

CETUXIMAB: From Bench to Bedside

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Abstract: Cetuximab (IMC-C225, Erbitux ImClone Systems Inc, New York, NY) is a recombinant, human/mouse chimeric monoclonal antibody (MAb) that binds specifically to the extracellular domain of the human epidermal growth factor receptor (EGFR) on both normal and tumor cells, and competitively inhibits the binding of epidermal growth factor (EGF) as well as other ligands.

Cetuximab binding to the EGFR blocks phosphorylation and activation of receptor-associated kinases and their associated downstream signalling (MAPK, PI3K/Akt, Jak/Stat pathways) resulting in inhibition of many cellular processes such as induction of apoptosis, cell growth, decreased Matrix Metallo-Proteinase (MMPs) and vascular endothelial growth factor (VEGF) production. Cetuximab is also able to display cytotoxic effect through antibody-dependent cellular cytotoxicity (ADCC).

In vitro and *in vivo* experiments elucidated a wide range of biological properties attributed to cetuximab, these include: direct inhibition of EGFR tyrosine kinase activity, inhibition of cell cycle progression, inhibition of angiogenesis, invasion and metastatization processes, activation of pro-apoptotic molecules and synergic cytotoxicity effect with chemotherapy and radiotherapy.

Several studies have shown cetuximab is able to inhibit growth of EGFR-expressing tumor cells *in vitro* as well as in nude mice bearing xenografts of human cancer cell lines.

Moreover, numerous clinical trials demonstrated cetuximab efficacy in different tumor types and it is approved by Food and Drugs Administration (FDA) for use in the treatment of metastatic colorectal cancer (mCRC) as single agent or in combination with chemotherapy, for locally/regionally advanced head and neck squamous cell carcinoma (HNSCC) in combination with radiotherapy, and as monotherapy for recurrent/metastatic HNSCC after failing platinum-based chemotherapy.

This review will illustrate pre-clinical and clinical data on biological properties of cetuximab focusing on the predictive markers of clinical response to this drug.

Keywords: Cetuximab, epidermal growth factor receptor, targeted therapy, predictive markers.

INTRODUCTION

With the emergence of new technologies and with an increasing knowledge of the molecular mechanisms that govern carcinogenesis and tumor progression came the discovery and the development of numerous new drugs designed to specifically target the relevant molecular pathways involved.

The term "targeted therapy" refers to a new generation of cancer drugs designed to interfere with a specific molecular target (typically a protein) that is believed to have a critical role in tumor growth or progression. The identification of appropriate targets is based on a detailed understanding of the molecular changes underlying cancer. This approach contrasts with the conventional, more empirical approach used to develop cytotoxic chemotherapeutics, the mainstay of cancer drug development in past decades.

The epidermal growth factor receptor (EGFR) represents one such target [1, 2]. Since one of the most studied molecular targets is the EGFR, a number of drugs targeting this receptor have been approved for cancer treatment, and others are being evaluated in late phase clinical trials [3-6].

Cetuximab is a chimeric anti-human EGFR monoclonal antibody (MAb) approved by Food and Drugs Administration (FDA) for use in the treatment of metastatic colorectal cancer (mCRC) as single agent or in combination with chemotherapy, for locally/regionally advanced head and neck squamous cell carcinoma (HNSCC) in combination with radiotherapy, and as monotherapy for recurrent/metastatic HNSCC after failing platinum-based chemotherapy.

This review will illustrate pre-clinical and clinical data on biological properties of cetuximab focusing on the predictive markers of clinical response to this drug.

CETUXIMAB: EGFR AS TARGET

Cetuximab is a chimeric anti-human EGFR MAb [7] that has recently been approved for use in mCRC as single agent or in combination with chemotherapy [8, 9], for locally/regionally advanced HNSCC in combination with radiotherapy, and as monotherapy for recurrent/metastatic HNSCC after failing platinum-based chemotherapy [10]. However, despite the recent success of targeted therapy in certain clinical oncology trials, including those using cetuximab, there still exist an urgent need for strategies to improve efficacy, as well as to reduce toxicity.

Cetuximab (IMC-C225, Erbitux ImClone Systems Inc, New York, NY) is a recombinant, human/mouse chimeric

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MAB that binds specifically to the extracellular domain of the human EGFR [11-13].

The first EGFR-targeting MABs were produced by John Mendelsohn and colleagues in 1983. M225, a MAB, was found to block ligand binding to EGFR, it binds EGFR with similar affinity to epidermal growth factor (EGF) and transforming growth factor- α (TGF- α) and it inhibits the proliferation of various epithelial tumor cell lines [12].

M225's potential was demonstrated by a pre-clinical study which reports that M225 and cisplatin used on their own had inhibitory effect on tumor graft. However, when both agents were administered in combination, a synergistic effect was observed, with significant tumor shrinkage and greatly increased survival [14].

Because of limited MAB technology at that time, M225 was a murine antibody and thus Human Anti-Mouse Antibodies development was expected in humans. To remedy this, the antigen-recognition sites of M225 were transferred into a human antibody backbone creating a new MAB called cetuximab (Erbiximab) with a very low incidence of hypersensitivity reaction [15].

Cetuximab binds specifically the extracellular domain of the EGFR on both normal and tumor cells [11-13], and competitively inhibits the binding of EGF as well as other ligands, such as TGF- α [7].

Cetuximab is produced in mammalian (murine myeloma) cell culture and it is composed of the Fv regions of a murine anti-EGFR antibody with human IgG1 heavy and kappa light chain constant regions and has an approximate molecular weight of 152 kDa [16]. The binding to EGFR is characterized by a higher affinity ($K_d = 0.1-0.2$ nM) than either EGF or TGF- α [7]. The selective action of cetuximab accounts for a minor toxicity instead experienced with other EGFR inhibitors acting on the intracellular domain of EGFR and on a vast number of tyrosine kinases.

Cetuximab binding to the EGFR blocks phosphorylation and activation of receptor-associated kinases and their associated downstream signalling (Mitogen-Activated Protein Kinase (MAPK), Phosphoinositide 3-kinase/Akt (PI3K/Akt) and the Janus kinases/Signal Transducers and Activator of Transcription (Jak/Stat) pathways) interfering with many cellular processes such as induction of apoptosis, inhibition of cell growth and vascular endothelial growth factor (VEGF) production, decreased matrix metalloproteinase [17].

Furthermore, cetuximab induces receptor down-regulation through its initial dimerization and internalisation [18].

The EGFR is a member of type I tyrosine kinase receptor (TKR) family including EGFR/human epidermal growth factor receptor (HER)1-4. It is constitutively expressed in many normal epithelial tissues and it is over-expressed in several human cancers as well as those of the colon and rectum.

The EGFR (c-ErbB-1; HER1 in humans) is a transmembrane receptor tyrosine kinase member that belongs to the HER receptor family [19]. Receptors of HER family structurally contain extracellular, transmembrane, and tyro-

sine kinase domains. Each domain is responsible for a different HER mediated signalling pathway: the extracellular portion contains a ligand-binding site, while the intracellular portion contains the tyrosine kinase domain [20].

The HER family consists of 4 structurally related cellular receptors, which interact in many ways: HER1, also known as EGFR or ErbB1, HER2, also known as ErbB2, HER3, also known as ErbB3, HER4 or ErbB4 [21-23].

In mammals, canonical EGFR activation involves the binding of seven peptide growth factors: EGF, TGF- α , heparin-binding EGF-like growth factor (HBEGF), amphiregulin (AREG), betacellulin (BTC), epiregulin (EREG), and epigen (EPGN) [24]. This bond results in homodimerization of two EGFR or heterodimerization through different members of the human EGFR family [25].

These events activate EGFR intracellular region resulting in initiating a cascade of events into the cell such as autophosphorylation and signal transduction [26-29].

EGFR activation leads to recruitment of a range of adaptor proteins (such as Shc, GRB2) [30] and activates a series of intracellular signalling cascades to affect gene transcription, which in turn results in cancer cell proliferation, reduced apoptosis, invasion and metastasis and also stimulates tumor-induced angiogenesis.

The pathways mediating EGFR downstream effects have been well studied and three major signalling pathways have been identified: the first involves RAS-RAF-MAPK pathway [31, 32]: phosphorylated EGFR recruits the guanine-nucleotide exchange factor *via* the GRB2 and Shc adapter proteins, activating RAS and subsequently stimulating RAF and the MAP kinase pathway to affect cell proliferation, tumor invasion, and metastasis; the second involves PI3K/AKT pathway [33], which activates the major cellular survival and anti-apoptosis signals *via* activating nuclear transcription factors such as nuclear factor-kappa B (NF- κ B) [34]; the third involves JAK/STAT pathway which is also implicated in activating transcription of genes associated with cell survival.

EGFR activation may also lead to hydrolysis of phosphatidylinositol 4,5 biphosphate (PIP2) into inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG), resulting in activation of protein kinase C (PKC) and Ca²⁺/calmodulin-dependent protein kinases (CaMK).

The activation of these systems starts several transcriptional programs that mediate a variety of cellular responses, including cell division, motility, invasion, adhesion, cellular repair and survival (or death) [35]. After signal transduction, ligand-receptor complex can be internalized and either down-regulated or recycling on the cell surface [36].

EGFR is expressed on normal and malignant epithelial cells and it plays an important role in tumor biology. In fact, it promotes proliferation, metastatization, angiogenesis and inhibition of apoptosis [37, 38]. The main signal transduction pathways associated with EGFR activation are summarized in Fig. (1).

The most common EGFR alteration in tumor cells is its over-expression, that may lead to ligand-independent receptor dimerization. EGFR is frequently over-expressed in hu-

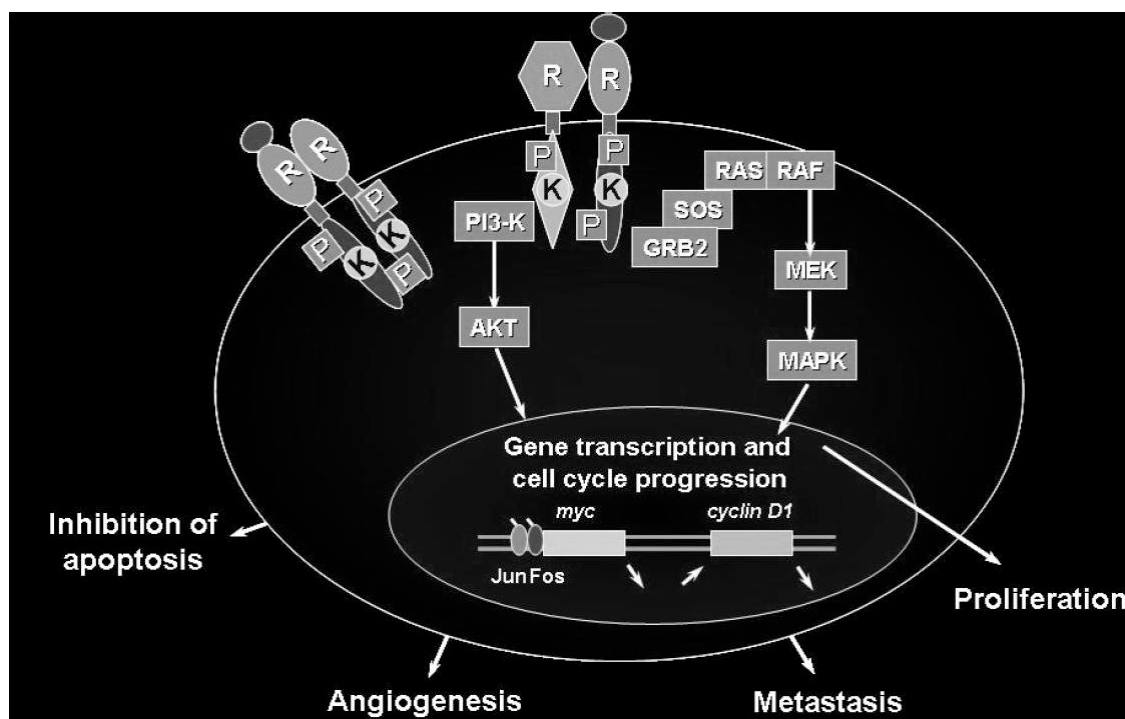


Fig. (1). The main signal transduction pathways associated with EGFR activation.

EGFR: epidermal growth factor receptor, GRB2: growth factor receptor-bound protein 2, K: kinase, MAPK: mitogen activated protein kinase, P: phosphate, PI3-K: phosphoinositide-3 kinase, R: receptor

man tumors including breast cancer, lung cancer, glioblastoma, bladder carcinoma, head and neck cancer, ovarian carcinoma, colorectal cancer and prostate cancer [39]. Overexpression correlates with disease progression, poor outcome and low response to therapy and resistance to cytotoxic treatments [40, 41].

In malignant cells there are other mechanisms related to the activation of EGFR like an increased production of ligands by the same tumor cells [41-43] or an expression of a mutant variant of the receptor (EGFR vIII) able to activate constitutively its own tyrosine kinase [44, 45].

Furthermore, metallo-proteinase-mediated cleavage of precursor membrane-bound EGF ligands seems to activate the EGFR through the stimulation of G-protein-coupled receptors [46].

Recently, a ligand-independent mechanism of EGFR activation *via* the urokinase plasminogen receptor has been identified [47].

CETUXIMAB: BIOLOGICAL PROPERTIES

As previously described, cetuximab binds to and inhibits EGFR activation. Through this mechanism cetuximab displays its antitumor activity.

In vitro and *in vivo* experiments elucidated a wide range of biological properties attributed to this MAb, these include the following: direct inhibition of EGFR tyrosine kinase activity [12], inhibition of cell cycle progression [48, 49], inhibition of angiogenesis, invasion and metastatization processes [50], activation of pro-apoptotic molecules [48, 51] and

synergic cytotoxicity effect with chemotherapy and radiotherapy [52]. Moreover, cetuximab is able to induce ADCC. The mechanisms by which antitumor activity is displayed are not completely clarified yet.

Different studies demonstrated that cetuximab mediates cell cycle arrest in various tumor cell lines, leading in some cases to apoptosis. It is known that cyclin dependent kinases are fine regulators of cell cycle progression particularly of G1 phase progression and subsequent S-phase entry. Moreover, S-phase progression is guided by growth factors expression.

Wu X *et al.* demonstrated that blockage of EGFR mediated by cetuximab induces G1 phase cell cycle arrest in DiFi human colon adenocarcinoma cells and that this blockage involves a cyclin/cyclin dependent kinase (CDK) inhibitor called p27KIP1 a kinase inhibitory protein [53].

Another interesting data derived from Kiyota *et al.* [54] that analysed the effect of mAb225 (an EGFR-blocking MAb 225) on human oral squamous cell carcinoma (SCC) cell lines. The results suggest that the antiproliferative effect of EGFR blockade may be mediated by cell cycle inhibitors p27KIP1 and p15INK4B with cell accumulation in G1 phase and cell decrease in S phase. In particular, G1 arrest was accompanied by a decrease in CDK2-, CDK4-, and CDK6-associated histone H1 kinase activities, and an increase in cell cycle inhibitors p27KIP1 and p15INK4B.

Peng D and colleagues [49] demonstrated that EGFR-blocking antibody mAb225 inhibits proliferation of androgen-independent DU145 prostatic cancer cells by arresting cell cycle progression in G1 though inhibition of CDK2 ac-

tivity. This effect is attributable to p27KIP1 up-regulation as demonstrated at mRNA and protein levels.

Experimental data have shown that cetuximab exerts a pro-apoptotic effect. It is known that G1 arrest is followed by apoptosis.

Wu X *et al.* reported that DiFi colorectal carcinoma cell line, which expresses high levels of EGFR on plasma membranes, can be induced to undergo G1 cell cycle arrest and apoptosis when cultured with mAb 225 at concentrations that saturate EGF receptors. Moreover, addition of Insulin-like Growth Factor-1 (IGF-1) or high concentrations of insulin can delay mAb C-225-induced apoptosis, while G1 arrest cannot be reversed by either IGF-1 or insulin [48]. These results indicate that EGF receptor activation is required both for cell cycle progression and for prevention of apoptosis in DiFi cells, and that a signal transduction pathway shared by receptors for insulin/IGF-1 and EGF may be involved in regulating apoptosis triggered by EGF receptor inhibitors.

Liu B *et al.* investigated the mechanisms by which fibroblast growth factor (bFGF) and IGF-1 modulate mAb 225-induced G1 arrest and apoptosis in DiFi cells. They found bFGF could strongly inhibit both the antibody-induced cell cycle arrest in G1 phase and the subsequent apoptosis. In contrast, although IGF-1 delayed the onset of apoptosis, it did not prevent mAb 225-induced G1 cell cycle arrest. Interestingly, either bFGF than IGF-1 acted on MEK pathway in DiFi cells [51].

Explore the mechanisms by which mAb 225 induces apoptosis in DiFi cells may be useful to identify new target(s) to maximize the antitumor effect of mAb 225 by inducing apoptosis in a variety of cancer cells. The precise molecular mechanisms by which mAb 225 is able to induce apoptosis in these cells remain not completely clear.

Another study conducted by Scwab and colleagues demonstrated that EGFR blockage through IMC-C225 MAb in MDA Panc-28 human pancreatic cancer cell line leads to a marked decrease of constitutive NF- κ B DNA binding activity and as consequence to a decrease in bcl-xl and bfl-1 transcription which are bcl-2 family members and are regulated by NF- κ B itself [55]. These results suggest an important role of EGFR in NF- κ B activation and that EGFR blockade by IMC-C225 may be effective in anti-apoptotic genes down-regulation.

Extensive experimental evidences reported that cetuximab displays antiangiogenetic activity decreasing tumor cell production of angiogenic growth factors such as VEGF, bFGF and interleukin-8 (IL-8).

Petit and colleagues were the first to shed light on the antiangiogenetic properties of cetuximab. The authors evaluated whether EGFR or ErbB2/neu may contribute to tumor angiogenesis by examining their effects on the expression of VEGF/vascular permeability factor (VPF). EGFR-positive A431 human epidermoid carcinoma cells, which are known to be heavily dependent on VEGF/VPF, C225 anti-EGFR neutralizing antibody treated down-regulate VEGF production by tumor cells themselves. Similar result was obtained from analysis conduct on other tumor cell lines [56].

Perrotte *et al.* evaluate whether EGFR-directed therapy affects angiogenesis by *in vitro* exposure of human transitional cell carcinoma (TCC) of the bladder cell line 253J B-V to cetuximab. The effect was the inhibition of mRNA and protein production of VEGF, IL-8 and bFGF by the cells, moreover anti-EGFR MAb C225 inhibits growth and metastasis of human TCCs established in the bladder wall of athymic nude mice [50].

Huang and co-workers examined the impact of cetuximab on invasive and metastatic potential of head and neck human squamous cell carcinoma (SCC) using *in vitro* and *in vivo* model systems [57]. *In vitro* treatment of human umbilical vascular endothelial cells (HUVEC) with cetuximab reduces cell-to-cell interaction and results in disruption of tube formation. The effect of C225 was additionally examined using an *in vivo* tumor xenograft neovascularization model of angiogenesis. Systemic treatment with C225 not only reduced tumor growth and the number of blood capillaries but also hindered the growth of established vessels toward the tumor.

These results provide evidence that C225 is capable to inhibit tumor-induced neovascularization and metastasis in SCC of the head and neck.

Endothelial cells within several neoplasms have been shown to express EGFR [58]. These cells proliferate following EGFR activation [59] and are instead induced to apoptosis and reduce neovascularization following EGFR blockade [60].

VEGF and IL-8 can act as survival factors for immature endothelial cells [61] and protect endothelial cells from apoptosis [62], so that decreased production of VEGF induced by cetuximab may enhance endothelial cell apoptosis, contributing to the reduction in neovascularity.

The results suggest that cetuximab displays its antiangiogenic effect by acting directly on endothelial cells.

Other studies supported the antiangiogenetic effect and demonstrated a cytostatic role of cetuximab. Moreover, the association with other biological or cytotoxic agents increases significantly cetuximab antitumor activity.

Ciardiello and colleagues evaluated the antiangiogenic and antitumoral activity of cetuximab alone and in combination with a human VEGF antisense (AS) 21-mer phosphorothioate oligonucleotide (VEGF-AS) in human GEO colon cancer cells [63].

Immunohistochemical analysis of GEO tumor xenografts showed a significant reduction of VEGF expression after treatment with VEGF-AS and a reduction of VEGF, bFGF, TGF- α expression after treatment with cetuximab, both determining a parallel reduction in microvessel count.

The combined treatment leads to a significant impact on VEGF expression but a slight or no impact on microvessels density.

In the clinical setting Vincenzi and colleagues investigated the impact of a combined weekly treatment of cetuximab plus irinotecan on VEGF, as pro-angiogenic factor, and interferon (IFN)- γ , as anti-angiogenic factor, modification in advanced colorectal cancer patients [64].

Cytokine levels were assessed at different time points before and after the beginning of the treatment. The median VEGF basal value showed a decrease that interestingly reached the highest statistical significance at the last time point with a reduction of 51.75%. Thus, cetuximab reduction of VEGF serum levels is a sudden and long lasting phenomenon.

The median IFN-gamma basal level significantly increased 1 day after treatment and persisted after 21 days. A precise analysis of this behaviour remains to be investigated.

A significant negative correlation between VEGF and IFN-gamma values at 1, 21 and 50 days after the first treatment cycle was shown by a linear regression model.

These data effort the concept of anti-angiogenetic role of cetuximab in cancer treatment, underlining the need of new antiangiogenic combination treatments with potential synergic activity.

In vitro and *in vivo* experiments stated that cetuximab inhibits the invasive and metastatic ability of different tumor types.

Perrotte *et al.* in an orthotopic bladder carcinoma xenograft model of tumor-bearing mice confirm that systemic treatment with a chimeric anti-EGFR MAb C225 results in tumor regression, inhibits tumor growth and prevents metastases to the regional lymph nodes and lungs [50].

Metallo proteinases (MMPs), a zinc-dependent endopeptidases family, are released by cancer cells and act by processing extra-cellular matrix components, cell surface proteins, and immune modulators. It is known that deregulation of proteolytic mechanisms has been implicated in tumor invasion and metastatic processes.

Cetuximab has been shown to inhibit the expression as well as the activity of several MMPs including the gelatinase MMP-9. Tumor metastasis represents a complex multistep process that requires migration, invasion, and angiogenesis.

Huang *et al.* observed that anti-EGFR antibody C225 treatment attenuated SCC-1 tumor cells migration through a chemotaxis chamber in a dose-dependent manner. Furthermore, in the presence of C225, SCC-1 ability to invade across a layer of extracellular matrix was significantly inhibited. Using an *in vivo* orthotopic floor-of-mouth xenograft model locoregional tumor invasion of SCC-1 into muscle, vessel, bone, and perineural tissues was inhibited in C225-treated mice. This inhibition was additionally characterized by down-regulation of MMP-9 expression. These data suggest that inhibition of metastatic potential by C225 may be mediated *via* decreased migration and invasion of SCC cells. These results show the capacity of C225 to block MMP-9 induction which plays a major role in invasion and metastatization tumor processes.

Experimental evidence demonstrated that cetuximab acts also on immune system even if the effects and its mechanisms have not been widely studied and understood yet.

The effects on immune system account for its indirect antitumor activity through cytotoxic effect mediated by effector cells such as monocytes and natural-killer cells and mediated by antibody-dependent cellular cytotoxicity (ADCC)

activity, as it has a human IgG1 backbone. However, except for one study that showed cetuximab capacity to mediate ADCC activity against a melanoma cell line [65], no published studies have focused on its ADCC activity.

The study by Kurai *et al.* analyzes cetuximab-mediated ADCC activity against lung cancer cell lines. In this study fresh peripheral blood mononuclear cells exhibited cetuximab-mediated ADCC activity against lung cancer cell lines at a low concentration of cetuximab and maximum ADCC activity was induced by very weak EGFR expression levels, which are faintly detectable by immunohistochemistry. ADCC activity was enhanced by interleukin-2 (IL-2) and it was less impaired by weekly chemotherapy with conventional cytotoxic drugs than was NK activity in lung cancer patients [66]. These data provide new insight into the possible antitumor mechanism of cetuximab and particularly regarding its immunomodulating effect.

Cetuximab ADCC activity has been described against several tumor cell lines expressing wild-type or mutant EGFR such as in HEK293 cells transfected with wild-type EGFR (293W) and a deletional mutant of EGFR (293D) and in the mock transfectant (293M). ADCC activity was detected in cells transfected with wild-type and mutant EGFR, in a dose-dependent manner, but not in the mock transfectant.

These results indicate that cetuximab affinity for the extra-cellular domain of EGFR and not its mutation status play a key role in triggering ADCC activity [67].

Moreover, the inhibition of transforming growth factor-beta (TGF-beta) could enhance cetuximab mediated ADCC against TGF-beta producing SCCs [68].

CHEMOTHERAPY AND RADIOTHERAPY ENHANCE THE ANTI-TUMOR EFFECTS OF CETUXIMAB: PRECLINICAL AND CLINICAL EVIDENCES

Experimental data reported that different chemotherapeutic agents can enhance EGFR phosphorylation and activation status [69-71] and instead EGF has been shown to increase sensitivity to doxorubicin and topoisomerase II inhibitors in human solid cancer cell lines [72, 73]. According to these results, EGFR inhibitors could increase chemotherapy-induced killing.

Fan *et al.* described a substantial xenograft growth inhibition (A431 SCC) in nude mice when treated with cetuximab and cisplatin in combination compared with a single modality [14].

Likewise, studies with doxorubicin in combination with anti-EGFR monoclonal antibodies demonstrated marked tumor regression of A431 and MDA-468 xenografts in athymic mice and the treatment with either doxorubicin or anti-EGF receptor MAb alone temporarily inhibited growth, but the combination of both agents substantially enhanced antitumor activity over that of doxorubicin [74].

In murine models of human colorectal carcinoma, cetuximab and irinotecan (CPT-11) combined treatment significantly inhibited cellular growth compared with either CPT-11 or IMC-C-225 monotherapy. The same benefit de-

rived from combined therapy was described in a CPT-11 refractory tumor model [75].

Cetuximab ability to overturn CPT-11 resistance could depend by inhibition of EGFR signalling pathways (MAPK and PI3K/AKT) leading to upregulation of proapoptotic stimuli and thus facilitating tumor cells death.

In human ovarian, breast and colon cancer cell lines which express functional EGFR the anti proliferative activity of MAb C225 (anti-EGFR humanized chimeric mouse MAb) in combination with topotecan was evaluated.

A dose-dependent supra additive increase of growth inhibition *in vitro* was observed when cancer cells were treated with topotecan and MAb C225 in a sequential schedule.

Treatment with MAb C225 also markedly enhanced apoptotic cell death induced by topotecan.

Treatment of mice bearing established human GEO colon cancer xenograft with topotecan or with MAb C225 determined a transient inhibition of tumor growth in contrast, an almost complete tumor regression was observed in all mice treated with the two agents in combination [52].

In vitro treatment of human pancreatic carcinoma L3.6pl cells with cetuximab plus gemcitabine resulted in additive cytotoxic effects that increased with increasing gemcitabine concentrations. Dose-dependent decreases in expression of the angiogenic factors VEGF and IL-8 (but not bFGF) were observed in the cetuximab-treated cells (mRNA and protein levels).

Moreover the combined treatment enhanced growth inhibition, tumor regression and metastasis. Significant differences in microvessel density and a direct correlation with the difference in percentage of apoptotic endothelial cells were observed after cetuximab or combination treatments (but not gemcitabine alone) [76].

Growth factors have been shown to mediate cellular responses to radiation in numerous tissues. For this reason growth factor receptors as well as proteins involved in cell signalling are the main targets investigated as radiosensitive. Experimental data demonstrated that radiation is able to upregulate EGFR expression on cancer cells, and EGFR signalling inhibition sensitises cells to radiation effects [77].

SCC tumor xenografts treated with cetuximab and radiation induces complete tumor regression in athymic mice [78]. *In vivo* and *in vitro* data obtained using different cell types such as HNSCC, colon, ovarian, non small cell lung cancer (NSCLC), and breast cancer lines demonstrated that cetuximab leads to enhanced killing in response to radiation [79-81].

The antitumor effect is comprehensive of different mechanisms such as cetuximab-induced inhibition of DNA damage repair, increased radio-sensitivity deriving from specific perturbations in cell cycle phase distribution, inhibition of tumor angiogenesis and enhancement of radiation-induced apoptosis.

As already described, cetuximab acts on cell cycle phases increasing the number of cells in G1 phase, which are radio-sensitive, and decreasing those in S phase, which is more radioresistant. Moreover, cetuximab is able to augment ra-

dion-induced apoptosis increasing proapoptotic (Bax) and decreasing antiapoptotic (Bcl-2) protein expression [82].

EGFR inhibition has been demonstrated to inhibit DNA repair mechanisms by acting on EGFR import into the nucleus and on DNA-PK, a protein implied in DNA damage repair [83].

These mechanisms could explain the *in vitro* sensitizer effect of cetuximab.

Another effect of EGFR inhibition is on angiogenesis process. Experimental data describe that radiation could increase VEGF expression but decrease of VEGF expression following radiation could enhance tumor control *in vivo* [84-86].

Supported by these results, many clinical trials have been conducted and other are ongoing.

Data from phase II and randomized phase II studies lead to the approval of cetuximab for the treatment of EGFR expressing metastatic colorectal carcinoma in patients who are refractory to irinotecan based chemotherapy either in combination with irinotecan or as monotherapy [8, 9, 87].

BOND trial demonstrated that cetuximab has clinically significant activity when given alone or in combination with irinotecan in patients with irinotecan-refractory colorectal cancer confirming the results of phase 2 studies.

The combination-therapy group had a significantly higher response rate and a significantly longer time to progression than the monotherapy group, suggesting that the combination of irinotecan and cetuximab should be preferred for patients with irinotecan-refractory cancer.

Patients whose disease had progressed during or within three months after treatment with an irinotecan-based regimen were randomly assigned to receive both cetuximab and irinotecan or cetuximab monotherapy. The rate of response in the combination-therapy group was significantly higher than that in the monotherapy group (22.9 vs. 10.8%). The median time to progression was significantly greater in the combination-therapy group (4.1 vs. 1.5 months). The median survival time was 8.6 months in the combination-therapy group and 6.9 months in the monotherapy group. Toxic effects were more frequent in the combination-therapy group, but their severity and incidence were similar to those that would be expected with irinotecan alone.

Moreover, the number of previous treatment regimens and previous use of oxaliplatin did not affect the efficacy of the cetuximab plus irinotecan combination. Cetuximab monotherapy also shows activity accompanied only by mild toxicity. These results suggest cetuximab monotherapy as a valuable alternative option for patients who are not considered candidates for further treatment with irinotecan-based chemotherapy or who decline such treatment [4].

The multicenter, open-label, phase III EPIC trial demonstrated that cetuximab added to irinotecan significantly improved progression free survival (PFS) (median, 4.0 v 2.6 months) and response rate (RR) (16.4% vs 4.2%), and resulted in significantly better scores in the quality of life (QOL) analysis of global health status in colorectal cancer patients refractory to oxaliplatin.

Median overall survival (OS) was comparable between treatments 10.7 months with cetuximab/irinotecan and 10.0 months with irinotecan alone [87].

Although cetuximab is approved in treatment of EGFR expressing colon cancer, Chung *et al.* showed that also EGFR-negative tumors have the potential to respond to cetuximab-based therapies [88].

Four out of 16 chemotherapy-refractory EGFR-negative colorectal cancer patients (14 treated with cetuximab plus irinotecan, and 2 with cetuximab monotherapy) objective responses in terms of response rate (25%; 95% CI, 4% to 46%) were seen.

As will be analysed, EGFR analysis by current immunohistochemical (IHC) techniques has low sensibility and thus does not seem to have predictive value. For these reasons, selection or exclusion of patients for cetuximab therapy on the basis of currently available EGFR IHC does not seem warranted.

Vincenzi *et al.* described VEGF modification in EGFR-negative patients treated with weekly cetuximab plus irinotecan and their correlation with clinical tumor response and outcome. Patient with a substantial VEGF reduction showed a response rate of 45.5% compared with 8.7% in the no reduced VEGF group, a median time to progression (TTP) of 6 versus 3.9 months and a greater OS (11.0 and 9.6 months, respectively). These data, even if obtained in a small population, suggest that cetuximab may induce VEGF modifications in EGFR-negative colorectal cancer patients and that these changes might at least in part be responsible for cetuximab efficacy observed in these patients. The impact on angiogenesis described in EGFR-negative colorectal cancer patients could lead to a reconsideration of cetuximab use in this subset of patients [89, 90]. The phase III CRYSTAL study was designed to explore the effectiveness of cetuximab addition to FOLFIRI regimen compared with FOLFIRI alone as first-line treatment of unselected mCRC patients expressing EGFR. The primary endpoint of the study was PFS. A total of 1,217 patients have been enrolled and randomized to receive FOLFIRI or FOLFIRI plus cetuximab. The subset of patients receiving cetuximab had a significantly prolonged PFS and increased RR (47% vs 39%) [91]. The large, randomized phase II oxaliplatin and cetuximab (OPUS) in first-line treatment of mCRC trial reported a 46% RR in patients treated with combination therapy (cetuximab plus FOLFOX-4) vs 36% RR in the control arm [92]. The RR percentages of OPUS study are lower than those described in other studies and those for FOLFOX-4 regimen. The cross-study variability in RRs might depend on patient selection criteria which are more stringent for small phase II studies than those applied for larger randomized studies. The addition of cetuximab to radiotherapy (RT) in locally advanced HNSCC patients improves locoregional disease control and survival (median survival of 20 months) [93]. These reported benefits lead to approval of cetuximab use in combination with RT in the treatment of unresectable HNSCC in the United States and in Europe. Promising benefits have been reported in terms of median TTP and OS following cetuximab monotherapy or in combination with a platinum agent in patients with platinum-refractory recurrent or metastatic HNSCC [94, 95], thus cetuximab is also approved in the United States as

monotherapy in patients with metastatic head and neck cancer refractory to standard chemotherapy.

The use of cetuximab as monotherapy or in combination with chemotherapy has been also analysed in advanced NSCLC patients. Docetaxel plus cetuximab as second-line treatment of advanced NSCLC patients showed a clinical benefit (25% RR with median TTP of 2.9 months) [96,97].

Xiong *et al.* conducted a phase III study to determine the response rate, time to disease progression, survival duration and rate, and toxicity with the combination of cetuximab and gemcitabine as I line therapy in patients with EGFR-expressing locally advanced or metastatic pancreatic cancer. The authors stated that cetuximab in combination with gemcitabine showed promising activity against advanced pancreatic cancer (partial response was 12.2%, stable disease was 63.4%, the median time to disease progression was 3.8 months, and the median overall survival duration was 7.1 months) [98].

Preclinical data have suggested a synergistic effect of cetuximab combined with gemcitabine and cisplatin and clinical data have shown a substantial improvement in response and survival when gemcitabine is combined with a platinum analogue compared with gemcitabine alone.

Aimed from these data Cascinu *et al.* have conducted a multicentre, randomised phase II trial to assess the activity and feasibility of a combination of cetuximab with gemcitabine and cisplatin compared with use of gemcitabine and cisplatin alone for the treatment of advanced pancreatic cancer. No significant difference was noted between the groups both for objective response, for disease control, for median progression-free survival or median overall survival. The authors concluded that the addition of cetuximab to a combination of gemcitabine and cisplatin does not increase response or survival for patients with advanced pancreatic cancer [99].

Another phase III study by Philip and colleagues analyzing the combination of cetuximab to gemcitabine versus gemcitabine alone did not demonstrated benefits in terms of OS, PFS and response in advanced pancreatic cancer patients [100].

EGFR STATUS AS A PROGNOSTIC FACTOR IN COLORECTAL CANCER

EGFR pathway plays a key role in tumorigenesis and in tumor progression, due to its importance its expression has been analysed as a possible prognosis marker in patients with colorectal cancer.

EGFR expression at variable degree was found in up to 82% of colorectal carcinomas [8, 13, 101] but its expression has been assessed in tumor samples by different methods that together with the heterogeneity in tumor biopsies and in enrolled patient populations have caused some discrepancy in the results of EGFR expression as a prognostic factor [102].

At least two studies linked EGFR expression with prognostic data including tumor staging, histology grade, vascular and lymphatic tumor cell invasion [21, 103, 104]. Moreo-

ver, EGFR overexpression and tumor invasion (TNM tumor stage) were significantly associated in CRC [105].

In contrast, other studies have no reported a difference in EGFR status and histological type or tumor grade, or stage [106,107].

Based on literature data Nicholson *et al.* examined the relationship between EGFR expression and cancer prognosis. More than 200 studies were considered to analyse relapse-free-interval or survival data directly in relation to EGFR levels in over 20000 patients. Analysis of the data showed that 10 cancer types both express elevated levels of EGFR relative to normal tissues. In colorectal cancer EGFR provided more modest prognostic information compared to others cancers, correlating to poor survival rates in 52% of studies [108].

The lack of a clear relationship between EGFR expression and prognosis is expected given that cetuximab activity and its influence on cancer cell survival can be amplified by a number of mechanisms other than increased receptor expression.

MOLECULAR PREDICTORS OF EFFICACY

With the development of modern molecular techniques and the increasing knowledge of chemotherapeutic mechanism(s) of action, the evaluation of chemotherapy-associated molecular markers is an approach most likely to supply novel “predictive” candidates for improved individualized patient management [109, 110].

The goal of this approach is to identify patients that will respond to a particular treatment and patients that will fail to respond to it.

Furthermore, since many anticancer drugs are associated with adverse side effects the identification of predictive markers could avoid unnecessary risk to those patients for whom treatment is likely to fail. Moreover, whereas the use of markers for assessing prognosis has been widely discussed in recent years there are few reviews on predictive factors [111].

We will summarize what we know on predictive markers of response to cetuximab.

Epidemiological data show that only 10-15% of patients benefit from mAb anti-EGFR target therapy, whereas about 85% receive mAb anti-EGFR target therapy without any advantage and with toxicity risk.

Clinical studies of cetuximab in mCRC failed to reveal an association between clinical outcome and EGFR protein expression as measured by immunohistochemistry [8, 9].

But, clinical responses have been demonstrated in patients with undetectable EGFR protein expression [88] and somatic mutations in the EGFR tyrosine kinase domain are associated with sensitivity to the tyrosine kinase inhibitors (TKIs) but not to cetuximab [112, 113].

Different markers have been identified in tumor samples from mCRC patients as potential predictors of response to cetuximab, i.e. activated EGFR, EGFR amplification, absence of KRAS mutations, PTEN expression, low VEGF

receptor (VEGFR) expression and loss of p21 or very recently the expression of EGFR ligands such as EREG and AREG [114].

The following are the studied makers and the related studies.

EGFR Status

As mentioned before, the use of cetuximab is currently restricted to those patients whose tumors express EGFR. Although tumor EGFR status can be quantified by several techniques (IHC, EIA, FACS, PCR, and FISH) in practice, IHC has by far been the most commonly used method, with most clinical cetuximab trials reported to date requiring EGFR IHC positivity for study entry.

This method is currently used despite continuous evidences in literature report that EGFR determination by immunohistochemistry do not correlate with clinical response to treatment and that EGFR expression on primary tumor may differ from metastatic sites.

The lack of correlation between IHC and clinical outcome could depend from different reasons some related to the methodology (time storage, tissue fixation technique used, storage conditions of biological material, scoring system, etc) other related to EGFR biology such as different EGFR expression on primary tumor from metastatic sites with an alternative/non-homogeneous pattern [8, 115, 116].

EGFR Amplification

The presence of an increased number of copies of EGFR, assessed by FISH, has been described in mCRC and it was associated with a better response to anti-EGFR therapies [17, 117].

Moroni *et al.* reported a possible predictive role of EGFR gene copy number in the treatment of mCRC with cetuximab or panitumumab. EGFR gene amplification evaluated by fluorescence in situ hybridization (FISH) was correlated with the objective response to treatment [118].

Romagnani *et al.* showed in 27 patients with mCRC treated with cetuximab that 6 out of 10 responder patients had an increased EGFR copy number compared to 2 out of 17 non-responder patients [119].

Nevertheless, Lenz *et al.* conducted a phase II trial of cetuximab in refractory mCRC showing that an EGFR gene copy number did not relate to response or progression-free survival but to survival. The evaluation was done by using polymerase chain reaction (PCR) technique [120].

The described discrepancies have to be related to the different assay methods: FISH in Romagnani's study and a PCR-based method in that of Lenz but this technique, as known, does not distinguish tumor from normal neighbouring tissue.

EGFR Mutations

Lynch *et al.* identified a subset of patients affected by non-small-cell lung cancer who exhibit EGFR gene muta-

tions and the presence of these mutations predicted for the response to EGFR-targeting therapies [121].

The presence of EGFR gene mutations was also reported in colorectal cancer but they were very rare and failed in predicting the response to anti-EGFR treatment [113, 122, 123].

In the Nagahara study, none of 11 colorectal carcinoma cell lines exhibited somatic mutations although 4 of 33 clinical tumors presented missense mutations clustered in the EGFR kinase domain in exons 19 and 20 [123]. The study by Ogino *et al.* confirms the rarity of activating EGFR mutation in CRC [124].

These studies suggest that for mCRC the impact of treatment with a TKI is likely to be limited [125].

Activated EGFR

A small subset of patients EGFR-positive mCRC refractory to irinotecan treated in the BOND study had activated/phosphorylated EGFR (pEGFR) expression as useful predictor of response to cetuximab based therapy [126]. Disease control rates differed between patients with a pEGFR immunohistochemical score with a trend toward higher disease control in patients with high levels of pEGFR who were treated with cetuximab with or without irinotecan. These preliminary data need further demonstrations in clinical trials.

EGFR Polymorphisms

The EGFR gene contains a highly polymorphic sequence in intron-1, which consists of a variable number of CA dinucleotide repeats ranging from 9 to 21 [127].

This sequence plays a key role in gene transcription as demonstrated by subjects or cell lines with a greater number of CA repeats that have lower levels of mRNA and protein expression [128, 129].

Graziano *et al.* reported that EGFR intron-1 S/S polymorphism (lower number of CA repeats) in germ-line cells of mCRC patients cetuximab treated was associated with favourable overall survival and treatment response [130].

In the clinical setting, the EGFR intron-1 (CA)_n could represent an easy and reproducible marker to be assessed even though its predictive role might be altered by genetic changes in cancer cells.

EGFR Ligands

The EGF signal pathway is activated by several stimuli. In particular, elevated expression of EREG and/or AREG may play an important role in tumor growth and survival by stimulating an autocrine loop through EGFR.

Khambata-Ford and colleagues reported a predictive role of EGFR ligands EREG and AREG showing that patients with a high versus low EREG expression in metastatic biopsies have superior progression-free survival rates and are more likely to have disease control with cetuximab based therapies [114].

Yamada *et al.* found that AREG expression in primary lesions of colorectal cancer is also an important predictive marker of liver metastasis [131].

A recent study by Mutsaers reported treatment-induced circulating ligands changes during EGFR inhibitors therapy and evaluated their potential as surrogate markers of the optimal biological dose. Data obtained showed that treatment-induced increases in circulating ligands, particularly TGF- α , should be serially assessed in clinical trials of anti-EGFR therapeutic antibodies as potential biomarkers of aid in determination of the optimal biological dose [132].

The RAS/RAF/MAPK and the PTEN-PI3K/Akt Signaling Pathways and Related Mutations

KRAS, a human homologue of the Kirsten rat sarcoma 2 virus gene, encodes a downstream G-protein that influences EGFR signalling cascade. In particular, it plays a crucial role in the RAS/MAPK pathway, and is involved in cell proliferation. When KRAS gene is mutated, the KRAS protein is constitutively active regardless of whether the EGFR is stimulated or not [133]. The presence of activating KRAS mutations might circumvent cetuximab's inhibitory activity.

Nowadays, several retrospective studies widely demonstrated the high prognostic and predictive value of KRAS mutations in mCRC patients treated with cetuximab.

A small retrospective study conducted by Lievre *et al.* with 30 cetuximab plus irinotecan refractory patients showed that 40% of the patients had a KRAS mutation. But patients with wild-type KRAS had a higher response rate and a much longer survival than patients with mutated KRAS [134].

Shirin Khambata-Ford *et al.* have conducted a study to identify markers that are associated with disease control in patients treated with cetuximab. They demonstrated that the majority of patients who achieved disease control following cetuximab monotherapy had wild-type KRAS [114].

Moreover, in another study they associated KRAS mutation status of 113 patients with irinotecan refractory mCRC treated with CTX in clinical trials to survival benefit which is even more pronounced in those patients with early radiological response. These characteristics may be exploited for response prediction [135].

Similar results were obtained by Di Fiore *et al.* from an analysis of 59 patients with chemotherapy-refractory mCRC treated with cetuximab plus chemotherapy, who had objective clinical responses had KRAS wild-type [125].

Based on these results, KRAS mutation status is currently the most important standard biomarker to predict response to anti-EGFR-based therapy in patients with mCRC, although its predictive value has to be confirmed by prospective studies since all existing data are from retrospective analyses. Ongoing trials will provide new data.

Another potential biomarker predictive of response to EGFR antibodies is lipid phosphatase and tensin homologue (PTEN). It is a key tumor suppressor gene located on chromosome 10 which regulates PI3K activation, a downstream effector of EGFR cascade. Mutations resulting in PTEN loss lead to uncontrolled activation of PI3K/AKT signalling

pathway that may result in resistance to EGFR-blockade, whether inactivation of this pathway is sufficient for EGFR resistance is not clear. Mutated form of PTEN is present in about 20 % of sporadic CRC. Recently, expression of PTEN and loss of gene function were analysed in CRC patients [136-138].

Starting from the preclinical evidence that Akt activation is an important resistance factor for anti-EGFR therapy [139]. Frattini *et al.* evaluated PTEN protein tumor expression in 27 cetuximab-treated mCRC patients and demonstrated for the first time that the loss of PTEN protein expression is associated with non responsiveness to cetuximab [137].

BRAF

BRAF is a serine/threonine kinase that belongs to the RAS/RAF/MEK/ERK/MAPK pathway, which is involved in the transduction of mitogenic signals from the cell membrane to the nucleus. BRAF activates MEK, which in turn activates the extracellular signal-regulated kinase (ERK). This kinase is another candidate biomarker for resistance in colorectal cancer, while BRAF mutations less frequent than KRAS mutations [138].

A recent study by Benvenuti *et al.* reported that BRAF status (activation) is associated with the lack of response to anti-EGFR mAb treatment in mCRCs patients [139]. These preliminary interesting results need to be confirmed in large clinical trials.

NF- κ B

NF- κ B is a protein complex that acts as a transcription factor and it is involved in cellular responses to stimuli such as stress, cytokines, growth factors, free radicals, ultraviolet irradiation, oxidised LDL and bacterial or viral antigen. NF- κ B target genes promote tumor cell proliferation, survival, migration, inflammation, and angiogenesis. Moreover, NF- κ B is specifically activated by EGFR signalling [140-142].

Scartozzi *et al.* analysed the NF- κ B role in predicting the efficacy of cetuximab therapy in advanced colorectal tumors. NF- κ B expression has been shown to be responsible for resistance to antineoplastic agents and it also plays a role in EGFR activation downstream signalling pathway in colorectal tumors. The authors by retrospectively analysing the nuclear immunoreactivity (defined as only distinct nuclear staining) for NF- κ B in EGFR-positive advanced colorectal cancer patients receiving cetuximab and irinotecan demonstrated that the response rate, the median time to progression and overall survival were significantly improved in NF- κ B-negative patients than in those positive [143]. Moreover, these findings seem to suggest that tumors that constitutively and aberrantly express NF- κ B are more likely to be refractory to cetuximab and irinotecan than those that do not show nuclear expression of this transcriptional factor.

Circulating Factors

Among circulating factors VEGF and magnesium are the most studied as predictor factors of cetuximab therapy efficacy.

Increasing evidence suggests that EGFR mediated pathways are linked to tumor angiogenesis through up-regulation of VEGF and other mediators of angiogenesis. Research continues to highlight the importance of VEGF in colorectal cancer and to generate new hypotheses about its role in angiogenesis throughout development of this cancer.

The importance of angiogenesis and its blockade by target therapies lead to an increasing interest in explaining the relationship between cetuximab and VEGF.

The precise mechanisms by which EGFR signalling pathways regulate VEGF are not completely clarified.

Perrotte and colleagues in 1999 identified, in an *in vitro* model, the role of angiogenesis inhibition as a new mechanism that contributes to the antitumor effect of EGFR blockade therapy with cetuximab. The authors observed that reduction in bladder cancer vascularization is secondary to down-regulation of VEGF, IL-8 and bFGF expression by EGFR blockade therapy with cetuximab [50].

Recently, Vallböhmer *et al.* reported the association between intratumoral VEGF gene expression (quantify by Real Time PCR method) and clinical outcome in terms to response to cetuximab in patients with mCRC cetuximab treated and EGFR-expressing. This evidence confirms the tight relationship between angiogenesis and EGFR pathway [144].

Vincenzi *et al.* in 2007 published the first evidence of VEGF reduction as predictive marker of efficacy of cetuximab plus weekly irinotecan therapy in heavily pretreated advanced colorectal cancer patients.

Patients with reduced circulating levels of VEGF during treatment showed a better response rate, a longer median time to progression and a greater overall survival than those without VEGF modifications [64, 89, 90].

Also magnesium has been studied as a circulating factor which is affected by cetuximab therapy.

The first description of hypomagnesemia cetuximab related was in 2005 [145].

After that, other cases have been reported and a growing interest about the role of magnesium (Mg) in cetuximab biological effects started to be investigated, by both retrospective and perspective clinical trials [146-148].

Several hypotheses have been done about the mechanisms of cetuximab-induced hypomagnesaemia [149-151]. They are mainly based on the high EGFR expression in the kidney, especially in the ascending limb of the loop of Henle where 70% of filtered Mg^{++} is reabsorbed, so that EGFR blockade could interfere with Mg^{++} transport.

An important study of monogenic disorders to understand this process has been conducted by Groenestege and colleagues [152]. They defined EGF as an autocrine/ paracrine magnesiotropic hormone that regulates renal Mg^{2+} reabsorption by regulating the activity of the Mg^{++} -permeable channel TRPM6 (transient receptor potential cation channel, subfamily M, member 6). The authors identified a point mutation in pro-EGF that disrupts sorting of the protein to the basolateral membrane of distal convoluted tubule cells in kidney nephrons. As a consequence, inhibition of the EGFR

by anti-EGFR antibodies led to suppressed activity of TRPM6 and renal Mg⁺⁺ wasting.

Moreover, they showed that colorectal cancer patients treated with cetuximab, an antagonist of the EGFR, develop hypomagnesaemia.

Schrag D *et al.* reported that patients with colorectal cancers treated with cetuximab may develop an Mg⁺⁺ wasting syndrome with hypomagnesaemia and inappropriate urinary excretion [145]. Tejpar *et al.* confirmed these findings, reporting that EGFR targeting antibodies (cetuximab and panitumumab) may lead to magnesium wasting in patients affected by colon cancer [148].

Another study reported a retrospective analysis of magnesium decreasing during cetuximab treatment and, in particular, a severe hypomagnesaemia was associated with treatment duration [147].

Vincenzi B *et al.* confirmed the previous findings by showing a progressive decrease in serum magnesium concentrations during a period of 92 days in patients affected by mCRC treated with cetuximab + irinotecan. These results offered the first evidence in considering magnesium decrease as a new predictor factor of efficacy and outcome in colorectal cancer patients treated with cetuximab + irinotecan [64].

Mg is involved in physiological enzymatic reactions as nucleic acid metabolism, protein synthesis and energy production. In cancer biology, it seems to be involved in the regulation of oxidative stress, carcinogenesis, tumor progression and angiogenesis (by acting on endothelial cells).

Moreover, it has been supposed that the interaction between anti-EGFR agents and Mg⁺⁺ homeostasis could be partially responsible of the anticancer activity of these agents [153].

Also the effect on cell motility is so striking that Mg⁺⁺ has been proposed to serve as a chemotactic factor for endothelial cells [154].

In conclusion, hypomagnesaemia could contribute to the antitumoral effect of cetuximab both by a direct action on endothelial cells and thus on angiogenesis and by an indirect influence on EGFR signalling and production of angiogenic molecules.

A complex cross-talk among magnesium, angiogenesis and EGFR signalling pathway is already been described and it need to be deeply analysed yet.

Among the available and previous presented molecular and genetic predictive markers, only KRAS mutational status seems to be the most accurate and reproducible predictive marker.

However, these studies are mostly based on retrospective analysis from non-randomized trials and/or small samples of patients.

For example, it cannot be excluded that EGFR amplification is in fact linked to a better outcome, independently of administered therapies. This phenomenon has been described for NSCLC where EGFR amplification has been associated with better survival for patients receiving either TKI or placebo, or TKI or chemotherapy. In this setting, EGFR ampli-

fication should not be considered as a predictive factor but in fact as an intrinsic prognostic factor. It will therefore be essential to validate these results in large-scale clinical prospective trials.

Moreover, the influence of the IGF-IR and PI3K/AKT pathways on EGFR-targeted therapy remains to be evaluated in CRC.

CONCLUSIONS

Anti-EGFR MAb cetuximab has been approved by FDA in the treatment of several tumor types and in different setting, both alone and in combination with chemo/radiotherapy.

Consistent and numerous preclinical trials have examined its effects in cancer cells from different tumors.

The mechanisms of action are multiple: inhibition of cell cycle progression, angiogenesis, invasion and metastatization, increase and activation of pro-apoptotic molecules and synergic cytotoxicity with chemotherapy and radiotherapy.

However, the biological activity of this drug is not completely known.

The interaction with Mg homeostasis is a recent active field of research and it may help to better understand the complex biological mechanisms of this agent.

Probably the key to maximize the benefit from cetuximab in the treatment of cancer is linked to find predictive factors of response and to design appropriate clinical trials to explore its potential role in the different settings of patients.

ABBREVIATIONS

ERK	=	extracellular signal-regulated kinase
GRB2	=	growth factor receptor-bound protein 2
IGF	=	Insulin-like Growth Factor-1
IGF-IR	=	Insulin-like Growth Factor-1 receptor
bFGF	=	fibroblast growth factor
MMP	=	Metallo proteinasi
TKI	=	tyrosine kinase inhibitor
TRPM6	=	transient receptor potential cation channel, subfamily M, member 6
TCC	=	transitional cell carcinoma
VPF	=	vascular permeability factor
PIP2	=	phosphatidylinositol 4,5 biphosphate
IP3	=	inositol 1,4,5-triphosphate
DAG	=	diacylglycerol
MEK	=	MAPK kinase
ERK	=	extracellular signal-regulated kinase
TKR	=	tyrosine kinase receptor
HER	=	human epidermal growth factor receptor
AREG	=	amphiregulin

EREG	=	epiregulin
mCRC	=	metastatic colorectal cancer
HNSCC	=	head and neck squamous cell carcinoma
TGF	=	transforming growth factor
MAPK	=	Mitogen-Activated Protein Kinase
PI3K	=	Phosphoinositide 3-kinase
EGFR	=	epidermal growth factor receptor
EGF	=	epidermal growth factor
MMP	=	matrix metallo-proteinase
VEGF	=	vascular endothelial growth factor

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