of *EGFR* coding region leading to Exon 12 in the variant 1 of mRNA transcript. This set allows determining if mutations R451C, S464L, G465R and K467T are present in the final resulting EGFR protein. More in particular if mutations

5   R451C and K467T are present in the final resulting EGFR protein.

Another aspect of the invention is a kit that comprises a set of primers consisting of: the set of primers of SEQ ID NOs: 6 and 7, and/or an oligonucleotide as defined in the second aspect of the invention.

10

This kit is a usable tool to detect the presence of the mutations (R451C, S464L, G465R and K467T) of SEQ ID NO: 13, and more particularly of mutations R451C and K467T of SEQ ID NOs: 1, or of any amino acid sequence comprising it, in an easy and fast way, since it includes the primers

15   for amplifying regions of EGFR gene that may include the disclosed mutations correlated with resistance to cetuximab treatment.

Also another aspect of the invention is, therefore, the kit as defined above, for use in the prediction of the response of a subject to a therapy regimen

20   comprising anti-EGFR monoclonal antibodies. Or the use of a kit as defined above for predicting response of a subject to a therapy regimen comprising anti-EGFR monoclonal antibodies.

Further, the invention also relates to an *in vitro* method of predicting the

25   response of a subject therapy regimen comprising cetuximab and/or panitumumab, wherein the method comprises:
(i) determining in a sample taken from the subject and by means selected from the group consisting of genotype methods, and/or protein sequencing methods if mutations are present or absent in a fragment defined by SEQ ID

30   NO: 12, which is a fragment from amino acid 450 to amino acid 470 of the consensus wild-type amino acid sequence of human EGFR of SEQ ID NO: 2; and ii) correlating the presence of any mutation identified in step i) with resistance of the subject to the therapy regimen comprising cetuximab, or correlating the absence of mutations in step i) with response of the subject to

35   therapy regimen comprising panitumumab.

Thus, SEQ ID NO: 12 corresponds to the wild-type amino acid fragment (or

sequence) of the human EGFR, and mutations in relation to this consensus wild-type amino acid sequence are determined within this SEQ ID NO: 12, that has been discovered as a meaningful fragment of EGFR regarding prediction of anti-EGFR moAb treatments. This SEQ ID NO: 12 forms also part of the

5   invention (LRSLKEISDGDVIISGNKNLC) as an isolated peptide.

The invention also relates to *in vitro* methods of predicting the response of a subject therapy regimen comprising cetuximab and/or panitumumab, wherein the method comprises:

10   i) determining by means selected from the group consisting of genotype methods, and/or protein sequencing methods, the presence or absence of at least one of the following amino acids:
     a cysteine at position 451 of the amino acid sequence corresponding to SEQ ID NO: 2, a leucine at position 464 of the amino acid sequence corresponding

15   SEQ ID NO: 2, an arginine at position 465 of the amino acid sequence corresponding SEQ ID NO: 2; and a threonine at position 467 of the amino acid sequence corresponding to SEQ ID NO: 2, in a sample taken from the subject; and ii) correlating the presence of any of the amino acids identified in step i) with resistance of the subject to the therapy regimen comprising

20   cetuximab, or correlating the absence of all of these amino acids in step i) with response of the subject to therapy regimen comprising panitumumab.

Further, the invention also relates, in a particular embodiment, to an *in vitro* method of predicting the response of a subject therapy regimen comprising

25   cetuximab and/or panitumumab, wherein the method comprises:
     i) determining by means selected from the group consisting of genotype methods, and/or protein sequencing methods, the presence or absence of at least one of the following amino acids:
     a cysteine at position 451 of the amino acid sequence corresponding to SEQ

30   ID NO: 2, and a threonine at position 467 of the amino acid sequence corresponding to SEQ ID NO: 2, in a sample taken from the subject; and ii) correlating the presence of any of the amino acids identified in step i) with resistance of the subject to the therapy regimen comprising cetuximab, or correlating the absence of all of these amino acids in step i) with response of

35   the subject to therapy regimen comprising panitumumab.

This in vitro method encompasses detecting if in exon 12 of the mRNA variant

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1 of the human *EGFR* gene finally coding for EGFR protein of SEQ ID NO: 2 there is a nucleotide change leading to a change of an amino acid with a side-chain comprising a terminal amino (-NH2) in the wild-type gene for a polar amino acid, whose carbohydrate side-chain comprises radicals with atoms from the oxygen group.

The put into practice of the *in vitro* method of predicting the response of a subject to a therapy regimen comprising cetuximab and/or panitumumab, implies the advantage of accommodating the more suitable therapy for the subject, and avoids wrong or not useful enough therapeutically approaches incurring waste time, which is an essential aspect for the subject and the success of the treatment, especially if the subject is affected with cancer.

In addition, detection of any of these mutations allows determining if a secondary resistance to cetuximab treatment has been developed in the subject, said subject not initially carrying the mutations in the *EGFR* gene.

Thus, another aspect of the invention is an in vitro method for determining the acquired resistance to a therapy regimen comprising cetuximab, the method comprising:
i) determining by means selected from the group consisting of genotype methods, and/or protein sequencing methods, the presence or absence of at least one of the following amino acids:
a cysteine at position 451 of the amino acid sequence corresponding to SEQ ID NO: 2, a leucine at position 464 of the amino acid sequence corresponding to SEQ ID NO: 2, an arginine at position 465 of the amino acid sequence corresponding to SEQ ID NO: 2, and a threonine at position 467 of the amino acid sequence corresponding to SEQ ID NO: 2, in a sample taken from the subject; and
ii) correlating the presence of any of the amino acids identified in step i) with acquired resistance of the subject to the therapy regimen comprising cetuximab, or correlating the absence of all of these amino acids in step i) with response of the subject to therapy regimen comprising panitumumab.

This *in vitro* method for determining the acquired resistance in a subject after treatment with cetuximab, advantageously allows stopping the treatment and further to avoid secondary or accompanying cetuximab adverse effects.

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Moreover, other approaches can be taken as fast as possible.

As above, this in vitro method for determining the acquired resistance
encompasses detecting if in exon 12 of the mRNA variant 1 of the human
5   EGFR gene finally coding for EGFR protein of SEQ ID NO: 2 there is a
nucleotide change leading to a change of an amino acid with a side-chain
comprising a terminal amino (-NH2) in the wild-type gene for a polar amino
acid, whose carbohydrate side-chain comprises radicals with atoms from the
oxygen group. This in vitro method encompasses also detecting if at least in
10   exon 12 of the mRNA variant 1 of the human EGFR gene finally coding for
EGFR protein fragment of SEQ ID NO: 12, there is a nucleotide change
leading to a change of wild-type amino acids to bulky amino acids (i.e. those
with a side-chain consisting of branched or unbranched C1-C4 hydrocarbons,
optionally with a terminal amino group) and/or to polar or charged amino
15   acids. Wild-type amino acid refers to the amino acid according to the
consensus amino acid sequence of human EGFR protein (SEQ ID NO: 2).

Another aspect of the invention is an in vitro method of identifying, in a sample
taken from a subject, the presence or absence of a cysteine at position 451 of
20   the amino acid sequence corresponding to SEQ ID NO: 2; and/or the
presence or absence of a leucine at position 464 of the amino acid sequence
corresponding to SEQ ID NO: 2; and/or the presence or absence of an
arginine at position 465 of the amino acid sequence corresponding to SEQ ID
NO: 2; and/or the presence or absence of a threonine at position 467 of the
25   amino acid sequence corresponding to SEQ ID NO: 2, the method comprising
determining the sequence of SEQ ID NO: 2, at least from position 450 to
position 470.

This later aspect can also be formulated as an in vitro method of identifying, in
30   a sample taken from a subject, the presence or absence of a cysteine at
position 451 of the amino acid sequence corresponding to SEQ ID NO: 2;
and/or the presence or absence of a leucine at position 464 of the amino acid
sequence corresponding to SEQ ID NO: 2; and/or the presence or absence of
an arginine at position 465 of the amino acid sequence corresponding to SEQ
35   ID NO: 2; and/or the presence or absence of a threonine at position 467 of the
amino acid sequence corresponding to SEQ ID NO: 2, the method comprising
determining the amino acid at positions 451 and/or 464 and /or 465 and/or

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467 by means selected from the group consisting of genotype methods, and/or protein sequencing methods.

BRIEF DESCRIPTION OF THE DRAWINGS

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FIG. 1 is the plot of two displays of sequencing results obtained by conventional Sanger sequencing (plot A) and Next Generation Sequencing (NGS) in a 454 GS Junior platform (Roche Applied Science, Mannheim, Germany) (plot B). It shows the acquisition of mutations in the EGFR

10 ectodomain following treatment with cetuximab in two samples. (A) In patient #31, the post-treatment tumor sample had acquired an A -> C substitution at nucleotide 1400 of *EGFR* gene that was not present in a pre-treatment biopsy, causing a substitution of a lysine to a threonine at amino acid 467 (K467T). (B) In patient #35, a C -> T substitution at nucleotide 1351 of the EGFR gene

15 was detected in the post-treatment sample, leading to a substitution of an arginine to a cysteine at amino acid 451 (R451C).

FIG. 2 is a Flow cytometry binding analysis of trypsinized NIH3T3 overexpressing wild-type EGFR (wt EGFR) and K467T EGFR mutant

20 incubated with cetuximab (FIG. 2A) or panitumumab (FIG. 2B) as primary antibodies and using a secondary antibody conjugated with phycoerythrin directed against human IgG. C means counts; FL2H denotes the maximal signal intensity in the second channel of fluorescence detection with a band pass of 585± 21 that is used to detect the phycoerythrin (PE) fluorescence; E

25 means empty.

FIG. 3 is also a Flow cytometry binding analysis of trypsinized NIH3T3 overexpressing wild-type EGFR (wt EGFR) and S464L EGFR mutant incubated with cetuximab (FIG. 3A) or panitumumab (FIG. 3B) as primary

30 antibodies and using a secondary antibody conjugated with phycoerythrin directed against human IgG. C means counts; FL2H denotes the maximal signal intensity in the second channel of fluorescence detection with a band pass of 585± 21 that is used to detect the phycoerythrin (PE) fluorescence; E means empty.

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FIG. 4 shows a Flow cytometry binding analysis of trypsinized NIH3T3 overexpressing wild-type EGFR (wt EGFR) and G465R EGFR mutant

incubated with cetuximab (FIG. 4A) or panitumumab (FIG. 4B) as primary antibodies and using a secondary antibody conjugated with phycoerythrin directed against human IgG. C means counts; FL2H denotes the maximal signal intensity in the second channel of fluorescence detection with a band

5 pass of 585± 21 that is used to detect the phycoerythrin (PE) fluorescence; E means empty.

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DETAILED DESCRIPTION OF THE INVENTION

In general, the following words or phrases have the indicated definition when
20 used in the description, examples and claims.

The term "therapy regimen" as used in the state of the art and also herein refers to any therapy intended to prevent, slow, arrest or reverse the growth of a precancerous lesion, cancer or a cancer metastasis. It includes
25 chemotherapy, radiation therapy, immunotherapy, monoclonal antibody therapy or other methods.

By "response" is to be understood any kind of improvement either clinical or non-clinical selected from, but not limited to, measurable reduction in tumour
30 size or evidence of disease or disease progression, stable disease, increase or elongation of progression of free survival or reduction in toxicity.

"Progression free survival" indicates the length of time during and after treatment that the cancer does not grow. Progression free survival includes
35 the amount of time patients have experienced a complete response or partial response, as well as the amount of time patients have experienced stable disease.

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"A complete response" to a therapy defines patients with valuable but non-measurable disease, whose tumour and all evidence of disease disappeared.

5   "A partial response" to a therapy defines patients with anything less than complete response.

"An anti-EGFR monoclonal antibody (anti-EGFR moAb)" relates to a monoclonal antibody and to a fragment thereof that are able to recognize

10   epitopes in the EGFR sequence protein. Approved moAb which recognize different epitopes of EGFR are cetuximab and panitumumab, but other moAb could be used in the therapy regimen for facing cancer disclosed in the present invention. Suitable antibody fragments include F(ab), F(ab'),Fv and nanobodies, among others.

15

The expression "genotype methods" includes all those methodologies and processes suitable for determining the genotype or, which is the same for identifying the nucleotide in a given position. Examples of said methodologies encompass Sanger sequencing, pyrosequencing, allele-specific PCR,

20   denaturing high pressure liquid chromatography (DHPLC), Allele Specific Primer Extension (ASPE), DNA biochips/microarrays and dynamic allele-specific hybridization (DASH).

For "protein sequencing methods" is to be understood any technique allowing

25   to determine the amino acid sequence of a protein, as well as which conformation the protein adopts and the extent to which it is complexed with any non-peptide molecules. The determination of amino acid composition may be performed by hydrolysis or separation of the amino acids. Known technologies include the Sanger sequencing, Edman degradation and mass

30   spectrometry.

If not indicated to the contrary, all sequences relating *EGFR* gene, mRNA variant and EGFR protein relate to the human one with the database accession numbers listed along the description. Also if not indicated to the

35   contrary, oligonucleotide sequences are shown in the 5'-3' direction, and peptide sequences are shown starting from the N-terminus amino acid ((also known as the amino-terminus, NH2-terminus, N-terminal end or amine-

terminus) of the peptide, according to the convention for writing peptide sequences.

All the amino acid sequences, as well as of oligonucleotides may be
5   synthesized following appropriate peptide or oligonucleotide chemical synthesis. Examples of peptide synthesis include solid-phase synthesis and liquid-phase synthesis, both processes coupling the carboxyl group or C-terminus of one amino acid to the amino group or N-terminus of another. Unintended reactions are avoided in solid-phase synthesis using protecting
10   groups, such as 9-fluorenylmethyloxycarbonyl (Fmoc) and Tert-butyloxycarbonyl (t-Boc). Alternatively, the peptides may be obtained by DNA recombinant technologies. Oligonucleotides may be obtained by solid-phase synthesis using phosphoramidite method and phosphoramidite building blocks derived from protected 2'-deoxynucleosides (dA, dC, dG, and T),
15   ribonucleosides (A, C, G, and U), or chemically modified nucleosides. Oligonucleotides may also be derived from DNA digestion with appropriate restriction enzymes.

As already explained above, prior art teachings show that mutations at domain
20   III of EGFR may help to map critical points for the interaction of moAbs (cetuximab and/or panitumumab). Nonetheless, these data may serve to detect specific epitopes but they are not concluding in terms of resistance to treatment, since only specific amino acid exchanges encompass this information (that of resistance, either primary or secondary resistance).
25   Particularly, acquired resistance to treatment is of great importance in order to modify the therapeutically approaches and avoid wasting time and efforts.

The present invention is based on novel mutations in the coding region of the *EGFR* gene. The novel mutations of the present invention are useful to predict
30   the response to moAb-based therapy of a patient with mCRC. In particular, they are useful to predict primary resistance and the appearance of a secondary resistance.

As already indicated above, each of the disclosed nucleotide changes lead to
35   the substitution to a cysteine at position 451 of SEQ ID NO: 2 (human EGFR protein), to a leucine at position 464 of SEQ ID NO: 2, to an arginine at position 465 of SEQ ID NO: 2, and to a threonine at position 467 of SEQ ID

NO: 2. All these particular mutations are located in a fragment from amino acid 450 to amino acid 470 of this SEQ ID NO: 2, said fragment hereweith named SEQ ID NO: 12.

5   The peptide according to the invention, with a length from 17 to 100 amino acids and comprising the sequence SEQ ID NO: 1 includes any of the mutations R451C or K467T.

In a particular embodiment, this peptide is selected from the group consisting

10   of: a sequence comprising SEQ ID NO: 1 wherein X^1 is R and X^2 is T; a sequence comprising SEQ ID NO: 1 wherein X^1 is C and X^2 is T; and a sequence comprising SEQ ID NO: 1 wherein X^1 is C and X^2 is K.

In a more particular embodiment, the peptide consists in SEQ ID NO: 1 and,

15   more particularly that SEQ ID NO: 1 selected from the group consisting of SEQ ID NO: 1 wherein X^1 is R and X^2 is T; SEQ ID NO: 1 wherein X^1 is C and X^2 is T; and SEQ ID NO: 1 wherein X^1 is C and X^2 is K. These sequences are represented by SEQ ID NO: 8 (RSLKEISDGDVIISGNT), SEQ ID NO: 9 (CSLKEISDGDVIISGNT) and SEQ ID NO: 10 (CSLKEISDGDVIISGNK).

20

In a particular embodiment, the peptide with a length from 17 to 100 amino acids and comprising the sequence SEQ ID NO: 1, further comprises SEQ ID NO: 4

25   NLCYANTINWKKLFGTSGGKTKIIX^3, wherein

X^3 is selected from S and R.

The peptide comprising both SEQ ID NO: 1 and SEQ ID NO: 4 corresponds,

30   in a particular embodiment, to a continuous amino acid sequence starting with SEQ ID NO: 1. This sequence has 42 amino acids and corresponds to a fragment of EGFR protein coded partially by exon 12 of the *EGFR* gene. It is represented by, or consists in SEQ ID NO: 5 (X^1SLKEISDGDVIISGNX^2NLCYANTINWKKLFGTSGGKTKIIX^3)

35

In another particular embodiment, the peptide with a length from 17 to 100 amino acids and comprising the sequence SEQ ID NO: 13, further comprises

SEQ ID NO: 4. This peptide comprising both SEQ ID NO: 13 and SEQ ID NO: 4 corresponds, in a particular embodiment, to a continuous amino acid sequence starting with SEQ ID NO: 13. It has 42 amino acids and corresponds to a fragment of EGFR protein coded partially by exon 12 of the

5 *EGFR* gene. It is represented by, or consists in SEQ ID NO: 14 (X^1SLKEISDGDVII$X^4$$X^5NX^2$NLCYANTINWKKLFGTSGGKTKII$X^3$)

Indeed, this SEQ ID NO: 5 includes any or all of the mutations R451C and K467T, and further it encompasses the option of including mutation S492R.

10 Thus, X^1, X^2 and X^3 have the same meaning as indicated above; and if X^1 is C, then X^2 is selected independently from K and T, and if X^1 is R, then X^2 is T.

Mutation S492R was firstly disclosed by the inventors in Montagut et al., (*supra*) as a key mutation for determining also resistance to moAb in cancers,

15 including metastasic colorectal cancer.

Besides, SEQ ID NO: 14 includes any or all of the mutations R451C, S464L, G465R and K467T, and further it encompasses the option of including mutation S492R. Thus, X^1, X^2, X^3, X^4 and X^5 have the same meaning as

20 indicated above, but at least one of X^1, X^2, X^4 or X^5 is, respectively, C, L, R or T.

In another particular embodiment, the peptide sequence comprising SEQ ID NO: 1 or SEQ ID NO: 13 has a length from 17 to 50 amino acids. In another

25 particular embodiment it has a length from 17 to 25 amino acids (that is 17, 18, 19, 20, 21, 22, 23, 24 or 25). In another most particular embodiment the peptide sequence has a length of 17 amino acids. In another particular embodiment it has a length of 21 amino acids, being any of SEQ ID NO: 1 or SEQ ID NO: 13 flanked in the N-terminal end by a leucine (L) and in the C-

30 terminal end by the tripeptide N-Asparagine-Leucine-Cysteine-C (abbreviated NLC)

In addition, and as will be depicted in the examples below, the inventors also detected a new mutation leading to resistance to moAb in cancers, including

35 metastasic colorectal cancer, namely a change of an isoleucine by a methionine at position 491 of SEQ ID NO: 2 (human EGFR protein). This mutation is herewith named I491M. The amino acid change I491M is the result

of the nucleotide change A→G at nucleotide 1473 (also known herein as A1473G) of the mRNA variant 1 of the *EGFR* gene (Codon ATA is changed to ATG)

5   New mutations identified in the present invention are alternatives, but may also be used in combination to assure a proper therapy selection.

The invention encompasses oligonucleotides coding for SEQ ID NO: 1 or SEQ ID NO: 13. In the particular embodiment of an oligonucleotide coding for SEQ
10   ID NO: 1 or SEQ ID NO: 13, optionally in combination with any embodiment above or below, said oligonucleotide further codes for SEQ ID NO: 4 and thus in another particular embodiment the oligonucleotide codes for SEQ ID NO: 5 or for SEQ ID NO: 14. Particular oligonucleotides are those consisting in nucleotide sequences coding for any of sequences SEQ ID NO: 8 to 10.
15   These oligonucleotides, as above exposed, may be used as hybridization probes for detecting the mutations.

The kit according to the invention comprises, besides the set of primers disclosed above, oligonucleotide probes for detecting wild-type or mutated
20   forms of *EGFR* gene coding for any of the mutations R451C and K467T. Examples of these probes for detecting mutated forms of *EGFR* gene, consists in oligonucleotides selected from those coding for any of SEQ ID NO: 1, 5, 8, 9 and 10.

25   The probes consisting in the oligonucleotides selected from those coding for any of SEQ ID NO: 1, 5, 8, 9 and 10 are nucleotide sequences comprising the several options of codon degeneracy in the corresponding mutation points.

Particular probes for the detection of the mutation R451C are those
30   complementary to the mutated region of the *EGFR*, wherein the nucleotide changes resulting in the mutation R451C of the present invention is located, being either the coding or complementary region of the gene. Thus, they hybridize with a fragment of the nucleotide sequence carrying the mutation, and allows detecting the nucleotide change C→T at position 1351 disclosed
35   above.

Particular probes for the detection of the mutation K467T are those

complementary to the mutated region of the *EGFR* wherein the nucleotide
changes resulting in the mutation K467T of the present invention is located,
being either the coding or complementary region of the gene. Thus, it
hybridizes with a fragment of the nucleotide sequence carrying the mutation,
5   and allows detecting the nucleotide change A→C at position 1400 disclosed
above.

Other particular oligonucleotide probes in the kit are for detecting wild-type or
mutated forms of *EGFR* gene coding for any of the mutations S464L and
10   G465R.

Particular probes for the detection of the mutation S464L are those
complementary to the mutated region of the *EGFR*, wherein the nucleotide
changes resulting in the mutation S464L of the present invention is located,
15   being either the coding or complementary region of the gene. Thus, they
hybridize with a fragment of the nucleotide sequence carrying the mutation,
and allows detecting the nucleotide change C→T at position 1391 disclosed
above.

20   Other particular probes for the detection of the mutation G465R are those
complementary to the mutated region of the *EGFR*, wherein the nucleotide
changes resulting in the mutation G465R of the present invention is located,
being either the coding or complementary region of the gene. Thus, they
hybridize with a fragment of the nucleotide sequence carrying the mutation,
25   and allows detecting the nucleotide change G→A at position 1393 disclosed
above.

For "nucleotide sequence carrying the mutation" is to be understood any of the
coding or complementary DNA chains in the DNA genomic structure, as well
30   as an mRNA chain which is going to be translated.

The kits of the invention may, optionally in combination with any embodiment
above or below, further comprise additional reagents for detecting mutations
in the *KRAS* and/or *PIK3CA*, and/or *BRAF* genes, and/or additional mutations
35   in *EGFR* gene. These reagents include specific primers for detecting particular
mutations in all these genes, and particularly mutations associated with
resistance to a therapy regimen comprising cetuximab and/or panitumumab.

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Other reagents included in the kit relate to oligonucleotide probes that can hybridize either with the wild-type or mutated forms of all these genes.

Thus, in a particular embodiment, optionally in combination with any of the
5   embodiments above or below, the kit comprises tools and means (reagents) to detect the mutations in KRAS selected from the group consisting of G12A; G12C; G12D; G12R; G12S; G12V; G13A; G13C, G13D; G13V as defined by Karapetis et al., "K-ras Mutations and Benefit from Cetuximab in Advanced Colorectal Cancer", The New England Journal of Medicine - 2008, Vol. 359,
10   pp.: 1757-1765. All these mutations are placed on codons 12 and 13 of the protein sequence of K-ras identified with the GenBank accession number NP_004976.2 from 24.07.2011 (named GTPase KRas isoform b precursor) and NP_203524.1 from 24.07.2011 (named GTPase KRas isoform a precursor. In another preferred embodiment the kit comprises reagents to
15   detect mutations in exons 9 and 20 of the PIK3CA gene that codifies for the PIK3CA protein with the GenBank accession number NP_006209.2 from 17.07.2011; and/or the V600E mutation placed on codon 600 of the protein sequence of BRAF identified with the GenBank accession number NP_004324.2 from 24.07.2011. In another preferred embodiment the kit
20   comprises means (reagents) to detect mutation S492R in EGFR protein of SEQ ID NO: 2. In another preferred embodiment the kit comprises means (reagents) to detect mutation I491M in EGFR protein of SEQ ID NO: 2.

The kits of the invention are in particular for use in the prediction of the
25   response of a subject to a therapy regimen comprising anti-EGFR monoclonal antibodies, in particular cetuximab and/or panitumumab. More particularly, the subject is affected with cancer, and the cancer is metastatic colorectal cancer.

The invention relates according to one aspect of the invention to an *in vitro*
30   method of predicting the response of a subject therapy regimen comprising cetuximab and/or panitumumab, wherein the method comprises:
(i) determining in a sample taken from the subject and by means selected from the group consisting of genotype methods, and/or protein sequencing methods if mutations are present or absent in a fragment defined by SEQ ID
35   NO: 12, which is a fragment from amino acid 450 to amino acid 470 of the consensus wild-type amino acid sequence of human EGFR of SEQ ID NO: 2; and ii) correlating the presence of any mutation identified in step i) with

resistance of the subject to the therapy regimen comprising cetuximab, or correlating the absence of mutations in step i) with response of the subject to therapy regimen comprising panitumumab.

5   In a particular embodiment of the *in vitro* method in step (i) there are determined within the SEQ ID NO: 12 if at least one of the following mutations are present or absent: a change of an arginine by a cysteine at corresponding position 451 of the SEQ ID NO:2; a serine by a leucine at corresponding position 464 of the SEQ ID NO: 2; a glycine by an arginine at the

10   corresponding position 465 of the SEQ ID NO: 2; and of a lysine by a threonine at the corresponding position 467 of the SEQ ID NO: 2.

In another particular embodiment, the in vitro method of predicting the response of a subject therapy regimen comprising cetuximab and/or

15   panitumumab comprises:
i) determining by means selected from the group consisting of genotype methods, and/or protein sequencing methods the presence or absence of at least one of the following amino acids:
a cysteine at position 451 of the amino acid sequence corresponding to SEQ

20   ID NO: 2, and a threonine at position 467 of the amino acid sequence corresponding to SEQ ID NO: 2, in a sample taken from the subject; and ii) correlating the presence of any of the amino acids identified in step i) with resistance of the subject to the therapy regimen comprising cetuximab, or correlating the absence of all of these amino acids in step i) with response of

25   the subject to therapy regimen comprising panitumumab.

This particular embodiment encompasses determining if SEQ ID NO: 1 is present in the sample of the subject, and further correlating in step ii) the presence of any of the mutations R451C and/or K467T with resistance of the

30   subject to the therapy regimen comprising cetuximab, or correlating the absence of all of these amino acids in step i) with response of the subject to therapy regimen comprising panitumumab.

In a more particular embodiment of the method, optionally in combination with

35   any embodiments above or below step i) encompasses determining if additionally SEQ ID NO: 4 is present in the sample of the subject. Thus, after determining if any of the mutations R451C and/or S464L and/or G465R and/or

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K467T is present, the method includes also determining if mutation S492R is present in EGFR protein.

Detection of mutation S492R relates to the particular embodiment of the in
5   vitro method, optionally in combination with any of the embodiments below or above, wherein step i) further comprises determining the presence or absence of an arginine at position 492 of the amino acid sequence corresponding to SEQ ID NO: 2, and wherein in step ii) the additional presence of the arginine identified in step i) is correlated with resistance of the subject to the therapy
10   regimen comprising cetuximab.

In another particular embodiment, optionally in combination with any embodiments above or below, the in vitro method of predicting the response of a subject therapy regimen comprising cetuximab and/or panitumumab,
15   further comprises determining in step (i) the presence or absence of a methionine at position 491 of the amino acid sequence corresponding to SEQ ID NO: 2, and wherein in step ii) the additional presence of the methionine identified in step i) is correlated with resistance of the subject to the therapy regimen comprising cetuximab.
20

In another particular embodiment, optionally in combination with any of the embodiments above or below, step i) is performed with a set of primers consisting of SEQ ID NOs: 6 and 7.

25   Further to the amplification with the above mentioned primers, in a particular embodiment step i) is performed by genotype methods. In another most particular embodiment, optionally in combination with any embodiment above or below, said genotype method is selected from Sanger sequencing, pyrosequencing, droplet digital PCR (ddPCR), allele-specific PCR, denaturing
30   high pressure liquid chromatography (DHPLC), Allele Specific Primer Extension (ASPE), DNA biochips/microarrays and dynamic allele-specific hybridization (DASH). In even a most particular embodiment, the genotype method is pyrosequencing.

35   Examples of pyrosequencing genotype methods include, among others, the next generation sequencing (NGS) methods known as 454 High though output pyrosequencing, sequencing by synthesis (Illumina), and Chain termination

sequencing (Sanger sequencing).

Alternatively, step i) includes specific probes to detect wild-type or mutated points as genotyping method of the amplified regions. Particular probes are

5   those oligonucleotides coding for SEQ ID NO: 1, SEQ ID NO: 5, SEQ ID NO: 8, SEQ ID NO: 9, and SEQ ID NO: 10. All these oligonucleotides are complementary to nucleotide sequences of mutated *EGFR* gene.

The *in vitro* method for predicting the response of a subject to a therapy

10   regimen comprising cetuximab and/or panitumumab, is carried out in a sample comprising the tumour, in which the nucleotide changes in the *EGFR* gene of the present invention can be detected. In cases of mCRC, the sample can be used directly as obtained from the source or following a pre-treatment of the sample. The sample may additionally comprise normal tissue adjacent to said

15   tumour. Accordingly, in case of mCRC the sample is selected from a primary colorectal cancer biopsy or a biopsy of a metastasis thereof. In other words, the sample may be a biopsy from colorectal cancer samples, including primary tumors and metastases. In a preferred embodiment, the metastasis is in the liver tissue.

20

Patients comprising any of the new identified mutations are likely to show response to a therapy regimen not comprising cetuximab as measured by any suitable clinical or sub-clinical increase or elongation in progression free survival.

25

In a preferred embodiment the therapy regimen is cetuximab alone or in combination with a chemotherapy regimen based on irinotecan, oxaliplatin and/or 5-fluorouracil (5-FU or 5FU). In a preferred embodiment the therapy regimen is panitumumab alone or in combination with a chemotherapy

30   regimen based on irinotecan, oxaliplatin and/or 5-fluorouracil.

The invention further provides also methods for deciding and/or recommending a therapy regimen for subjects affected with cancer, preferably mCRC, comprising: i) determining by means selected from the group

35   consisting of genotype methods, and/or protein sequencing methods the presence or absence of at least one of the following amino acids:
a cysteine at position 451 of the amino acid sequence corresponding to SEQ

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ID NO: 2, and a threonine at position 467 of the amino acid sequence corresponding to SEQ ID NO: 2, in a sample taken from the subject; and ii) recommending the administration to said subject of an effective amount of cetuximab, or a composition thereof, if all the mutations are absent, or
5   panitumumab, or a composition thereof, if at least one of the mutations is present.

The invention encompasses also an in vitro method for determining the acquired resistance to a therapy regimen comprising cetuximab, the method
10 comprising, in a particular embodiment of this aspect:
i) determining by means selected from the group consisting of genotype methods, and/or protein sequencing methods, the presence or absence of at least one of the following amino acids:
a cysteine at position 451 of the amino acid sequence corresponding to SEQ
15 ID NO: 2, and a threonine at position 467 of the amino acid sequence corresponding to SEQ ID NO: 2, in a sample taken from the subject; and
ii) correlating the presence of any of the amino acids identified in step i) with acquired resistance of the subject to the therapy regimen comprising cetuximab, or correlating the absence of all of these amino acids in step i) with
20 response of the subject to therapy regimen comprising panitumumab.

As above exposed, the invention provides also an in vitro method of identifying, in a sample taken from a subject, the presence or absence of a cysteine at position 451 of the amino acid sequence corresponding to SEQ ID
25 NO: 2; and/or the presence or absence of a threonine at position 467 of the amino acid sequence corresponding to SEQ ID NO: 2 by means selected from the group consisting of genotype methods, and/or protein sequencing methods. In a preferred embodiment the in vitro method of identifying the presence or absence of one or both of these mutations in SEQ ID NO: 2,
30 further comprises identifying the presence or absence of an arginine at position 492 of SEQ ID NO: 2 by means selected from the group consisting of genotype methods, and/or protein sequencing methods.

In a particular embodiment, optionally in combination with any embodiment
35 above or below, the method of identifying the presence or absence of a cysteine at position 451 of the amino acid sequence corresponding to SEQ ID NO: 2; and/or the presence or absence of a leucine at position 464 of the

amino acid sequence corresponding to SEQ ID NO: 2; and/or the presence or absence of an arginine at position 465 of the amino acid sequence corresponding to SEQ ID NO: 2; and/or the presence or absence of a threonine at position 467 of the amino acid sequence corresponding to SEQ

5   ID NO: 2, is carried out by determining the sequence of SEQ ID NO: 2 up to position 467 by means selected from the group consisting of genotype methods, and/or protein sequencing methods. In a preferred embodiment, the method is carried out by determining the sequence of SEQ ID NO: 2 from position 450 to 470 (SEQ ID NO: 12), and more preferably from 451 to

10   position 467. For "determining a sequence up to a position" is to be understood that the sequencing is performed from oligonucleotide or amino acid from position 1 of said sequence to the position (nucleotide or amino acid) of interest (in this particular case, to amino acid 467 or to the nucleotide leading to this amino acid).

15

Throughout the description and claims the word "comprise" and variations of the word, are not intended to exclude other technical features, additives, components, or steps. Furthermore, the word "comprise" encompasses the case of "consisting of". Additional objects, advantages and features of the

20   invention will become apparent to those skilled in the art upon examination of the description or may be learned by practice of the invention. The following examples are provided by way of illustration, and they are not intended to be limiting of the present invention. Furthermore, the present invention covers all possible combinations of particular and preferred embodiments described

25   herein.

EXAMPLES

Example 1.Tumor samples and patients

30

There was performed a proof-of-concept approach to study and characterize the presence of heterogeneous mutations emerging after cetuximab-based therapy in routine clinical practice. All mCRC consenting patients treated with anti-EGFR moAb at Parc de Salut Mar Biobank (MARBiobanc, Barcelona,

35   Spain) Hospital del Mar institution between January 2010 and June 2013 were included in this study. In 34 patients the specimens were prospectively collected for this study and in 3 patients there were analyzed sequential

biopsies taken in the past in the context of their routine clinical management. In the analysis, there were only included patients that had good quality paired pre- and post- treatment biopsies and that had acquired resistance to anti-EGFR based-therapy defined as progression disease following a) complete

5   response or partial response or b) stable disease for more than 16 weeks (7-9). Response was evaluated according to the Response Evaluation Criteria in Solid Tumors (RECIST)(Eisenhauer et al., "New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1)", Eur J Cancer 2009, Vol. 45(2):228-247). Tumoral biopsy obtained during the regular diagnosis

10   procedure was used as the pre-treatment (initial) sample. In most cases this sample was obtained from the primary tumor during routine colonoscopy. A second initial biopsy from a metastatic site is not routine and was not performed unless necessary for pathologic diagnosis. The study included re-biopsy following treatment failure in patients that consented to this extra

15   procedure. Re-biopsies at the time of progression were obtained from the most accessible lesion with less potential risk of related complications for the patient according to ethical considerations. Serum samples were collected before starting the cetuximab- based therapy and at the time of progression. When a mutation was detected in the post-treatment biopsy sample, the

20   serum sample from that same patient was analyzed for that specific mutation. In this study, there are included nine cases (patients #21 to #28 and patient #36) that had been previously assessed for *EGFR* S492R, *KRAS* exon 2, *BRAF* V600E and *PIK3CA* mutations by direct sequencing and that in the current work were analyzed for the mutations reported above (R451C and

25   K467T) using deep-sequencing technology. Biological samples were obtained from Parc de Salut Mar Biobank (MARBiobanc). This study was approved by the local Ethics Board (CEIC-2012/4741/I). All participating patients signed written informed consent.

30   For the KRAS, BRAF, NRAS, PIK3CA and EGFR sequencing, DNA extraction from tumoral samples was performed as previously described by Diaz et al. "The molecular evolution of acquired resistance to targeted EGFR blockade in colorectal cancers", Nature-2012, Vol No. 486, pp.:537-40. Mutational analysis of *KRAS* (exons 2, 3 and 4), *BRAF* (exon15), *NRAS* (exons 2 and 3), *PIK3CA*

35   (exons 9 and 20) and *EGFR (exon 12, 13)* was performed by Sanger sequencing using BigDye v3.1 (Applied Biosystems, Foster City, CA) following the manufacturer's instructions and analyzed on a 3500Dx Genetic Analyzer

(Applied Biosystems). All cases were also screened by pyrosequencing using a Next Generation Sequencing (NGS) 454 GS Junior platform (Roche Applied Science, Mannheim, Germany). Moreover, processed and quality-filtered reads were analyzed using the GS Amplicon Variant Analyzer software

5   version 2.5p1 (Roche). Mutations detected by NGS were confirmed by competitive allele-specific TaqMan® PCR (CAST-PCR, Applied Biosystems) when specific assays were available.

Primers for EGFR sequences were those disclosed above and defined by the

10   set of primers consisting in SEQ ID NOs: 6 and 7. The pair of SEQ ID NO: 6 and SEQ ID NO: 7 served for amplifying entirely exon 12 that could contain mutations R451C and K467T, and some intron flanking regions. This sequence is represented by SEQ ID NO: 11:

15   <u>caaagttttcagggatacattgtttttatattttcaccacatgatttttcttctctccaatgtag</u>TGGTCAGTTT
TCTCTTGCAGTCGTCAGCCTGAACATAACATCCTTGGGATTACGCTCCCT
CAAGGAGATAAGTGATGGAGATGTGATAATTTCAGGAAACAAAAATTTGT
GCTATGCAAATACAATAAACTGGAAAAAACTGTTTGGGACCTCCGGTCAG
AAAACCAAAATTATAAGCAACAGAGGTGAAAACAGCTGCAgtaagtcaccgcttt
20   ctgtttagtttatggagttggttctaatgggtcctttatttgtatttag<u>aatattgaagggctattcccatttaa</u>;

wherein underwritten nucleotides correspond to the sequences identical (for SEQ ID NO: 6) or complementary (for SEQ ID NO: 7) to the primers of the set, capital letters relate to exon 12 and non-capital are intron fragments.

25

Amplification was performed under the following conditions: 95°C for 10 minutes ; 40 cycles of 95°C, 1 minute, 60°C, 1' 30'' and 72°C 1 minute; and a final extension of 10 minutes at 72°C.

30   Besides, it was performed a Fluorescence in situ Hybridization (FISH). FISH was performed whenever there was sufficient remaining material following analysis of mutations. Amplification of *EGFR* was assessed by fluorescent *in situ* hybridization (FISH) using the LSI *EGFR/CEP7* probe (Abbott Molecular Inc., DesPlaines, IL), as previously described (for example in document such

35   as Salido et al., "Increased ALK gene copy number and amplification are frequent in non-small cell lung cancer", J Thorac Oncol - 2011, Vol. No. 6, pp.:21-7). *KRAS* amplification was analyzed using a dual colour FISH assay

with *KRAS*/CEP12 probe (Abnova). Samples with a ratio *KRAS*/CEP12 greater than 3, in at least 10% of 50 analysed nuclei were scored. When the average number of chromosome 12 exceeded 2.5 or 4 per cell, the case was considered polysomic or high-polysomic respectively.

Example 2. Presence of R451C and K467T EGFR mutations and acquired resistance to cetuximab

As depicted in FIG 1, which is a plot of two different displays of a Next Generation Sequencing (NGS) 454 GS Junior platform (Roche Applied Science, Mannheim, Germany), shows that some patients acquired mutations in *EGFR* ectodomain following treatment with cetuximab. FIG. 1 (A) shows a patient #31, in which the post-treatment tumor sample had acquired an A -> C substitution at nucleotide 1400 of *EGFR* gene that was not present in the pre-treatment biopsy, causing a substitution of a lysine to a threonine at amino acid 467 (K467T). The substitution is detected by the genotyping method and visualized (arrow) by means of a double pick (band or curve) in this position. Lower pick corresponded to the C nucleotide.

On the other side, in FIG. 1 (B) it is shown the display of the sequencing process (Read minus the Reference) from patient #35, in which a C -> T substitution at nucleotide 1351 of the EGFR gene was detected in the post-treatment sample, leading to a substitution of a arginine to a cysteine at amino acid 451 (R451C). Substitution is also marked with an arrow and in this case the change is visualized by a negative value.

Example 3. Mutations in SEQ ID NO: 12 (fragment from amino acid 450 to amino acid 470 of SEQ ID NO: 2) involve resistance to cetuximab treatment.

3A: EGFR ectodomain mutations and acquired resistance to cetuximab in CRC cell models

It was previously reported that acquisition of resistance in CRC cells is associated with emergence of KRAS, BRAF and NRAS activating mutation.. To discover additional mechanisms of resistance to EGFR blockade 5 CRC

cell lines were exploited (DiFi, LIM1215, HCA-46, NCIH508, OXCO-2 and CCK81), which are highly sensitive to cetuximab. All these cell lines are wild type for KRAS, NRAS, BRAF and PIK3CA with the exception of NCIH508, which displays the p.E545K PIK3CA mutation. Altogether, these cell models

5   recapitulate the molecular features of tumors from CRC patients likely to respond to anti EGFR therapies. For each line, at least five million cells were exposed continuously to cetuximab until resistant populations emerged. To define molecular mechanisms underlying acquisition of resistance, it was initially performed Sanger sequencing of genes involved in regulation of the

10   EGFR signalling pathway (EGFR, KRAS, BRAF, NRAS, and PIK3CA). In accordance with previous reports, resistant populations often displayed KRAS, BRAF and NRAS mutations (See Misale et al. "Blockade of egfr and mek intercepts heterogeneous mechanisms of acquired resistance to anti-egfr therapies in colorectal cancer", Sci Transl Med- 2014;6:224ra226). All of

15   these alleles were detected in the resistant cells but not in the corresponding parental population from which they originated. Importantly, in several occasions multiple genetic alterations were concomitantly present in the resistant cell population indicating their polyclonal status. To assess the molecular features of individual clones it was therefore performed limited cell

20   dilutions of LIM1215 and CCK81 as these cell lines are amenable to this procedure. Single clones were then subjected to Sanger sequencing for candidate genes (EGFR, KRAS, BRAF, NRAS, and PIK3CA). Notably, mutation profiling of clones identified three novel EGFR variants: S464L, G465R and I491M. Mutations S464L, G465R, together with mutations of

25   Example 2 (R541C and K467T) are located in SEQ ID NO: 12 (a fragment defining part of the cetuximab binding epitope). Considering that the resistant derivatives are polyclonal, and in light of the limited sensitivity of the Sanger sequencing method, it was postulated that variants present in less than 20% of the cell populations might have remained undetected. To identify mutations

30   present at low frequency it was employed droplet digital PCR (ddPCR) which is known to have a mutant/wild type sensitivity of 1:20000. ddPCR probes were designed and individually validated using control mutant DNA to detect EGFR variants previously identified in tumor biopsy or cell lines. This analysis unveiled the presence 3 new EGFR variants (S464L, G465R, and I491M) that

35   were not detected by Sanger sequencing in resistant cell populations. ddPCR could not be performed in tissue samples because there was no sufficient material left. Overall, the mutational landscape of cell lines with acquired

resistance to cetuximab, recapitulate the molecular profiles of tumors that relapsed upon cetuximab treatment.

ddPCR™ Supermix for Probes (Bio-Rad™) using *KRAS, NRAS, BRAF* and *EGFR* assay (PrimePCR™ ddPCR™ Mutation Assay, Bio-Rad and custom designed). ddPCR was performed according to manufacturer's protocol and the results reported as percentage or fractional abundance of mutant DNA alleles to total (mutant plus wild type) DNA alleles. 8 to 10 μl of DNA template was added to 10 μl of ddPCR™ Supermix for Probes (Bio-Rad) and 2 μl of the primer/probe mixture. This 20 μl sample was added to 70 μl of Droplet Generation Oil for Probes (Bio-Rad) and used for droplet generation. Droplets were then thermal cycled with the following conditions: 5 minutes at 95°C, 40 cycles of 94°C for 30s, 55°C for 1 minute followed by 98°C for 10 minutes (Ramp Rate 2°C/sec). Samples were then transferred to a QX200™ Droplet Reader (Bio-Rad) for fluorescent measurement of FAM and HEX probes. Gating was performed based on positive and negative controls, and mutant populations were identified. Fractional Abundances of the mutant DNA in the wild-type DNA background were calculated for each sample using QuantaSoft software (Bio-Rad). Multiple replicates (minimum of four) were performed for each sample. ddPCR analysis of normal control gDNA from cell lines and no DNA template (water) controls were performed in parallel with all the samples, including again multiple replicates as a contamination-free control.
EGFR probes and primers sequences are available upon request.

Cell culture and generation of resistant cells utilized herein has already been previously described (see Misale S et al. Emergence of KRAS mutations and acquired resistance to anti-EGFR therapy in colorectal cancer. Nature. 2012;486:532-6; Misale S, Arena S et al Blockade of EGFR and MEK intercepts heterogeneous mechanisms of acquired resistance to anti-EGFR therapies in colorectal cancer. Sci Transl Med. 2014;6:224ra26). CCK81 cells were cultured in MEM medium (Invitrogen) supplemented with 5 % FBS, 2mM L-glutamine, antibiotics (100U/mL penicillin and 100 mg/mL streptomycin) and grown in a 37°C and 5% CO2 air incubator. CCK81 cetuximab-resistant derivatives were obtained by increasing the cetuximab dosage stepwise from 680 nM to 1.4 μM during the course of six months.

3B: Presence of S464L, G465R and K467T EGFR mutation and resistance to cetuximab

To establish whether the S464L, G465R and K467T EGFR mutations of the invention were responsible for the observed resistance to cetuximab, full-length wild-type EGFR and any of the S464L, G465R or K467T EGFR mutations were ectopically expressed in cultured NIH3T3 mouse embryonic fibroblast cell line that lack detectable endogenous EGFR expression.

EGFR was stimulated with its natural ligand EGF in the presence of cetuximab or panitumumab in transfected cells. Antibody binding was analyzed by flow cytometry using a secondary antibody to human IgG conjugated with phycoerythrin (PE). NIH 3T3 cells expressing the empty vector were used as a negative control (EMPTY). The percentage of cells binding to the antibody are shown in the two-dimensional dot plots of FIGs. 2-4. In this FIGs. 2-4, cell counts (C of "counts",Y-axis) in the FL2H channel of the fluorescence detection are plotted for the assay with cetuximab (FIG. 2 A, 3A and 4A) and for the assay with panitumumab (FIG. 2B, 3B and 4B).

In wild-type EGFR cells (EGFRWT in FIGs. 2-4 A/B), both cetuximab and panitumumab inhibited EGFR activation, whereas in cells carrying the K467T mutation (EGFR_K467T in FIG. 2 A/B), S464L (EGFR_S464L in FIG. 3 A/B) and G465R (EGFR_G465R in FIG. 4 A/B) panitumumab, but not cetuximab, effectively blocked EGF-induced EGFR activation. EMPTY is the negative control (EGFR non-expressing cells)
For the DNA constructs, the pLX301-EGFR WT construct, a generous gift from Dr. C. Sun and Prof R. Bernards (NKI, Amsterdam), was constructed from pLX301 (Addgene®). *EGFR* mutants containing the 4 point mutations (R451C, S464L, G465R, and K467T) were constructed using the QuikChange® II site-directed mutagenesis kits from Agilent™ Technologies with pLX301-EGFR WT plasmid as the template DNA. The presence of mutations was confirmed by DNA sequencing.

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CLAIMS

1. A peptide with a length from 17 to 100 amino acids and comprising the
sequence SEQ ID NO: 13

X^1SLKEISDGDVII$X^4$$X^5NX^2$, wherein

X^1 is selected from R and C;
X^4 is selected from S and L;
X^5 is selected from G and R;
X^2 is selected from K and T; and wherein at least one of X^1, X^4, X^5
and X^2 is, respectively, C, L, R or T.

2. The peptide according to claim 1, with a length from 17 to 100 amino acids
and comprising the sequence SEQ ID NO: 1

X^1SLKEISDGDVIISGNX^2, wherein:

X^1 is selected from R and C;
X^2 is selected from K and T; and

wherein if X^1 is C, then X^2 is selected independently from K and T, and
if X^1 is R, then X^2 is T.

3. The peptide according to claim 1 or 2, said peptide further comprising SEQ
ID NO: 4

NLCYANTINWKKLFGTSGGKTKIIX^3, wherein

X^3 is selected from S and R.

4. The peptide according to claim 3, which comprises sequence SEQ ID NO: 5

X^1SLKEISDGDVIISGNX^{2}NLCYANTINWKKLFGTSGGKTKIIX^3, wherein

X^1, X^2 and X^3 have the same meaning as in claims 1 and 2; and

wherein if X^1 is C, then X^2 is selected independently from K and T, and if X^1 is R, then X^2 is T.

5. The peptide according to any one of claims 1-3, which comprises sequence SEQ ID NO: 14

X^1SLKEISDGDVIIX^{4}X^{5}NX^{2}NLCYANTINWKKLFGTSGGKTKIIX^3, wherein

X^1, X^2 and X^3, X^4, and X^5 have the same meaning as in claims 1, 2 and 3; and

wherein at least one of X^1, X^4, X^5 and X^2 is, respectively, C, L, R or T.

6. An oligonucleotide comprising a sequence coding for a peptide with an amino acid sequence SEQ ID NO: 1 or for a peptide with an amino acid sequence SEQ ID NO: 13.

7. The oligonucleotide according to claim 6, further coding for a peptide with an amino acid sequence SEQ ID NO: 4.

8. The oligonucleotide according to claim 6 or 7, comprising a sequence coding for a peptide with an amino acid sequence SEQ ID NO: 5 or for a peptide with an amino acid sequence SEQ ID NO: 14.

9. A set of primers consisting of SEQ ID NOs: 6 (CAAAGTTTTCAGGGATACATTGTTTTT) and 7 (TTAAATGGGAATAGCCCTTCAATATT).

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10. A kit which comprises a primer consisting of SEQ ID NO: 6 and a primer consisting of SEQ ID NO: 7.

11. The kit of claim 10, further comprising an oligonucleotide as defined in
any one of claims 6-8.

12. The kit according to claim 10 or 11, further comprising specific primers or probes for detecting other mutations in the EGFR signaling pathway, the mutations selected from:
- *KRAS* mutations G12A; G12C; G12D; G12R; G12S; G12V; G13A; G13C, G13D; or G13V;
- *PIK3CA* mutations in exon 9 or exon 20;
- *BRAF* mutation V600E, or
- *EGFR* mutations S492R or I491M.

13. The kit as defined in any one of claims 10 to 12, for use in the prediction of the response of a subject to a therapy regimen comprising cetuximab and/or panitumumab.

14. An *in vitro* method of predicting the response of a subject therapy regimen comprising cetuximab and/or panitumumab, wherein the method comprises
(i) determining in a sample from the subject and by means selected from the group consisting of genotype methods, and/or protein sequencing methods, if mutations are present or absent in a fragment defined by SEQ ID NO: 12, which is a fragment from amino acid 450 to amino acid 470 of the consensus wild-type amino acid sequence of human EGFR of SEQ ID NO: 2, said mutations being at least one of a change of an arginine by a cysteine at corresponding position 451 of the SEQ ID NO:2; a serine by a leucine at corresponding position 464 of the SEQ ID NO: 2; a glycine by an arginine at the corresponding position 465 of the SEQ ID NO: 2; and of a lysine by a threonine at the corresponding position 467 of the SEQ ID NO: 2; and
ii) correlating the presence of any mutation identified in step i) with resistance of the subject to the therapy regimen comprising cetuximab, or correlating the absence of mutations in step i) with response of the subject to therapy regimen comprising panitumumab.

15. The *in vitro* method according to claim 14, wherein step i) is performed with a set of primers as defined in claim 9, or with a kit as defined in any one of claims 10 to 12.

16. The *in vitro* method according to any one of claims 14-15, comprising in step i) further determining whether a change of a serine by an arginine is present or absent at position 492 of the amino acid sequence corresponding to SEQ ID NO: 2, and wherein in step ii) the additional presence of the arginine identified in step i) is correlated with resistance of the subject to the therapy regimen comprising cetuximab.

17. An *in vitro* method of selecting therapy for a subject comprising (i) determining in a sample from the subject and by means selected from the group consisting of genotype methods, and/or protein sequencing methods, if mutations are present or absent in a fragment defined by SEQ ID NO: 12, which is a fragment from amino acid 450 to amino acid 470 of the consensus wild-type amino acid sequence of human EGFR of SEQ ID NO: 2, said mutations being at least one of a change of an arginine by a cysteine at corresponding position 451 of the SEQ ID NO:2; a serine by a leucine at corresponding position 464 of the SEQ ID NO: 2; a glycine by an arginine at the corresponding position 465 of the SEQ ID NO: 2; and of a lysine by a threonine at the corresponding position 467 of the SEQ ID NO: 2; and ii) selecting a therapy regimen that does not include cetuximab if any mutations are identified as present in step i), or selecting a therapy regimen comprising panitumumab in the absence of mutations identified in step i).

18. The *in vitro* method according to claim 17, wherein step i) is performed with a set of primers as defined in claim 9, or with a kit as defined in any one of claims 10 to 12.

19. The *in vitro* method according to any one of claims 17-18, wherein step i) further comprises determining the presence or absence of an arginine at position 492 of the amino acid sequence corresponding to SEQ ID NO: 2, and ii) selecting a therapy regimen that does not include cetuximab if presence of an arginine at position 492 is determined.

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20. An *in vitro* method of identifying, in a sample from a subject, the presence or absence of a cysteine at position 451 of the amino acid sequence corresponding to SEQ ID NO: 2; the presence or absence of a leucine at position 464 of the amino acid sequence corresponding to SEQ ID NO: 2; the presence or absence of an arginine at position 465 of the amino acid sequence corresponding to SEQ ID NO: 2; or the presence or absence of a threonine at position 467 of the amino acid sequence corresponding to SEQ ID NO: 2, the method comprising determining the sequence of SEQ ID NO: 2, at least from position 450 to position 470.

21. An *in vitro* method of identifying, in a sample from a subject, the presence or absence of a cysteine at position 451 of the amino acid sequence corresponding to SEQ ID NO: 2; the presence or absence of a leucine at position 464 of the amino acid sequence corresponding to SEQ ID NO: 2; the presence or absence of an arginine at position 465 of the amino acid sequence corresponding to SEQ ID NO: 2; or the presence or absence of a threonine at position 467 of the amino acid sequence corresponding to SEQ ID NO: 2, the method comprising determining the amino acid at positions 451, 464, 465, or 467 by means selected from the group consisting of genotype methods, and protein sequencing methods.

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(A)
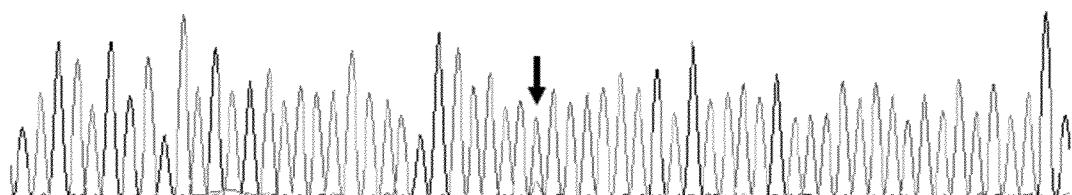
A>C
AAA>ACA
Lys>Thr
K467T

(B)
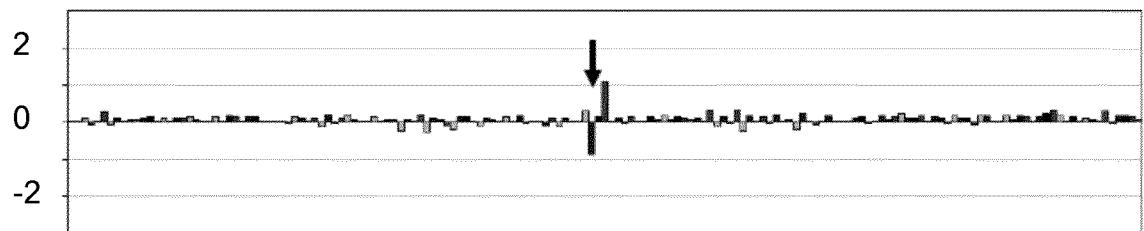
C>T
CGC>TGC
Arg>Cys
R451C

<u>**FIG. 1**</u>

(A)

EMPTY   EGFRWT

EGFR K467T

(B)

EMPTY   EGFRWT

EGFR_K467T

FIG. 2

(A)
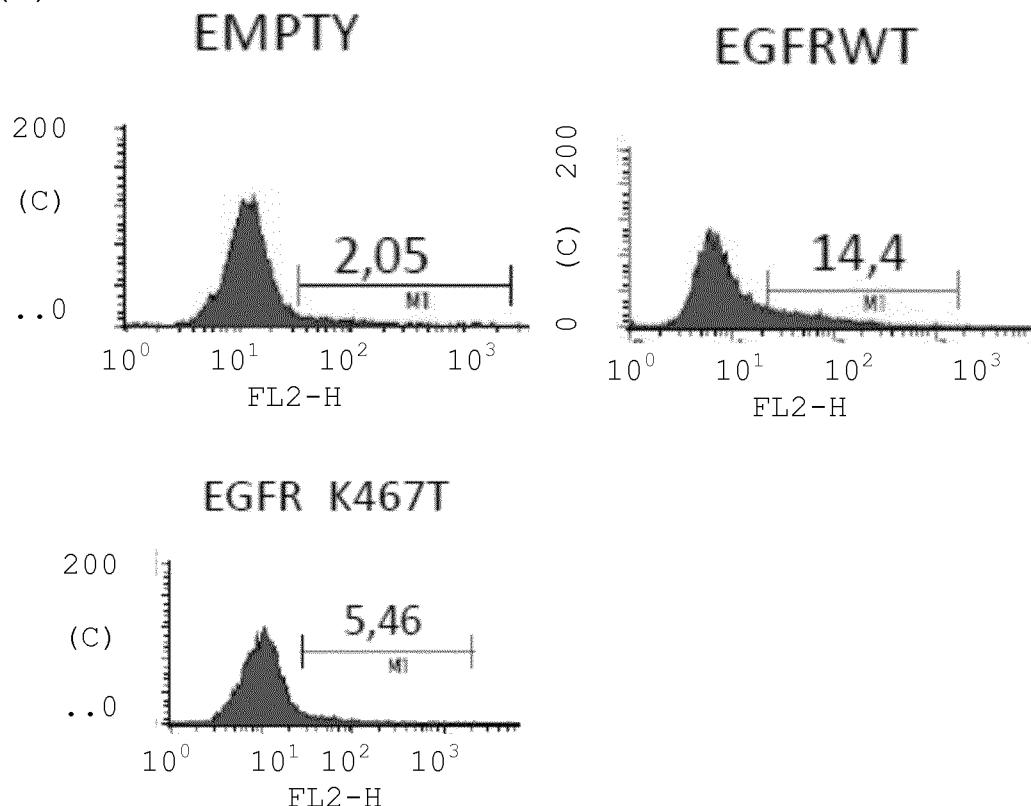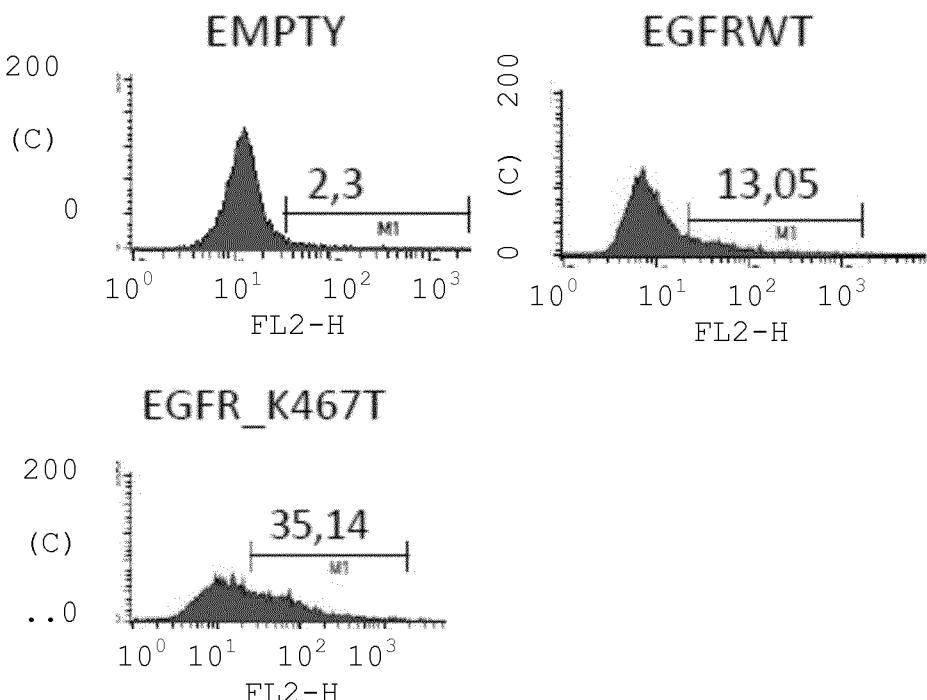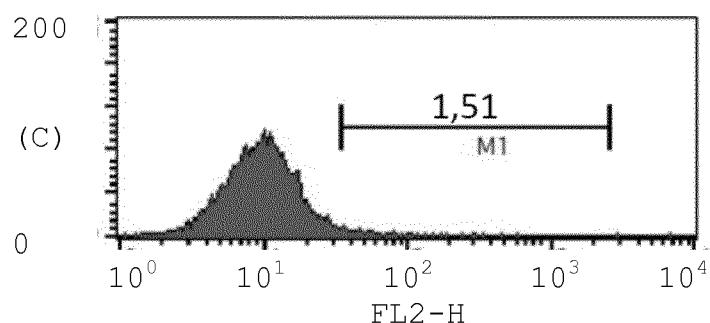
Empty

EGFRWT

EGFR_S464L

FIG. 3

(B)
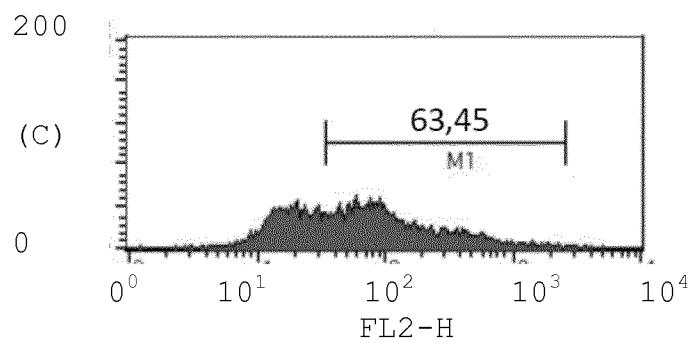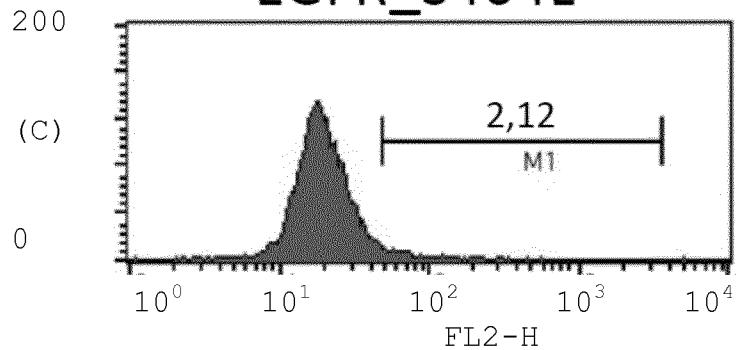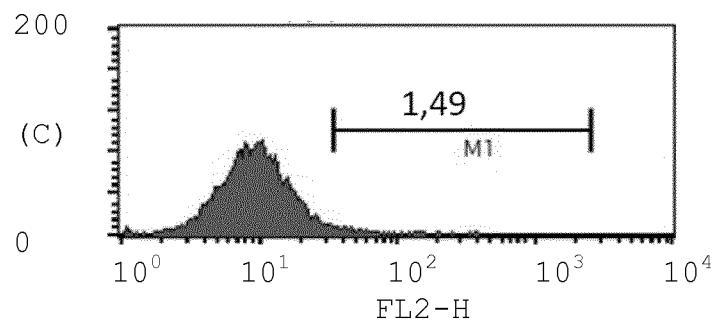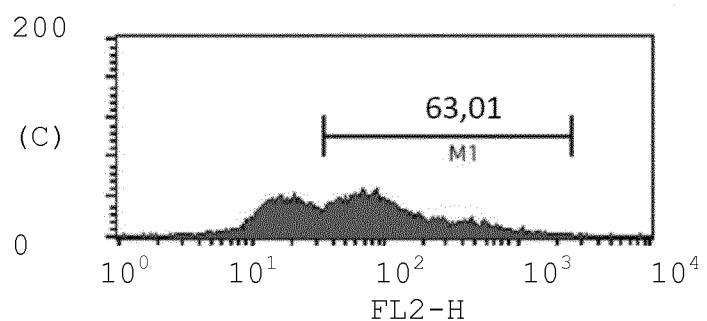
Empty

EGFRWT

EGFR_S464L

Cont. FIG. 3

(A)
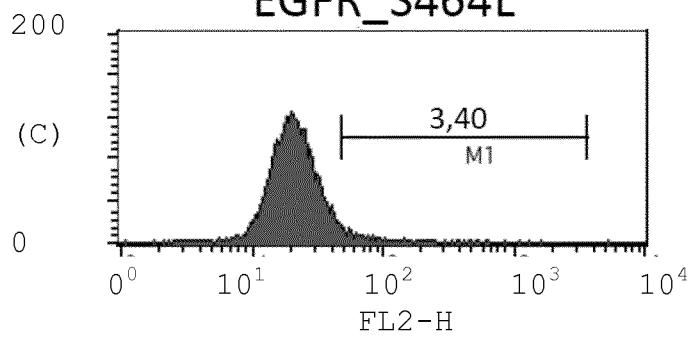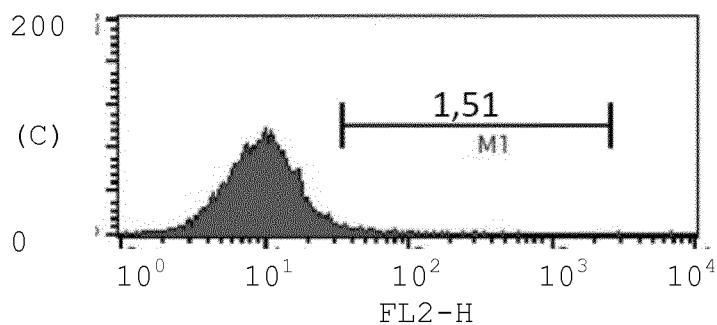
Empty


EGFRWT

EGFR_G465R

FIG. 4

(B)
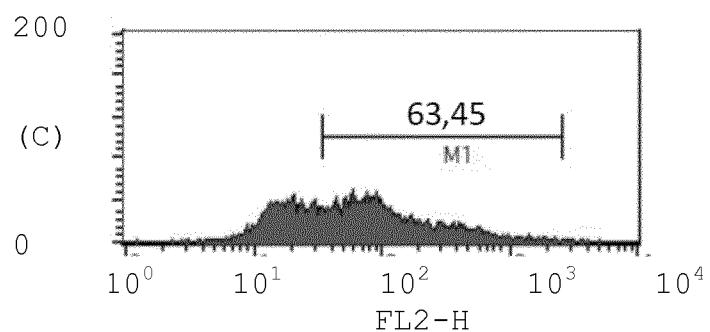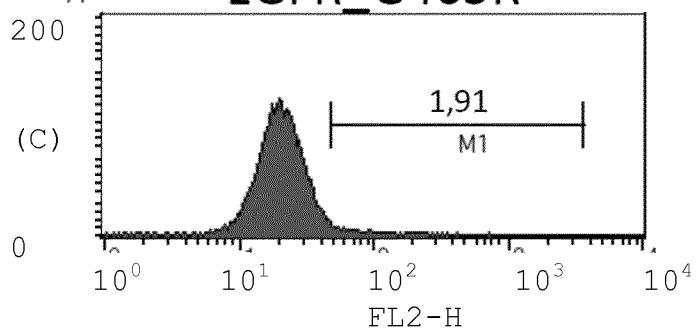
Empty
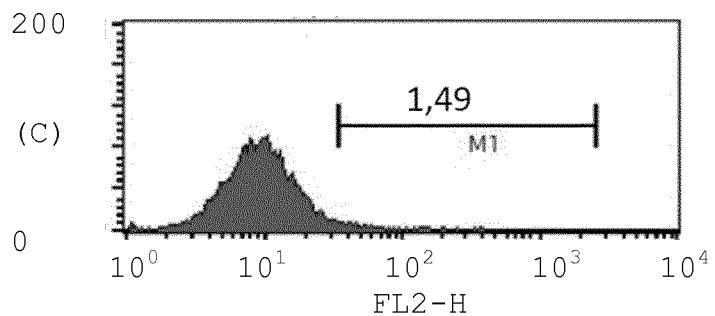
EGFRWT

EGFR_G465R

Cont. FIG. 4