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(54) Title: PREDICTION OF THE TREAMENT RESPONSE TO AN ANTI-EGFR MOLECULE OF A SUBJECT SUFFERING OF RENAL CANCER

(57) Abstract: The present invention relates to the field of medicine and in particular to the treatment of renal cancer and to the prediction of the response to treatment of a renal cancer subject to an anti-epidermal growth factor receptor (EGFR) molecule. More particularly, the inventors herein describe a single nucleotide polymorphism influencing the response to anti-EGFR therapy and methods to distinguish a sensitive subject from a resistant subject and/or for providing the subject with optimized and personalized anti- EGFR therapy. It further relates to products, including compositions and kits, as well as uses thereof in the context of the invention.

PREDICTION OF THE TREAMENT RESPONSE TO AN ANTI-EGFR MOLECULE OF A SUBJECT SUFFERING OF RENAL CANCER

FIELD OF THE INVENTION

The present invention relates to the field of medicine and in particular to the treatment of renal cancer and to the prediction of the response to treatment of a renal cancer subject to an anti-epidermal growth factor receptor (EGFR) molecule. More particularly, the inventors herein describe a single nucleotide polymorphism (SNP) influencing the response to anti-EGFR therapy and methods to distinguish a sensitive subject from a resistant subject and/or for providing the subject with optimized and personalized anti-EGFR therapy. It further relates to products, including compositions and kits, as well as uses thereof in the context of the invention.

BACKGROUND

Renal cell carcinoma (RCC, also known as hypernephroma) is a renal cancer that originates in the lining of the proximal convoluted tubule, the very small tubes in the kidney that filter the blood and remove waste products. Clear cell metastatic renal cell carcinoma (ccRCC) is the most common type of kidney cancer in adults, responsible for approximately 80% of cases. It is also known to be the most lethal of all the genitourinary tumors. Initial treatment is most commonly a radical or partial nephrectomy and remains the mainstay of curative treatment. Where the tumor is confined to the renal parenchyma, the 5-year survival rate is 60-70%, but this is lowered considerably where metastases have spread. It is resistant to radiation therapy and chemotherapy, but some cases respond to immunotherapy. Targeted cancer therapies such as sunitinib, temsirolimus, bevacizumab, interferon-alpha, and sorafenib have improved the outlook for RCC (progression-free survival), although they have not yet demonstrated improved survival.

Before the development of anti-angiogenic therapies (AAT), the outcome of metastatic clear cell renal cell carcinomas (mRCC) was poor. The first treatment approved for mRCC was the humanized monoclonal antibody bevacizumab/Avastin (BVZ) in combination with the standard treatment interferon alpha (IFN), the only treatment that showed a modest efficacy [1]. These drugs are aimed at asphyxiating the tumours, so they should be curative but the results of pivotal clinical trials were disappointing and gave only an increase in the time to progression and in the quality of life without a major improvement in overall survival [2-8]. The reasons for this poor efficacy depend on compensative mechanisms that allow tumour cells to escape drug-mediated cell death. Acquisition of dependence on alternative signaling pathways favouring cell proliferation and invasion has been described including the c-MET [9] and the neuropilin (NRP1/NRP2) [10, 11] pathways. Myeloid cells have also been involved in the refractoriness to AAT [12]. The presence of redundant pro-angiogenic

factors is also one of the causes of relapse to treatments targeting the VEGF/VEGFR pathway especially the ELR+CXCL pro-angiogenic/pro-inflammatory cytokines [13-15]. Identification of markers of response to treatment is an important challenge and may favour the discovery of new potent therapeutic targets [16, 17]. The epidermal growth factor receptor (EGFR) is over-expressed in mRCC probably via EGR-1 dependent activation of its promoter [18]. The hypoxia-inducible factors 1, 2 (HIF-1, 2) are constitutively active in the majority of mRCC because of frequent loss of function of the von Hippel-Lindau gene that stimulates the expression of the transforming growth factor α (TGF-α), an activator of the EGFR pathway [19]. Inventors' previous results showed that the pressure of selection exerted by BVZ induced down-regulation of the phospho tyrosine phosphatase receptor kappa (PTPRκ), a natural inhibitor of EGFR activity [20] resulting in the acquisition of increased proliferation of tumour cells [14]. These cells were driven by over-activation of EGFR as attested by the level of phosphorylation and of the subsequent activation of the ERK/MAP kinase and PI3 kinase/AKT pathways. *In vitro*, the EGFR inhibitor erlotinib/Tarceva (also herein identified as « ERLO »), which is approved for the treatment of lung cancers harbouring specific mutations in EGFR [21, 22], strongly inhibited proliferation of cells derived from BVZ-resistant tumours [14].

Identifying patients likely to respond to treatment represents a therapeutic challenge to limit the administration of ineffective toxic products. Thus, the discovery of predictive markers of response to treatment would improve the current therapeutic practices by avoiding a waste of time at diagnostic and would anticipate treatment gains. The clinician would therefore choose an alternative or optimized treatment among those available (usually administered in the second or third line treatment) [3]. The accurate selection of the patients capable of responding to a particular chemotherapy is a solution for them to receive the most appropriate therapy as soon as possible and typically as soon as they are diagnosed.

SUMMARY OF THE INVENTION

The present invention is based on the discovery by inventors that a single-nucleotide polymorphism (SNP G2618>A) having the number rs 1050171 (according to the NCBI SNP database) that modifies the codon coding to glutamine 787 (Q787) in the human epidermal growth factor receptor (EGFR) protein from CAG to CAA [NP_005219, amino acid sequence corresponding to SEQ ID NO: 54 encoded by mRNA nucleic acid sequence, identified as NM_005228, and by wild-type deoxyribonucleic acid (DNA) sequence corresponding to SEQ ID NO: 53] is indicative for a negative treatment response (resistance) to an anti-EGFR molecule of a subject suffering of renal cancer, more specifically renal cell carcinoma (RCC), in particular clear cell renal cell carcinoma (ccRCC) or metastatic ccRCC (mccRCC) when the mutation is a homozygous mutation (A/A), and is indicative for a positive treatment response (sensitivity) to an anti-EGFR molecule of such a subject when the mutation is a heterozygous mutation (G/A). On the other end, the absence of said SNP is indicative for

a positive treatment response (sensitivity) to an anti-EGFR molecule of the wild-type subject suffering of renal cancer, more specifically renal cell carcinoma (RCC), in particular ccRCC or mccRCC. Thus, the present invention includes methods and kits for predicting or assessing the response of a subject having a renal tumor or cancer as herein identified, to an anti-EGFR molecule, in particular to a small molecule directed to EGFR, typically a tyrosine kinase inhibitor (TKI) such as erlotinib (Tarceva®) or AZD3759, an anti-EGFR antibody and/or an inhibitory polynucleotide capable of interfering with the expression and/or function of EGFR.

In the context of the present invention, the expressions "cancer treatment" or "renal cancer treatment", and more specifically "chemotherapy" or "chemotherapeutic treatment", generally designate a "cancer treatment", including for example an "anti-epidermal growth factor receptor (EGFR) molecule" such as for example a small molecule directed to EGFR, an anti-EGFR antibody and an inhibitory polynucleotide capable of interfering with the expression and/or function of EGFR; an anti-angiogenic molecule such as an anti-VEGF molecule; an anti-PD1 or anti-PDL1 molecule; an immune checkpoint inhibitor such as interferon alpha (also herein identified as "IFN"); or any combination thereof.

Herein disclosed is thus in particular an *in vitro* or *ex vivo* method for assessing or predicting the treatment response to an anti-epidermal growth factor receptor (EGFR) molecule of a subject suffering from renal cancer, or in other words to distinguish a sensitive subject from a resistant subject, the method comprising (i) providing a biological sample comprising genetic material from the subject suffering from renal cancer and (ii) performing a single nucleotide polymorphism (SNP) genotyping analysis in the EGFR gene locus in said sample, wherein the presence of a SNP corresponding to a homozygous mutation that modifies in the EGFR wild-type deoxyribonucleic acid (DNA) sequence the codon coding to glutamine 787 (Q787) in the EGFR protein from CAG to CAA is indicative for a negative treatment response of the subject to the anti-EGFR molecule, and the presence of a SNP corresponding to a heterozygous mutation that modifies in the EGFR wild-type DNA sequence the codon coding to glutamine 787 (Q787) in the EGFR protein from CAG to CAA, or the presence of a EGFR wild-type DNA sequence, is indicative for a positive treatment response of the subject to the anti-EGFR molecule.

Also herein described is a method, typically an *in vitro* or *ex vivo* method, of selecting an appropriate or optimal chemotherapeutic treatment of cancer for a subject having a renal cancer, typically a renal cell carcinoma (RCC), in particular a clear cell renal cell carcinoma (ccRCC) or metastatic ccRCC, which method comprises a step a) of performing, in a biological sample of said subject, a single nucleotide polymorphism (SNP) genotyping analysis in the EGFR gene locus, the presence of a homozygous mutation that modifies in the EGFR wild-type DNA sequence the codon coding to glutamine 787 (Q787) in the EGFR protein from CAG to CAA being the indication that the anti-

epidermal growth factor receptor (EGFR) molecule will not be efficient alone against cancer in the subject, and a step b) of selecting an appropriate or optimal chemotherapeutic treatment of cancer in the subject; and, on the contrary, the presence of a heterozygous mutation that modifies in the EGFR wild-type DNA sequence the codon coding to glutamine 787 (Q787) in the EGFR protein from CAG to CAA being the indication that the EGFR molecule will be efficient alone (optimal) against cancer in the subject (said molecule being an appropriate chemotherapeutic treatment of the cancer in the subject).

In a particular aspect wherein the herein above described method of selecting an appropriate or optimal chemotherapeutic treatment of cancer for a subject having a renal cancer reveals the absence of the herein described SNP (this meaning that the subject is a wild-type subject), said method indicates that the EGFR molecule is efficient alone against cancer in the subject, and thus may be considered as an appropriate chemotherapeutic treatment of cancer, but is less efficient than in a subject exhibiting a heterozygous mutation that modifies the codon coding to glutamine 787 (Q787) in the EGFR protein from CAG to CAA and thus is not as such an optimal treatment of cancer. For such wild-type subjects an optimal chemotherapeutic treatment of cancer combines the EGFR molecule with an anti-angiogenic molecule, preferably bevacizumab.

Further herein described are an anti-EGFR molecule for use in the treatment of a subject suffering from renal cancer, wherein the subject exhibits genotype G/A or G/G at rs 1050171, and an anti-EGFR molecule for use in the treatment of a subject suffering from renal cancer, wherein the subject exhibits genotype A/A at rs 1050171 and wherein said molecule is used in combination with bevacizumab (Avastin®), and preferably in addition in combination with interferon alpha (IFN) and/or with a molecule targeting PD-1 and/or PDL1.

Another aspect of the description relates to a kit or diagnostic composition for the analysis of rs 1050171, as a biomarker SNP usable for assessing/predicting the treatment response to an anti-EGFR molecule such as herein described, in a subject suffering from renal cancer.

A typical kit comprises products for detecting in a biological sample from a subject suffering from renal cancer the presence of a SNP modifying the codon coding to glutamine 787 (Q787) in the EGFR protein from CAG to CAA in the EGFR gene locus.

Another typical kit comprises molecules recognizing specifically (usable to detect) the herein described SNP.

A further typical kit comprises detection means selected from the group comprising, or consisting in, at least two of primer(s), probe(s), enzyme(s) for primer elongation, nucleotide(s) and/or labeling agent(s), preferably a pair of primers selected from SEQ ID NOs: 27 and 28, SEQ ID NOs: 31 and 32, and SEQ ID NOs: 51 and 52; and optionally a leaflet or notice providing instructions or describing protocols on how the kit and its components should be used, typically instructions for

performing/carrying out (nucleic acid) hybridization and/or amplification, and/or all or part of anyone of the herein described methods.

The description also relates to the use of such a kit for assessing or predicting the treatment response (sensitivity or resistance) to an anti-EGFR molecule of a subject suffering from renal cancer and/or ii) for determining the potential toxicity of said anti-EGFR molecule in such a subject.

DETAILED DESCRIPTION OF THE INVENTION

In the present description, the cancer is a renal (or kidney) cancer, and can be more specifically a renal cell carcinoma (RCC), in particular a clear cell renal cell carcinoma (ccRCC) or a metastatic ccRCC (mccRCC).

In the present description, the renal cancer is conventionally treated with one of the following cancer therapy: immunotherapy, specific kinase inhibitor-based therapy, antiangiogenic agent based-therapy, antibody-based therapy and surgery.

In the present description, the conventional anti-renal cancer drug, agent or molecule (also herein generally identified as "chemotherapeutic drug, agent or molecule" or "chemotherapy") encompasses among others kinase inhibitors such as a tyrosine kinase inhibitor (TKI); antiangiogenic agents such as an anti-VEGF molecule; cytokines; monoclonal antibodies such as bevacizumab (Avastin®) or anti-PD1 or anti-PDL1 antibodies; immune checkpoint inhibitors such as interferon alpha (also herein identified as "IFN"), and any combination thereof used in the treatment of renal cancer.

In a particular aspect, the conventional renal cancer treatment involves an "anti-epidermal growth factor receptor (EGFR) molecule" such as for example a small molecule directed to EGFR, an anti-EGFR antibody or an inhibitory polynucleotide capable of interfering with the expression and/or function of EGFR.

The anti-epidermal growth factor receptor (EGFR) protein (also known as ErbB-1 or HER-1) referred to in the context of the present invention is a transmembrane glycoprotein tyrosine kinase, which upon activation stimulates various downstream mediators, related to different biological processes such as cell proliferation, angiogenesis, invasion, metastasis and apoptosis. It is often found to be upregulated in cancers and is a key modulator in the process of cell proliferation in both normal and malignant epithelial cells. The EGFR protein amino acid sequence is SEQ ID NO: 54 (also herein identified as NP_005219) and the corresponding mRNA sequence (also herein identified as NM_005228) is encoded by SEQ ID NO: 53. EGFR is found at abnormally high levels in cancer cells, and EGFR activation appears to be important in tumor growth and progression. Some types of cancers show mutations in their EGFRs, which may cause unregulated cell division through continual or abnormal activation of the EGFR.

"Anti-EGFR molecules" also herein identified as "EGFR inhibitors" as used herein refers to any compound capable of interfering with the expression and/or function of EGFR. Compounds interfering with the function of EGFR are compounds which bind directly or indirectly to the EGFR so as to modulate the receptor mediated activity, typically slow down or stop cell growth, while compounds interfering with the expression of EGFR relates to compounds interfering at any stage of EGFR gene expression so as to reduce the number of EGFR obtained.

Anti-EGFR molecules used in the context of the present invention include small molecules directed to EGFR, anti-EGFR antibodies and inhibitory polynucleotides capable of interfering with the expression and/or function of EGFR. Anti-EGFR molecules generally include any anti-EGFR molecule which can be used for renal cancer therapy such as anti-EGFR molecules, in particular small molecules, anti-EGFR antibodies and inhibitory polynucleotides which were tested in clinical trials as well as anti-EGFR molecules currently studied in clinical trials and/or to be developed.

In an aspect of the methods as well as of the uses herein described, the anti-EGFR molecule is a small molecule. Small molecules directed to EGFR include any organic compound having a low molecular weight, in particular a molecular weight not exceeding 600 Da or 800 Da, typically of about 500 Da, not being a polymer and capable to bind to EGFR, thus interfering with its function.

A small molecule directed to EGFR, being an anti-EGFR molecule in the context of the invention, can be for example selected from erlotinib (Tarceva®), AZD3759 (also known as (R)-4-((3-chloro-2-fluorophenyl)amino)-7-methoxyquinazolin-6-yl 2,4-dimethylpiperazine-1-carboxylate; CAS number 1626387-80-1), gefitinib (Iressa®), lapatinib (Tykerb®), afatinib (Giotrif®), dacomitinib, neratinib (Nerlynx®), vandetanib (Caprelsa®) and osimertinib (Tagrisso®), in particular from erlotinib (Tarceva®), AZD3759, gefitinib (Iressa®), lapatinib (Tykerb®) and afatinib (Giotrif®). A preferred anti-EGFR molecule is selected from erlotinib, afatinib and lapatinib. A particularly preferred anti-EGFR molecule is erlotinib.

In a preferred aspect, the small molecule directed to EGFR is erlotinib (Tarceva®, also herein identified as "ERLO").

In another aspect of the method as well as of the use described herein, the anti-EGFR molecule is an anti-EGFR antibody. Anti-EGFR antibodies denote any antibody or fragment thereof that binds specifically to EGFR.

In the context of the present invention the term "antibody" relates to full length antibodies, human antibodies, humanized antibodies, fully human antibodies, genetically engineered antibodies and multispecific antibodies, as well as to fragments of such antibodies retaining the characteristic properties of the full length antibody. In one embodiment of the method as well as of the use described herein, the antibody is a humanized antibody. A "humanized antibody" is an antibody which has been modified in order to provide an increased similarity to antibodies produced in humans, e.g. by grafting a murine CDR into the framework region of a human antibody. In another embodiment of the method

as well as of the use described herein, the antibody is a fully human antibody. Anti-EGFR antibodies may be monoclonal or polyclonal antibodies. Monoclonal antibodies are monospecific antibodies (i.e. binding to the same epitope) derived from a single cell line. Hence, monoclonal antibodies are, except for variants arising during their production, substantially identical antibodies. In contrast thereto, "polyclonal antibodies" relates to a variety of antibodies directed to different epitopes of an antigen. Methods for production of monoclonal and polyclonal antibodies are known in the art and include e.g. the hybridoma technology and recombinant DNA methods.

In one embodiment of the methods as well as of the uses described herein, the anti-EGFR antibody is a monoclonal antibody.

An anti-EGFR antibody, being an anti-EGFR molecule in the context of the invention, can be for example selected from cetuximab (Erbitux®), panitumumab (Vectibix®) and necitumumab (Portrazza®).

In a preferred aspect, the anti-EGFR antibody is cetuximab (Erbitux®).

In yet another aspect of the methods as well as of the uses described herein, the anti-EGFR molecule is an inhibitory polynucleotide molecule capable of interfering with the expression and/or function of EGFR. Such inhibitory polynucleotides include antisense oligonucleotide specific for EGFR, small interfering RNA (siRNA) specific for EGFR, or a microRNA specific for EGFR. The terms "antisense oligonucleotide specific for EGFR" refers to nucleic acids corresponding to complementary strand of the EGFR mRNA. Preferably, the antisense oligonucleotide comprises a sequence complementary to at least a portion of the EGFR gene expression product. Generally, antisense technology can be used to control, i.e. reduce or abolish gene expression through antisense DNA or RNA, or through triple-helix formation. In one embodiment, an antisense molecule may be generated internally by the organism, for example intracellularly by transcription from an exogenous sequence. A vector or a portion thereof may be transcribed, producing an antisense nucleic acid of the invention. Such a vector can remain episomal or become chromosomally integrated, as long as it can be transcribed to produce the desired antisense molecule. Corresponding vectors can be constructed by recombinant DNA technology methods known to the person skilled in the art. Vectors can be plasmid, viral, or others known in the art, used for replication and expression in vertebrate cells, e.g. vectors as defined herein above.

The term "siRNA specific for EGFR" as mentioned herein above refers to a particular type of small molecules, namely small inhibitory RNA duplexes that induce the RNA interference (RNAi) pathway to negatively regulate gene expression of EGFR. Methods for designing suitable siRNAs directed to a given target nucleic acid are known to person skilled in the art. The term "mi RNA specific for EGFR" as used herein refers to a short single-stranded RNA molecule of typically 18-27 nucleotides in length, which regulate gene expression of EGFR. miRNAs are encoded by genes from whose DNA they are transcribed but are not translated into a protein. Mature miRNA molecules are typically at least partially complementary to mRNA molecules corresponding to the expression product of the present invention, and fully or partially down-regulate gene expression. Preferably, miRNAs according to the

present invention may be 100% complementary to their target sequences. Alternatively, they may have 1, 2 or 3 mismatches, e.g. at the terminal residues or in the central portion of the molecule.

Metastatic clear cell renal cell carcinomas (mRCC) over-express the vascular endothelial growth factor (VEGF). Hence, the anti-VEGF antibody bevacizumab (Avastin®), also herein identified as "BVZ", combined with interferon alpha, also herein identified as "IFN", was approved for the treatment of mRCC. However, approval was lost in July 2016 due to the absence of sustained efficacy. Surprisingly, inventors previously showed that BVZ accelerates tumour growth in experimental models of mRCC in mice. This unexpected result was in part explained by down-regulation of the phospho tyrosine phosphatase receptor kappa (PTPRκ) in tumour cells. The epidermal growth factor receptor (EGFR) is a direct target of PTPRκ. Therefore, its down-regulation leads to constitutive activation of EGFR. Inventors then tested the effect of the currently approved EGFR inhibitor erlotinib (Tarceva®), also herein identified as "ERLO", in addition to BVZ/IFN on the growth of two experimental mRCC models in mice and discovered that tumour growth was strongly reduced by the triple combination BVZ/IFN/ERLO in a sub-group of patients. Tumour growth inhibition was linked to reduced levels of pro-angiogenic/pro-inflammatory cytokines of the ELR+CXCL family and to subsequent inhibition of vascularization, a decreased number of lymphatic vessels and polarization of macrophages towards the M1 phenotype.

Do *et al.* [36] teach that non-small cell lung carcinoma patients with EGFR mutations present in the cancer show a favorable clinical prognosis that is associated with an increased sensitivity to tyrosine kinase inhibitors such as gefitinib, and assume that osteosarcomas may also be sensitive to tyrosine kinase inhibitors considering the presence of EGFR mutations and the positive expression of EGFR.
The synonymous mutation (c.2361G>A encoding p. Gln787Gln) [corresponding to the SNP of interest in the context of the present invention, said SNP being herein described in relation with the EGFR sequences identified thanks to the NCBI nomenclature] has been described by Tan *et al.* [37] in patients with head and neck squamous-cell cancers (SCCs) who have been identified as "exceptional responders to gefitinib".
Surprisingly, inventors have discovered, and now herein describe for the first time, that a single-nucleotide polymorphism (SNP G2618>A) having the number rs 1050171 (according to the NCBI SNP database) that modifies the codon coding to glutamine 787 (Q787) in the human epidermal growth factor receptor (EGFR) protein from CAG to CAA (NP_005219, amino acid sequence corresponding to SEQ ID NO: 54 encoded by mRNA nucleic acid sequence, identified as NM_005228, and by wild-type deoxyribonucleic acid (DNA) sequence corresponding to SEQ ID NO: 53) is indicative for a negative treatment response (resistance) to an anti-EGFR molecule of a subject suffering of renal cancer, more specifically renal cell carcinoma (RCC), in particular clear cell renal cell carcinoma (ccRCC) or metastatic ccRCC (mccRCC) when the mutation is a homozygous

mutation (A/A), and is indicative for a positive treatment response (sensitivity) to an anti-EGFR molecule of such a subject when the mutation is a heterozygous mutation (G/A). On the other end, the absence of said SNP is indicative for a positive treatment response (sensitivity) to an anti-EGFR molecule of the wild-type subject suffering of renal cancer, more specifically renal cell carcinoma (RCC), in particular ccRCC or mccRCC. The SNP mutation of interest is a silent/synonymous mutation, i.e. a mutation which does not result in an amino acid exchange in the encoded protein (the encoded protein will still be in the "wild-type status"). The amino acid involved is located in the kinase domain (Q 787) of the EGFR protein. This specific mutation (G2618>A) is particularly interesting since it modifies a frequently used codon for Q to a rare codon (CAG code for Q in 73% of cases, CAA in 27% of cases) without an amino acid substitution. Contrary to the present invention which distinguishes the homozygous form of the SNP from the heterozygous form thereof and further distinguishes wild-type subjects from mutant subjects, in US2015/344964 this mutation has been correlated to a positive treatment response to anti-EGFR molecule in colorectal cancer patients.

In a particular aspect, genotype AA (also herein identified as "homozygous mutation A/A" or "A/A") at rs 1050171 is indicative for a negative response, genotype AG (also herein identified as "heterozygous mutation A/G", "A/G", "G/A" or GA) at rs 1050171 is indicative for a positive response (typically for an optimal response), and genotype GG (also herein identified as "G/G", "GG" or as "wild-type genotype) is indicative for a positive response (typically for a partially, or intermediary, positive response), of a subject suffering of renal cancer, more specifically renal cell carcinoma (RCC), in particular clear cell renal cell carcinoma (ccRCC) or metastatic ccRCC (mccRCC), to anti-EGFR molecules such as erlotinib or AZD3759.

In a particular aspect, the SNP modifying the codon coding to glutamine 787 (Q787) in the EGFR protein from CAG to CAA (at rs 1050171) responsible for a negative response to an anti-EGFR molecule of a subject suffering from renal cancer, is a homozygous mutation (A/A).

In another particular aspect, the SNP modifying the codon coding to glutamine 787 (Q787) in the EGFR protein from CAG to CAA (at rs 1050171) responsible for a positive response to an anti-EGFR molecule of a subject suffering from renal cancer, is a heterozygous mutation (G/A).

In a further particular aspect, genotype G/G at rs 1050171 is indicative for a positive (but not optimal when compared to the response of subject having a "G/A" genotype) response to an anti-EGFR molecule of a subject suffering from renal cancer.

The present description includes methods and kits for predicting or assessing the response of a subject having a renal tumor or cancer as herein identified, to an anti-EGFR molecule, in particular to a small molecule directed to EGFR, typically a tyrosine kinase inhibitor (TKI) such as erlotinib (Tarceva®) or AZD3759, an anti-EGFR antibody such as cetuximab (Erbitux®), and/or an inhibitory polynucleotide capable of interfering with the expression and/or function of EGFR.

Herein described is in particular an *in vitro* or *ex vivo* method for assessing or predicting the treatment response to an anti-epidermal growth factor receptor (EGFR) molecule of a subject suffering from renal cancer, typically a renal cell carcinoma (RCC), in particular a clear cell renal cell carcinoma (ccRCC) or a metastatic ccRCC (mccRCC), or in other words to distinguish a sensitive subject from a resistant subject, the method comprising (i) providing a biological sample comprising genetic material from the subject suffering from renal cancer and (ii) performing a single nucleotide polymorphism (SNP) genotyping analysis in the EGFR gene locus in said sample, wherein the presence of a SNP corresponding to a homozygous mutation that modifies in the EGFR wild-type DNA sequence the codon coding to glutamine 787 (Q787) in the EGFR protein from CAG to CAA is indicative for a negative treatment response (resistance) of the subject to the anti-EGFR molecule, and the presence of a SNP corresponding to a heterozygous mutation that modifies in the EGFR wild-type DNA sequence the codon coding to glutamine 787 (Q787) in the EGFR protein from CAG to CAA, or the presence of a EGFR wild-type DNA sequence, is indicative for a positive treatment response (sensitivity) of the subject to the anti-EGFR molecule.

It is to be understood that the subject to be treated with an anti-EGFR molecule may solely be treated with said anti-EGFR molecule or additionally with an adjuvant therapy, such as e.g. a distinct chemotherapy.

By “sensitivity” or “responsiveness” is intended herein the likelihood that a patient will positively respond (“sensitive subject” or “responsive subject”) to a chemotherapeutic treatment, typically to the anti-EGFR molecule. The SNP of interest in the context of the present invention in addition allows a distinction between subjects who will optimally respond to the treatment (heterozygous mutant subject) and subjects who will positively (but not optimally, for example partially or incompletely) respond to the treatment (wild-type subject).

By “resistant” is intended herein the likelihood that a patient will not respond (“resistant subject”) to a chemotherapeutic treatment, typically to the anti-EGFR molecule.

Predictive methods of the invention can be used clinically by the medicinal practitioner to make treatment decisions by choosing as soon as possible the most appropriate treatment modalities/regimens for a particular patient. These predictive methods allow determining the likelihood that a patient will exhibit a (at least partially) positive clinical response or a negative clinical response to treatment with an anti-EGFR molecule and constitute a valuable tool for predicting whether a patient is likely to respond favorably to anti-EGFR molecule treatment.

The term "treatment response in a patient suffering from metastatic renal cancer in the sense of the invention refers to a positive clinical response to the treatment in a patient having been diagnosed with

metastatic renal cancer. This treatment response may occur during and/or after the treatment with one or more anti-EGFR molecule(s). Such a positive clinical response may range from stopping the progression of the tumor and/or metastasis thereof to a partial or full remission of the tumor, but also includes an increase of the time of the progression free interval, of the time of the overall survival and/or of the time of the disease-free survival of RC. Overall survival (OS) as used herein refers to the time span from starting the treatment until renal cancer specific death of the patient. Disease free survival refers to the time span of survival of patients having been disease free due to a treatment against renal cancer (e.g. by surgery, anti-EGFR molecule treatment) until the next relapse. In contrast thereto, progression free interval denotes the time span after treatment during which the renal cancer and/or metastasis thereof do not worsen or progress. Treatment response, however, also includes a partial alleviation of the symptoms or a complete remission of the symptoms, indicated by a change of symptoms strength and/or frequency. In a particular aspect, metastatic renal cancer patient's treatment response is evaluated according to RECIST criteria. Metastasis or metastatic foci are typically evaluated/monitored by CT scan, a stabilization or an at least 20% decrease of the tumor's or metastasis' size being typically indicative of a positive response to treatment, and an increase of at least 30% of the tumor's or metastasis' size being typically indicative of a negative response to treatment

In the context of the present invention, the patient or subject is a mammal. In a particular embodiment, the mammal is a human being, whatever its age or sex. The mammal may also be a dog or a cat. The patient typically has a renal tumor. Unless otherwise specified in the present disclosure, the renal tumor is a cancerous or malignant tumor. In a particular aspect, the subject is a subject who has not been previously exposed to a treatment of cancer or a subject who has received the first administration of a chemotherapeutic drug. In another particular aspect, the subject is a subject who has been previously exposed to a treatment of cancer. In a further particular aspect, the subject is a subject who has undergone at least partial resection of the cancerous renal tumor. A particular subpopulation of subjects is composed of subjects suffering from non-metastatic renal cancer. Another particular subpopulation of subjects is composed of subjects having metastases.

In the context of the present invention, a particular subpopulation of subjects suffering from renal cancer is composed of high or intermediate risk of relapse subjects according to the Motzer classification (Motzer RJ et, al., 1999, J Clin Oncol, 17:2530).

In a particular aspect of the invention, the subject is suffering of metastatic renal cancer, in particular of metastatic renal cell carcinoma (RCC), more particularly of metastatic clear cell RCC (mccRCC). In a particular aspect, the subject suffering of metastatic renal cancer is for example a relapsed patient, the patient being, or having been, treated with a reference treatment, i.e. a treatment involving an antiangiogenic or immunotherapeutic molecule.

If the subject is identified, using a method according to the present invention, as resistant to a particular treatment of renal cancer, typically an anti-EGFR molecule, or in a particular aspect, as responding positively but not optimally to such a treatment, the method advantageously further comprises a step of selecting a distinct chemotherapeutic treatment, typically involving a "compensatory molecule", to be used instead of or in combination with the originally preselected chemotherapeutic drug or with a distinct chemotherapeutic drug, as the appropriate therapeutic treatment of cancer for the subject.

In a preferred aspect, the anti-EGFR molecule for which a treatment response is to be predicted is selected from the group consisting of a small molecule directed to EGFR, an anti-EGFR antibody and an inhibitory polynucleotide capable of interfering with the expression and/or function of EGFR such as herein above described.

In an even more preferred aspect, the anti-EGFR molecule is a tyrosine kinase inhibitor (TKI) such as herein above described, in particular selected from erlotinib (Tarceva®), AZD3759, gefitinib (Iressa®), lapatinib (Tykerb®), afatinib (Giotrif®) and dacomitinib, for example selected from erlotinib (Tarceva®), AZD3759, gefitinib (Iressa®), lapatinib (Tykerb®) and afatinib (Giotrif®). A preferred anti-EGFR molecule may be selected from erlotinib, afatinib or lapatinib. In a particularly preferred aspect, the anti-EGFR molecule is erlotinib.

In a distinct particular aspect, the anti-EGFR molecule is an anti-EGFR antibody, such as cetuximab (Erbitux®), panitumumab (Vectibix®) or necitumumab (Portrazza®). In a preferred aspect, the anti-EGFR antibody is cetuximab (Erbitux®).

In a particular embodiment, the herein above described method is performed prior to initiation of treatment (which is typically a renal cancer treatment involving an anti-EGFR molecule), i.e. before any administration to the subject of a chemotherapy for treating the subject's cancer. Less preferably but also possible, this step can be performed after the any (for example the first) administration of a chemotherapeutic drug to the subject. This step can be performed before any tumor surgical resection. It can also be performed on a subject who has undergone at least partial resection of the cancerous tumor.

The genotyping analysis is typically performed at rs 1050171 in the EGFR gene locus (of SEQ ID NO: 53). Methods for determining the genotype at rs 1050171, or for determining mutational status, are known to the person skilled in the art and include amongst others DNA sequencing, real-time PCR with specific primers and probes, RT-PCR, fluorescence *in situ* hybridisation, immunohistochemistry, semi-nested PCR and/or nested PCR.

Implementations of the methods of the invention first involve obtaining a (biological) sample from a subject, typically a sample from which a nucleic acid sample may be obtained. The sample is preferably a fluid sample and may include blood, urine, plasma, serum, lymphatic fluid, spinal fluid, pleural effusion, ascites, sputum, or a combination thereof. The sample is typically a blood sample or a

derivative thereof. The sample may further be a solid sample, typically a tumor sample. A preferred sample is a blood sample (or a derivative thereof), a sputum sample, or a tumor sample, even more preferably a blood or sputum sample.

Typically, the DNA is subsequently extracted or purified from the sample prior to SNP genotyping analysis. Any method known in the art may be used for DNA extraction or purification. Suitable methods comprise inter alia steps such as centrifugation steps, precipitation steps, chromatography steps, dialyzing steps, heating steps, cooling steps and/or denaturation steps. For some embodiments, a certain DNA content in the sample may have to be reached. DNA content can be measured for example via UV spectrometry as described in the literature. DNA amplification may be useful prior to the SNP analysis step. Any method known in the art can be used for DNA amplification. The sample can thus be provided in a concentration and solution appropriate for the SNP analysis.

For the SNP genotyping analysis, SNP-specific primers and/or probes, a primer extension reaction, SNP microarrays, restriction analysis and/or DNA-sequencing may be used. Reagents and methods for performing SNP genotyping analyses are known in the art.

In one aspect of the description, the SNP genotyping analysis performed in step ii) of the method disclosed herein includes a PCR followed by restriction analysis. More specifically, after extraction of the DNA from the biological sample from the subject, a PCR amplification is made to cover all or part (typically the appropriate exon) of the EGFR gene (exemplary primers are herein provided, for example a first couple comprising as forward sequence: -5'-GAAGCCTACGTGATGGCCAG-3' (SEQ ID NO: 51) and as reverse sequence: -5'-GGTACTGGGAGCCAATATTGTC-3' (SEQ ID NO: 52), and a second couple comprising as forward sequence: -5'-ATGCGACCCTCCGGGACGGC-3' (SEQ ID NO: 27) and as reverse sequence: -5'-CAAACGGTCACCCCTTTCTTTTCC-3' (SEQ ID NO: 28)). The PCR-amplicon is then submitted to restriction analysis according to the manufacturer's instructions. In an alternative aspect, the SNP genotyping analysis of step b) of the method disclosed herein is performed by DNA-sequencing. DNA sequencing usually employs a primer designed as flanking the region to be analysed together with labelled nucleotides in a PCR-like setup. By analysing the labels at the corresponding positions, it is possible to determine the sequence of DNA starting from the regions to which the primer is hybridizing. Furthermore, it is possible to determine the genotype of an allele by sequencing since a peak corresponding to two different bases or a peak indicating an identical base at a certain position may be detected. DNA-microarray techniques may also be used in step ii); the techniques are based on hybridization events between the test-DNA and so-called "probes" immobilized on defined spots of a Microarray in a chamber. Today, such microarrays are routinely used to determine DNA-sequences even down to the level of a single base and thus for the detection of SNPs. This is possible by selecting the probes accordingly and using specific hybridization conditions. The DNA may be labelled for detecting purposes. Routinely, probes covering the different sequences

at the position of an SNP may be used in combination with corresponding controls; thus, also the genotype of the corresponding SNP may be analysed.

Further, real-time PCR methods may also be used in step ii), wherein real-time PCR is based on the incorporation of double strand specific dyes into DNA while said DNA is amplified. Said dyes are detected only in case they are incorporated. Thus, the more DNA amplified, the higher the detection signal of the corresponding dye. By designing primers accordingly and/or by adding suited probe-nucleotides hybridizing to a specific DNA-sequence only (which are able to discriminate between SNPs) and using specific hybridization conditions, polymorphisms may be analyzed.

Also, mass-spectrometry (MS) may be used in step ii) of the present method. In MALDI-MS, a sample is mixed with a solution containing a matrix material and a drop of the liquid is placed on the surface of a probe. The matrix solution then e.g. co-crystallizes with the biological sample and the probe is inserted into the mass spectrometer and laser energy is then directed to the probe surface where it absorbs and ionizes the biological molecules without significantly fragmenting them.

In another aspect of the method described herein the SNP genotyping analysis of step ii) includes a combination of the above described methods, in particular restriction analysis with at least one further method for identifying the genotype at rs l050171 of the subject described herein, in particular DNA-sequencing.

Also herein described is a method, typically an *in vitro* or *ex vivo* method, of selecting an appropriate or optimal chemotherapeutic treatment of cancer for a subject having a renal cancer, typically a renal cell carcinoma (RCC), in particular a clear cell renal cell carcinoma (ccRCC) or metastatic ccRCC. This method typically comprises a step a) of performing, in a biological sample of said subject, a single nucleotide polymorphism (SNP) genotyping analysis in the EGFR gene locus, the presence of a homozygous mutation that modifies in the EGFR wild-type DNA sequence the codon coding to glutamine 787 (Q787) in the EGFR protein from CAG to CAA being the indication that the anti-epidermal growth factor receptor (EGFR) molecule will not be efficient alone against cancer in the subject, and a step b) of selecting an appropriate or optimal chemotherapeutic treatment of cancer in the subject; and, on the contrary, the presence of a heterozygous mutation that modifies in the EGFR wild-type DNA sequence the codon coding to glutamine 787 (Q787) in the EGFR protein from CAG to CAA being the indication that the EGFR molecule will be efficient alone (optimal) against cancer in the subject (said molecule being an appropriate chemotherapeutic treatment of the cancer in the subject).

In a particular aspect wherein the herein above described method of selecting an appropriate or optimal chemotherapeutic treatment of cancer for a subject having a renal cancer reveals the absence of the herein described SNP (this meaning that the subject is a wild-type subject), said method indicates that the EGFR molecule is efficient alone against cancer in the subject, and thus may be considered as an appropriate chemotherapeutic treatment of cancer, but is less efficient than in a

subject exhibiting a heterozygous mutation that modifies the codon coding to glutamine 787 (Q787) in the EGFR protein from CAG to CAA and thus is not as such an optimal treatment of cancer. For such wild-type subjects an optimal chemotherapeutic treatment of cancer combines the EGFR molecule with an anti-angiogenic molecule, preferably bevacizumab.

Inventors have evaluated and describe in details in the experimental part of the present description the relevance of combinations of ERLO/BVZ/IFN to prevent acquired resistance and to improve the current therapeutic practices. The description highlights the molecular mechanisms associated with the efficacy of *in vivo* combined treatments and the relevance of their use in a specific fraction of subjects.

In a particular aspect, the appropriate treatment of cancer as determined in the context of the previously described method may comprise the administration to a subject as herein defined suffering of a renal cancer, typically a renal cell carcinoma (RCC), in particular a clear cell renal cell carcinoma (ccRCC) or metastatic ccRCC, typically in addition of the anti-EGFR molecule, preferably in addition of erlotinib, of AZD3759, of bevacizumab (Avastin®) and/or interferon alpha (IFN), preferably of bevacizumab and IFN, optionally in combination with a molecule targeting PD-1 and/or PDL1, when erlotinib has been identified as not efficient, or not optimal, when administered alone to the subject. Bevacizumab, IFN and/or a molecule targeting PD-1 and/or PDL1 can indeed advantageously be used as compensatory molecule(s) allowing the anti-cancer treatment to be effective, preferably optimal, in the subject.

Typical examples of molecules targeting PD-1 which can be used in the context of the present invention are nivolumab and/or pembrolizumab.
Typical examples of molecules targeting PDL1 which can be used in the context of the present invention are atezolizumab and/or avelumab.
In a particular embodiment, a molecule targeting PD-1 can be used in combination with a molecule targeting PDL1.

A further aspect herein described thus also relates to an anti-EGFR molecule as herein described, for example erlotinib or AZD3759, for use in the treatment of a subject suffering from renal cancer, wherein the subject exhibits genotype G/A or G/G at rs 1050171.

Another aspect herein described relates to anti-EGFR molecule as herein described, for example erlotinib or AZD3759, for use in the treatment of a subject suffering from renal cancer, wherein the subject exhibits genotype A/A at rs 1050171 and wherein said molecule is used in combination with an antiangiogenic agent such as bevacizumab (Avastin®), and preferably in addition in combination

with an immune checkpoint inhibitor such as interferon alpha (IFN) and/or with a molecule targeting PD-1 and/or PDL1.

In a further distinct aspect, the appropriate treatment of cancer does not involve the cancer treatment, typically the anti-EGFR molecule which has been identified as inefficient alone in the subject thanks to the herein above described method of the invention. In this aspect, the appropriate treatment of cancer will preferably be a distinct cancer treatment ("alternative treatment") as herein described, typically selected from a distinct anti-EGFR molecule or from an anti-angiogenic molecule, for example a herein described monoclonal antibody (preferably used in combination with an immune checkpoint inhibitor such as IFN).

In the methods herein described of assessing the sensitivity of a subject having a renal tumor to a chemotherapy, typically an anti-EGFR molecule, as well as in the methods herein described of selecting an appropriate chemotherapeutic treatment, any classical method known by the skilled person of determining the presence of the herein described SNP, such as typically those herein described, can be used.

The present invention also includes kits containing reagents necessary for determining the treatment response to a chemotherapy, typically to an anti-EGFR molecule, of a subject having a renal cancer, typically a renal cell carcinoma (RCC), in particular a clear cell renal cell carcinoma (ccRCC) or metastatic ccRCC (e.g. diagnostic compositions) which are packed so as to allow their transport and storage. The kit may also contain a package leaflet describing how the kit and its components should be used.

The present invention typically includes kits, or diagnostic compositions, for assessing or predicting the sensitivity or resistance of a subject having such a renal cancer, to chemotherapy, typically to an anti-EGFR molecule.

The kits, or diagnostic compositions, are typically for the analysis of (for determining the genotype at) rs 1050171, as SNP indicative for, usable for assessing/predicting, the treatment response to an anti-EGFR molecule such as herein described, in a subject suffering from renal cancer.

In a particular aspect, the kit comprises products for detecting in a biological sample from a subject suffering from renal cancer the presence of a SNP modifying the codon coding to glutamine 787 (Q787) in the EGFR protein from CAG to CAA in the EGFR gene locus.

A typical kit comprises molecules recognizing specifically (usable to detect) the herein described SNP.

Another typical kit of the invention comprises detection means selected from the group comprising, or consisting in, at least two of primer(s), probe(s), enzyme(s) for primer elongation, nucleotide(s) and/or labeling agent(s); and optionally a leaflet or notice providing instructions or describing protocols on how the kit and its components should be used, typically instructions for performing/carrying out

(nucleic acid) hybridization and/or amplification, and/or all or part of anyone of the herein described methods.

The term "primer" as used herein denotes an oligonucleotide that acts as an initiation point of nucleotide synthesis under conditions in which synthesis of a primer extension product complementary to a nucleic acid strand is induced.

The term "probe" as used herein denotes an oligonucleotide that selectively hybridizes to a target nucleic acid under suitable conditions.

The primers and probes may be generated such that they are able to discriminate between wild-type allele or mutated allele of the position of the SNP to be analyzed, i.e. of rs 1050171. Methods for the design of sequence specific primers and probes are known in the art. Exemplary primers which may be used are those shown in SEQ ID NOs: 27, 28, 31, 32, 51 and 52. A preferred pair of primers is preferably selected from SEQ ID NOs: 27 and 28, SEQ ID NOs: 31 and 32, and SEQ ID NOs: 51 and 52.

The present description also relates to the use of a kit as herein described i) for assessing or predicting the treatment response (sensitivity or resistance) to an anti-EGFR molecule of a subject suffering from renal cancer, typically of renal cell carcinoma (RCC), in particular of clear cell renal cell carcinoma (ccRCC) or metastatic ccRCC, and/or ii) for determining the potential toxicity of said anti-EGFR molecule in such a subject.

Throughout this application, various references describe the state of the art to which this invention pertains. The disclosures of these references are hereby incorporated by reference into the present disclosure.

Further aspects and advantages of the present invention are described in the following experimental section (with reference to figures 1 to 12), which should be regarded as illustrative and not limiting the scope of the present application.

LEGENDS TO THE FIGURES

Figure 1. 786-O and A498 cells present different sensitivities to ERLO. **a** 786-O cells were treated with increasing concentrations of ERLO. The percentage of live and dead cells is indicated. *p<0.05; **p<0.01. **b** A498 cells were treated with increasing concentrations of ERLO. The percentage of live and dead cells is indicated. * p < 0.05; *** p < 0.001. **c** 786-O or A498 cells were treated with increasing concentrations of ERLO. EGF levels were evaluated in cell supernatants by ELISA. *** p < 0.001. **d** 786-O or A498 cells were treated with increasing concentrations of ERLO and were evaluated for the presence of total and active form of EGF receptor (EGFR/pEGFR), HER3, the total and active form of ERK (ERK:pERK) and the active form of AKT (pAKT) by immuno-blotting.

HSP90 is shown as a loading control. **e** 786-O or A498 cells were treated with increasing concentrations of ERLO. VEGF levels were evaluated in cell supernatants by ELISA. *** $p < 0.001$. **f** 786-O or A498 cells were treated with increasing concentrations of ERLO. CXCL8 levels were evaluated in cell supernatants by ELISA. ** $p < 0.01$; *** $p < 0.001$.

Figure 2. The role of the BVZ IFN and ERLO combination on RCC xenograft tumour growth. **a** 3.10^6 cells 786-O cells were subcutaneously injected into nude mice. Seven days after injections all mice developed tumours. 49 days after cell injection, mice were treated twice a week with control or ERLO (E, 50 mg/kg) or BVZ (B, 7.5 mg/kg) plus IFN (I, 9MIU) plus or minus ERLO (50 mg/kg). The tumour volume is presented as the means ± s.d. (n = 10). Statistical differences to the untreated mice are shown: *p <0.05; *** p<0.001. **b** Same experiment as described in a but using A498 cells. * $p < 0.05$; ** $p< 0.01$; *** $p< 0.001$. * $p < 0.05$; *** $p < 0.001$.

Figure 3. The BVZ/IFN/ERLO combination decreased the tumour blood vessel density and prevented/inhibited the development of lymphatic vessels. The tumour vasculature in each experimental group was detected by mmuno-staining for CD31 (endothelial cells, green) and α-SMA i (pericytes, red); **a** 786-O cell model; **c** A498 cell model. LYVE-1 immuno-staining (green) shows lymphatic endothelial cells. Lymphatic vessels with lumens (L) are indicated. **b** 786-O model; **d** A498 model. Tumour sections were counterstained with 40,6-diamidino-2-phenylindole (DAPI) (nucleus, blue). **e** The intra-tumour amount of haemoglobin (Hg), a global read out of the blood supply, is given for both model systems and for the different experimental conditions.

Figure 4. The capacity to proliferate and the sensitivity to ERLO of cells from experimental tumours. **a** The capacity to proliferate of 786-O cells isolated from three independent tumours from each group was tested using the MTT assay (C cells from untreated mice; B+I; cells from BVZ/IFN-treated mice; B+I+E; cells from BVZ/IFN/ERLO-treated mice) in the absence (-) or presence (+) of ERLO. **b** The proliferative capacity of A498 cells isolated from three independent tumours for each group in the absence (-) or presence (+) of ERLO was tested using MTT assays. For both cell types, results are presented as the mean fold increase ± s.d. Statistical differences in the fold increase of tumour cells isolated from control mice were taken as reference values. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. **c** Representative 786-O cells from the four experimental groups were tested for the presence of the total and active form of EGFR (EGFR/pEGFR) in the absence (-) or presence (+) of ERLO (10 μM). HSP90 is shown as a loading control. Quantification of the relative level of EGFR (EGFR/HSP90) and pEGFR (pEGFR/EGFR) is shown. The reference values (100%) correspond to the levels of EGFR and pEGFR in cells of tumours derived from untreated mice in the absence of ERLO. **d** Equivalent experiments as described in **c** for the A498 model.

Figure 5. The presence of a silent mutation in the kinase domain of EGFR is indicative of ERLO and AZD3759 efficacy. **a** Sequence chromatogram analysis of the EGFR coding region of genomic DNA obtained from RCC4, 786-O and A498 cells. **b** *In vitro* transcription and translation of two independent wild-type (WT1, WT2) and mutated (Mut1, Mut2) EGFR expression plasmids. Upper

panel: equal amounts of DNA were used for *in vitro* reactions, and the quality of the plasmids was verified on agarose gels coloured with ethidium bromide. Lower panel: proteins resulting from the *in vitro* transcription/translation reaction were analyzed by immuno-blotting. **c** 200 ng of two independent expression vectors carrying wild-type (WT1, WT2) and mutated (Mut1, Mut2) EGFR expression plasmids were transfected into HEK293 and total protein lysates were analysed by immune-blotting. Comparison between samples was performed after the calculation of the transfection efficiency. HSP90 is shown as a loading control. **d** The IC50 for ERLO of the different primary cells wild-type (WT) heterozygous (HET) or homozygous (HOM) for the G 2618 A mutation was tested by MTT assays. * $p < 0.05$; *** $p < 0.001$. **e** The IC50 for AZD3759 of the different primary cells wild-type (WT) heterozygous (HET) or homozygous (HOM) for the G 2618 A mutation was tested by MTT assays. * $p < 0.05$; *** $p < 0.001$.

Figure 6. 786-O and A498 cells show different sensitivities to ERLO. Quantification of immuno-blots (three independent experiments), the representative images was shown on Fig. 1d. 786-O or A498 cells were treated with increasing concentrations of ERLO and were tested for the presence of total (EGFR/HSP90), the active form of the EGF receptor (pEGFR/EGFR), the active form of AKT (pAKT/HSP90) and the active form of ERK (pERK/ERK). The statistical significance for the different ERLO concentrations for a specific cell line is shown by * (* $p < 0.05$; ** $p < 0.01$). The statistical significance for the comparison of the two cell lines is shown by # (# $p < 0.05$; ## $p < 0.01$).

Figure 7. BVZ/IFN/ERLO on the vascular/lymphatic networks. The tumour vascular/lymphatic networks in each experimental group (control, B+I, E, B+I+E) were evaluated by CD31 immuno-staining and coverage of the vessels using an anti-αSMA antibody as presented on Fig. 2. Vascular/lymphatic density (vessels/mm^2) and the number of vessels covered with αSMA labelled cells were determined using the Image J program. Quantification (means ± SD) resulted from analysis of four independent tumours and considered at least ten fields for each tumour. Statistically significant differences are indicated: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figure 8. Differential inhibition of proliferation-mediating signaling pathways by ERLO in cells derived from experimental tumours. **a** Representative 786-O cells from the four experimental groups were tested for the presence of the total and active form of the EGF receptor (EGFR/pEGFR, see Fig. 3) and active forms of ERK (pERK) and AKT (pAKT). HSP90 is shown as a loading control. Quantification of the relative level of EGFR (EGFR/HSP90), pEGFR (pEGFR/EGFR), pERK (pERK/HSP90) and pAKT (pAKT/HSP90) is shown (Fig. 3). The reference values (100%) correspond to the levels of the different parameters in cells of tumours derived from untreated mice in the absence of ERLO. **b** Equivalent experiments as described in **a** for the A498 model.

Figure 9. Parental 786-O cells proliferated faster than A498 parental cells. The proliferative capacity of parental 786-O and A498 cells was tested using the MTT assay. The results are presented as the mean percent increase ± s.d. Statistical differences are indicated; ** $p < 0.01$; *** $p < 0.001$.

Figure 10. RCC4, 786-O and A498 cells expressed different EGFR levels. **a** Representative immunoblots showing the EGFR protein level in the different RCC cell lines after treatment in the absence or presence of increasing concentrations of ERLO. HSP90 is shown as a loading control. **b** Quantification in three independent experiments for EGFR levels in the different cell lines and in response to ERLO. ** $p < 0.01$; *** $p < 0.001$.

Figure 11. High EGFR and EGFR-AS1 levels correlated to RCC aggressiveness. **a** Graph showing relative levels of EGFR transcript in RCC tumors of different stages versus normal measured by RNA-seq data available from TCGA. *** $p < 0.001$. **b** The Kaplan–Meier analysis of overall survival of patients with metastatic RCC from the TCGA data base. Overall survival was calculated from patient subgroups with baseline levels of EGFR that were less or greater than the third quartile value. **c** Graph showing relative levels of EGFR-AS1 transcript in RCC tumors versus normal (http://gepia.cancer-pku.cn). **d** Graph showing relative levels of EGFR-AS1 transcript in RCC tumors of different stages (http://gepia.cancer-pku.cn) [63]. **e** The Kaplan–Meier analysis of disease-free survival of patients with metastatic RCC from the TCGA data base.

Figure 12. Sensitivity to anti-EGFR inhibitors depending on the mutation of the EGFR.
Wild-type (RCC4), heterozygous (786-O) or mutant (A498) RCC cells were incubated with different concentrations of the indicated inhibitors. The IC50 for the different drugs was tested by MTT assays. ¤ indicates a statistically significant difference between RCC4 and 786-O cells; * indicates a statistically significant difference between 786-O and A498 cells; § indicates a statistically significant difference between RCC4 and A498 cells. ¤, *, § $p < 0.05$; ¤¤, §§ $p < 0.01$; *** $p < 0.001$.

EXPERIMENTAL SECTION

Materials and methods

Cell lines

The Ethics departments of the University hospital, the Cancer Centre (Centre Antoine Lacassagne), Nice, France and the Princess Grace Hospital of Monaco approved this study and participants provided their written informed consent. Cells were isolated from tumours as previously described [35]. Cells were suspended in cell culture medium specific for renal cells (PromoCell). Further experiments were performed after passage ten when the cell line was established. RCC4, 786-O and A498 cells were from the American Type Culture Collection and were cultured in the same defined medium.

RNA extraction and RT-PCR

Quantitative PCR (qPCR) experiments were performed after cell passage 11. One microgram of total RNA was used for reverse transcription, using the QuantiTect Reverse Transcription kit (QIAGEN, Hilden, Germany), with blend of oligo(dT) and random primers to prime first-strand synthesis. For real-time PCR, inventors used the master mix plus for SYBR assay (Eurogentec, Liege, Belgium). To

calculate the relative expression of the different mRNAs, the 2[2DDC(T)] method was used [61]. The PCR conditions were 10 minutes at 95°C followed by 40 cycles 15 seconds at 95°C, 1 minute at 60°C. The sequences of the different couples of oligo-nucleotides (list of oligo-nucleotides used in qPCR experiments) are detailed below:

	FORWARD	REVERSE
Housekeeping genes		
36B4	CAGATTGGCTACCCAACTGTT	GGCCAGGACTCGTTTGTACC
m-36B4	AGATTCGGGATATGCTGTTGGC	TCGGGTCCTAGACCAGTGTTC
GAPDH	TGC ACC ACC AAC TGC TTA GC	GGC ATG GAC TGT GGTCAT GAG
Pro/Anti-angiogenesis genes		
m-CD31	ACGCTGGTGCTCTATGCAAG	TCAGTTGCTGCCCATTCATCA
m-NG2	ACTAACCCATGCACTACATCAAG	ACTTTTCCAGACAGAGAGCCTT
m-αSMA	GTC CCA GAC ATC AGG GAG TAA	TCG GAT ACT TCA GCG TCA GGA
IL6	CCTGAACCTTCCAAAGATGGC	TTCACCAGGCAAGTCTCCTCA
CXCL5	AGCTGCGTTGCGTTTGTTTAC	TGGCGAACACTTGCAGATTAC
CXCL4	AGCCACGCTGAAGAATGGAA	CACACACGTAGGCAGCTAGT
CXCL10	GTGGCATTCAAGGAGTACCTC	TGATGGCCTTCGATTCTGGATT
Lymphangiogenesis genes		
mLYVE	CAG CACACTAGCCTGGTGTTA	CGCCCATGATTCTGCATGTAGA
m-VEGF-C	CTCTGTGGGACCACATGGTAA	TCCTCTCCCGCAGTAATCCA
Proliferation genes		
PTPRk	AATGCTCCTCCTCAGCTTCTTGGT	AGGACCATCGCCATTGATCGAGTT
EGFR	ATGCGACCCTCCGGGACGGC	CAAACGGTCACCCC TTTCTTTTCC
EGF	TGTCCACGCAATGTGTCTGAA	CATTATCGGGTGAGGAACAACC
m-EGFR	GCCATCTGGGCCAAAGATACC	GTCTTCGCATGAATAGGCCAAT
m-EGF	AGCATCTCTCGGATTGACCCA	CCTGTCCCGTTAAGGAAAACTCT
CSF1	TGGCGAGCAGGAGTATCAC	AGGTCTCCATCTGACTGTCAAT
m-CSF1	ATGAGCAGGAGTATTGCCAAGG	TCCATTCCCAATCATGTGGCTA
m-CSF1R	TGTCATCGAGCCTAGTGGC	CGGGAGATTCAGGGTCCAAG
Immune tolerance genes		
PDL1	TGGCATTTGCTGAACGCATTT	TGCAGCCAGGTCTAATTGTTTT
Macrophage M1 genes		
miNOS	TCACCTTCGAGGGCAGCCGA	TCCGTGGCAAAGCGAGCCAG
mIL6	ATCCAGTTGCCTTCTTGGGACTGA	TTGGATGGTCTTGGTCCTTAGCCA
Macrophage M2 genes		
mARG1	GATTATCGGAGCGCCTTTCT	CCACACTGACTCTTCCATTCTT
mCD206	CTGCAGATGGGTGGGTTATT	GGCATTGATGCTGCTGTTATG

The herein below described additional couple of oligo-nucleotides has been used by inventors in relation with the EGFR sequence:

Forward sequence: GAAGCCTACGTGATGGCCAG (SEQ ID NO: 51)

Reverse sequence: CAACTCCCAAACGGTCACCC (SEQ ID NO: 52)

Antibodies

The following antibodies were used for immuno-blotting: anti-phospho ERK 1,2 and anti-tubulin (Sigma St Louis, MO), anti-phospho S6 Kinase, total anti-EGFR/HER1 and anti-pEGFR/HER1 (Cell Signaling, Cambridge, UK) and anti ERKs (Santa Cruz Biotechnology, Santa Cruz, CA references sc 93).

Immuno-fluorescence

Tumour sections were handled as described previously [14, 62]. Sections were incubated with anti-mouse LYVE-1 polyclonal (Ab 14817, 1:200; Abcam, Cambridge, MA, USA) or monoclonal anti-α-

smooth muscle actin Sigma (αSMA A2547, 1:1000; Sigma, France), and rat monoclonal anti-mouse CD31 (clone MEC 13.3, 1:1000; BD Pharmingen, Franklin Lakes, NJ, USA) antibodies.

Measurement of haemoglobin and cytokines

Frozen tumour tissues were homogenised using a Precellys tissue homogeniser (Bertin, Montigny-le-Bretonneux, France) in cell extraction buffer (Biosource, Villebon sur Yvette, Belgium). The intra-tumour haemoglobin content was measured using the Drabkin reagent kit 525 (Sigma, France). CXCL cytokines and VEGF were measured by using PeproTech ELISA kits according to the manufacturer's recommendations (PeproTech, Neuilly-sur-Seine, France). VEGFC were measured with a Human DuoSet ELISA kits (R&D Systems, Minneapolis, MN, USA).

Statistical analysis

Statistical analyses were two-sided and were performed using R-2.12.2 for Windows. Statistical comparisons were performed using the Student *t*-test or Wilcoxon test for quantitative data.

Results

ERLO exerts a strong cytostatic and cytotoxic effect that depends on the mRCC cell line and inhibits the production of pro-angiogenic cytokines.

Activation of the EGFR pathway in response to bevacizumab/Avastin (BVZ) was demonstrated previously in experimental mRCC in mice when using different model cell lines of increasing aggressiveness [14]. However, the intrinsic sensitivity to EGFR inhibitors of mRCC cells was poorly investigated. Therefore, inventors evaluated sensitivity using two model cell lines of highly aggressive mRCC, 786-O and A498 cells. They obtained a dose-dependent decrease in the proliferation rate with both cell lines. The maximal reduction was of 60% and 33% for 786-O and A498 cells, respectively for the highest erlotinib/Tarceva (ERLO) concentration (10 μM). Regardless of the ERLO concentration, the percentage of dead cells was equivalent (10% and 2% for 786-O and A498 cells, respectively, Fig. 1a, b). Therefore, ERLO is cytostatic rather than cytotoxic and the cytostatic effect was stronger for 786-O cells. ERLO induced dose-dependent inhibition of EGF production by 786-O cells whereas this was not modified in A498 cells (Fig. 1c). Therefore, the more potent effect of ERLO on cell proliferation observed for 786-O cells may be explained by inhibition of an EGF/EGFR autocrine pathway. Consistent with this, the phosphorylated/active form of EGFR (pEGFR) was dose-dependently inhibited by ERLO in 786-O cells. In A498 cells, the EGFR levels were higher compared to 786-O cells and ERLO had no incidence on pEGFR, which remained low whether or not ERLO was present, as compared to basal levels in 786-O cells (Fig. 1d and Fig. 6). Inventors observed a significant decrease in the activity of the ERK/MAP kinase proliferation pathway for both cell lines. However, the ERK activity was lower in 786-O cells and was more strongly inhibited by ERLO as compared to A498 cells. The AKT activity (pAKT) was high and was inhibited by ERLO in 786-O cells but almost undetectable in A498 cells. This result may explain the differential effect exerted by ERLO on proliferation for the two independent cell lines (Fig. 1d and Fig. 6).

Gefitinib, another EGFR inhibitor used to treat lung cancers [21], or cetuximab, a monoclonal antibody against EGFR, reduced the production of VEGF and CXCL8 in different cancer cells, which may explain their therapeutic efficacy [23, 24]. Therefore, the effect of EGFR inhibition on secreted cytokines involved in angiogenesis was evaluated. ERLO, even at a low concentration (1 μM), inhibited VEGF production in 786-O cells but this was not modified in A498 cells (Fig. 1e). The opposite result was observed for CXCL8 (dose-dependent inhibition in A498 cells and no effect in 786-O cells, Fig. 1f). These results suggest that ERLO may indirectly inhibit angiogenesis through down-regulation of pro-angiogenic factors by tumour cells in mRCC.

Combining BVZ/IFN with ERLO inhibited the growth of experimental mRCC in mice.

Considering that activation of the EGFR pathway is one of the causes of relapse when on anti-angiogenic treatment with BVZ [14], inventors tested the effect of the combination of BVZ/IFN, one of the first approved anti-angiogenic therapies [5], with the EGFR inhibitor ERLO on the growth of two experimental mRCC tumour cell lines 786-O and A498 cells in mice. Tumour growth was equivalent in the control and the BVZ/IFN groups for 786-O cells while transient inhibition was observed for A498 cells. These results reflect the intrinsic or acquired resistance observed in patients [5]. ERLO alone had a modest effect on tumour growth and relapse was observed after 45 days of treatment with 786-O cells. This observation is consistent with the results of clinical trials showing the lack of anti-tumour activity associated with anti-EGFR treatments [25, 26]. However, a sustained inhibitory effect was observed for A498 cells suggesting that inhibition of the EGFR pathway may hold some benefit depending on the genetic characteristics of the tumour. The triple association BVZ/IFN/ERLO was the most efficacious showing strong inhibition of tumour growth with 786-O and A498 cells although the effect of the triple combination was equivalent to ERLO alone for the latter cells (Fig. 2a, b). These results highlight the differences in response to anti-angiogenic therapies (AAT) and EGFR pathway–targeting treatments, which probably reflects tumour heterogeneity [27] or different subclasses of kidney tumours (clear cell (786-O) or papillary (A498) carcinomas [28]).

BVZ/IFN/ERLO strongly reduced tumour vessel density and prevented the development of lymphatic vessels.

Inventors showed previously that BVZ alone stimulated experimental tumour growth. This unexpected result correlated with tumour vessel normalization and the development of a lymphatic network involved in tumour cell dissemination [14, 29]. Considering these observations, inventors hypothesized that the triple combination may eradicate blood vessels and prevent dissemination via the lymphatics. The number of blood vessels decreased for 786-O tumours treated with BVZ/IFN and ERLO (Fig. 3a and Fig. 7a) but was not different for A498 tumours (Fig. 3c and Fig. 7b). However, these treatments increased the number of vessels (CD31 positive) lined with αSMA-positive cells suggesting vessel normalization (Fig. 3a, c and Fig. 7a, b). The triple combination decreased the

number of blood vessels but also increased coverage with αSMA labelled cells for 786-O and A498 tumours (Fig. 3a, c and Fig. 7a, b). The amount of tumour haemoglobin was significantly decreased for only the triple combination suggesting that the treatment reduced tumour perfusion and/or haemorrhagic vessels (Fig. 3e). As previously reported, BVZ stimulated the development of a lymphatic network in 786-O tumours [14]. A similar result was observed when BVZ was coupled with IFN for 786-O and A498 tumours although lymphatic vessels were already present in A498 tumours in untreated mice (Fig. 3b, d and Fig. 7a, b). ERLO stimulated the development of lymphatics for both tumour model systems. However, the triple combination strongly reduced the BVZ/IFN- or ERLO-dependent development of the lymphatic network for both model systems (Fig. 3b, d and Fig. 7a, b) and the basal level of lymphatics for the A498 tumours. These results suggest that the triple combination inhibited tumour growth and prevented anti-angiogenic-dependent metastatic dissemination via the lymphatics.

Analysis of genes related to tumour angiogenesis and lymphangiogenesis, cell proliferation, immune tolerance and polarization of macrophages

To understand the better efficacy of BVZ/IFN/ERLO, inventors investigated the genes involved in the adaptation of cancer cells (proliferation genes) and cells of the tumour environment (immune tolerance, macrophages, pro/anti-angiogenic genes) to a given treatment. Table 1 summarizes the modifications to the mRNA analyzed by qPCR or proteins analyzed by ELISA.

qPCR	786-O				A498			
	Control	BVZ/INF	ERLO	BVZ/INF + ERLO	Control	BVZ/INF	ERLO	BVZ/INF + ERLO
Housekeeping genes								
36B4	100	100	100	100	100	100	100	100
m-36B4	100	100	100	100	100	100	100	100
Proliferation genes								
PTPRκ	100	289 (***)	88	54 (***)	100	185 (**)	156 (**)	33 (**)
EGFR	100	158 (*)	116	133	100	172 (**)	210 (**)	82
m-EGFR	100	313 (*)	222 (**)	96	100	106	151 (*)	156 (*)
CSF1	100	467 (**)	72	104	100	159	39 (**)	75
CSF1R	100	43 (*)	50 (*)	26 (**)	100	71	73	59 (*)
m-CSF1	100	17 (**)	242 (***)	135	100	27 (***)	44 (**)	50 (*)
m-CSF1R	100	122	110	81	undetected			
Immune tolerance gene								
PDL1	100	1332 (***)	130	768 (**)	undetected			
Macrophage M1 genes								
m-iNOS	100	93	121	104	100	127	80	75
m-IL6	100	110	73	127	100	107	78	70
Macrophage M2 genes								
m-ARG1	100	8 (***)	334 (***)	19 (***)	100	139 (*)	143 (**)	55 (***)
m-CD206	100	38 (***)	644 (***)	59 (*)	100	74	104	52 (*)
Pro/anti-angiogenesis genes								
IL6	100	99	146	33 (*)	undetected			
CXCL5	100	100	309 (**)	49 (*)	100	74 (**)	52 (***)	80 (**)
CXCL4	100	394 (***)	73	119	undetected			
CXCL10	100	87	166 (*)	216 (*)	undetected			

ELISA (pg/mg)	786-O				A498			
	Control	BVZ/INF	ERLO	BVZ/INF + ERLO	Control	BVZ/INF	ERLO	BVZ/INF + ERLO
hVEGF	2065	2280	3192 (**)	3287 (**)	1646	1945	2591 (**)	2620 (***)
mVEGF	2395	2214	1755 (*)	2007	2108	2206	1431 (**)	1930
hVEGF-C	63.5	56.4	50.2	102.1 (**)	43.1	46.2	53.1	84.7 (**)
hEGF	221	264 (**)	272 (**)	711 (***)	173	192	128 (**)	294 (**)
mEGF	697	766	363 (**)	683	267	313	286 (*)	468 (**)
CXCL1	376	267 (*)	302	289 (*)	238	300	333	349
CXCL7	26.4	29	34.4 (*)	38.4 (**)	25.8	25.8	31.7 (*)	32.6 (*)
CXCL8	2560	1620 (***)	1659 (**)	2019 (**)	1835	1675	1503 (*)	1765
Good		8	5	8		3	7	5
Bad		-5	-8	-6		-2	-6	-7
Score		3	-3	2		1	1	-2

Table 1: Analysis of pro-angiogenic/pro-lymphangiogenic/pro-inflammatory genes/proteins in tumors from mice treated with ERLO; BVZ/INF or BVZ/INF/ERLO. The percentage expression of the different genes evaluated by qPCR and the amounts of cytokines detected by ELISA are show. The indication "m" stands for mouse genes. If not indicated the genes are human ones. For the measured

genes, the reference values (100) correspond to the content of a given gene in tumors of the placebo-treated mice. The amounts of cytokine in tumor extracts are given in pictograms (pg) or nanograms (ng) per milligrams (mg) of total proteins. The statistically significant differences are shown. * $p < 0.05$: ** $p < 0.01$: *** $p < 0.001$. A good prognostic marker is presented in black characters on a grey background; a poor prognostic marker is presented in white characters on a black background and markers with no significant modification are presented in black characters on a white background. The number of good or bad prognostic markers and markers that are not influenced by a given treatment are shown. A score of +1 is given to a good prognostic marker whereas a score of -1 is given to a poor prognostic marker. The final score corresponds to the addition of good and poor prognostic markers. For 786-O cells, BVZ/INF and BVZ/INF/ERLO treatments gave positive scores (3 and 2 respectively) with the highest number of good prognostic indicators (8), whereas ERLO gave a negative score (-3) with the highest number of bad prognostic factors (-8). For A498 cells, BVZ/INF/ERLO treatments gave the worst score (-2) with the highest number of poor prognostic indicators (-7), whereas BVZ/INF and ERLO gave equivalent positive scores (1) with the highest number of good prognostic indicators for ERLO (7).

First, it was obvious that modifications to gene expression differed for the two cell lines highlighting the differences in the genetic background of the tumour. However, some genes were consistently modified by the different treatments in both cell lines. Phospho tyrosine phosphatase receptor kappa (PTPRκ) mRNA levels were decreased by BVZ [14], but were up-regulated by BVZ/IFN in 786-O and A498 cells. Strikingly, inhibition of EGFR by ERLO induced PTPRκ only in A498 tumours. However, PTPRκ levels were down-regulated by the triple combination. These results suggest that the association of IFN with BVZ prevented compensatory activation of proliferation pathways mediated by a decrease in PTPRκ. However, concomitant inhibition of the VEGF and EGFR pathways resulted in down-regulation of PTPRκ. Human EGFR levels were increased by BVZ/IFN in both cell lines indicating that the compensatory mechanisms linked to VEGF/VEGFR inhibition involved the EGFR pathway. Induction of EGFR in cells of the microenvironment was also observed in response to ERLO with both cell lines indicating that EGFR inhibition was compensated by over-expression of the receptor. In both cell lines, the inhibition of the EGFR pathway was also compensated by over-expression of EGF by tumour cells only for the triple combination. The colony stimulating factor 1 and its receptor (CSF1/CSF1R) were then investigated since CSF1R is highly expressed in RCC cells because of chromosome 5q22qter amplification [30, 31]. The triple combination inhibited CSF1R expression in both cell lines suggesting that the treatment indirectly targeted an autocrine proliferation pathway. Inventors' previous observation showed that BVZ had no effect on expression of its target VEGF produced either by tumour cells or cells of the microenvironment [14]. Unfortunately, the triple combination stimulated VEGF expression by tumour cells in both model systems. Moreover, VEGFC, a key player involved in metastatic dissemination via the lymphatics, was enhanced by the triple

combination in both model systems. Increased VEGFC expression was consistent with the presence of lymphatic vessels observed in Fig. 2b, d. The expression of angiogenic factors redundant for VEGF was suspected to promote BVZ resistance. The CXCL family of cytokines was investigated because of its involvement in RCC aggressiveness, as inventors previously shown [13, 14]. The CXCL family of cytokines is divided into pro- and anti-angiogenic members. Only CXCL5 and CXCL7, two pro-angiogenic members, are consistently down- and up-regulated in both cell lines, respectively by the triple combination. The inflammatory context is a key player in adaptation to treatment particularly for polarization of macrophages [32]. Only the triple combination down-regulated expression of M2 markers of macrophages (arginase and CD206). Finally, immune tolerance was investigated because of the efficacy of anti-programmed death ligand (PDL1) antibody treatment, especially for the most aggressive tumours [33]. PDL1 was only detected in 786-O cells and BVZ/IFN and BVZ/IFN/ERLO strongly induced its expression suggesting that BVZ/IFN plus nivolumab (anti-PD1) or atezolizumab (anti-PDL1 [34]) may have clinical benefit. According to these differences, inventors attempted to quantify the good and bad prognostic markers. They gave a score of 1 for a good prognostic marker, a score of -1 for a bad marker and 0 for unchanged or undetected markers. The best score (3) was obtained with BVZ/IFN treatment whereas the worst score (-3) was assigned to ERLO treatment of 786-O cells. For the A498 cells BVZ/IFN or ERLO generated the best scores. Surprisingly, triple treatment did not give the best score although tumour growth was strongly impaired. These results suggest that the triple association may select tumour cells with a more aggressive phenotype that are kept in check by the drugs. They also reflect again the tumour heterogeneity between individuals.

Cells derived from mice tumours treated with BVZ/IFN/ERLO are still sensitive to ERLO

The different treatments generated a wide range of profiles of tumour growth. Therefore, inventors hypothesized that due to the selection pressure exerted by the different drugs, tumour cells acquired specific genotypic/phenotypic profiles. Thus, they analyzed their proliferation after amplification and selection from the tumours, as previously described [14]. The proliferation rates forty-eight hours after seeding of cells from control, BVZ/IFN and ERLO 786-O treated-tumours were low or similar (125, 175 and 160 %, respectively, Fig. 4a). However, cells from BVZ/IFN/ERLO 786-O treated-tumours proliferated three times more than those from control tumours (350 %, Fig. 4a), which probably reflected their strong level of EGF production (Table 1). The proliferation rates of A498 cells extracted from the different tumours were higher than that of 786-O cells (200 %) whereas they were lower for parental cells (Fig. 9). However, they were similar whatever the treatment (Fig. 4b). In these cells, the intra-tumour levels of human/mouse EGF and EGFR varied according to the treatment (Table 1). Inventors showed previously that exposure to BVZ sensitized resistant cells to ERLO because of PTPRκ down-regulation [14]. Consistently, 786-O and A498 cells from BVZ/IFN tumours were more sensitive to ERLO than cells from control tumours (28 % versus 43 % inhibition for 786-O cells and 17 % versus 32 % for A498 cells). This result is also consistent with increased expression of

EGFR in both model systems. 786-O cells from ERLO tumours were still highly sensitive to ERLO (40 % inhibition) whereas A498/ERLO cells became insensitive (only 7 % inhibition). This result may be explained by increased expression of EGF in 786-O cells and its down-regulation in A498 cells (Table 1). Cells from triple-treated tumours were still sensitive to ERLO whatever the model. This persistent response to ERLO was probably linked to increased expression of EGF in both model systems (Table 1 and Fig. 4a, b). Hence, the chronic inhibition of the EGF/EGFR compensatory proliferation pathway probably explains the *in vivo* efficacy of the triple combination. Inventors then analyzed the level and activity of EGFR and of downstream signalling pathways involved in cell proliferation (ERK/MAP Kinase and PI3Kinase/AKT) and their sensitivity to ERLO. Total EGFR levels were increased in cells following treatment of the 786-O model system and were slightly decreased in the A498 model. Basal levels of the phosphorylated/active form of EGFR (pEGFR) decreased in 786-O and A498 cells after BVZ/IFN treatment. This result is consistent with increased levels of PTPRκ (Table 1 and Fig. 4c, d). However, the decreased level of PTPRκ in cells from the triple-treated tumours resulted in a modest increase in basal pEGFR levels for both systems. ERLO inhibited pEGFR in the different cells for both cellular models except for A498 cells from the triple-treated tumours. This result reflects an alternative mechanism of EGFR activation probably through the increased expression of EGF by cells of the microenvironment (Table 1). Inhibition of the EGFR activity correlated with inhibition of ERK and preferentially with the AKT activity (Fig. 8a, b). However, the persistence of ERK and AKT activity independently of the EGFR activity may also reflect activation of alternative proliferation pathways independent of the EGF/EGFR pathway after chronic exposure to treatments.

Primary cells present a different sensitivity to ERLO

Inventors showed previously that treatment response to anti-angiogenic drugs was equivalent in metastatic patients and in primary cells derived from the patients' surgically removed tumour after cultured in a medium specific for kidney cells [35]. The half-maximal inhibitory concentration (IC50) for ERLO and for the first-line treatment of metastatic tumors with sunitinib, is reported in Table 2 for inventors' reference 786-O and A498 cell lines and the already described primary cells [35].

	CELL LINES		PRIMARY CELLS		
	786-O	**A498**	**CC**	**M**	**TF**
Sunitinib	6± 1	6 ± 0.3	4.1 ± 0.3	4 ± 0.5	11.3 ± 0.8
Erlotinib	5.3 ± 0.9	9± 0.5	3.2 ± 0.1	11.5 ± 1.2	12.2 ± 2

Table 2: Sensitivity of the primary cells to the different treatments. IC50 for the different drugs ± SD is shown. 786-O cells are sensitive to sunitinib and erlotinib and serve as the reference. Inventors considered the cells to be sensitive to a drug if the concentration giving 50% inhibition of cell

proliferation (IC50) was lower than or equal to the IC50 in 786-O cells and considered to be resistant if the IC50 was higher than in 786-O cells. CC, M and TF cells were derived from metastatic patients. When cells are sensitive to a given treatment, it is presented on a white background but if cells are insensitive it appeared on a black background.

Three primary cell cultures were derived from metastatic tumours (CC, M, TF). Some cells were sensitive to both treatments (sunitinib, ERLO; 786-O, CC), to only sunitinib (A498, M), or to none of these treatments (TF). Only one primary culture (CC) was more sensitive to ERLO compared to 786-O cells (IC50 1.65 lower). M and TF cells presented a 2.2 and a 2.3-fold higher IC50 for ERLO compared to 786-O cells. Once again, this result may highlight the tumour cell heterogeneity.

To explain the relative sensitivity to ERLO of the primary cultures, inventors compared their relative amount of EGFR to that of inventors' reference cell lines 786-O and A498. They also added an additional cell line obtained from the ATCC, RCC4 cells. These cells were not used for *in vivo* experiments because they are probably the least aggressive and they do not form tumours in nude mice. Whereas the three cell lines expressed equivalent mRNA levels, the amounts of EGFR protein were very different and ERLO did not influence the level. The lowest levels were observed in 786-O cells and the highest in A498 cells, with RCC4 cells showing an intermediate level (Fig. 10).

Therefore, EGFR levels and the subsequent activity varied a lot from tumour to tumour, a situation that may explain the relative sensitivity to ERLO in clinical trials.

A silent mutation in the EGFR sequence correlated with EGFR levels and ERLO/AZD3759 sensitivity

Analysis of the online database cBioportal showed that EGFR is overexpress in RCC and that over-expression of EGFR is indicative of shorter overall survival for patients with metastatic RCC (Fig. 11a, b). Moreover, ERLO is efficient for treatment of lung tumours but only if EGFR has mutations in the kinase domain [21, 22]. To determine whether specific mutation(s) may explain the relative expression and sensitivity to ERLO of the different cell lines and primary cultures, inventors performed exome sequencing of the *EGFR* gene. The different mutations/deletions determining ERLO sensitivity in lung cancers were not detected in RCC cells [21, 22]. They only detected a single-nucleotide polymorphism (SNP) that modifies the codon corresponding to glutamine from CAG to a CAA (NM_005228; G 2618 to A, rs1050171), a mutation described in osteosarcoma [36] and in head and neck squamous cell carcinoma [37]. RCC4 cells are wild-type (CAG codon) on both alleles, 786-O cells are heterozygous for the mutation and A498 cells are homozygous for the mutation (CAA on both alleles, Fig. 5a). The amino acid involved is located in the kinase domain (Q 787). This specific mutation is particularly interesting since it modifies a frequently used codon for Q to a rare codon (CAG, frequent codon for Q (73%) to rare codon CAA (27%)). This may explain the difference in the total amounts of EGFR detected in the different cell lines and the relative sensitivity to ERLO (Fig

10). Inventors then obtained additional samples of surgically removed tumours from which they derived primary cultures. 3 out of 31 primary cells (9.7%) were wild-type, 13 out of 31 (41.9%) were heterozygous and 15 out of 31 (48.4%) were homozygous for the silent mutation. For the majority of patients' samples, they obtained normal renal tissue from which they derived primary cultures. Normal cells were carrying the mutation suggesting its presence in the germinal state. This result was consistent with the allele distribution of this SNP in the European population (https://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=1050171). The first primary cells that they obtained were heterozygous (CC, M) or homozygous (TF) for the G 2618 to A mutation. Sensitivity to ERLO was tested in the different primary cells. The IC50 for ERLO was the lowest for cells with the heterozygous mutation and the highest for the cells with the homozygous mutation and intermediate for wild-type cells (Fig. 5d). The same experiment was carried out using AZD3759 instead of ERLO. Likewise, the IC50 for AZD3750 was the lowest for cells with the heterozygous mutation and the highest for the cells with the homozygous mutation and intermediate for wild-type cells (Fig. 5e). Considering these results, inventors investigated whether the G2618A mutation could be responsible for the discrepancy between the mRNA and protein levels observed in the different cell lines. Thus, they hypothesized that a higher efficiency of translation of mRNA carrying the A mutation occurs. To functionally test this hypothesis, they performed an *in vitro* transcription and translation assay using an EGFR construct for both the wild-type and the mutated allele. The results clearly show that the wild-type construct was translated less than the variant (Fig. 5c). To confirm these results, they set up an experimental model by cloning the two full length coding regions (the wild-type G allele, the mutated A allele) into an eukaryotic expression vector to perform transient transfection in HEK293 cells expressing very low EGFR levels. A luciferase construct was co-transfected as a control for transfection efficiency [38]. Comparing only samples with the same transfection efficiency, they found that the wild-type EGFR plasmid produced a lower amount of protein than the polymorphic one (Fig. 5d).

Discussion

The presence of high amounts of EGFR in mRCC cells suggested that inhibitors of EGFR may have a potent therapeutic effect on patients. However, a phase II clinical trial with the EGFR inhibitor gefitinib/Iressa [26] and a phase I/II clinical trial using the EGFR-directed antibody cetuximab/Erbitux gave disappointing results [39]. Kuroda et al., suggested that these negative results were due to constitutive stimulation of the AKT pathway probably through activation of alternative tyrosine kinase receptors [40]. However, both clinical trials did not associate EGFR inhibitors with the FDA approved combination of BVZ and IFN. While remaining cautious, the differences between the results of the clinical trials and inventors' preclinical models suggest that interferon alpha (IFN) plays a pivotal role and enhances the therapeutic effect of BVZ and ERLO. The recent development of immune

checkpoint inhibitors for kidney cancer [41] strongly suggests that IFN, the first generation of immuno-therapies, is a key player for combined treatment and should be associated with anti-EGFR inhibitors for a maximal effect.

To gain insight into the molecular mechanisms associated with the success of inventors' preclinical models, inventors scrutinized the different pathways that were involved in relapse on treatment with BVZ alone in their previous study [14], in particular: modification to the network of blood and lymphatic vessels, compensation by redundant angiogenic factors, selection of more aggressive tumour cells and adaptation to the microenvironment. Hence, Table 1 indicates the different pathways that may explain the relative success of BVZ/IFN/ERLO treatment. Inventors' previous study highlighted the strong impact of BVZ on the normalization of the vascular network and the development of a VEGFC dependent lymphatic network. In the present experiment, a striking difference between ERLO and BVZ/IFN treatments, alone or in combination was observed for both networks. Whereas single treatment normalized the blood vessels and stimulated the development of a lymphatic network, the triple combination was associated with a decrease in the number of blood vessels, an increase in α-SMA labelled cells and the presence of fewer or equivalent numbers of Lyve-1 positive cells. Despite the stabilization of tumour growth, the presence of lymphatic [42] and probably α-SMA-labelled tumour associated fibroblasts [43, 44] is perhaps indicative of further tumour evolution. The pressure of selection mediated by the treatment, stimulated VEGFC expression by human tumour cells within tumours. Such differences have already been observed and are implicated in mechanisms of resistance [45]. VEGFC-dependent induction by a specific treatment may also serve to define the best concentration of a drug that avoids such compensatory mechanisms [46].

Inventors' current study was based on the fact that BVZ induced a strong decrease in the amounts of mRNA of PTPRκ, the major down-regulator of EGFR activity. However, BVZ/IFN increased PTPRκ levels. Hence, IFN may indirectly decrease the activity of major tyrosine kinase receptors including EGFR. EGFR is not only expressed by tumour cells but also by endothelial cells and the EGF/EGFR pathway participates in processes of tumour vascularization [47]. Induction of human or mouse EGF and/or EGFR with single treatment with ERLO or BVZ/IFN may explain the increase in the number of mature blood vessels.

The decrease in the amount of CSF1R with the triple treatment argues strongly for a decrease in tumour growth since the CSF1/CSF1R pathway exerts an autocrine proliferation loop in RCC [48] and CSF1R is indicative of poor prognosis [31]. Moreover, the EGF produced by tumour cells stimulated the secretion of CSF1 by cells of the microenvironment, which amplified proliferation of tumour cells [48]. Any decrease in either EGF or CSF1 will prevent tumour growth, a situation encountered with only the triple combination.

Triple treatment also played a prominent role on the polarization of macrophages that can alternate between pro-inflammatory (M1) and pro-tumourigenic (M2) phenotypes [49, 50]. Whereas M1 markers were not affected by the different treatments, M2 markers were down-regulated, especially

with BVZ/IFN/ERLO for the 786-O and A498 cellular models. M2 macrophages have been implicated in increased angiogenesis [51]. Hence, down-regulation of M2 macrophages may explain the decrease in microvessel density in tumours with BVZ/IFN/ERLO. It is interesting to note that the M2 phenotype is stimulated by the CSF1 pathway [52], which may explain up-regulation of M2 markers in the presence of ERLO alone.

The increase in PDL1, participates in evasion of immune surveillance [53]. Since treatments targeting the PD-1/PDL1 axis have been approved for the treatment of mRCC [54], it may be used at relapse when on the triple combination. However, despite expression of PDL1 by tumour cells, the presence of IFN may still induce proliferation of cytotoxic T lymphocytes and therefore may maintain immune surveillance.

EGFR inhibitors are currently used for the treatment of lung cancers but treatment is efficient only if the receptor has specific mutations in the kinase domain [21, 22]. Moreover, a mutation that antagonizes the efficacy of the major EGFR inhibitor ERLO was recently discovered [55]. Although these mutations are more or less frequent depending on the cancer types, their presence is very rare in samples of mRCC [56-58]. A specific mutation of the kinase domain of EGFR was recently described in mRCC but in another position than that described in the literature [59]. The discovery of a specific mutation in EGFR in mRCC may constitute an efficient predictive marker of sensitivity/resistance to EGFR inhibitors to increase the treatment arsenal in case of therapeutic impasse. However, it was unexpected that a silent mutation may affect the amount of EGFR protein and sensitivity to ERLO.

The analysis of genome sequences in cancer revealed that silent mutations can control the speed of mRNA translation, mRNA folding, pre-mRNA splicing, and through translational pausing, the folding of proteins [60]. Moreover, mRNA containing CAG codons are less translated than those with the CAA codon coding for glutamine. Hence, silent mutations that are considered to be irrelevant may be driver mutations for tumour development. They may also constitute predictive markers of resistance to a given treatment, as inventors described recently [38]. The G2618A mutation is particularly interesting because it modifies a frequently used codon for Q to a rare codon (CAG code for Q in 73%, CAA in 27% of cases). Its presence in the germinal state suggests that the patients with kidney cancers carrying a homozygous mutation (A/A) are intrinsically resistant to EGFR inhibitors. However, the opposite situation was observed for patients with head and neck cancers (higher sensitivity to ERLO if A/A), a phenotype depending on the presence of the long non coding RNA EGFR-AS [37] which is highly expressed in RCC and is correlated with tumor stage (Fig. 11c, d). Moreover, overexpression of EGFR-AS1 is correlated with a longer disease-free survival in kidney cancers (Fig. 11e) whereas it has no incidence on survival in head and neck tumors which reflects a different genetic situation and which may explain an opposite EGFR inhibitor sensitivity.

In conclusion, EGFR is a relevant therapeutic target for mRCC in combination with anti-angiogenic treatment but only when a relevant mutation, different to those described in lung cancer, is present. Association of first generation immunotherapy with IFN should be revisited because of the associated

debilitating side effects and new associations with immune checkpoint inhibitors may have a strong therapeutic impact.

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CLAIMS

1. A method for predicting the treatment response to an anti-epidermal growth factor receptor (EGFR) molecule of a subject suffering from renal cancer, preferably of a human subject, the method comprising (i) providing a biological sample comprising genetic material from the subject suffering from renal cancer and (ii) performing a single nucleotide polymorphism (SNP) genotyping analysis in the EGFR gene locus in said sample, wherein the presence of a homozygous mutation that modifies in the EGFR wild-type deoxyribonucleic acid (DNA) sequence the codon coding to glutamine 787 (Q787) in the EGFR protein from CAG to CAA is indicative for a negative treatment response of the subject to the anti-EGFR molecule, and the presence of a heterozygous mutation that modifies in the EGFR wild-type DNA sequence the codon coding to glutamine 787 (Q787) in the EGFR protein from CAG to CAA, or the presence of a EGFR wild-type DNA sequence, is indicative for a positive treatment response of the subject to the anti-EGFR molecule.

2. The method according to claim 1, wherein the genotyping analysis is performed at rs 1050171 in the EGFR gene locus.

3. The method according to claim 1 or 2, wherein the SNP modifying the codon coding to glutamine 787 (Q787) in the EGFR protein from CAG to CAA is a homozygous mutation (A/A) or a heterozygous mutation (G/A).

4. The method according to anyone of the preceding claims, wherein the genotyping analysis is performed by a technique selected from the group consisting of terminator sequencing, restriction digestion, allele-specific polymerase reaction, single- stranded conformational polymorphism analysis, genetic bit analysis, temperature gradient gel electrophoresis ligase chain reaction and ligase/polymerase genetic bit analysis.

5. The method according to anyone of the preceding claims, wherein the biological sample is selected from a blood sample, a sputum sample, a urine sample, a plasma sample and a tumor sample.

6. The method according to anyone of the preceding claims, wherein the method is performed prior to initiation of treatment with an anti-EGFR molecule.

7. The method according to anyone of the preceding claims, wherein the anti-EGFR molecule for which a treatment response is to be predicted is selected from the group consisting of a small molecule directed to EGFR, an anti-EGFR antibody and an inhibitory polynucleotide capable of interfering with the expression and/or function of EGFR.

8. The method according to claim 7, wherein the anti-EGFR molecule is a tyrosine kinase inhibitor (TKI), in particular selected from erlotinib (Tarceva®), AZD3759, gefitinib (Iressa®), lapatinib (Tykerb®) and afatinib (Giotrif®).

9. The method according to claim 8, wherein the anti-EGFR molecule is erlotinib, afatinib or lapatinib, preferably erlotinib.

10. An anti-EGFR molecule for use in the treatment of a subject suffering from renal cancer, preferably of a human subject, wherein the subject exhibits genotype G/A or G/G at rs 1050171, preferably genotype G/A.

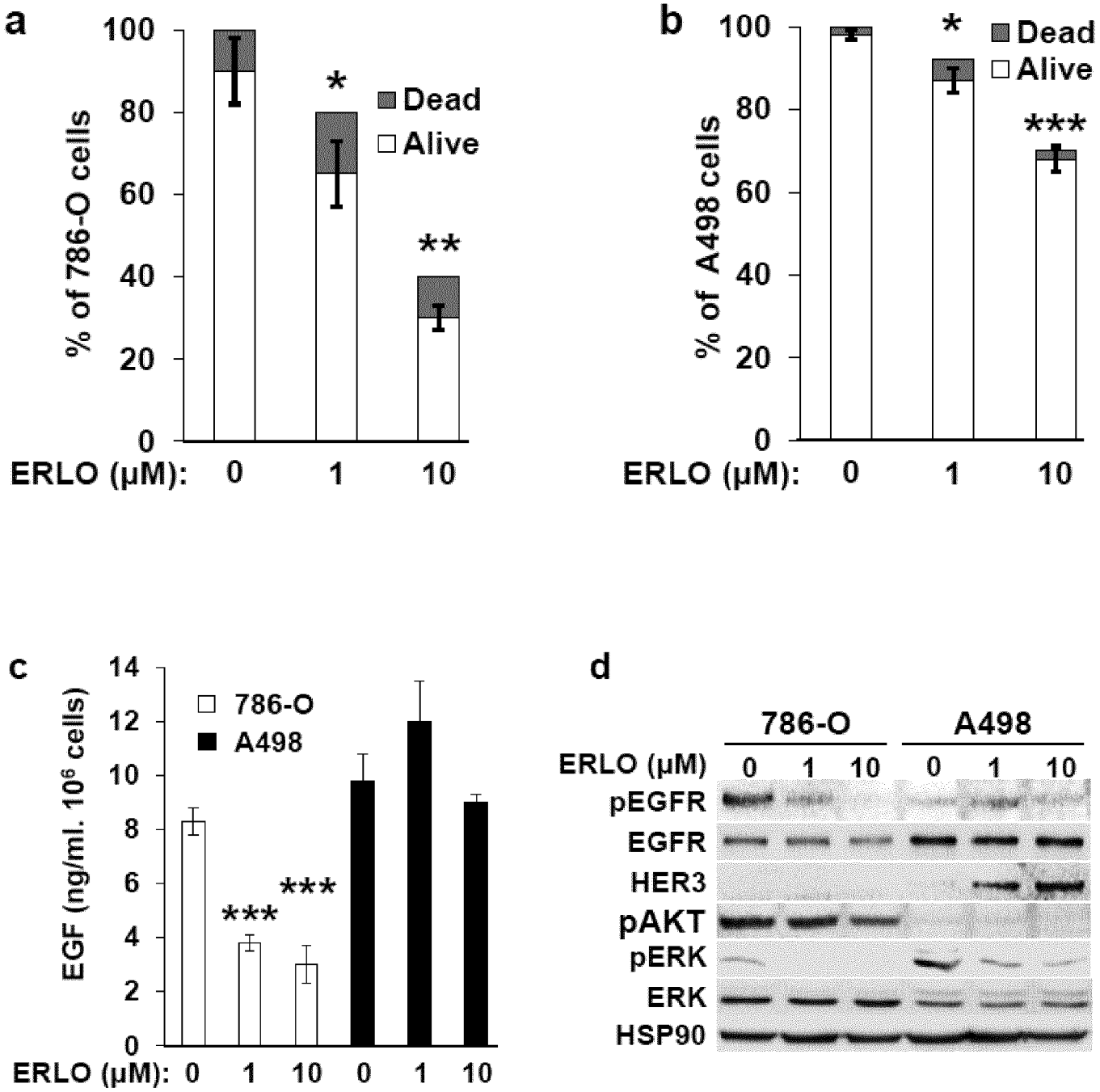
11. An anti-EGFR molecule for use in the treatment of a subject suffering from renal cancer, preferably of a human subject, wherein the subject exhibits genotype A/A at rs 1050171 and wherein said molecule is used in combination with bevacizumab (Avastin®), and preferably in addition in combination with interferon alpha (IFN) and/or with a molecule targeting PD-1 and/or PDL1.

12. A kit comprising products for detecting in a biological sample from a subject suffering from renal cancer, preferably of a human subject, the presence of a SNP modifying the codon coding to glutamine 787 (Q787) in the EGFR protein from CAG to CAA in the EGFR gene locus.

13. The kit according to claim 12, wherein the kit comprises at least two of primer(s), probe(s), enzyme(s) for primer elongation, nucleotide(s) and/or labeling agent(s), preferably a pair of primers selected from SEQ ID NOs: 27 and 28, SEQ ID NOs: 31 and 32, and SEQ ID NOs: 51 and 52, and, optionally a leaflet providing instructions on how the kit and its components should be used.

14. Use of the kit according to claim 12 or 13 for predicting the treatment response to an anti-epidermal growth factor receptor (EGFR) molecule of a subject suffering from renal cancer, preferably of a human subject.

15. The method according to anyone of claims 1-9, the anti-EGFR molecule for use according to claim 10 or 11, the kit according to claim 12 or 13, and the use of the kit according to claim 14, wherein the renal cancer is a clear cell renal cell carcinoma (ccRCC), in particular metastatic ccRCC (mccRCC).


a
% of 786-O cells
100
80
60
40
20
0
*
**
Dead
Alive
ERLO (μM): 0 1 10
b
% of A498 cells
100
80
60
40
20
0
*
***
Dead
Alive
ERLO (μM): 0 1 10
c
EGF (ng/ml. 10^6 cells)
14
12
10
8
6
4
2
0
786-O
A498
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***
ERLO (μM): 0 1 10 0 1 10
d
786-O
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ERLO (μM) 0 1 10 0 1 10
pEGFR
EGFR
HER3
pAKT
pERK
ERK
HSP90

FIGURE 1

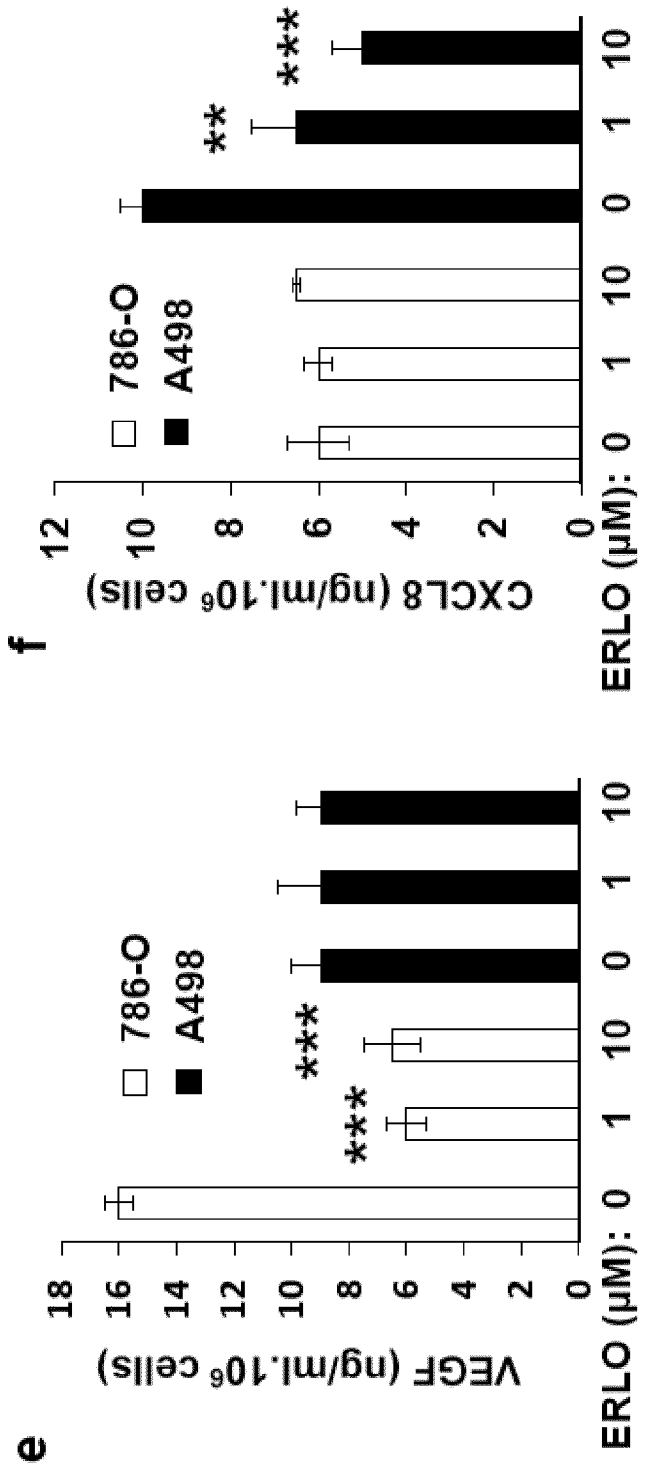
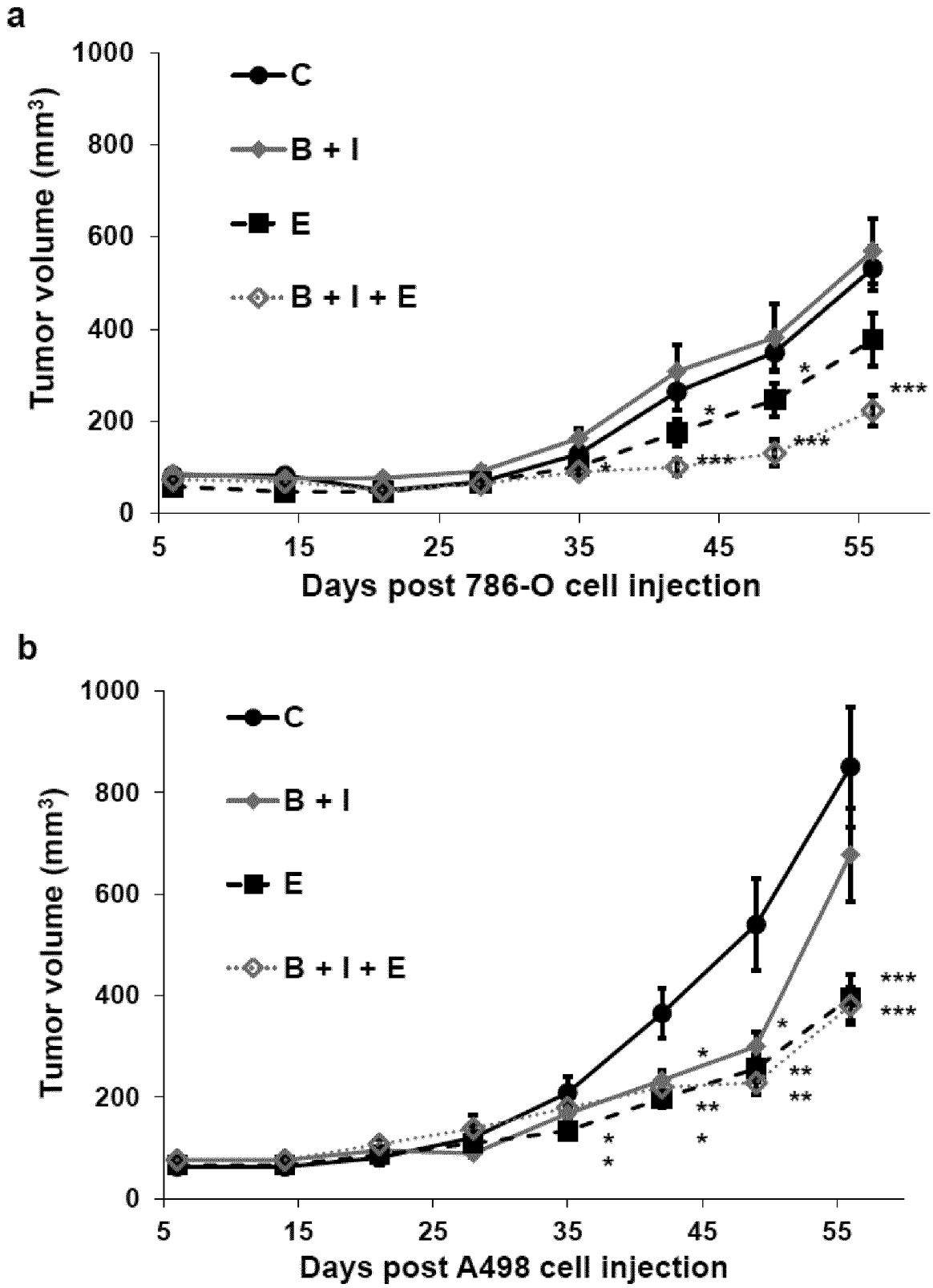


FIGURE 1 (Following)


a
Tumor volume (mm³)
1000
800
600
400
200
0
C
B + I
E
B + I + E
5
15
25
35
45
55
Days post 786-O cell injection
b
Tumor volume (mm³)
1000
800
600
400
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C
B + I
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B + I + E
5
15
25
35
45
55
Days post A498 cell injection

FIGURE 2

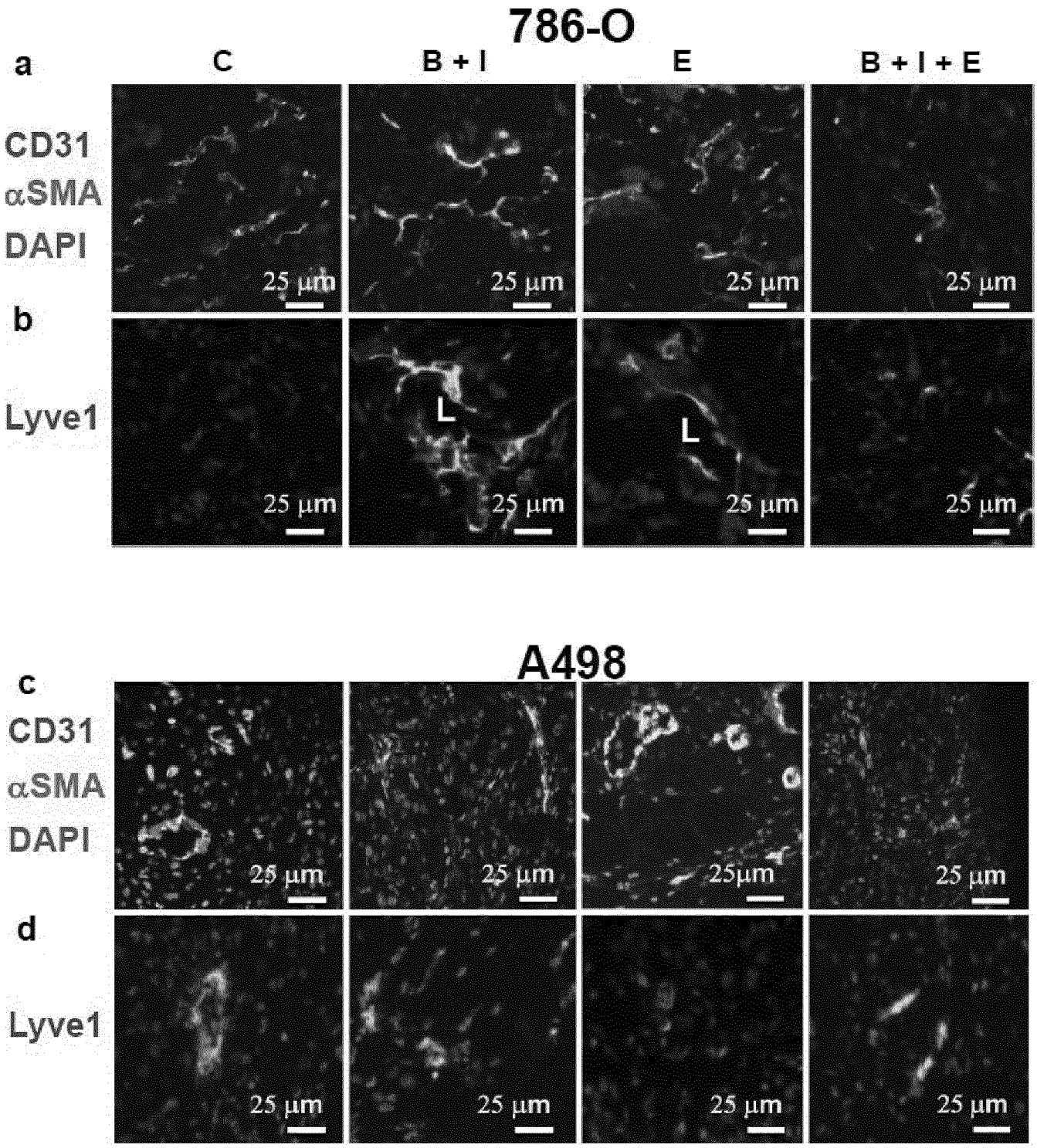


FIGURE 3

e

Haemoglobin (mg/ml/µg protein)	C	B + I	E	B + I + E
786-O tumors	552	634	580	402 (***)
A498 tumors	954	783	818	612 (**)

FIGURE 3 (Following)

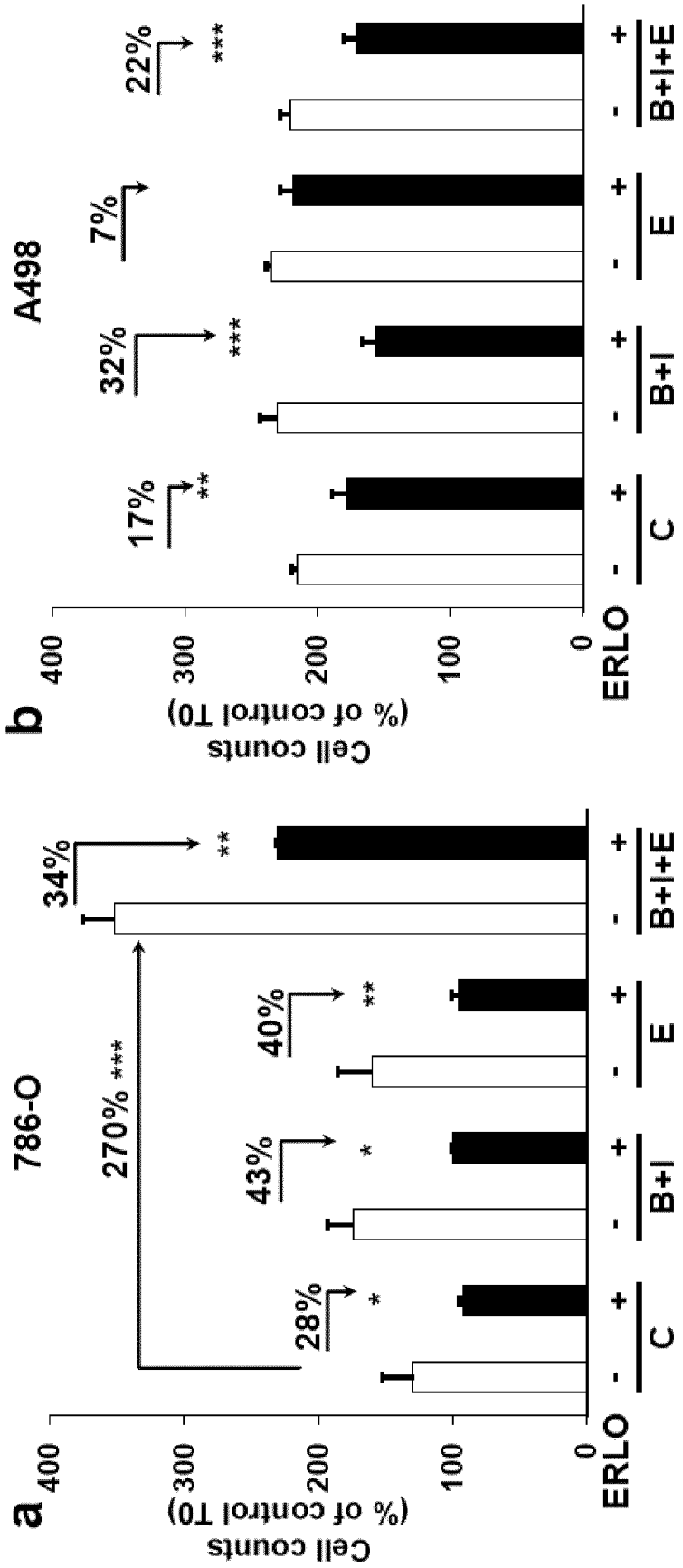

a
786-O
Cell counts
(% of control T0)
400
300
200
100
0
ERLO
C
B+I
E
B+I+E
-
+
28%
*
43%
*
40%
**
270%
***
34%
**
b
A498
Cell counts
(% of control T0)
400
300
200
100
0
ERLO
C
B+I
E
B+I+E
-
+
17%
**
32%
***
7%
22%
***

FIGURE 4

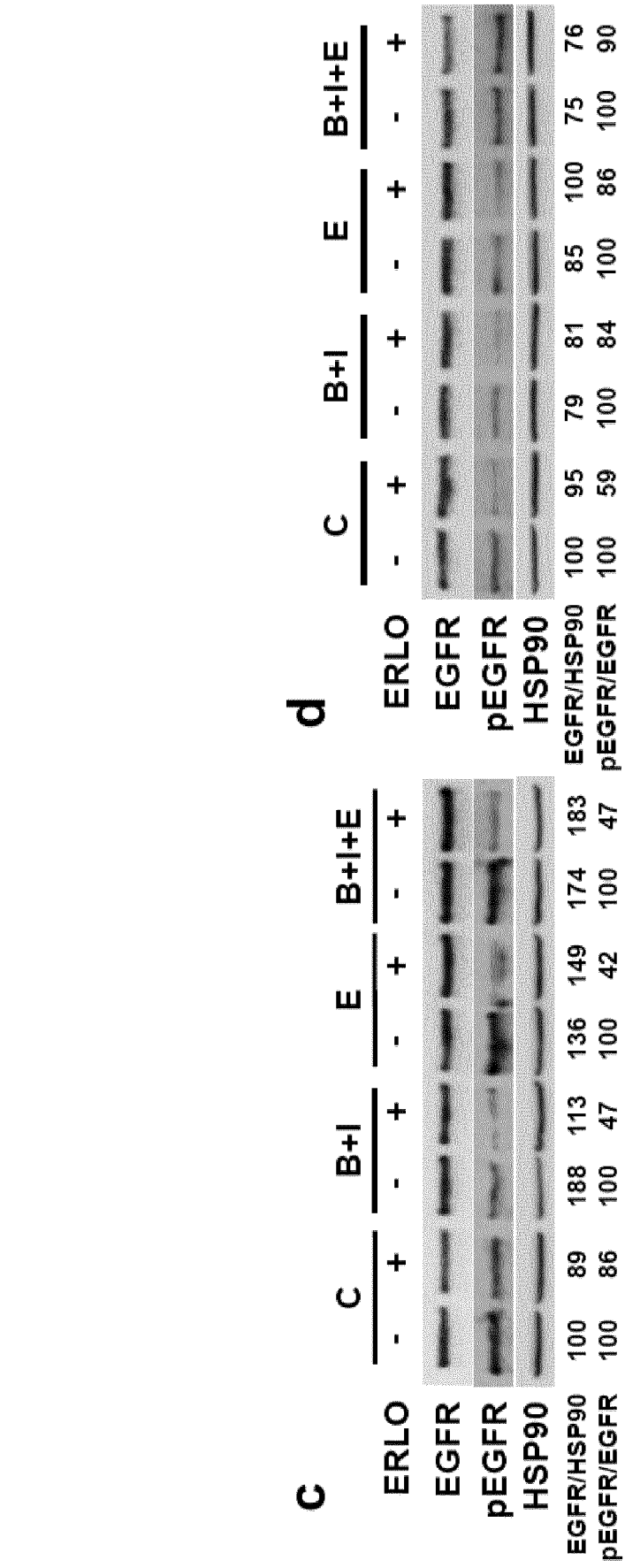


FIGURE 4 (following)

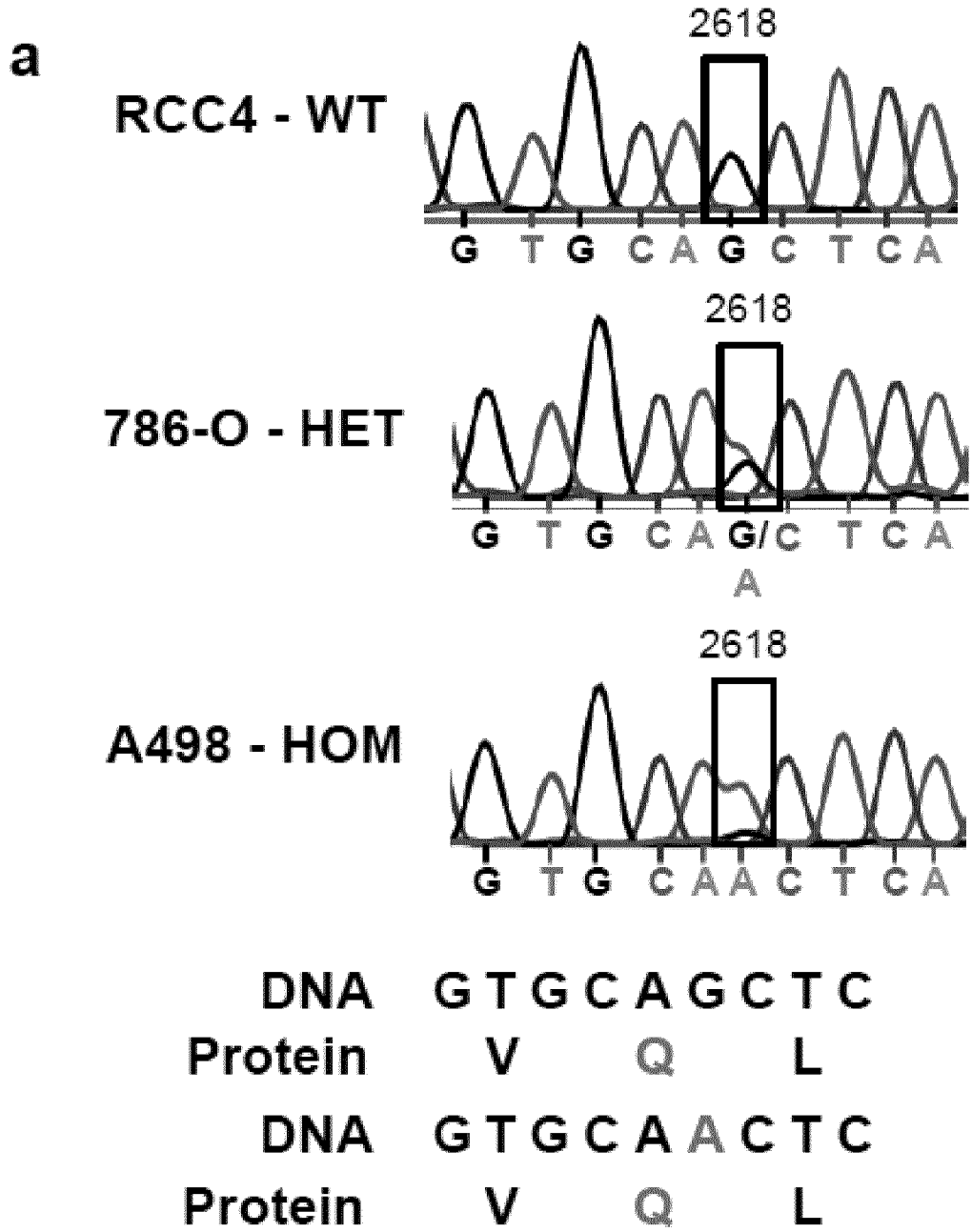

a
RCC4 - WT
2618
G T G C A G C T C A
786-O - HET
2618
G T G C A G/C T C A
A
A498 - HOM
2618
G T G C A A C T C A
DNA G T G C A G C T C
Protein V Q L
DNA G T G C A A C T C
Protein V Q L

FIGURE 5

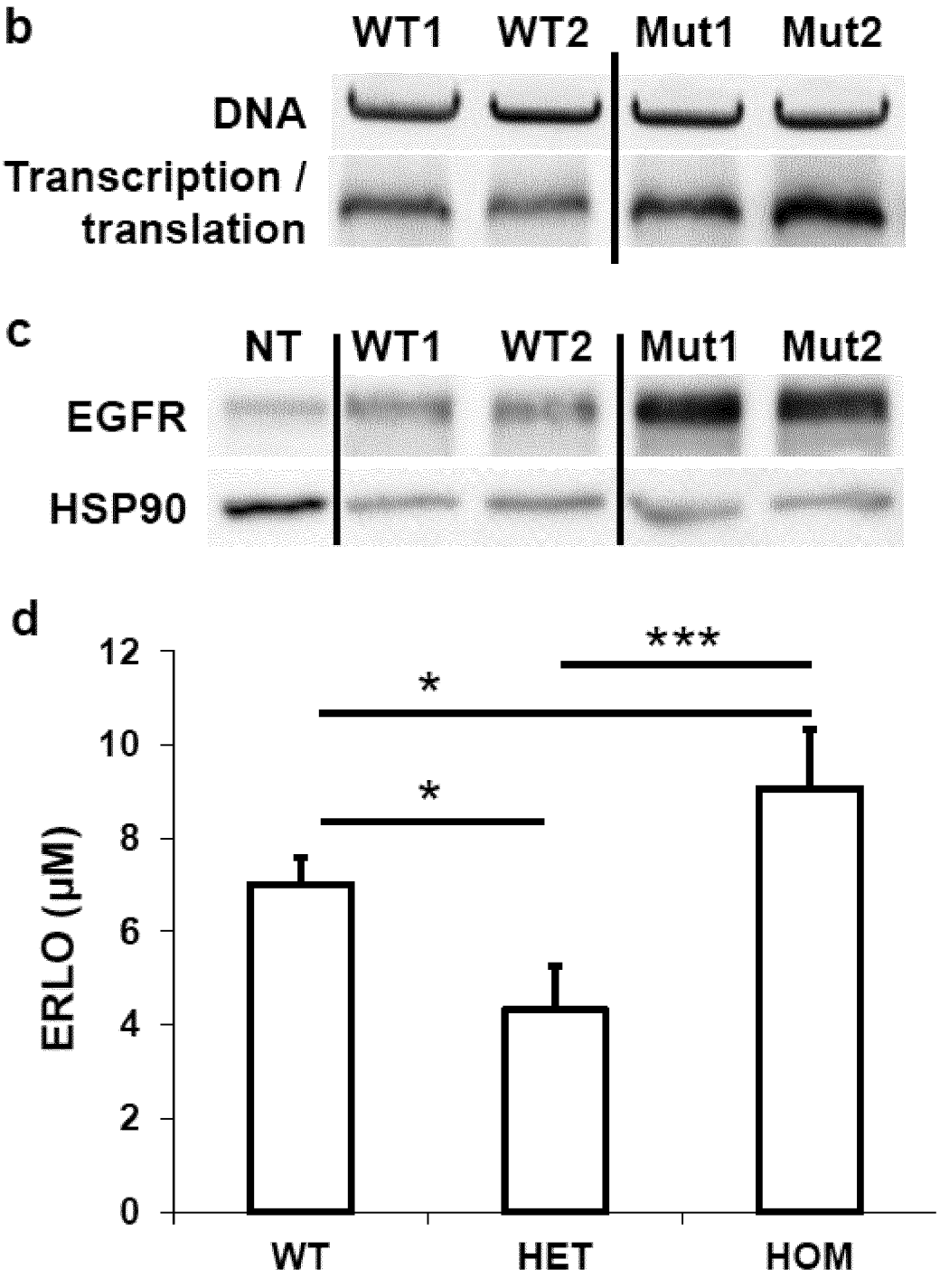


FIGURE 5 (Following)

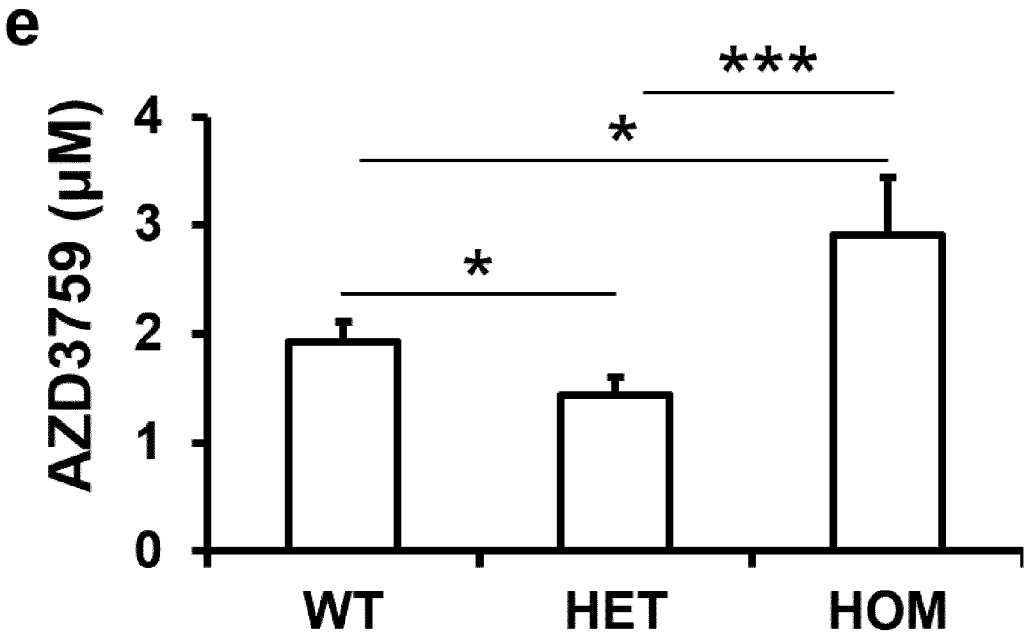
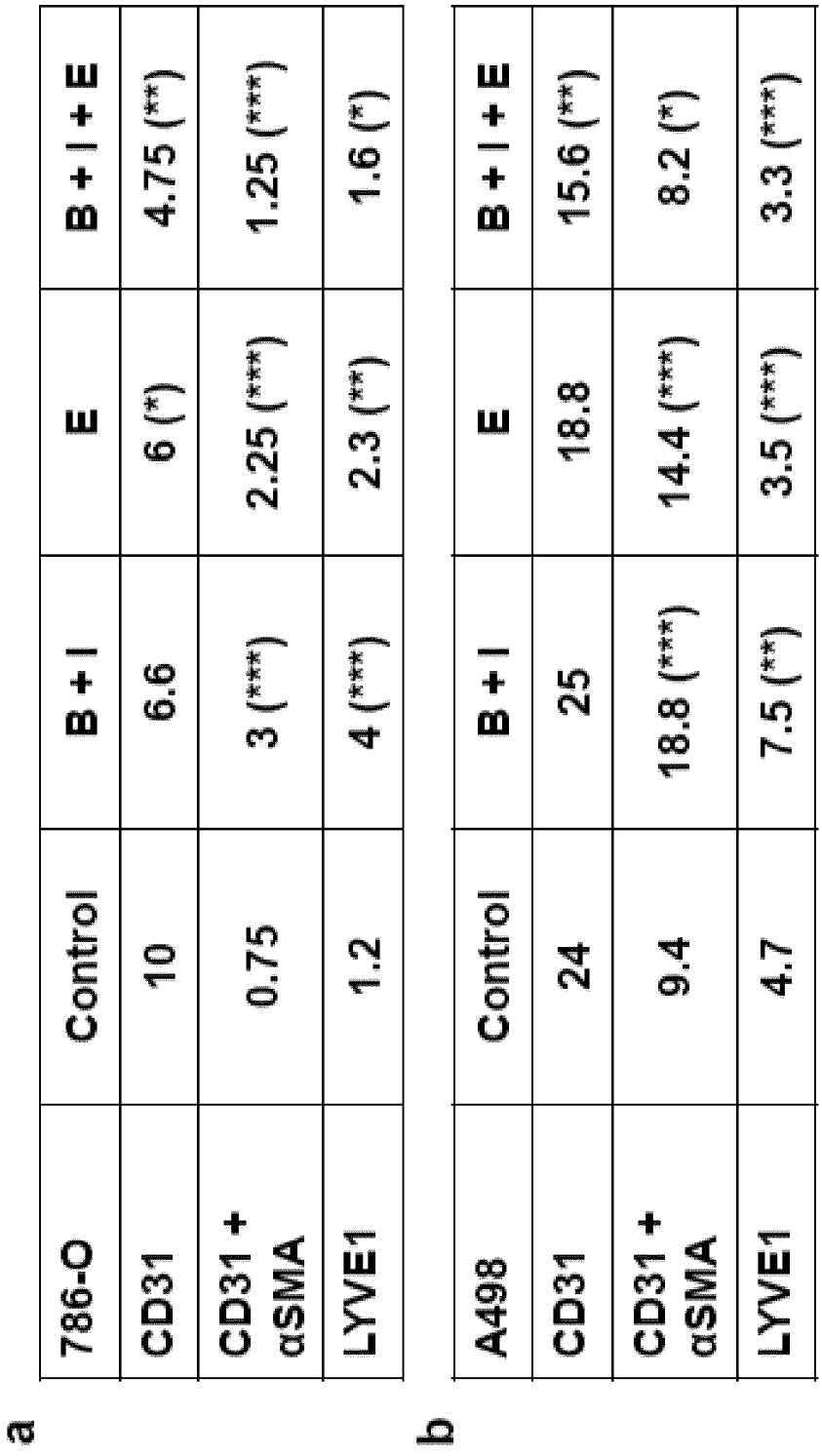


FIGURE 5 (Following)

	ERLO (µM)	EGFR/HSP90	pEGFR/EGFR	pAKT/HSP90	pERK/ERK
786-O	0	100	100	100	100
	1	120	17 **	77 *	30 **
	10	140	1 **	53 *	30 **
A498	0	247 ##	1 #	14 #	279 ##
	1	184	5	23	198 *
	10	240	1	23	151 *

FIGURE 6



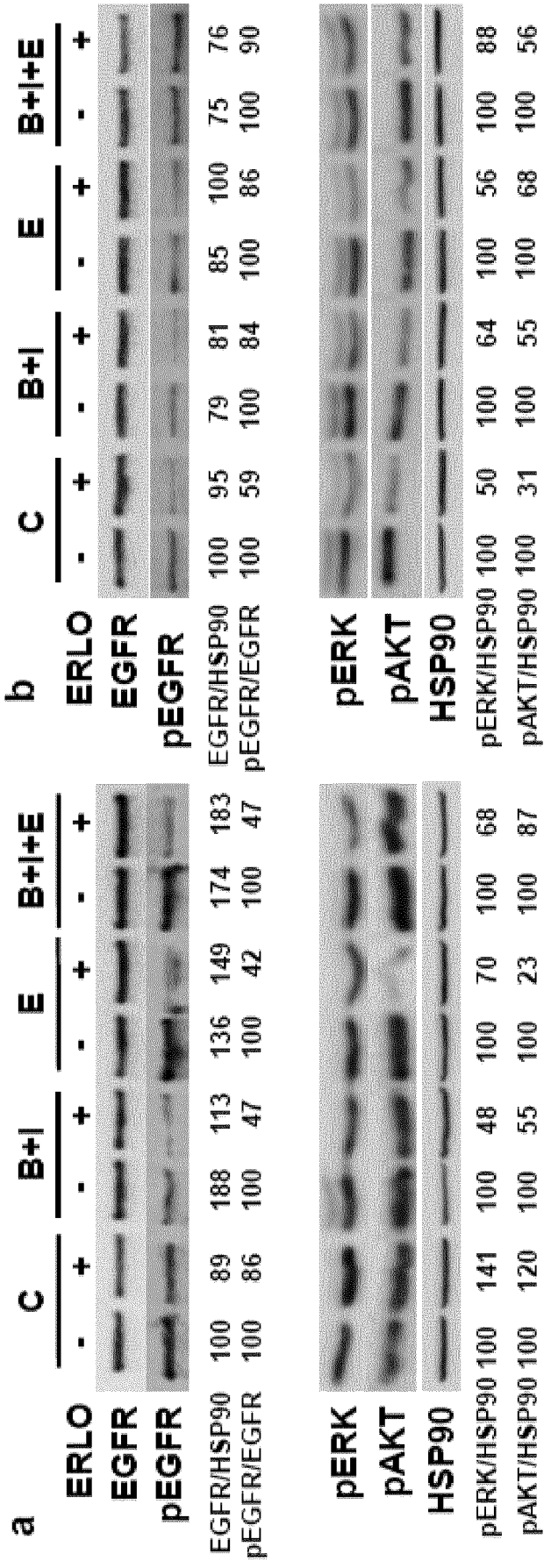
a

786-O	Control	B + I	E	B + I + E
CD31	10	6.6	6 (*)	4.75 (**)
CD31 + αSMA	0.75	3 (***)	2.25 (***)	1.25 (***)
LYVE1	1.2	4 (***)	2.3 (**)	1.6 (*)

b

A498	Control	B + I	E	B + I + E
CD31	24	25	18.8	15.6 (**)
CD31 + αSMA	9.4	18.8 (***)	14.4 (***)	8.2 (*)
LYVE1	4.7	7.5 (**)	3.5 (***)	3.3 (***)

FIGURE 7


a
C
B+I
E
B+I+E
- + - + - + - +
ERLO
EGFR
pEGFR
EGFR/HSP90 100 89 188 113 136 149 174 183
pEGFR/EGFR 100 86 100 47 100 42 100 47
pERK
pAKT
HSP90
pERK/HSP90 100 141 100 48 100 70 100 68
pAKT/HSP90 100 120 100 55 100 23 100 87
b
C
B+I
E
B+I+E
- + - + - + - +
ERLO
EGFR
pEGFR
EGFR/HSP90 100 95 79 81 85 100 75 76
pEGFR/EGFR 100 59 100 84 100 86 100 90
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pAKT/HSP90 100 31 100 55 100 68 100 56

FIGURE 8

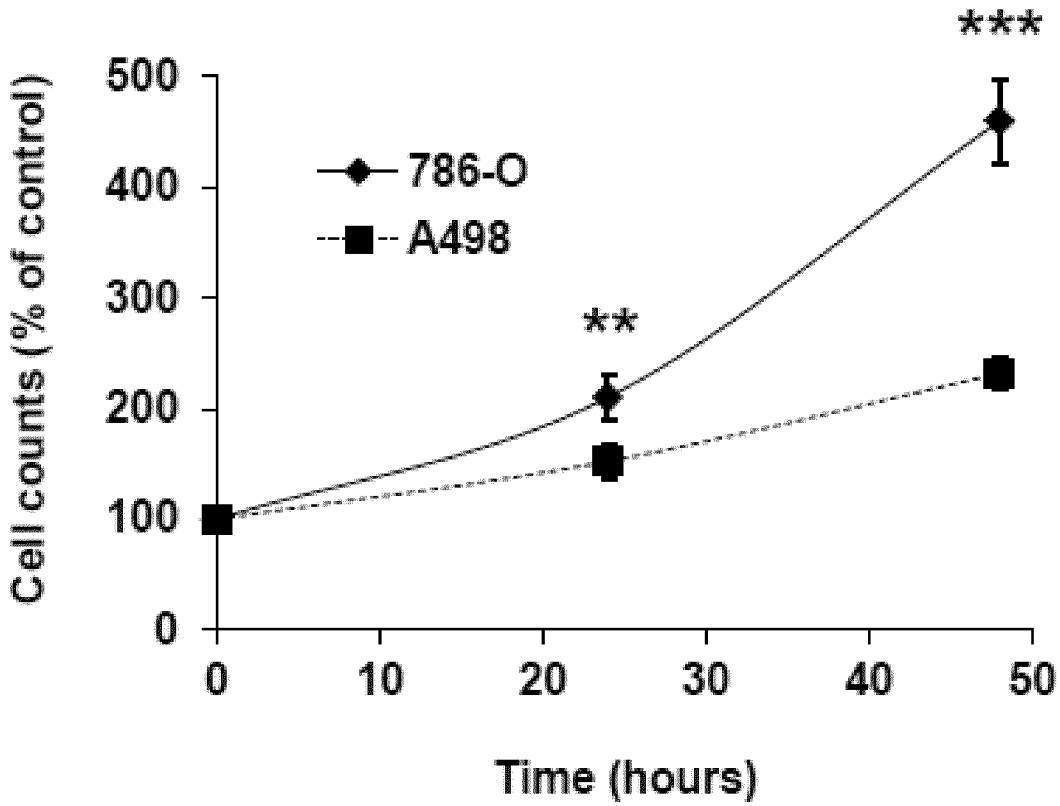
***
**
786-O
A498
Cell counts (% of control)
500
400
300
200
100
0
0
10
20
30
40
50
Time (hours)

FIGURE 9

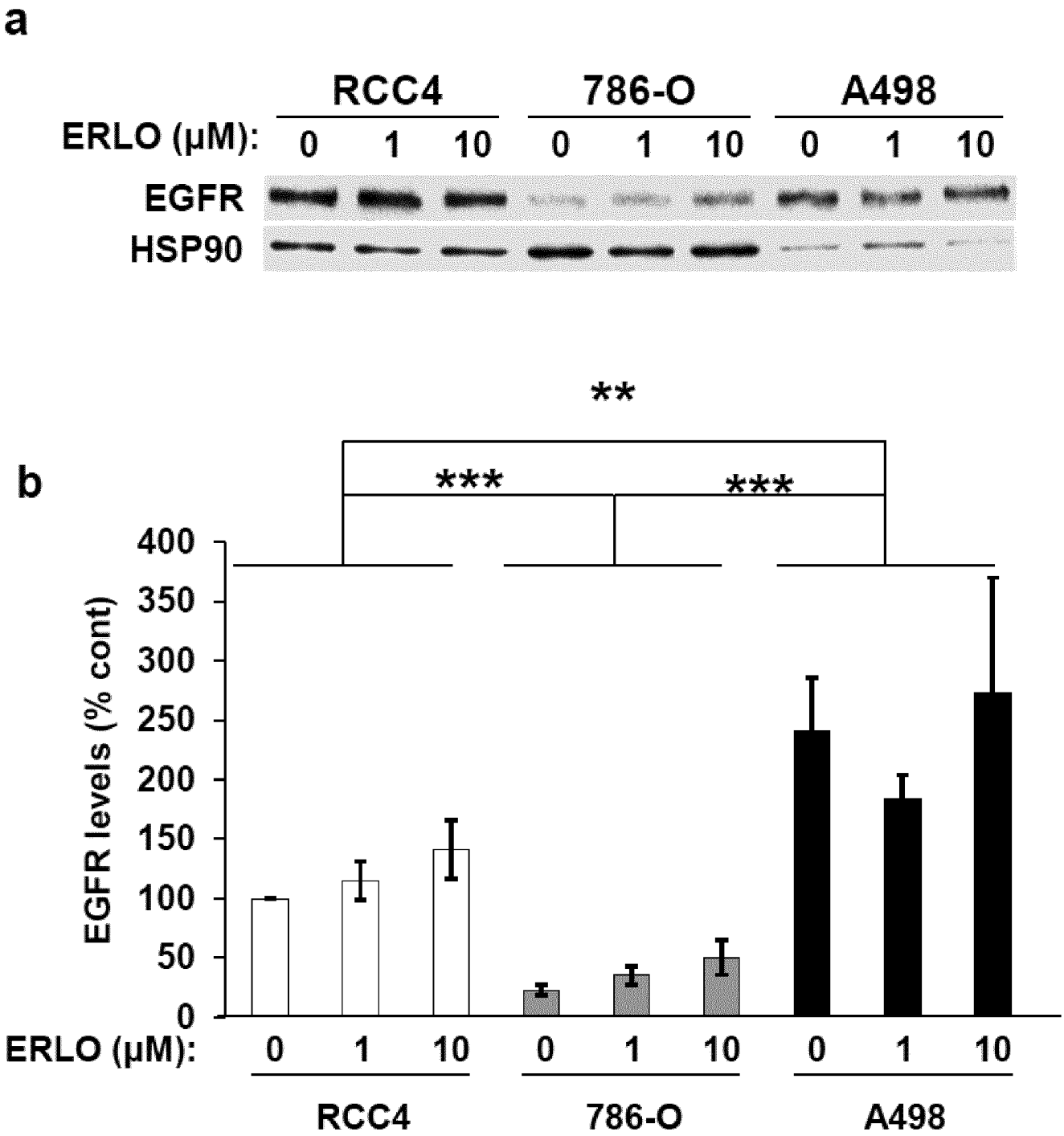
a
RCC4
786-O
A498
ERLO (μM):
0 1 10 0 1 10 0 1 10
EGFR
HSP90
b
**
***
***
EGFR levels (% cont)
400
350
300
250
200
150
100
50
0
ERLO (μM):
0 1 10 0 1 10 0 1 10
RCC4
786-O
A498

FIGURE 10

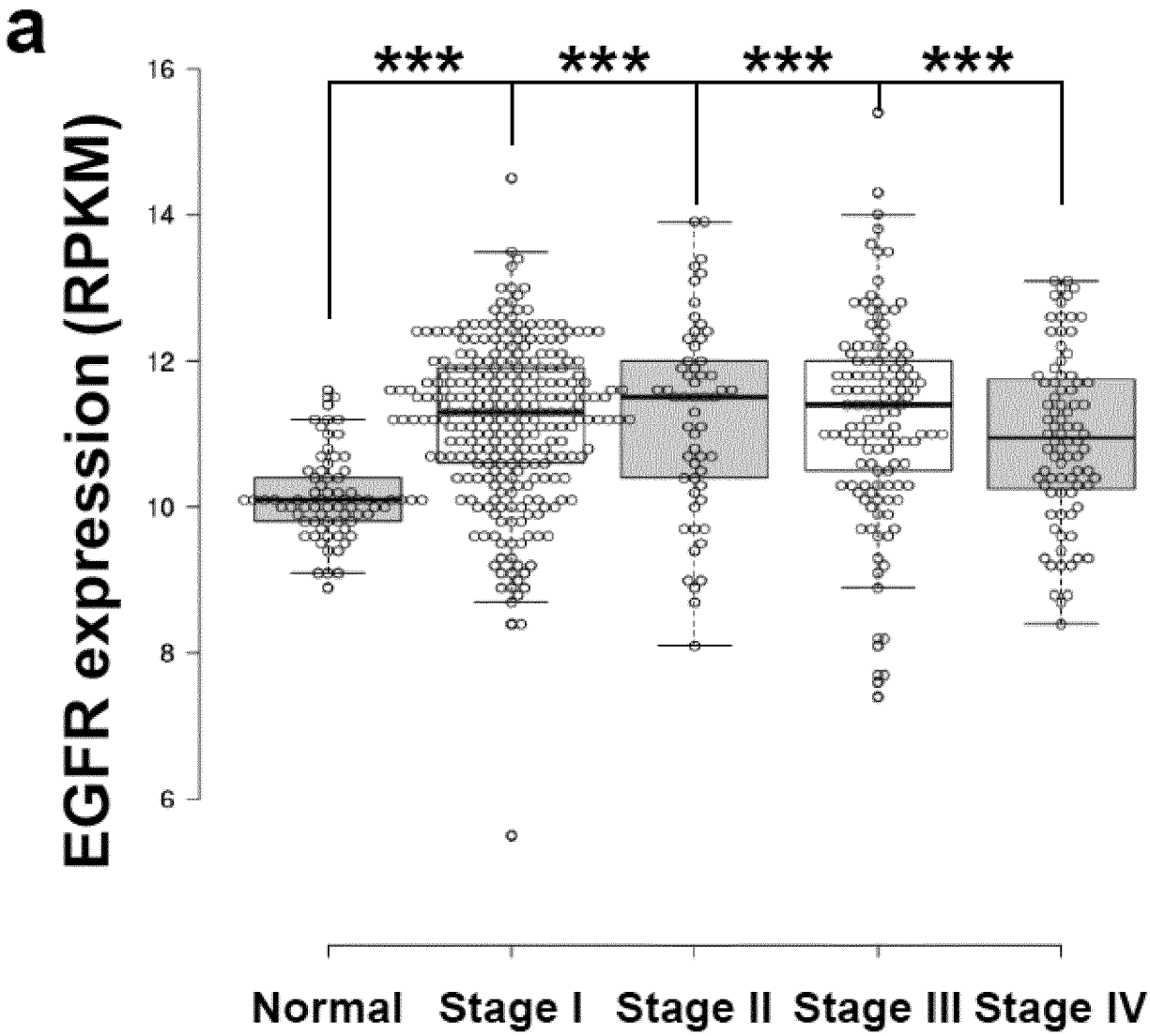
a
***
***
***
***
16
14
12
10
8
6
EGFR expression (RPKM)
Normal
Stage I
Stage II
Stage III
Stage IV

FIGURE 11

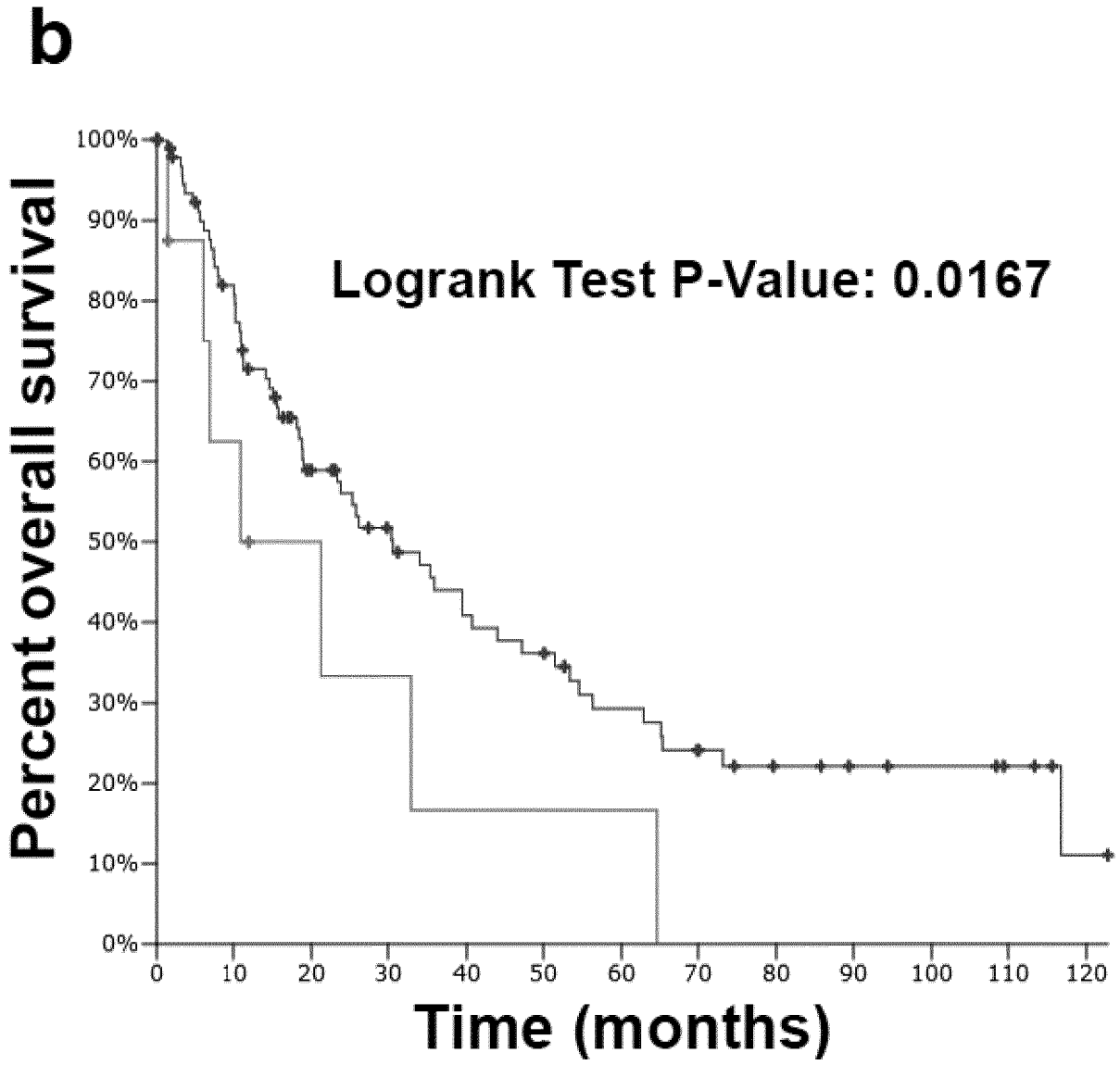

FIGURE 11 (Following)

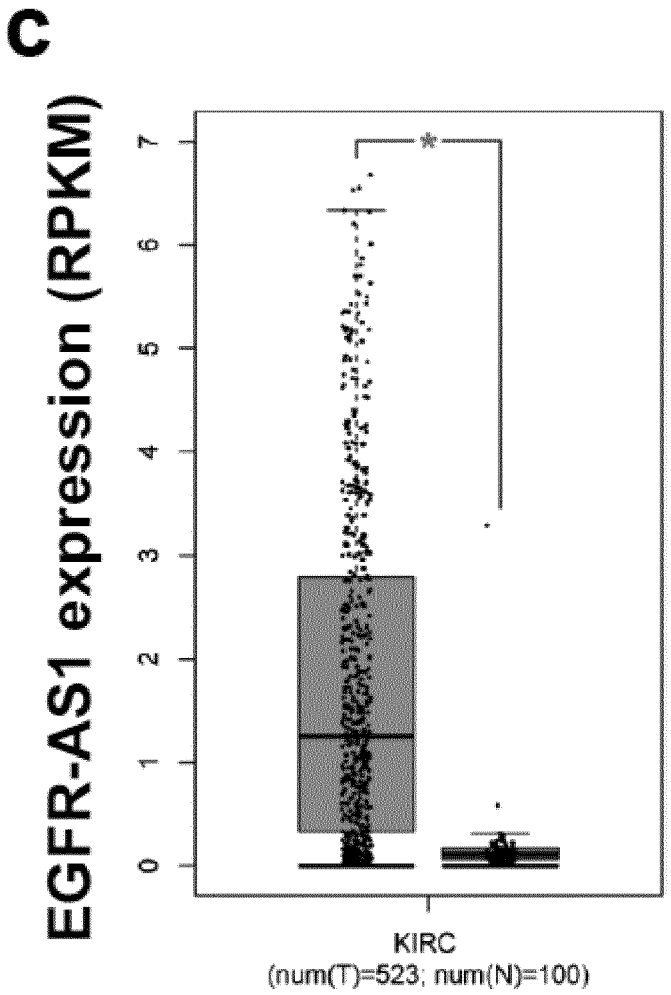

FIGURE 11 (Following)

D

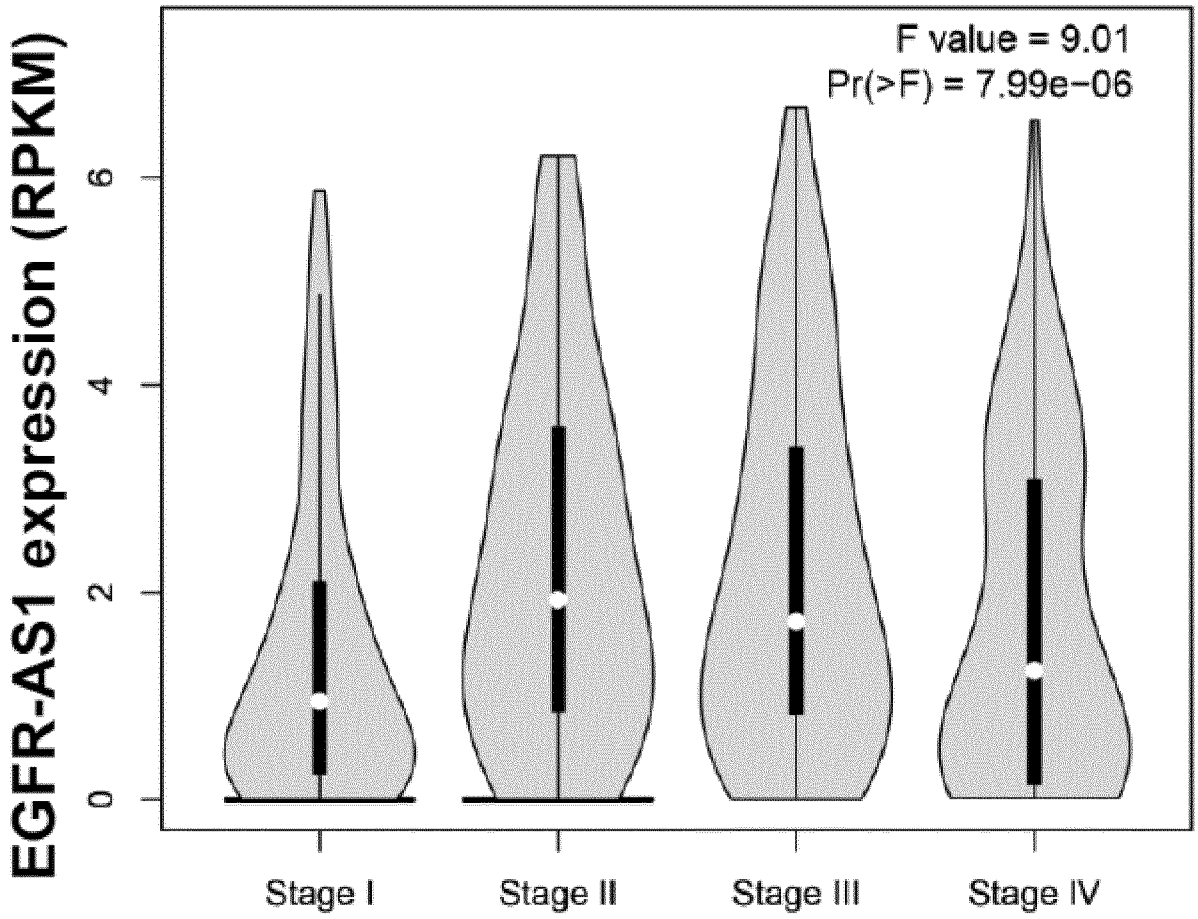

FIGURE 11 (Following)

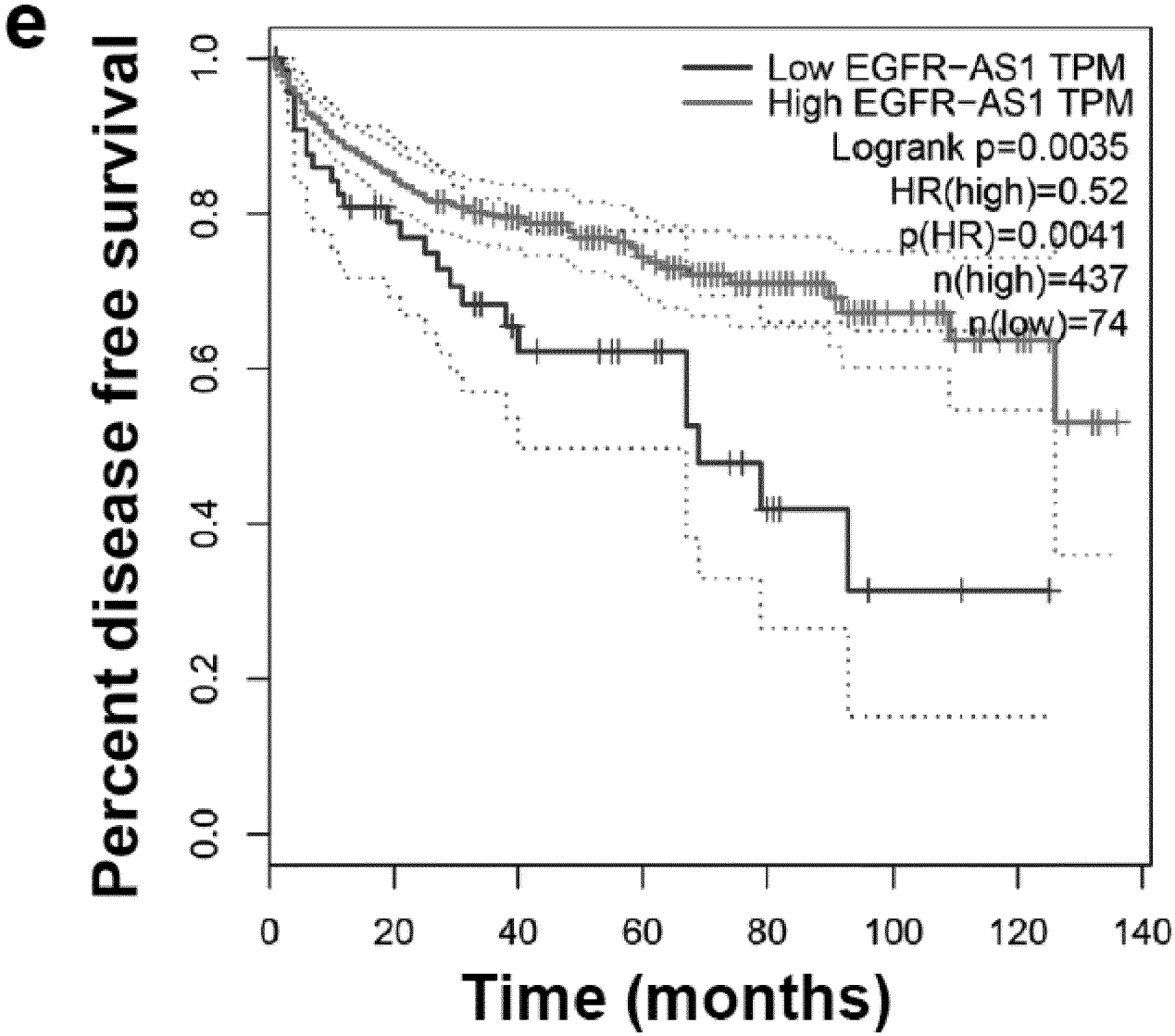

FIGURE 11 (Following)

IC50 µmol/L	G/G (RCC4)	G/A (786-O)	A/A (A498)
Afatinib	2.6	2.1(*)	2.8
Erlotinib	7(¤)	4(***)	9(§)
Lapatinib	4.9(¤¤)	4.3(***)	5.6(§§)

FIGURE 12

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2019/072918

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/6886 A61K39/395
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, BIOSIS, Sequence Search, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BONIN SERENA ET AL: "A synonymous EGFR polymorphism predicting responsiveness to anti-EGFR therapy in metastatic colorectal cancer patients", TUMOR BIOLOGY, KARGER, BASEL, CH, vol. 37, no. 6, 15 December 2015 (2015-12-15), pages 7295-7303, XP036096255, ISSN: 1010-4283, DOI: 10.1007/S13277-015-4543-3 [retrieved on 2015-12-15]	12,13
Y	abstract page 7297 page 7301	1-11,14, 15
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[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search	Date of mailing of the international search report
26 September 2019	10/10/2019

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Reuter, Uwe

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	YONGCHANG LAI ET AL: "Crosstalk between VEGFR and other receptor tyrosine kinases for TKI therapy of metastatic renal cell carcinoma", CANCER CELL INTERNATIONAL, vol. 18, no. 1, 5 March 2018 (2018-03-05), XP055558647, DOI: 10.1186/s12935-018-0530-2 pages 2-4 abstract; figure 1; tables 1,3 -----	1-11,14, 15
A	Anonymous: "Patients With Advanced Papillary Kidney Cancer Respond Well to Bevacizumab/Erlotinib Combination Therapy - The ASCO Post", The ASCO Post, 1 December 2014 (2014-12-01), XP055558945, Retrieved from the Internet: URL:http://www.ascopost.com/issues/december-1-2014/patients-with-advanced-papillary-kidney-cancer-respond-well-to-bevacizumaberlotinib-combination-therapy/ [retrieved on 2019-02-19] abstract -----	1-15
A	PETER MAKHOV ET AL: "Resistance to Systemic Therapies in Clear Cell Renal Cell Carcinoma: Mechanisms and Management Strategies", MOLECULAR CANCER THERAPEUTICS, vol. 17, no. 7, 1 July 2018 (2018-07-01), pages 1355-1364, XP055558661, US ISSN: 1535-7163, DOI: 10.1158/1535-7163.MCT-17-1299 abstract -----	1-15

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