Mechanisms of acquired resistance to anti-EGFR therapies in squamous cell carcinoma

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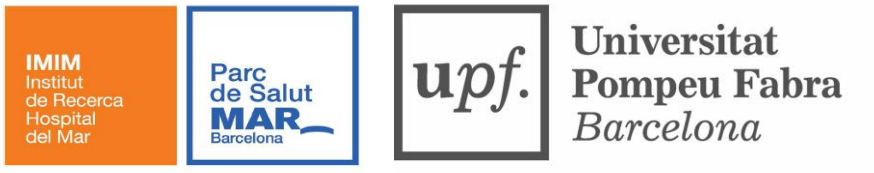

Dedicatòria

Als meus pares, que m'han ensenyat
el valor immens de l'esforç.

A la Laia i l'Oriol,
que no es cansin mai
de preguntar:
"Per què, mami?"
La curiositat és la llavor del coneixement.

Al Jorge, per ser-hi incondicionalment.

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malalts, metges i científics.

A tots els que s'enfronten a un càncer,
ja sigui en pròpia pell
o en la d'algú a qui estimen.

Agraïments

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Dank je wel

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ABSTRACT

Targeted therapies against the Epidermal Growth Factor Receptor (EGFR) are useful to treat many human cancers such as non-small cell lung cancer, colorectal cancer and head and neck cancer. However, the efficacy of such treatments is always compromised by resistance. This doctoral thesis has focused in the mechanisms of acquired resistance to targeted therapies against the EGFR (such as the small tyrosine kinase inhibitors gefitinib and erlotinib, or the monoclonal antibody cetuximab) in squamous cell carcinomas. In the first part of the thesis, preclinical studies with cellular and xenograft models were developed to elucidate the molecular mechanisms of resistance; the second part of the thesis was performed in tumor samples from patients with advanced squamous cell carcinomas of the head and neck. The main finding from the preclinical analysis was that the activation of the insulin-like growth factor receptor 1 system, mainly through downregulation of insulin-like growth factor binding proteins, is responsible for the acquired resistance to anti-EGFR therapies. However, these results could not be validated in a small sample set of advanced head and neck cancer patients.

RESUM

Els tractaments dirigits contra el receptor del factor de creixement epidèrmic (EGFR) són útils en diversos càncers en l'home, com el càncer de pulmó de cèl·lula no petita, el càncer colorrectal o els tumors de cap i coll. Però l'eficàcia d'aquests tractaments sempre està limitada per l'aparició de resistències. Aquesta tesi doctoral s'ha centrat en investigar els mecanismes de resistència adquirida a tractaments dirigits contra l'EGFR (com els inhibidors tirosina quinasa gefitinib i erlotinib o l'anticòs monoclonal cetuximab) en carcinomes escamosos. En la primera part de la tesi s'han desenvolupat estudis preclínics amb models cel·lulars i xenoinjerts per desxifrar els mecanismes moleculars de resistència; la segona part de la tesi ha inclòs estudis en mostres de carcinomes escatosos de cap i coll de pacients amb tumors avançats. La troballa principal dels estudis preclínics ha estat que l'activació del sistema del receptor del factor de creixement semblant a la insulina, principalment a través de la disminució dels nivells de les proteïnes d'unió als factors de creixement semblants a la insulina, és la responsable de l'aparició de resistència adquirida als tractaments anti-EGFR. Posteriorment, però, aquests resultats no han estat validats en una petita cohort de pacients amb tumors avançats de cap i coll.

PROLOGUE

This doctoral thesis reflects my personal growth as a physician scientist in the past ten years, and my will to work in this bench-to-bedside loop that has repeatedly proven to be useful in the management of cancer patients. As the US National Cancer Institute Dictionary defines it, “bench-to-bedside” is a term used to describe the process by which the results of research done in the laboratory are directly used to develop new ways to treat patients.

After completing my training as a medical oncologist at Hospital Vall d’Hebron, I moved to the laboratory of Dr. Carlos L. Arteaga at Vanderbilt University in Nashville, Tennessee. As a research fellow, I was committed to learn how to study a clinical relevant question by taking it to the laboratory, applying useful preclinical models and unraveling the complex molecular biology of cancer.

One of the areas in which I focused was on acquired mechanisms of resistance to targeted therapies. By then, the anti-EGFR directed antibody cetuximab was being successfully used in patients with advanced colorectal cancer, and the anti-EGFR TKI small molecule gefitinib was being used to treat patients with advanced non-small cell lung cancer. From the very beginning of clinical development, though, it was clear that not all patients benefited from these targeted agents, so the quest to understand the mechanisms

of both primary and acquired resistance became of major importance. The seminal discovery of the existence of sensitizing mutations mainly in exons 19 and 21 of the EGFR in a subpopulation of non-small cell lung cancer patients became fundamental in identifying the subset of patients most likely to benefit from anti-EGFR therapies. Furthermore, the discovery of a secondary mutation developing in patients receiving these drugs, mainly the T790M point mutation, was the responsible of the appearance of acquired resistance in the majority of cases. However, little was known by then regarding mechanisms of acquired resistance to anti-EGFR therapies in tumors expressing the wild type receptor. The work done in this area led to the publication that represents Chapter 1 of this doctoral thesis. It describes the participation of the IGF system as the main mechanism of acquired resistance to both small molecule TKIs and antibodies against the EGFR in a variety of cellular models dependent on wild type EGFR.

Years later, back in Barcelona and working as a medical oncologist at Hospital del Mar, I designed a clinical research protocol to explore mechanisms of acquired resistance in patients with squamous cell carcinoma of the head and neck (SCCHN) that had received treatment with cetuximab. Cetuximab is an approved drug in this disease that has shown to have single-agent activity in platinum-refractory recurrent/metastatic tumors, and to prolong overall survival when added to radiation therapy for locally advanced tumors

or to first-line platinum/5-fluorouracil chemotherapy for recurrent/metastatic disease. Although more than 90% of head and neck tumors are squamous cell carcinomas that overexpress the EGFR, and cetuximab is widely used, prognosis of advanced tumors is very poor, and we lack predictive biomarkers for EGFR-targeted therapies. Both primary and acquired resistance to cetuximab is frequently encountered in the clinic when treating these patients and little is known on how to prevent or reverse this resistance. The results of this clinical study represent Chapter 2 of this doctoral thesis

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ABBREVIATIONS AND ACRONYMS

%: percentage

5-FU: 5-fluorouracil

ADCC: antibody-dependent citotoxicity

ASCO: American Society of Clinical Oncology

ATP: adenosine triphosphate

CDKN2A: cyclin-dependent kinase inhibitor 2A

CRC: colorectal cancer

CNV: copy number variations

CT: chemotherapy

ctDNA: circulating tumor DNA

DCR: disease control rate

DNA: deoxyribonucleic acid

E2F1: E2F transcription factor 1

EGF: epithelial growth factor

EGFR: epidermal growth factor receptor

ErbB: erythroblastic leukemia viral oncogene homolog

ERK: extracellular regulated kinase

ESMO: European Society of Medical Oncology

FDA: Food and Drug Administration

FFPE: formalin-fixed paraffin-embedded

GDP: guanosine di-phosphate

GTP: guanosine tri-phosphate

Grb2: growth factor receptor bound 2

HB-EGF: heparin-binding epithelial growth factor

HER: human epidermal growth factor receptor

HIV: human immunodeficiency virus

HPV: human papillomavirus

HR: hazard ratio

IDH: isocitrate deshydrogenase

IGF: insulin-like growth factor

IGF1R: insulin-like growth factor receptor 1

IGF2R: insulin-like growth factor receptor 2

IGFBP: insulin-like growth factor binding protein

IHC: immunohistochemistry

IR: insulin receptor

IRS: insulin receptor substrate

JAK: Janus Kinase

KRAS: Kirsten rat sarcoma viral oncogene

MAPK: mitogen activated protein kinase

MAPKKK: mitogen activated protein kinase kinase kinase

mCRC: metastatic colorectal cancer

mRNA: messenger ribonucleic acid

mTOR: mammalian target of rapamycin

NGS: next-generation sequencing

NRG: neuregulin

NSCLC: non-small cell lung cancer

ORR: overall response rate

OS: overall survival

p-: phospho-

PD1: programmed cell death protein 1

PDK1: 3-phosphoinositide dependent protein kinase-1

PFS: progression-free survival

PI3K: phosphoinositide 3-kinase

PIK3CA: phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha

PIP_2: phosphatidylinositol-4,5-biPhosphate

PIP_3: phosphatidylinositol-3,4,5-triPhosphate

PS: performance status

QoL: quality of life

Ref.: reference

Rb: retinoblastoma

R/M: recurrent or metastatic

RNA: ribonucleic acid

RT: radiation therapy

RTK: receptor tyrosine kinase

SCCHN: squamous cell carcinoma of the head and neck

Shc: src homology

Sos: son of sevenless

STAT: signal transducer and activator of transcription

TGFα: transforming growth factor alpha

TK: tyrosine kinase

TKI: tyrosine kinase inhibitor

TNF: tumor necrosis factor

TNM: tumor-node-metastasis staging system

TPF: docetaxel, cisplatin and 5-fluorouracil regimen

TRAF3: TNF receptor-associated factor 3

Vs.: versus

WT: wild type

1. EGFR IN CANCER

The EGFR (also known as HER1 or ErbB1) belongs to the HER family which includes three other closely related type 1 transmembrane RTK: HER2 (ErbB2), HER3 (ErbB3) and HER4 (ErbB4) (1, 2). It was first linked to cancer in the early 1980's, when it was identified as a cellular homolog of the *v-erbB* oncogene of avian erythroblastosis virus and found to be amplified in A431 human carcinoma cells (3-5). Its dysregulation is associated with a vast number of epithelial tumors such colon, head-and-neck, breast, ovarian, and non-small cell lung cancer.

1.1. The EGFR protein

The *EGFR* gene is located in chromosome 7 and encodes for a 1210-residue polypeptide precursor chain which is cleaved to release a 1186-residue protein that is glycosilated and inserted into the cell membrane. The extracellular region of the EGFR contains 4 different domains: domain I and III are homologous ligand binding domains (also referred as L1 and L2), while domains II and IV are cystine rich domains (also known as CR1 and CR2) (6, 7). The transmembrane and juxtamembrane domain is an α-helix with regulatory functions for receptor internalization and basolateral sorting in polarized cells (8-10). The TK domain comprises residues 713 to 979 where ATP binds. Finally, the C-terminal domain contains

tyrosine and serine/threonine residues that can get phosphorylated to modulate EGFR-signal transduction pathways (11).

1.2. The HER family of receptors

The receptors of the HER family function as either homo- or heterodimers. Upon ligand binding, residues in the C-terminal intracellular domain are transphosphorylated and serve as docking sites for adaptor proteins or enzymes, which initiate a complex and tightly controlled array of signaling cascades (12). These receptors are critical for the regulation of various important aspects of cellular functions such as cell proliferation, differentiation, migration, invasion, and apoptosis (Reviewed in 13). Dysregulation of HER signaling is crucial for the initiation, maintenance and progression of many epithelial cancers, and targeting HER signaling has been intensively investigated and has proven useful for the treatment of several malignancies (14, 15).

Although all four members of the HER family have similar essential domains, their functional activity is not identical (16-19) so each individual member has unique properties (Table 1). In brief, all have known ligands except HER2 (20); all except HER3 have considerable intrinsic tyrosine kinase activity (21); and HER2 is constitutively available for dimerization due to its resting “active” conformation. These characteristics make HER2 a *favorable* dimerization partner, and HER3 an *obligate* heterodimerization partner (22).

Table 1. The HER family: comparison among individual members

	EGFR (HER1, ErbB1)	**HER2 (ErbB2)**	**HER3 (ErbB3)**	**HER4 (ErbB4)**
Ligand	EGF, HB-EGF,TGF-α, Amphiregulin, betacellulin, epiregulin, etc	None	NRG1, NRG2	NRG1-4, HB-EGF, betacellulin, epiregulin
Kinase activity	Yes	Yes	None to minimal	Yes
Dimerization	Homo, Hetero-	Homo, Hetero-, favorble	Hetero-, mandatory	Homo, Hetero-
Effectors and adaptors	Ras > PI3K No p85 site	Ras > PI3K No p85 site	PI3K > Ras 6 x p85 sites	Ras ~ PI3K 1 x p85 site

Ras > PI3K: activates Ras signaling more easily than PI3K, p85: binding site for p85 regulatory subunit of PI3K, Homo: homodimerization, Hetero-: heterodimerization, Ligands: EGF: epidermal growth factor; HB-EGF: heparin-binding epidermal growth factor, TGF-α: transforming growth factor alpha, NRG: neuregulin.

There is a wide array of HER ligands with different affinities for every family member. EGF, TGFα and amphiregulin specifically bind EGFR. B-cellulin, HB-EGF and epiregulin bind both EGFR and HER4. NRG1 and NRG2 bind both HER3 and HER4, while NRG3 and NRG4 are only able to bind to HER4 (Reviewed in 23).

1.3. EGFR signaling pathways

The main signaling pathways activated by EGFR are represented in Figure 1 and described below. There are some differences and similarities among the four HER family members. Intracellularly, all activate ERK 1/2 via the recruitment of Grb2 or Shc adaptors (2). PI3K is more readily activated through HER3 and HER4 due to their ability to directly bind the p85 regulatory subunit of PI3K (2, 14). Together, these features confer great plasticity to the HER signaling network, which results in various biological consequences in the behavior of a cell.

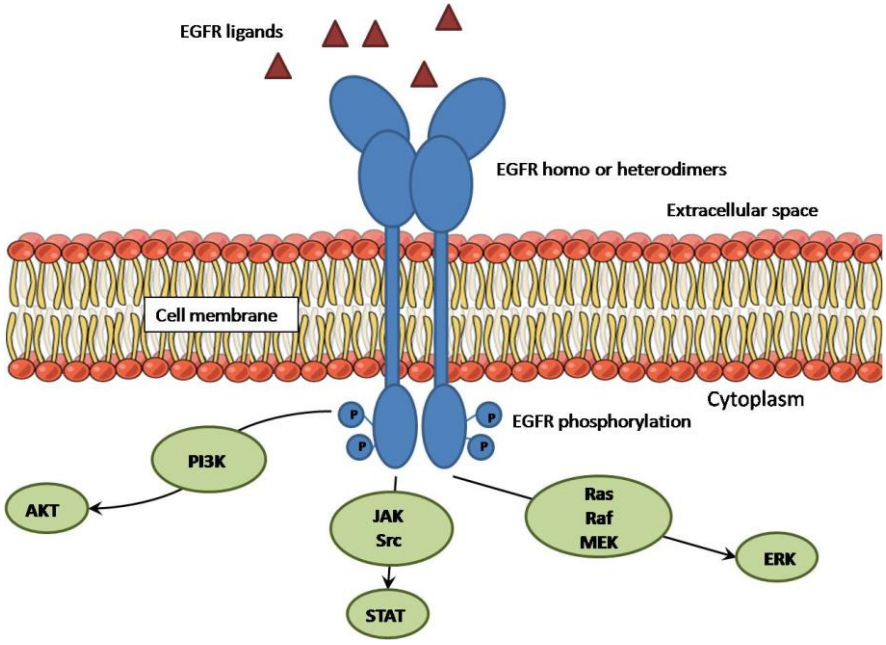

Figure 1. Schematic representation of EGFR signaling pathways.
The figure represents the main signaling pathways activated upon EGFR stimulation by its ligands: the RAS-RAF-MEK-ERK pathway, the PI3K-AKT pathway, the STATs signaling pathway and Src activation.

1.3.1. The RAS/RAF/MEK/ERK pathway

The RAS/RAF/MEK/ERK pathway is one of the most important pathways activated by EGFR. The key player that leads to the activation of this signaling cascade RAS-RAF-MEK1/2-ERK1/2 is Grb2 adaptor protein. In unstimulated conditions, Grb2 is localized in the cytoplasm. Upon receptor phosphorylation, the complex Grb2/Sos relocalizes at the plasma membrane and facilitates the interaction of GDP-RAF with Sos, resulting in exchange from GDP to GTP activated RAS (24, 25).

There are three *RAS* genes (*H-RAS, K-RAS* and *N-RAS*) which encode for three G-proteins that are controlled by its GDP/GTP bound state. Activated RAS binds with high affinity to one of the MAPKKK of the RAF family (RAF-1, A-RAF and B-RAF) which then becomes activated (26). Activated RAF phosphorylates MEK1 and MEK2 that finally phosphorylate the downstream MAPK ERK1/2. This is the effector kinase that translocates to the nucleus and activates several transcription factors. (27). Activation of the MAPK signaling cascade provides a negative pathway feedback loop by phosphorylating Sos and disrupting the Grb2/Sos complex. (28).

The RAS/RAF/MEK/ERK pathway has generally been associated with increased proliferation, survival, angiogenesis, migration and invasion (Reviewed in 29). The oncogenic capability of *RAS* was discovered more than 30 years ago, in the early 1980's, by transforming mouse

embryonic fibroblasts with cDNA of different tumor cell lines. Using viral transforming genes as probes for hybridization, *HRAS* and *NRAS* genes were identified as the responsible of the transforming process (30). Mutation in position G12V in *KRAS* was also found to induce cellular transformation of mouse embryonic fibroblasts (31, 32). Since these early discoveries, *RAS* has become one of the most important oncogenes in human cancers and mutations in these cellular GTPases are among the most common mutations associated with human cancers (Reviewed in 33, 34) (Table 2).

Table 2. Frequency of *RAS* mutations in selected human solid cancers.

Human cancer	***KRAS***	***NRAS***	***HRAS***
Pancreas	90	0.5	--
Colorectal	34.6	4	0.6
Lung	16.5	0.6	0.5
Small intestine	22.6	0.7	--
Stomach	6.2	1	1.3
Esophagus	2	--	0.6
Biliary	24.6	2.6	--
Ovarian	11	0.7	0.1
Endometrial	14.5	2.3	0.5
Cervix	6.6	0.8	5.9
Skin	2.2	15.6 (melanoma)	11.5 (non-melanoma)
Prostate	5	0.8	3
Urothelial	4.4	1.2	0.3
Head and neck	2	1.6	6.2
Thyroid	1.8	6.7	3.7

Frequency is expressed in percentages. (Adapted from Ref. 33)

1.3.2. The PI3K/AKT pathway

The PI3K/AKT pathway plays a central role in a variety of cellular functions such as survival, proliferation, motility, cell growth, apoptosis and metabolism (Reviewed in 35).

PI3K are heterodimers formed by a catalytic subunit (p110) and a regulatory subunit (p85). When EGFR is activated by its ligands, PI3K is recruited to the cell membrane, although the major binding partner of PI3K is not EGFR, but HER3 (36). The catalytic subunit then phosphorylates PIP_2 in the cell membrane to PIP_3. PTEN catalyzes the opposite reaction inhibiting PI3K signaling. PIP_3 then recruits AKT and PDK1. AKT phosphorylation is a key step in the activation of several pathways (Reviewed in 37, 38).

Human cancers are rich in genetic alterations in the PI3K/AKT pathway (39, 40). Loss of PTEN function by mutation or promoter methylation has been described (41). The PI3KCA gene encoding for the catalytic subunit p110α is frequently amplified or mutated, mainly in exons 9 and 20. These mutations give rise to constitutive AKT activation which has transforming capabilities both in vivo and in vitro.

1.3.3. The JAK and STATs pathway

The STAT family of proteins has seven members: STAT1 to STAT4, STAT5a, STAT5b and STAT6. However, only STAT1, STAT3, STAT5a and STAT5b are known to play important roles in human cancer (42).

STATs are transcription factors. Classically, STATs are activated by cytokine receptors and this activation is mediated by JAK kinases. However, upon ligand binding to the EGFR, STATs activation does not require JAK kinases (43, 44). Once activated through phosphorylation of key residues, STATs form homo- or heterodimers and translocate into the cell nucleus to activate gene transcription (45). STAT3 and STAT5 regulate gene expression that control cell cycle progression, survival, angiogenesis, migration and invasion. STAT1 functions as a tumor suppressor by inducing cell cycle arrest and apoptosis.

1.3.4. The Src family of kinases

The Src family of kinases has nine different members. They are cytosolic tyrosine kinases involved in signals transduction pathways from growth factor receptors such as the EGFR (Reviewed in 46). Both proteins share many substrates and their complex interactions are not completely understood. Src contributes to EGFR signaling by binding to the receptor and phosphorylating several targets recruited to the receptor, and by phosphorylating the receptor itself to increase docking sites for other proteins.

2. TARGETED THERAPIES AGAINST EGFR AND MECHANISMS OF RESISTANCE

Although a comprehensive revision of all targeted agents against the EGFR (those available for routine clinical practice as well as those under development) and of the mechanisms of both primary and acquired resistance is far beyond the scope of this doctoral thesis, a brief description of selected drugs and a few illustrative examples from the literature regarding biomarkers predictive for treatment response or resistance will be useful to understand the hypothesis and objectives of this work.

2.1. Small molecule TKIs and antibodies against EGFR

Given the role of EGFR dysregulation through multiple mechanisms in many epithelial tumors, a great number of drugs have been developed to target it and a few have been eventually approved for the treatment of human malignancies. These drugs target the receptor through two basic mechanisms:

a) Binding to the intracellular TK domain of the receptor to inhibit TK activation.

   Drugs in this category are small compounds that can be given orally to patients. They can be further divided in first, second and third generation TKI based, on the reversibility of their binding to the receptor as well as

their inhibitory potency on the WT or mutant forms of EGFR and binding to other member of the HER family of receptors.

Gefitinib (47) and erlotinib (48) belong to the first generation of TKIs. They are reversible ATP analogues that inhibit WT EGFR as well as the mutant EGFR forms more frequently detected in untreated NSCLC (see Section 2.2 Acquired resistance to anti-EGFR TKIs in NSCLC for more details).

Afatinib (49) and dacomitinib (50) are second-generation irreversible EGFR TKIs. Osimertinib (51) is a third generation irreversible EGFR TKI that binds to the cysteine-797 residue in the kinase domain via covalent bond formation. It was engineered to specifically target the mutant T790M EGFR and EGFR with TKI sensitizing mutations, with little activity on WT EGFR.

b) Binding to the extracellular domain of the receptor to prevent ligand binding and/or receptor dimerization.

Drugs in this category are large antibodies that need to be administered to patients intravenously.

Cetuximab is a chimeric murine-human monoclonal antibody that competes with ligands for EGFR binding (52) and it also has immune-mediated activity through ADCC. Panitumumab is a fully human IgG2 antibody that recognizes a different epitope from cetuximab on

the extracellular domain of EGFR to prevent ligand binding to the receptor (53).

2.2. Acquired resistance to anti-EGFR TKIs in NSCLC

The first identification of a molecular mechanism responsible of acquired resistance to anti-EGFR TKIs in a subset of NSCLC was the trigger to initiate the research now presented in this doctoral thesis, so some detailed additional background on this aspect is next presented.

In the past 15 years, treatment has radically evolved in a subgroup of molecularly defined patients within the NSCLC population. In this subgroup of patients, mutations in the *EGFR* gene are a key driver event and these tumors become oncogene-addicted. The detection of *EGFR* mutations was particularly enriched in lung adenocarcinomas affecting Asiatic, female, never smoker patients (54, 55). The most common *EGFR* mutations (>90% cases) identified were small multi-nucleotide in-frame deletions in exon 19 (ex19del) and a point mutation in exon 21 leading to substitution of leucine for arginine at position 858 (L858R). Preclinical work demonstrated that these EGFR mutants were highly sensitive to EGFR blockade using small molecule TKIs and these findings were soon translated into large phase III clinical trials demonstrating the benefit of several EGFR TKIs over standard platinum-based chemotherapy in terms of response rate, PFS, toxicity profile and QoL (56-64).

Meaningful tumor regression is observed in 60-80% of patients treated with EGFR TKIs, but inevitably, after a median time of 9 to 12 months, resistance develops and the tumors become refractory. Among the different mechanisms of acquired resistance, a secondary mutation in the exon 20 of the *EGFR* gene (mutation *T790M*) was the first described (65) and is the most frequent event, occurring in ~50-60% of cases.

The *T790M* mutation consists of the substitution of threonine at the "gatekeeper" amino acid 790 by methionine. This mutation makes the receptor refractory to the inhibition by reversible first generation EGFR TKIs such as gefitinib and erlotinib. Second generation irreversible TKIs (afatinib and dacomitinib) show activity against *T790M* in vitro, but concentrations required to block *T790M* activity preclinically are no achievable in patients without reaching significant toxicity. Osimertinib is an oral, irreversible, third generation TKI targeting *T790M* and *EGFR* TKI-sensitizing mutations but sparing the activity of WT *EGFR*. A large phase III trial has recently demonstrated that osimertinib is the best option in the acquired resistance setting (66).

All the above reviewed mechanisms of resistance are "target dependent", characterized by the development of secondary mutations in the "target", the *EGFR*. However, mechanisms are complex and can also be "target independent", characterized by activation of alternative pathways. Target independent mechanisms include *MET* amplification, *HER2*

amplification, *PIK3CA* mutations, *BRAF* mutations, histological transformation from NSCLC to SCLC, or epithelial to mesenchymal transition. Despite extensive research, still about 20% of cases have an unknown mechanism of resistance (Figure 2).

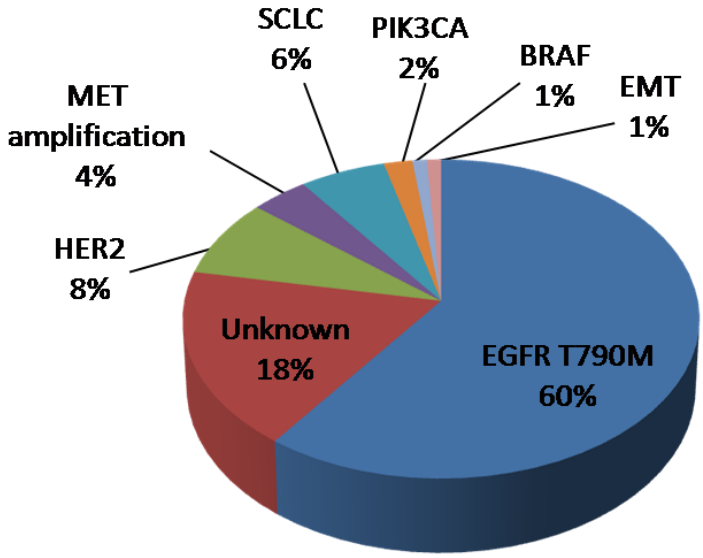

Figure 2. Mechanisms of acquired resistance to EGFR TKIs in NSCLC.
NSCLC: non-small cell lung cancer, EGFR TKI: epidermal growth factor receptor tyrosine kinase inhibitor, EMT: epithelial to mesenchymal transition, SCLC: histological transformation to small cell lung cancer (Adapted from Ref. 67).

Histological and biological interrogation of tissue samples - taken both before therapy and after the development of drug resistance- has been essential to unravel the complex mechanisms of resistance in EGFR-driven NSCLC and to establish predictive biomarkers of sensitivity and resistance. It

also highlights the importance of repeated tumor biopsies to guide treatment decisions in patients.

2.3. Primary and acquired resistance to anti-EGFR in mCRC

Important lessons on primary and acquired resistance to EGFR targeted therapies have also been learnt from other tumor types in the past 10 years. Such is the case of CRC, the second most frequently diagnosed malignancy in Europe, responsible for about 11-13% of cancer-related deaths (68).

Active systemic therapies to treat mCRC include fluoropyrimidines (5-FU and oral derivatives such as capecitabine or S1), oxaliplatin, irinotecan, trifluridine/tipiracil, regorafenib, targeted agents against the EGFR (cetuximab, panitumumab) and anti-angiogenic drugs (bevacizumab, aflibercept, ramucirumab). Treating mCRC patients require multidisciplinary teams to decide not just the best option for a patient at a given time, but also the best sequence of therapies in the continuum of care of the disease. To take optimal decisions, treatment goals, patient characteristics and tumor biology has to be taken into account (Reviewed in 69).

In today's clinical practice, the molecular biomarkers that are routinely analyzed to guide treatment decisions in mCRC are related to the EGFR pathway.

Initial retrospective analyses of pivotal clinical trials for the EGFR monoclonal antibodies, cetuximab and panitumumab, showed that patients with mCRC, whose tumors contain

activating mutations in *KRAS* exon 2 (codons 12 and 13), do not derive benefit from EGFR monoclonal antibody therapy (70-75). More recently, evidence from the PRIME study with panitumumab (76) and from the CRYSTAL study with cetuximab (77) showed that mutations other than those in *KRAS* exon 2 [i.e. exons 3 and 4 of *KRAS* and exons 2, 3 and 4 of *NRAS* (globally known as "expanded *RAS* analysis")] also predicted a lack of response to EGFR targeted agents. Moreover, these therapies may in fact even have a detrimental effect in patients with *RAS*-mutant disease (76-81).

Today, expanded *RAS* analyses must be conducted on all eligible patients being considered for EGFR antibody therapy, but a list of biomarkers beyond *RAS* mutational status is emerging which may impact the future of mCRC treatment with anti-EGFR antibodies. Mutations in *KRAS*, *NRAS,* and probably *BRAF;* and amplification of *HER2* and *MET* drive primary (or "de novo") resistance to anti-EGFR treatments. With the exception of *EGFR* extracellular domain mutations (82), which are described only in the acquired setting, all of the genetic alterations defined as a mechanism of "de novo" resistance may also be responsible for acquired resistance. The prognostic role of *PIK3CA* mutations is uncertain (83), but a *PIK3CA* exon 20 mutation may predict resistance to EGFR-antibody therapy (84-88), although the correlation is not strong enough to be applied as a negative predictive marker (89). *PIK3CA* and *PTEN* alterations often co-occur

with *KRAS* or *BRAF* mutations (85, 90). There is no clear evidence for HER3 overexpression and *HER3* mutations, epithelial to mesenchymal transition, *MET* alterations (overexpression or gene amplification) or *KRAS* amplification, *EGFR* mutations in the TK domain (such as those described in NSCLC) or *EGFR* amplification in the resistance to EGFR antibody therapies. Emerging data indicate that *HER2* activating mutations or *HER2* amplification may mediate in some instances resistance to EGFR antibodies (91, 92). A phase II clinical trial also showed *HER2* amplification to be a predictive biomarker of response to HER2 dual inhibition with trastuzumab and lapatinib in a cohort of mCRC patients failing EGFR antibody therapy (93).

As in the case of NSCLC with sensitizing and resistance mutations to EGFR TKIs, management of mCRC patients requires information on changing tumor biology over the course of the disease, and once again, highlights the importance of repeated tumor tissue analysis to guide patient therapies.

In the most recent years, efforts are being developed to use ctDNA as an alternative source of material where mutations and other molecular changes can be detected and monitored. Genomic alterations in solid tumors can be characterized by studying the ctDNA released from cancer cells into the plasma. This approach -popularly known as “liquid biopsy”- represents a safe, convenient and minimally invasive procedure that may eventually substitute the need for

repeated tumor tissue biopsies which are associated with higher risks of complications. Recently reported results in mCRC patients are showing that there is a good concordance between mutations detected in plasma ctDNA and tissue samples and that “liquid biopsies” are useful in monitoring treatment for mCRC patients (94, 95).

3. THE IGF SYSTEM IN CANCER

Another important growth factor system in cancer is the IGF system, also highly relevant for the research presented in this thesis. The ligands and receptors that make up the signaling network of the IGF system are complex (Reviewed in 96). This system is composed of the three circulating ligands (IGF-I, IGF-II and insulin), multiple receptors; and six binding proteins (the IGFBPs).

The type 1 IGF receptor (IGF1R) is a RTK closely related to the insulin receptor (IR). Their kinase domains exhibit 84% homology (97). In normal physiology, ligand activation of IGF1R plays a role in fetal growth and linear growth of the skeleton and other organs, whereas insulin acts via IR to regulate glucose homeostasis (98, 99).

The IGF1R is a heterotetramer. The IGF1R gene transcript is translated as a single polypeptide chain and is then processed into an extracellular domain (the α-subunit) and a transmembrane or cytoplasmic domain (the β-subunit) which possesses TK activity (100). The IGF1R is transported to the membrane fully assembled in the dimeric form, and ligand

binding of IGF-I or IGF-II to IGF1R results in a conformational change leading to transphosphorylation of one β-subunit by the other. Activated IGF1R recruits and phosphorylates adaptor proteins belonging to the IRS family or SHC. The phosphorylated adaptor proteins then serve as docking sites for other signaling molecules, resulting in the activation of downstream pathways such as the PI3K and MAPK pathways.

The type 2 IGF receptor (IGF2R) binds IGF-II, among other proteins, but lacks TK activity and does not transduce signals (101). It seems to serve as a sink for IGF-II.

The IGFBPs are a family of six proteins (IGFBP1 to IGFBP6) that function to regulate bioavailability of IGF-I and IGF-II to interact with the receptors (102). The binding affinity of IGFBPs for the ligands is higher than that of IGF1R for the ligands. IGFBP3 is the dominant binding partner of IGFs, accounting for 70-80% of IGF-I binding. In this complex, IGF-I cannot bind to the IGF1R. In times of stress, IGFBP3 is proteolytically cleaved and releases IGF-I to its receptor. IGFBPs have long been established as potent negative regulators of IGF1R activation. In addition, many of the IGFBPs have IGF-independent effects (103).

The main components of the IGF system and signaling pathways are summarized in Figure 3.

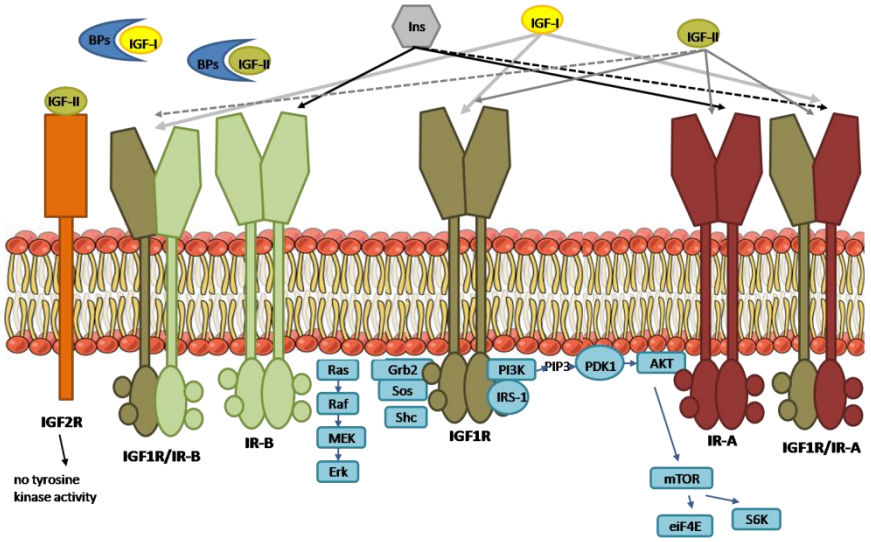

Figure 3. The main components of the IGF system and signaling pathways.

For simplicity, only signaling initiated by the activated IGF1R is shown. Activation of these downstream signaling pathways leads to enhanced proliferation, survival, and metastasis in cancer cells. *Solid arrows*: high-affinity binding; *dotted arrows*: low affinity binding. IGF-I: insulin-like growth factor I, IGF-II: insulin-like growth factor II, IGF1R: insulin-like growth factor receptor 1, IGF2R, insulin-like growth factor receptor 2, IR-A: insulin-receptor isoform A, IR-B: insulin-receptor isoform B, Ins: insulin, BPs: insulin-growth factor binding proteins, PI3K: phosphatidylinositol 3'-kinase, PDK1: phosphoinositide dependent kinase, mTOR: mammalian target of rapamycin, eIF4E: eukatyotic translation initiation factor 4E, S6K: p70S6 kinase (Adapted from Ref. 96).

Multiple pieces of evidence have shown that IGF1R plays a role in maintaining the malignant phenotype, providing an excellent rationale for targeting the IGF system in cancer (Reviewed in 104). The proliferative and antiapoptotic effects of IGF1R signaling are mediated through the adaptor protein IRS1, which functions as a scaffold protein and facilitates the activation of a number of downstream signaling molecules. The adaptor protein IRS2 is also activated by IGF1R. Upon

activation, IRS2 facilitates focal adhesion kinase phosphorylation, dissolution of both focal adhesions and actin stress fibers, and enhances cell motility and invasion.

4. HEAD AND NECK CANCER

This doctoral thesis started by exploring mechanisms of acquired resistance to anti-EGFR targeted therapies in squamous cell carcinoma models that are dependent on WT EGFR, and then continued to explore these mechanisms in a clinical setting. Head and neck cancer seemed the best choice for several reasons as it will be further presented in this section. First, squamous cell carcinoma is by far the most abundant histology in tumors arising in these anatomic areas. Second, *EGFR* mutations are rare in head and neck cancers, so the majority of tumors depend on WT *EGFR*. Third, no predictive biomarker for anti-EGFR targeted treatment is available for clinical use, although cetuximab is widely administered both in locally advanced and recurrent or metastatic disease. And last, little was known on the mechanisms of resistance in head and neck cancer.

4.1. Epidemiology and risk factors

Head and neck cancers include a variety of tumors originating in the lips, oral cavity, hypopharynx, oropharynx, nasopharynx and larynx. Together they represent the 6th most common malignancy worldwide, with more than 500,000 new patients diagnosed per year, accounting for approximately 6% of all

cancer cases (105). The approximate distribution of head and neck cancer is oral cavity, 44%; larynx, 31%; and pharynx, 25%. These cancers originate in the epithelium of the upper aerodigestive tract and 90% of them are squamous cell carcinomas (106, 107).

The most important independent risk factors for SCCHN are tobacco and alcohol consumption, and SCCHN incidence trends have been strongly influenced by patterns of tobacco use over time and across countries (108). Approximately 90% of patients have a history of tobacco use. Compared to non-smokers, tobacco users have a 4-5 fold increased risk for cancer in the oral cavity, oropharynx and hypopharynx and a 10-fold increased risk of laryngeal cancer. Alcohol intake independently increases the risk, especially of hypopharyngeal cancer. It acts synergistically with tobacco, resulting in an approximately 35-fold increase in risk in heavy smokers (>2pack/day) and drinkers (>4drinks/day). Men have a 2- to 5-fold greater risk than women, and risk also increases with age, with a median age of diagnosis in the late 60s and 70s.

In recent years HPV infection has been recognized as an increasingly important oncogenic agent especially in developed northern countries, with the degree of risk varying among tumor sites (109-113). The rising incidence of HPV-related cancers is changing the epidemiology and demographics of SCCHN with an increase in the proportion of oropharyngeal tumors occurring in a younger patient

population of higher socioeconomic status (114). HPV type 16 (HPV16) is responsible for more than 90% of HPV positive oropharyngeal cancers. The time from first oral HPV infection to the development of cancer is estimated to be more than a decade. Measures of sexual behavior (number of vaginal and oral partners, history of genital warts) have been associated with HPV positive oropharyngeal cancer.

HPV positive tumors are characterized by an earlier T stage at presentation but with extensive nodal involvement. However, prognosis is better compared to tobacco-related SCCHN (115-117). Differences in patient characteristics and prognosis from HPV negative and HPV positive SCCHN are summarized in Table 3.

Table 3. Comparison between HPV negative and HPV positive SCCHN.

Parameter	HPV negative	HPV positive
Gender	2-3 fold more common in men	4-5 fold more common in men
Median age at diagnosis	Late 60s and 70s	Early 50s
Race	--	More common in whites
Smoking	90% smoking history	50-65% smoking history
Sexual behavior	Not a significant risk factor	Number of oral and vaginal sex partners and genital warts are risk factors
Site	Oral cavity and larynx most commonly	Oropharynx, (<20% HPV positive at other sites)

Clinical picture	Varies	Early T stage, enlarged lymph nodes
Incidence trends	Decreasing	Increasing
Survival rates	All sites: 65% 5y OS, Oropharynx: 25% 5y OS	60-80% 5y OS

HPV: human papillomavirus, SCCHN: squamous cell carcinoma of the head and neck, Y: years, OS: overall survival. Adapted from "Head and neck cancers: essentials for clinicians" ESMO Press 2017 (ISBN: 978-88-941795-2-1).

Other risk factors for SCCHN include immunosupression (organ transplant recipients, HIV infection) and certain genetic diseases such as Fanconi anemia. Nasopharyngeal and paranasal sinus cancers are associated with the Epstein-Barr virus.

4.2. Pathogenesis

In tobacco-related SCCHN tumors, transformation of normal mucosa into invasive carcinoma follows a molecular progression model of multistep carcinogenesis. Loss of genetic material from chromosome region 9p21 and inactivation of p16 are the earliest alterations identified in hyperplastic mucosa. Subsequent transition to dysplasia is characterized by loss of 3p and 17p and by p53 inactivation. Loss of 11q, 13q and 14q precedes transition to carcinoma in situ. Losses of 6p, 8p and 4p are identified during transformation to invasive carcinoma (Reviewed in 118, 119). HPV related SCCHN is molecularly driven by the host

genome disruption of factor E2 expression, the transcriptional repressor of E6 and E7 viral proteins. E6 and E7 encode oncoproteins that bind and degrade p53 and Rb tumor suppressor, respectively. Degradation of Rb induces expression of $p16^{INK4A}$ (120).

Recently, the Cancer Genome Atlas Network published a comprehensive analysis of the somatic mutations of almost 280 SCCHN and showed that HPV associated tumors are dominated by helical domain mutations of the oncogene *PIK3CA*, alterations involving loss of *TRAF3* and amplification of the cell cycle gene *E2F1*. Smoking-related SCCHN demonstrate near universal loss-of-function *TP53* mutations and *CDKN2A* inactivation with frequent copy number alterations including amplification of 3q26/28 and 11q13/22 (121).

4.3. EGFR as a therapeutic target in SCCHN

The most widely studied growth factor receptor in SCCHN is the EGFR whose overexpression in the majority of tumors of the head and neck was first described over 30 years ago (122). EGFR overexpression has been linked to malignant progression, resistance to radiotherapy and poor prognosis (123, 124), and in many SCCHN one or more EGFR ligands are often overexpressed either by host cells or tumor cells themselves (autocrine signaling) (125, 126). Overexpression of EGFR in SCCHN is often caused by *EGFR* gene copy

number increases (10-30% cases), either amplification at the 7p11 locus or polysomy (127).

EGFR mutations in SCCHN are relatively rare with the exception of the EGFRvIII, a constitutively active, ligand-independent RTK variant which has an in-frame deletion of exons 2-7 that yield a functional receptor with a truncated extracellular domain. EGFRvIII frequency in SCCHN varies widely with incidences ranging from 0 to 40%, with similarly conflicting reports as to its impact on patient survival.

Alterations in other HER family members are also described in SCCHN. HER2 is overexpressed in approximately 6% of cases, but its importance in this malignancy is uncertain, with reports both of an association with poor prognosis (128) and of no relationship with outcome (129). Similarly, HER3 can be overexpressed and has been found to correlate with reduced survival (130, 131). There have been conflicting reports of HER4 expression in SCCHN (132).

As it will be next discussed in the section regarding clinical management of head and neck cancer patients, cetuximab has become a standard of treatment for the management of both locally advanced and R/M disease. However, in contrast to other tumor types such as CRC or NSCLC (as briefly reviewed previously), no predictive biomarkers of response or resistance to anti-EGFR therapies are available for SCCHN.

4.4. Clinical management of patients with SCCHN

The anatomic areas where SCCHN develop hold important physiologic functions such as breathing, swallowing, speech and hearing. Clinical symptoms at presentation are often related to primary tumor site, although about 5% of patients present only with enlarged neck lymph nodes (133). A multidisciplinary team is required to optimize diagnosis, clinical work-up and treatment decisions for these patients (134).

Approximately one third of patients are diagnosed at early stage, localized disease (TNM stages I and II), but the majority present as locally advanced disease (TNM stages III and IV, M0). Distant site metastases, more commonly affecting lungs and bones, are unusual at initial presentation (< 10% cases).

The specific site of disease, stage, and pathologic findings guide treatment, but other considerations have to be taken into account, such as patient preferences, comorbidities and prior utilized therapies (135). Similarly, managing and preventing sequelae after surgery, RT and systemic therapy (i.e. pain, xerostomia, speech and swallowing problems) requires many different health professionals. Finally, health-related QoL issues are paramount in head and neck cancer patients since these tumors affect basic physiologic functions, the senses (taste, smell, and hearing) and uniquely human traits (physical appearance, voice). Medical scientific societies such as ASCO provide guidelines for the

management of adults surviving to head and neck cancer (136).

4.4.1. Early stage disease

Single-modality treatment with either surgery or RT is generally recommended for patients who present with early stage disease. These two modalities are curative in about 80% of stage I patients and 60% of stage II patients. The choice of surgery or RT is often based on local institutional expertise and/or morbidities of these therapeutic options depending on primary tumor site location. Overall survival in this population is often limited due to heart, lung, liver and vascular comorbidities associated with tobacco and alcohol consumption, as well as the risk of second malignancies (137).

4.4.2. Locally advanced disease

Prognosis for patients with locally advanced disease is poor, with about only 30-40% patients surviving beyond 5 years (137). Local recurrences account for the majority of deaths. Therapeutic decisions require discussion in multidisciplinary teams to plan and execute multi-modality treatments that often include surgery, RT and systemic therapy.

No clinical studies have compared surgery followed by RT +/- CT versus concurrent CT and RT, although survival results are probably similar. In the last 20 years, CT has demonstrated to increase OS when added to RT, but this

benefit has only been observed when given concurrently. The meta-analysis of multiple clinical trials concludes that the survival benefit is not seen when CT is administered as induction therapy before RT or in the adjuvant setting following local treatment (138, 139). Several randomized trials have demonstrated that the addition of CT concurrently to RT improves locoregional control (15-25% increase) and OS (10-15% increase) compared to RT alone (140-144). Currently, concomitant CT+RT is a standard therapy in inoperable stage III-IV, M0 SCCHN. In locally advanced tumors initially treated with surgery, complementary RT or CT+RT (in tumors with features for high risk of relapse such as positive surgical margins or extranodular lymph node invasion) are recommended (145, 146). The recommended CT regimen is cisplatin 100mg/m^2 given days 1, 22 and 43 concurrent with RT.

Another strategy developed to manage locally advanced disease has been RT administered concomitantly with other systemic therapies different from classical CT. Several positive early clinical trials combining the anti-EGFR monoclonal antibody cetuximab with RT (bio-RT) finally led to a large phase III trial that demonstrated the superiority of bio-RT compared to RT alone (147). In this study, bio-RT increased 10% OS at 5 years compared to RT alone, the magnitude of the benefit being similar to what is observed in the concomitant CT+RT trials, although these two strategies (concomitant CT+RT vs. bio-RT) have never been directly

compared in the setting of a large phase III randomized clinical trial. Median OS was 49 months for bio-RT treated patients compared to 29 months for RT alone (p=0.02, HR 0.74 [0.56-0.76]) (148). Today, RT concomitant with weekly cetuximab is a standard of care option for locally advanced head and neck cancer patients.

Induction CT before definitive concurrent CT+RT or bio-RT has been largely investigated in many clinical trials as an organ-preservation strategy as well as a potential strategy to increase both distant site and locoregional disease control, and OS. However, no benefit on OS has been clearly demonstrated and induction CT should not be considered routine standard practice in unselected patients (149-151).

4.4.3. R/M disease

About one half of patients treated for stage III-IV, M0 disease and one third of patients treated for early stage disease will develop locoregional relapses or have locally persistent disease. Most locoregional or distant relapses are usually detected within the first two years following prior treatment. (152).

Surgery is recommended for resectable recurrent or persistent disease, and adjuvant therapy (RT or concomitant CT+RT) should be considered, if feasible, after local salvage therapy. For patients with recurrent disease who are not candidates for curative-intent salvage therapy with either surgery or RT (+/- systemic therapy), treatment management

is similar to patients with metastatic disease. The standard of care for these patients is systemic therapy and the main objectives are to prolong survival and/or to provide symptom palliation and improve QoL. However, prognosis is extremely poor with a median overall survival of less than a year.

4.4.3.1. First line therapy

Active agents for the treatment of R/M SCCHN include cisplatin and carboplatin, 5-FU, methotrexate, paclitaxel and docetaxel, vinblastine, bleomycin, ifosfamide, doxorubicin, cyclophosphamide and hydroxyurea. Combination CT has not produced better survival outcomes than CT monotherapy. Combination CT regimens are associated with higher response rates than single-agent therapy, but at the expense of a higher incidence of severe toxicities (Reviewed in 153).

A wide variety of targeted therapies either alone or in combination with standard CT has been largely explored in SCCHN, and many new agents are under current development. These compounds target the main cellular and molecular drivers of SCCHN, including both the cancer cells and the tumor microenvironment: preventive and therapeutic anti-HPV vaccines, cell-cycle inhibitors, PI3K inhibitors, mTOR inhibitors, AKT inhibitors, TKIs and antibodies against EGFR and other HER family members, histone deacetylase inhibitors, immune checkpoint inhibitors and other immunotherapies, Src inhibitors, IGF1R inhibitors, MET inhibitors, and antiangiogenics, just to name a few (Reviewed

in 154-156). However, results have been mostly disappointing with the exception of cetuximab, the anti-EGFR directed monoclonal antibody that gained FDA approval in 2011 for R/M patients in combination with platinum-based CT.

Initial evidence of cetuximab activity in the R/M setting came from monotherapy studies in platinum-refractory patients, showing an ORR of 13%, DCR of 45.5% and median OS of 5.5 months. (157). Further clinical development finally led to a large phase III trial -known as the EXTREME trial- that compared an active standard of care CT combination (6 platinum-based CT cycles, either cisplatin or carboplatin, + 5FU) with the same regimen together with weekly cetuximab, followed by cetuximab maintenance. The addition of weekly cetuximab resulted in a 2.7 month increase of median OS from 7.4 to 10.1 months (HR for death: 0.80, [0.64-0.99], P=0.04), a 2.3 month prolongation of PFS (HR for progression: 0.54, P<0.001) and increased the response rate from 20 to 36% (P<0.001) without adversely affecting QoL (158, 159).

For frail patients (PS2 and/or elderly patients) or for those unfit for platinum-based therapies, the combination of weekly paclitaxel and cetuximab is an alternative regimen with significant tumor activity and a favorable toxicity profile. Despite the lack of evidence from phase III randomized trials, this combination is widely used in everyday clinical practice and both phase II and real world data support its use (160, 161).

4.4.3.2. Second line therapy

Numerous trials have evaluated drugs in second line for R/M SCCHN. However, no significant benefit has been demonstrated in terms of OS, and response rates are low (below 20%) and short-lived. Recently, though, immunotherapeutical approaches using checkpoint inhibitors such as anti-PD1 antibodies have shown promising results. A phase III trial comparing nivolumab to standard of care investigator's choice therapy (methotrexate, cetuximab or docetaxel) in platinum-refractory patients demonstrated a 2 month significant improvement in median OS for nivolumab-treated patients without deterioration in QoL parameters (162). Although a clear survival benefit could not be demonstrated for another anti-PD1 antibody, pembrolizumab, the FDA has recently granted approval of both drugs (163, 164).

HYPOTHESIS

Acquired resistance to anti-EGFR therapies in squamous carcinomas expressing wild type EGFR emerges due to the selective pressure imposed to malignant cells by continuous drug exposure. Resistant cells can be generated in the laboratory and then studied to elucidate the mechanisms of acquired resistance. Finally, these and other mechanisms of resistance can be explored in tumor tissue from cancer patients in the clinic.

OBJECTIVES

The general objective of this doctoral thesis was to study and identify mechanisms that lead to resistance to EGFR-targeted therapies in systems with WT EGFR.

The specific objectives were:

1. To determine the molecular mechanisms of acquired resistance to anti-EGFR targeted therapies in preclinical models of squamous cell carcinoma expressing WT EGFR:

- To generate resistant cell lines derived from parental highly sensitive cells by chronically exposing them to increasing concentrations of EGFR TKIs or antibodies against EGFR.
- To study the altered signaling pathways responsible of the resistant phenotype by comparing genes and/or protein expression in the parental versus resistant cells.
- To confirm the molecular mechanisms of resistance with functional studies.

2. To explore the molecular mechanisms of resistance in a cohort of patients with SCCHN treated with cetuximab:

- To collect tumor samples from SCCHN patients before initiation of cetuximab treatment and after failure to cetuximab.

- To validate in these tumor samples the mechanisms identified in objective 1.

- To investigate potential alternative mechanisms of resistance through the analysis of genomic alterations in these tumors.

CHAPTER 1:

Guix M, Faber AC, Wang SE, Olivares MG, Song Y, Qu S, et al. Acquired resistance to EGFR tyrosine kinase inhibitors in cancer cells is mediated by loss of IGF-binding proteins. J Clin Invest. 2008 Jun 1;118(7):2609–19. DOI: 10.1172/JCI34588

CHAPTER 2:

Exploratory analysis of mechanisms of acquired resistance to cetuximab in tumors from patients with advanced SCCHN

2.1. MATERIALS AND METHODS

2.1.1. Clinical research protocol

In order to explore potential mechanisms of resistance to cetuximab in the clinic, a research protocol was written and submitted for IRB approval at Hospital del Mar in November 2013. All patients signed informed consent. All samples were obtained from the Hospital del Mar Tumor Bank.

Briefly, patients had to be 18 years of age or older, had a cytologically or histologically diagnosed squamous cell carcinoma of the head and neck and had received cetuximab therapy, either concomitant with RT for locally advanced disease, or in combination with CT (platinum-5FU doublet CT -with either cisplatin or carboplatin- or weekly paclitaxel) or as cetuximab maintenance monotherapy in the R/M setting. Patients were excluded if they had received any investigational agent.

The initial FFPE tissue sample from the diagnostic biopsy from every patient was used for biomarker evaluation. Upon progression to cetuximab therapy, a second biopsy for research purposes was obtained. In those patients in whom a salvage surgery was planned, the surgical specimen was used to avoid an unnecessary additional biopsy. This second biopsy was also used for biomarker evaluation, so that paired samples from every patient would be available for comparisons. Biopsies were mostly obtained from the primary

tumor site, although a few came from distant metastatic sites (easily accessible skin metastases).

2.1.2. Tissue handling

Tumor biopsies were fixed in formalin and embedded in paraffin according to standard pathology procedures. If enough tissue was available, a portion was fresh frozen for further molecular studies. An expert pathologist in head and neck cancer reviewed all samples.

2.1.3. IHC for IGFBP3 and P-IGF1R

In brief, a standard two-step indirect avidin-biotin complex (ABC) method was used for visualization. Following deparaffinization in xylenes, the tissue sections were rehydrated in graded alcohols. The sections were place in 0.01M sodium citrate buffer (pH 6.0) and heated at 120°C for 3 min for antigen retrieval. For IGFBP3, the primary antibody, the anti-human IGFBP-3 mouse monoclonal antibody (Clone 84728) (R&D Systems, Minneapolis, MN), was then applied at final concentration of 20µg/ml and incubated for 1h at room temperature. For P-IGF1R, the primary antibody, the anti-human phospho-Y1161 anti-IGF1 Receptor (Ab39398) (Abcam, Cambridge, UK) was then applied at 1:50 dilution for 1h at room temperature. Tissue sections were next incubated with biotinylated secondary IgG and signal developed using the chromagen diamino-benzidine (DAB).

A semi-quantitative assessment of the antibody staining on the tissue sections was performed by a single study pathologist blinded to the clinicopathological variables. For both proteins, cytoplasmic and nuclear expression were scored using two measures: intensity on a 0-3 scale (0=negative, 1=weakly positive, 2=moderately positive, 3=strongly positive), and percentage of positively stained target cells (range 0-100% positive) at each intensity. To better represent overall protein levels, we combined the frequency and intensity measures into an integrated Histo-score following the formula: [(percent staining at intensity 3*3)+(percent staining at intensity 2*2)+(percent staining at intensity 1*1)]/100.

2.1.4. HPV status

2.1.4.1. HPV-DNA

Briefly, we used SPF-10 PCR and a DNA enzyme immunoassay (DEIA) to test for the presence of HPV-DNA. Virus genotyping was performed using reverse hybridization line probe assay (LiPA25_v1) on all samples testing positive for viral DNA, targeting 25 HPV types with different oncogenic risk. DNA quality was evaluated in all HPV-DNA negative samples by testing for the human tubulin gene.

2.1.4.2. IHC for $p16^{INK4a}$

Protein expression patterns were evaluated for $p16^{INK4a}$ under the manufacturer's standards: Roche Laboratories AG (Heidelberg). Overexpression, intensity of tumor nuclear and

cytoplasmic staining was scored and those with strong nuclear and cytoplasmic staining in >70% of the tumor cells were considered positive.

2.1.4.3. HPV E6*I mRNA Detection

All HPV-DNA positive samples underwent RNA extraction and E6*I mRNA detection. Briefly, the assays target a total of 20 HPV types. For each sample, type-specific E6*I mRNA real-time PCR (RT-PCR) was performed for all available HPV types detected at the DNA level and additionally for HPV16.

2.1.5. Analysis of mutations and CNV

DNA was extracted from FFPE tissue using the Qiamp DNA Mini kit (Qiagen) and quantified using the Qubit™ dsDNA HS Assay Kit in a Qubitfluorometre (Thermofisher). 10 ng of DNA were used to amplify selected regions of 52 cancer related genes using the commercial kit Oncomine Focus Assay (Thermofischer). The resulting library was templated with the Ion Chef and sequenced on a NGS platform Ion PGM System (Thermofischer). Sequences were analysed and annotated with the Ion Reporter software v5.4 and visualised with the Integrative Genomics Viewer v2.3. This method is capable of detecting mutations down to an admixture level of 5% and CNV of selected genes. The genes included with hotspot mutations are: *AKT1, ALK, AR, BRAF, CDK4, CTNNB1, DDR2, EGFR, ERBB2, ERBB3, ERBB4, ESR1, FGFR2, FGFR3, GNA11, GNAQ, HRAS, IDH1, IDH2, JAK1, JAK2, JAK3, KIT, KRAS, MAP2K1, MAP2K2, MET, MTOR, NRAS,*

PDGFRA, PIK3CA, RAF1, RET, ROS1, and *SMO.* The genes with focal CNV gains are: *ALK, AR, BRAF, CCND1, CDK4, CDK6, EGFR, ERBB2, FGFR1, FGFR2, FGFR3, FGFR4, KIT, KRAS, MET, MYC, MYCN, PDGFRA,* and *PIK3CA.*

2.1.6. Statistical analysis

Clinical variables recorded were age, sex, tumor TNM classification, HPV status (in oropharyngeal tumors), tobacco and alcohol use, received therapies, response to cetuximab, duration of response, PFS and OS. Standard descriptive statistics were used for clinical variables. A Spearman's correlation test was used to analyze any relationship between the expression levels of different markers and a Wilcoxon signed rank test was used to compare marker expression levels in paired basal and post-progression samples. Results were considered significant at P value < 0.05.

2.2. RESULTS

2.2.1. Patients characteristics

From November 2013 to February 2017, 22 patients were included in the study protocol. Baseline characteristics are summarized in Table 1. Median age was 60 years (range 41-84), the vast majority of patients were male (91%). The oropharynx was the most common localization of the primary

tumor in 27% of cases (4 patients with primary tonsil tumors, 2 patients with base of tongue tumors), followed by larynx, hypopharynx (5 pyriform sinus tumors), oral cavity (5 patients) and one major salivary gland tumor. At the time of initial diagnosis, most patients had locally advanced tumors (stage IVA in 50% of cases, and stage III in 27%). Three patients had distant metastasis (stage IVC) at presentation: bilateral lung nodules in two patients and skin metastasis in one patient.

Table 1. Patients demographics and tumor characteristics.

		N	%
Sex			
Male		**20**	***91***
Female		**2**	***9***
Age (years)			
Median	60		
Range	41-84		
Tumor localization and subsite			
Larynx		**5**	***23***
• Supraglotis		4	
• Glotis		1	
Oropharynx		**6**	***27***
• Tonsil		4	
• Base of tongue		2	
Hypopharynx		**5**	***23***
• Pyriform sinus		5	
Oral cavity		**5**	***23***
• Floor of mouth		2	
• Buccal mucosa (retromalar trigone)		2	
• Hard palate		1	
Major salivary glands		**1**	***4***
HPV status (oropharyngeal cancer only)			
Positive		**1**	***17***
Negative		**1**	***17***
Unknown		**4**	***66***

Clinical stage at diagnosis		
II	**2**	***9***
III	**6**	***27***
IVA	**11**	***50***
IVC	**3**	***14***

N: number, %: percentage, HPV: human papillomavirus.

Twelve patients received cetuximab for recurrent or metastatic disease in combination with chemotherapy. The chemotherapy regimen was cisplatin + 5FU in 6 patients, carboplatin + 5FU in 5 patients and weekly paclitaxel in one patient. In all cases, cetuximab was administered using the standard regimen of a starting loading dose of 400mg/m^2 followed by a weekly dose of 250mg/m^2, and continued until progression or unacceptable toxicity. Median time from the beginning of cetuximab therapy to disease progression was 9.5 months (range 3-22 months).

The remaining ten patients had received cetuximab in combination with radiotherapy (bio-RT) for the management of locally advanced disease, and had subsequently progressed or relapsed. Three patients had received induction chemotherapy (3 cycles of TPF regimen) prior to bio-RT.

2.2.2. Pharmacodynamic analysis by IHC

From the 22 patients, 17 had paired tumor biopsies for biomarker analysis by IHC. There were no viable tumor cells in the post-progression biopsy in two patients, two patients

had only a cytological sample at initial diagnosis (with not enough tumor cells for reliable biomarker IHC analysis) and one patient had been sent to another center for participation in a clinical trial where the tumor sample was needed for inclusion.

Using the Wilcoxon signed rank test, there were no statistically significant differences in the levels of cytoplasmic or nuclear IGFBP3 upon cetuximab progression compared to the basal levels (P=0.45 for nuclear IGFBP3, and P=0.69 for cytoplasmic IGFBP3) (Figure 1). No differences were observed when all tumor samples were compared globally, or when only the subset of patients with R/M disease was considered (data not shown).

Regarding P-IGF1R, there was a significant reduction in the level of cytoplasmic P-IGF1R upon progression to cetuximab compared to the basal levels (P=0.028). Differences were not statistically significant for nuclear P-IGF1R (P=0.24) (Figure 2).

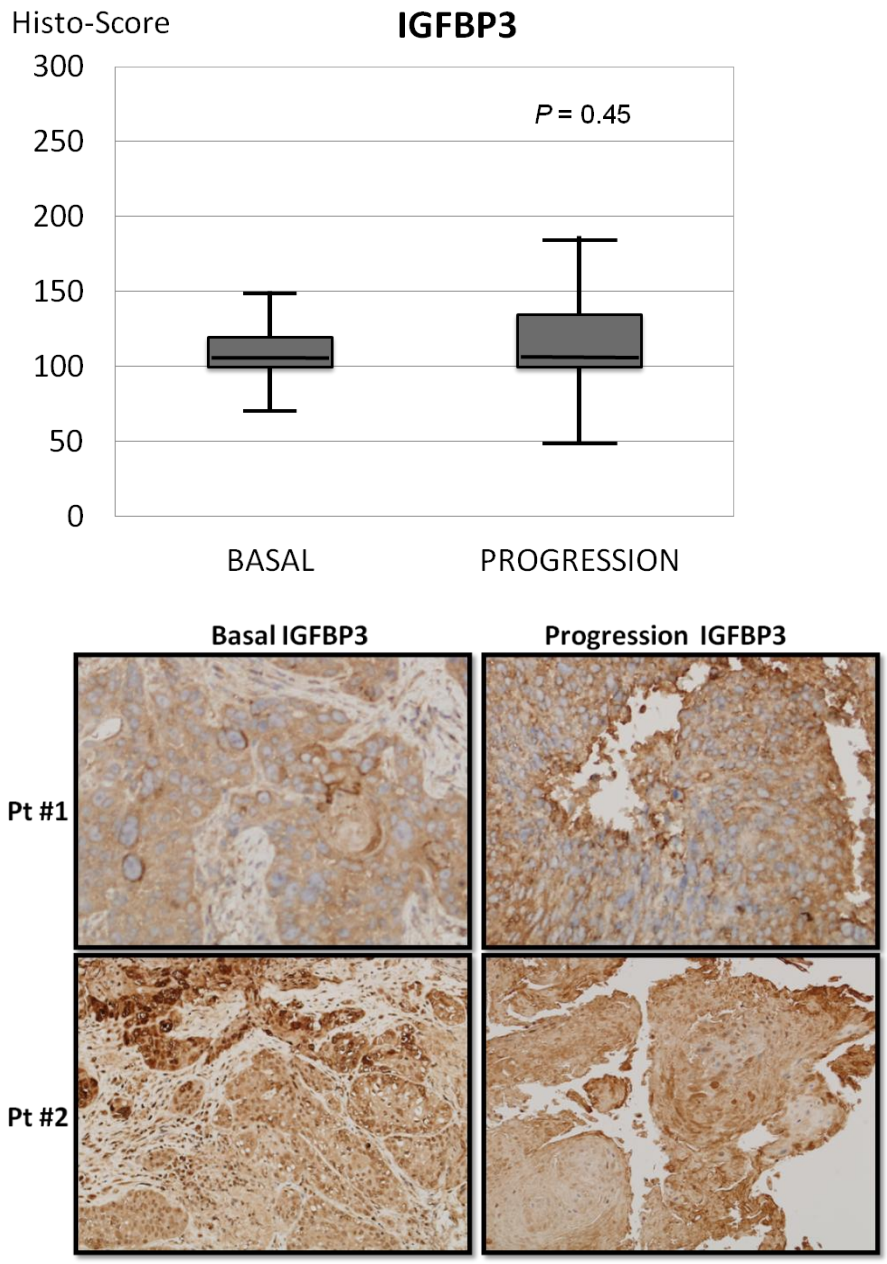

Figure 1. IGFBP3 expression by IHC in paired tumor biopsies.

Top. Boxplot graph showing no significant changes in cytoplasmic IGFBP3 expression (N=17 patients)**. Bottom**. Photographs of IGFBP3 expression in two patients.
IHC: immunohistochemistry, P-IGF1R: phosphorylated insulin-growth factor receptor 1. Pt: patient.

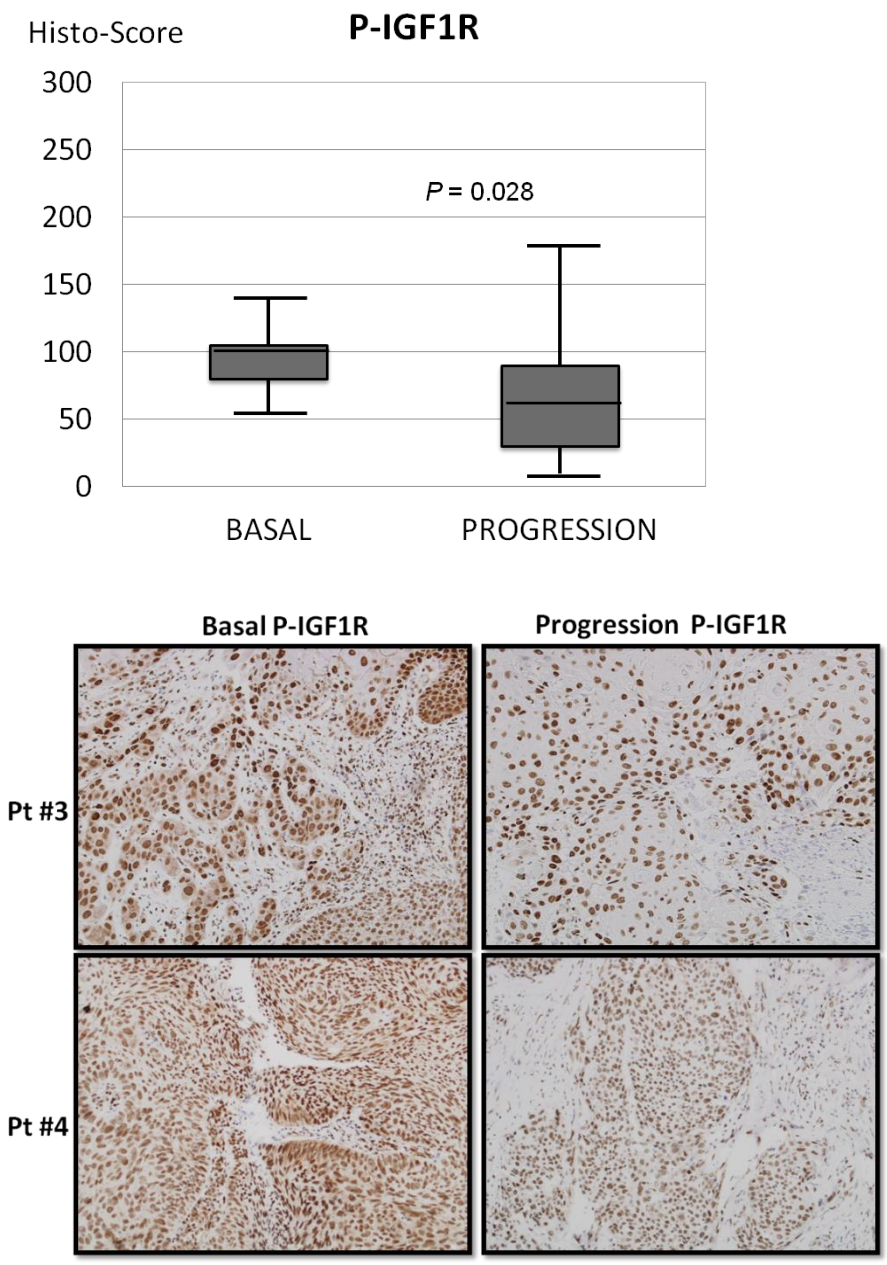

Figure 2. P-IGF1R expression by IHC in paired tumor biopsies.

Top. Boxplot graph showing a significant reduction in cytoplasmic P-IGF1R expression (N=17 patients)**. Bottom**. Photographs of P-IGF1R expression in two patients.
IHC: immunohistochemistry, P-IGF1R: phosphorylated insulin-growth factor receptor 1. Pt: patient.

There was no significant correlation between IGFBP3 and P-IGF1R expression neither in the basal samples nor in the samples after cetuximab progression (Spearman correlation test for basal IGFBP3 vs. P-IGF1R, P=0.55; and post-progression IGFBP3 vs. P-IGF1R, P=0.70).

2.2.3. Analysis of genomic alterations

High quality DNA was available from 12 basal tumors, and from 18 samples post-progression to cetuximab. Overall, mutations were detected in 67% of basal samples and 55% of post-progression samples; and CNV in 42% and 33%, respectively. The most frequently reported alterations were *PIK3CA* mutations in 30% of samples, CNV in *CCND1* in 23% of samples, and *MET* mutations in 20% of samples. We also found lower frequency of mutations (<10% of cases) in *BIRC2, FGFR3, RET, KIT, ERBB2, ERBB3, CDK4, IDH2, BRAF, MTOR* and *RET*. *PIK3CA* mutations included E542Q, E545K, H1047Q, R93Q and K337E, all except the last one have annotations in the COSMIC catalogue of somatic mutations in cancer. We found no mutations in *KRAS*, *NRAS*, *HRAS* and *EGFR*.

We had only paired basal and post-progression DNA from 10 patients. The only 2 patients that had no somatic genomic alterations detected in the basal sample remained alterations-free in the post-progression sample. Three patients had only *MET* mutations in the basal samples, and these were also the only mutations detected in the post-progression samples. A

mutation in *BRAF* and *IDH2* appeared in one post-progression sample that was not found in the basal biopsy; and one CNV appeared in *CDK6* in an additional post-progression biopsy.

DISCUSSION

Given the plethora of targeted agents and the heterogeneity of human tumors, one of the areas in oncology of growing importance is the finding of predictive biomarkers to better match patients and drugs. And we will probably need to perform this matching process patient-drug several times along the course of the disease, since almost inevitably tumors develop resistance to targeted therapies in a highly dynamic process. This is one of the fundamental aims of personalized medicine (165, 166).

The focus of this doctoral thesis has been on unravelling mechanisms of acquired resistance to anti-EGFR targeted therapies in human cancers. In its first chapter, preclinical models were generated to study the molecular mechanisms of acquired resistance to anti-EGFR TKIs (gefitinib and erlotinib) and to the monoclonal antibody cetuximab. In the second chapter, mechanisms of resistance were explored in a small cohort of head and neck cancer patients that had initially responded to cetuximab and eventually progressed.

At the time this research work was started, two mechanisms of acquired resistance to anti-EGFR therapies had been described both in preclinical models and in NSCLC patients harboring *EGFR* TKI sensitizing mutations. One of these mechanisms was the development of a secondary mutation in the *EGFR* gene, the T790M mutation, which had been detected in approximately 50% of NSCLCs with acquired

resistance to EGFR-TKIs (167). The other one was the amplification of the *MET* oncogene (168). However, little was known regarding treatment resistance in models dependent on WT *EGFR*.

We chose two model systems to investigate: the A431 cell line (derived from a human squamous cell carcinoma of vulvar origin which harbors WT *EGFR* gene amplification) and the HN11 cell line (derived from a human squamous cell carcinoma of a human oral cavity tumor that also expresses WT *EGFR*). Both cell lines are highly sensitive to EGFR-targeted therapies. Resistant cells were generated by chronically exposing the cells to increasing doses of gefitinib, erlotinib or cetuximab. All resistant cells shared a common phenotype: they activated PI3K signaling (and downstream Akt) not only through EGFR/ErbB-3, but they also adopted the IGF1R pathway in addition to signaling through the HER family of receptors. The gene expression profiles of the resistant cell lines compared to the sensitive parental cells suggested that their increased IGF1R activation was not due to ligand overexpression but to downregulation of the IGFBPs. Concomitant blockade of EGFR and IGF1R signaling was required to reverse the acquired resistance to anti-EGFR therapies.

Together with the findings described in Chapter 1 of this doctoral thesis, there is additional evidence in the literature to support that the IGF1R system may play a role in mediating resistance to anti-EGFR therapies in squamous cell

carcinomas (reviewed next in this discussion), so we were encouraged to continue with this line of research in tumor samples from patients with SCCHN.

Barnes *et al*. showed for the first time that the IGF1R was overexpressed in a panel of human SCCHN cell lines compared to normal human epidermal keratinocytes, and that IGF1R levels measured by Western blot were higher in a sample set of 12 head and neck cancers compared to paired normal mucosa from the same patients. Combined treatment with A12 (an anti-IGF1R blocking antibody) and cetuximab was more effective than either agent alone at reducing cell proliferation and migration in head and neck cancer cells. Xenograft experiments also showed that the combination of A12 plus cetuximab resulted in complete tumor regression in 44% of cases, compared to only 31% of cases with either A12 or cetuximab monotherapy (169).

Jameson *et al.* showed that the sensitivity of five SCCHN cell lines to the EGFR-TKI gefitinib was reduced when the IGF1R was activated. The apoptotic response and reduction in cell number that resulted from gefitinib treatment in the sensitive cell lines was blocked by IGF1R activation. The IGF1R-TKI PQ401 acted synergistically with gefitinib in inhibiting the growth of these cell lines (170).

Iyer *et al.* showed that heterodimerization of EGFR with IGF1R was increased in cetuximab resistant SCCHN cell lines, and this could lead to increased activity of the EGFR (171).

Mechanistically, Wilsbacher *et al.* showed that IGF1R and EGFR kinase inhibitor combinations block proliferation and induce apoptosis through cyclin D1 reduction and Bax activation (172).

Interestingly, the role of IGFBP3 mediating resistance to other anti-HER targeted therapies has also been reported in a breast cancer model of BT474 cells (characterized by HER2 amplification) with acquired resistance to trastuzumab (a monoclonal antibody directed against HER2). Treatment with recombinant human IGFBP3 increased the sensitivity of BT474 trastuzumab resistant cells to trastuzumab in vitro and showed potent single-agent activity in mice bearing BT474 trastuzumab-resistant xenografts (173).

However, the relevance of the IGF system in the biology of SCCHN is complex and still not completely understood. Conflicting results on the carcinogenic and prognostic role of the IGF system have been reported in the literature.

Zhong *et al.* found a positive correlation between IGFBP3 positivity and increased tumor size as well as lymph node metastasis in tumor tissue from patients with oral SCCHN (174). Similarly, Bao *et al.* found that IGFBP3 was significantly elevated in patients with nasopharyngeal carcinoma and its expression level was correlated with nodal invasion, distant metastasis and TNM clinical stage (175). Sun *et al*. explored the prognostic role of both IGF1R and IGFBP3 positivity in 131 patients with SCCHN who had undergone surgical resection, and showed that IGFBP3

positivity was associated with shorter time to progression, whereas the IGF1R itself failed to show prognostic relevance (176).

On the other hand, Dale *et al.* studied 64 cases of SCCHN with matched normal tonsillar epithelium and found that IGF1R was overexpressed in SCCHN compared to normal mucosa and OS and disease-specific survival were reduced in patients whose tumors contained high membranous expression of IGF1R measured by IHC, suggesting a prognostic role for IGF1R expression in SCCHN (177). Papadimitrakopoulou *et al.* studied 34 tongue squamous cell carcinomas and 30 premalignant lesions of the oral cavity and larynx, and found that reduced IGFPB3 expression was associated with significantly shorter disease-specific survival and disease-free survival (178).

In the A431 and HN11 resistant cell lines, single agent treatment with either AEW541 (a small molecule TKI of IGF1R) or MK-0646 (an antibody against the IGF1R) had no effect on cell proliferation. Dual anti-EGFR and IGF1R blockade was necessary to decrease cell numbers. Moreover, the dual blockade was required to delay or prevent the development of acquired resistance. If these findings were to be translated into the clinic, one would hypothesize that single agent treatment using anti-IGF1R drugs may be insufficient to elicit tumor responses, that dual blockade would be necessary to revert acquired resistance, and that dual blockade in patients at “high risk” of developing this “IGF-

dependent anti-EGFR resistant phenotype" could help reach long-term remissions or completely abrogate the development of resistance. In fact, a published single case report suggests that a short course of an anti-IGF1R agent in a patient with a SCCHN tumor that had initially responded to cetuximab and later developed acquired resistance was enough to revert the resistant phenotype and re-sensitize the tumor when rechallenged with cetuximab (179).

Despite the strong evidence on the role of IGF system in the development and maintenance of a malignant phenotype, targeting it in humans using either antibodies against the IGF1R or small molecule IGF1R-TKIs has largely been unsuccessful (reviewed in 180).

A phase II clinical trial of figitumumab (a fully human monoclonal antibody IgG2 subtype targeting the IGF1R) in patients with R/M SCCHN (GORTEC 2008-02) after failure to platinum-based therapy was a negative trial, showing no meaningful activity of the drug as single agent in an unselected patient population (181).

A phase II clinical trial of cixutumumab (another monoclonal antibody targeting the IGF1R) alone or with cetuximab for refractory R/M SCCHN did not result in improvement in PFS compared to historical data of cetuximab monotherapy in the population of platinum-refractory patients. Patients were stratified according to prior cetuximab exposure, but results of these two subpopulations of patients were not reported. Results of this trial have only been released in abstract form

and are not yet published (182). Globally, median PFS was 1.9 months in the cixutumumab monotherapy arm vs. 2.0 months in the cixutumumab plus cetuximab arm. However, there was an increase from 5.9% to 15.3% in the 6-month PFS in the cixutumumab plus cetuximab arm compared to the cixutumumab monotherapy arm, and an increased clinical benefit rate from 19% to 38%. Although not significant, the trend towards and increased percentage of patients achieving longer disease control before resistance development in the combination arm compared to cixutumumab single agent would support the findings in our work. Unfortunately, the clinical development of cixutumumab has been stopped.

A hypothetical important reason for failure is that we have not been able to identify a biomarker of “IGF-dependency” in tumors, so we cannot select the right subgroup of patients with the highest probabilities of benefiting from these therapies. Results from the experiments in Chapter 1 of this doctoral thesis may suggest that downregulation of IGFBP3 may be used as a surrogate marker of IGF1R activation and “IGF-dependency” in cetuximab resistant tumors. This hypothesis has only been explored in a small population of NSCLC patients with EGFR TKI sensitizing mutations. Serum levels of IGFBP3 were measured in 20 patients before and after the development of EGFR-TKI resistance. There were no significant changes in IGFBP3 serum levels before and after TKI treatment, so the authors concluded that the IGFBP3 serum level was not a reliable indicator of resistance

(183). IGFBP3 serum levels have not been studied in cetuximab-refractory SCCHN patients.

In Chapter 2 of this doctoral thesis, mechanisms of resistance to cetuximab treatment were explored in a small cohort of 22 patients with advanced head and neck cancer. We based our studies in the comparison of the tumor samples taken at initial diagnosis (prior to cetuximab therapy) to the biopsies taken after progression to cetuximab therapy.

Aim number one was to try to validate the findings from the preclinical models in the patients' paired samples. Following on the results of Chapter 1, changes in IGFBP3 and P-IGF1R expression were measured using IHC. We found no significant changes in IGFBP3 expression in basal samples compared to post-progression tumors in the complete cohort of patients, nor in the subset of patients who had received cetuximab in the R/M setting. Regarding P-IGF1R, there was a significant decrease in the Histo-score of the samples after progression on cetuximab therapy compared to the initial samples.

These findings are in disagreement with the changes described in the preclinical models of A431 and HN11 cells. Several reasons may account for this discrepancy.

First, the preclinical models were developed treating cells exclusively with anti-EGFR and anti-IGF1R agents, whereas in SCCHN cetuximab is often administered in combination with CT. It is well established that many cytotoxic agents have complex interactions with the IGF-system (184). IGF

stimulation activates prosurvival signaling in cells and causes cells to progress through the cell cycle. These processes are greatly affected when cells are exposed to cytotoxic chemotherapy. As a result, the scenario of basal activation of the IGF1R signaling system may be different in tumors that have received chemotherapy compared to untreated tumors. In our cohort, more than 50% of patients had received chemotherapy concomitant with cetuximab.

Second, IGFBP3 levels are known to change in response to physiological stressful conditions (185). We did not control for stressful events in the analyzed patient population. However, in all likelihood some of them could have been submitted to stressful circumstances such as recent major surgery for treatment of their tumors, or infections, just to name a few.

Third, we only analyzed changes in IGFBP3 levels, thus we may have missed the potential role of other IGFBPs, such as IGFBP4. We had decided on analyzing only IGFBP3 since there was a complete lack of data in the literature regarding IGFBP4 in SCCHN.

Fourth, the sample size analyzed was small and very heterogeneous (i.e. only 17 patients had paired tumor samples, biopsies after progression to cetuximab had been obtained in an interval from 4 days to 9 months after the last dose of cetuximab, some were surgical specimens and others were small diagnostic biopsies).

Lastly, resistance to anti-EGFR targeted therapies in SCCHN may arise through multiple different mechanisms, not only

through the IGF-system. In fact, many other mechanisms of resistance have been described in preclinical models of SCCHN (reviewed in 186-190) and will next be briefly discussed.

Cross-talk with several other RTK signaling pathways beyond the IGF and HER network have been implicated as mediators of resistance to EGFR-targeted agents, including the VEGFR pathway and angiogenesis (191), the Notch pathway (192, 193) and the HGF/c-MET pathway (127, 194-196). The cross-talk of the EGFR with other growth factor receptors is represented in Figure 1.

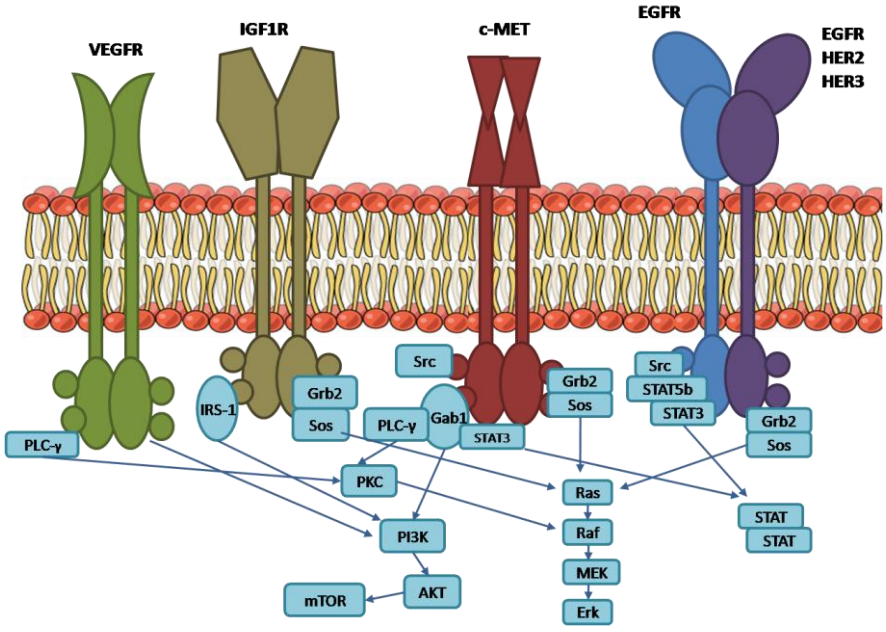

Figure 1. Resistance mechanisms originating from cross-talk among growth factor receptors.

Growth factor receptors such as the VEGF, the IGF1R and c-MET provide compensatory activation of cell survival and proliferation pathways when EGFR is inhibited. VEGFR: vascular endothelial growth factor receptor, IGF1R: insulin-like growth factor receptor 1, EGFR: epithelial growth factor receptor, IRS: insulin receptor substrate, PI3K: phosphatidylinositol

3'-kinase, mTOR: mammalian target of rapamycin, STAT: single transducer and activator of transcription (Adapted from Ref. 197).

Mutations in the Notch pathway have been documented in 10-20% of SCCHN. MET is overexpressed in roughly 80% of SCCHN, amplified in 13% of tumors, and mutations have also been described. Both clinical and experimental data suggest that MET expression predicts resistance to radiation, cisplatin and cetuximab.

Other mechanisms of resistance in SCCHN can be mediated through alterations on the EGFR itself or some downstream effectors of its signaling pathway, or through overexpression of its ligands.

Despite abundant evidence from preclinical experiments, only a few studies have reported results coming from patients with SCCHN receiving cetuximab. Biomarker analysis of the phase III EXTREME trial of cetuximab plus first-line platinum/5FU CT for advanced SCCHN found that improvements in survival and efficacy outcomes were not influenced by tumor EGFR expression levels or *EGFR* gene copy number, so these have no utility as predictive biomarkers (198, 199). The detection of mutant EGFRvIII and high levels of amphiregulin in tumors have been linked to resistance to the combination of docetaxel plus cetuximab in a small phase II clinical trial with 47 patients (200). A recent paper has reported that acquired mutations in *HRAS* can be detected in circulating ctDNA of a subset of patients with advanced SCCHN with acquired resistance to cetuximab,

suggesting its role as a potential biomarker of resistance (201).

Additional mechanisms of resistance may be mediated by alterations in other members of the HER family of receptors, mainly HER2 and HER3, although once again, evidence coming from patients is scarce and their value as predictive biomarkers for cetuximab therapy has not been established.

The final work in this thesis was to further explore mechanisms of resistance in our cohort of SCCHN samples, by testing for mutations and CNV gains in 52 cancer related genes using a commercially available NGS assay (202). We found high abundance of alterations in genes previously known to be involved in SCCHN such as mutations in *PIK3CA* and *MET*, as well as CNV gains in *CCND1* (121). Overall, 40% of samples had 2 or more genetic abnormalities. We also found lower frequency of mutations in additional genes such as *BIRC2, FGFR3, RET, KIT, ERBB2, ERBB3, CDK4, IDH2, BRAF, MTOR* and *RET*; and CNV gains in *EGFR, MYC, KRAS, FGFR1* and *CDK6*.

In the majority of patients from whom we had paired DNA from the initial and post-progression biopsies (8 out of 10 patients) we saw no change in the profile of genomic alterations. Interestingly, however, we had two patients in whom we found new mutations and CNV in the post-progression samples.

The first case had a CNV in *CDK6*, a key regulator gene during the G1/S cell cycle transition. Aberrant expression of

CDK6 protein has been observed in many cancer types. In SCCHN high cytoplasmic and nuclear expression of CDK6 has been found to be significantly correlated with higher T classification and more advanced tumor status (203). However, whether or not CDK6 overexpression can play a role in acquired resistance to anti-EGFR therapies remains unknown.

The second case had 2 new mutations in the post-progression biopsy, in *BRAF* and *IDH2* genes. *BRAF* mutations, mainly the *V600E* mutant, are most probably linked to resistance to anti-EGFR targeted antibodies in mCRC (204, 205). Our patient had a *G606R* mutation, which has only been reported 3 times in the COSMIC database (one lung, one colon and one sebaceous gland skin tumor), so the potential causality of this mutation on cetuximab resistance will need further characterization. The second mutation was *R172M* in the *IDH2* gene. IDH1 and IDH2 are metabolic enzymes catalyzing the conversion of isocitrate to α-ketoglutarate. Multiple preclinical models have provided evidence for the oncogenic potential of *IDH1/IDH2* mutations, which alter epigenetic regulation, cancer cell differentiation and metabolism (Reviewed in 206). Point mutations in *IDH1/2* define distinct subsets of glioblastomas and low-grade gliomas, chondrosarcomas, intrahepatic cholangiocarcinomas and hematologic malignancies. In head and neck tumors, they have only been described in undifferentiated sinonasal carcinomas, a rare entity with a very aggressive behavior

(207). Whether or not this mutation is linked to response to anti-EGFR therapies remains to be determined.

Interestingly, we found no acquired mutations in *KRAS* and *HRAS*, or in the *EGFR* gene itself (neither in the extracellular domain nor in the intracellular TK domain), suggesting that the mechanisms of resistance to anti-EGFR therapies in SCCHN are distinct to what is frequently observed in SCLC or mCRC.

In summary, in this doctoral thesis we have walked from the bed to the benchside and back as a strategy to try to improve personalized medicine. We developed preclinical models to study acquired resistance to anti-EGFR therapies in carcinoma cells WT for EGFR and identified potential mechanisms of resistance. We then tried to validate those findings in the clinic, in tumors of advanced head and neck cancer patients; and finally, we have established potential areas of interest to guide future research. In this journey, we highlight the importance of obtaining biopsies at the time of cancer recurrence or progression to targeted agents. By identifying how a patient's cancer becomes refractory to a targeted agent, we will be well positioned to devise rational treatment strategies to improve cancer control and re-induce remissions. Eventually, the goal may shift to determine if therapies that block the resistance mechanisms can be used earlier in the natural history of tumors to prevent or delay cancer recurrences or progressions.

CONCLUSIONS

1. Squamous carcinoma cells acquire resistance to anti-EGFR targeted therapies (erlotinib, gefitinib and cetuximab) through a cross-talk with the IGF-system.

2. Resistant cells activate the IGF1R through downregulation of the expression levels of IGFBPs.

3. Concomitant inhibition of signaling through the EGFR and the IGF1R is necessary to overcome resistance to anti-EGFR targeted therapies.

4. Combined treatment with anti-EGFR and anti-IGF1R inhibitors can prevent or delay the development of acquired resistance to anti-EGFR targeted therapies.

5. The activation of IGF1R through decreased expression of IGFBP3 has not been linked to the acquired resistance to cetuximab in our exploratory analysis of a small cohort of head and neck cancer patients.

6. New genomic alterations (mutations in *IDH2* and *BRAF*, and a CNV in *CDK6*) have been detected in a subset of advanced head and neck cancer patients after progression to cetuximab-based treatments.

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