Refining EGFR-MONOCLONAL ANTIBODY TREATMENT *in colorectal cancer*

Lisanne Krens

The research presented at this thesis was performed at the department of Clinical Pharmacy and Toxicology and Clinical Oncology of Leiden University Medical Center, Leiden, The Netherlands.

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Chapter 1

General introduction

Colorectal cancer and KRAS

Colorectal cancer (CRC) is the second most common tumor type worldwide and accounts for more than 5,000 cancer deaths each year in the Netherlands (www.cijfersoverkanker.nl). The *KRAS* gene has a key role in carcinogenesis, signal transduction and proliferation. Mutations in the *KRAS* gene are found in 40 percent of the CRC tumors. The most frequent mutations in *KRAS* are guanine to adenine transitions and guanine to thymine transversions with 90% of the somatic point mutations occurring in hotspot codon 12 (70%) or 13 (30%) in exon 1. Other, less frequent, mutations are found in codon 61, 62 and 146[1].

KRAS protein prenylation

The activating *KRAS* mutation results in uncontrolled cell growth. To be active, the KRAS protein requires posttranslational prenylation, by binding to a farnesyl- (C-15) or geranylgeranylgroup (C-17). After prenylation KRAS becomes more hydrophobic and associates with the plasma membrane. Membrane association is crucial for the function of the KRAS protein in the RAS-RAF-MAPK pathway. Inactivated KRAS is bound to GDP; activation occurs by the conversion of GDP to GTP by guanine exchange factors. The ratio of GDP and GTP is controlled by guanine exchange factors and GTPase-activating proteins (GAPs). Active KRAS is hydrolyzed by GAPs to return to an inactive state [2].

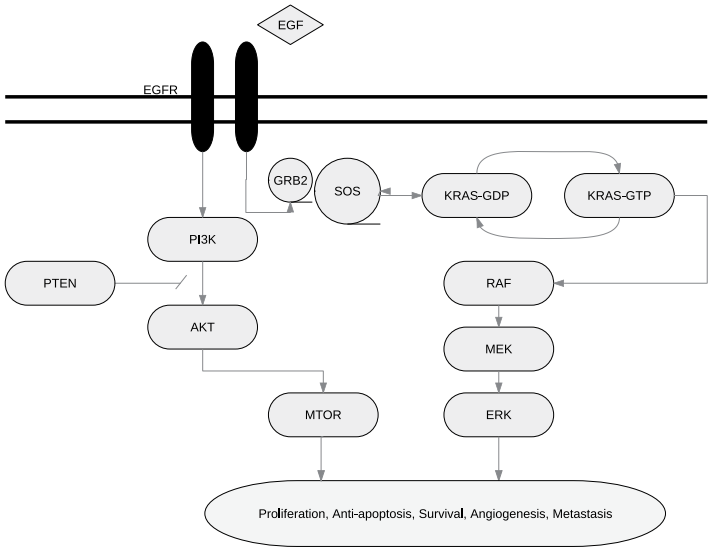

Figure 1: Overview of EGFR-dependent intracellular signaling. Abbreviations: AKT, protein kinase B; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ERK, extracellular signal-related kinase; GRB2, growth factor bound protein 2; KRAS-GDP, KRAS bound to guanine diphosphate; KRAS-GTP, KRAS bound to guanine triphosphate; MEK, mitogen-activated protein kinase; MTOR, mammalian target of rapamycin; PI3K, phosphatidyl-inositide-3-kinase; PTEN, phosphatase and tensin homolog; RAF, V-raf murine sarcoma viral oncogene homolog; SOS, son of sevenless.

EGFR antibodies cetuximab and panitumumab

Binding of a ligand to the Epidermal Growth Factor Receptor (EGFR) activates important downstream processes such as the RAS-RAF-MAPK and the PI3 kinase pathway (figure 1). The EGFR is an important target in the treatment of CRC. Blockage of the EGFR leads to inhibition of cancer cell growth. The two registered EGFR antibodies, cetuximab and panitumumab are both indicated for the treatment of metastatic CRC in *RAS* wild type patients only. In *KRAS* mutant patients, KRAS is permanently activated, leading to constant cell signaling and proliferation independent of the EGFR [3].

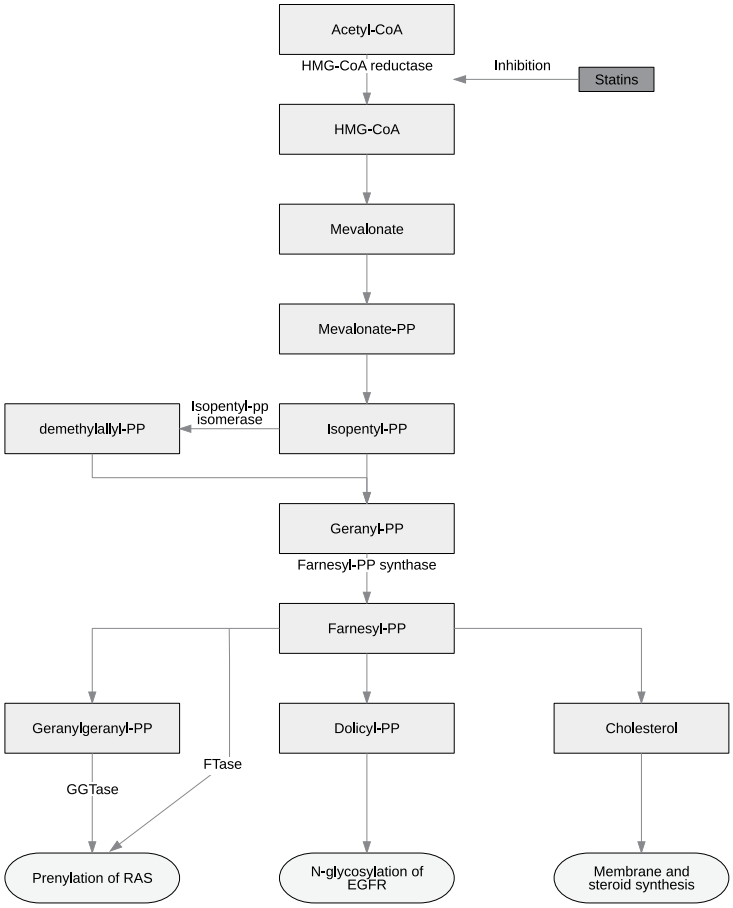

Figure 2: Overview of the mevalonate pathway and the inhibition of HMG-CoA by statins. Abbreviations: Acetyl-CoA (acetyl coenzyme A); EGFR, epidermal growth factor receptor; FTase, farnesyltransferase; GGTase, geranylgeranyltransferase; HMG-CoA (3-hydroxy-3-methylglutaryl-coenzyme A) (reductase); -PP, -pyrophosphate.

Statins and colorectal cancer

Statins inhibit cholesterol synthesis via inhibition of HMG-CoA-reductase in the mevalonate pathway and also prevent protein prenylation (figure 2). We hypothesize that statins may be useful for modulating *KRAS* mutant tumors. It is well known that statins can be used to lower cholesterol and have shown to reduce the number of cardiovascular events and mortality in patients with cardiovascular risks [4]. In addition, the use of statins has been associated with a reduced risk in a variety of malignancies such as colon, rectum, lung and liver cancer [5]. To date, several studies and meta-analysis have investigated statin use and the risk of developing CRC and outcomes but with inconclusive findings[6-8]. Fewer studies focus on effects of statin after diagnosis during treatment [9-14].

Phenoconversion of KRAS to overcome EGFR antibody resistance

Statins may inhibit the expression of the mutant KRAS phenotype by preventing the prenylation of the KRAS protein and as a consequence preventing plasma membrane association and so inhibiting the overactivated KRAS protein. We theorised that the inhibitory effect of statins may normalise the phenotype into a more KRAS wild type phenotype and render *KRAS* mutant colorectal cancers sensitive to EGFR antibodies(5;6).

FCGR3A and cetuximab

An important mechanism of action for some monoclonal antibodies, including cetuximab is antibody-dependent cellular cytotoxicity (ADCC). Monoclonal antibodies are generally molecules of the IgG class and have an antigen-binding fragment (Fab). Fc gamma receptors (FCGR) on effector cells, for example macrophages and natural killer cells, bind to the Fab fragment and this causes lysis of the cancer cell. Germline polymorphisms in the genes encoding the Fc gamma receptor 2A (FCGR2A) c.535A>G, resulting in a change of histidine to arginine at codon 131 and 3A (FCGR3A) c.818A>C resulting in a change of phenylalanine to valine at position 158 have been associated with decreased therapeutic activity of cetuximab[15].

Use of EGFR antibodies in patients with hepatic or renal impairment

Panitumumab and cetuximab are both used for the treatment of metastatic CRC, and a part of the patients will present with liver metastasis and subsequent hepatic impairment. On the other hand, some patients are heavily pre-treated with chemotherapy and radiotherapy and may have decreased renal function. This is especially the case in patients with head and neck cancer where cetuximab is being used in patients that cannot be treated with cisplatin, e.g. due to renal impairment. Knowledge on the dosing in these specials populations is highly relevant; nonetheless the pharmacokinetics and safety of both cetuximab and panitumumab are, to date, not studied in these populations.

Aims and outline of this thesis

The general aims of this thesis with the common denominator 'optimization of EGFR targeted monoclonal antibody therapy in cancer' are to study:

1. *The phenoconversion effects of statins on KRAS mutant colorectal cancer both in vitro and in CRC patient populations and their ability to render KRAS mutant colorectal cancer cells sensitive for the EGFR monoclonal antibodies cetuximab or panitumumab*

2. *The effect of the germline polymorphisms in FCGR3A on cetuximab efficacy*

3. *The pharmacokinetics of the EGFR antibodies cetuximab and panitumumab in patients with renal or hepatic impairment*

In **chapter 2** a systematic review is presented on clinical and preclinical studies with compounds, which interfere with the mevalonate pathway and the prenylation of KRAS, published before April 2009. The novel concept of modulation of the KRAS protein by altering the phenotype and the consequent sensitising for EGFR antibodies is discussed.

In **chapter 3** the effects of the combined treatment with simvastatin and cetuximab are studied *in vitro* in different *KRAS* mutant and wild type cell lines. The sulforhodamine assay is used to study the effects of treatment on survival and proliferation. Upregulated and downregulated tyrosine and serine/threonine kinases and corresponding pathways influenced by concomitant treatment with simvastatin and cetuximab in *KRAS* mutant and *KRAS* wild type cell lines are explored. The aim of this study is to explore the responsible pathways which are affected by simvastatin and cetuximab treatment.

Our hypothesis is that *KRAS* mutant cetuximab treated patients with concurrent statin use may have a favourable outcome from EGFR therapy compared to non-users. **Chapter 4** describes a retrospective evaluation of the effects of statin use in the CAIRO2 study cohort in *KRAS* mutant metastatic CRC patients treated with cetuximab. The primary objective in this study is to determine whether statin use during chemotherapy with CAPOX-bevacizumab and cetuximab is associated with improved progression free survival as compared to non-(statin) users.

In the RASTAT-C and RASTAT-P studies, described in **chapter 5 and 6**, treatment with 80 mg simvastatin daily combined with panitumumab two-weekly or cetuximab weekly is studied in a Simon two stage design single arm clinical trials in patients with *KRAS* mutant CRC. The primary objective is to investigate whether the percentage of patients free from progression and alive 12.5 weeks after the first administration of cetuximab is similar to the results of the *KRAS* wild type population of phase III studies treated with cetuximab or panitumumab.

In the cetuximab arm of the CAIRO2 study the *FCGR3A* 818C (VF plus VV) allele was associated with decreased PFS in the entire group of KRAS mutant and wild type patients. The predictive role of this polymorphism may be independent of *KRAS* status. In **chapter 7** these findings of *FCGR3A* status (in relation to KRAS status) on the progression free survival and overall survival in three cohorts of metastatic colorectal cancer patients treated are combined. In this meta-analysis individual patient data are pooled.

Many metastatic CRC patients will present with liver metastases and some with liver dysfunction. The pharmacokinetics of panitumumab in patients with hepatic impairment has not been investigated, and dosage adjustments are undetermined. **Chapter 8** describes a case

of a patient with progressive metastatic CRC and liver dysfunction treated with panitumumab. Pharmacokinetic data and toxicity of this patient are compared to historical data from a population with adequate liver functions.

In the literature the effect of renal impairment on the pharmacokinetics of anticancer drugs are scarce. **Chapter 9** reports a 68 year old metastatic osteosarcoma patient with impaired renal function due to prior chemotherapy, who was treated on compassionate use basis with 400 mg/m^2 cetuximab. Pharmacokinetic parameters are compared to pharmacokinetic data from a study population with normal kidney function.

This thesis ends with concluding remarks and future perspectives in **chapter 10** and a summary of the results in **chapter 11**.

References

1 Brink M, de Goeij AF, Weijenberg MP, Roemen GM, Lentjes MH, et al. (2003) KRAS oncogene mutations in sporadic colorectal cancer in The Netherlands Cohort Study. Carcinogenesis 24: 703-710.

2 Fang JY, Richardson BC. (2005) The MAPK signaling pathways and colorectal cancer. Lancet Oncol 6: 322-327.

3 Allegra CJ, Jessup JM, Somerfield MR, Hamilton SR, Hammond EH, et al. (2009) American Society of Clinical Oncology provisional clinical opinion: testing for KRAS gene mutations in patients with metastatic colorectal carcinoma to predict response to anti-epidermal growth factor receptor monoclonal antibody therapy. J Clin Oncol 27: 2091-2096.

4 Eisenberg DA. (1998) Cholesterol lowering in the management of coronary artery disease: the clinical implications of recent trials. Am J Med 104: 2S-5S.

5 Nielsen SF, Nordestgaard BG, Bojesen SE. (2012) Statin use and reduced cancer-related mortality. N Engl J Med 367: 1792-1802.

6 Liu Y, Tang W, Wang J, Xie L, Li T, et al. (2013) Association between statin use and colorectal cancer risk: a meta-analysis of 42 studies. Cancer Causes Control .

7 Graaf MR, Beiderbeck AB, Egberts AC, Richel DJ, Guchelaar HJ. (2004) The risk of cancer in users of statins. J Clin Oncol 22: 2388-2394.

8 Lochhead P, Chan AT. (2013) Statins and colorectal cancer. Clin Gastroenterol Hepatol 11: 109-118.

9 Mace AG, Gantt GA, Skacel M, Pai R, Hammel JP, et al. (2013) Statin therapy is associated with improved pathologic response to neoadjuvant chemoradiation in rectal cancer. Dis Colon Rectum 56: 1217-1227.

10 Ng K, Ogino S, Meyerhardt JA, Chan JA, Chan AT, et al. (2011) Relationship between statin use and colon cancer recurrence and survival: results from CALGB 89803. J Natl Cancer Inst 103: 1540-1551.

11 Lee J, Jung KH, Park YS, Ahn JB, Shin SJ, et al. (2009) Simvastatin plus irinotecan, 5-fluorouracil, and leucovorin (FOLFIRI) as first-line chemotherapy in metastatic colorectal patients: a multicenter phase II study. Cancer Chemother Pharmacol 64: 657-663.

12 Katz MS, Minsky BD, Saltz LB, Riedel E, Chessin DB, et al. (2005) Association of statin use with a pathologic complete response to neoadjuvant chemoradiation for rectal cancer. Int J Radiat Oncol Biol Phys 62: 1363-1370.

13 Theodoropoulos G, Wise WE, Padmanabhan A, Kerner BA, Taylor CW, et al. (2002) T-level downstaging and complete pathologic response after preoperative chemoradiation for advanced rectal cancer result in decreased recurrence and improved disease-free survival. Dis Colon Rectum 45: 895-903.

14 Siddiqui AA, Nazario H, Mahgoub A, Patel M, Cipher D, et al. (2009) For patients with colorectal cancer, the long-term use of statins is associated with better clinical outcomes. Dig Dis Sci 54: 1307-1311.

15 Mellor JD, Brown MP, Irving HR, Zalcberg JR, Dobrovic A. (2013) A critical review of the role of Fc gamma receptor polymorphisms in the response to monoclonal antibodies in cancer. J Hematol Oncol 6: 1-10.

Part 1

Colorectal cancer, KRAS, FCGR3A and statins

Chapter 2

L.L. Krens, J.M. Baas, H. Gelderblom and H.J. Guchelaar

Drug Discov Today. 2010 Jul;15(13-14):502-16

Therapeutic modulation of KRAS signaling in colorectal cancer

Abstract

KRAS has an important role in colorectal carcinogenesis and mutant *KRAS* leads to a permanently activated KRAS protein. To exert its biological activity, KRAS requires post-translational modification by prenylation.

KRAS modulation has become a promising concept for new therapies, mostly by interference with the mevalonate pathway and subsequently by the prenylation of KRAS. Clinical data of agents interfering with the mevalonate pathway and the prenylation of RAS are summarized and suggest that these agents might be effective when administered in combination with anticancer drugs that target KRAS. Here, we discuss the novel concept that modulation of KRAS might potentiate EGFR therapy by altering the KRAS phenotype.

Introduction

Colorectal cancer (CRC) is the second most common tumor type in the USA and accounts for 49,920 cancer deaths each year. It is, therefore, the second most common cause of cancer-related mortality in the USA, causing nearly 9% of all cancer-related deaths [1].

If diagnosed early, colorectal tumors can be cured by radical resection. Unfortunately, many patients are diagnosed with (distant) metastasis either during follow-up or at first presentation. A small subset of patients with metastasis confined to a single organ (mostly the liver) can be cured by resection. For the majority of patients with metastasized disease, however, the only treatment option is palliative systemic treatment. In the past decade, new chemotherapeutic agents for CRC have become available, such as irinotecan and oxaliplatin. For advanced or metastasized CRC patients failing 5-FU (or capecitabine or UFT (ftorafur plus uracil)), oxaliplatin and irinotecan, therapy with a monoclonal antibody against the epidermal growth factor receptor (EGFR) is advised, but only in patients with tumors not harboring an activating mutation in the *KRAS* gene. *RAS* has a key role in carcinogenesis, signal transduction and proliferation in colorectal carcinoma. Mutations in *RAS* are found in 30% of all cancers and are a potential target for therapy. This review focuses on the role of KRAS and the novel concept of modulating KRAS with statins, farnesyltransferase inhibitors, geranylgeranyltransferase inhibitors and bisphosponates in human colorectal carcinomas.

Search strategy

A systematic literature search in PubMed was conducted on 3 April 2009 using the following keywords and combinations: *KRAS*, (colorectal) carcinoma, farnesyltransferase inhibitors, geranylgeranyltransferase inhibitor, bisphosphonates, statins, EGFR inhibitors, cetuximab and panitumumab. Results were assessed by reviewing titles and abstracts, and relevant articles were retrieved. Cited references in these articles were used to find further relevant articles.

RAS proto-oncogenes

The *RAS* gene family consists of proto-oncogenes, which control cell growth in mammalian cells. Three different kinds of *RAS* oncogenes are known: *Kirsten RAS (KRAS), Harvey RAS (HRAS)* and *Neuroblastoma RAS (NRAS)*; these members of the *RAS* gene family are closely related and function in a similar way [2]. The *KRAS* gene encodes for a 21 kDa membrane-bound guanosine triphosphate (GTP)/guanosine diphosphate (GDP)-binding G protein. The KRAS protein serves as a switch between the EGFR and the nucleus, controlling downstream processes. To be active, hydrophilic KRAS requires post-translational modification by prenylation. Ras terminates in a CAAX sequence: a cysteine (C), two aliphatic amino acids (A) and any amino acid (X). The CAAX sequence is subject to post-translational farnesylation or geranylgeranylation. A 15-carbon chain from farnesylpyrophosphate (FPP) is added to the cysteine residue close to the carboxyl terminus, and this process is catalyzed by the enzyme farnesyl protein transferase (FTase). When FTase is inhibited, KRAS will be geranylgeranylated, thereby a 20-carbon chain of geranylgeranylpyrophosphate (GGPP) is added to ras catalyzed by geranylgeranyltransferase (GGTase) [3,4]. After isoprenylation of ras, the endopeptidase RCE1 protease removes the

AAX amino acids at the end of the carboxyl terminus. The new terminus is methylated by isoprenylcysteine carboxyl methyltranferase (ICMT) before RAS is transported to the cellular membrane. In NRAS and KRAS, the SH-group of cysteine residue is palmitoylated before transport to the membrane. As a consequence of post-translational modifications, KRAS becomes more hydrophobic and translocates from the cytosol to attach to the cell membrane by its farnesylgroup or geranylgeranylgroup [5–7] (figure 1). Membrane association of KRAS is crucial for its function in signaling and transforming activities.

Both FPP and GGPP are isoprenoids formed during the mevalonate pathway. FPP is a precursor for cholesterol, heme A, dolichols and ubiquinones, and GGPP can be formed out of FPP [8]. Inactivated KRAS is bound to GDP; activation occurs by the conversion of GDP to GTP by guanine exchange factors. In normal cells, the ratio of GDP and GTP is controlled by guanine exchange factors and GTPase-activating proteins (GAPs). Active KRAS is hydrolyzed by GAPs to return to an inactive state [9].

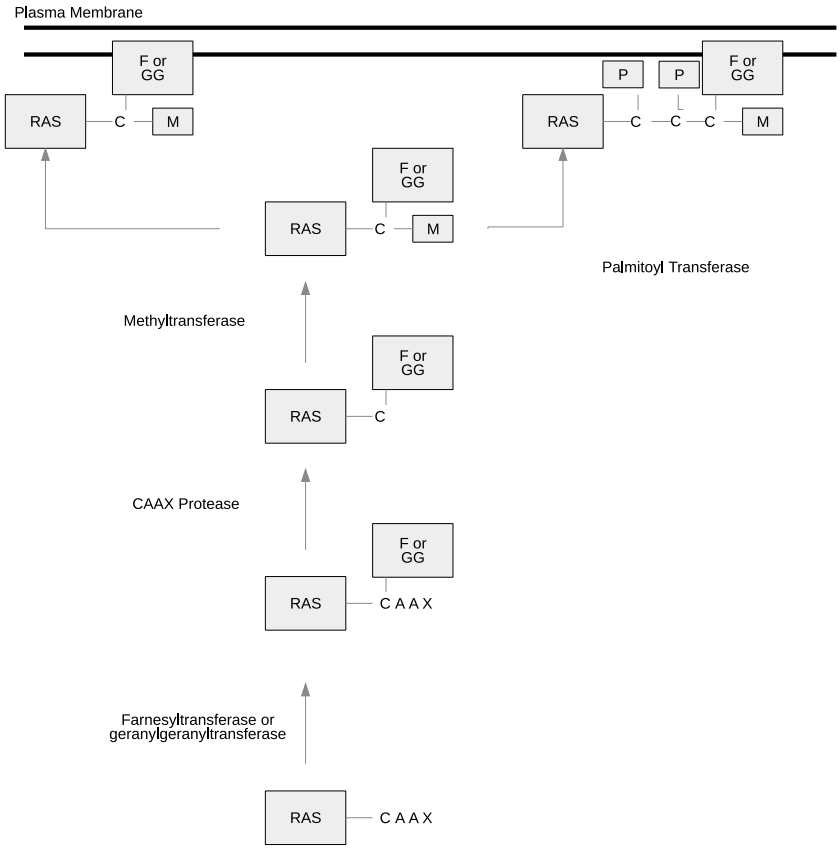

Figure 1: Post-translational modification of RAS. Abbreviations: F, farnesyl pyrophosphate; GG, geranylgeranylpyrophosphate; M, methylgroup; P, palmitoylgroup.

KRAS signaling

KRAS is situated in the inner cell membrane. Binding of a ligand to the EGFR activates a downstream process to the nucleus. This process activates major pathways in the cell: the RAS–RAF–mitogen-activated protein kinase (MAPK) and the PI3 kinase pathway (figure 2). KRAS has a key role in the RAS–RAF–MAPK pathway. Son of sevenless (SOS) is conformationally modificated by interaction with growth factor receptor bound protein 2. Activated SOS induces the KRAS pathway [10]. In RAS-RAF-MAPK signaling, KRAS activates serine–threonine kinase raf 1, which phosphorylates two MAPK kinases. These in turn phosphorylate other MAPKs. MAPKs translocate to the nucleus and activate transcription factors involved in proliferation [8,11]. Signaling via the PI3 kinase pathway activates AKT and thereby phosphoproteins, for example, p-GSK3 and p-AKT [12]. The tumor suppressor gene PTEN inhibits the PI3 kinase pathway.

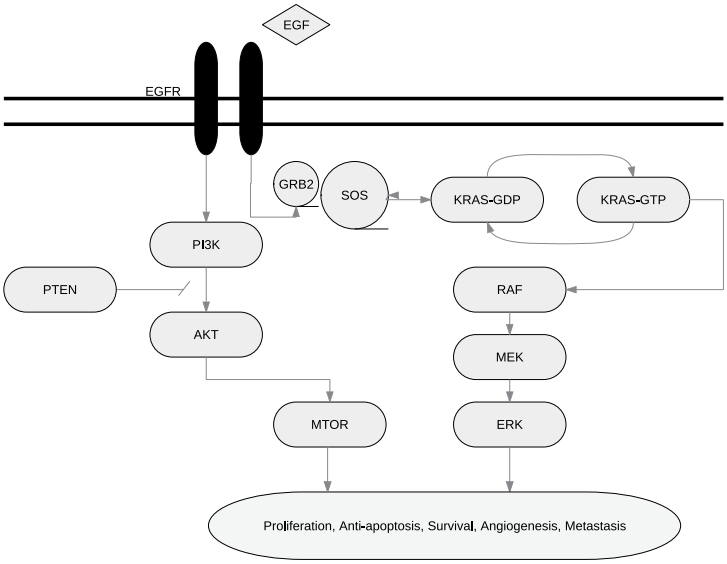

Figure 2: Overview of EGFR-dependent intracellular signaling. Abbreviations: AKT, protein kinase B; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ERK, extracellular signal-related kinase; GRB2, growth factor bound protein 2; KRAS-GDP, KRAS bound to guanine diphosphate; KRAS-GTP, KRAS bound to guanine triphosphate; MEK, mitogen-activated protein kinase; MTOR, mammalian target of rapamycin; PI3K, phosphatidyl-inositide-3-kinase; PTEN, phosphatase and tensin homolog; RAF, V-raf murine sarcoma viral oncogene homolog; SOS, son of sevenless.

KRAS mutations in cancer

KRAS mutations have an important role in tumorigenesis. In CRC, *KRAS* somatic mutations are thought to be involved in the transition of adenoma into carcinoma, contributing to tumor growth and atypia [13,14]. Mutant *RAS* is present in approximately 30% of all human cancers. *KRAS* mutational rate is high in some tumors; however, it is low in others (Table 1). Approximately 40% of CRCs have mutations in *KRAS*.

Table 1: Mutations of *NRAS, KRAS* and HRAS in different tumor types [32,93].

Tumor type	*RAS*	Frequency (%)
Colorectal carcinoma	*KRAS*	50
Lung adenocarcinoma (NSCLC)	*KRAS*	30
Pancreatic carcinoma	*KRAS*	90
Melanoma	*NRAS*	20
Thyroid carcinoma	*KRAS*, *NRAS*, *HRAS*	50
Myeloid disorders	*NRAS* (less frequently *KRAS*, *HRAS*)	30

Abbreviations: KRAS, Kirsten RAS gene; NSCLC, non-small cell lung carcinoma; NRAS, neuroblastoma RAS gene; HRAS, Harvey RAS.

Mutations are found in primary tumors and matched metastases. Most mutations are found in the primary tumor, indicating a role in early tumorigenesis. Mutations are occasionally found only in metastases; however, thus indicating such mutations can also occur during a later stage of disease [15].

The most frequent mutations in *KRAS* are guanine to adenine transitions and guanine to thymine transversions [16] with 90% of the somatic point mutations occurring in hotspot codon 12 (70%) or 13 (30%) in exon 1. Other, less frequent, mutations are known in codon 61, 62 and 146. The most frequent mutations in codon 12 and 13 are listed in Table 2. 6.6% of the somatic mutations are found outside codon 12 or 13 in codons 8, 9, 10, 15, 16, 19, 20 or 25[16]. A recent study showed mutations in codon 59, 61, 117 and 163 [17]. During tumor progression, more *KRAS* codon 12 mutations and fewer codon 13 mutations are found. In normal tissue, however, there is a balanced codons 12 and 13 mutation ratio [18].

Table 2: Common transitions and transversions in KRAS codon 12 and 13

Codon 12 mutations		
GGT (glycine) → AGT (serine)	G–A transition	G12S
GGT (glycine) → GAT (aspartate)	G–A transition	G12D
GGT (glycine) → TGT (cysteine)	G–T transversion	G12C
GGT (glycine) → GTT (valine)	G–T transversion	G12V
GGT (glycine) → CGT (arginine)	G–C transversion	G12R
GGT (glycine) → GCT (alanine)	G–C transversion	G12A
Codon 13 mutations		
GGC (glycine) → GAC (aspartate)	G–A transition	G13D
GGC (glycine) → TGC (cysteine)	G–T transversion	G13C
GGC (glycine) → GTC (valine)	G–T transversion	G13V
GGC (glycine) → CGC (arginine)	G–C transversion	G13R
GGC (glycine) → GCC (alanine)	G–C transversion	G13A
GGC (glycine) → AGC (serine)	G–A transition	G13S

Abbreviations: A, adenine; C, cytosine; G, guanine; T, thymine.

Different mutations in codon 12 or 13 have various effects on disease progression [19]. Guanine to adenine point mutations are associated with methylguanine methyltransferase epigenetic silencing [20]. Mutations leading to a 12-glycine residue (without a side chain) toward a residue with a side chain interfere with the geometry of KRAS and the ability of GTP to be hydrolyzed to return to an inactive state. These mutations cause impaired GTPase activity: KRAS binds GAP, but there is no activation of the GAP because of steric hindrance [21], and they permit a permanently active state causing growth and proliferation [22,23]. Consequently, mutant KRAS operates independently of activation of the EGFR and causes downstream processes [24].

No clear conclusions can be drawn from the studies regarding the influence of *KRAS* on the progression of colon cancer and, thus, the prognostic impact of *KRAS* mutation in colorectal carcinoma is unclear. Several studies link *KRAS* to worse prognosis, whereas others do not implicate a prognostic role for *KRAS* [25–31]. The RASCAL study was initiated to determine whether the presence of KRAS mutations in CRC patients is associated with poor prognosis. Initial results of this study suggested that *KRAS* mutational status is indeed associated with poorer disease-free survival and overall survival. The RASCAL II study, however, reported that only one specific mutation reduces disease-free and overall survival statistically significant and that KRAS mutational status in general is not a prognostic marker. Nevertheless, mutational status of *KRAS* is of great clinical relevance in CRC patients in predicting response to EGFR-inhibitor-based therapy. The RASCAL II study showed that only glycine to valine transversion on codon 12 had a statistically significant influence on interval between operation and relapse or death from any cause and on overall survival [19,32]. Post hoc analyses of two trials evaluating the EGFR inhibitors panitumumab and cetuximab in CRC showed lack of response to these agents in *KRAS* mutant patients [33,34]. Nowadays, EGFR inhibitor therapy in CRC is indicated only in patients free of mutations in codons 12 and 13 of the *KRAS* gene.

Testing for *KRAS* gene mutations

Currently, testing for *KRAS* mutations is not standardized. For the identification of *KRAS* mutations, different methods are being used; however, data about the accuracy of different tests are limited [12]. *KRAS* testing currently focuses on codon 12 or 13 mutations. Seven mutations in these codons contribute to more than 95% of all *KRAS* mutations. In real-time polymerase chain reactions, probes for the most common mutations in codons 12, 13 and sometimes 61 are applied. In direct sequencing analysis, all possible mutations of *KRAS* can be identified [35]. Many methods of *KRAS* testing are laboratory-based methods. The following methods are used for *KRAS* testing: gel electrophoresis assays, sequencing, allele-specific PCR assays and allele-discrimination-based allele-specific ligation detection reaction.

Allele discrimination is based on discrimination amplification efficiencies at low melting temperatures. Some assays are commercially available [36,37]. Juan et al. [38] compared testing methods (Histogenex, Genzyme, Invitek and Gentrix) from four independent commercial laboratories with their internal direct sequencing, and all but one (Invitek) were comparable with the internal direct sequencing method.

Tol et al. [36] compared two commonly used *KRAS* mutation tests, real time PCR and sequencing in DNA extracted from CRC samples. Both sequencing and real-time PCR are reliable *KRAS* testing assays with a sensitivity of 95.5% (95% confidence interval 91.7–97.9%) and 96.5% (95% confidence interval 93.0–98.6%), respectively.

A difficulty in *KRAS* testing occurs when a low volume of tumor material is available, for example because of pre-treatment with radiotherapy. In samples with less than 30% tumor cells, a *KRAS* mutation can be missed by sequencing. Obviously, high-quality *KRAS* testing is necessary because the *KRAS* status of a patient is used to determine clinical opportunities. The European Society of Pathology has started a Quality Assessment program for *KRAS* testing because of the lack of procedures and standardization (http://esp-pathology.org).

KRAS and pathogenetic pathways in CRC

In the progression toward CRC, pathological genetic changes occur. This review focuses on *KRAS*; however, other genetic changes have an important role and interplay in colorectal carcinogenesis. Early genetic abnormalities arise in adenomatous polyposis coli, *KRAS* and *BRAF* (v-raf murine sarcoma viral oncogene homolog B1). Mismatch repair gene mutation and *MLH1* mutation contribute to microsatellite instability. These pathological genetic changes lead to dysplastic crypt and (early) adenoma formation.

Further positive selection occurs for the mutation of TGFb receptor 2, insulin-like growth factor 2 receptor, *BAX*, loss of *SMAD4*, *TP53* and *PIK3CA*, which lead to further progression to carcinoma.

KRAS, BRAF, PTEN and *PIK3CA* are mediators of the down- stream signaling of the EGFR. Genetic alterations in these genes contribute to a different EGFR signaling. Oncogenic mutations in *RAS* and *BRAF* activate the MAPK signaling pathway. *BRAF* mutations occur in 13% of CRCs. *PIK3CA* encodes for PI3 kinase. PI3 kinase is controlled by PTEN, which could be lost in colorectal carcinoma. Figure 3 overviews the pathogenic changes and interplay in colorectal carcinoma [39,40].

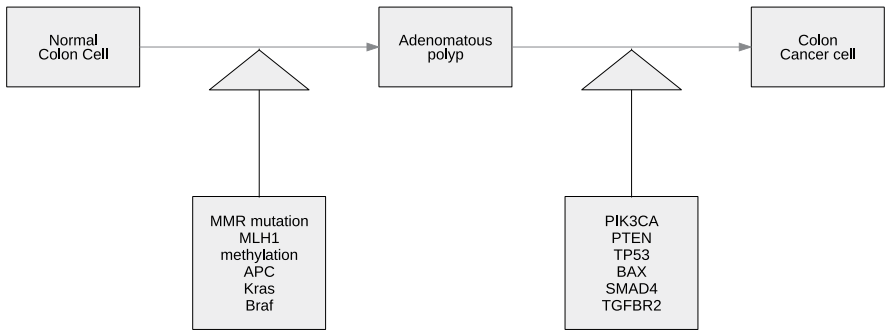

Figure 3: Genetic alterations in colorectal carcinoma. Abbreviations: APC, adenomatous polyposis coli; BAX, BCL2-associated X protein; BRAF, V-raf murine sarcoma viral oncogene homolog; KRAS, Kirsten RAS gene; MMR, mismatch repair; MLH1, human mutL homolog 1; PIK3CA, phosphoinositide-3-kinase; catalytic, alpha polypeptide; PTEN, phosphatase and tensin homolog; SMAD4, SMAD family member 4; TGFBR2, transforming growth factor, beta receptor II; TP53, tumor protein p53.

Targeting KRAS as an anticancer therapy

Modulating KRAS signaling has become a promising concept for new cancer therapies. A variety of approaches, mostly interfering with the mevalonate pathway, 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA) reductase and prenylation of KRAS have been studied [41]. The mevalonate metabolites, FPP and GGPP, play an important part in the post-translational modification of KRAS and have become a target for different anticancer approaches. The effects of statins, bisphosphonates, FTIs, GGTIs, RAS converting enzyme 1 (Rce1) inhibitors and (soprenylcysteine carboxyl methyltransferase) ICMT inhibitors on the mevalonate pathway and indirectly on prenylation of KRAS (Fig. 4) and the results of phase I, II and III clinical studies are discussed.

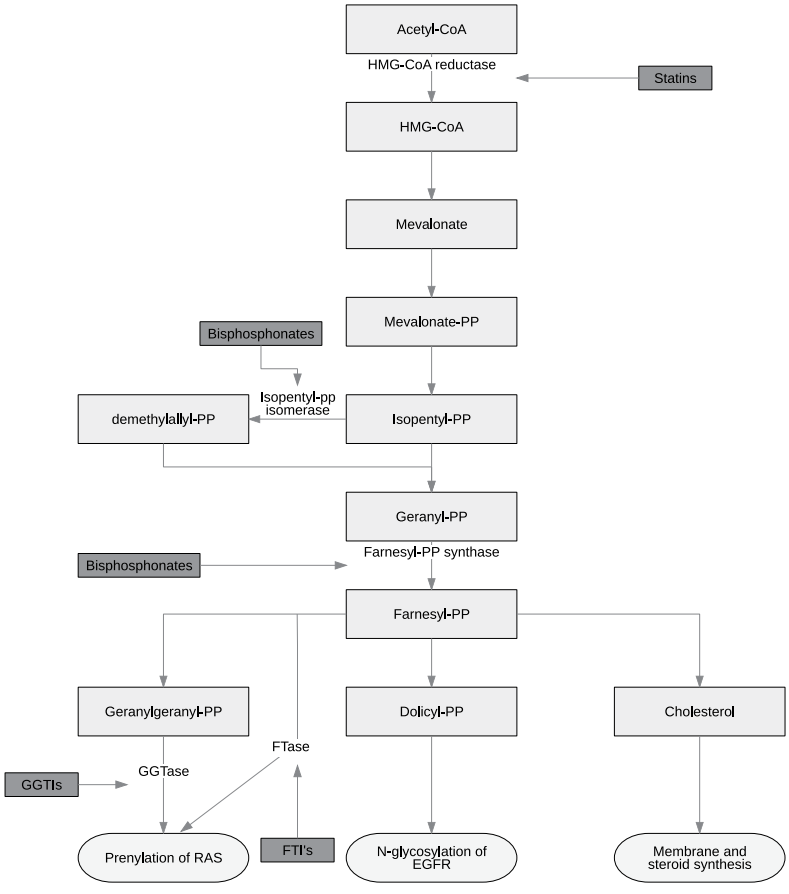

Figure 4: Overview of the mevalonate pathway and inhibitors. The mevalonate pathway causes prenylation of ras, N-glycosylation of EGFR and membrane and steroid synthesis. Statins, bisphosphonates, farnesyltransferase inhibitors and geranylgeranyltransferase inhibitors have inhibitory effects on the mevalonate pathway and thus on prenylation of KRAS. Abbreviations: Acetyl-CoA (acetyl coenzyme A); EGFR, epidermal growth factor receptor; FTase, farnesyltransferase; FTIs, farnesyltransferase inhibitors; GGTase, geranylgeranyltransferase; GGTIs, geranylgeranyltransferase inhibitors; HMG-CoA (3-hydroxy-3-methylglutaryl-coenzyme A) (reductase),; -PP, -pyrophosphate.

Statins

Statins are HMG-CoA inhibitors, which suppress the cholesterol biosynthesis in humans by their inhibitory effect on the mevalonate pathway, thereby inhibiting the formation of low-density lipoprotein (LDL). Owing to upregulation of LDL receptors, the blood clearance of LDL also enhances, increasing the lipid-lowering effect of statins.

Besides the cholesterol-lowering effects, statins are believed to inhibit tumor cell growth and angiogenesis, induce apoptosis and impair tumor metastasis. Through inhibition of HMG-CoA, statins inhibit the formation of mevalonate, thereby affecting the synthesis of the isoprenoids FPP and GGPP. These substrates are used for farnesylation and geranylgeranylations of RAS and RHO. In addition, statins affect both angiogenesis and inflammation processes [5,42] and exert a role in chemoprevention by the inhibition of HMG-CoA reductase, which is upregulated in colon cancer cells [43]. *In vitro* studies have shown that statins suppress growth and induce apoptosis [44,45]. The clinical characteristics of colon cancer among statin users differ from non-users. The former have a lower tumor state, have a lower frequency of metastases, more frequently have a right-sited location of the tumor and have a significantly improved five-year survival rate (37% versus 33%, P-value < 0.01) [46].

The anticancer effects of statins have been studied in phases I, II, and III clinical trials in various malignancies (Table 3), with statin doses from 20 mg/day up to 45 mg/kg/day. Results vary, showing no (additional) effect of statins in multiple myeloma [47–49] and promising results in hepatocellular carcinoma [50]. Graf et al. [50] studied the addition of statins to transcatheter arterial chemoembolization (TACE) in hepatocellular carcinoma and found a significant gain in overall survival compared to TACE alone (median overall survival 20.9 months versus 12.0 months, P = 0.003).

Lee et al. [51] recently reported results of a trial adding simvastatin to irinotecan, leucovorin and 5-FU (FOLFIRI) as first-line therapy in CRC patients. They based the hypothesis on a synergistic effect of these therapies in preclinical research. Response rates and overall survival were similar to historical results of FOLFIRI alone, but time to progression was prolonged (9.9 months versus 6.7–8.5 months), and there was no additional toxicity.

These trials show promising activity of statins in solid tumors, yet further studies on statins in cancer therapy are needed.

Farnesyltransferase inhibitors

Prenylation is a necessary post-translational step for functional KRAS; for that reason, farnesyltransferase inhibitors (FTIs) and geranylgeranyltranferase inhibitors (GGTIs) have been developed as anticancer therapy. Besides KRAS, other GTPases that promote tumor progression are prenylated. FTase can recognize and prenylate tetrapeptides with a CAAX sequence. FTIs act through two mechanisms. FPP analogs selectively compete with FPP for binding to FTase and the CAAX sequence of KRAS. The peptidemimetics competes with RAS-CAAX for FTase; some FTIs compete via both mechanisms. By these mechanisms, FTIs inhibit farnesylation of not only ras proteins but also various other polypeptides, such as nuclear lamins A and B, skeletal muscle phophorylase kinase, transducin, cGMP phophodiesterase and the cell regulatory protein tyrosine phosphatases [52].

Table 3: Phase I, II, and III trials evaluating statins in cancer treatment

Study	Refs	Study design	Tumor type	Agent	Additional agent	n	Main results
Lee	[51]	Phase II	CRC	Simvastatin	FOLFIRI	49	TTP possibly prolonged; no effect on RR or OS
Graf	[50]	Phase III	HCC	Pravastatin	TACE	183	mOS 20.9 months versus 12.0 months
Lopes-Aguilar	[94]	Phase II	Brain stem tumors (pediatric)	Fluvastatin	Chemotherapy + thalidomide	9	RR 78%
Sondergaard	[47]	Phase II	Multiple myeloma	Simvastatin	None	6	RR 0%
van der Speck	[48]	Phase II	Multiple myeloma	Simvastatin	VAD	12	RR 8%
Schidmaier	[49]	Phase II	Multiple myeloma	Simvastatin	Bortezomib or bendamustine	6	RR 0%
Knox	[95]	Phase I	SCCHN/cervical carcinoma	Lovastatin	None	26	RR 0%; CBR 23%
Lersch	[96]		HCC	Pravastatin versus octreotide versus gemcitabine		58	mOS 7.2 versus 5 versus 3.5 months
Kim	[97]	Phase II	Gastric adenocarcinoma	Lovastatin	None	16	RR 0%
Kawata	[98]	Phase III	HCC	Pravastatin	TAE + oral 5FU	91	mOS 18 months versus 9 months
Larner	[99]	Phase I/II	Astrocytoma/ GBM	Lovastatin	±Radiation	18	RR 11%; CBR 17%
Thibault	[100]	Phase I	Solid tumors	Lovastatin	None	88	Lovastatin well tolerated up to 25 mg/ kg/day

Abbreviations: (m)OS, (median) overall survival; CBR, clinical benefit rate (i.e. complete and partial remission and stable disease); CRC, colorectal carcinoma; FOLFIRI, irinotecan, leucovorin and 5-FU; GBM, glioblastoma multiforme; HCC, hepatocellular carcinoma; RR, response rate (i.e. complete and partial remission); SCCHN, squamous cell carcinoma of head and neck; TA(C)E, transcatheter arterial (chemo)embolization; TTP, time to progression; VAD, vincristine, adriamycin, dexamethasone.

Four FTIs were tested in clinical trials worldwide: lonafarnib and tipifarnib (both oral compounds) have been tested in phase II and phase III studies (listed in Table 4), and BMS-214662 and L-778,123, administered intravenously, were tested in phase I studies. Some of the trials listed in Table 4 tested tipifarnib and lonafarnib in solid tumors, such as breast, pancreatic, colorectal, urothelial and brain tumors, but the results of these trials were disappointing. Sparano et al. recently published the results of a phase II trial testing the addition of tipifarnib to neo-adjuvant doxorubicin–cyclophosphamide in patients with clinical stage IIB–IIIC breast cancer. The trial included 44 patients, and a pathological complete remission was seen in 25%,

compared to 10–15% for chemotherapy alone according to historical results. Still, the role of tipifarnib in the treatment of solid tumors remains unclear and further study is needed. In hematologic malignancies, however, tipifarnib did show some single-agent activity, especially in elderly patients with poor risk and previously untreated acute myeloid leukemia. Lancet et al. [54] tested tipifarnib monotherapy in this population and observed a response rate of 23%. Tipifarnib was submitted to the FDA for the treatment of acute myeloid leukemia in elderly patients not applicable for standard chemotherapy in January 2005. In June 2005, however, the FDA filed a Not Approvable Letter, awaiting the results of subsequent phase III trials of tipifarnib for this indication [55–57]. Recently, the results of a phase III trial comparing tipifarnib with best supportive care in newly diagnosed acute myeloid leukemia in patients of 70 years or older were published. The results showed no effect of tipifarnib on survival (median survival, 107 days versus 109 days; P-value, 0.843) [58].

Activation of *KRAS* by mutation is associated with radiotherapy resistance. Preclinical studies *in vitro* and *in vivo* with FTIs showed that the radiosensitivity of cells might be improved. The potential synergistic effect for radiosensitization might be the inhibition of activated KRAS by the FTIs [59–61].

A phase I trial of L-778,123 (an FTI and GGTI) and radiotherapy in 12 patients with pancreatic cancer showed acceptable toxicity. In a patient-derived pancreatic cell line, radiosensitization was observed. In total, eight patients completed treatment, one patient showed partial response for six months, five patients showed stable disease (>2 months) and two patients were progressive [62].

Another phase I trial with L-788,123 with radiotherapy in nine patients with locally advanced head and neck or lung cancer showed a complete response in one patient and five patients with a partial response [63].

Table 4: Phase II and III trials evaluating FTIs in cancer treatment

Author	Refs	Study design	Tumor	Agent	Additional agent	n	Endpoints and results
Harrousseau	[58]	Phase III	AML	Tipifarnib	None	457	No effect on survival
Sparano	[53]	Phase II	Breast cancer	Tipifarnib	Doxorubicin and cyclophosphamide	44	RR 77%
Li	[101]	Phase II	Breast cancer	Tipifarnib	Fulvestrant	33	CBR 52%; target CBR (70%) not achieved
Lustig	[102]	Phase II	GBM	Tipifarnib	Radiotherapy	28	RR 0%; CBR 29%
Eckhardt	[103]	Phase II	Pancreatic cancer	Tipifarnib versus placebo	Gemcitabine	244	No effect of the addition of tipifarnib on survival
Ravoet	[104]	Phase II	MDS/AML	Lonafarnib	None	16	RR 6%
Feldman	[105]	Phase II	MDS/CML	Lonafarnib	None	67	RR 4%; HI 19%
Karp	[106]	Phase II	AML	Tipifarnib	None (maintenance)	48	mDFS 13.5 months
Fouladi	[107]	Phase II	Glioma	Tipifarnib	None	97	RR 2%
Johnston	[108]	Phase II	Breast cancer	Tipifarnib	None	120	RR 12%

Author	Refs	Study design	Tumor	Agent	Additional agent	n	Endpoints and results
Harousseau	[109]	Phase II	AML	Tipifarnib	None	252	RR 4%
Lancet	[54]	Phase II	AML	Tipifarnib	None	158	RR 23%
Cloughesy	[110]	Phase II	Glioma	Tipifarnib	None versus + EIAEDs	89	10% had PFS > 6 months; RR > 7%
Whitehead	[111]	Phase II	CRC	Tipifarnib	None	55	RR 7%
Borthakur	[112]	Phase II	CML	Lonafarnib	None	13	RR 18%
Macdonald	[113]	Phase II	Pancreatic cancer	Tipifarnib	None	53	mOS 2.6 months
Kim	[114]	Phase II	NSCLC	Lonafarnib	Paclitaxel	33	RR 10%; CBR 48%
Theodore	[115]	Phase II	Urothelial cancer	Lonafarnib	Gemcitabine	31	RR 32%
Winquist	[116]	Phase II	Urothelial cancer	Lonafarnib	None	19	RR 0%
Rosenberg	[117]	Phase II	Urothelial cancer	Tipifarnib	None	34	RR 6%; CBR 44%
Rao	[118]	Phase III	CRC	Tipifarnib versus placebo	None	268	CBR 24% versus 13%; no effects on PFS and OS
Heymach	[119]	Phase II	SCLC	Tipifarnib	None	22	RR 0%; mPFS 1.4 months
Van Cutsem	[120]	Phase III	Pancreatic cancer	Tipifarnib versus placebo	Gemcitabine	688	mOS 193 days versus 182 days
Kurzrock	[121]	Phase II	MDS	Tipifarnib	None	28	RR 11%; severe toxicity
Alsina	[122]	Phase II	Multiple myeloma	Tipifarnib	None	43	RR 0%; CBR 64%
Johnston	[108]	Phase II	Breast cancer	Tipifarnib	None	76	RR up to 14%
Adjei	[123]	Phase II	NSCLC	Tipifarnib	None	44	RR 0%; CBR 16%
Cohen	[124]	Phase II	Pancreatic cancer	Tipifarnib	None	20	RR 0%; mOS 19.7 weeks
Cortes	[125]	Phase II	Multiple myeloma/ CML	Tipifarnib	None	40	RR 18%
Sharma	[126]	Phase II	CRC	Lonafarnib	None	21	RR 0%; CBR 14%

Abbreviations: (m)DFS, (median) disease-free survival; (m)OS, (median) overall survival; (m)PFS, (median) progression-free survival; (N)SCLC, (non) small cell lung carcinoma; AML, acute myeloid leukemia; CBR, clinical beneficial rate (i.e. complete remission, partial remission and stable disease); CML, chronic myeloid leukemia; CRC, colorectal cancer; EIAEDs, enzyme-inducing antiepileptic drugs; GBM, glioblastoma multiforme; MDS, myelodysplastic syndrome; HI, hematologic improvement; RR, response rate (i.e. complete and partial remission).

GGTase inhibitors

Only inhibition of the farnesylation of KRAS by FTIs does not considerably affect its function, because KRAS can be geranylgeranylated as well. GGTase I geranylgeranylates KRAS when FTases are inhibited by FTIs. This fact triggered the development of GGTIs. GGPP analogs and CAAL peptidomimetics both act as GGTIs. Inhibition of KRAS prenylation might require co-treatment of FTIs with GGTIs and might explain the limited efficacy of the FTIs as single drug [56]. Moreover, in contrast to FTIs, GGTIs are able to block phosphorylation of both PDGF- and EGF-dependent tyrosine kinase receptors. GGTase inhibitors have been tested in preclinical studies and showed decreased tumor growth (cell-cycle arrest in G1 and apoptosis) in vivo and *in vitro* [65–67]. Possibly because of the preclinical toxicity of GGTase I inhibitors, up till now they have not proceeded to clinical stages.

Bisphosphonates

Bisphosphonates (BPs) inhibit isopentenyl diphosphatase isomerase and FPP synthase and probably also GGPP synthase, two metabolites in the mevalonate pathway. The newer nitrogen- containing BPs (e.g. pamidronate and zoledronic acid), inhibited farnesylation and geranylgeranylation of KRAS, resulting in a decrease of downstream signaling, inducing apoptosis [5,64]. Other observed effects of BPs on tumor cells are inhibition of migration through and adhesion and invasion to the extracellular matrix, so-called 'MMP activity'. At low concentrations, BPs inhibit the mevalonate pathway, whereas at higher concentrations, MMP activity is inhibited [68]. Furthermore, BPs reduce complications such as osteoporosis and skeletal morbidity caused by metastatic bone disease in metastatic and non-metastatic disease. In non-metastatic disease, BPs might prevent bone metastasis [69]; in metastatic disease, BPs might delay or prevent the complications caused by bone metastasis [70,71]. Clinical studies on BPs in cancer treatment have been performed, mainly focusing on endpoints regarding skeletal-related events such as fractures and bone pain. Some of these trials also focus on response-related endpoints, to investigate the role of BPs in survival in cancer.

Table 5 shows the phase II/III clinical trials on BPs in cancer treatment, not (only) focusing on skeletal-related events. The largest and most recent trial was published by Gnant et al. [72], who tested the effects of the addition of zoledronic acid to either goserelin and tamoxifen or goserelin and anastrozole in pre-menopausal women with endocrine-responsive early breast cancer. After a median follow-up of 47.8 months, a disease-free survival rate of 94.0% was seen in the group receiving endocrine therapy with zoledronic acid, compared to 90.8% in the group receiving only endocrine therapy (P = 0.01) [72].

Nowadays, BPs are known to reduce bone loss owing to hormone therapy (such as for breast and prostate cancer) and prevent skeletal-related events [70]. Despite the results published by Gnant et al. [72], however, there is no consensus about the effect of BPs on survival.

Table 5: Phase II and III clinical trials evaluating the effect of bisphosphonates on response related endpoints in malignancies.

Author	Refs	Study design	Tumor	Agent	Additional agent	n	Endpoints and results
Gnant	[72]	Phase III	Breast	Zoledronic acid	Tamoxifen and goserelin versus anastrozole and goserelin	1803	Significantly longer disease-free survival with zoledronic acid
James	[127]	Phase III	Prostate	Zoledronic acid	Androgen suppression ± docetaxel / ±celecobrix		Ongoing trial
Diel	[128]	Phase III	Breast	Clodronate	Adjuvant therapy	290	At 55 months follow up significantly improved PFS and OS with clodronate
Kristensen	[129]	Phase III	Breast	Pamidronate	Adjuvant chemotherapy and/or radiotherapy	953	No effect on occurrence of bone metastases
Kattan	[130]	Phase II	Prostate	Zoledronic acid	Docetaxel estramustine	27	PSA response in 52% RR 21%
Mason	[131]	Phase III	Prostate	Clodronate versus placebo	None	508	No effects on OS and bone metastases-free survival
Pavlu	[132]	Phase I/II	CML	Zoledronic acid	Imatinib	10	RR 0%
Di Lorenzo	[133]	Phase II	Prostate	Zoledronic acid	Docetaxel vinorelbine	40	PSA response in 32% RR in 40%
Di Lorenzo	[134]	Phase II	Prostate	Zoledronic acid	Gemcitabine prednisone	22	PSA response in 23% RR in 14%
Mitsiades	[135]	Phase III	Prostate	Zoledronic acid	None versus somatostatin analog and dexamethasone	38	RR 0% versus 65%. PFS and OS significantly improved
Lewis	[136]	Phase II	Melanoma	Apomine	None	42	RR 0%, mPFS 6.1 months
Bertelli	[137]	Phase II	Prostate	Zoledronic acid	Docetaxel	25	PSA response in 48%, mild toxicity
Figg	[138]	Phase II	Prostate	Alendronate	Ketoconazole and hydrocortisone	72	No significant differences in PFS, OS and RR
Tiffany	[139]	Phase II	Prostate	Zoledronic acid	Imatinib	15	No effects on pain and PSA
Dearnaley	[140]	Phase III	Prostate	Clodronate	None	311	Non-significant better BPFS and OS
Mardiak	[141]	Phase III	Breast	Clodronate versus placebo	Standard chemotherapy	73	Time to development of (bone) metastases 13 months versus 28 months

Abbreviations: (m)PFS, (median) progression-free survival; BPFS, bone progression-free survival; CML, chronic myeloid leukemia; OS, overall survival; PSA, prostate-specific antigen; PSA response, >50% PSA decline; RR, response rate (i.e. complete and partial remission).

Other post-prenylation inhibitors

After prenylation, KRAS undergoes endoproteolytic processing by the RCE1 protease and carboxyl methylation by ICMT. These enzymes, which act on both farnesylated and geranylgeranylated enzymes, could be targets for anticancer therapy.

Few small-molecule inhibitors of RCE1 and ICMT have been described so far. RPI, a prenylated CAAX peptide, competitively inhibits RCE1 as substrate analogs. Two types of ICMT inhibitors have been developed; both types act as mimics of substrates. The S-adenosylhomocysteines bind to methyltransferases and competitively inhibit the enzyme. In preclinical studies with cell lines, a partial block of proliferation was shown. Membrane-associated KRAS was reduced by 66% in one study, resulting in a decrease of downstream MEK/ERK signaling [73,74]. The second group of ICMT inhibitors contains derivatives of prenylcysteine: for example, N-acetyl-S-farnesyl-L-cysteine and N-acetyl-S-geranylgeranyl- L-cysteine. These substrates act also as substrates for ICMT; however, they target other processes in the cell as well [75].

EGFR antibodies and *KRAS*

The EGFR is a target for anticancer therapy. EGFR is expressed in normal tissues and different tumors. The EGFR is a 170-kDa transmembrane receptor with an extracellular ligand binding domain, a transmembrane domain and an intracellular tyrosine kinase membrane. There are four EGFR-related receptors; EGFR (HER1), HER2, HER3 and HER4. The binding of the ligand to the ligand-binding domain results in a conformational change, enabling the receptor to form an EGFR–EGFR homodimer or an EGFR–HER2, EGFR–HER3 or EGFR–HER4 heterodimer (figure 5). The active dimer cause ATP-dependent phosphorylation of EGFR through tyrosine kinases, which cause proliferation, inhibition of apoptosis, invasion and metastasis [76].

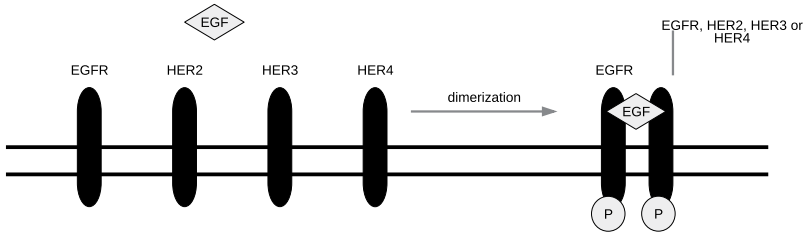

Figure 5: Dimerization of the EGFR. The binding of a specific ligand (e.g. EGF) causes a conformational change and results in homodimer or heterodimer formation. Abbreviations: EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; HER, human epidermal growth factor receptor.

Monoclonal EGFR antibodies bind the extracellular domain of EGFR, thereby blocking the ligand-binding region, and as a result, the EGFR tyrosine kinase activation is halted and ras signaling is inhibited [76,77]. Cetuximab can induce antibody-dependent cell-mediated cytotoxicity (ADCC) and downregulation and degradation of EGFR and in this way exerts its anti-tumor activity. For panitumumab, no ADCC has been described [78].

Two EGFR antibodies, cetuximab and panitumumab, have been registered. Cetuximab is registered for the treatment of metastasized colorectal carcinoma with EGFR overexpression in

KRAS wild type patients (monotherapy or in combination with chemotherapy), head and neck squamous cell carcinomas in combination with radiotherapy, and metastasized head and neck squamous cell carcinomas in combination with cisplatin-based chemotherapy. Panitumumab is registered for colorectal carcinoma with EGFR overexpression in *KRAS* wild type patients. Retrospective analysis of clinical trials showed a lack of clinical activity of cetuximab and panitumumab in patients with mutant *KRAS* because mutant *KRAS* operates independently of activation of the EGFR [24,33,34,79–88]. Table 6 represents clinical studies on the efficacy of cetuximab or panitumumab in patients with CRC with either mutant or wild type *KRAS* tumors. These results indicate that the efficacy of panitumumab and cetuximab (mono-) therapy is limited to patients with wild type *KRAS* tumors [33,34,89,90].

Table 6: Studies investigating KRAS and cetuximab and panitumumab and *KRAS* status in colorectal carcinoma.

Study	Refs	Treatment	*KRAS* status	RR	Median PFS	Median OS
Douillard	[91]	FOLFOX4 ± panitumumab	*KRAS* mutant	N/A	7.3 months	N/A
			KRAS wild type	-55	9.6 months	N/A
Peeters	[142]	FOLFIRI ± panitumumab	*KRAS* mutant	N/A	N/A	N/A
			KRAS wild type	-35	5.9 months	14.5 months
Van Cutsem	143 and 144	FOLFIRI ± cetuximab	*KRAS* mutant	102 (59.3)	7.6 months	17.5 months
			KRAS wild type	38 (36.2)	9.9 months	24.9 months
Bokemeyer	[81]	FOLFOX-4 ± cetuximab	*KRAS* mutant	17 (33)	5.5 months	N/A
			KRAS wild type	37 (60)	7.7 months	N/A
Tol	[88]	Capecitabine + oxaliplatin + bevacizumab ± cetuximab	*KRAS* mutant	(45.9)	8.1 months	17.2 months
			KRAS wild type	(61.4)	10.5 months	21.8 months
Amado	[33]	Panitumumab versus BSC	*KRAS* mutant	0 (0)	7.4 months	4.5 months
			KRAS wild type	21 (17)	12.3 weeks	6.8 months
Karapetis	[34]	Cetuximab versus BSC	*KRAS* mutant	(1.2)	1.9 months	4.8 months
			KRAS wild type	(1.28)	3.7 months	9.5 months
Lievre 2008		Cetuximab ± chemotherapy	*KRAS* mutant	0 (0)	9 weeks	10.1 months
			KRAS wild type	34 (43.6)	31.4 weeks	14.3 months
Lievre 2006	[85]	Cetuximab ± chemotherapy	*KRAS* mutant	0	N/E	6.9 months
			KRAS wild type	-65	N/E	16.3 months

Study	Refs	Treatment	*KRAS* status	RR	Median PFS	Median OS
De Roock	[82]	Cetuximab ± irinotecan	*KRAS* mutant	0 (0)	12 weeks	27.3 weeks
			KRAS wild type	27 (21)	24 weeks	43 weeks
Khambata-Ford	[24]	Cetuximab	*KRAS* mutant	3 (10)	59 days	N/E
			KRAS wild type	24 (48)	61 days	N/E
Di Fiore	[145]	Cetuximab plus chemotherapy	*KRAS* mutant	0 (0)	3 months	N/E
			KRAS wild type	12 (27.9)	5.5 months	N/E
Benvenuti	[79]	Cetuximab/panitumumab	*KRAS* mutant	1 (6.2)	N/A	N/E
			KRAS wild type	10 (31.2)	N/A	N/E
Frattini	[146]	Cetuximab	*KRAS* mutant	1 (10)	N/A	N/E
			KRAS wild type	9 (53)	N/A	N/E
Hecht	[147]	Bevacizumab + irinotecan based chemotherapy ± panitumumab	*KRAS* mutant	30	8.3 months	17.8 months
			KRAS wild type	54	10 months	N/A
		Bevacizumab + oxaliplatin based chemotherapy ± panitumumab	*KRAS* mutant	47	10.4 months	19.3 months
			KRAS wild type	50	9.8 months	20.7 months
Garm Spindler	[84]	Irinotecan + cetuximab	*KRAS* mutant	0 (0)	2.3 months	8.7 months
			KRAS wild type	-40	8.0 months	11.1 months
Bibeau	[80]	Panitumumab versus BSC	*KRAS* mutant	1 (4)	3.0 months	8.7 months
			KRAS wild type	10 (27)	5.5 months	10.8 months
Prenen	[87]	Irinotecan ± cetuximab	*KRAS* mutant	1 (1.3)	12 weeks	26 weeks
			KRAS wild type	37 (30.3)	24 weeks	45 weeks
Laurent-Puig	[148]	Cetuximab, remaining therapy unspecified	*KRAS* mutant	0 (0)	8.6 weeks	
			KRAS wild type	24 (68.4)	32 weeks	
Moroni	[149]	Chemotherapy ± cetuximab/ panitumumab	*KRAS* mutant	2 (20)	N/E	N/E
			KRAS wild type	8 (38)	N/E	N/E
Loupakis	[150]	Irinotecan + cetuximab	*KRAS* mutant	N/A	3.1 months	6.1 months

Study	Refs	Treatment	*KRAS* status	RR	Median PFS	Median OS
			KRAS wild type	N/A	4.2 months	13.5 months
Cappuzzo	[151]	Chemotherapy ± cetuximab	*KRAS* mutant	4 (9.5)	4.4 months	9.5 months
			KRAS wild type	10 (26.3)	5.4 months	10.8 months
Finocchiaro	[152]	Cetuximab	*KRAS* mutant	(6.3)	3.7 months	8.3 months
			KRAS wild type	(26.5)	6.3 months	10.8 months
Freeman	[153]	Panitumumab	*KRAS* mutant	0 (0)	N/A	N/A
			KRAS wild type	(10.5)	N/A	N/A
Di Nicolantonio	[83]	Chemotherapy ± cetuximab/ panitumumab	*KRAS* mutant	2 (6)	N/A	N/A
			KRAS wild type	22 (28)	N/A	N/A
Tabernero	[154]	Cetuximab	*KRAS* mutant	0 (0)		
			KRAS wild type	(27.6)		
		Chemotherapy + cetuximab	*KRAS* mutant	(31.6)	5.6 weeks	
			KRAS mutant	(55.2)	9.4 weeks	

Abbreviations: BSC, best supportive care; N/A, not available (yet); N/E, not evaluated; OS, overall survival; PFS, progression-free survival; RR, response rate. The values in parentheses are the percentages of patients with RR.

Alternative strategies

An alternative strategy to attack *KRAS* mutated cells would be to inhibit targets downstream of ras, such as mTOR (using RAD001), PI3 kinase (using BEZ235) or raf (using BAY 43-9006). One could consider combining inhibitors of targets within the RAS-RAF-MAPK and PI3 kinase pathway, thereby possibly creating inhibition comparable to targeting of the EGFR. Inhibitors of various targets within these pathways have been tested *in vivo* and are currently being studied in phase I/II clinical trials (http:// www.clinicaltrials.gov). Because the efficacy of these agents has not been proved yet, however, none of them are standard in cancer therapy. Such alternative strategies might be relevant in the future in the treatment of patients harboring *KRAS* mutations.

Future perspectives

KRAS mutation status has an impact on the therapeutic opportunities for patients with colorectal carcinoma. Both cetuximab and panitumumab are effective only in *KRAS* wild type patients,

and in *KRAS* mutant patients, a worse response has been reported [81,91]. Modulation of KRAS prenylation in *KRAS* mutant tumors might potentiate EGFR therapy [92] because the metabolites formed during the mevalonate pathway have a key role in prenylation and thereby post-translational activation of KRAS. Indeed, inhibition of the mevalonate pathway could influence the potential of KRAS to translocate from the cytosol toward the membrane and, thus, alter the KRAS phenotype toward the wild type. Combinations of EGFR antibodies to target the EGFR with KRAS modulators such as statins, BPs, FTIs or GGTIs inhibitors targeting RAS-RAF-MAPK signaling might augment the effect in patients with *KRAS* mutations. In (pre)clinical studies, further investigation should be done to elucidate the role of statins, FTIs, GGTIs, BPs, RCE1 inhibitors and ICMT inhibitors in CRC and the possibilities of therapeutic modulation of *KRAS* mutations.

References

1 Jemal, A. et al. (2009) Cancer statistics, 2009. CA Cancer J. Clin. 59, 225–249

2 Shimizu, K. et al. (1983) Three human transforming genes are related to the viral ras oncogenes. Proc. Natl. Acad. Sci. U. S. A. 80, 2112–2116

3 Rowell, C.A. et al. (1997) Direct demonstration of geranylgeranylation and farnesylation of Ki-Ras in vivo. J. Biol. Chem. 272, 14093–14097

4 Whyte, D.B. et al. (1997) K- and N-Ras are geranylgeranylated in cells treated with farnesyl protein transferase inhibitors. J. Biol. Chem. 272, 14459–14464

5 Konstantinopoulos, P.A. et al. (2007) Post-translational modifications and regulation of the RAS superfamily of GTPases as anticancer targets. Nat. Rev. Drug Discov. 6, 541–555

6 Vogelstein, B. et al. (1988) Genetic alterations during colorectal-tumor development. N. Engl. J. Med. 319, 525–532

7 Downward, J. (2003) Targeting RAS signaling pathways in cancer therapy. Nat. Rev. Cancer 3, 11–22

8 Graaf, M.R. et al. (2004) Effects of statins and farnesyltransferase inhibitors on the development and progression of cancer. Cancer Treat. Rev. 30, 609–641

9 Buday, L. and Downward, J. (1993) Epidermal growth factor regulates p21ras through the formation of a complex of receptor, Grb2 adapter protein, and Sos nucleotide exchange factor. Cell 73, 611–620

10 Roberts, P.J. and Der, C.J. (2007) Targeting the Raf–MEK–ERK mitogen-activated protein kinase cascade for the treatment of cancer. Oncogene 26 3291–3310

11 Fang, J.Y. and Richardson, B.C. (2005) The MAPK signaling pathways and colorectal cancer. Lancet Oncol. 6, 322–327

12 Heinemann, V. et al. (2009) Clinical relevance of EGFR- and KRAS-status in colorectal cancer patients treated with monoclonal antibodies directed against the EGFR. Cancer Treat. Rev. 35, 262–271

13 Bos, J.L. et al. (1987) Prevalence of ras gene mutations in human colorectal cancers. Nature 327, 293–297

14 Forrester, K. et al. (1987) Detection of high incidence of KRAS oncogenes during human colon tumorigenesis. Nature 327, 298–303

15 Artale, S. et al. (2008) Mutations of KRAS and BRAF in primary and matched metastatic sites of colorectal cancer. J. Clin. Oncol. 26, 4217–4219

16 Brink, M. et al. (2003) KRAS oncogene mutations in sporadic colorectal cancer in The Netherlands Cohort Study. Carcinogenesis 24, 703–710

17 Wojcik, P. et al. (2008) KRAS mutation profile in colorectal carcinoma and novel mutation–internal tandem duplication in KRAS. Pol. J. Pathol. 59, 93–96

18 Kraus, M.C. et al. (2006) The balanced induction of KRAS codon 12 and 13 mutations in mucosa differs from their ratio in neoplastic tissues. Int. J. Oncol. 29, 957–964

19 Andreyev, H.J. et al. (1998) Kirsten ras mutations in patients with colorectal cancer: the multicenter "RASCAL" study. J. Natl. Cancer Inst. 90, 675–684

20 Sanchez-de-Abajo, A. et al. (2007) Molecular analysis of colorectal cancer tumors from patients with mismatch repair proficient hereditary nonpolyposis colorectal cancer suggests novel carcinogenic pathways. Clin. Cancer Res. 13, 5729–5735

21 Adjei, A.A. (2001) Blocking oncogenic Ras signaling for cancer therapy. J. Natl. Cancer Inst. 93, 1062–1074

22 Tong, L.A. et al. (1991) Crystal structures at 2.2 A resolution of the catalytic domains of normal ras protein and an oncogenic mutant complexed with GDP. J. Mol. Biol. 217, 503–516

23 Scheffzek, K. et al. (1997) The Ras–RasGAP complex: structural basis for GTPase activation

and its loss in oncogenic Ras mutants. Science 277, 333–338

24 Khambata-Ford, S. et al. (2007) Expression of epiregulin and amphiregulin and K- ras mutation status predict disease control in metastatic colorectal cancer patients treated with cetuximab. J. Clin. Oncol. 25, 3230–3237

25 Anwar, S. et al. (2004) Systematic review of genetic influences on the prognosis of colorectal cancer. Br. J. Surg. 91, 1275–1291

26 Graziano, F. and Cascinu, S. (2003) Prognostic molecular markers for planning adjuvant chemotherapy trials in Dukes' B colorectal cancer patients: how much evidence is enough? Ann. Oncol. 14, 1026–1038

27 Klump, B. et al. (2004) Molecular lesions in colorectal cancer: impact on prognosis? Original data and review of the literature. Int. J. Colorectal Dis. 19, 23–42

28 Conlin, A. et al. (2005) The prognostic significance of KRAS, p53, and APC mutations in colorectal carcinoma. Gut 54, 1283–1286

29 Unknown author, (2005) ASCO conference highlights: potential markers of response in NSCLC. Signal 6, 12–16

30 Russo, A. et al. (2005) Prognostic and predictive factors in colorectal cancer: Kirsten Ras in CRC (RASCAL) and TP53CRC collaborative studies. Ann. Oncol. 16 (Suppl. 4), iv44–iv49

31 Castagnola, P. and Giaretti, W. (2005) Mutant KRAS, chromosomal instability and prognosis in colorectal cancer. Biochim. Biophys. Acta 1756, 115–125

32 Andreyev, H.J. et al. (2001) Kirsten ras mutations in patients with colorectal cancer: the 'RASCAL II' study. Br. J. Cancer 85, 692–696

33 Amado, R.G. et al. (2008) Wild type KRAS is required for panitumumab efficacy in patients with metastatic colorectal cancer. J. Clin. Oncol. 26, 1626–1634

34 Karapetis, C.S. et al. (2008) KRAS mutations and benefit from cetuximab in advanced colorectal cancer. N. Engl. J. Med. 359, 1757–1765

35 Plesec, T.P. and Hunt, J.L. (2009) KRAS mutation testing in colorectal cancer. Adv. Anat. Pathol. 16, 196–203

36 Tol J. et al. (2009) High sensitivity of both sequencing and real-time PCR analysis of KRAS mutations in colorectal cancer tissue. J. Cell Mol. Med. [Epub ahead of print]

37 van Krieken, J.H. et al. (2008) KRAS mutation testing for predicting response to anti-EGFR therapy for colorectal carcinoma: proposal for an European quality assurance program. Virchows Arch. 453, 417–431

38 Juan, T. et al. (2008) A comparability study of 4 commercial KRAS tests. AACR Meeting Abstracts 2008 1811

39 Markowitz, S.D. and Bertagnolli, M.M. (2009) Molecular origins of cancer: molecular basis of colorectal cancer. N. Engl. J. Med. 361, 2449–2460

40 Walther, A. et al. (2009) Genetic prognostic and predictive markers in colorectal cancer. Nat. Rev. Cancer 9, 489–499

41 Friday, B.B. and Adjei, A.A. (2005) KRAS as a target for cancer therapy. Biochim. Biophys. Acta 1756, 127–144

42 Demierre, M.F. et al. (2005) Statins and cancer prevention. Nat. Rev. Cancer 5, 930–942

43 Hentosh, P. et al. (2001) Sterol-independent regulation of 3-hydroxy-3- methylglutaryl coenzyme A reductase in tumor cells. Mol. Carcinog. 32, 154–166

44 Sassano, A. and Platanias, L.C. (2008) Statins in tumor suppression. Cancer Lett. 260, 11–19

45 Jakobisiak, M. et al. (1991) Cell cycle-specific effects of lovastatin. Proc. Natl. Acad. Sci. U. S. A. 88, 3628–3632

46 Siddiqui, A.A. et al. (2009) For patients with colorectal cancer, the long-term use of statins is associated with better clinical outcomes. Dig. Dis. Sci. 54, 1307–1311

47 Sondergaard, T.E. et al. (2009) A phase II clinical trial does not show that high dose simvastatin has beneficial effect on markers of bone turnover in multiple myeloma. Hematol. Oncol. 27, 17–22

48 van der Spek, S.E. et al. (2007) High dose simvastatin does not reverse resistance to vincristine, adriamycin, and dexamethasone (VAD) in myeloma. Haematologica 92, e130–e131

49 Schmidmaier, R. et al. (2007) First clinical experience with simvastatin to overcome drug resistance in refractory multiple myeloma. Eur. J. Haematol. 79, 240–243

50 Graf, H. et al. (2008) Chemoembolization combined with pravastatin improves survival in patients with hepatocellular carcinoma. Digestion 78, 34–38

51 Lee, J. et al. (2009) Simvastatin plus irinotecan, 5-fluorouracil, and leucovorin (FOLFIRI) as first-line chemotherapy in metastatic colorectal patients: a multicenter phase II study. Cancer Chemother. Pharmacol. 64, 657–663

52 Crul, M. et al. (2001) Ras biochemistry and farnesyl transferase inhibitors: a literature survey. Anticancer Drugs 12, 163–184

53 Sparano, J.A. et al. (2009) Phase II trial of tipifarnib plus neoadjuvant doxorubicin–cyclophosphamide in patients with clinical stage IIB–IIIC breast cancer. Clin. Cancer Res. 15, 2942–2948

54 Lancet, J.E. et al. (2007) A phase 2 study of the farnesyltransferase inhibitor tipifarnib in poor-risk and elderly patients with previously untreated acute myelogenous leukemia. Blood 109, 1387–1394

55 Basso, A.D. et al. (2006) Lipid posttranslational modifications. Farnesyl transferase inhibitors. J. Lipid Res. 47, 15–31

56 Sebti, S.M. and Hamilton, A.D. (2000) Farnesyltransferase and geranylgeranyltransferase I inhibitors and cancer therapy: lessons from mechanism and bench-to-bedside translational studies. Oncogene 19, 6584–6593

57 Cox, A.D. (2001) Farnesyltransferase inhibitors: potential role in the treatment of cancer. Drugs 61, 723–732

58 Harousseau, J.L. et al. (2009) A randomized phase 3 study of tipifarnib compared with best supportive care, including hydroxyurea, in the treatment of newly diagnosed acute myeloid leukemia in patients 70 years or older. Blood 114, 1166– 1173

59 Brunner, T.B. et al. (2003) Farnesyltransferase inhibitors as radiation sensitizers. Int. J. Radiat. Biol. 79, 569–576

60 Cengel, K.A. et al. (2007) Oncogenic KRAS signals through epidermal growth factor receptor and wild type H-Ras to promote radiation survival in pancreatic and colorectal carcinoma cells. Neoplasia 9, 341–348

61 Brunner, T.B. et al. (2004) Radiation sensitization by inhibition of activated Ras. Strahlenther. Onkol. 180, 731–740

62 Martin, N.E. et al. (2004) A phase I trial of the dual farnesyltransferase and geranylgeranyltransferase inhibitor L-778,123 and radiotherapy for locally advanced pancreatic cancer. Clin. Cancer Res. 10, 5447–5454

63 Hahn, S.M. et al. (2002) A Phase I trial of the farnesyltransferase inhibitor L-778,123 and radiotherapy for locally advanced lung and head and neck cancer. Clin. Cancer Res. 8, 1065–1072

64 Walker, K. and Olson, M.F. (2005) Targeting Ras and Rho GTPases as opportunities for cancer therapeutics. Curr. Opin. Genet. Dev. 15, 62–68

65 Sun, J. et al. (1999) The geranylgeranyltransferase I inhibitor GGTI-298 induces hypophosphorylation of retinoblastoma and partner switching of cyclin- dependent kinase inhibitors. A potential mechanism for GGTI-298 antitumor activity. J. Biol. Chem. 274, 6930–6934

66 Sun, J. et al. (2003) Geranylgeranyltransferase I inhibitor GGTI-2154 induces breast carcinoma apoptosis and tumor regression in H-Ras transgenic mice. Cancer Res. 63, 8922–8929

67 Vogt, A. et al. (1997) The geranylgeranyltransferase-I inhibitor GGTI-298 arrests human tumor cells in G0/G1 and induces p21(WAF1/CIP1/SDI1) in a p53- independent manner. J. Biol. Chem. 272, 27224–27229

68 Woodward, J.K. et al. (2005) Preclinical evidence for the effect of bisphosphonates and cytotoxic drugs on tumor cell invasion. Anticancer Drugs 16, 11–19

69 Diel, I.J. (2001) Bisphosphonates in the prevention of bone metastases: current evidence. Semin. Oncol. 28, 75–80

70 Aapro, M. et al. (2008) Guidance on the use of bisphosphonates in solid tumors: recommendations of an international expert panel. Ann. Oncol. 19, 420–432

71 Gralow, J. and Tripathy, D. (2007) Managing metastatic bone pain: the role of bisphosphonates. J. Pain Symptom Manage. 33, 462–472

72 Gnant, M. et al. (2009) Endocrine therapy plus zoledronic acid in premenopausal breast cancer. N. Engl. J. Med. 360, 679–691

73 Wang, H. et al. (1997) Inhibition of growth and p21ras methylation in vascular endothelial cells by homocysteine but not cysteine. J. Biol. Chem. 272, 25380–25385

74 Winter-Vann, A.M. et al. (2003) Targeting Ras signaling through inhibition of carboxyl methylation: an unexpected property of methotrexate. Proc. Natl. Acad. Sci. U. S. A. 100, 6529–6534

75 Winter-Vann, A.M. and Casey, P.J. (2005) Post-prenylation-processing enzymes as new targets in oncogenesis. Nat. Rev. Cancer 5, 405–412

76 Ciardiello, F. and Tortora, G. (2008) EGFR antagonists in cancer treatment. N. Engl. J. Med. 358, 1160–1174

77 Li, S. et al. (2005) Structural basis for inhibition of the epidermal growth factor receptor by cetuximab. Cancer Cell 7, 301–311

78 Imai, K. and Takaoka, A. (2006) Comparing antibody and small-molecule therapies for cancer. Nat. Rev. Cancer 6, 714–727

79 Benvenuti, S. et al. (2007) Oncogenic activation of the RAS/RAF signaling pathway impairs the response of metastatic colorectal cancers to anti-epidermal growth factor receptor antibody therapies. Cancer Res. 67, 2643–2648

80 Bibeau, F. et al. (2009) Impact of FcgRIIa–FcgRIIIa polymorphisms and KRAS mutations on the clinical outcome of patients with metastatic colorectal cancer treated with cetuximab plus irinotecan. J. Clin. Oncol. 27, 1122–1129

81 Bokemeyer, C. et al. (2009) Fluorouracil, leucovorin, and oxaliplatin with and without cetuximab in the first-line treatment of metastatic colorectal cancer. J. Clin. Oncol. 27, 663–671

82 De Roock, W. et al. (2008) KRAS wild type state predicts survival and is associated to early radiological response in metastatic colorectal cancer treated with cetuximab. Ann. Oncol. 19, 508–515

83 Di Nicolantonio, F. et al. (2008) Wild type BRAF is required for response to panitumumab or cetuximab in metastatic colorectal cancer. J. Clin. Oncol. 26, 5705–5712

84 Garm Spindler, K.L. et al. (2009) The importance of KRAS mutations and EGF61A > G polymorphism to the effect of cetuximab and irinotecan in metastatic colorectal cancer. Ann. Oncol. 20, 879–884

85 Lievre, A. et al. (2006) KRAS mutation status is predictive of response to cetuximab therapy in colorectal cancer. Cancer Res. 66, 3992–3995

86 Lievre, A. et al. (2008) KRAS mutations as an independent prognostic factor in patients with advanced colorectal cancer treated with cetuximab. J. Clin. Oncol. 26, 374–379

87 Prenen, H. et al. (2009) PIK3CA mutations are not a major determinant of resistance to the epidermal growth factor receptor inhibitor cetuximab in metastatic colorectal cancer. Clin. Cancer Res. 15, 3184–3188

88 Tol, J. et al. (2009) Chemotherapy, bevacizumab, and cetuximab in metastatic

colorectal cancer. N. Engl. J. Med. 360, 563–572

89 Allegra, C.J. et al. (2009) American Society of Clinical Oncology Provisional Clinical Opinion: testing for KRAS gene mutations in patients with metastatic colorectal carcinoma to predict response to anti-epidermal growth factor receptor monoclonal antibody therapy. J. Clin. Oncol. 27, 2091–2096

90 Linardou, H. et al. (2008) Assessment of somatic KRAS mutations as a mechanism associated with resistance to EGFR-targeted agents: a systematic review and meta- analysis of studies in advanced non-small-cell lung cancer and metastatic colorectal cancer. Lancet Oncol. 9, 962–972

91 Douillard, J. et al. (2009) Phase III study (PRIME/20050203) of panitumumab with FOLFOX4 compared to FOLFOX4 alone in patients with previously untreated metastatic colorectal cancer (mCRC): preliminary safety data. ASCO Meeting Abstracts

92 Mantha, A.J. et al. (2005) Targeting the mevalonate pathway inhibits the function of the epidermal growth factor receptor. Clin. Cancer Res. 11, 2398–2407

93 Bos, J.L. (1989) ras oncogenes in human cancer: a review. Cancer Res. 49, 4682–4689

94 Lopez-Aguilar, E. et al. (2008) Phase II study of metronomic chemotherapy with thalidomide, carboplatin–vincristine–fluvastatin in the treatment of brain stem tumors in children. Arch. Med. Res. 39, 655–662

95 Knox, J.J. et al. (2005) A phase I trial of prolonged administration of lovastatin in patients with recurrent or metastatic squamous cell carcinoma of the head and neck or of the cervix. Eur. J. Cancer 41, 523–530

96 Lersch, C. et al. (2004) Treatment of HCC with pravastatin, octreotide, or gemcitabine – a critical evaluation. Hepatogastroenterology 51, 1099–1103

97 Kim, W.S. et al. (2001) Phase II study of high-dose lovastatin in patients with advanced gastric adenocarcinoma. Invest. New Drugs 19, 81–83

98 Kawata, S. et al. (2001) Effect of pravastatin on survival in patients with advanced hepatocellular carcinoma. A randomized controlled trial. Br. J. Cancer 84, 886–891

99 Larner, J. et al. (1998) A phase I-II trial of lovastatin for anaplastic astrocytoma and glioblastoma multiforme. Am. J. Clin. Oncol. 21, 579–583

100 Thibault, A. et al. (1996) Phase I study of lovastatin, an inhibitor of the mevalonate pathway, in patients with cancer. Clin. Cancer Res. 2, 483–491

101 Li, T. et al. (2009) Phase II trial of the farnesyltransferase inhibitor tipifarnib plus fulvestrant in hormone receptor-positive metastatic breast cancer: New York Cancer Consortium Trial P6205. Ann. Oncol. 20, 642–647

102 Lustig, R. et al. (2008) Phase II preradiation R115777 (tipifarnib) in newly diagnosed GBM with residual enhancing disease. Neuro-oncology 10, 1004–1009

103 Eckhardt, S.G. et al. (2009) Patient-reported outcomes as a component of the primary endpoint in a double-blind, placebo-controlled trial in advanced pancreatic cancer. J. Pain Symptom Manage. 37, 135–143

104 Ravoet, C. et al. (2008) Farnesyl transferase inhibitor (lonafarnib) in patients with myelodysplastic syndrome or secondary acute myeloid leukaemia: a phase II study. Ann. Hematol. 87, 881–885

105 Feldman, E.J. et al. (2008) On the use of lonafarnib in myelodysplastic syndrome and chronic myelomonocytic leukemia. Leukemia 22, 1707–1711

106 Karp, J.E. et al. (2008) Phase II trial of tipifarnib as maintenance therapy in first complete remission in adults with acute myelogenous leukemia and poor-risk features. Clin. Cancer Res. 14, 3077–3082

107 Fouladi, M. et al. (2007) A phase II study of the farnesyl transferase inhibitor, tipifarnib, in children with recurrent or progressive high-grade glioma, medulloblastoma/primitive neuroectodermal tumor, or brainstem glioma:

a Children's Oncology Group study. Cancer 110, 2535–2541

108 Johnston, S.R. et al. (2003) Phase II study of the efficacy and tolerability of two dosing regimens of the farnesyl transferase inhibitor, R115777, in advanced breast cancer. J. Clin. Oncol. 21, 2492–2499

109 Harousseau, J.L. et al. (2007) A phase 2 study of the oral farnesyltransferase inhibitor tipifarnib in patients with refractory or relapsed acute myeloid leukemia. Blood 109, 5151–5156

110 Cloughesy, T.F. et al. (2006) Phase II trial of tipifarnib in patients with recurrent malignant glioma either receiving or not receiving enzyme-inducing antiepileptic drugs: a North American Brain Tumor Consortium Study. J. Clin. Oncol. 24, 3651–3656

111 Whitehead, R.P. et al. (2006) Phase II trial of R115777 (NSC #70818) in patients with advanced colorectal cancer: a Southwest Oncology Group study. Invest. New Drugs 24, 335–341

112 Borthakur, G. et al. (2006) Pilot study of lonafarnib, a farnesyl transferase inhibitor, in patients with chronic myeloid leukemia in the chronic or accelerated phase that is resistant or refractory to imatinib therapy. Cancer 106, 346–352

113 Macdonald, J.S. et al. (2005) A phase II study of farnesyl transferase inhibitor R115777 in pancreatic cancer: a Southwest oncology group (SWOG 9924) study. Invest. New Drugs 23, 485–487

114 Kim, E.S. et al. (2005) Phase II study of the farnesyltransferase inhibitor lonafarnib with paclitaxel in patients with taxane-refractory/ resistant nonsmall cell lung carcinoma. Cancer 104, 561–569

115 Theodore, C. et al. (2005) Multicentre EORTC study 16997: feasibility and phase II trial of farnesyl transferase inhibitor & gemcitabine combination in salvage treatment of advanced urothelial tract cancers. Eur. J. Cancer 41, 1150–1157

116 Winquist, E. et al. (2005) A multinomial Phase II study of lonafarnib (SCH 66336) in patients with refractory urothelial cancer. Urol. Oncol. 23, 143–149

117 Rosenberg, J.E. et al. (2005) A phase II trial of R115777, an oral farnesyl transferase inhibitor, in patients with advanced urothelial tract transitional cell carcinoma. Cancer 103, 2035–2041

118 Rao, S. et al. (2004) Phase III double-blind placebo-controlled study of farnesyl transferase inhibitor R115777 in patients with refractory advanced colorectal cancer. J. Clin. Oncol. 22, 3950–3957

119 Heymach, J.V. et al. (2004) Phase II study of the farnesyl transferase inhibitor R115777 in patients with sensitive relapse small-cell lung cancer. Ann. Oncol. 15, 1187–1193

120 Van Cutsem, E. et al. (2004) Phase III trial of gemcitabine plus tipifarnib compared with gemcitabine plus placebo in advanced pancreatic cancer. J. Clin. Oncol. 22, 1430–1438

121 Kurzrock, R. et al. (2004) Phase II study of R115777, a farnesyl transferase inhibitor, in myelodysplastic syndrome. J. Clin. Oncol. 22, 1287–1292

122 Alsina, M. et al. (2004) Farnesyltransferase inhibitor tipifarnib is well tolerated, induces stabilization of disease, and inhibits farnesylation and oncogenic/tumor survival pathways in patients with advanced multiple myeloma. Blood 103, 3271–3277

123 Adjei, A.A. et al. (2003) Phase II study of the farnesyl transferase inhibitor R115777 in patients with advanced non-small-cell lung cancer. J. Clin. Oncol. 21, 1760–1766

124 Cohen, S.J. et al. (2003) Phase II and pharmacodynamic study of the farnesyltransferase inhibitor R115777 as initial therapy in patients with metastatic pancreatic adenocarcinoma. J. Clin. Oncol. 21, 1301–1306

125 Cortes, J. et al. (2003) Efficacy of the farnesyl transferase inhibitor R115777 in chronic myeloid leukemia and other hematologic malignancies. Blood 101, 1692–1697

126 Sharma, S. et al. (2002) A phase II trial

of farnesyl protein transferase inhibitor SCH 66336, given by twice-daily oral administration, in patients with metastatic colorectal cancer refractory to 5-fluorouracil and irinotecan. Ann. Oncol. 13, 1067– 1071

127 James, N.D. et al. (2009) Systemic therapy for advancing or metastatic prostate cancer (STAMPEDE): a multi-arm, multistage randomized controlled trial. BJU Int. 103, 464–469

128 Diel, I.J. et al. (2008) Adjuvant oral clodronate improves the overall survival of primary breast cancer patients with micrometastases to the bone marrow: a long- term follow-up. Ann. Oncol. 19, 2007–2011

129 Kristensen, B. et al. (2008) Bisphosphonate treatment in primary breast cancer: results from a randomised comparison of oral pamidronate versus no pamidronate in patients with primary breast cancer. Acta Oncol. 47, 740–746

130 Kattan, J.G. et al. (2008) Weekly docetaxel, zoledronic acid and estramustine in hormone-refractory prostate cancer (HRPC). Invest. New Drugs 26, 75–79

131 Mason, M.D. et al. (2007) Oral sodium clodronate for nonmetastatic prostate cancer – results of a randomized double-blind placebo-controlled trial: Medical Research Council PR04 (ISRCTN61384873). J. Natl. Cancer Inst. 99, 765–776

132 Pavlu, J. et al. (2007) Dual inhibition of ras and bcr-abl signaling pathways in chronic myeloid leukaemia: a phase I/II study in patients in complete haematological remission. Br. J. Haematol. 137, 423–428

133 Di Lorenzo, G. et al. (2007) Docetaxel, vinorelbine, and zoledronic acid as first-line treatment in patients with hormone refractory prostate cancer: a phase II study. Eur. Urol. 52, 1020–1027

134 Di Lorenzo, G. et al. (2007) Phase II trial of gemcitabine, prednisone, and zoledronic acid in pretreated patients with hormone refractory prostate cancer. Urology 69, 347–351

135 Mitsiades, C.S. et al. (2006) Randomized controlled clinical trial of a combination of somatostatin analog and dexamethasone plus zoledronate vs. zoledronate in patients with androgen ablation-refractory prostate cancer. Anticancer Res. 26, 3693–3700

136 Lewis, K.D. et al. (2006) A phase II open-label trial of apomine (SR-45023A) in patients with refractory melanoma. Invest. New Drugs 24, 89–94

137 Bertelli, G. et al. (2006) Weekly docetaxel and zoledronic acid every 4 weeks in hormone-refractory prostate cancer patients. Cancer Chemother. Pharmacol. 57, 46–51

138 Figg, W.D. et al. (2005) A randomized, phase II trial of ketoconazole plus alendronate versus ketoconazole alone in patients with androgen independent prostate cancer and bone metastases. J. Urol. 173, 790–796

139 Tiffany, N.M. et al. (2004) Imatinib mesylate and zoledronic acid in androgen- independent prostate cancer. Urology 63, 934–939

140 Dearnaley, D.P. et al. (2003) A double-blind, placebo-controlled, randomized trial of oral sodium clodronate for metastatic prostate cancer (MRC PR05 Trial). J. Natl. Cancer Inst. 95, 1300–1311

141 Mardiak, J. et al. (2000) Adjuvant clodronate therapy in patients with locally advanced breast cancer – long term results of a double blind randomized trial. Slovak Clodronate Collaborative Group. Neoplasma 47, 177–180

142 Peeters, M. et al. (2009) Randomized phase 3 study of panitumumab with FOLFIRI vs FOLFIRI alone as second-line treatment (tx) in patients (pts) with metastatic colorectal cancer (mCRC). Eur. J. Cancer Suppl. 7, 9

143 Van Cutsem, E. et al. (2008) Kras status and efficacy in the Crystal Study: 1st-line treatment of patients with metastatic colorectal cancer (mCRC) receiving FOLFIRI with or without cetuximab. Ann. Oncol. 19 (Suppl. 8), viii4

144 Van Cutsem, E. et al. (2009) Cetuximab and chemotherapy as initial treatment for metastatic colorectal cancer. N. Engl. J. Med. 360, 1408–1417

145 Di Fiore, F. et al. (2007) Clinical relevance of KRAS mutation detection in metastatic colorectal cancer treated by Cetuximab plus chemotherapy. Br. J. Cancer 96, 1166–1169

146 Frattini, M. et al. (2007) PTEN loss of expression predicts cetuximab efficacy in metastatic colorectal cancer patients. Br. J. Cancer 97, 1139–1145

147 Hecht, J.R. et al. (2009) A randomized phase IIIB trial of chemotherapy, bevacizumab, and panitumumab compared with chemotherapy and bevacizumab alone for metastatic colorectal cancer. J. Clin. Oncol. 27, 672–680

148 Laurent-Puig, P. et al. (2007) Kras mutations in colorectal cancer is a predictive factor of response and survival in patient treated with cetuximab. Ann. Oncol. 18, vii81

149 Moroni, M. et al. (2005) Gene copy number for epidermal growth factor receptor (EGFR) and clinical response to antiEGFR treatment in colorectal cancer: a cohort study. Lancet Oncol. 6, 279–286

150 Loupakis, F. et al. (2008) Analysis of Pten expression and Kras mutations on primaries (prim) and metastases (mets) to predict benefit from cetuximab plus irinotecan (Cetiri) in metastatic colorectal cancer (mCRC) patients (pts). Ann. Oncol. 19 (Suppl. 8), viii133–viii134

151 Cappuzzo, F. et al. (2008) Primary resistance to cetuximab therapy in EGFR FISH- positive colorectal cancer patients. Br. J. Cancer 99, 83–89

152 Finocchiaro, G. et al. (2007) EGFR, HER2 and Kras as predictive factors for cetuximab sensitivity in colorectal cancer. J. Clin. Oncol. 25, 4021

153 Freeman, D.J. et al. (2008) Association of KRAS mutational status and clinical outcomes in patients with metastatic colorectal cancer receiving panitumumab alone. Clin. Colorectal Cancer 7, 184–190

154 Tabernero, J. et al. (2008) Correlation of efficacy to KRAS status (wt vs. mut) in patients (pts) with metastatic colorectal cancer (mCRC), treated with weekly (q1w) and q2w schedules of cetuximab combined with FOLFIRI. Presented at the 2008 Gastrointestinal Cancers symposium of the American Society of Clinical Oncology (ASCO), 25–27 January 2008, Orlando, USA

Chapter 3

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Simvastatin in G13D KRAS mutated colorectal cancer cells render cells susceptible for cetuximab

Abstract

Introduction

Statins are commonly used to reduce cholesterol levels and lower the cardiovascular risk. Beside cholesterol, also the formation of farnesylpyrophophate (a C15-group) and geranylgeranylpyrophosphate (a C17-group) are inhibited. These groups are used to activate the KRAS protein by prenylation (addition of a C15 or C17 group). After prenylation, KRAS becomes more lipophilic and translocates from the cytosol to the membrane. In this study, we hypothesized that the cetuximab resistant phenotype of *KRAS* mutant cancer cells could be converted to a more KRAS wild type phenotype rendering the cells susceptible for cetuximab, by co-incubating cells with simvastatin.

Method

Survival assays to measure proliferation were performed to study the effect of simvastatin, cetuximab and combination in the *KRAS* wild type A431 and KRAS mutant LoVo, HCT116 and SW480 cell lines. Furthermore KRAS localization assays as well as kinase activity assays were performed for all cell lines, to further explore the potential mechanisms.

Results

Simvastatin combined with cetuximab resulted in decreased proliferation in *KRAS* codon 13 mutated cell lines. Especially in the *KRAS* G13D mutant LoVo cells a synergistic effect on inhibition of proliferation by the combination treatment was observed. After incubation with simvastatin more KRAS protein was situated in the cytoplasm in *KRAS* mutant cells compared to control cells. The EGFR target pathway is controlled by tyrosine kinase activities, which showed synergistic inhibition in LoVo cells. Simvastatin elevated Serine/Threonine kinase activities, including AKT and NOS phosphosites, which is in accordance to previous reports.

Conclusion

In summary, we observed a synergistic effect of adding simvastatin to cetuximab treatment in inhibition of *KRAS* codon 13 mutated CRC cell proliferation. The mechanism of action appears to involve aberrant translocation of KRAS likely due to lack of prenylation and enhanced inhibition of tyrosine kinase signaling.

Introduction

Epidermal growth factor receptor (EGFR) signaling plays an important role in proliferation of cells, and therefore blocking this pathway has emerged as an effective drug target in oncology. Cetuximab and panitumumab are two registered EGFR antibodies. Patients with advanced or metastasized colorectal carcinoma (CRC) failing fluorouracil (or alternatives such as capecitabine or uracil and tegafur, oxaliplatin and irinotecan) can be treated with chemotherapy combined with one of these anti-EGFR monoclonal antibodies. However, cetuximab and panitumumab have both been registered for the use in patients with *KRAS* wild type tumors only, since retrospective analyses of clinical trials showed a lack of efficacy in *KRAS* mutated colorectal tumors. Unfortunately, approximately 40% of patients with colorectal cancer have a somatic *KRAS* mutation, which leads to a constant expression of KRAS protein, operating independently of EGFR and thus EGFR antibody resistance. As a result, a considerable group of CRC patients is excluded from therapy with cetuximab or panitumumab and does not benefit from EGFR antibody treatment [1].

KRAS can be mutated at several positions, 90 % of the activating *KRA*S mutations occur at codon 12 and 13[2]. Membrane association of the KRAS protein is crucial for its function as a switch in the signal transduction pathway between EGFR and the nucleus. To achieve this, the hydrophilic KRAS protein is farnesylated (addition of farnesylpyrophosphate, a farnesylgroup), or geranylgeranylated (addition of a geranylgeranylpyrophosphate, a geranylgeranylgroup). As a result, KRAS becomes more lipophilic and translocates from the cytosol to associate with the cell membrane. This so-called prenylated active KRAS exerts its function in the cellular membrane [3,4]. Statins are known to inhibit the conversion of 3-hydroxy-3-methyl-glutaryl Co-enzyme A (HMG-CoA) to mevalonate, a precursor for cholesterol synthesis; this reduces the cholesterol synthesis and thus the formation of low-density lipoprotein. Beyond their lipid lowering effects, statins are extensively studied for their effects on cellular proliferation in cancer. As shown in *in vitro* studies, simvastatin affects angiogenesis, apoptosis as well as the inflammation processes. [5-8] Another important effect of statins, although less studied, is the interference with the formation of farnesyl- and geranylgeranylgroups. These groups are formed as part of the mevalonate cascade and are crucial for the prenylation of proteins, such as KRAS. Since farnesyl- and geranylgeranyl moieties are essential for post-translational prenylation, and thus for activation of KRAS. Statins may have the potential to phenoconvert *KRAS* mutated tumors into a more KRAS wild type and thus EGFR inhibitor sensitive phenotype [9,10].

In this proof of concept study we hypothesized that phenoconversion of *KRAS* mutant colorectal cancer cells could be achieved by simvastatin, rendering colorectal cancer cells sensitive for cetuximab. In this study, cell growth survival assays were used to investigate a possible synergistic effect of simvastatin on cetuximab sensitivity in several *KRAS* mutant colorectal cancer cell lines and in wild type EGFR overexpressing cell line. The effect of simvastatin on membrane association of KRAS was investigated, and in addition, overall kinase activity upon treatment was explored.

Material and Methods

Cell lines

The human colorectal cancer cell lines LoVo (*KRAS* mutation G13D), HCT116 (*KRAS* mutation G13D), SW480 (*KRAS* mutation G12V), and the human epidermoid carcinoma cell line A431 (wild type for *KRAS* and over-expressing EGFR) were obtained from American Type Culture Collection-ATCC (Manassas VA, USA). These cell lines were selected using the information about *KRAS* mutations in the Cancer Genome Project (http://www.sanger.ac.uk/genetics/CGP/CellLines/). Cell lines harbouring a *KRAS* mutation in codon 12 or 13 were selected because these mutations are most common in colorectal cancer. Cells were grown in RPMI 1640 medium (Invitrogen, Breda, The Netherlands) supplemented with 10 % fetal bovine serum (Greiner Bio-One GmbH, Frickenhausen, Germany) and 1% penicillin/streptomycin (Invitrogen, Breda, The Netherlands) in a 5% CO_2 atmosphere at 37 °C. When applicable, cells were detached from flasks with Trypsin-EDTA solution (Invitrogen, Breda, the Netherlands). Cells were cultured for a maximum of 20 passages. Cetuximab was kindly provided by Merck (Darmstadt, Germany). Simvastatin was obtained from Fagron (Nieuwerkerk a/d IJssel, The Netherlands) and was chemically activated by alkaline hydrolysis prior to use as described before [11]. Simvastatin was selected because this statin is most commonly prescribed in Europe.

Cell survival

The effects of simvastatin and cetuximab as single agents or in combination on survival of the cells, were evaluated using the sulphorhodamine binding (SRB) colorimetric assay as described by Skehan et al. [12] with minor modifications. Cells were harvested by trypsinization, counted and plated in 96-well plates at a density of 5 x 10^3 cells per well (100 µL/well). Following overnight incubation, cells were pre-treated with simvastatin (2 µM). After 24 hours of incubation, cells were co-treated with cetuximab (500 µg/ml) and incubated for another 48 hours. At the end of the incubation, the SRB assay was performed as described below. Cells were fixed by addition of 25 µl ice-cold 50% trichloroacetic acid (TCA) to the growth medium. The plate was incubated at 4°C for 1 hour and then the cells were gently washed three times with milli-Q water. After drying at room temperature, cells were stained with 50 µl of 4% sulphorhodamine (w:v dissolved in 1% acetic acid) for 30 min. At the end of the staining period, unbound sulphorhodamine was removed by washing three times with 1% acetic acid. The plates were air dried and bound SRB was dissolved in 200 µl of 10 mM Tris-base (pH 10.5). Next, the plate was shaken followed by reading the optical density (OD) at 550 nm in a microplate spectrophotometer (Spectramax 190, Molecular Devices, Sunnyvale, USA). Results are expressed as the relative percentages of absorbance compared to controls, which were not exposed to drugs. Results of OD measurements are expressed as a percentage of cell proliferation of the controls. Results are expressed as means with corresponding standard deviations of three independent experiments in at least fourfold. Statistical analysis was performed using SPSS, a two-sided t-test were performed and p-values <0.05 were considered significant.

Membrane association of KRAS

Cell were seeded in a 6 wells plate at a density of 5 x 10^5cells per well. After overnight incubation, cells were incubated for 24 hours according to the following conditions: vehicle as negative control, 2 µM simvastatin or with 2 µM of the geranylgeranyltransferase inhibitor GGTI-298 (Merck, Darmastadt, Germany). GGTI-298 is known to inhibit prenylation and therefore taken

as positive control. Next, cells were trypsinized and membrane and cytoplasm fractions were separated using Subcellular Protein Fractionation Kit for Cultured Cells (Thermo Scientific, Breda, The Netherlands). Protein concentration was determined using BCA protein assay kit (Thermo Scientific, Breda, The Netherlands). Presence of KRAS protein in membrane and cytoplasm fractions was analysed by western blotting. Equal amounts of cell lysates were loaded on 11% SDS-PAGE gels. After blotting on nitrocellulose membrane, the blot was cut in two to detect KRAS and Actin (as internal control) in the same lane. Primary antibodies against KRAS (RAS (D2C1), Cell Signaling Technology, Leiden, The Netherlands) and Actin (actin (13E5), Cell Signaling Technology, Leiden, The Netherlands)) were used in this study. Anti-rabbit IgG, HRP-linked antibody and LumiGlo (Cell Signaling Technology, Leiden, The Netherlands) was used to visualize the proteins bands on Chemidoc from Biorad (Veenendaal, the Netherlands).

Kinase activity profiling

Cells, counted and plated in 6-wells plates at a density of 1 x 10^6 cells per well (2 mL/well) were harvested by trypsinization. Following overnight incubation, cells were pre-treated with 2.0 μM simvastatin or vihicle for 24 hours and another 24 hours with wicicle, simvastatin, cetucimab or combination. At the end of the incubation, cells were lysed using M-PER Lysis buffer (Fischer Thermo Scientific, Rockford, USA) supplemented with Protease inhibitor Cocktail and phosphatase Inhibitor cocktail (Fischer Thermo Scientific, Rockford, USA) (1 million cells per 100 μl). The protein content was determined with Pierce BCA Protein Assay Kit (Thermo Scientific, Breda, The Netherlands). Protein serine/threonine kinase activity and tyrosine kinase activity was determined in triplicate using Pamgene's Serine/Threonine Kinase (STK) peptide or tyrosine kInase (PTK) microarrays (Pamgene, 's-Hertogenbosch, the Netherlands), according to the manufacturers' instructions [13]. All microarray data processing and visualizations were performed using Bionavigator and Matlab software (R2010B, The Mathworks, Natick, MA) as described before[14]. For the serine/threonine kinase assay 109 peptides were included in the analysis, for the PTK assay, 89 peptides were included and analysed in the R-package 'multcomp' using Dunnett contrasts. For pathway analysis, substrate peptides that showed a significantly different ($p<0.05$) phosphorylation pattern between vehicle and simvastatin, cetuximab or combination were used as input to identify pathways with differential activity using GeneGo Metacore (Thomson Reuters). The peptides were linked to the UniProt ID's of the proteins that they were derived from Uni-Prot database (www.expasy.org) and these were used for a pathway analysis.

Results

Effect of simvastatin and cetuximab on survival of KRAS mutant and wild type cell lines

The cytotoxicity of simvastatin was first tested in a concentration range from 0.1 to 2.0 μM (data not shown). A minimal cytotoxic effect was observed and therefore a concentration of 2.0 μM of simvastatin was used in the experiments to inhibit the mevalonate pathway and the formation of farnesyl- and geranylgeranylgroups. Incubation of the *KRAS* wild type A431 cell line with 500 μg/ml cetuximab resulted in a decrease of survival to 66 ± 3.3 % compared to untreated cells ($p=0.045$) (figure 1). The combination of both simvastatin and cetuximab diminished cell growth further to 56 ± 4.1 %, but this was not significantly different from the growth inhibitory effect of cetuximab as single agent ($p=0.228$). Incubation of LoVo and HCT116 cells, both

harbouring a KRAS G13D mutation, with 500 µg/ml cetuximab resulted in a non-significant survival reduction of 84 ± 11.1 % and 99 ± 5.5 %, respectively compared to control. In these cell lines, compared to control, simvastatin monotherapy (2 µM) resulted in a survival of 76 ± 9.0 % for LoVo and 84 ± 7.5 % for HCT116. Combined treatment with simvastatin and cetuximab reduced the proliferation in LoVo further to 47 ± 4.9 %, which is significantly different from the growth inhibitory effect of either cetuximab ($p<0.001$) or simvastatin ($p<0.001$) as single agents. In HCT116 the same synergistic effect was seen, combination therapy significantly reduced the survival to 68 ± 5.6 % compared to simvastatin alone ($p<0.002$) or cetuximab alone ($p<0.001$). No synergistic effect was observed in SW480 cells (*KRAS* G12V) when treated with simvastatin and cetuximab as well. Incubation with 500 µg/ml cetuximab did not result in a decrease of survival (102 ± 1.7 % $p=1.00$). Simvastatin (2 µM) as single agent resulted in survival of 79 ± 7.5%. Compared to treatment with simvastatin alone, the combination with cetuximab, did not result in an additional reduction in the survival of the SW480 cells (73 ± 10.9 %).

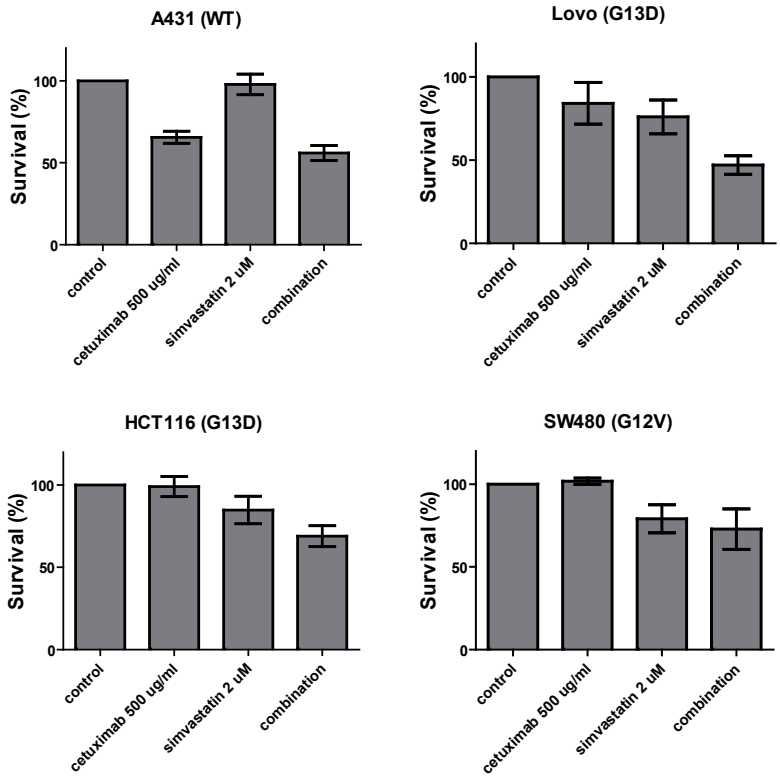
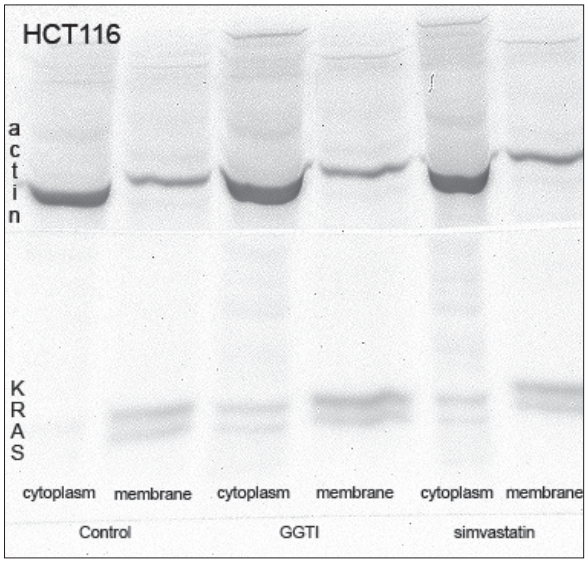


Figure 1: Cell proliferation assays (Sulforhodamine-B assays) of three *KRAS* mutant (LoVo, HCT 116 and SW480) and one *KRAS* wild type cell line treated with simvastatin, cetuximab or the combination for three days. The results of the measured optical density are expressed as a percentage of cell proliferation compared to the untreated controls. Results are means and the 95 % confidence intervals of three independent experiments in at least fourfold.

KRAS translocation

To test whether the observed synergistic effect was the result of decreased KRAS activation, the effect of simvastatin on the prenylation of mutated *KRAS* was analysed in LoVo and HCT116 cells. Membrane and cytoplasm fractions of cells incubated with simvastatin were tested for KRAS localization. In both cell lines, KRAS was mainly localized in membranes of the cell. Upon simvastatin treatment, an increase of KRAS concentrations in the cytoplasm of LoVo and HCT116 cells was observed (figure 2a and 2b). This was also seen in presence of the positive control GGTI-298 (Sigma-Aldrich, the Netherlands) an established prenylation inhibitor.



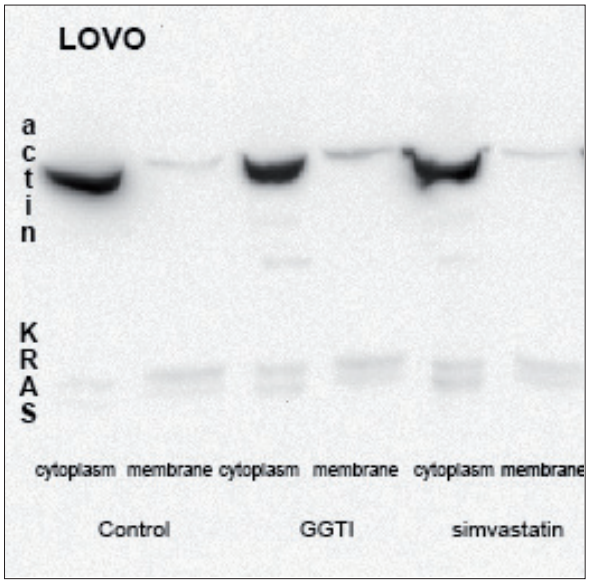


Figure 2 a and b: Western blot analysis showing the amount of KRAS in the cytoplasm and membrane after treatment with the positive control GGTI-298 or simvastatin in KRAS mutant G13D HCT116 cells (a) and LoVo cells (b).

Kinase activity profiling

As described above, a clear synergistic effect of simvastatin on cetuximab treatment was observed in LoVo cells. Therefore, PTK and STK activity was explored for these cells at different treatment conditions. STK activity reflects downstream signaling of KRAS. In LoVo cells, treatment with cetuximab or simvastatin as single agents display a strong activated STK activity, nearly all peptides on the STK array became significantly phosphorylated. Because of this strong effect, no conclusion could be drawn. PTK activity reflects EGFR signaling, upstream of KRAS. Cetuximab and simvastatin as single agents showed only a slight effect on PTK activity, however, combination of both drugs show a clear synergistic effect displayed by decreased PTK activity (figure 3A and 3B). In all other cell lines (A431, HCT116 and SW480), this synergy was not observed.

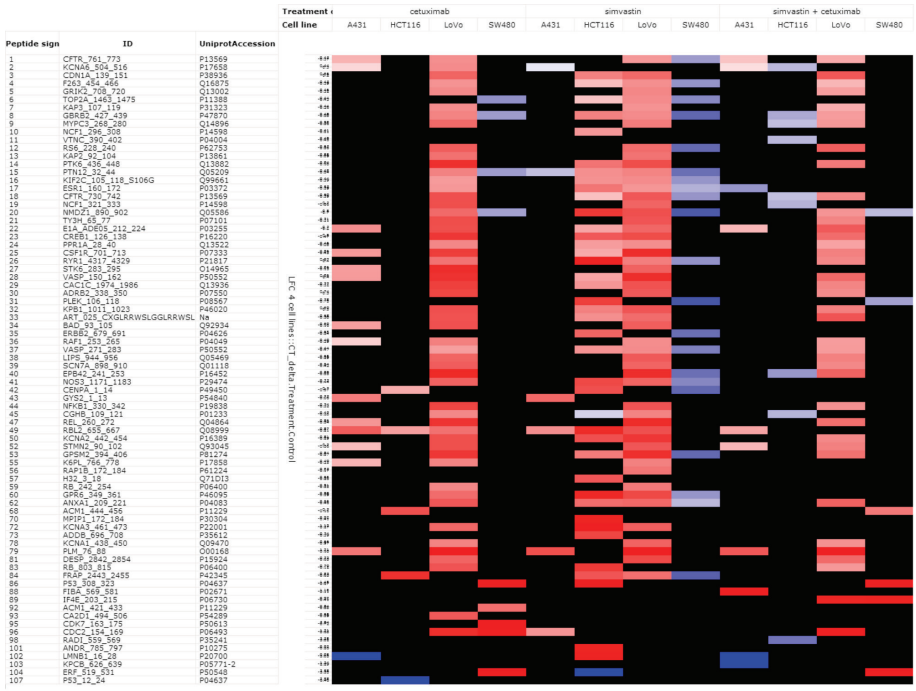


Figure 3a: STK activity profiles. Significant ($p < 0.05$) log ratio of signal intensity versus the control sample for cell lines A431, HCT116 and LoVo, treated with simvastatin, cetuximab or combination. Each column represents a sample, each row a peptide. The relative signal with respect to control is indicated by the color intensity: red implies higher, blue lower than control.

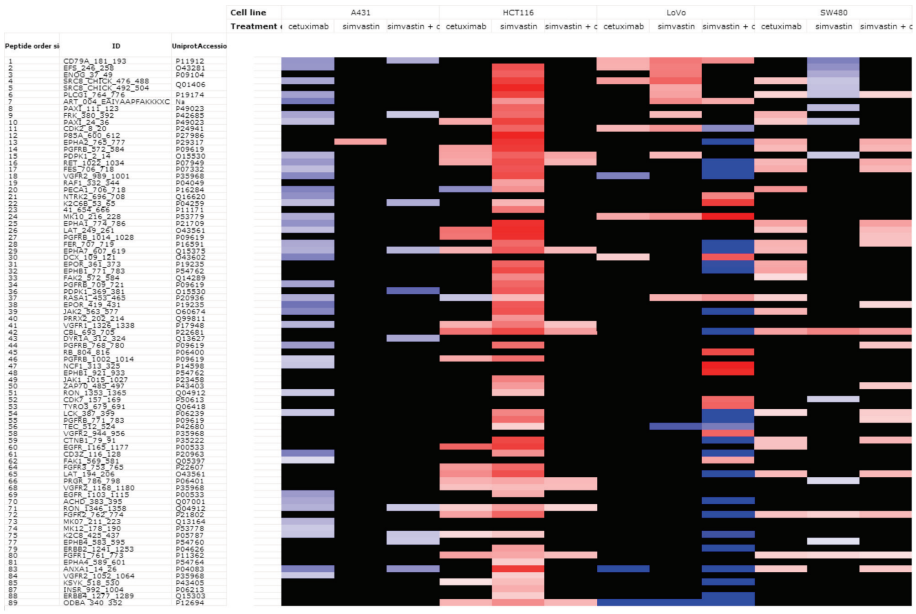


Figure 3b: PTK activity profiles. Significant ($p < 0.05$) log ratio of signal intensity versus the control sample for cell lines A431, HCT116 and LoVo, treated with simvastatin, cetuximab or combination. Each column represents a sample, each row a peptide. The relative signal with respect to control is indicated by the color intensity: red implies higher, blue lower than control.

Discussion

In this study we investigated if phenoconversion of *KRAS* mutant colorectal cancer cells by simvastatin could be achieved in order to restore sensitivity of KRAS mutant cells to anti-EGFR treatment. We also explored overall kinase activity upon treatment. Our study shows that simvastatin affects KRAS localization and that simvastatin treatment of cells results in decreased membrane association of KRAS. Furthermore, *KRAS* codon 13 mutant cells become sensitive for cetuximab in presence of simvastatin. Exploring kinase activity revealed a decrease in PTK activity in LoVo cells upon treatment with simvastatin together with cetuximab, explaining the significant decrease in cell survival.

The proliferation of *KRAS* G13D mutated LoVo and HCT116 cell lines was significantly inhibited in presence of both drugs, whereas the individual drugs have no or only a minimal effect on cell proliferation. However, in the SW480 cell line, harbouring a *KRAS* codon 12 mutation the combined therapy of cetuximab and simvastatin did not significantly affect the proliferation. This indicates that not all *KRAS* mutations in colorectal cancer cells have a similar effect on anti-EGFR sensitivity, although other mutations should also be taken into account. In a recent study computational analysis revealed that the codon 13 mutated *KRAS* protein has a similar structure compared to the wild type protein [15]. This similar structure of KRAS may contribute to difference in effect between *KRAS* 12 and 13 mutations observed in this study. Also retrospective analysis in

patients showed that patients with G13D mutant colorectal tumor might respond to cetuximab treatment[16,17]. Our findings are in agreement with the preclinical study of Lee et al. [18] who also showed that simvastatin might overcome cetuximab resistance in colorectal cancer by modulating BRAF activity, an important kinase in the RAS-RAF–MAPK signaling pathway[19]. Other studies showed that simvastatin also affects angiogenesis, apoptosis and the inflammation processes [5-8] but at higher concentrations (up to 50 μM). In our experiments a low simvastatin concentration of 2 μM was used, aiming at affecting only the prenylation of KRAS without a direct cytotoxic effect. Of note, *KRAS* mutant cells are more susceptible simvastatin than the wild type *KRAS* cell line. A possible explanation for this is a strong dependence (addiction) of the mutant cells on permanently activated KRAS and its corresponding pathways. A recent preclinical study [20] showed that simvastatin indeed inhibited the geranylgeranylation of the KRAS protein and this inhibition could be reversed with addition of geranylgeranylpyrophosphate. The geranylgeranyltransferase inhibitor GGTI-298 was used as a positive control in the Westernblot experiments. Incubation with this inhibitor indeed resulted in elevated levels of KRAS in the cytoplasm. Western blot analysis used here showed that membrane association of mutant KRAS is inhibited by simvastatin in the *KRAS* G13D cell lines LoVo and HCT116. This effect is most likely caused by a decrease in prenylation of the KRAS protein after simvastatin treatment.

The phosphorylation pattern was clearly affected by simvastatin in the *KRAS* mutated cell lines. Whereas this effect was absent in the wild type cell line. The differences in kinome profiles after simvastatin treatment also supports the aforementioned dependence on KRAS signaling in *KRAS* mutant cell lines. The results from the kinome analysis showed that simvastatin altered the KRAS dependant signaling in the cancer cell lines, especially in *KRAS* codon 13 mutated cells. Pamchip STK data revealed, most clearly seen in LoVo cells, an abundant STK activity (of which PKB/AKT). These findings are in accordance with the findings of Kureishi et al.[21]. Skaletez-Rorowski[22] showed that low doses of statins promote AKT activation while high doses result in toxicity and cell death[23]. Because of the tremendous STK activity detected in LoVo cells, more subtle effects downstream of *KRAS* could not be observed by the Pamchip array. However, the observed synergistic effect of simvastatin and cetuximab on *KRAS* codon 13 mutated cells, could well be explained by Pamchip PTK data. EGFR is a tyrosine kinase receptor of which signaling could be blocked by cetuximab. As expected, LoVo cells show a decreased PTK activity upon incubation with cetuximab. Hardly any effect was seen upon simvastatin treatment, also as expected since simvastatin was considered to affect only STKs. Interestingly, the synergistic effect of both drugs on cell survival was also seen on PTK activity. As mentioned before, conform the results of Lee et al [24] simvastatin and cetuximab synergise after BRAF (effector protein downstream of KRAS) modulation. A recent report by Prahallad et al. [25] showed that blocking mutated *BRAF* by vemurafenib resulted in feedback activation of EGFR. Indeed, these cells became sensitive for cetuximab. Taken together, their and our findings, interfering in the signaling pathway downstream of *KRAS* (by simvastatin or vemurafinib), results in feedback activation of EGFR. Consequently, PTK activity is diminished upon treatment by both drugs.

In conclusion, the cetuximab resistant phenotype of *KRAS* codon 13 mutated cell lines, could be converted to a more KRAS wild type cell line (which is moderately sensitive to cetuximab) by co-treatment with simvastatin. The mechanism behind this phenoconversion is interfering in membrane association of KRAS most likely by inhibition of prenylation of KRAS. Interfering in the pathway downstream of KRAS results in a feedback mechanism of EGFR, resulting in a synergistic effect of simvastatin on cetuximab treatment. The latter conclusion is confirmed by the observed decreased PTK activity.

References

1 Allegra CJ, Jessup JM, Somerfield MR, Hamilton SR, Hammond EH, et al. (2009) American Society of Clinical Oncology provisional clinical opinion: testing for KRAS gene mutations in patients with metastatic colorectal carcinoma to predict response to anti-epidermal growth factor receptor monoclonal antibody therapy. J Clin Oncol 27: 2091-2096.

2 Brink M, de Goeij AF, Weijenberg MP, Roemen GM, Lentjes MH, et al. (2003) KRAS oncogene mutations in sporadic colorectal cancer in The Netherlands Cohort Study. Carcinogenesis 24: 703-710.

3 Krens LL, Baas JM, Gelderblom H, Guchelaar HJ. (2010) Therapeutic modulation of KRAS signaling in colorectal cancer. Drug Discov Today 15: 502-516.

4 Graaf MR, Richel DJ, van Noorden CJ, Guchelaar HJ. (2004) Effects of statins and farnesyltransferase inhibitors on the development and progression of cancer. Cancer Treat Rev 30: 609-641.

5 Konstantinopoulos PA, Karamouzis MV, Papavassiliou AG. (2007) Post-translational modifications and regulation of the RAS superfamily of GTPases as anticancer targets. Nat Rev Drug Discov 6: 541-555.

6 Demierre MF, Higgins PD, Gruber SB, Hawk E, Lippman SM. (2005) Statins and cancer prevention. Nat Rev Cancer 5: 930-942.

7 Sassano A, Platanias LC. (2008) Statins in tumor suppression. Cancer Lett 260: 11-19.

8 Jakobisiak M, Bruno S, Skierski JS, Darzynkiewicz Z. (1991) Cell cycle-specific effects of lovastatin. Proc Natl Acad Sci U S A 88: 3628-3632.

9 Zhang FL, Casey PJ. (1996) Protein prenylation: molecular mechanisms and functional consequences. Annu Rev Biochem 65: 241-269.

10 Amado RG, Wolf M, Peeters M, Van CE, Siena S, et al. (2008) Wild type KRAS is required for panitumumab efficacy in patients with metastatic colorectal cancer. J Clin Oncol 26: 1626-1634.

11 Dai Y, Khanna P, Chen S, Pei XY, Dent P, et al. (2007) Statins synergistically potentiate 7-hydroxystaurosporine (UCN-01) lethality in human leukemia and myeloma cells by disrupting Ras farnesylation and activation. Blood 109: 4415-4423.

12 Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, et al. (1990) New colorimetric cytotoxicity assay for anticancer-drug screening. J Natl Cancer Inst 82: 1107-1112.

13 Sikkema AH, Diks SH, den Dunnen WF, ter EA, Scherpen FJ, et al. (2009) Kinome profiling in pediatric brain tumors as a new approach for target discovery. Cancer Res 69: 5987-5995.

14 Eriksson A, Kalushkova A, Jarvius M, Hilhorst R, Rickardson L, et al. (2014) AKN-028 induces cell cycle arrest, downregulation of Myc associated genes and dose dependent reduction of tyrosine kinase activity in acute myeloid leukemia. Biochem Pharmacol 87: 284-291.

15 Chen CC, Er TK, Liu YY, Hwang JK, Barrio MJ, et al. (2013) Computational analysis of KRAS mutations: implications for different effects on the KRAS p.G12D and p.G13D mutations. PLoS One 8: e55793.

16 Tejpar S, Celik I, Schlichting M, Sartorius U, Bokemeyer C, et al. (2012) Association of KRAS G13D Tumor Mutations With Outcome in Patients With Metastatic Colorectal Cancer Treated With First-Line Chemotherapy With or Without Cetuximab. J Clin Oncol 30: 3570-3577.

17 Mao C, Huang YF, Yang ZY, Zheng DY, Chen JZ, et al. (2013) KRAS p.G13D mutation and codon 12 mutations are not created equal in predicting clinical outcomes of cetuximab in metastatic colorectal cancer: a systematic review and meta-analysis. Cancer 119: 714-721.

18 Lee J, Jung KH, Park YS, Ahn JB, Shin SJ, et al. (2009) Simvastatin plus irinotecan, 5-fluorouracil, and leucovorin (FOLFIRI) as first-line chemotherapy in metastatic colorectal patients: a multicenter phase II study. Cancer

Chemother Pharmacol 64: 657-663.

19 Berndt N, Hamilton AD, Sebti SM. (2011) Targeting protein prenylation for cancer therapy. Nat Rev Cancer 11: 775-791.

20 Al-Haidari AA, Syk I, Thorlacius H. (2014) HMG-CoA reductase regulates CCL17-induced colon cancer cell migration via geranylgeranylation and RhoA activation. Biochem Biophys Res Commun 446: 68-72.

21 Kureishi Y, Luo Z, Shiojima I, Bialik A, Fulton D, et al. (2000) The HMG-CoA reductase inhibitor simvastatin activates the protein kinase Akt and promotes angiogenesis in normocholesterolemic animals. Nat Med 6: 1004-1010.

22 Skaletz-Rorowski A, Lutchman M, Kureishi Y, Lefer DJ, Faust JR, et al. (2003) HMG-CoA reductase inhibitors promote cholesterol-dependent Akt/PKB translocation to membrane domains in endothelial cells. Cardiovasc Res 57: 253-264.

23 Sassano A, Platanias LC. (2008) Statins in tumor suppression. Cancer Lett 260: 11-19.

24 Lee J, Lee I, Han B, Park JO, Jang J, et al. (2011) Effect of simvastatin on cetuximab resistance in human colorectal cancer with KRAS mutations. J Natl Cancer Inst 103: 674-688.

25 Sun C, Wang L, Huang S, Heynen GJ, Prahallad A, et al. (2014) Reversible and adaptive resistance to BRAF(V600E) inhibition in melanoma. Nature 508: 118-122.

Chapter 4

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Statin use is not associated with improved progression free survival in cetuximab treated KRAS mutant metastatic colorectal cancer patients: results from the CAIRO2 study

Abstract

Introduction

Statins may inhibit the expression of the mutant KRAS phenotype by preventing the prenylation and thus the activation of the KRAS protein. This study was aimed at retrospectively evaluating the effect of statin use on outcome in *KRAS* mutant metastatic colorectal cancer patients (mCRC) treated with cetuximab.

Method

Treatment data were obtained from patients who were treated with capecitabine, oxaliplatin bevacizumab ± cetuximab in the phase III CAIRO2 study. A total of 529 patients were included in this study, of whom 78 patients were on statin therapy.

Results

In patients with a KRAS wild type tumor (n=321) the median PFS was 10.3 vs. 11.4 months for non-users compared to statin users and in patients with a KRAS mutant tumor (n=208) this was 7.6 vs. 6.2 months, respectively. The hazard ratio (HR) for PFS for statin users was 1.12 (95% confidence interval 0.78 -1.61) and was not influenced by treatment arm, KRAS mutation status or the KRAS*statin interaction. Statin use adjusted for covariates was not associated with increased PFS (HR= 1.01, 95% confidence interval 0.71 – 1.54). In patients with a KRAS wild type tumor the median OS for non-users compared to statin users was 22.4 vs. 19.8 months and in the KRAS mutant tumor group the OS was 18.1 vs. 14.5 months. OS was significantly shorter in statin users versus non-users (HR =1.54; 95% confidence interval 1.06-2.22). However, statin use, adjusted for covariates was not associated with increased OS (HR= 1.41, 95% confidence interval 0.95 – 2.10).

Conclusion

In conclusion, the use of statins at time of diagnosis was not associated with an improved PFS in *KRAS* mutant mCRC patients treated with chemotherapy and bevacizumab plus cetuximab.

Introduction

Statins are widely prescribed to lower blood cholesterol concentration and have shown to reduce the risk of cardiovascular events and mortality [1]. In addition, the use of statins have been associated with a reduced risk of malignancies in a variety of organ sites, such as colon, rectum, lungs and liver[2]. Statins inhibit cholesterol synthesis via inhibition of the mevalonate pathway but also lower protein prenylation (figure 1). As a posttranscriptional process, protein prenylation is crucial for several cancer cell growth related proteins, such as KRAS. The KRAS protein is activated by post-translational prenylation by binding farnesyl (C15) and geranylgeranyl (C17) moieties, both products of the mevalonate pathway. After prenylation the KRAS protein becomes lipophilic and translocates to the cellular membrane to exerts its function[3].

Epidermal Growth Factor Receptor (EGFR) inhibitors, such as cetuximab and panitumumab, have shown survival benefit in combination with chemotherapy and as monotherapy in metastatic colorectal cancer (mCRC) patients [4]. Their benefit is restricted to patients with a *KRAS* exon 2 wild type tumor[5], which recently was further narrowed to *RAS* wild type exon 2-4 tumors [6]. In patients with a *KRAS* mutated tumor, the RAS pathway is permanently activated, leading to constant cell signaling and proliferation independent of the EGFR.

Statins may inhibit the expression of the mutant KRAS phenotype by preventing the prenylation of the KRAS protein and normalize the phenotype into KRAS wild type and therefore render *KRAS* mutant colorectal cancers sensitive to EGFR antibodies[7]. We hypothesize that *KRAS* mutant cetuximab treated CRC patients with concurrent statin use have a favourable outcome from EGFR therapy compared to non-users. This study was aimed at retrospectively evaluating the effect of statin use in *KRAS* mutant mCRC patients treated with cetuximab.

Materials and methods

Patients

For this analysis prospectively collected data were obtained from mCRC patients participating in the CAIRO2 study of the Dutch Colorectal Cancer Group (DCCG). Patients were randomised between capecitabine plus oxaliplatin (CAPOX) and bevacizumab, study arm A, and the same regimen plus cetuximab, study arm B (ClinicalTrials.gov NCT00208546 [8]). Cetuximab was administered at a dose of 400 mg/m^2 on the first day followed by 250 mg/m^2 weekly thereafter. Details of eligibility criteria and results have been reported elsewhere[8]. Patients with a tumor with an unknown *KRAS* mutation status were excluded from this analysis.

Drug exposure

Statin use was defined as the use of a statin at visit 0, the randomisation or at visit 1, 3 weeks after start of treatment. All statins (ATC-codes C10AAXX), commercially available in The Netherlands within the study period were included: simvastatin, pravastatin, atorvastatin, rosuvastatin and fluvastatin.

Potential confounders

Use of drugs related to progression and development of colorectal carcinoma such as non-steroidal anti-inflammatory drugs (NSAID's), aspirin, fibrates and bisphosphonates at visit 0 or 1 were considered as potential confounders. The use of these drugs was recorded. If the use of

these drugs in the study population was less then <1%, the drug was excluded from the further analysis. The use of fibrates was excluded, from the analysis because of the low prevalence (<1%).

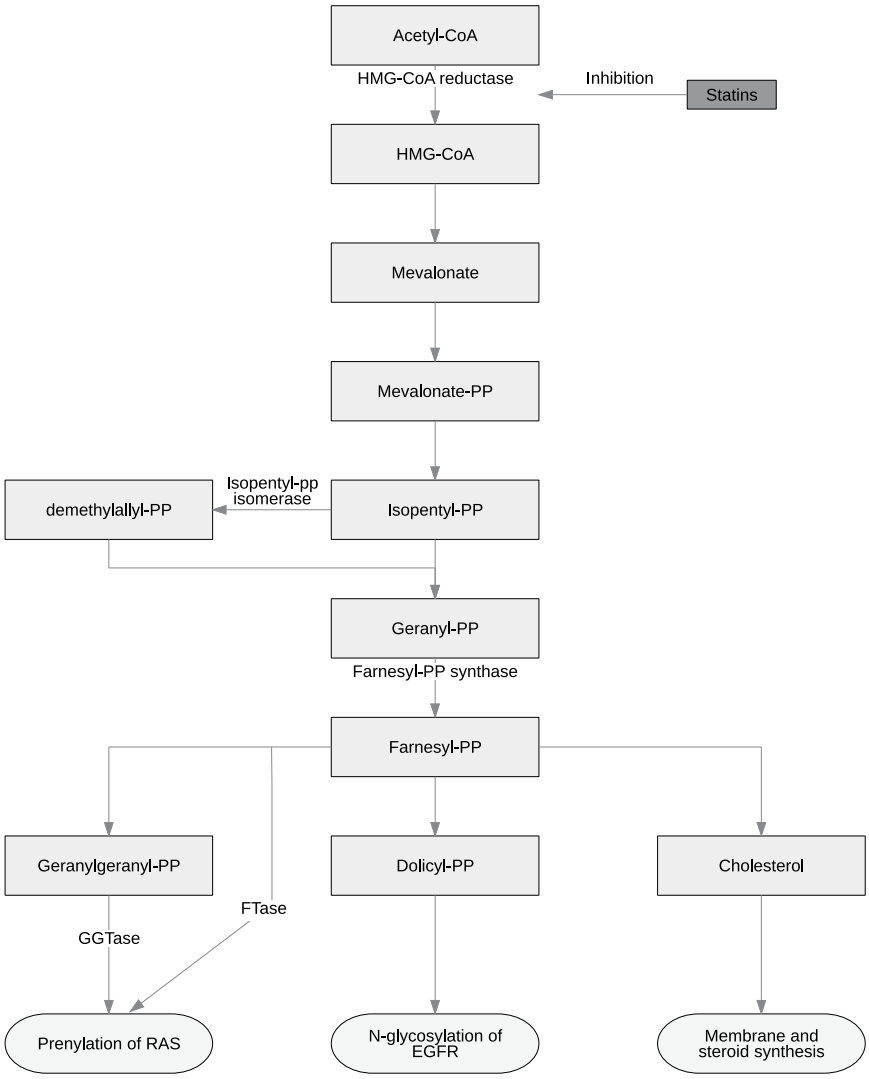

Figure 1: Overview of the mevalonate pathway and the inhibition of HMG-CoA by statins

Mevalonate pathway causes prenylation of ras, N-glycosylation of EGFR and membrane and steroid-synthesis. Statins inhibit HMG-CoA reductase and thus the prenylation of KRAS. Abbreviations: Acetyl-CoA, Acetyl coenzyme A ; EGFR, epidermal growth factor receptor; FTase, farnesyltransferase; GTase, geranylgeranyltransferase; HMG-CoA (reductase), 3-hydroxy-3-methyl-glutaryl-CoA reductase; -PP, -pyrophosphate.

Outcome measures

The primary outcome measure in this study was to assess the influence of statin use during chemotherapy with CAPOX-bevacizumab and cetuximab on progression free survival (PFS) in patients with *KRAS* mutant CRC. Furthermore, we examined the influence on overall survival (OS).

Statistical analysis

Baseline characteristics were compared between statin users and nonusers using a χ^2 test for categorical comparisons and for continuous variables the Student's t-test was used.

PFS was calculated as time from randomisation to the first documented progression, death or last follow up, whichever came first. OS was calculated as time from randomisation to death or last follow up. Kaplan-Meier survival estimates were calculated to determine the effect of statin use on PFS and OS in the cetuximab treated group by stratifying the study population into two groups according to *KRAS* status. For comparison between the statin users and non-users a log-rank test was used.

Cox proportional hazard models were used to determine whether the statin use in patients with *KRAS* mutant tumors treated with cetuximab was a significant predictor of PFS and OS. Instead of a subgroup analysis based on KRAS status and treatment arm, we used a Cox proportional hazard model, to study the effects of statins in cetuximab treated patients and compare it to non-cetuximab users to exclude a general statin effect. The following parameters were used in the model, statin use, *KRAS* mutation status, treatment arm, allowing for a different effect of statins between *KRAS* mutant and wild type tumors by means of an effect modifier in the model. In the multivariate analysis we included potential confounders with a p-value of <0.10 from the baseline univariate analysis, between statin user and non-users.

The deviating baseline characteristics between statin users and non-users with a p-value of <0.1 were also included in the multivariate analysis, e.g. prior adjuvant therapy, number of affected organs, and age.

The data are expressed as hazard ratios (HR), 95% CI intervals and P values. All statistical tests were two sided and p values <0.05 were considered statistically significant unless stated otherwise. All statistical analyses were performed using SPSS version 20 (SPSS for Windows, SPSS Inc., Chicago, IL, USA).

Results

Baseline patient characteristics according to statin use

795 patients were enrolled in the CAIRO2 study. A total of 529 patients from the CAIRO2 study were included in this analysis, 266 patients were excluded based on unknown *KRAS* mutation status, due to retrospective genotyping of the *KRAS* mutation status of the tumor, because the CAIRO2 study was performed in the pre *KRAS* era. A total of 78 patients were on statin therapy, of whom 43 patients were classified in treatment group A CAPOX-B and 35 in group B, CAPOX-B with cetuximab. 451 patients did not use a statin, of whom 225 patients were in group A and 226 to group B. The study population is described in table 1. It is noteworthy that patients in the statin group were older (67.1 vs. 61.9 p<0.001), more likely to be an aspirin user (44.9% vs. 6.4% p<0.001) and had a lower number of affected organs (>1 organ: 48.7% vs. 60.3% p=0.049) compared to patients who were not on statins. These deviating baseline characteristics between statin users and non-users with a p-value of <0.1 were included in the multivariate analysis.

Table 1: Patient Characteristics

Parameter	Statin users N (%)	Non-statin users N (%)	P value
Patients			
total	78 (14.0)	451 (86.0)	
KRAS status			0.112
Wild type	41 (52.6)	280 (62.1)	
Mutant	37 (47.4)	171 (37.9)	
Sex			0.269
Male	50 (64.1)	259 (57.4)	
female	28 (35.9)	192 (42.6)	
Arm			0.393
CAPOX-B	43 (55.1)	225 (49.9)	
CAPOX-B + cetuximab	35 (44.9)	226 (50.1)	
Serum LDH			0.624
Normal	48 (61.5)	288 (63.9)	
Above normal	30 (38.5)	159 (35.3)	
WHO performance status			0.467
0	29 (62.8)	306 (67.8)	
1	28 (35.9)	145 (32.2)	
Prior adjuvant therapy			0.055
No	70 (89.7)	364 (80.7)	
Yes	8 (10.3)	87 (19.3)	
Number of affected organs			0.049
1 organ	40 (51.3)	177 (39.2)	
> 1 organ	38 (48.7)	272 (60.3)	
Site of primary tumor			0.871
Colon	34 (43.6)	209 (46.3)	
Rectum	19 (24.4)	115 (25.5)	
Recto sigmoid	25 (32.1)	126 (27.9)	
Age			<0.001
Mean	67.1	61.9	
Range	46.1 – 83.6	27.6 -80.0	
Statin			
Pravastatin	13 (16.7)		
Simvastatin	28 (35.9)		
Atorvastatin	23 (29.5)		
Rosuvastatin	11 (14.1)		
Fluvastatin	3 (3.8)		
NSAID user	6 (7.7)	45 (10.0)	0.528
Aspirin user	35 (44.9)	29 (6.4)	<0.001
Bisphophonate user	2 (2.6)	5 (1.1)	0.299
Fibrate user	1 (1.3)	1 (0.2)	-

Effect of statin use on progression free survival

Statin use alone did not have a statistically significant effect on PFS of cetuximab treated patients with a *KRAS* mutant tumor (figure 2). In patients with a *KRAS* wild type tumor, the median PFS was 10.3 vs. 11.4 months (p=0.882) for nonusers compared to statin users, and in the *KRAS* mutant group 7.6 vs. 6.2 months (p=0.291), respectively. The hazard ratio (HR) of PFS was 1.12 (95% confidence interval 0.78 -1.61) and was not influenced by treatment arm, *KRAS* mutation status or the *KRAS**statin interaction.

In the multivariate analysis, the covariate adjusted HR for PFS was 1.01 (95%CI 0.71-1.54) for statin users.

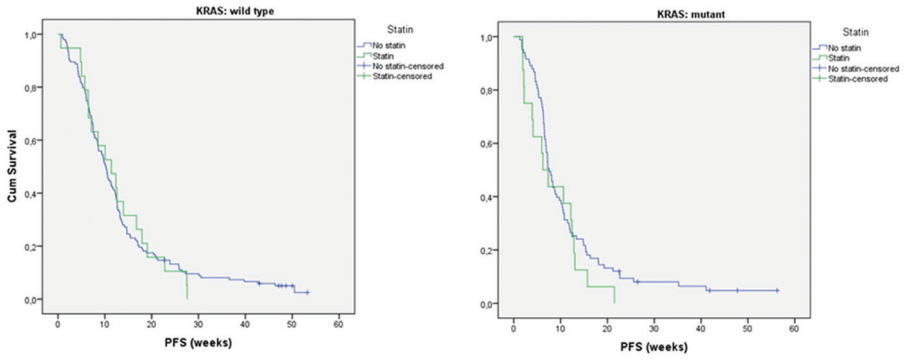

Figure 2: Kaplan-Meier plots for progression free survival for patients with *KRAS* wild type (19 statin users and 145 nonusers) and *KRAS* mutant (16 statin users and 83 nonusers) tumors treated with capecitabine, oxaliplatin, bevacizumab and cetuximab.

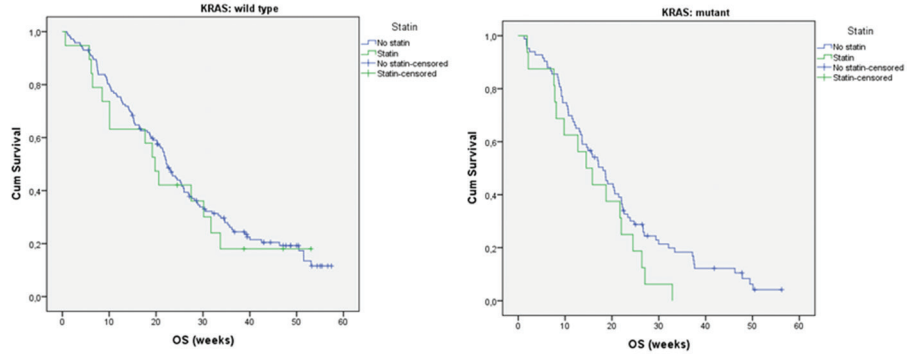

Figure 3: Kaplan-Meier plots for overall survival for patients with *KRAS* wild type (19 statin users and 145 nonusers) and *KRAS* mutant tumors (16 statin users and 83 nonusers) treated with capecitabine, oxaliplatin, bevacizumab and cetuximab.

Effect of statin use on overall survival

Among patients with a *KRAS* wild type tumor, the median OS for non-users compared to statin users was 22.4 vs. 19.8 months (p=0.650), in patients with a *KRAS* mutant tumor the median OS was 18.1 vs. 14.5 months (p=0.125) (figure 3), respectively. The OS was different between statin users and non-users (HR =1.54 for statin users 95% confidence interval 1.06-2.22) in the crude analysis. However, the covariate adjusted hazard ratio for OS was not associated with increased survival in the statin users (HR= 1.41 for statin users, 95% confidence interval 0.95 – 2.10).

Discussion

The results of this cohort study of patients diagnosed with metastatic CRC show that the use of statins is not associated with an improved PFS in patients with *KRAS* mutant tumors treated with cetuximab.

To the best of our knowledge, this is the first study investigating the effects of statin use on outcome in metastatic CRC patients in relation to *KRAS* mutation status and use of cetuximab. Preclinical studies have shown the antitumor effect of statins in CRC by a variety of mechanisms on cell proliferation. The leading hypothesized mechanism of statins is the inhibition of farnesylation of the KRAS protein[7,9]. We hypothesized that *KRAS* mutant CRC treated with cetuximab benefit from statin use, because statins may phenoconvert the overactive KRAS protein to a more wild type KRAS phenotype and thereby render these tumors sensitive to cetuximab treatment. Instead of stratifying for *KRAS* status and treatment arm and performing a subgroup analysis, a Cox proportional hazard model in the complete cohort of 529 patients was used, allowing for a different effect of statins between KRAS mutant and wild type tumors by means of an effect modifier. This study design allows to exclude a possible "generic" effect of statins on survival, because patients with a *KRAS* wild type tumor and patients in the arm without cetuximab were also included in the analysis. We did not observe an effect of statin use on the wild type *KRAS* tumors and thus no effect on cetuximab sensitivity. Moreover, we found no association between statin use and progression-free or overall survival in patients with a mutant *KRAS* tumor and therefore our study results do not support our hypothesis.

A possible explanation for the lack of effect of statins is that the cohort existed of patients with CRC with metastatic disease and hence a relatively short progression-free survival to demonstrate a modulating effect of statins on the efficacy of cetuximab. Secondly, in preclinical studies high doses of statins are used to treat cancers, aiming at inducing a cytotoxic treatment effect. The high concentrations used in those *in vitro* cell cultures are most likely not reached if the registered dose of statin for cardiovascular prevention is prescribed[10]. On the other hand, the registered doses decrease cholesterol levels and subsequently, the formation of prenylgroups, is reduced and as a consequence the prenylation of KRAS is inhibited[11].

This retrospective cohort study has some limitations. The included patients used different doses and statin types. We did not analyse the type, duration or dose of statin given, so we were unable to access the individual effect of these characteristics on the endpoints of the study. The bioavailability, pharmacokinetics and pharmacodynamics could be significantly different, however, we expect that patients were adequately treated for hypercholesterolemia as it is common practice to titrate patients based upon monitoring of their cholesterol levels. The proposed effect of the statins in this study is inhibition of formation of farnesyl- and geranylgeranylgroups which are essential for KRAS activation and also closely related to the main statin effect namely HMG-CoA reductase inhibition. Therefore, all statins could be combined in this study. Since patients included in this study were on stable statin dose, we assumed that target levels of cholesterol were reached. Consequently, this also implies that effective inhibition of formation of farnesyl- and geranylgeranylgroups was reached at the individualized statin dose. We thoroughly screened the patients' co-medication to minimize the exposure misclassifications, nonetheless, the uncertainty of patients' compliance to the prescribed regimen and the lack of prescription information may influence the study results. Patients with statin use at randomisation or first visit were included in the statin user group. We did neither record patients with prior statin use, nor new users after randomisation. Therefore, no cumulative statin dose could be calculated, which might be an

important factor, because it gives more information about the potential dose relationships and causality. The effect of different statins was not studied, because of the limited number of patients per subgroup. Differences between statins may exist since the hydrophilic statins, rosuvastatin and atorvastatin, have a decreased ability to penetrate cell membranes[12].

Another important limitation of this study is that patients were treated with the combination of chemotherapy, bevacizumab and cetuximab. Hypertension, a common site effect of bevacizumab is correlated with a better survival in CRC patients treated with bevacizumab[13]. A possible negative interaction between bevacizumab and cetuximab may have caused less hypertension in the cetuximab treated group, which contributed to the negative outcome of the CAIRO2 study[8]. So, for this study it means that the outcomes in the cetuximab treated group may have been influenced by the negative interaction between bevacizumab and cetuximab.

Obviously, PFS may be confounded by many factors. However, in our study outcomes were controlled for the main potential drug confounders, NSAID's, aspirin and bisphosphonates as well as for prior adjuvant therapy, number of organs effected and age. Nonetheless, confounding from unknown variables is still possible.

For testing a difference in effect on treatment between statin user and nonuser, PFS is the preferred primary endpoint. By studying PFS, a direct drug effect of statins on the cetuximab efficacy can be determined. A pronounced disadvantage of overall survival as an endpoint for this study is that this endpoint is less closely related to the drug effects. In the secondary analysis the use of statins in the unadjusted model was associated with a decrease of overall survival in the statin user group. A feasible explanation for the observed effect is that the statin users tend to be older and seemed to be less healthy, with a higher incidence of comorbidities then non-statin users, a confounding by indication. In the covariate adjusted cox regression this decrease of survival was not significant.

Due to the retrospective nature of this study we were not able to present data about KRAS prenylation levels of patients on statin therapy. In studies were the effects of statins are researched, data on prenylation levels of KRAS would be of great value, however at the moment a good assay to determine prenylation levels is lacking. To date, a number of studies have investigated statin use, CRC risk and clinical outcomes with inconclusive findings. Numerous studies and meta-analysis have investigated whether statin use reduces the risk of developing CRC[14,15]. Fewer studies focus on effects of statin after diagnosis during treatment [16-21]. The study of Mace et al.[16] showed that rectal cancer patients in the statin cohort treated with neo-adjuvant chemoradiation had a better response (65.7% versus 48.7%, $p = 0.004$) and lower median regression rate (1 versus 2, $p = 0.01$). Two other studies [19,20] in patients with rectal cancer treated with neo-adjuvant chemoradiation showed similar results indicating an association between statin use and response. However, in a study of Ng et al.[17], statin use during and after adjuvant chemotherapy among patients with stage 3 colon cancer was not associated with improved disease free survival, recurrent free survival or overall survival. In a prospective study of Lee et al. [18] the addition of simvastatin 40 mg, daily, to irinotecan, 5-fluorouracil, and leucovorin (FOLFORI) to first-line treatment in metastatic CRC patients showed promising antitumor activity and no additional adverse effects. These studies show that statin use in combination with systemic treatment for CRC may have some effect, but do not allow definite conclusions. However, all the above mentioned studies adressed the general cytotoxic effects of statins regardless of the *KRAS* status of the tumors. In our cohort we had the unique opportunity to study the effect of statins on cetuximab efficacy in CRC in relation to *KRAS* mutation status. Molecular data is warranted to study the exact mechanism of statins and their ability to potentiate chemotherapeutic agents. In

new studies with statins, molecular data from tumors and patients should be collected, this data help to understand the involved mechanisms.

In conclusion, the use of statins at time of diagnosis was not associated with an improved PFS or OS in metastatic colorectal cancer patients with a *KRAS* mutant tumor treated with combination chemotherapy bevacizumab and cetuximab.

References

1 Eisenberg DA. (1998) Cholesterol lowering in the management of coronary artery disease: the clinical implications of recent trials. Am J Med 104: 2S-5S.

2 Nielsen SF, Nordestgaard BG, Bojesen SE. (2012) Statin use and reduced cancer-related mortality. N Engl J Med 367: 1792-1802.

3 Konstantinopoulos PA, Karamouzis MV, Papavassiliou AG. (2007) Post-translational modifications and regulation of the RAS superfamily of GTPases as anticancer targets. Nat Rev Drug Discov 6: 541-555.

4 Tol J, Punt CJ. (2010) Monoclonal antibodies in the treatment of metastatic colorectal cancer: a review. Clin Ther 32: 437-453.

5 Lievre A, Bachet JB, Le CD, Boige V, Landi B, et al. (2006) KRAS mutation status is predictive of response to cetuximab therapy in colorectal cancer. Cancer Res 66: 3992-3995.

6 Douillard JY, Oliner KS, Siena S, Tabernero J, Burkes R, et al. (2013) Panitumumab-FOLFOX4 treatment and RAS mutations in colorectal cancer. N Engl J Med 369: 1023-1034.

7 Krens LL, Baas JM, Gelderblom H, Guchelaar HJ. (2010) Therapeutic modulation of KRAS signaling in colorectal cancer. Drug Discov Today 15: 502-516.

8 Tol J, Koopman M, Cats A, Rodenburg CJ, Creemers GJ, et al. (2009) Chemotherapy, bevacizumab, and cetuximab in metastatic colorectal cancer. N Engl J Med 360: 563-572.

9 Graaf MR, Richel DJ, van Noorden CJ, Guchelaar HJ. (2004) Effects of statins and farnesyltransferase inhibitors on the development and progression of cancer. Cancer Treat Rev 30: 609-641.

10 Chan KK, Oza AM, Siu LL. (2003) The statins as anticancer agents. Clin Cancer Res 9: 10-19.

11 Maciejak A, Leszczynska A, Warchol I, Gora M, Kaminska J, et al. (2013) The effects of statins on the mevalonic acid pathway in recombinant yeast strains expressing human HMG-CoA reductase. BMC Biotechnol 13: 68.

12 Gonyeau MJ. (2014) The spectrum of statin therapy in cancer patients: is there a need for further investigation? Curr Atheroscler Rep 16: 383.

13 Scartozzi M, Galizia E, Chiorrini S, Giampieri R, Berardi R, et al. (2009) Arterial hypertension correlates with clinical outcome in colorectal cancer patients treated with first-line bevacizumab. Ann Oncol 20: 227-230.

14 Liu Y, Tang W, Wang J, Xie L, Li T, et al. (2013) Association between statin use and colorectal cancer risk: a meta-analysis of 42 studies. Cancer Causes Control .

15 Graaf MR, Beiderbeck AB, Egberts AC, Richel DJ, Guchelaar HJ. (2004) The risk of cancer in users of statins. J Clin Oncol 22: 2388-2394.

16 Mace AG, Gantt GA, Skacel M, Pai R, Hammel JP, et al. (2013) Statin therapy is associated with improved pathologic response to neoadjuvant chemoradiation in rectal cancer. Dis Colon Rectum 56: 1217-1227.

17 Ng K, Ogino S, Meyerhardt JA, Chan JA, Chan AT, et al. (2011) Relationship between statin use and colon cancer recurrence and survival: results from CALGB 89803. J Natl Cancer Inst 103: 1540-1551.

18 Lee J, Jung KH, Park YS, Ahn JB, Shin SJ, et al. (2009) Simvastatin plus irinotecan, 5-fluorouracil, and leucovorin (FOLFIRI) as first-line chemotherapy in metastatic colorectal patients: a multicenter phase II study. Cancer Chemother Pharmacol 64: 657-663.

19 Katz MS, Minsky BD, Saltz LB, Riedel E, Chessin DB, et al. (2005) Association of statin use with a pathologic complete response to neoadjuvant chemoradiation for rectal cancer. Int J Radiat Oncol Biol Phys 62: 1363-1370.

20 Theodoropoulos G, Wise WE, Padmanabhan A, Kerner BA, Taylor CW, et al. (2002) T-level downstaging and complete pathologic response after preoperative chemoradiation for advanced rectal cancer result in decreased recurrence and improved disease-free survival. Dis Colon Rectum 45: 895-903.

21 Siddiqui AA, Nazario H, Mahgoub A, Patel M, Cipher D, et al. (2009) For patients with colorectal cancer, the long-term use of statins is associated with better clinical outcomes. Dig Dis Sci 54: 1307-1311.

Chapter 5

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*Contributed equally

Submitted

Safety and efficacy of the addition of simvastatin to cetuximab in previously treated KRAS mutant metastatic colorectal cancer patients

Abstract

Introduction

Cetuximab is registered for use in CRC patient with **RAS** wild type tumours only. Simvastatin blocks the mevalonate pathway and thereby interferes with the post-translational modification (prenylation) of KRAS. We hypothesize that the activitated KRAS pathway in *KRAS* mutant tumors can be inhibited by simvastatin rendering these tumors sensitive to the EGFR inhibitor cetuximab.

Methods

A Simon two-stage, single-arm, phase II study was performed to test the efficacy and safety of the addition of simvastatin to cetuximab in patients with a *KRAS* mutation in their tumour who were previously treated with fluoropyrimidine, oxaliplatin and irinotecan based regimens. The primary endpoint of this study was to test the percentage of patients alive and free from progression 12.5 weeks after the first administration of cetuximab. Our hypothesis was that at least 40% was free from progression, comparable to, though slightly lower than in **KRAS** wild type patients.

Results

Four of 18 included patients (22.2%) were free from progression at the primary endpoint time. The time to progression in these 4 patients ranged from 20.3 to 47 weeks.

Conclusion

Based on the current study we conclude that the theoretical concept of KRAS modulation with simvastatin was not applicable in the clinic, as we were not able to restore sensitivity to cetuximab in patients harbouring a *KRAS* mutation.

Introduction

Colorectal cancer (CRC) is a major healthcare issue. Each year over 940.000 patients are diagnosed with CRC world-wide and over 500.000 people die of this disease[1]. In patients with advanced or metastatic colorectal treatment with monoclonal antibodies directed against the epidermal growth factor receptor (EGFR), cetuximab and panitumumab are proven to be active after failing fluoropyrimidine, oxaliplatin and irinotecan based regimens, though only in patients with tumours without a mutation in the *KRAS*[2;3] or more recently *RAS* gene[4]. At time of design of this study, patients with a *KRAS* mutation in their tumour were left with no therapeutic options after failing conventional therapy. This led to the question whether increased activation of KRAS signaling by KRAS mutations can be modulated, thereby making KRAS mutated tumours sensitive to EGFR inhibitor therapy. One possible target for modulation is the mevalonate pathway, as we have previously discussed[5].

The mevalonate pathway is a metabolic cascade with various end-products including cholesterol. Other end-products are farnesylated and geranylgeranylated proteins (C15 and C17), both essential for posttranslational prenylation of the RAS protein and its association with the cytoplasmic membrane, and thereby activation of the RAS protein (Figure 1). By using HMG-CoA reductase inhibitors not only the synthesis of cholesterol is inhibited, but also the formation of C15 and C17, thereby inhibiting posttranslational modification of RAS[5;6]. By blocking the mevalonate pathway in CRC patients with *KRAS* mutated tumours, the activated KRAS pathway might be inhibited. This would theoretically lead to increased sensitivity to cetuximab, potentially comparable to tumours with wild type *KRAS*.

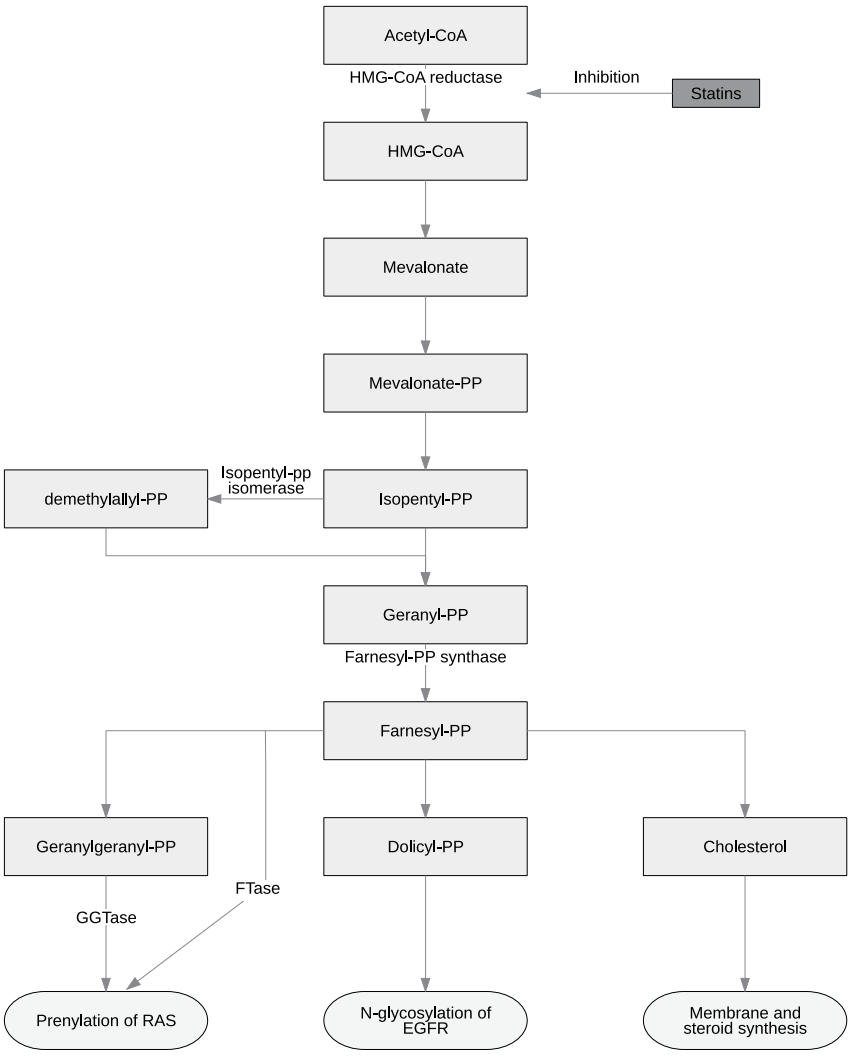

Figure 1: Mevalonate pathway

This single-arm, phase II study was designed to test the safety and efficacy of the addition of simvastatin to cetuximab in patients with a *KRAS* mutation in their tumour who were previously treated with fluoropyrimidine, oxaliplatin and irinotecan based regimens.

Methods

Patients

Eligible patients had advanced or metastatic colorectal cancer with a mutation in codon 12, 13 or 61 of the *KRAS* gene (either on tissue of the primary tumour or of a metastasis), after failing fluoropyrimidine, oxaliplatin and irinotecan based regimens, or after failure of oxaliplatin based therapy in patients who cannot be treated with irinotecan. In case of progressive disease within 6 months after start of adjuvant fluoropyrimidine, oxaliplatin, and irinotecan containing regimens the adjuvant therapy was considered to be treatment for metastatic disease.

Other eligibility criteria included age 18 years or older, World Health Organisation (WHO) performance score of 0 to 2 and progression of disease in the past three months prior to inclusion. Exclusion criteria included symptomatic brain metastases, previous treatment with EGFR inhibitors, history of toxicity during statin use and another malignancy during the past four years (with the exception of non-melanoma skin cancer and adequately treated pre-invasive carcinoma of the cervix).

The study protocol was approved by the Ethics Committees of all participating hospitals. Written informed consent was obtained prior to any study-related interventions.

Study design

This phase II, single-arm, multi-center study was performed using a Simon two-stage design[7]. In the first stage, 15 patients were included, followed by an interim analysis. Results of this analysis would determine whether the combination of simvastatin and cetuximab may have clinical benefit in this group of CRC patients, thus justifying the second stage and including up to 41 patients. If the first stage would suggest that this combination does not indicate clinical benefit, no additional patients would be exposed to this combination.

Treatment schedule

Cetuximab was first administered at least one week after start of simvastatin therapy. The initial cetuximab dose was 400 mg/m^2 (over 120 minutes) with subsequent weekly infusions of 250 mg/m^2 (over 60 minutes). Pretreatment with an antihistamine and a corticosteroid was mandatory before the first infusion of cetuximab and recommended for all subsequent infusions.

Simvastatin 80 mg orally once daily was started at start of study participation and continued throughout the entire study. This dose was chosen taken into consideration the need for continuous administration of the statin during the entire study, inhibitory effect on the mevalonate pathway and tolerability. Statins in cancer therapy have been studied in clinical trials in solid[8-18] and haematologic [19-21] malignancies, both as monotherapy as well as additional to chemotherapy. Statin doses from 20 mg/day up to 35 mg/kg/day were used, with only continuous use of statins when dosed at a maximum of 80 mg/day. Since the aim of this study is to modulate KRAS during the entire treatment with cetuximab and therefore a continuous exposure to simvastatin is needed, a dose of 80 mg/day was selected in order to obtain maximum effect while minimizing the risk of toxicity. Patients who were already using statins prior to inclusion had to switch to simvastatin in the above mentioned dose.

Treatment was continued until progression of disease, clinical signs of progression according to the investigators assessment, unacceptable toxicity or cetuximab toxicity requiring withholding of more than two subsequent infusions.

Tumour response was every six weeks using CT-scans and according to Response Evaluation Criteria In Solid Tumors (RECIST) version 1.1. Scans of patients free from progression at time of primary endpoint were centrally reviewed. All patients were followed for survival once every 3 months after termination of study participation. All patients were assessed for toxicity prior to every administration of cetuximab.

Endpoints

Primary objective was to test the percentage of patients with *KRAS* mutant advanced or metastatic colorectal cancer alive and free from progression and alive at 12.5 weeks after the first administration of cetuximab in combination with simvastatin. Our hypothesis was that at least 40% of patients was free from progression, comparable to though slightly lower than in *KRAS* wild type patients[2].

Secondary objectives were to investigate overall survival (OS), objective response rate (ORR), progression free survival (PFS), and safety of simvastatin combined with cetuximab in this population and to evaluate the correlation between skin toxicity and response to treatment. Exploratory endpoints were to investigate the role of cholesterol as a possible biomarker during this treatment and whether PIK3CA status correlate with response to cetuximab in this population.

Mutational analysis

KRAS mutational status was reconfirmed centrally, testing for the seven most frequent mutations in codon 12 and 13 as described in detail elsewhere[22]. In addition, we tested for the three most common mutation in the *PIK3CA* gene; in exon 9 (c.1624G>A (p.E542K) and c.1633G>A (pE545K)) and exon 20 (c.3140A>G (p.H1047R). Though *KRAS* and *BRAF* mutations are known to be mutually exclusive[23], we did test for the activating hotspot mutation p.V600E.

Statistics

Sample size was chosen based on previous published data of CRC patients with *KRAS* wild type tumours treated with cetuximab[2], aiming for a at least six out of 15 patients free from progression at 12.5 weeks after start of cetuximab treatment in patients with *KRAS* mutant type tumours (i.e., slightly lower than the effect in *KRAS* wild type patients). Combined with an alpha of 0.05 and a power of 0.80, an interim size of 15 and a total sample size of 46 patients were required. An interim analysis was to be performed after the inclusion of 15 evaluable patients. Only when at least 40% (i.e. 6 patients) were free from progression at the 12.5 weeks, another 31 patients would be enrolled during the second stage of the study.

Results

Patients

During the first stage of the study 18 instead of 15 patients were enrolled to account for patients that were thought to unevaluable for the primary endpoint. Baseline characteristics are listed in Table 1. Fifteen patients had previously been receiving two lines of chemotherapy, two patients were only treated with oxaliplatin/5FU based therapy prior to inclusion and 1 patient had received

three lines of chemotherapy (oxaliplatin and irinotecan based therapy and regorafenib during participation in a different trial). None of the patients were using statins prior to inclusion.

Table 2 shows type of *KRAS* mutation per patient, along with *PIK3CA* mutational status.

Table 1: Baseline characteristics

Age – years	
Mean	62
Range	52 – 75
Gender – n (%)	
Male	13 (72)
Female	5 (18)
WHO performance score – n (%)	
0	13 (72)
I	5 (18)
Site of primary tumour – n (%)	
Colon	12 (67)
Rectum	6 (33)
Prior lines of chemotherapy – n (%)	
1	2
2	15
3	1
Prior surgery – n (%)	13 (72)
Prior radiotherapy – n (%)	4 (22)

Table 2: *KRAS* and *PIK3CA* mutational status per patient

Study number	*KRAS* mutation	*PIK3CA* mutational status
1	G12D	Wild type
2	G12V	Wild type
3	G12V	Wild type
4	G12C	Wild type
5	G12V	Wild type
6	G12S	Wild type
7	*missing*	*Missing*
8	G12V	Wild type
9	G13D	Wild type
10	G13D	Wild type
11	G12D	Wild type
12	G12D	Wild type
13	*missing*	*Missing*
14	G12V	Wild type
15	G12A	Wild type
16	G12A	Wild type
17	G13D	Mutation in exon 9
18	G12D	Mutation in exon 9

Efficacy

Four of 18 patients were free from progression at the primary endpoint time, therefore the percentage of patients alive and free from progression 12.5 weeks after the first administration of cetuximab was 22%. The time to progression in these four patients ranged from 20.3 to 47 weeks. Drug exposure to simvastatin and cetuximab was equal for all patients since none of the patients required dose reductions while on study.

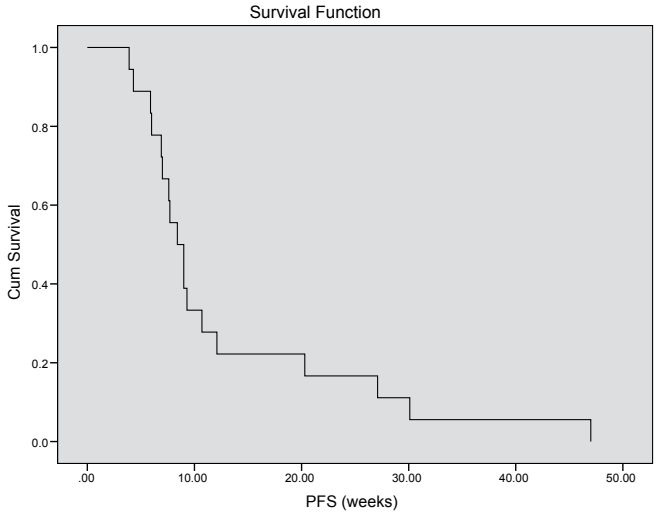

Figure 2a: Progression free survival in weeks for the addition of simvastatin to cetuximab in CRC patients failing standard therapy

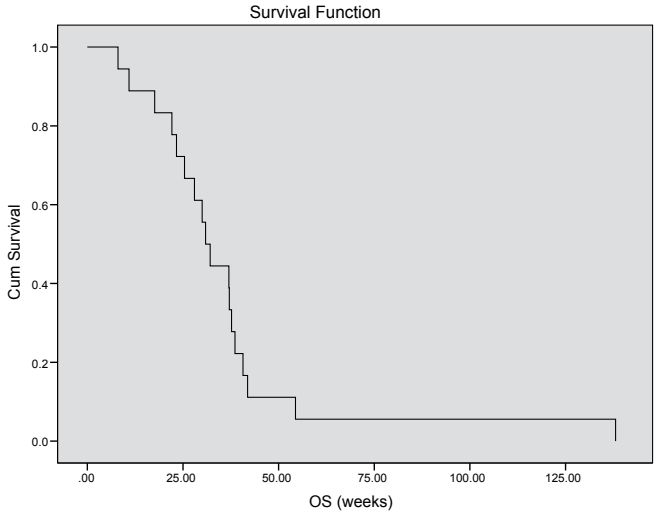

Figure 2b: Overall survival in weeks for the addition of simvastatin to cetuximab in CRC patients failing standard therapy

Figure 2 shows progression free (panel A) and overall survival (panel B). Median progression free survival was nine weeks (mean 12.9 weeks, range 3.9 - 47 weeks). Median overall survival was 31.5 weeks (mean 36.3, range 8-138.1). Objective response rate was 6% (partial remission in one patient). A true relation between skin toxicity and efficacy of treatment was not observed in this study though this may (partly) be due to the low number of patients and due to the improved knowledge of the efficacy of pre-emptive skin toxicity management.

Safety

Main symptoms and adverse events reported on study reported were fatigue (n=11), acne (n=10) and rash (n=6). Myopathy was not reported. Three patients had elevation of creatine kinase (CK) levels on study (grade 4 in one patient). Table 3 shows the most frequent reported adverse events. Skin toxicity occurred in 10 patients; the worst grade of acneiform rash was grade 3 in one patient, grade 2 in four patients and grade 1 in the remaining five patients. One patient experienced a severe (i.e. grade 3) allergic reaction during the first infusion of cetuximab. This was the only grade 3 infusion reaction reported during the entire study, and this patient did not experience any further reactions while on study. Hypomagnesaemia was reported in four patients, no cases of hypocalcaemia were reported.

Table 3: Adverse events occurring in > 10%

Event	Any grade	Grade 3-4
Fatigue	11 (61%)	1 (6%)
Acneiform rash	10 (56%)	1 (6%)
Anemia	9 (50%)	-
Rash (not acneiform)	6 (33%)	1 (6%)
Tumour-related pain	6 (33%)	1 (6%)
Pruritis	5 (28%)	-
Nausea	5 (28%)	-
Dyspnea	4 (22%)	-
Hypomagnesaemia	4 (22%)	-
Creatine kinase elevation	3 (17%)	1 (6%)
Constipation	3 (17%)	-
Fever	2 (11%)	-
Infusion related reaction	2 (11%)	1 (6%)
Thrombocytopenia	2 (11%)	-
Weight loss	2 (11%)	-
Thrombosis	2 (11%)	1 (6%)

One of the serious adverse events did precede the death of a participant. Upon the scheduled laboratory examination severe elevation of liver enzymes were observed soon after start of study medication. Rhabdomyolysis due to simvastatin was considered, (though on study CK levels were below 3.000 U/l) and so was progression of liver metastases with impaired liver function. Study medication was interrupted immediately, however the patient's situation did not improve and it was decided to terminate study participation permanently. Specific SNPs associated with increased risk of statin-induced myopathy (i.e. SLCO1B1 variants[24]) were considered though none were identified in this patient. The patient deceased few weeks later. Post-mortem examination did not occur.

Exploratory endpoints

Serum cholesterol was measured in all patients at baseline and in 15 patients on study. All showed cholesterol reduction, ranging from a maximum reduction of 0.8% to 64.4%. Cholesterol reduction on study did not differ between patients free from progression at time of primary endpoint compared to those who were not (mean reduction 37.1% versus 30.5%, p-value = 0.55). The percentage of cholesterol reduction did not correlate with progression free survival.

Tumour tissue of 15 patients was available for central review. Table 3 shows mutational status of KRAS and PIK3CA per patient. Thirteen patients had a mutation in codon 12 (most often G12D and G12V) and three in codon 13. A mutation in the *PIK3CA* gene was detected in 2 patients (both exon 9). Of the four patients responding to treatment, 3 had a *KRAS* mutation in codon 12 and one had a *PIK3CA* mutation. As expected in patients with a *KRAS* mutation in their tumour, all patients were *BRAF* wild type.

Discussion

To our knowledge, this is the first clinical trial testing the addition of simvastatin to cetuximab monotherapy in CRC patients harbouring a KRAS mutation in tumour tissue as an attempt to restore cetuximab sensitivity. While it was remarkable to notice a durable progression free survival in four patients, the interim analysis showed that the predefined criteria to proceed to the second stage of this study were not reached. Therefore, the current study suggests that high dose simvastatin does not render cetuximab sensitivity in *KRAS* mutant CRC.

Statines are one of several potential agents to modulate *KRAS* signaling, as we have previously reviewed[5]. The current study is not the first to hypothesize on statins and their inhibitory effect on the activity of RAS and its downstream pathway. However, all but one previous reports include only preclinical data. Lovastatin showed to inhibit RAS activation in KRAS transformed thyroid cells through inhibition of its farnesylation, and thereby inhibiting activity of the downstream pathway[25]. Furthermore, lovastatin and simvastatin inhibit downstream activity in breast cells with mutated *HRAS*, known to induce an invasive phenotype, possibly by inhibiting membrane localization of *HRAS*. The effect was reversed when adding farnesyl pyrophosphate, indicating the effect was related to prenylation of *RAS*[26]. More recently, simvastatin was shown to restore cetuximab resistance *in vitro* and *in vivo*[27]. Based on these results, one might wonder whether the negative outcome of the current study would have been different if using higher doses of simvastatin. As mentioned above, statin doses up to 35 mg/kg/day have been prescribed in clinical trials, though higher doses were not used continuously as was essential in the current design. Preclinical data showed a significant reduction in cell growth of *KRAS* mutant CRC cell lines using 0.2 μM simvastatin, the equivalent of 2mg/kg/day in humans[27]. Moreover, in cardiovascular disease the registered dose of 80 mg of simvastatin is significantly lowers cholesterol serum levels. It is reasonable that this dose will also affect the formation of the C15 and C17 groups and subsequently the prenylation of the KRAS protein. Furthermore, we question whether higher doses will be feasible in terms of safety.

A recent study of Lee et al tested the efficacy of the addition of the same dose of simvastatin (i.e. 80mg once daily) to cetuximab and irinotecan in *KRAS* mutant CRC patients failing prior oxaliplatin, fluoropyrimidine and irinotecan based therapy[28]. The initially reported PFS and OS (median 7.6 months and 12.8 months respectively) were considerably higher than historical results in chemotherapy refractory CRC patients with *KRAS* mutated tumours[29]

and chemotherapy refractory CRC patients in general[30-32]. Moreover, these results were in contrast with our findings and the authors concluded that simvastatin may overcome cetuximab resistance in patients with *KRAS* mutant tumours. However, a recent erratum published by this group showed that initial survival data were incorrect[33]. The corrected PFS and OS are in line with our results, providing no evidence for a modulating effect of simvastatin on the *KRAS* mutant phenotype.

The majority of patients had a *KRAS* mutation in codon 12 and only 3 in codon 13. It has been reported that tumours harbouring a G13D mutation in the *KRAS* gene might be sensitive to EGFR-inhibitors[34]. Moreover, none of our patients had a *PIK3CA* mutation in exon 20. Mutations in exon 20 of the PIK3CA gene might also be more likely to be sensitive to EGFR-inhibitors, contrary to mutations in exon 9[35]. However, while the number of patients in our study is low, of the four patients who were free from progression at time of the primary endpoint only one patients had a G13D mutation in the *KRAS* gene and none had a *PIK3CA* mutation in exon 20.

While one patient developed impaired liver function, it remains unclear whether this was related to simvastatin. Nonetheless, CK levels were clearly increased in this particular patient. However, none of the other patients reported statin related adverse events (e.g. myopathy) and CK levels were only mildly elevated in two patients. There was no need for dose reduction. Overall, simvastatin 80 mg once daily was considered well tolerated in the current population.

Conclusion

Based on the current study we conclude that the concept of KRAS modulation with simvastatin was not applicable in the clinic. Other strategies are needed for CRC patients with tumours harbouring a *KRAS* mutation who failed standard therapy. Recently regorafenib was registered for CRC patients failing standard therapy (including EGFR inhibitors if wild type *KRAS*), but the gain in survival is limited to 6 weeks[30]. Better treatment strategies are needed for this patient population.

Compliance with ethical standards

Conflict of interest

Jara M, Baas: none; Lisanne L. Krens: none; A.J. ten Tije: none; F. Erdkamp: none; Tom van Wezel: none; Hans Morreau: none; Henk-Jan Guchelaar: research funding by Amgen Inc and Merck BV; Hans Gelderblom: research funding by Amgen Inc en Merck BV.

Informed consent

The informed consent form was signed by the patient (and physician) prior to inclusion and according to the ICH guidelines on Good Clinical Practice.

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References

1 Denters MJ, Deutekom M, Fockens P, Bossuyt PM, Dekker E. (2009) Implementation of population screening for colorectal cancer by repeated fecal occult blood test in the Netherlands. BMC Gastroenterol 9: 28.

2 Karapetis CS, Khambata-Ford S, Jonker DJ, O'Callaghan CJ, Tu D, et al. (2008) KRAS mutations and benefit from cetuximab in advanced colorectal cancer. N Engl J Med 359: 1757-1765.

3 Amado RG, Wolf M, Peeters M, Van CE, Siena S, et al. (2008) Wild type KRAS is required for panitumumab efficacy in patients with metastatic colorectal cancer. J Clin Oncol 26: 1626-1634.

4 Sorich MJ, Wiese MD, Rowland A, Kichenadasse G, McKinnon RA, et al. (2014) Extended RAS mutations and anti-EGFR monoclonal antibody survival benefit in metastatic colorectal cancer: a meta-analysis of randomized, controlled trials. Ann Oncol .

5 Krens LL, Baas JM, Gelderblom H, Guchelaar HJ. (2010) Therapeutic modulation of KRAS signaling in colorectal cancer. Drug Discov Today 15: 502-516.

6 Swanson KM, Hohl RJ. (2006) Anti-cancer therapy: targeting the mevalonate pathway. Curr Cancer Drug Targets 6: 15-37.

7 Simon R. (1989) Optimal two-stage designs for phase II clinical trials. Control Clin Trials 10: 1-10.

8 Hong JY, Nam EM, Lee J, Park JO, Lee SC, et al. (2014) Randomized double-blinded, placebo-controlled phase II trial of simvastatin and gemcitabine in advanced pancreatic cancer patients. Cancer Chemother Pharmacol 73: 125-130.

9 Manoukian GE, Tannir NM, Jonasch E, Qiao W, Haygood TM, et al. (2011) Pilot trial of bone-targeted therapy combining zoledronate with fluvastatin or atorvastatin for patients with metastatic renal cell carcinoma. Clin Genitourin Cancer 9: 81-88.

10 Han JY, Lim KY, Yu SY, Yun T, Kim HT, et al. (2011) A phase 2 study of irinotecan, cisplatin, and simvastatin for untreated extensive-disease small cell lung cancer. Cancer 117: 2178-2185.

11 Han JY, Lee SH, Yoo NJ, Hyung LS, Moon YJ, et al. (2011) A randomized phase II study of gefitinib plus simvastatin versus gefitinib alone in previously treated patients with advanced non-small cell lung cancer. Clin Cancer Res 17: 1553-1560.

12 Konings IR, van der Gaast A, van der Wijk LJ, de Jongh FE, Eskens FA, et al. (2010) The addition of pravastatin to chemotherapy in advanced gastric carcinoma: a randomised phase II trial. Eur J Cancer 46: 3200-3204.

13 Lee J, Jung KH, Park YS, Ahn JB, Shin SJ, et al. (2009) Simvastatin plus irinotecan, 5-fluorouracil, and leucovorin (FOLFIRI) as first-line chemotherapy in metastatic colorectal patients: a multicenter phase II study. Cancer Chemother Pharmacol 64: 657-663.

14 Graf H, Jungst C, Straub G, Dogan S, Hoffmann RT, et al. (2008) Chemoembolization combined with pravastatin improves survival in patients with hepatocellular carcinoma. Digestion 78: 34-38.

15 Knox JJ, Siu LL, Chen E, Dimitroulakos J, Kamel-Reid S, et al. (2005) A Phase I trial of prolonged administration of lovastatin in patients with recurrent or metastatic squamous cell carcinoma of the head and neck or of the cervix. Eur J Cancer 41: 523-530.

16 Lersch C, Schmelz R, Erdmann J, Hollweck R, Schulte-Frohlinde E, et al. (2004) Treatment of HCC with pravastatin, octreotide, or gemcitabine--a critical evaluation. Hepatogastroenterology 51: 1099-1103.

17 Kim WS, Kim MM, Choi HJ, Yoon SS, Lee MH, et al. (2001) Phase II study of high-dose lovastatin in patients with advanced gastric adenocarcinoma. Invest New Drugs 19: 81-83.

18 Kawata S, Yamasaki E, Nagase T, Inui Y, Ito N, et al. (2001) Effect of pravastatin on survival in patients with advanced hepatocellular carcinoma. A randomized controlled trial. Br J Cancer 84: 886-891.

19 Ahmed TA, Hayslip J, Leggas M. (2013)

Pharmacokinetics of high-dose simvastatin in refractory and relapsed chronic lymphocytic leukemia patients. Cancer Chemother Pharmacol 72: 1369-1374.

20 Hus M, Grzasko N, Szostek M, Pluta A, Helbig G, et al. (2011) Thalidomide, dexamethasone and lovastatin with autologous stem cell transplantation as a salvage immunomodulatory therapy in patients with relapsed and refractory multiple myeloma. Ann Hematol 90: 1161-1166.

21 van der Spek E, Bloem AC, Sinnige HA, Lokhorst HM. (2007) High dose simvastatin does not reverse resistance to vincristine, adriamycin, and dexamethasone (VAD) in myeloma. Haematologica 92: e130-e131.

22 van Eijk R., Licht J, Schrumpf M et al. (2011) Rapid KRAS, EGFR, BRAF and PIK3CA mutation analysis of fine needle aspirates from non-small-cell lung cancer using allele-specific qPCR. PLoS.One.; 6: e17791.

23 Tol J, Nagtegaal ID, Punt CJ. (2009)BRAF mutation in metastatic colorectal cancer. N.Engl.J.Med.; 361: 98-9.

24 Link E, Parish S, Armitage J, Bowman L, Heath S, et al. (2008) SLCO1B1 variants and statin-induced myopathy--a genomewide study. N Engl J Med 359: 789-799.

25 Laezza C, Fiorentino L, Pisanti S, Gazzerro P, Caraglia M, et al. (2008) Lovastatin induces apoptosis of KRAS-transformed thyroid cells via inhibition of ras farnesylation and by modulating redox state. J Mol Med (Berl) 86: 1341-1351.

26 Kang S, Kim ES, Moon A. (2009) Simvastatin and lovastatin inhibit breast cell invasion induced by H-Ras. Oncol Rep 21: 1317-1322.

27 Lee J, Lee I, Han B, Park JO, Jang J, et al. (2011) Effect of simvastatin on cetuximab resistance in human colorectal cancer with KRAS mutations. J Natl Cancer Inst 103: 674-688.

28 Lee J, Hong YS, Hong JY, Han SW, Kim TW, et al. (2014) Effect of simvastatin plus cetuximab/irinotecan for KRAS mutant colorectal cancer and predictive value of the RAS signature for treatment response to cetuximab. Invest New Drugs 32: 535-541.

29 De Roock RW, Claes B, Bernasconi D, De SJ, Biesmans B, et al. (2010) Effects of KRAS, BRAF, NRAS, and PIK3CA mutations on the efficacy of cetuximab plus chemotherapy in chemotherapy-refractory metastatic colorectal cancer: a retrospective consortium analysis. Lancet Oncol 11: 753-762.

30 Grothey A, Van CE, Sobrero A, Siena S, Falcone A, et al. (2013) Regorafenib monotherapy for previously treated metastatic colorectal cancer (CORRECT): an international, multicentre, randomised, placebo-controlled, phase 3 trial. Lancet 381: 303-312.

31 Jonker DJ, O'Callaghan CJ, Karapetis CS, Zalcberg JR, Tu D, et al. (2007) Cetuximab for the treatment of colorectal cancer. N Engl J Med 357: 2040-2048.

32 Van Cutsum E, Peeters M, Siena S, Humblet Y, Hendlisz A, et al. (2007) Open-label phase III trial of panitumumab plus best supportive care compared with best supportive care alone in patients with chemotherapy-refractory metastatic colorectal cancer. J Clin Oncol 25: 1658-1664.

33 Lee J, Hong YS, Hong JY, Han SW, Kim TW, et al. (2014) Erratum to: Effect of simvastatin plus cetuximab/irinotecan for KRAS mutant colorectal cancer and predictive value of the RAS signature for treatment response to cetuximab. Invest New Drugs .

34 Tejpar S, Celik I, Schlichting M, Sartorius U, Bokemeyer C, Van CE. (20120 Association of KRAS G13D tumor mutations with outcome in patients with metastatic colorectal cancer treated with first-line chemotherapy with or without cetuximab. J.Clin.Oncol.; 30: 3570-7.

35 Karapetis CS, Jonker D, Daneshmand M et al. (2014) PIK3CA, BRAF, and PTEN status and benefit from cetuximab in the treatment of advanced colorectal cancer--results from NCIC CTG/AGITG CO.17. Clin.Cancer Res.; 20: 744-53.

Chapter 6

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Accepted

Safety and efficacy of the addition of simvastatin to panitumumab in previously treated KRAS mutant metastatic colorectal cancer patients

Abstract

Introduction

Panitumumab has proven efficacy in patients with metastatic or locally advanced colorectal cancer patients provided they have no activating *KRAS* mutation in their tumour. Simvastatin blocks the mevalonate pathway and thereby interferes with the post-translational modification of KRAS. We hypothesize that the activity of the RAS induced pathway in patients with a *KRAS* mutation might be inhibited by simvastatin. This would theoretically result in increased sensitivity to panitumumab, potentially comparable to tumours with wild type *KRAS*.

Methods

A Simon two-stage design single-arm, phase II study was designed to test the safety and efficacy of the addition of simvastatin to panitumumab in colorectal cancer patients with a *KRAS* mutation after failing fluoropyrimidine, oxaliplatin and irinotecan based therapy. The primary endpoint of this study was the proportion of patients alive and free from progression 11 weeks after the first administration of panitumumab, aiming for at least 40% which is comparable to though slightly lower than in KRAS wilde-type patients in this setting. If this 40% was reached then the study would continue into the second step up to 46 patients. Explorative correlative analysis for mutations in the KRAS and related pathways was performed.

Results

One of 14 patients was free from progression at the primary endpoint time. Median progression free survival was 8.4 weeks, median overall survival status was 19.6 weeks.

Conclusion

We conclude that the concept of mutant KRAS phenotype expression modulation with simvastatin was not applicable in the clinic.

Introduction

The epidermal growth factor receptor (EGFR) inhibitors panitumumab and cetuximab have proven efficacy in third-line treatment of colorectal cancer (CRC) patients failing 5-FU, oxaliplatin and irinotecan based regimens [1;2], but only in patients with tumours not harbouring an activating *KRAS* mutation in codon 12, 13 or 61 [3-5] or, more recently published, several other *RAS* mutations [6]. At the time of design of this study the available literature showed that *KRAS* mutations are found in tumour tissue of 40% of CRC patients, at least 90% located on codon 12 or 13 of the *KRAS* gene [4]. Patients harbouring these mutations in their tumour were left with little therapeutic options after failing standard therapy. This raised the question whether *KRAS* mutations can be modulated, thereby making *KRAS* mutated tumours sensitive to EGFR inhibitor therapy. The possible target for the simvastatin modulation is the mevalonate pathway, as we have previously discussed [7].

Statins (HMG-CoA-reductase inhibitors) inhibit cholesterol synthesis by inhibiting the mevalonate pathway, a metabolic cascade also responsible for syntheses of farnesylated and geranylgeranylated proteins (C15 and C17), both essential for post-translation activation of the KRAS protein [8]. As statins also inhibit the synthesis of C15 and C17, they may inhibit post-translational activation of RAS proteins. Therefore, statins may inhibit the expression of the mutant *KRAS* phenotype and normalize the phenotype into *KRAS* wild type, rendering sensitivity to panitumumab.

This single-arm, multicenter phase II study was designed to test safety and efficacy of the addition of simvastatin to panitumumab in previously treated CRC patients with a *KRAS* mutation in their tumour.

Methods

Patients

Eligible patients had advanced or metastatic colorectal cancer with a mutation in codon 12, 13 or 61 of the *KRAS* gene (either on tissue of the primary tumour or of a metastasis), after failure of fluoropyrimidine, oxaliplatin and irinotecan based regimens, or after failure of oxaliplatin therapy and unable to tolerate irinotecan. In patients with progressive disease within six months after start of adjuvant therapy, these therapies were considered to be treatment for metastatic disease.

Other eligibility criteria included: age 18 years or older, World Health Organisation (WHO) performance score of 0 to 2 and progression of disease in the three months prior to inclusion. Exclusion criteria included symptomatic brain metastases, previous treatment with EGFR inhibitors, history of toxicity during statin use or an other malignancy during the past four years (with the exception of non-melanoma skin cancer and adequately treated pre-invasive carcinoma of the cervix).

The study protocol was approved by the Ethics Committees of all participating hospitals and all study procedures were in accordance with the 1964 Helsinki declaration and its later amendments. Written informed consent from the patient was obtained prior to any study-related interventions.

Treatment schedule

Panitumumab 6 mg/kg was administered intravenously once every two weeks. The first administration was scheduled at least one week after start of simvastatin. Simvastatin 80 mg once daily was started at baseline and continued throughout entire study participations, though dose reductions or temporary interruptions were allowed in case of toxicity. This starting dose of simvastatin was chosen for the following reasons: inhibitory effect on the mevalonate pathway (and not high dose antitumour effect by itself), tolerability and the need for continuous administration of the statin during the entire study. Statins in cancer therapy have been studied in clinical trials in solid [9-19] and haematologic [20-22] malignancies, both as monotherapy as well as additional to standard therapy. Statin doses from 20 mg/day up to 35 mg/kg/day were used in various intermittent schedules. In continuous dosing schedules, simvastatin was used at a maximum of 80mg/day. The aim of this study was to modulate KRAS during the entire treatment with panitumumab, therefore a continuous exposure to simvastatin was needed and a dose of 80 mg/day was selected in order to obtain maximum effect while minimizing the risk of toxicity. Patients who were already using statins prior to inclusion had to switch to simvastatin Treatment was continued until progression of disease according to Response Evaluation Criteria In Solid Tumors (RECIST) version 1.1, clinical signs of progression according to the investigators assessment, unacceptable toxicity, signs of rhabdomyolysis or panitumumab toxicity requiring interruption of treatment.

Tumour response was measured seven weeks after baseline and every two cycles thereafter using CT-scans and according to RECIST version 1.1. These intervals were based on historical data on PFS of *KRAS* wild type colorectal cancer patients treated with panitumumab [3]. Scans of patients free from progression at the time of primary endpoint were centrally reviewed. All patients were followed for survival once every three months after termination of study participation. Adverse events were monitored on an ongoing basis per cycle and toxic effects were categorized using the NCI Common Terminology Criteria for Adverse Events (CTCAE), Version 3.0.

Endpoints

Primary endpoint was the proportion of patients alive and free from progression at 11 weeks after the first administration of panitumumab in combination with simvastatin. Our hypothesis was that at least 40% of patients would be free from progression at 11 weeks, comparable to though slightly lower than the proportion of *KRAS* wild type patients that remains free from progression at 11 weeks when treated with panitumumab [3].

Secondary endpoints were overall survival (OS), objective response rate (ORR), progression free survival (PFS), and safety of simvastatin combined with panitumumab in this population and to evaluate the correlation between skin toxicity and response to treatment. Exploratory endpoints were to investigate the role of cholesterol as a possible biomarker during this treatment and whether *PIK3CA* status correlates with response to panitumumab in this population.

Mutational analysis

KRAS mutational status was reconfirmed centrally, testing for the seven most frequent mutations in codon 12 and 13 as described in detail elsewhere[23]. In addition, we tested for the three most common mutation in the *PIK3CA* gene; in exon 9 (c.1624G>A (p.E542K) and c.1633G>A (pE545K)) and exon 20 (c.3140A>G (p.H1047R). Though *KRAS* and *BRAF* mutations are known to be mutually exclusive[24], all tissue was tested for the activating hotspot mutation p.V600E.

Design and statistics

This phase II, single-arm, multi-center study was performed using a Simon two-stage design [25]. In the first stage, 15 patients were included, after which an interim analysis was performed. Results of this analysis would determine whether the combination of simvastatin and panitumumab may have clinical benefit in this group of CRC patients, thus justifying the second stage up to 46 patients in total.

The sample size was chosen based on previously published data of CRC patients with *KRAS* wild type tumours treated with panitumumab [3], aiming for at least 6 out of 15 patients free from progression at 11 weeks after start of combination panitumumab and simvastatin treatment in patients with *KRAS* mutant type tumours. Combined with an alpha of 0.05 and a power of 0.80, an interim size of 15 and a total sample size of 46 patients were required. An interim analysis was to be performed after the inclusion of 15 evaluable patients. Only when at least 40% (i.e. six patients) were free from progression at the 11 weeks, another 31 patients would be enrolled during the second stage of the study.

Results

Patients

From April 2010 to May 2012, 17 patients were included. Notably, 17 instead of 15 patients were included, due to the fact that two patients were considered to be unevaluable (both showed clinical signs of progression prior to the first infusion of panitumumab). However, after review, three instead of two patients were unevaluable (Figure 1). The third unevaluable patient had a second malignancy which was first discovered at the baseline CT-scan. As none of the three unevaluable patients received panitumumab, all three were excluded in the efficacy and safety analysis. Baseline characteristics of the remaining 14 patients are listed in Table 1. One patient only received oxaliplatin/5FU based chemotherapy prior to study participation. None were receiving any kind of statin prior to study participation. Table 2 shows type of *KRAS* mutation per patient, along with *PIK3CA* mutational status. Tumour tissue was available in all but one patient. Two patients had a *PIK3CA* mutation on tumour tissue, one located in exon 20 and one in exon 9. Eleven patients had a *KRAS* codon 12 mutant tumour and two patients had a *KRAS* codon 13 mutant tumour.

Efficacy

One study participant was free from progression at the primary endpoint time. The percentage of patients alive and free from progression 11 weeks after the first administration of panitumumab is therefore 7%. The predefined criteria to proceed to the second stage of the study were not met, therefore no further patients were included. Time to progression in this particular patient was 17 weeks, median time to progression was 8.4 weeks (mean 8.7, range 5-17, Figure 2 panel A). Median overall survival was 19.6 weeks (mean 24.2, range 8.3-71.1, Figure 2 panel B). Objective response rate was 0% as none of the patients had a (partial) remission. Analysis of a correlation between skin toxicity and efficacy was not feasible due to absence of responders.

Exposure to panitumumab was equal in all patients; none required dose reductions or delays. Two patients needed 50% dose reduction of simvastatin, both after the second infusion of panitumumab. Reason for dose reduction was elevation of liver enzymes in one patient. In the other patient reason for dose reduction was not specified, though liver enzymes were stable in this specific patient and myalgia was not reported.

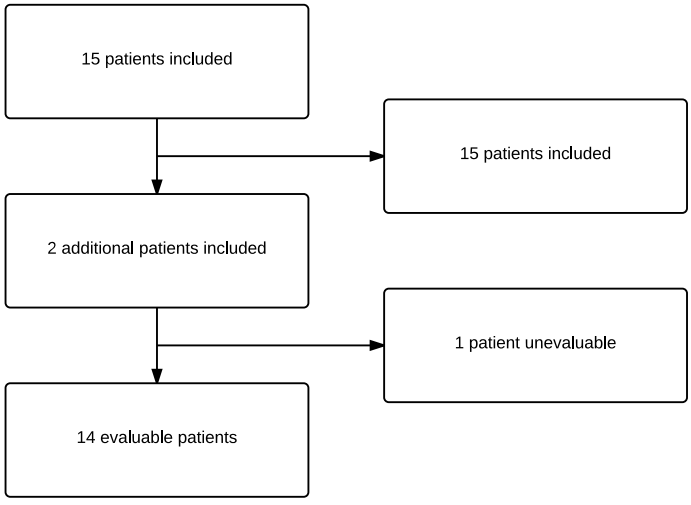

Figure 1: Flow chart of the inclusion of patients

Table 1: Baseline characteristics

Age – years	
Mean	59
Range	33 – 77
Gender – n (%)	
Male	5 (36)
Female	9 (64)
WHO performance score – n (%)	
0	7 (50)
I	5 (36)
II	2 (14)
Site of primary tumour – n (%)	
Colon	8 (57)
Rectum	6 (43)
Prior lines of chemotherapy – n (%)	
1	1 (7)
2	8 (57)
3	2 (14)
Not reported	3 (22)
Prior surgery – n (%)	9 (64)
Prior radiotherapy – n (%)	5 (36)

Table 2: Mutational status per patient

Study nr	*KRAS*	*PIK3CA*
1	G12V	Wild type
2	G12D	Wild type
3	G12C	Wild type
4	G12V	Wild type
5	G12D	Wild type
7	G12V	Wild type
8	G12A	Wild type
9	G12A	Wild type
10	G12V	Mutation in exon 20
11	G12D	Wild type
13	G13D	Mutation in exon 9
14	G13D	Wild type
15	G12V	Wild type
17	*missing*	*missing*

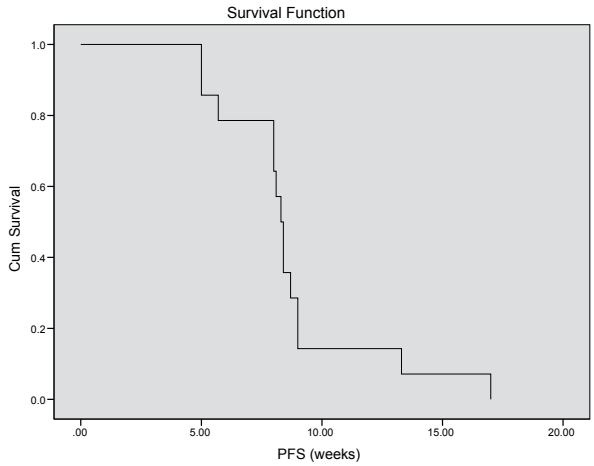

Figure 2a: Kaplan meier plot for progression free survival in weeks of CRC patient treated with 80 mg of simvastatin in combanation with panitumumab.

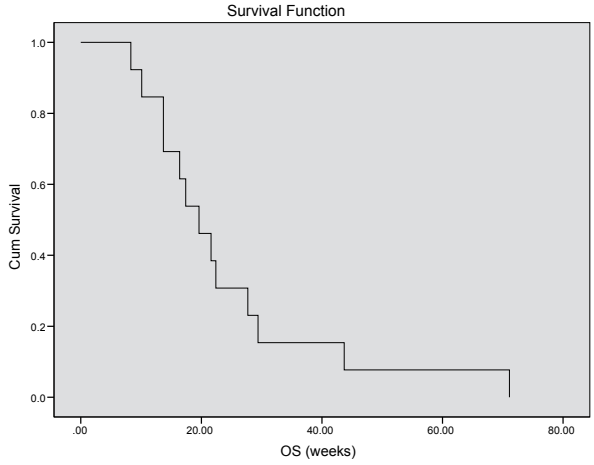

Figure 2b: Kaplan meier plot for overall survival in weeks of CRC patient treated with 80 mg of simvastatin in combination with panitumumab.

Toxicity

The most frequently reported adverse events on study were fatigue (n=10), anemia (n=9) and hypomagnesaemia (n=9). The incidence of severe adverse events is provided in Table 3. Skin toxicity occurred in 10 patients. Acneiform rash was reported in seven patients, none had grade 3 acneiform rash, though one case of grade 3 folliculitis was reported. Myopathy occurred in three patients. Grade 3 myopathy was reported in one patient and the patient terminated study participation for this reason. Elevation of CK was reported in all three patients with myopathy (up to 3917U/l in one patient) and in two additional patients.

Table 3 Severe (grade 3) adverse events

Event	Number (percentage)
Fatigue	3 (21)
Nausea	2 (14)
Pruritis	1 (7)
Vomitus	1 (7)
Myalgia	1 (7)
Folliculitis	1 (7)
Paronychia	1 (7)

Discussion

To the best of our knowledge this is the first clinical trial of combined treatment with simvastatin and panitumumab in CRC patients with a *KRAS* mutation in tumour tissue, testing the theoretical concept of *KRAS* modulation by statins. As only one out of 14 patients was alive and free from progression at time of the primary endpoint, study enrolment was terminated after the first stage of the study and it was concluded that simvastatin does not render sensitivity to panitumumab in this specific population.

The current study is not the first to hypothesize on statins and their inhibitory effect on the activity of RAS and its downstream pathway. However, all but one previous report are on preclinical research. Lovastatin was shown to inhibit RAS activation in *KRAS* transformed thyroid cells through inhibition of its farnesylation, and thereby inhibiting activity of the downstream pathway [26]. Furthermore, it was shown that lovastatin and simvastatin inhibit downstream activity in breast cells with mutated *HRAS*, known to induce an invasive phenotype, possibly by inhibiting membrane localization of HRAS. The effect was reversed by farnesyl pyrophosphate, indicating the effect was related to prenylation of RAS [27]. More recently, simvastatin was shown to restore cetuximab resistance *in vitro* and *in vivo* [28]. Based on these results, it may be questioned if a higher dose of simvastatin would have been necessary to overrule *KRAS* mutation and render sensitivity to EGFR inhibitor therapy. As mentioned above, statin doses up to 35mg/kg/day have been prescribed in clinical trials, though higher doses were not used continuously as was essential in the current design. Preclinical data research showed a significant reduction in cell growth of *KRAS* mutant CRC cell lines using 0.2 μM simvastatin, the equivalent of 2 mg/kg/day in humans [28]. Moreover, in cardiovascular disease the registered dose of 80 mg of simvastatin is significantly lowerings cholesterol serum levels. It is reasonableto assume that this dose will also affect the formation of the C15 and C17 groups and subsequently the prenylation of the KRAS protein. Furthermore, we question whether higher doses will be feasible in terms of safety.

The lack of effect in the current study is in striking contrast with the original reported data by Lee et al [29], testing the addition of simvastatin 40mg once daily to third-line therapy with cetuximab plus irinotecan in CRC patients harbouring a *KRAS* mutation. Their original report showed indeed a low response rate ((one out of 52 patients had a partial remission), however PFS was 7.6 months, which is even higher than historical results of third-line cetuximab plus irinotecan in *KRAS* wild type CRC patients [30;31]. However, in a recent erratum Lee et al [32] reported corrected measurements of PFS in their population. Corrected mean PFS was 3.7 months (range 2.1-5.3), significantly lower than previous reports of cetuximab plus irinotecan as third-line therapy in *KRAS* wild type [30;31]. In summary, both our study as well as the study by Lee et al show that simvastatin does not render sensitivity to EGFR inhibitor therapy.

Mutational status of *PIK3CA* is also related to response to EGFR inhibitor based therapy. The majority of *PIK3CA* mutations are located in exon 9 and exon 20, and those mutations may occur in patients with or without *KRAS* mutation in tumour tissue. Only *PIK3CA* mutations in codon 20 are associated with lower ORR and PFS [4]. If statins would be able to induce a *KRAS* wild type phenotype in our population, a high incidence of *PIK3CA* mutations might still lead to low PFS. However, since only two patients harboured a *PIK3CA* mutation (one in exon 9 and one in exon 20), this is not likely to (partly) explain the results of the current study.

Toxicity of this dose of simvastatin in CRC patients failing standard chemotherapy was relatively mild, with only two patients in need for dose reduction and only one patients experiencing severe myopathy. Panitumumab was also well tolerated, in line with previous data of panitumumab as third-line therapy [2].

Conclusion

The present study showed that simvastatin 80 once daily does not render sensitivity to panitumumab in CRC patients with a *KRAS* mutation failing oxaliplatin, 5-FU and irinotecan based therapy. The theoretical concept of *KRAS* modulation using statins does not seem feasible in the clinic. Recently, regorafenib was registered for these patients (and for *KRAS* wild type patients after failing third-line therapy with an EGFR inhibitor), however PFS gain is limited [33]. New therapeutic strategies for these patients are needed.

References

1 Jonker DJ, O'Callaghan CJ, Karapetis CS, Zalcberg JR, Tu D, et al. (2007) Cetuximab for the treatment of colorectal cancer. N Engl J Med 357: 2040-2048.

2 Van Cutsum E, Peeters M, Siena S, Humblet Y, Hendlisz A, et al. (2007) Open-label phase III trial of panitumumab plus best supportive care compared with best supportive care alone in patients with chemotherapy-refractory metastatic colorectal cancer. J Clin Oncol 25: 1658-1664.

3 Amado RG, Wolf M, Peeters M, Van CE, Siena S, et al. (2008) Wild type KRAS is required for panitumumab efficacy in patients with metastatic colorectal cancer. J Clin Oncol 26: 1626-1634.

4 De Roock W, Claes B, Bernasconi D, De SJ, Biesmans B, et al. (2010) Effects of KRAS, BRAF, NRAS, and PIK3CA mutations on the efficacy of cetuximab plus chemotherapy in chemotherapy-refractory metastatic colorectal cancer: a retrospective consortium analysis. Lancet Oncol 11: 753-762.

5 Karapetis CS, Khambata-Ford S, Jonker DJ, O'Callaghan CJ, Tu D, et al. (2008) KRAS mutations and benefit from cetuximab in advanced colorectal cancer. N Engl J Med 359: 1757-1765.

6 Sorich MJ, Wiese MD, Rowland A, Kichenadasse G, McKinnon RA, et al. (2014) Extended RAS mutations and anti-EGFR monoclonal antibody survival benefit in metastatic colorectal cancer: a meta-analysis of randomized, controlled trials. Ann Oncol .

7 Krens LL, Baas JM, Gelderblom H, Guchelaar HJ. (2010) Therapeutic modulation of KRAS signaling in colorectal cancer. Drug Discov Today 15: 502-516.

8 Swanson KM, Hohl RJ. (2006) Anti-cancer therapy: targeting the mevalonate pathway. Curr Cancer Drug Targets 6: 15-37.

9 Graf H, Jungst C, Straub G, Dogan S, Hoffmann RT, et al. (2008) Chemoembolization combined with pravastatin improves survival in patients with hepatocellular carcinoma. Digestion 78: 34-38.

10 Han JY, Lim KY, Yu SY, Yun T, Kim HT, et al. (2011) A phase 2 study of irinotecan, cisplatin, and simvastatin for untreated extensive-disease small cell lung cancer. Cancer 117: 2178-2185.

11 Han JY, Lee SH, Yoo NJ, Hyung LS, Moon YJ, et al. (2011) A randomized phase II study of gefitinib plus simvastatin versus gefitinib alone in previously treated patients with advanced non-small cell lung cancer. Clin Cancer Res 17: 1553-1560.

12 Hong JY, Nam EM, Lee J, Park JO, Lee SC, et al. (2014) Randomized double-blinded, placebo-controlled phase II trial of simvastatin and gemcitabine in advanced pancreatic cancer patients. Cancer Chemother Pharmacol 73: 125-130.

13 Kawata S, Yamasaki E, Nagase T, Inui Y, Ito N, et al. (2001) Effect of pravastatin on survival in patients with advanced hepatocellular carcinoma. A randomized controlled trial. Br J Cancer 84: 886-891.

14 Kim WS, Kim MM, Choi HJ, Yoon SS, Lee MH, et al. (2001) Phase II study of high-dose lovastatin in patients with advanced gastric adenocarcinoma. Invest New Drugs 19: 81-83.

15 Knox JJ, Siu LL, Chen E, Dimitroulakos J, Kamel-Reid S, et al. (2005) A Phase I trial of prolonged administration of lovastatin in patients with recurrent or metastatic squamous cell carcinoma of the head and neck or of the cervix. Eur J Cancer 41: 523-530.

16 Konings IR, van der Gaast A, van der Wijk LJ, de Jongh FE, Eskens FA, et al. (2010) The addition of pravastatin to chemotherapy in advanced gastric carcinoma: a randomised phase II trial. Eur J Cancer 46: 3200-3204.

17 Lee J, Jung KH, Park YS, Ahn JB, Shin SJ, et al. (2009) Simvastatin plus irinotecan, 5-fluorouracil, and leucovorin (FOLFIRI) as first-line chemotherapy in metastatic colorectal patients: a multicenter phase II study. Cancer Chemother Pharmacol 64: 657-663.

18 Lersch C, Schmelz R, Erdmann J, Hollweck R, Schulte-Frohlinde E, et al. (2004) Treatment of HCC with pravastatin, octreotide,

or gemcitabine--a critical evaluation. Hepatogastroenterology 51: 1099-1103.

19 Manoukian GE, Tannir NM, Jonasch E, Qiao W, Haygood TM, et al. (2011) Pilot trial of bone-targeted therapy combining zoledronate with fluvastatin or atorvastatin for patients with metastatic renal cell carcinoma. Clin Genitourin Cancer 9: 81-88.

20 Ahmed TA, Hayslip J, Leggas M. (2013) Pharmacokinetics of high-dose simvastatin in refractory and relapsed chronic lymphocytic leukemia patients. Cancer Chemother Pharmacol 72: 1369-1374.

21 Hus M, Grzasko N, Szostek M, Pluta A, Helbig G, et al. (2011) Thalidomide, dexamethasone and lovastatin with autologous stem cell transplantation as a salvage immunomodulatory therapy in patients with relapsed and refractory multiple myeloma. Ann Hematol 90: 1161-1166.

22 van der Spek E, Bloem AC, Sinnige HA, Lokhorst HM. (2007) High dose simvastatin does not reverse resistance to vincristine, adriamycin, and dexamethasone (VAD) in myeloma. Haematologica 92: e130-e131.

23 van Eijk R., Licht J, Schrumpf M et al. Rapid KRAS, EGFR, BRAF and PIK3CA mutation analysis of fine needle aspirates from non-small-cell lung cancer using allele-specific qPCR. PLoS.One. 2011; 6: e17791.

24 Tol J, Nagtegaal ID, Punt CJ. BRAF mutation in metastatic colorectal cancer. N.Engl.J.Med. 2009; 361: 98-9.

25 Simon R. (1989) Optimal two-stage designs for phase II clinical trials. Control Clin Trials 10: 1-10.

26 Laezza C, Fiorentino L, Pisanti S, Gazzerro P, Caraglia M, et al. (2008) Lovastatin induces apoptosis of KRAS-transformed thyroid cells via inhibition of ras farnesylation and by modulating redox state. J Mol Med (Berl) 86: 1341-1351.

27 (Kang S, Kim ES, Moon A. (2009) Simvastatin and lovastatin inhibit breast cell invasion induced by H-Ras. Oncol Rep 21: 1317-1322.

28 Lee J, Lee I, Han B, Park JO, Jang J, et al. (2011) Effect of simvastatin on cetuximab resistance in human colorectal cancer with KRAS mutations. J Natl Cancer Inst 103: 674-688.

29 Lee J, Hong YS, Hong JY, Han SW, Kim TW, et al. (2014) Effect of simvastatin plus cetuximab/irinotecan for KRAS mutant colorectal cancer and predictive value of the RAS signature for treatment response to cetuximab. Invest New Drugs 32: 535-541.

30 Sohn BS, Kim TW, Lee JL, Ryu MH, Chang HM, et al. (2009) The role of KRAS mutations in predicting the efficacy of cetuximab-plus-irinotecan therapy in irinotecan-refractory Korean metastatic colorectal cancer patients. Oncology 77: 224-230.

31 Spindler KL, Pallisgaard N, Lindebjerg J, Frifeldt SK, Jakobsen A. (2011) EGFR related mutational status and association to clinical outcome of third-line cetuximab-irinotecan in metastatic colorectal cancer. BMC Cancer 11: 107.

32 Lee J, Hong YS, Hong JY, Han SW, Kim TW, et al. (2014) Erratum to: Effect of simvastatin plus cetuximab/irinotecan for KRAS mutant colorectal cancer and predictive value of the RAS signature for treatment response to cetuximab. Invest New Drugs .

33 Tejpar S, Celik I, Schlichting M, Sartorius U, Bokemeyer C, et al. (2012) Association of KRAS G13D tumor mutations with outcome in patients with metastatic colorectal cancer treated with first-line chemotherapy with or without cetuximab. J Clin Oncol 30: 3570-3577.

34 Grothey A, Van CE, Sobrero A, Siena S, Falcone A, et al. (2013) Regorafenib monotherapy for previously treated metastatic colorectal cancer (CORRECT): an international, multicentre, randomised, placebo-controlled, phase 3 trial. Lancet 381: 303-312.

Chapter 7

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Submitted

Effect of the Fc gamma receptor polymorphism V158F status on the survival of metastatic colorectal cancer patients treated with cetuximab: a meta-analysis

Abstract

Background

The use of cetuximab in metastatic colorectal cancer (CRC) is limited to patients with wild type *KRAS* tumors and more recently to *RAS* wild type tumors only. Antibody-dependent cellular cytotoxicity (ADCC), mediated by the Fc gamma receptor (FCGR) is assumed to be an important mechanism for induction of tumor cell death by cetuximab. Several studies explored the role of FCGR3A (rs396991) genetic polymorphism in cetuximab efficacy in mCRC patients, but the results from these studies are discordant.

Method

An individual patient data meta-analysis was performed, to better understand the effect of FCGR3A FF versus non FF (FV and VV) polymorphism on progression free survival (PFS) and overall survival (OS) in patients with *KRAS* mutant or wild type metastatic CRC, treated with cetuximab. Three studies were included in this meta-analysis.

Results

The hazard ratio (HR) for the primary endpoint progression free survival for FCGR3A non FF, adjusted for *KRAS* and the interaction between FCGR3A and *KRAS* was equal to 1.07 (95% confidence interval 0.89 - 1.29, p = 0.45). For overall survival, the HR for FCGR3A non FF, adjusted for *KRAS* and the interaction between FCGR3A and *KRAS* was equal to 0.91 (95% confidence interval 0.77 - 1.07).

Conclusion

The results of the present analysis suggest that FCGR3A rs396991 is not associated with progression free or overall survival in cetuximab treated mCRC patients.

Introduction

Cetuximab is an IgG1-type chimeric monoclonal antibody (MoAb) that targets the epidermal growth factor receptor (EGFR). Cetuximab is mainly used for treatment of metastatic colorectal cancer. Blocking of EGFR results in decreased proliferation, cell survival and angiogenesis. However, about 40% of colorectal cancers harbor a mutation in *KRAS* and these tumors do not respond to anti-EGFR therapy[1-3]. For this reason, the use of cetuximab is limited to patients with wild type *KRAS* tumors and more recently to patients with *RAS* wild type tumors only[4].

Antibody-dependent cellular cytotoxicity (ADCC), mediated by the Fc gamma receptor (FCGR) is assumed to be an important mechanism for induction of tumor cell death by cetuximab[5]. MoAbs are molecules of the IgG class and have an antigen-binding fragment (Fab) and a constant fragment (Fc). Fc gamma receptors (FCGRs) are expressed on immune effector cells, such as macrophages and natural killer lymphocytes. ADCC is induced when FCGRs bind to the monoclonal Fc fragment, since this interaction leads to the activation and degranulation of the effector cells and the subsequent lysis of the tumor[6].

Several germline single nucleotide polymorphisms (SNP) in the *FCGR* gene have been identified that confer a different binding affinity of the FCGR to the Fc fragment of the MoAb. The polymorphism in the Fc gamma receptor 3A gene (*FCGR3A*) c.818A>C results in a change of phenylalanine (F) to valine (V) at position 158 (rs396991)[6,7]. The C allele coding for valine of FCGR3A has a much higher affinity for binding to Fc than the wild type A allele coding for phenlyalanine. Importantly, the V phenotype has been related with a more extensive IgG1-induced ADCC[8,9]. Several studies, especially in large B-cell and follicular lymphoma patients treated with the MoAb rituximab, show a better clinical outcome for patients with FCGR3A VV phenotype, a finding that might be explained by the higher binding affinity conferred by this V phenotype[5,10-12].

The possible advantage of FCGR3A VV phenotype is less clear in mCRC patients treated with cetuximab. Indeed, the results from several studies investigating the association between *FCGR3A* genotype and cetuximab efficacy in mCRC patients are discordant (Table 1). A total of 13 published studies have investigated the association between *FCGR3A* and response, progression free or overall survival in mCRC patients treated with cetuximab. Seven studies[13-19] did not find any significant association between *FCGR3A* and outcome. The studies by Bibeau et al.[20] and Calemma et al.[21] reported that patients with the FCGR3A VV phenotype had a longer PFS. In contrast, four other studies reported a higher likelihood of cetuximab induced progression free or overall survival for patients with the FCGR3A F phenotype. In the study of Zhang et al.[22] a significantly higher response rate (RR) was seen in cetuximab plus bevacizumab treated patients with the FF group (RR = 56%) compared to FV (RR = 25%) and VV (RR = 8%) phenotypes. Dahan et al. [23] (58 patients) reported a decreased overall survival for patients with the *FCGR3A* VV phenotype whereas Pander et al. showed that the C allele coding for valine was associated with a shorter progression free survival[24]. Finally, a small study performed by Zhang et al. in 2007 with only 39 mCRC patients showed that those with the F-containing phenotype (FF or FV) had a longer PFS [31]. These conflicting results could be explained by a limited sample size of the different studies, genotyping errors (distribution of the genotypes is not always consistent with the Hardy-Weinberg equilibrium)[25-26] and different clinical scenarios.

As mentioned, most studies did have some drawbacks regarding the number of patients per study. The studies of Bibeau et al.[27], Zhang et al.[22] and Park et al.[28] reported genotype distributions of *FCGR3A* which deviated from Hardy-Weinberg equilibrium. Some of these

studies reported an altered outcome for the different *FCGR3A* genotypes, however, deviation from the Hardy-Weinberg equilibrium raises concerns in interpreting the outcome of these studies. All studies conducted retrospective analysis and positive/significant associations were found in relatively small patient cohorts, ranging from 32 to 270 patients. In these small studies many associations may have been studied and due to multiplicity, false positive associations may have occurred.

In an earlier study of our research group, the study of Pander et al.[24], we showed that the V-phenotype was associated with worse progression. In addition, in an *in vitro* study we showed that an extensive binding of *FCGR3A* with the C allele coding for valine, expressed by type 2 macrophages, resulted in the release of tumor promoting factors[29]. This effect of the *FCGR3A* genotype appeared independent of the *KRAS* mutation status of the tumor[24]. This preclinical finding, resulting in extensive release of tumor promoting factors after extensive binding by the C allele coding for V, was the basis to further investigate the association between *FCGR3A* polymorphisms, *KRAS* mutational status and survival. A dominant model was used to study the differences between FCGR3A wild type FF versus FCGR3A heterozygous mutant FV plus homozygous mutant VV. Interestingly, in both our preclinical study and the CAIRO2 study an effect of FCGR3A was seen independent of *KRAS* mutational status. Interestingly, the effect of the FCGR3A polymorphism was substantial and in patients with a KRAS mutant tumor and a favourable FCGR3A polymorphism survival was comparable to patients with a KRAS wild type tumor but unfavourable FCGR3A polymorphism. For this reason, we aimed to study the effect of the *FCGR3A* (rs396991) polymorphism in patients with *KRAS* wild type and mutant CRC despite the fact that cetuximab is nowadays only used in RAS wild type patients. Consequently, for our meta-analysis we selected studies in which patients were included with KRAS mutant and wild type tumors, performed at the time when the use of cetuximab was not yet restricted to (K) RAS wild type tumors.

We conducted an individual patient data meta-analysis combining 1,301 patients from three independent studies, to study and FCGR3A FF versus non FF (FV and VV) phenotypes on the progression free survival (PFS) and overall survival (OS) of metastatic colorectal cancer in patients with a *KRAS* mutant or wild type tumors treated with cetuximab. The other studies were excluded due to unknown *KRAS* status, *KRAS* wild type patients only, genotyping method, missing survival data or inability to provide the data.

Table 1: Overview of previous published studies, which studied the association between *FCGR3A* polymorphisms and cetuximab response in metastatic colorectal cancer.

Study	Patients	Distribution of FCGR3A phenotypes [1]	Treatment	*KRAS* status of tumor	Results
Zhang et al. 2007	39	FF: 16 FV: 14 VV: 5 $X^2 = 0.44$ p = 0.51	cetuximab	Unknown	Patients with any F phenotype showed favourable response (median PFS 3.7 vs. 1.1 months p = 0.004).
Graziano et al. 2008	110	FF: 38 FV: 50 VV: 22 $X^2 = 0.56$ p = 0.45	Irinotecan + cetuximab	Whole population	No association found on progression free or overall survival.
Bibeau et al. 2009	68	FF: 15 FV: 43 VV: 10 $X^2 = 5.02$ P = 0.03	Irinotecan + cetuximab	Whole population and subgroup analysis in *KRAS* wild type and *KRAS* mutant	VV phenotype associated with longer PFS. VV phenotype 6.9 months vs. FV or VV phenotype 3.2 months in whole population. VV phenotype 5.5 months vs. FV or VV phenotype 2.8 months in *KRAS* mutant patients
Zhang et al. 2010	31	FF: 11 FV: 9 VV:11 $X^2 = 5.45$ p = 0.02	Cetuximab + bevacizumab + Irinotecan	In whole population	No association found on response rate.
	32	FF: 10 FV: 12 VV: 12 $X^2 = 2.89$ p = 0.09	Cetuximab + bevacizumab	Independent of *KRAS* status. Effect seen in whole population and *KRAS* wild type patients.	FF associated with a better response rate (56%) compared to FV (25%) and VV (8%) p = 0.05
Pander et al. 2010	270	FF: 119 FV and VV: 157	CAPOX + bevazizumab + cetuximab	Whole population and subgroup analysis in *KRAS* wild type and *KRAS* mutant	V allele associated with decrease in PFS (VV and FV 8.2 vs 12.8 months in FF and HR 1.56, p = 0.006) regardless of *KRAS* status
Paez et al. 2010	104	FF: 47 FV: 41 VV: 16 $X^2 = 1.89$ p = 0.17	Chemotherapy + cetuximab or panitumumab or panitumumab alone	Whole population and subgroup analysis in *KRAS* wild type and *KRAS* mutant	No association found for response rate or PFS.

Study	Patients	Distribution of FCGR3A phenotypes [1]	Treatment	*KRAS* status of tumor	Results
Dahan et al. 2011	58	FF: 30 FV: 20 VV: 6 $X^2 = 0.88$ $p = 0.34$	Irinotecan + cetuximab	Whole population and in subgroup of *KRAS* wild type patients	Median OS was 9.8 months in FF vs. 9.0 in FV vs. 2.6 in VV patients $p < 0.001$
Calemma et al. 2012	49	FF: 5 FV: 26 VV: 18 $X^2 = 0.98$ $p = 0.32$	Cetuximab or panitumumab	*KRAS* wild type patients only	Unfavourable prognosis for FF phenotypeMedian PFS VV, FV,FF; 18.2 vs. 17.3 vs. 9.4 months $p = 0.04$.
Rodriguez et al. 2012	44	FF: 13 FV and VV: 31	cetuximab	In mutated phenotype population (any *KRAS*, *BRAF*, *NRAS* or *PI3CA* mutation)	No association found, adjusted odds ratio for VV +FV was 3.8 (95% CI 0.5 -26)
Park et al. 2012	118	FF: 36 FV: 65 VV: 6 $X^2 = 10.86$ $p < 0.001$	Chemotherapy + cetuximab	Whole population and subgroup analysis in *KRAS* wild type, and *KRAS* mutant	No significant differences between RR, OS or PFS
Negri et al.	86	FF: 27 FV: 40 VV: 19 $X^2 = 0.33$ $p = 0.85$	cetuximab	*KRAS* wild type only	No significant differences between response rate or time to tumor progression
Kjersem et al. 2014	328	FF: 162 FV: 131 VV: 35 $X^2 = 1.19$ $p = 0.27$	FLOX +cetuximab	Subgroup analysis in patients with *KRAS* wild type and *KRAS* mutant	None of the FCGR3A phenotype were associated with altered response
Geva et al. 2014	1024	FF: 391 FV: 466 VV: 167 $X^2 = 1.99$ $p = 0.16$	Chemotherapy + cetuximab or cetuximab monotherapy	Whole population and subgroup analysis in *KRAS* wild type, exploratory analysis in *KRAS* mutant	No differences between median PFS between VV vs. FF +FV, better DCR and median OS in *KRAS* mutant subgroup in exploratory setting

Abbreviations: PFS, progression free survival; OS, overall survival; DCR, disease control rate; RR, response rate; CAPOX, capecitabine and oxaliplatin; FLOX, fluorouracil and oxaliplatin
1. If $p < 0.05$ not consistent with Hardy-Weinberg equilibrium.

Material and Methods

Individual patient data acquisition

To study the association between survival times and *FCGR3A* polymorphism, a literature search was performed in June 2014 on PubMed, by using the keywords cetuximab, FCGR polymorphisms, KRAS and (metastatic) colorectal cancer. We used the following criteria to select publications:

1. Treatment with cetuximab in mCRC;

2. Individual patient data regarding overall survival (OS) and progression free survival (PFS) or the individual patient data reconstruction using Kaplan Meier curves;

3. Availability of *FCGR3A* (rs396991) genotype;

4. Genotyping methods of eligible studies were reviewed to prevent inclusion of patients with an unreliable *FCGR3A* genotype. (Several methods do not discriminate between *FCGR3A* and *FCGR3B*, which may result in genotyping errors[26]);

5. Availability of *KRAS* mutational status (KRAS codon 12, 13 and if possible 61) of the tumor (both *KRAS* wild type and mutant patient were included in this study).

This resulted in the inclusion of patients from three studies, the CAIRO2 [24], Rodriguez et al.[15] and the study of the European colorectal cancer consortium [30]. The FCGR3A polymorphism data from the European colorectal cancer consortium, was not published at time of analysis but the authors provided us with the data. Recently, the FCGR3A polymorphisms data from the European colorectal cancer consortium was published by Geva et al.[18]. All studies were approved by the local ethics committees and all included patients gave informed consent. Articles were excluded due to unknown *KRAS* status[13,31]. *KRAS* wild type patients only[19,21], genotyping method[16,20,22,23], missing survival data[14] and inability to provide the data[17].

Study 1: cohort CAIRO2 study

Data from 193 patients were available from the CAIRO2 study, which started in the pre-*KRAS* era. These patients with mCRC were treated with firstline capecitabine, oxaliplatin and bevacizumab (CAPOX-B) or the same regimen plus cetuximab. Cetuximab was administered intravenously at a dose of 400 mg/m2 on the first day, followed by 250 mg/m2 weekly thereafter. Dose reductions were carried out according to the study protocol. The duration of a treatment cycle was three weeks. Treatment was continued until disease progression, death or unacceptable toxicity, whichever occurred first.

Study 2: cohort Rodriguez et al.

Data were available from 99 patients. Patients with mCRC were treated with cetuximab administered on an every-second week schedule at a dose of 500 mg/m2 combined with standard irinotecan or oxaliplatin based chemotherapy. Patients were treated in either first (31%) or second line therapy

Study 3: cohort European colorectal cancer consortium

From the European colorectal cancer consortium data were available from 1009 patients. Patients with mCRC were treated with irinotecan or oxaliplatin based chemotherapy and cetuximab or cetuximab monotherapy.

KRAS tumor status and FCGR3A rs396991 polymorphism

In all three studies, genotyping of the *FCGR3A* was performed on a validated realtime PCR system with a predesigned assay for *FCGR3A* rs396991 (C__25815666_10). Details about the used methods are described elsewhere [15,30,32].

Outcome measures

The association between *FCGR3A* rs396991 genotype and the primary endpoint PFS and the secondary endpoint OS were investigated. PFS was calculated as time from randomisation to the first documented progression, death or loss to follow up, whichever occurred first. OS was estimated from time since randomisation to death or loss to follow up.

Statistical analysis

Meta-analysis based on the survival outcomes coming from the three studies described above was performed, for two studies individual patient data were available while for the third study individual patient data were reconstructed from the estimated PFS and OS. Reconstruction of the relevant data is discussed by Fiocco et al. [33,34]. Further details on data analysis are described in appendix 1. A multivariate mixed effects Cox proportional hazard model with study as random effects was employed to investigate the effects of *FCGR3A*, *KRAS* mutation status and the interaction between FCGR3A and KRAS on the primary endpoint PFS and secondary endpoint OS.

Results

Individual patient data meta-analysis

A total of 1,301 patients were included in the analysis. In table 2 an overview of the incidence of *FCGR3A* polymorphism and *KRAS* tumor status is shown. For all three studies, the reported *FCGR3A* genotypes were in Hardy-Weinberg equilibrium. To study the effect of *FCGR3A* polymorphisms, we used a dominant genetic model (FF vs non FF). Table 3 shows an overview of the median PFS and OS for the three different studies for *FCGR3A* FF and non FF.

Progression free survival

The hazard ratio (HR) for FCGR3A non FF, adjusted for *KRAS* and the interaction between FCGR3A and *KRAS* was equal to 1.07 (95% confidence interval 0.89 – 1.29, p = 0.45). The estimated pooled Kaplan Meier curves, for patients with *KRAS* mutant and wild type tumors and FCGR3A FF and non FF status, are shown in Figure 1. A small, non-significant effect is seen between FCGR3A FF and non FF, stratified for KRAS status of the tumor. For patients with *KRAS* wild type tumors, median PFS was equal to 14.0 (95% CI interval 12.5 – 15.9) and 15.2 (95% CI interval 14.0 – 17.1) months for FCGR3A FF and non FF respectively. For patients

with a *KRAS* mutant tumor PFS was 10.6 (95% CI interval 9.0 – 13.1) and 9.2 (95% CI interval 8.0 – 11.3) months for FCGR3A FF and non FF, respectively.

Table 2: distribution of FCGR3A polymorphisms

Polymorphism or mutation	Study 1: CAIRO2 N (%)	Study 2: Rodriguez N (%)	Study 3: European colorectal cancer consortium N (%)	Total
total	193	99	1009	1301
KRAS wild type	125 (64.8)	56 (56.7)	676 (67.0)	857 (65.9)
KRAS mutant	68 (35.2)	43 (43.4)	333 (33.0)	444 (34.1)
FCGR3A – FF	84 (43.5)	43 (43.4)	384 (38.1)	511 (39.3)
FCGR3A – VF	83 (43.0)	41 (41.4)	459 (45.5)	583 (44.8)
FCGR3A – VV	26 (13.5)	15 (15.1)	166 (16.5)	207 (15.9)
HWE p-value[1]	0.45	0.32	0.15	0.06

Abbreviation: HWE: Hardy-Weinberg Equilibrium.
1. If $p < 0.05$ not consistent with Hardy-Weinberg Equilibrium.

Table 3: Median PFS and OS for cetuximab treated KRAS wild type and mutant patients

Study	Treatment	FCGR3A	Median PFS	Median OS
1: CAIRO2	CAPOX + bevacizumab and cetuximab	FF non FF	11.6 months 8.1 months	21.7 months 21.9 months
2: Rodriguez	Cetuximab 2-weekly	FF non FF	5.0 months 4 months	44.5 months 25.9 months
3: European colorectal cancer consortium	Irinotecan/ oxaliplatin based chemotherapy +cetuximab or cetuximab alone.	FF non FF	4.1 months 3.8 months	9.9 months 10.1 months

Overall survival

The HR for FCGR3A non FF, adjusted for *KRAS* and the interaction between FCGR3A and *KRAS* was 0.91 (95% confidence interval 0.77 – 1.07). In figure 2 the pooled Kaplan Meier curves for patients with KRAS mutant and wild type tumors, and FCGR3A FF and non-FF status are depicted. A difference between patients with a KRAS wild type and KRAS mutant tumor was observed in the plots, although this difference is not significant. For patients with KRAS wild type tumor, median OS was equal to 37.3 (95% CI interval 33.1 – 45.3) and 46.3 (95% CI interval 39.0 – 54.0) months for *FCGR3A* FF and non FF respectively. For patients with KRAS mutant tumor median OS was equal to 27.7 (95% CI interval 22.6-35.2) and 21.5 (18.9-26.4) months for FCGR3A FF and non FF respectively.

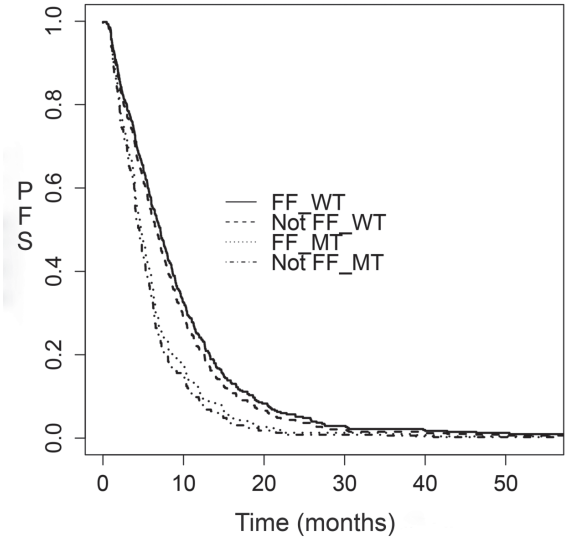

Figure 1: Estimated Kaplan Meier curves for progression free survival in patients treated with cetuximab, stratified by *KRAS* tumor status and FCGR3A status.
Abbreviations: **PFS:** progression free survival, FF_WT: FCGR3A FF and *KRAS* wild type; Not FF_WT, FCGR3A not FF and *KRAS* wild type; FF_MT, FCGR3A FF and *KRAS* mutant; Not FF_MT, FCGR3A not FF and *KRAS* mutant

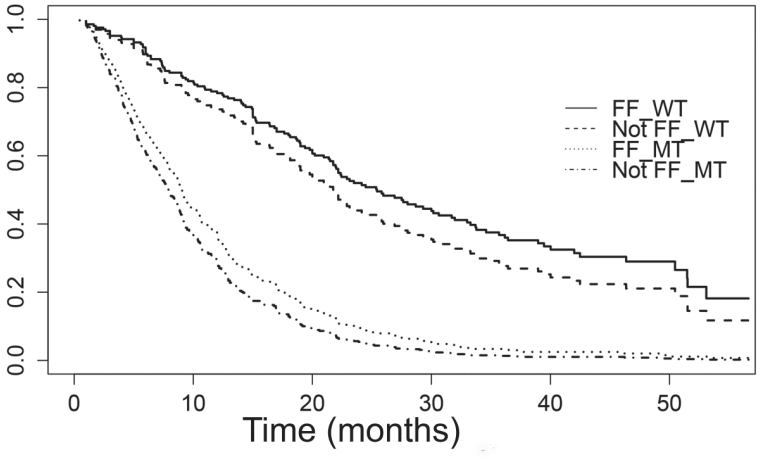

Figure 2: Estimated Kaplan Meier curves for overall survival in patients treated with cetuximab, stratified by *KRAS* tumor status and FCGR3A status.
Abbreviations: **FF_WT:** FCGR3A FF and *KRAS* wild type; Not FF_WT, FCGR3A not FF and *KRAS* wild type; FF_MT, FCGR3A FF and *KRAS* mutant; Not FF_MT, FCGR3A not FF and *KRAS* mutant

Discussion

The meta-analysis performed in this study indicates that the *FCGR3A* (rs396991) polymorphism is not associated with progression free or overall survival in patients with metastatic colorectal cancer treated with cetuximab in patients with either a *KRAS* mutant or *KRAS* wild type tumor. In our meta-analysis we did not find any advantage for the FF genotype in terms of clinical efficacy.

Both *KRAS* wild type and mutant metastatic colorectal cancer patients were included in the analysis, since the interesting results seen in the preclinical[29] and CAIRO 2[24] study. This meta-analysis however shows that there is no difference between the KRAS wild type and mutant populations.

Nowadays cetuximab is used in RAS wild type patients only. In the three included studies only the KRAS status of the tumor was known. Unfortunately, we were not able to extend our research to the effect of FCGR3A in the RAS mutant colorectal cancer patient due to the retrospective design of the studies included in this meta-analysis. Most probably the including RAS mutant colorectal patients in the analysis would not have altered the outcome of this study

Differences between studies due to a specific design and methodology, clinical procedures, different lines of chemotherapy and patients' characteristics can contribute to variability in treatment effect among studies. Heterogeneous studies are a common problem in meta-analysis[36]; to take into account the inter-trial heterogeneity caused by different treatments and lines of therapy used in these studies, we performed a meta-analysis by including studies as random effects, allowing for differences in the treatment effect and different regimens used from study to study and providing a more efficient estimate of the average treatment effect[37].

The inconsistent finding in studies concerning *FCGR3A* polymorphism and cetuximab efficacy shows the importance of genotyping methods, appropriate sample size and proper use of statistical methodology. We pooled data concerning 1,301 patients to improve the statistical power to detect the presence of a treatment effects on survival. Instead of reporting the classical forest plot based on hazard ratio for each individual study, we performed a meta-analysis based on individual patient data, which gave a better estimation of the potential benefit of FCGR3A FF status by using the individual patient data. An individual patient data meta-analysis approach of time to event outcomes, although usually more demanding, allows a deeper investigation.

Advanced and metastasizing CRC and prior lines of chemotherapy may be linked to decreased immune responses and impaired natural killer cell dysfunction and consequently failure of cetuximab treatment[38-40]. This may result in a more limited role of ADCC in cetuximab treated patients with advanced disease. Noteworthy, patients from CAIRO2 received concomitant chemotherapy and in this study, a difference in median PFS between FCGR3A FF and non FF was observed. Nonetheless, no difference was seen for *FCGR3A* on median OS.

In this meta-analysis we have studied the *FCGR3A* polymorphism rs396991 but ADCC is a complex biological process and a more in-depth analysis of alternative crucial steps in the immunological pathway may be of influence. Consideration of other FCGRs, MHC expression, IFN-gamma pathway components and antigen processing machinery genes might provide a broader insight into the role of immunity in cetuximab efficacy.

In conclusion, our results do not support a predictive role for the *FCGR3A* polymorphism (rs396991) in cetuximab efficacy.

References

1 Lievre A, Bachet JB, Le CD, Boige V, Landi B, Emile JF, Cote JF, Tomasic G, Penna C, Ducreux M, Rougier P, Penault-Llorca F, Laurent-Puig P (2006) KRAS mutation status is predictive of response to cetuximab therapy in colorectal cancer. Cancer Res 66: 3992-3995.

2 Karapetis CS, Khambata-Ford S, Jonker DJ, O'Callaghan CJ, Tu D, Tebbutt NC, Simes RJ, Chalchal H, Shapiro JD, Robitaille S, Price TJ, Shepherd L, Au HJ, Langer C, Moore MJ, Zalcberg JR (2008) K-ras mutations and benefit from cetuximab in advanced colorectal cancer. N Engl J Med 359: 1757-1765.

3 Allegra CJ, Jessup JM, Somerfield MR, Hamilton SR, Hammond EH, Hayes DF, McAllister PK, Morton RF, Schilsky RL (2009) American Society of Clinical Oncology provisional clinical opinion: testing for KRAS gene mutations in patients with metastatic colorectal carcinoma to predict response to anti-epidermal growth factor receptor monoclonal antibody therapy. J Clin Oncol 27: 2091-2096. J

4 Douillard JY, Oliner KS, Siena S, Tabernero J, Burkes R, Barugel M, Humblet Y, Bodoky G, Cunningham D, Jassem J, Rivera F, Kocakova I, Ruff P, Blasinska-Morawiec M, Smakal M, Canon JL, Rother M, Williams R, Rong A, Wiezorek J, Sidhu R, Patterson SD (2013) Panitumumab-FOLFOX4 treatment and RAS mutations in colorectal cancer. N Engl J Med 369: 1023-1034.

5 Weng WK, Levy R (2003) Two immunoglobulin G fragment C receptor polymorphisms Iindependently predict response to rituximab in patients with follicular lymphoma. J Clin Oncol 21: 3940-3947.

6 van Sorge NM, van der Pol WL, van de Winkel JG (2003) FcgammaR polymorphisms: Implications for function, disease susceptibility and immunotherapy. Tissue Antigens 61: 189-202.

7 Mellor JD, Brown MP, Irving HR, Zalcberg JR, Dobrovic A (2013) A critical review of the role of Fc gamma receptor polymorphisms in the response to monoclonal antibodies in cancer. J Hematol Oncol 6: 1. 1756-8722-6-1

8 Koene HR, Kleijer M, Algra J, Roos D, von dem Borne AE, de HM (1997) Fc gammaRIIIa-158V/F polymorphism influences the binding of IgG by natural killer cell Fc gammaRIIIa, independently of the Fc gammaRIIIa-48L/R/H phenotype. Blood 90: 1109-1114.

9 de Haas M., Koene HR, Kleijer M, de VE, Simsek S, van Tol MJ, Roos D, von dem Borne AE (1996) A triallelic Fc gamma receptor type IIIA polymorphism influences the binding of human IgG by NK cell Fc gamma RIIIa. J Immunol 156: 2948-2955.

10 Ahlgrimm M, Pfreundschuh M, Kreuz M, Regitz E, Preuss KD, Bittenbring J (2011) The impact of Fc-gamma receptor polymorphisms in elderly patients with diffuse large B-cell lymphoma treated with CHOP with or without rituximab. Blood 118: 4657-4662. blood-2011-04-346411

11 Persky DO, Dornan D, Goldman BH, Braziel RM, Fisher RI, Leblanc M, Maloney DG, Press OW, Miller TP, Rimsza LM (2012) Fc gamma receptor 3a genotype predicts overall survival in follicular lymphoma patients treated on SWOG trials with combined monoclonal antibody plus chemotherapy but not chemotherapy alone. Haematologica 97: 937-942.

12 Kim DH, Jung HD, Kim JG, Lee JJ, Yang DH, Park YH, Do YR, Shin HJ, Kim MK, Hyun MS, Sohn SK (2006) FCGR3A gene polymorphisms may correlate with response to frontline R-CHOP therapy for diffuse large B-cell lymphoma. Blood 108: 2720-2725. blood-2006-01-009480

13 Graziano F, Ruzzo A, Loupakis F, Canestrari E, Santini D, Catalano V, Bisonni R, Torresi U, Floriani I, Schiavon G, Andreoni F, Maltese P, Rulli E, Humar B, Falcone A, Giustini L, Tonini G, Fontana A, Masi G, Magnani M (2008) Pharmacogenetic profiling for cetuximab plus irinotecan therapy in patients with refractory advanced colorectal cancer. J Clin Oncol 26: 1427-1434. 26/9/1427

14 Paez D, Pare L, Espinosa I, Salazar J, del RE, Barnadas A, Marcuello E, Baiget M (2010) Immunoglobulin G fragment C receptor

polymorphisms and KRAS mutations: are they useful biomarkers of clinical outcome in advanced colorectal cancer treated with anti-EGFR-based therapy? Cancer Sci 101: 2048-2053.

15 Rodriguez J, Zarate R, Bandres E, Boni V, Hernandez A, Sola JJ, Honorato B, Bitarte N, Garcia-Foncillas J (2012) Fc gamma receptor polymorphisms as predictive markers of Cetuximab efficacy in epidermal growth factor receptor downstream-mutated metastatic colorectal cancer. Eur J Cancer 48: 1774-1780.

16 Park SJ, Hong YS, Lee JL, Ryu MH, Chang HM, Kim KP, Ahn YC, Na YS, Jin DH, Yu CS, Kim JC, Kang YK, Kim TW (2012) Genetic polymorphisms of FcgammaRIIa and FcgammaRIIIa are not predictive of clinical outcomes after cetuximab plus irinotecan chemotherapy in patients with metastatic colorectal cancer. Oncology 82: 83-89.

17 Kjersem JB, Skovlund E, Ikdahl T, Guren T, Kersten C, Dalsgaard AM, Yilmaz MK, Fokstuen T, Tveit KM, Kure EH (2014) FCGR2A and FCGR3A polymorphisms and clinical outcome in metastatic colorectal cancer patients treated with first-line 5-fluorouracil/folinic acid and oxaliplatin +/- cetuximab. BMC Cancer 14: 340.

18 Geva R, Vecchione L, Kalogeras KT, Vittrup JB, Lenz HJ, Yoshino T, Paez D, Montagut C, Souglakos J, Cappuzzo F, Cervantes A, Frattini M, Fountzilas G, Johansen JS, Hogdall EV, Zhang W, Yang D, Yamazaki K, Nishina T, Papamichael D, Vincenzi B, Macarulla T, Loupakis F, De SJ, Spindler KL, Pfeiffer P, Ciardiello F, Piessevaux H, Tejpar S (2014) FCGR polymorphisms and cetuximab efficacy in chemorefractory metastatic colorectal cancer: an international consortium study. Gut Epub ahead of print. gutjnl-2014-307234

19 Negri FV, Musolino A, Naldi N, Bortesi B, Missale G, Laccabue D, Zerbini A, Camisa R, Chernyschova N, Bisagni G, Loupakis F, Ruzzo A, Neri TM, Ardizzoni A (2014) Role of immunoglobulin G fragment C receptor polymorphism-mediated antibody-dependant cellular cytotoxicity in colorectal cancer treated with cetuximab therapy. Pharmacogenomics J 14: 14-19. tpj201254 [pii];10.1038/tpj.2012.54 [doi].

20 Bibeau F, Lopez-Crapez E, Di FF, Thezenas S, Ychou M, Blanchard F, Lamy A, Penault-Llorca F, Frebourg T, Michel P, Sabourin JC, Boissiere-Michot F (2009) Impact of Fc{gamma}RIIa-Fc{gamma}RIIIa polymorphisms and KRAS mutations on the clinical outcome of patients with metastatic colorectal cancer treated with cetuximab plus irinotecan. J Clin Oncol 27: 1122-1129.

21 Calemma R, Ottaiano A, Trotta AM, Nasti G, Romano C, Napolitano M, Galati D, Borrelli P, Zanotta S, Cassata A, Castello G, Iaffaioli VR, Scala S (2012) Fc gamma receptor IIIa polymorphisms in advanced colorectal cancer patients correlated with response to anti-EGFR antibodies and clinical outcome. J Transl Med 10: 232. 1479-5876-10-232

22 Zhang W, Azuma M, Lurje G, Gordon MA, Yang D, Pohl A, Ning Y, Bohanes P, Gerger A, Winder T, Hollywood E, Danenberg KD, Saltz L, Lenz HJ (2010) Molecular predictors of combination targeted therapies (cetuximab, bevacizumab) in irinotecan-refractory colorectal cancer (BOND-2 study). Anticancer Res 30: 4209-4217.

23 Dahan L, Norguet E, Etienne-Grimaldi MC, Formento JL, Gasmi M, Nanni I, Gaudart J, Garcia S, Ouafik L, Seitz JF, Milano G (2011) Pharmacogenetic profiling and cetuximab outcome in patients with advanced colorectal cancer. BMC Cancer 11: 496. 1471-2407-11-496

24 Pander J, Gelderblom H, Antonini NF, Tol J, van Krieken JH, van der Straaten T, Punt CJ, Guchelaar HJ (2010) Correlation of FCGR3A and EGFR germline polymorphisms with the efficacy of cetuximab in KRAS wildtype metastatic colorectal cancer. Eur J Cancer 46: 1829-1834. S0959-8049(10)00248-0

25 Mellor JD, Brown MP, Irving HR, Zalcberg JR, Dobrovic A (2013) A critical review of the role of Fc gamma receptor polymorphisms in the response to monoclonal antibodies in cancer. J Hematol Oncol 6: 1. 1756-8722-6-1

26 van der Straaten T, Martijn R, el HT, Baak-Pablo R, Guchelaar HJ (2013) A novel specific pyrosequencing method for genotyping FCGR3A rs396991 without coamplification of homologous gene FCGR3B. Pharmacogenet

Genomics 23: 631-635.

27 Bibeau F, Lopez-Crapez E, Di FF, Thezenas S, Ychou M, Blanchard F, Lamy A, Penault-Llorca F, Frebourg T, Michel P, Sabourin JC, Boissiere-Michot F (2009) Impact of Fc{gamma}RIIa-Fc{gamma}RIIIa polymorphisms and KRAS mutations on the clinical outcome of patients with metastatic colorectal cancer treated with cetuximab plus irinotecan. J Clin Oncol 27: 1122-1129.

28 Park SJ, Hong YS, Lee JL, Ryu MH, Chang HM, Kim KP, Ahn YC, Na YS, Jin DH, Yu CS, Kim JC, Kang YK, Kim TW (2012) Genetic polymorphisms of FcgammaRIIa and FcgammaRIIIa are not predictive of clinical outcomes after cetuximab plus irinotecan chemotherapy in patients with metastatic colorectal cancer. Oncology 82: 83-89.

29 Pander J, Heusinkveld M, van der Straaten T, Jordanova ES, Baak-Pablo R, Gelderblom H, Morreau H, van der Burg SH, Guchelaar HJ, van HT (2011) Activation of tumor-promoting type 2 macrophages by EGFR-targeting antibody cetuximab. Clin Cancer Res 17: 5668-5673.

30 De Roock W., Claes B, Bernasconi D, De SJ, Biesmans B, Fountzilas G, Kalogeras KT, Kotoula V, Papamichael D, Laurent-Puig P, Penault-Llorca F, Rougier P, Vincenzi B, Santini D, Tonini G, Cappuzzo F, Frattini M, Molinari F, Saletti P, De DS, Martini M, Bardelli A, Siena S, Sartore-Bianchi A, Tabernero J, Macarulla T, Di FF, Gangloff AO, Ciardiello F, Pfeiffer P, Qvortrup C, Hansen TP, Van CE, Piessevaux H, Lambrechts D, Delorenzi M, Tejpar S (2010) Effects of KRAS, BRAF, NRAS, and PIK3CA mutations on the efficacy of cetuximab plus chemotherapy in chemotherapy-refractory metastatic colorectal cancer: a retrospective consortium analysis. Lancet Oncol 11: 753-762.

31 Zhang W, Gordon M, Schultheis AM, Yang DY, Nagashima F, Azuma M, Chang HM, Borucka E, Lurje G, Sherrod AE, Iqbal S, Groshen S, Lenz HJ (2007) FCGR2A and FCGR3A polymorphisms associated with clinical outcome of epidermal growth factor receptor expressing metastatic colorectal cancer patients treated with single-agent cetuximab. J Clin Oncol 25: 3712-3718.

32 Tol J, Koopman M, Cats A, Rodenburg CJ, Creemers GJ, Schrama JG, Erdkamp FL, Vos AH, van Groeningen CJ, Sinnige HA, Richel DJ, Voest EE, Dijkstra JR, Vink-Borger ME, Antonini NF, Mol L, van Krieken JH, Dalesio O, Punt CJ (2009) Chemotherapy, bevacizumab, and cetuximab in metastatic colorectal cancer. N Engl J Med 360: 563-572. 360/6/563

33 Fiocco M, Putter H, van Houwelingen JC (2009) Meta-analysis of pairs of survival curves under heterogeneity: a Poisson correlated gamma-frailty approach. Stat Med 28: 3782-3797.

34 Fiocco M, Stijnen T, Putter H (2012) Meta-analysis of time-to-event outcomes using a hazard-based approach: Comparison with other models, robustness and meta-regression. Computational Statistics & Data Analysis 56: 1028-1037.

35 Lievre A, Bachet JB, Le CD, Boige V, Landi B, Emile JF, Cote JF, Tomasic G, Penna C, Ducreux M, Rougier P, Penault-Llorca F, Laurent-Puig P (2006) KRAS mutation status is predictive of response to cetuximab therapy in colorectal cancer. Cancer Res 66: 3992-3995.

36 Ioannidis JP, Patsopoulos NA, Rothstein HR (2008) Reasons or excuses for avoiding meta-analysis in forest plots. BMJ 336: 1413-1415. 336/7658/1413 [

37 Michiels S, Baujat B, Mahe C, Sargent DJ, Pignon JP (2005) Random effects survival models gave a better understanding of heterogeneity in individual patient data meta-analyses. J Clin Epidemiol 58: 238-245.

38 de Souza AP, Bonorino C (2009) Tumor immunosuppressive environment: effects on tumor-specific and nontumor antigen immune responses. Expert Rev Anticancer Ther 9: 1317-1332.

39 Scartozzi M, Galizia E, Chiorrini S, Giampieri R, Berardi R, Pierantoni C, Cascinu S (2009) Arterial hypertension correlates with clinical outcome in colorectal cancer patients treated with first-line bevacizumab. Ann Oncol 20: 227-230.

40 Kono K, Takahashi A, Ichihara F, Sugai H, Fujii H, Matsumoto Y (2002) Impaired antibody-dependent cellular cytotoxicity mediated by herceptin in patients with gastric cancer. Cancer Res 62: 5813-5817.

41 Negri FV, Musolino A, Naldi N, Bortesi B, Missale G, Laccabue D, Zerbini A, Camisa R, Chernyschova N, Bisagni G, Loupakis F, Ruzzo A, Neri TM, Ardizzoni A (2014) Role of immunoglobulin G fragment C receptor polymorphism-mediated antibody-dependant cellular cytotoxicity in colorectal cancer treated with cetuximab therapy. Pharmacogenomics J 14: 14-19.

Appendix 1: data reconstruction

Starting point for the meta-analysis are the estimated survival curve reported for each study and the minimum and the maximum follow up (min_{FUP} and max_{FUP}) of patients. These quantities may be given directly but most often they will need to be estimated from the manuscript by looking at dates of accrual (if given) and from the date of submission, or perhaps publication of the manuscript. A model for the censoring mechanism based on the minimum and the maximum follow up is assumed here for computing the number at risk and person years for each time. Let C(t) be the function that models the censoring mechanism. Based on the available information we choose the function C(t) as follows

$$C(t) = \begin{cases} 1 & if\ t \leq minFUP \\ 1 - \dfrac{t - minFUP}{maxFUP - minFUP} & if\ minFUP < t < maxFUP \\ 0 & if\ t \geq maxFUP \end{cases} \qquad (1)$$

This function expresses the proportion of patients at time t that have at least t time units of follow-up. Given the number of eligible patients (n), the effective number at risk, the number of revisions at time j and the number of censored are estimated, respectively, as

$$\tilde{r}_j = nS_j C_j \ , \qquad (2)$$

$$d_j = n(S_{j-1} - S_j)\ \frac{C_{j-1} + C_j}{2} \qquad (3)$$

and

$$c_j = n(C_{j-1} - C_j)\ \frac{S_{j-1} + S_j}{2} \qquad (4)$$

This assumes that the censored observations are distributed uniformly over the interval. Under the same assumption, from the number of patients at risk r_j , we can determine the number of person-years over interval I_j , as $r_j = \Delta_j (r_j - c_j / 2)$, where $\Delta_j = t_j - t_{j-1}$ the length of I_j . Following the methodology described the data for each study involved in the meta-analysis have been reconstructed. A model with study as random effects has been fitted to the reconstructed data, to estimate the hazard ratio of progression free and overall survival and its associate confidence interval.

Part II

EGFR antibodies in special populations

Chapter 8

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Pharmacokinetics of panitumumab in a patient with liver dysfunction: a case report

Abstract

Introduction

Panitumumab is used for the treatment for metastatic *RAS* wild type colorectal cancer (mCRC). It is likely that many of these patients will present with liver metastases and some with liver dysfunction. The pharmacokinetics in patients with hepatic impairment has not been investigated, and dosage adjustments are undetermined. Here, we present a case of a patient with progressive mCRC and liver dysfunction.

Method

A heavily pretreated *KRAS* wild type mCRC patient with liver disease Child-Pugh class B was treated with 2-weekly intravenous panitumumab (6 mg/kg). The patient received 2 doses of 490 mg i.v. panitumumab after which progressive disease was documented. Toxicities were graded using CTCAEv4.0. Serum samples were collected, and panitumumab concentrations were determined using a validated immunoassay. Pharmacokinetic parameters after the first dose, including dose-normalized AUC from time zero–day 14, clearance (CL), and elimination half-life (T1/2), were estimated via trapezoidal noncompartmental methods. Data were compared to historical data from a population with adequate liver function, as reported by Stephenson (Clin Colorectal Cancer, 8:29–37, 2009). Values within the range of the mean ± 1 standard deviation (SD) were considered not deviant.

Results

Calculated AUC after the first dose of 6 mg/kg panitumumab in this patient with hepatic dysfunction was 877 μg day/mL (Stephenson's cohort 1: 744 ± 195 μg day/mL). Estimated T1/2 was 3.58 days (5.28 ± 1.90 days), and CL was 6.9 mL/day/kg (8.21 ± 3.79 mL/day/kg). Estimated PK parameters during the first cycle were inside reported mean ± 1 SD of historical controls without liver dysfunction. No toxicity was reported during treatment; particularly, no diarrhea and skin toxicity were noticed.

Conclusion

The pharmacokinetics of panitumumab in this patient suffering from metastatic colorectal cancer with liver dysfunction Child-Pugh class B was similar compared to patients with adequate liver function. Moreover, no substantial toxicity was detected. The here-presented data may help clinical decision making in real-life practice. Two-weekly panitumumab monotherapy seems to be safely applicable in patients with *KRAS* wild type mCRC and hepatic dysfunction, without the need for any dose adjustments.

Introduction

Panitumumab is a fully human IgG2 monoclonal antibody targeting the EGFR receptor. Panitumumab is approved for the treatment for patients with wild type *RAS* metastatic colorectal cancer (mCRC). In the first line, panitumumab is indicated in combination with FOLFOX and in the second line with FOLFIRI for patients who have received first-line fluoropyrimidine-based chemotherapy (excluding irinotecan). Panitumumab as monotherapy is indicated after failure of fluoropyrimidine, oxaliplatin, and irinotecan containing regimens.

As panitumumab is used in treatment for metastatic colorectal cancer, it is likely that many of these patients will present with liver metastases and hence some even with significant liver dysfunction. In the pharmacokinetic studies of Weiner et al., Rowinsky et al., Ma et al., and Stephenson et al. [1–5], the pharmacokinetics of panitumumab have been described comprehensively. However, panitumumab has not been studied in patients with hepatic dysfunction.

Knowledge on the dosing in liver impaired patient is highly relevant; particularly, since in the panitumumab productinformation guidelines, advices for dosing in hepatic failure are lacking [6]. The clearance of panitumumab occurs via two pathways. Panitumumab can be cleared via an EGFR sink, which results in saturation of the receptor with panitumumab and consequent clearance. Secondly, the clearance via the reticuloendothelial system is also present in the liver. The capacity of this system is extensive, due to large numbers of receptors in the body. So dose adjustments may not be necessary in case of liver dysfunction.

Here, we report on the pharmacokinetics of panitumumab in a single patient with hepatic dysfunction treated with single agent 6 mg/kg panitumumab intravenously administered. The objective of this case study is to describe and discuss the effects of hepatic impairment on the pharmacokinetics of panitumumab and to compare the pharmacokinetic data with data from patients without impaired hepatic function.

Subject and methods

Case presentation

In December 2005, a 60-year-old Caucasian male was diagnosed with a T3N + M0 colon carcinoma. The tumor was completely resected, and the patient was treated adjuvantly with eight cycles of capecitabine combined with oxaliplatin (CAPOX). In March 2009, the patient presented with metastatic disease and received a UFT/leucovorin plus bevacizumab regimen, followed by three-weekly irinotecan from January until April 2010.

In August 2010, the patient presented with progressive disease and liver dysfunction, Child-Pugh class B, with bilirubine, gamma GT, ALAT, and ASAT all elevated (Table 1). Treatment with two-weekly 6 mg/kg panitumumab was suggested despite the present hepatic dysfunction. Panitumumab is not contra-indicated in patients with hepatic dysfunction; however, it has not been studied in patients with hepatic impairment [6]. Since there is no clinical data supporting dose adjustments in patients with hepatic impairment, it was decided to start at the regular dose and to measure the panitumumab serum levels in this patient. In total, the patient received two cycles of panitumumab, after which disease progression was documented.

Table 1: Overview of the patient's laboratory tests results during the first cycle of panitumumab treatment.

Marker	Ref. value	13-08 2010	16-08 2010	25-08-2010 day 1 cycle 1 panitumumab	01-09 2010	08-09-2010 day 15 cycle 2 panitumumab	19-09 2010
Sodium (mmol/)	136–144	137	130	125	130	140	140
Potassium (mmol/L)	3.6–4.8	4.4	4.2	4.2	3.3	3	4.6
Ureum (mmol/L)	2.5–7.5	9.3	10.7	14.4	10.2	3	14.4
Creatinine	62–106	112	90	96	98	63	127
eGFR (mL/min)	>60	58	>60	>60	>60	>60	50
Albumin	34–48			44	43	34	18
Bilirubin total (µmol/L)	0–17	148	136	63	40	31	165
Bilirubin conjugated (µmol/L)	0–5	109	95	43	25	19	125
Alkaline phosphatase (U/L)	40–120	232	184	133	213	293	635
Gamma GT (U/L)	5–55	230	175	164	408	498	348
ASAT (U/L)	5–35	89	91	114	69	62	207
ALAT (U/L)	5–45	110	106	165	88	61	87
INR		1.3					2.8

Methods

To study the effects of panitumumab in this patient with hepatic dysfunction, serum samples were collected to determine the serum drug concentrations. The patient, with a body weight of 81 kilograms, received two cycles, 14 days separated (day 1 and day 15), of 490 mg panitumumab, according to the approved dosing instructions of 2-weekly 6 mg/kg body weight. Further dosing was stopped due to early disease progression.

In both instances, panitumumab was administered intravenously in 1 hour. Serum samples were collected at 0.5, 1, 2, 4, 8, 24 hour, 4 days, and 7 days after the first panitumumab infusion. In addition, just before the second infusion (day 15) and 30 minutes and 1 hour after the second infusion, blood samples were drawn [3].

The samples were allowed to clot for 30 minutes, followed by centrifuging at 3,000 rounds per minutes. The serum was transferred to a tube and stored at −80 °C until analysis. Panitumumab serum drug concentrations were performed by PPD (Richmond, VA, USA) using a validated immunoassay with electrochemiluminescence detection as follows. Microplate wells were coated with mouse panitumumab antibody to capture the panitumumab. Standards, quality controls, study samples, and blank were loaded into the wells after pretreating 1:100 with 1 × PBS containing 1 % BSA, 1 M NaCl, and 0.5 % Tween-20. The panitumumab in the standards, controls, and samples was captured in the wells, and unbound materials were removed by washing the cells. Horseradish peroxidase labeled rabbit panitumumab antibody was added to the wells for detection. After washing, tetramethylbenzidine peroxidase substrate was added to the wells. The produced colorimetric signal produced after the reaction was proportional to the amount of panitumumab. The color development was stopped by addition of 2 N sulfuric acid, and the optical density was measured at 450–650 nm.

For an analytical run to be acceptable, a minimum of six acceptable calibration standard levels was required to generate an acceptable calibration curve, and a minimum of four out of six controls with at least one control at each level must meet the method acceptance criteria (difference ±20 % and coefficient of variation ≤15 %). The nominal assay range was 400–20,000 ng/mL. If the sample was outside the upper limit, the sample was repeated at an increased dilution. If the sample was below the lower limit and the dilution factor was one, the sample was reported as below the quantification limit (<400 ng/mL).

Pharmacokinetic parameters

Pharmacokinetic parameters were estimated by trapezoidal noncompartmental methods using MW/PHARM 3.5 of Mediware (Groningen, The Netherlands) [7]. Pharmacokinetic parameters for panitumumab i.e., area under the serum concentration–time curve from time zero to 14 days (AUC0–14), maximum observed serum concentration (Cmax), and minimum observed serum concentration (Cmin)—were determined. Half-life (T½) and clearance (CL) were calculated.

For comparison, historical data from the Summary of Product Characteristics (SPC) [6] and cohort 1 of Stephenson et al. [4] were used. From this study, the dose-normalized (for the first dose of 6 milligram per kilogram) AUC, clearance, elimination half-life, minimum and maximum concentrations were used. In case the value was within the reported serum level ±1 standard deviation, the found value was considered not to be clinically relevant or clinically different.

Toxicity

Information on toxicities was scheduled to be collected at baseline, just before each course, at the day of infusion and 7 days after infusion. Information on toxicities was also scheduled to be collected during each unplanned hospital visit or contact. Toxicities were graded using CTCAEv4.0.

Results

The Cmax measured in this patient was 176 μg/mL after the first infusion and 164 μg/mL after the second infusion. The Cmin (10.5 μg/mL) was determined just before the second administration of panitumumab. The reported serum concentrations of panitumumab have been used to calculate the AUC0–14 (μg day/mL), half-life (days), and clearance (mL/day/kg) (Table 2).

In Table 2, the pharmacokinetic parameters of panitumumab in this patient with Child-Pugh class B liver dysfunction are reported. In this table, the historical pharmacokinetic data of panitumumab in patients with normal liver function are shown as well [4, 6]. In Figure 1, the plasma concentration versus time curves are shown. In summary, in our patient, the calculated AUC was 877 μg day/mL.

In Stephenson's cohort 1 [4], after the first dose of 6 mg/kg, a mean AUC of 744 ± 195 μg day/mL was calculated. The half-life calculated in our single patient was 3.58 days with a calculated clearance of 6.9 mL/day/kg. The study of Stephenson reported a half-life of 5.28 ± 1.90 days and a clearance of 8.21 ± 3.79 mL/day/kg. All these parameters following the first administration of panitumumab reported in our patient with severe liver dysfunction are within the range

of one standard deviation around the mean of the data reported for cohort 1 in the study of Stephenson [4]. Likewise, the maximum and minimum concentration, elimination half-life, and clearance were comparable (Figure 1 and Table 2). A difference between the data from the SPC and parameters after the third dose was noted; however, it should be noted that these parameters were not determined following single dose but after multiple dosing, in the steady state phase.

Table 2: Historical comparison of pharmacokinetic parameters of panitumumab of the single patient with severe liver dysfunction with patients with adequate liver function

Descriptive statistic	Cmax (µg/mL)	Cmin (µg/mL)	AUC0-tau (µg day/mL)	T1/2 (days)	CL (mL/day/kg)
Cohort 1 first dose 6 mg/kg (2-weekly) Stephenson	152 (29.2)	18.1 (8.6)	744 (195)	5.28 (1.90)	8.21 (3.79)
Cohort 1 third dose 6 mg/kg (2-weekly) Stephenson	232 (71.2)	46.6 (16.9)	1,310 (375)	9.08 (3.61)	4.96 (1.49)
SPC 6 mg/kg (2-weekly)	213 (59)	39 (14)	1,306 (374)	7.5	4.9
Case first dose	179	10.5	877	3.58	6.9
Case second dose	164	n/a	n/a	n/a	n/a

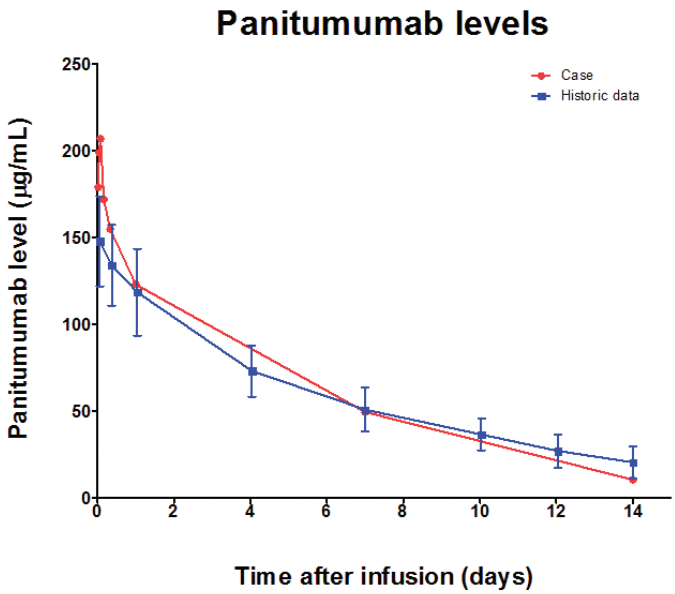

Figure 1: Time curve of serum panitumumab serum concentrations following 1 h infusion of 690 mg (6 mg/kg) panitumumab in a metastasized colon cancer patient with Child-Pugh B liver dysfunction

In our patient with Child-Pugh class B liver dysfunction, no toxicity was recorded after the first two doses of panitumumab; specifically, no diarrhea and no skin toxicity were seen.

Discussion

No advice on the necessity of adjusting the dose of panitumumab, a fully human antibody targeting the EGFR that is dosed two-weekly, in special populations, such as patients with hepatic dysfunction, is available. Also, the safety and pharmacokinetics have not been studied specifically in patients with liver impairment. Like other EGFR targeting agents, panitumumab only has been tested in clinical trials in metastatic colorectal cancer in selected populations with adequate laboratory tests and good performance characteristics. In real-life practice, however, many patients do not match these criteria and may present for example with severe liver dysfunction. There is a clear need for studies in different populations to guide the clinician in real-life practice[8].

The here-reported results of our pharmacokinetic study in a single, heavily pre-treated patient suffering from metastasized colorectal cancer treated with 6 mg/kg 2-weekly monotherapy panitumumab do not indicate the necessity of any dose adjustments in patients with liver dysfunction and appear to be tolerable and safe. Pharmacokinetic parameters reported are within the range of one single standard deviation of previously reported data in patients with adequate liver functions. In addition, no substantial toxicity was noticed. However, larger studies of panitumumab in liver impaired patients are needed before firm conclusions can be drawn, and a more solid advice on the necessity of dose adjustments in patients with various degrees of hepatic dysfunction can be given.

The side-effect profile of cetuximab in a liver impaired patient has been presented recently by Moosman et al. [9]. In that particular case report, Moosman and colleagues report on a 57-year-old metastasized colorectal cancer patient with severe liver dysfunction that was successfully treated with cetuximab, a weekly administered chimeric monoclonal antibody targeting the EGFR as well. Based on their observation, it was concluded by the authors that cetuximab is an effective treatment in patients who cannot be treated with cytotoxic agents due to hepatic dysfunction. However, no pharmacokinetic data are presented. The here reported data on panitumumab add to their conclusion. However, it should be noted that there are important differences between the EGFR antibodies cetuximab and panitumumab. For example, cetuximab is a chimeric antibody, whereas panitumumab is a fully human antibody. Next, cetuximab is an IgG1 antibody, whereas panitumumab is an IgG2 antibody. As a consequence, the serum clearance, Fc domain interactions, and potential initiation of ADCC differ between both antibodies. Therefore, it is not possible to directly extrapolate the pharmacokinetic profiles of one of these antibodies to the other [10].

Successful treatment for solid tumors relies on the ability of EGFR inhibitors to penetrate into the tumor tissue. Clearance of both panitumumab and cetuximab occurs by the EGFR sink and the reticuloendothelial system. Their clearance may also partly depend on the EGFR-positive tumor burden and antigen density in the tumor, i.e., a high tumor burden and/or a high density of EGFR may lead to subsequent higher clearance of panitumumab. In our patient, unfortunately, the tumor burden and the antigen density in the tumor were not known. The impact of EGFR binding sites in the liver on serum clearance of EGFR antibodies remains to be fully clarified. A study of Schechter et al. with 99MTC-cetuximab, however, indicated that the liver may not have any EGFR binding sites, but simply extracts EGFR antibodies, which are not cleared elsewhere in the body [11]. This aforementioned extraction by the liver and the impact of liver dysfunction on total EGFR antibody clearance needs further clarification.

In conclusion, the pharmacokinetics of panitumumab in our single patient suffering from metastatic colorectal cancer with liver dysfunction Child-Pugh class B do not seem to be altered compared with patients with adequate liver function. Moreover, no substantial toxicity was noticed. Based on these data, panitumumab can be considered safe for treatment in patients with hepatic dysfunction without any dose adjustment. However, more studies seem warranted before firm conclusions can be drawn to guide clinical decision in daily practice.

Acknowledgments

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References

1 Weiner LM, Belldegrun AS, Crawford J, Tolcher AW, Lockbaum P, Arends RH, Navale L, Amado RG, Schwab G, Figlin RA (2008) Dose and schedule study of panitumumab monotherapy in patients with advanced solid malignancies. Clin Cancer Res 14:502–508

2 Rowinsky EK, Schwartz GH, Gollob JA, Thompson JA, Vogelzang NJ, Figlin R, Bukowski R, Haas N, Lockbaum P, Li YP, Arends R, Foon KA, Schwab G, Dutcher J (2004) Safety, pharmacokinetics, and activity of ABX-EGF, a fully human anti-epidermal growth factor receptor monoclonal antibody in patients with metastatic renal cell cancer. J Clin Oncol 22:3003–3015

3 Ma P, Yang BB, Wang YM, Peterson M, Narayanan A, Sutjandra L, Rodriguez R, Chow A (2009) Population pharmacokinetic analysis of panitumumab in patients with advanced solid tumors. J Clin Pharmacol 49:1142–1156

4 Stephenson JJ, Gregory C, Burris H, Larson T, Verma U, Cohn A, Crawford J, Cohen RB, Martin J, Lum P, Yang X, Amado RG (2009) An open-label clinical trial evaluating safety and pharmacokinetics of two dosing schedules of panitumumab in patients with solid tumors. Clin Colorectal Cancer 8:29–37

5 Yang BB, Lum P, Chen A, Arends R, Roskos L, Smith B, Perez Ruixo JJ (2010) Pharmacokinetic and pharmacodynamic perspectives on the clinical drug development of panitumumab. Clin Pharmacokinet 49:729–740

6 Vectibix (Panitumumab) (2008) Summary of Product Characteristics: Amgen Inc Thousand Oaks, CA

7 Proost JH, Meijer DK (1992) MW/Pharm, an integrated software package for drug dosage regimen calculation and therapeutic drug monitoring. Comput Biol Med 22:155–163

8 Sorbye H, Pfeiffer P, Cavalli-Bjorkman N, Qvortrup C, Holsen MH, Wentzel-Larsen T, Glimelius B (2009) Clinical trial enrollment, patient characteristics, and survival differences in prospectively registered metastatic colorectal cancer patients. Cancer 115:4679–4687

9 Moosmann N, Laessig D, Michaely HJ, Schulz C, Heinemann V (2007) Effective second-line treatment with cetuximab and bevacizumab in a patient with hepatic metastases of colorectal cancer and hyperbilirubinemia. Onkologie 30:509–512

10 Noguchi T, Ritter G, Nishikawa H (2013) Antibody-based therapy in colorectal cancer. Immunotherapy 5:533–545

11 Schechter NR, Wendt RE III, Yang DJ, Azhdarinia A, Erwin WD, Stachowiak AM, Broemeling LD, Kim EE, Cox JD, Podoloff DA, Ang KK (2004) Radiation dosimetry of 99mTc-labeled C225 in patients with squamous cell carcinoma of the head and neck. J Nucl Med 45:1683–1687

Chapter 9

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Pharmacokinetics and safety of cetuximab in a patient with renal dysfunction

Abstract

In the literature data on the effect of renal impairment on the pharmacokinetics of anticancer drugs are scarce. Here, we report a 68 year old metastatic osteosarcoma patient with impaired renal function due to prior chemotherapy, who was treated on compassionate use basis with 400 mg/m^2 cetuximab. Pharmacokinetic parameters after the first dose, including dose normalised AUC from time zero to day 7 (AUC_{0-7}), clearance (Cl), elimination half-life ($t_{1/2}$) were estimated using trapezoidal non compartmental methods and compared to pharmacokinetic data from a study population with normal kidney function. These results showed that the pharmacokinetics of cetuximab in this patient with renal failure was similar to that with adequate renal function and suggests that cetuximab can be safely used in cancer patients with renal impairment without dose adjustment.

Introduction

Cetuximab is a monoclonal antibody, targeting epidermal growth factor receptor (EGFR) and registered for the treatment of colorectal and head and neck cancer. During its development, the drug has been investigated in patients with adequate renal and hepatic function only and a dose of 250 mg/m^2 every week, after an initial loading dose of 400 mg/m^2, is defined in the summary of product characteristics.

No specific dose recommendations are given for patients with renal impairment (http://packageinserts.bms.com/pi/pi_erbitux.pdf). The elimination of antibodies occurs via both nonspecific intracellular catabolism, following fluid-phase endocytosis, and receptor-mediated elimination after binding to their target antigen. Part of cetuximab clearance is therefore explained by binding to EGFR. Clearance of the EGFR antibody cetuximab seems independent of the liver and kidney function[1]. In addition, there are four case reports[2-5] of haemodialysis patients who could safely be treated with standard doses of cetuximab. The aim of this study was to determine the pharmacokinetics of the conventional dose cetuximab in patients with impaired renal function and to compare it to published data obtained in populations of cetuximab treated patients with normal renal function.

Method

Case presentation

We treated a 68 year old metastatic osteosarcoma patient with impaired renal function due to prior chemotherapy on compassionate use basis with 400 mg/m^2 cetuximab. The serum creatinine of this patient was 128 µmol/L. The glomerular filtration rate (GFR) was 35 mL/min/173 m^2, calculated with the MDRD formula (0.742 * 175 * Serum creatinine$^{-1.154}$ * Age$^{-0.203}$). The GFR, calculated with the Cockcroft-Gault formula was 41 mL/min (0.85 * (140 - Age) * Weight) / (72 * Serum creatinine). This treatment was based on preclinical data on cetuximab activity in osteosarcoma[6] and the lack of other treatment options. The medical ethical committee approved the treatment and the pharmacokinetic analysis and the patient gave informed consent. The starting dose was 740 mg, preceded by the recommended 2 mg of the antihistamine clemastine, to avoid an allergic reaction to cetuximab.

Sample collection

Cetuximab was infused over two hours. Serum samples were collected 2, 3.5, 4.5, 44 and 168 hours after the end of the infusion in line with a previous pharmacokinetic study[7]. Cetuximab serum concentrations were measured using a validated immunoassay[8]. Limit of detection was 0.012 µg/mL and lower limit of quantitation (LLOQ) was 0.75 µg/mL.

Pharmacokinetic parameters

Pharmacokinetic parameters after the first dose, including dose normalised AUC from time zero to day 7 (AUC_{0-7}), clearance (Cl), elimination half-life ($t_{1/2}$) were estimated by trapezoidal non compartmental methods using MW/PHARM 3.5 (Mediware, Groningen, The Netherlands). Results were compared to historical data on cetuximab in patients with normal renal function as reported by Tan et al.[9] Fracasso et al.[10] and Thariat et al.[2].

Results

The maximum concentration (Cmax), measured at the end of the 2 hour infusion, in this patient was 297 µg/mL, the minimum concentration (Cmin or through level) was 34.4 µg/mL. The reported serum concentration profile, shown in figure 1, was used to calculate the AUC, Cl and $t_{½}$. In table 1, an overview of the pharmacokinetics of cetuximab in study populations with normal renal function and in this case are shown (2;9;10). In this table the pharmacokinetics after a single dose of 400 mg/m^2 are depicted, and are used for comparison.

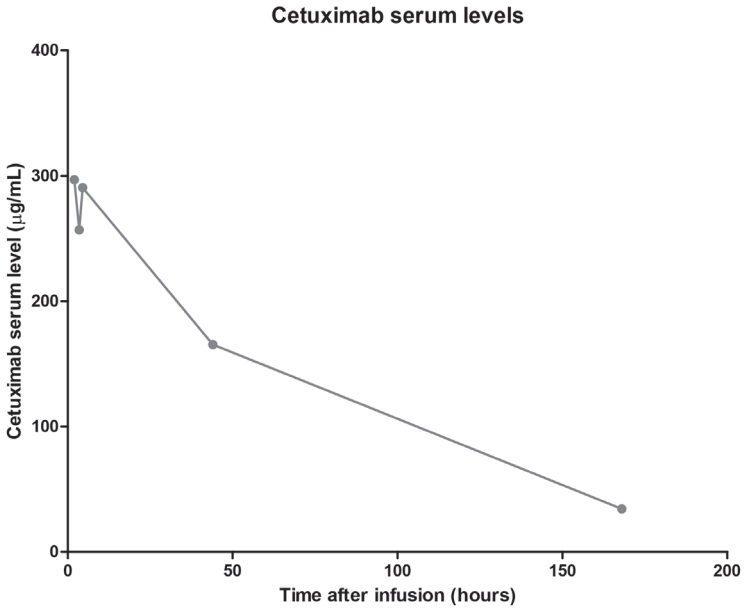

Figure 1: Time curve of cetuximab serum concentration following two hour infusion of 740 mg cetuximab in a patient with a glomerular filtration rate of approximately 35 mL per minute.

In this patient the calculated AUC after the first dose was 20,280 µg*day/mL. The half-life after a single dose of cetuximab was 53.2 hours with a calculated clearance of 32.6 mL/h. The Cmax was approximately 30% higher compared to the Cmax in the studies of Tan et al. and Fracasso et al. (9;10)The other parameters Cl, AUC, $T_{1/2}$ and V are comparable as reported in those 2 studies. The half-life of cetuximab in our patient is 30% shorter than that calculated in the studies.

During the first course of cetuximab, the patient experienced adverse effects: reversible grade 2 hallucinations and fatigue. After careful considerations these symptoms were deemed to be most likely caused by the 2 mg clemastine. Due to these side effects the patient refused further treatment with cetuximab. Little is known about the pharmacokinetics of clemastine, nonetheless, normally no dose reductions are advised in patient with renal impairment.

Cetuximab-related side effects such as skin toxicity and diarrhoea did not occur in this patient during the first course.

Table 1: Pharmacokinetic parameters of the case and historical data from three studies.

Study	GFR/ serum creatinin range	model	Dose	Cmax µg/mL	Cmin µg/mL	AUC_{0-7} h*mg/L	CL mL/h	$T_{½}$ Hours	V Litre
Case	±35 mL/min	Non compartmental model	740 mg (400 mg/m^2)	297.0	34.4	20.280	32.6	53.2	2.48
Tan et al. 2006	Creatinine ≤ 1.5 times upper limit of normal	Non compartmental model	400 mg/m^2 single dose	205.0 (65.7)	n/a	19,000 (7,802)	21.5 (7.68)	75.10 (15.9)	2.44 (0.43)
Fracasso et al. 2007	Creatinine ≤ 1.5 times upper limit of normal	Non compartmental model	400 mg/m^2 single dose	228.9 (6,5)	n/a	24,620 (9,555)	43.6 (15.8)	97.5 (20.7)	4.8 (2.2)
Thariat et al. 2008	hemodialysis	Two-compartment model with first order elimination	250 mg/m^2	n/a	n/a	n/a	25	285	7.56

Abbreviation: n/a = not applicable

Discussion

This case report shows that the pharmacokinetics of cetuximab in a patient with renal failure is similar to that with adequate renal function. Different studies investigated the pharmacokinetics of cetuximab in population with adequate renal function. For the comparison, studies with single doses were used. Most of the studies reported cetuximab pharmacokinetic parameters at steady state after a loading dose of 400 mg/m^2 followed by weekly doses of 250 mg/m^2. Using a loading dose, steady state is usually reached within three weeks. Since this patient discontinued therapy after one single infusion, we can only compare this single dose to similar administrations available in the literature.

Our study shows some difference between the estimated parameters in our patient and patients with normal renal function. Due to inter patient variability, nonetheless, overall the kinetic profile is in line with the population with adequate renal function. Cetuximab pharmacokinetics are not studied in patients with impaired renal function, four case reports[2-5] studied the kinetics of cetuximab in patients undergoing haemodialysis. These four case studies showed that cetuximab in a patient with haemodialysis may be safely used. The estimated pharmacokinetic parameters were comparable to cetuximab patients with normal kidney function. Our study shows, for the first time, that pharmacokinetic parameters were also not altered in a patient with decreased renal function without haemodialysis.

At 3.5 hours, a lower cetuximab concentration was measured compared to the concentration measured at 4.5 hours. This appears to be noise from a single patient because these time points were very close to each other, these concentrations were measured shortly after infusion of cetuximab and the concentration of cetuximab decreases only slowly at this interval. Many drugs that enter the market are studied in a patient population with normal renal function and no dose recommendations are made for patients with impaired organ function and formal organ impairment studies are lacking. This is also the case for cetuximab. Other than from common

sense and four case reports in haemodialysis patients there was no information available to guide our decision on how to use cetuximab in this patient. As renal impairment is a common problem in head and neck cancer patients due to prior cisplatin chemotherapy and in colorectal cancer patients due to the high incidence of the disease our finding of no clinically relevant alteration of cetuximab pharmacokinetics in our patient with impaired renal function has clinical importance for dose guidance in future patients.

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References

1 Blick SK, Scott LJ. (2007) Cetuximab: a review of its use in squamous cell carcinoma of the head and neck and metastatic colorectal cancer. Drugs; 67(17):2585-607.

2 Thariat J, Azzopardi N, Peyrade F, Launay-Vacher V, Santini J, Lecomte T et al. (2008) Cetuximab pharmacokinetics in end-stage kidney disease under hemodialysis. J Clin Oncol; 26(25):4223-5.

3 Inauen R, Cathomas R, Boehm T, Koeberle D, Pestalozzi BC, Gillessen S et al. (2007) Feasibility of using cetuximab and bevacizumab in a patient with colorectal cancer and terminal renal failure. Oncology; 72(3-4):209-10.

4 Aldoss IT, Plumb T, Zhen WK, Lydiatt DD, Ganti AK. (2009) Cetuximab in hemodialysis: a case report. Head Neck; 31(12):1647-50.

5 Fontana E, Pucci F, Ardizzoni A. (2013) Colorectal cancer patient on maintenance dialysis successfully treated with cetuximab. Anticancer Drugs.

6 Pahl JH, Ruslan SE, Buddingh EP, Santos SJ, Szuhai K, Serra M et al. (2012) Anti-EGFR antibody cetuximab enhances the cytolytic activity of natural killer cells toward osteosarcoma. Clin Cancer Res 18(2):432-41.

7 Azzopardi N, Lecomte T, Ternant D, Boisdron-Celle M, Piller F, Morel A et al. (2011) Cetuximab pharmacokinetics influences progression-free survival of metastatic colorectal cancer patients. Clin Cancer Res 17(19):6329-37.

8 Ceze N, Ternant D, Piller F, Degenne D, Azzopardi N, Dorval E et al. (2009) An enzyme-linked immunosorbent assay for therapeutic drug monitoring of cetuximab. Ther Drug Monit; 31(5):597-601.

9 Tan AR, Moore DF, Hidalgo M, Doroshow JH, Poplin EA, Goodin S et al. (2006) Pharmacokinetics of cetuximab after administration of escalating single dosing and weekly fixed dosing in patients with solid tumors. Clin Cancer Res; 12(21):6517-22.

10 Fracasso PM, Burris H, III, Arquette MA, Govindan R, Gao F, Wright LP et al. (2007) A phase 1 escalating single-dose and weekly fixed-dose study of cetuximab: pharmacokinetic and pharmacodynamic rationale for dosing. Clin Cancer Res; 13(3):986-93.

Chapter 10

General discussion

Introduction

The epidermal growth factor receptor (EGFR) antibodies cetuximab and panitumumab are both registered for the treatment of *RAS* wild type metastatic colorectal cancer (CRC). About 40% of the patients have a tumor with a mutation in the *KRAS* gene. In patients with a *KRAS* mutant tumor KRAS is permanently activated which lead to constant cell signaling and proliferation independent of the EGFR. Patients with a *(K)RAS* mutant tumor are considered not to benifit from anti-EGFR therapy. The use is limited to patients with *KRAS* wild type tumors and more recently in *RAS* wild type only[1,2]. After having become chemotherapy refractory, treatment options are limited for this substantial patient group [3]. This means there is an urgent need to optimize anti-EGFR therapy.

This thesis investigates several strategies to refine EGFR targeted monoclonal antibody therapy in CRC by:

- statins and their ability to phenoconvert *KRAS* mutant CRC;
- exploration of polymorphisms in the gene encoding *FCGR3A* and their association with cetuximab efficacy;
- Investigating the pharmacokinetics of cetuximab and panitumumab in patients with renal or hepatic dysfunction.

Statins and their ability to phenoconvert KRAS mutant CRC

Statins are commonly used to reduce cholesterol levels in patients in order to reduce the risk for cardiovascular events. Statins inhibit HMG-CoA-reductase in the mevalonate pathway and subsequently the formation of cholesterol. Besides cholesterol the formation of farnesylpyrophophate (a C15-group) and geranylgeranylpyrophosphate (a C17-group) are also inhibited. These C15 and C17 groups are used to prenylate the KRAS protein. Prenylation is an essential step in the activation of the KRAS protein. After prenylation, KRAS becomes more lipophilic and associates with the plasma membrane. By preventing prenylation and plasma membrane association the over-activated KRAS protein may be inhibited. We theorised that the inhibitory effect of statins may normalise the KRAS mutant phenotype into a more KRAS wild type phenotype and render *KRAS* mutant colorectal cancers sensitive to EGFR antibodies. Thus, statins may inhibit the expression of the mutant *KRAS* phenotype by preventing prenylation (the addition of C15 or C17 groups) of the KRAS protein.

Besides statins, other KRAS modulators, such as bisphosphonates, farnesyltransferase inhibitors or geranylgeranyltransferase inhibitors, also affect the mevalonate pathway. These KRAS modulators might augment the effect of EGFR antibodies in patients with *KRAS* mutations. In **chapter 2**, clinical studies with these KRAS modulators and their outcomes were reviewed. This review indicates that combinations of EGFR antibodies to target the EGFR with KRAS modulators may be effective in patients with *KRAS* mutant tumors.

To understand the role of statins in CRC and to explore the potential of therapeutic modulation of *KRAS* mutated CRC tumors, an *in vitro* study with *KRAS* wild type and mutant

cell lines was performed. A survival assay was used to study the effects of simvastatin and cetuximab on proliferation in colorectal cancer cell lines. In *KRAS* G13D mutated HCT116 and LoVo cell lines a combination of simvastatin pre-treatment and cetuximab induced a small reduction in growth (**chapter 3**). This effect was not observed in the SW480 cell line harboring a codon 12 *KRAS* mutation. The observation that codon 13 *KRAS* mutated tumors are responsive to EGFR-antibody therapy is in line with other retrospective studies[4-6], which also showed a positive association on outcome for the *KRAS* codon 13 mutations. An *in vitro* study showed that codon 13 *KRAS* mutation may confer weaker transforming capacities on cancer cells, compared to other *KRAS* mutations[7]. Computational analysis revealed that the codon 13 mutated KRAS protein has a similar structure compared to the wild type protein [8]. Our studies revealed that all *KRAS* mutant cells showed a pronounced cytotoxic effect after simvastatin monotherapy. The effect of simvastatin on growth was not observed in the *KRAS* wild type A431 cell line. A possible explanation for the cytotoxic effect may be a strong dependence ("addiction") of the mutant cells on permanently activated KRAS and its corresponding pathways. These pathways are possibly highly activated due to mutant *KRAS*. The observed effect of combination treatment with simvastatin and cetuximab was relatively small; therefore other pathways besides the RAS-RAF-MAPK pathway may play important roles which are not affected by blocking the EGFR receptor.

As the *in vitro* studies (**chapter 3**) showed promising results, we decided to perform a retrospective analysis to evaluate the effect of statin use on outcome in *KRAS* mutant metastatic colorectal cancer patients (mCRC) treated with cetuximab. In the CAIRO2 study metastatic CRC patients were treated with capecitabine, oxaliplatin, bevacizumab with or without cetuximab. We retrospectively analysed the effect of statin use at time of diagnosis on progression free survival (PFS) in CRC patients with *KRAS* mutant tumors treated with cetuximab. **Chapter 4** showed that the use of statins in patients with a *KRAS* mutant tumor was not associated with an improved PFS.

To exclude a possible "generic" effect of statins on survival, patients with *KRAS* wild type tumors and patients in the study-arm without cetuximab were also included in the analysis. Additionally, the study design with an effect modifier helps to identify possible different effects of statins between patients with *KRAS* mutant and wild type tumors.

Possible explanations for the lack of a modulating effect are that the cohort consisted of patients with metastatic disease and hence a relatively short PFS. The detection of a small to moderate effect on PFS may be difficult in this patient group. Another explanation for the absence of an effect was the concomitant use of bevacizumab in the CAIRO2 study. The combination of cetuximab and bevacizumab is not used nowadays, due to an unfavourable outcome. Hypertension, a common side effect of bevacizumab is also a prognostic factor for a better overall survival in CRC patients treated with bevacizumab [9]. A possible negative interaction between bevacizumab and cetuximab may have caused less hypertension in the cetuximab treated group, which contributed to the negative outcome of the CAIRO2 study.

In two prospective studies we investigated the possibility of simvastatin to phenoconvert mutant *KRAS* in CRC patients treated with cetuximab or panitumumab. In the RASTAT C and P studies (**chapter 5 and 6**) metastatic CRC patients who failed on first- and second-line therapy, were treated with 80 mg of simvastatin daily and cetuximab (RASTAT C) or panitumumab (RASTAT P). Both studies were terminated after a planned interim analysis of the Simon two stage design, because similar survival as seen in KRAS wild type patients was not observed. In a similar study, Lee et al.[10] tested the addition of 80 mg of simvastatin to cetuximab and irinotecan. The disease controle rate in this study was 65.4%. Their original report indeed showed a low response rate (1 out of 52 patients had a partial remission), however PFS was 7.6 months,

which is even higher than historical results of third-line cetuximab plus irinotecan in KRAS wild type CRC patients. However, in a recent erratum Lee et al[30] reported corrected measurements of PFS in their population. Corrected mean PFS was 3.7 months (range 2.1-5.3), significantly lower than previous reports of cetuximab plus irinotecan as third-line therapy in KRAS wild type [10]. In summary, both our study as well as the study by Lee et al. shows that simvastatin does not render sensitivity to EGFR inhibitor therapy.

Polymorphisms FCGR3A gene and their association with cetuximab efficacy

An important mechanism of cetuximab induced cell-killing is antibody-dependent cellular cytotoxicity (ADCC). Fc gamma receptors (FCGR) on effector cells, for example macrophages and natural killer cells, bind to the Fc fragment of the cetuximab molecule and this causes lysis of the cancer cell. The germline polymorphism (rs396991) in the Fc gamma receptor 3A (*FCG3A*) c.818A>C results in a change of phenylalanine to valine at codon 158. [11]. Previous results from studies investigating the association between *FCGR3A* polymorphisms and cetuximab efficacy are highly variable. A firm conclusion or direction of the effect of *FCGR3A* polymorphisms on cetuximab efficacy cannot be drawn from these studies. These inconsistent findings in studies may have been caused by incorrect genotyping methods[12] or insufficient statistical power[13]. In some studies [14-16], the observed allele frequencies were not in Hardy-Weinberg equilibrium, possibly because genotyping errors may have occurred due to *FCGR3B* co-amplification. The meta-analysis (**chapter 7**) performed on individual patient data shows that the *FCGR3A* polymorphism is not associated with improved survival in cetuximab treated CRC patients and there is no significant difference between patients with *KRAS* wild type and mutant tumors. Lack of effect of the *FCGR3A* polymorphisms may be explained by the fact that most patients were also co-treated with classic chemotherapy or patients received previous lines of chemotherapy, which suppresses macrophages and natural killer cells[17]. Moreover, all included patients had metastatic CRC, which may lead to decreased immune responses and impaired natural killer cell dysfunction in end stage CRC[18] and consequently failure of cetuximab treatment.

Pharmacokinetics of cetuximab and panitumumab in patients with renal or hepatic dysfunction

Both panitumumab and cetuximab are used in patients with metastatic CRC. Some of these patients will present with hepatic impairment, due to liver metastasis. In addition, head and neck cancer patients are heavily pre-treated with chemotherapy and radiotherapy and may have decreased renal function. Cetuximab is used in these head and neck cancer patients because they cannot be treated with cisplatin, due to renal impairment.

Knowledge of dosing in these specials populations with impaired renal or hepatic function is highly relevant. The pharmacokinetics and safety of both cetuximab and panitumumab have not been studied in these populations. In two case reports, with panitumumab and cetuximab in cancer patients with liver and kidney dysfunction respectively, we showed that dose adjustments in patients with liver or kidney failure are not necessary and that treatment appears to be tolerable and safe (**chapters 8, 9**). However, larger studies in patients with hepatic or renal dysfunction are

needed before firm conclusions can be drawn to guide clinical decision making in daily practice. Successful treatment of solid tumors relies on the ability of EGFR inhibitors to penetrate into the tumor tissue. Clearance of both panitumumab and cetuximab occurs by the EGFR sink and the reticuloendothelial system. Their clearance may also partly depend on the EGFR-positive tumor burden and antigen density in the tumor, i.e. a high tumor burden and/or a high density of EGFR may lead to subsequent higher clearance of EGFR antibodies. In our patients the tumor burden and the antigen density in the tumor were not known. The impact of EGFR binding sites in the liver on serum clearance of EGFR antibodies remains to be fully clarified.

Future research prospectives

In this thesis, the possibility of statins to phenoconvert the KRAS mutant protein to a more wild type protein to overcome EGFR-monoclonal antibody resistance in CRC was studied. The apparently promising results from the preclinical study, however, were not translated to the clinic. Statin use in the CAIRO2 study was not associated with a better progression free survival in cetuximab treated metastatic CRC patients with a *KRAS* mutant tumor. In the RASTAT C en P studies, the combination of cetuximab or panitumumab with simvastatin did not lead to a survival comparable with KRAS wild type patient. In these three studies we were not able to show that statins can phenoconvert the mutant KRAS protein and render these tumors sensitive for cetuximab. In this thesis, the hypothesis that statins can phenoconvert the mutant KRAS to a more favourable phenotype was studied only in patients with advanced colorectal cancer and thus with a short life expectancy. Recent evidence suggest that other *RAS* mutations (in exons 3 and 4 of *KRAS* and exons 2, 3 and 4 of a related gene, *NRAS*) may also be predictive of anti-EGFR resistance. These other RAS proteins also requires post-translational farnesylation to become active. In further studies all *RAS* mutant patients need to be included.

A "perfect world" study where the effect of statins and concomitant chemotherapy and EGFR antibodies is studied in recently diagnosed (*RAS* mutant) patients who did not receive a statin before CRC diagnosis would be of great value. Since these patients have a longer life expectancy, lower tumor burden and possibly a phenotype, which could be modified by statin use.

Besides statins, farnesyl- and geranylgeranyltransferase inhibitors also have a crucial role in the mevalonate pathway and consequently the prenylation of (K)RAS. Inhibiting of (K) RAS prenylation might require combined treatment with these inhibitors. Possibly because of preclinical toxicity geranylgeranyltransferase inhibitors did not proceed to clinical stages. A combination of statin and low doses farnesyl- and geranylgeranyltransferase inhibitors may be an effective treatment in CRC patients with a *(K)RAS* mutant tumor.

In this thesis we studied the effect of the *FCGR3A* gene, which is involved in antibody dependent cellular toxicity (ADCC). Other effector mechanisms and accompanying polymorphisms, such as complement-dependant cytotoxicity, phagocytosis and apoptosis may play a crucial and still unknown role in efficacy. The enormous complexity of cancer makes it debatable whether a single mutation or germ-line polymorphism might have a noteworthy effect on the tumor sensitivity to targeted therapies. Innovative technologies, such as next-generation sequencing, kinase activity profiling of tumors, computational biology and genome-wide association studies, will be useful in achieving a better overview of involved pathways and in further optimizing and personalizing the use of anti-EGFR monoclonal antibodies. By sequencing tumor DNA, targeted treatment can be optimized for the specific characteristics of the tumor.

Many drugs that enter the market are studied in a patient population with adequate organ functions only[19]. No dose recommendations are made for patients with impaired liver or kidney function and formal organ impairment studies are lacking. This is also true for cetuximab and panitumumab. Other than from common sense and some case reports in haemodialysis patients[20-23], liver failure (**chapter 8**) and kidney failure (**chapter 9**), there is no information available to guide our decision on how to use cetuximab or panitumumab in these populations. Larger studies, where the efficacy and safety of EGFR antibodies and chemotherapies is studied in patients with liver and kidney dysfunction, will be of great value. Liver and kidney dysfunction will become more common, since patients are getting older and more treatments become available, which will substantially increase in the incidence of these cases. Development of new drugs should include studies in organ failure patients that reflect clinical dilemmas often encountered in routine patient care. Mandatory additional research with FDA or EMA approval would ensure this process on a timely basis.

Conclusions

The goal of this thesis was to refine EGFR monoclonal antibody treatment in CRC by four different strategies. The first strategy, focused on statins and their ability to phenoconvert KRAS mutant CRC Statin use in metastatic CRC patients with *KRAS* mutant tumors, did not affect progression free or overall survival. The second strategy explored polymorphisms in the gene encoding FCGR3A and their association with cetuximab efficacy. Neither of the *FCGR3A* polymorphisms showed a significant association with improved PFS. Although some studies reported that the effect of the *FCGR3A* polymorphisms on cetuximab efficacy is independent of *KRAS*. The meta-analysis showed that there is no significant difference between patients with *KRAS* wild type or mutant tumors. In conclusion, the result from these two strategies shows that the options for CRC patients with a *KRAS* mutation after failing first line chemotherapy and bevacizumab still remain poor. The outcomes from these strategies demonstrate that the involved pathways are very complex and urgently need further exploration.

The final strategy to optimize anti-EGFR therapy focused on the pharmacokinetics of cetuximab and panitumumab in patients with renal or hepatic dysfunction. The described case reports help clinical decision making in real-life practice. Cetuximab and panitumumab monotherapy seems to be safely applicable in patients with *KRAS* wild type metastatic CRC and hepatic or renal dysfunction, without the need for dose adjustments.

References

1 Douillard JY, Oliner KS, Siena S, Tabernero J, Burkes R, et al. (2013) Panitumumab-FOLFOX4 treatment and RAS mutations in colorectal cancer. N Engl J Med 369: 1023-1034.

2 Perkins G, Pilati C, Blons H, Laurent-Puig P. (2014) Beyond KRAS status and response to anti-EGFR therapy in metastatic colorectal cancer. Pharmacogenomics 15: 1043-1052.

3 Laurent-Puig P, Lievre A, Blons H. (2009) Mutations and response to epidermal growth factor receptor inhibitors. Clin Cancer Res 15: 1133-1139.

4 De RW, Jonker DJ, Di NF, Sartore-Bianchi A, Tu D, et al. (2010) Association of KRAS p.G13D mutation with outcome in patients with chemotherapy-refractory metastatic colorectal cancer treated with cetuximab. JAMA 304: 1812-1820.

5 Tejpar S, Celik I, Schlichting M, Sartorius U, Bokemeyer C, et al. (2012) Association of KRAS G13D Tumor Mutations With Outcome in Patients With Metastatic Colorectal Cancer Treated With First-Line Chemotherapy With or Without Cetuximab. J Clin Oncol 30: 3570-3577.

6 Bando H, Yoshino T, Yuki S, Shinozaki E, Nishina T, et al. (2012) Clinical outcome of Japanese metastatic colorectal cancer patients harbouring the KRAS p.G13D mutation treated with cetuximab + irinotecan. Jpn J Clin Oncol 42: 1146-1151.

7 Guerrero S, Figueras A, Casanova I, Farre L, Lloveras B, et al. (2002) Codon 12 and codon 13 mutations at the KRAS gene induce different soft tissue sarcoma types in nude mice. FASEB J 16: 1642-1644.

8 Chen CC, Er TK, Liu YY, Hwang JK, Barrio MJ, et al. (2013) Computational analysis of KRAS mutations: implications for different effects on the KRAS p.G12D and p.G13D mutations. PLoS One 8: e55793.

9 Tol J, Koopman M, Cats A, Rodenburg CJ, Creemers GJ, et al. (2009) Chemotherapy, bevacizumab, and cetuximab in metastatic colorectal cancer. N Engl J Med 360: 563-572.

10 Lee J, Hong YS, Hong JY, Han SW, Kim TW, et al. (2014) Effect of simvastatin plus cetuximab/irinotecan for KRAS mutant colorectal cancer and predictive value of the RAS signature for treatment response to cetuximab. Invest New Drugs 32: 535-541.

11 Mellor JD, Brown MP, Irving HR, Zalcberg JR, Dobrovic A. (2013) A critical review of the role of Fc gamma receptor polymorphisms in the response to monoclonal antibodies in cancer. J Hematol Oncol 6: 1.

12 van der Straaten T, Martijn R, el HT, Baak-Pablo R, Guchelaar HJ. (2013) A novel specific pyrosequencing method for genotyping FCGR3A rs396991 without coamplification of homologous gene FCGR3B. Pharmacogenet Genomics 23: 631-635.

13 Geva R, Vecchione L, Kalogeras KT, Vittrup JB, Lenz HJ, et al. (2014) FCGR polymorphisms and cetuximab efficacy in chemorefractory metastatic colorectal cancer: an international consortium study. Gut Epub ahead of print.

14 Bibeau F, Lopez-Crapez E, Di FF, Thezenas S, Ychou M, et al. (2009) Impact of Fc{gamma}RIIa-Fc{gamma}RIIIa polymorphisms and KRAS mutations on the clinical outcome of patients with metastatic colorectal cancer treated with cetuximab plus irinotecan. J Clin Oncol 27: 1122-1129.

15 Park SJ, Hong YS, Lee JL, Ryu MH, Chang HM, et al. (2012) Genetic polymorphisms of FcgammaRIIa and FcgammaRIIIa are not predictive of clinical outcomes after cetuximab plus irinotecan chemotherapy in patients with metastatic colorectal cancer. Oncology 82: 83-89.

16 Zhang W, Azuma M, Lurje G, Gordon MA, Yang D, et al. (2010) Molecular predictors of combination targeted therapies (cetuximab, bevacizumab) in irinotecan-refractory colorectal cancer (BOND-2 study). Anticancer Res 30: 4209-4217.

17 de Souza AP, Bonorino C. (2009) Tumor immunosuppressive environment: effects on tumor-specific and nontumor antigen immune responses. Expert Rev Anticancer Ther 9: 1317-1332.

18 Kono K, Takahashi A, Ichihara F, Sugai H, Fujii H, et al. (2002) Impaired antibody-dependent cellular cytotoxicity mediated by herceptin in patients with gastric cancer. Cancer Res 62: 5813-5817.

19 Sorbye H, Pfeiffer P, Cavalli-Bjorkman N, Qvortrup C, Holsen MH, et al. (2009) Clinical trial enrollment, patient characteristics, and survival differences in prospectively registered metastatic colorectal cancer patients. Cancer 115: 4679-4687.

20 Thariat J, Azzopardi N, Peyrade F, Launay-Vacher V, Santini J, et al. (2008) Cetuximab pharmacokinetics in end-stage kidney disease under hemodialysis. J Clin Oncol 26: 4223-4225.

21 Fontana E, Pucci F, Ardizzoni A. (2013) Colorectal cancer patient on maintenance dialysis successfully treated with cetuximab. Anticancer Drugs .

22 Inauen R, Cathomas R, Boehm T, Koeberle D, Pestalozzi BC, et al. (2007) Feasibility of using cetuximab and bevacizumab in a patient with colorectal cancer and terminal renal failure. Oncology 72: 209-210.

23 Aldoss IT, Plumb T, Zhen WK, Lydiatt DD, Ganti AK. (2009) Cetuximab in hemodialysis: a case report. Head Neck 31: 1647-1650.

Chapter 11

Summary

The use of the epidermal growth factor receptor (EGFR) antibodies cetuximab and panitumumab is limited to colorectal cancer (CRC) patients with *KRAS* wild type tumors and more recently in *RAS* wild type only. After having become chemotherapy refractory, treatment options are limited for this substantial patient group. This means that there is an urgent need to optimize anti-EGFR therapy. The work presented in this thesis aimed at optimising EGFR targeted monoclonal antibody therapy in metastatic CRC.

This thesis investigates several strategies to refine EGFR targeted monoclonal antibody therapy in CRC by:

- statins and their ability to phenoconvert *KRAS* mutant CRC;
- exploration of polymorphisms in the gene encoding *FCGR3A* and their association with cetuximab efficacy;
- and investigating the pharmacokinetics of cetuximab and panitumumab in patients with renal or hepatic dysfunction.

In patients with *KRAS* mutant tumors, the KRAS protein is highly active and these patients' tumors do not respond to anti-EGFR therapy. Before the KRAS protein exerts its important function in the cell signaling cascade, prenylation of the KRAS protein is required. Prenylation is the addition of C15 and C17 fatty acid chains to the KRAS protein. Prenylated KRAS is more lipophilic and can easily associate with the membrane. Membrane association of KRAS is crucial for its function in the RAS-RAF-MAPK signaling pathway. Statins and other KRAS modulators, such as bisphosphonates, farnesyltransferase inhibitors or geranylgeranyltransferase inhibitors affect the prenylation of the KRAS protein. Inhibition of the prenylation may lead to a more wild type KRAS phenotype. The modification of the KRAS mutant phenotype to a more KRAS wild type phenotype may augment the effect of EGFR antibodies in patients with *KRAS* mutations.

In **chapter 2**, clinical studies with statins and other KRAS modulators and their use in cancer treatment are reviewed. This review indicates that combinations of EGFR antibodies to target the EGFR with KRAS modulators may be an effective approach in patients with *KRAS* mutant tumors.

Chapter 3 describes an *in vitro* study using *KRAS* wild type and mutant cell lines. The aim of this study was to understand the role of statins in CRC cells and to explore the potential of therapeutic modulation of *KRAS* mutated CRC tumor cell lines. Western blot analysis showed that simvastatin inhibited the prenylation of the KRAS protein. The inhibition by simvastatin resulted in less membrane association of KRAS. A survival assay was used to study the effects of simvastatin and cetuximab on proliferation in colorectal cancer cell lines. In *KRAS* G13D mutated HCT116 and LoVo cell lines a combination of simvastatin pre-treatment and cetuximab resulted in less proliferation. This effect was not observed in the SW480 cell line harbouring a codon 12 *KRAS* mutation.

Since the *in vitro* studies showed promising results, we decided to perform a retrospective analysis to evaluate the effect of statin use on outcome in *KRAS* mutant metastatic CRC patients treated with cetuximab. In the CAIRO2 study by the Dutch Colorectal Study Group Metastatic CRC patients were treated with capecitabine, oxaliplatin, bevacizumab with or without cetuximab. We retrospectively analysed the effect of statin use at time of diagnosis on progression free survival (PFS) in CRC patients with *KRAS* mutant tumors treated with cetuximab and described the results in **chapter 4.** In our study we showed that the use of statins in patients with a *KRAS* mutant tumor did not lead to an improved progression free survival.

In two prospective studies we investigated the potential of simvastatin to phenoconvert mutant KRAS in CRC patients treated with cetuximab or panitumumab. In the RASTAT C and P studies described in **chapter 5 and 6**, metastatic CRC patients who failed on first- and second-line therapy, were treated with 80 mg of simvastatin daily and cetuximab (RASTAT C) or panitumumab (RASTAT P). Both studies were terminated after a planned interim analysis of the Simon two-stage design, because similar survival as seen in *KRAS* wild type patients was not observed.

An important mechanism of cetuximab induced cell-killing is antibody-dependent cellular cytotoxicity (ADCC). Fc gamma receptors (FCGR) on effector cells, for example macrophages and natural killer cells, bind to the Fc fragment of the cetuximab molecule and this causes lysis of the cancer cell. The germline polymorphism (rs396991) in the Fc gamma receptor 3A (*FCG3A*) c.818A>C results in a change of phenylalanine to valine at codon 158. Previous results from studies investigating the association between F158V *FCGR3A* polymorphisms and cetuximab efficacy are highly variable and firm conclusions cannot be drawn. To clarify the effect of the *FCGR3A* F158V polymorphism on efficacy a meta-analysis was performed. The individual patient data meta-analysis (**chapter 7**) shows that *FCGR3A* polymorphism is not associated with improved survival in cetuximab treated CRC patients. Some earlier studies showed that patients with specific *FCGR3A* polymorphisms might benefit from cetuximab treatment regardless of their *KRAS* mutational status. In this study, there is no significant difference in cetuximab efficacy between patients with *KRAS* wild type and mutant tumors.

Both cetuximab and panitumumab are used in patients with advanced or metastatic disease. Due to previous treatment or metastatic disease these patient are likely to have renal or hepatic insufficiency. Knowledge of dosing in specials populations with impaired renal or hepatic function is highly relevant. The pharmacokinetics and safety of both cetuximab and panitumumab have not been studied in these special populations. In two case reports, with panitumumab and cetuximab in cancer patients with liver and kidney dysfunction respectively, we showed that dose adjustments in patients with liver or kidney failure are not necessary and that treatment seems to be tolerable and safe (**chapters 8 and 9**).

In **chapter 10** the results from the performed research are discussed and future perspective are presented. Despite the promising results from the preclinical study, KRAS modulation with simvastatin is not applicable in the clinic and other strategies are needed for colorectal cancer patients with tumors harbouring a *KRAS* mutation who failed standard therapy. Besides statins, farnesyl- and geranylgeranyltransferase inhibitors also have a crucial role in the mevalonate pathway and consequently the prenylation of KRAS. A combination of statin and low doses farnesyl- and geranylgeranyltransferase inhibitors may be an effective treatment in CRC patients with a *KRAS* mutant tumor. Some studies reported that the effect of the *FCGR3A* polymorphisms on cetuximab efficacy is independent of *KRAS* status. The *FCGR3A* polymorphisms did not show a significant association with PFS. Moreover, no differences in cetuximab efficacy were found between patients with a *KRAS* mutant and *KRAS* wild type tumor. The results from these two approaches show that treatment options for CRC patients with a *(K)RAS* mutant tumor after failing chemotherapy and bevacizumab still remain poor.

The described case reports in this thesis help clinical decision making in real-life practice. Cetuximab and panitumumab monotherapy seems to be safely applicable in patients with *RAS* wild type metastatic CRC and hepatic or renal dysfunction, without the need for dose adjustments.

Chapter 12

Nederlandse samenvatting

In Nederland wordt jaarlijks bij circa 10.900 mensen dikkedarmkanker vastgesteld. De meest toegepaste behandelingen bij dikkedarmkanker zijn: een operatie, bestraling (radiotherapie) en behandeling met chemotherapie en antilichamen. Vaak is een combinatie van behandelmethoden nodig. Epidermale groei factor receptor (EGFR) antilichamen, zoals cetuximab en panitumumab zijn niet effectief gebleken bij de behandeling van patiënten met een *KRAS* mutante dikkedarmkanker. Ongeveer 40 % van de patiënten met een dikkedarmtumor heeft een mutatie in het *KRAS* gen. Indien behandeling met klassieke chemotherapie niet meer werkt zijn er voor deze patiëntengroep nog maar weinig behandelopties over. In dit promotieonderzoek is onderzoek gedaan naar drie verschillende manieren om de behandeling met EGFR-antilichamen te optimaliseren. Hierbij lag het accent op:

1. de effecten van statines bij de behandeling van dikkedarmtumoren met EGFR antilichamen;

2. de effecten van het FCGR3A polymorfisme en de effectiviteit van cetuximab;

3. de farmacokinetiek van cetuximab en panitumumab in patiënten met nier- of leverfunctiestoornissen.

De *KRAS* mutatie in de tumor van deze patiëntengroep leidt tot een voortdurende productie van het KRAS eiwit en een continue activatie van de eiwitten: RAS, RAF en MAPK, zodat 'upstream' remming van de EGF-receptor geen zin heeft. Cholesterol verlagende statines remmen HMG-CoA reductase en hierdoor de vorming van farnesylgroepen (C15-groepen) en geranyl-geranylgroepen (C17- groepen). Deze twee groepen worden gebruikt om eiwitten, waaronder KRAS te prenyleren en te activeren. Bij de prenylatie worden aan het KRAS eiwit C15- of C17 groepen gekoppeld. Door deze prenylatie wordt het KRAS eiwit lipofieler en verplaatst het zich naar het celmembraan, waar het zich verankert en zijn functie kan uitoefenen.

Statines en andere KRAS modulatoren, zoals bisfosfonaten en prenyltransferase remmers hebben mogelijk een effect op de expressie van het mutante KRAS eiwit omdat door een afgenomen prenylering minder KRAS eiwit geactiveerd wordt. (Deze fenotypische conversie leidt tot een meer wild type tumor en de hypothese is dat deze tumoren daardoor weer gevoelig worden voor de behandeling met EGFR-antilichamen.

In **hoofdstuk 2** worden de behandeleffecten van statines en verschillende andere KRAS modulatoren bij patiënten met kanker beschreven. Dit overzicht laat zien dat combinatie van KRAS modulatoren en EGFR-antilichamen mogelijk effectief kan zijn bij patiënten met een KRAS mutante tumor.

In de preklinische "proof of concept" studie beschreven in **hoofdstuk 3** wordt het effect van de combinatie van simvastatine en cetuximab onderzocht in vier verschillende *KRAS* mutante en wildtype tumor cellijnen. Het doel van deze studie was om te onderzoeken of deze tumor cellijnen door gelijktijdige behandeling met simvastatine weer gevoelig worden voor de behandeling met EGFR antilichamen. Met behulp van de Westernblot analyse werd aangetoond dat simvastatine de verplaatsting van KRAS eiwit van het cytoplasma naar het celmembraan remt. Het KRAS eiwit is hierdoor waarschijnlijk minder actief. In de *KRAS* G13D mutante cellijnen, LoVo en HCT 116, resulteerde combinatie behandeling met simvastatine en cetuximab in verminderde groei. Dit effect op de cel groei werd niet gezien in de *KRAS* wildtype cellijn A431 en de KRAS mutante SW480 cellijn (G12V).

In een retrospectieve cohortanalyse van de CAIRO2 studie (**hoofdstuk 4**) is het effect van statinegebruik op de progressievrije overleving in de patiënten met *KRAS* mutante dikkedarmkanker behandeld met cetuximab onderzocht. Bij deze studie zijn alle in Nederland geregistreerde statines meegenomen. Statinegebruik leidde in deze patiëntengroep niet tot een betere progressievrije overleving ten opzichte van niet-statinegebruikers.

In twee multicenter klinische trials (**hoofdstuk 5 en 6**) zijn patiënten met een gemetastaseerd KRAS mutant colorectaal carcinoom in de derde lijn behandeld met dagelijks 80 mg simvastatine en daarnaast wekelijks cetuximab (RASTAT-C studie) of tweewekelijks panitumumab (RASTAT-P). De hypothese was dat de behandeling met de combinatie simvastatine met een EGFR- antilichaam een progressie vrije overleving geeft van 12.5 weken; deze overleving is gelijk aan de overleving die gezien wordt in *KRAS* wildtype patiënten. Deze studies werden opgezet volgens het Simon two-stage design. Dit betekent dat halverwege de studie, na inclusie van de helft van de benodigde patiënten een geplande interim analyse van het effect van de behandeling plaatsvindt. Bij een positief resultaat, mag de studie gecontinueerd worden en worden de overige patiënten geïncludeerd in de studie. Bij de geplande interim analyse van de RASTAT-C en RASTAT-P was de progressie vrije overleving bij patiënten behandeld met simvastatine en een EGFR-antilichaam niet gelijk aan de progressievrije overleving die gezien wordt in *KRAS* wildtype patiënten. Beide studies werden gestopt na de geplande interim analyse.

Antilichaam-afhankelijke cellulaire cytotoxiciteit (ADCC) zorgt er voor dat de kankercel door afweercellen, waaronder macrofagen, kapot gemaakt wordt. Dit wordt ook wel lysis genoemd. De Fc-gamma receptoren op macrofagen herkennen het Fc-fragment van het antilichaam en binden hieraan. Deze binding veroorzaakt lysis van de kankercel. Een belangrijk verondersteld werkingsmechanisme van cetuximab is ADCC en vervolgens lysis van de dikkedarmkankercel. Het kiembaan polymorfisme (rs396991) in het gen dat codeert voor de Fc-gamma receptor 3A (*FCGR3A*) zorgt er voor dat het aminozuur fenylalanine verandert in valine. Deze verandering van aminozuur veroorzaakt mogelijk veranderingen in de affiniteit van de FCGR3A voor het EGFR antilichaam cetuximab. Verschillende studies hebben gekeken naar het effect van het polymorfisme op de effectiviteit van cetuximab. De uitkomsten waren echter zeer variabel en dit leidde niet tot duidelijke conclusies. Met behulp van een individuele patiëntendata meta-analyse (**hoofdstuk 7**) werd gekeken naar het effect van het *FCGR3A* polymorfisme en cetuximab effectiviteit in patiënten met *KRAS* mutante en wild type dikkedarmtumoren. Het *FCGR3A* polymorfisme had geen invloed op de progressievrije overleving van patiënten met een KRAS mutante dikkedarm tumor.

Cetuximab en panitumumab worden beiden toegepast bij patiënten met vergevorderde of gemetastaseerde ziekte. Door een uitgebreide voorbehandeling met bijvoorbeeld chemotherapie of gemetastaseerde ziekte presenteert een deel van deze patiënten zich met nier- en leverfunctiestoornissen. De toepassing van panitumumab en cetuximab is echter alleen onderzocht bij patiënten met een goede nier- of leverfunctie. Deze twee case reports (**hoofdstuk 8 en 9**) laten zien dat de farmacokinetiek van cetuximab en panitumumab in patiënten met nier- of leverfunctiestoornissen niet verschilt van patiënten zonder nier- of leverfunctiestoornissen. Dosis verlaging is niet noodzakelijk en de behandeling lijkt veilig.

Tot slot worden in **hoofdstuk 10** de resultaten van het gehele onderzoek in dit proefschrift bediscussieerd en wordt een toekomstperspectief voor verder onderzoek geschetst. Ondanks de aanwijzingen vanuit het preklinisch *in vitro* onderzoek dat statines, *KRAS* mutante dikkedarmkanker cellen gevoelig kunnen maken voor EGFR antilichamen leidde het gebruik van statines in de prospectieve studies en de cohortanalyse echter niet tot een verbeterde

progressie vrije overleving. Daarnaast was de effectiviteit van cetuximab niet verschillend voor de verschillende FCGR3A polymorfismen. De behandelingsmogelijkheden van patiënten met een *KRAS* mutante tumor blijven uiterst beperkt en andere therapieën zijn nodig om patiënten met een *KRAS* mutante dikkedarmtumor te behandelen.

De bijwerking van huidtoxiciteit voorkomen is belangrijk om het effect van de EGFR-antilichamen te maximaliseren. De identificatie van SNPs geassocieerd met huidtoxiciteit dragen mogelijk bij aan een verdere optimalisatie en nieuwe inzichten van de therapie met EGFR-antilichamen.

Tenslotte laten de gepresenteerde case reports zien dat dosisaanpassingen niet noodzakelijk zijn bij lever- en nierfunctiestoornissen. Dit zal echter in de toekomst in grotere patiëntenstudies onderzocht moeten worden.

Dankwoord

Na ruim zes jaar is mijn proefschrift eindelijk af. Graag wil ik iedereen bedanken die een bijdrage geleverd heeft aan mijn promotie onderzoek,

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Celkweek onderzoek is niet altijd makkelijk en gaat vaak met teleurstellingen gepaard. Tahar, mijn co-promotor, mede door jouw doorzettingsvermogen en altijd weer nieuwe experimentele ideeën zijn we ver gekomen. Jouw toewijding en vertrouwen in een goede afloop in de afrondende fase heb ik als erg waardevol ervaren. Renee, dank voor alle hulp, ondersteuning en gezelligheid gedurende de lab experimenten.

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Curriculum Vitae

Lisanne Laura Krens was born in Pijnacker, the Netherlands on May 26th 1984. She finished her secondary school (gymnasium) in 2002 at the Erasmus College in Zoetermeer.

In 2002 she started her studies in pharmacy at the Utrecht University in Utrecht, the Netherlands. After completing her bachelors' degree in 2005, she continued her master's degree in pharmacy. As part of her education she worked at the hospital pharmacy of Nsambya Hospital in Kampala, Uganda (supervisor: Dr. Willem Rutten). She was part of a project that aimed to help to implement the pharmaceutical management system Msupply.

In 2008 she started working as a pharmacist at the hospital pharmacy of the Zuwe Hofpoort hospital in Woerden, the Netherlands. In 2009 she started her specialisation as a hospital pharmacist (supervisor: Prof. dr. Henk-Jan Guchelaar) in combination with a PhD research at the department of Clinical Pharmacy and Toxicology at the Leiden University Medical Center (supervisors: Prof. dr. Henk-Jan Guchelaar and Prof. dr. Hans Gelderblom and Dr. Tahar van der Straaten). Her training as a hospital pharmacist was completed in 2014. In 2011, during her specialisation she visited the hospital pharmacy of the Magbenteh hospital in Magbenteh, Sierra Leone. In this rural hospital she trained the pharmacy staff the basics of pharmaceutical management system Msupply.

After completion of her thesis, she will continue her career as a hospital pharmacist at the department of Clinical Pharmacy of the Ziekenhuisgroep Twente (ZGT) hospital in Hengelo and Almelo, The Netherlands.

Lisanne Krens is married to Lukas Vermeer.

List of publications

Related to this thesis

Krens LL, Simkens LH, Baas JM, Koomen ER, Gelderblom H, Punt CJ, Guchelaar HJ. Statin Use Is Not Associated with Improved Progression Free Survival in Cetuximab Treated KRAS Mutant Metastatic Colorectal Cancer Patients: Results from the CAIRO2 Study. PLoS One. 2014 Nov 6;9(11):e112201.

Krens LL, Baas JM, Verboom MC, Paintaud G, Desvignes C, Guchelaar HJ, Gelderblom Pharmacokinetics and safety of cetuximab in a patient with renal dysfunction. Cancer Chemother Pharmacol. 2014 Jun;73(6):1303-6.

Krens LL, Baas JM, de Jong FA, Guchelaar HJ, Gelderblom H. Pharmacokinetics of panitumumab in a patient with liver dysfunction: a case report. Cancer Chemother Pharmacol. 2014 Feb;73(2):429-33.

Krens LL, Baas JM, Gelderblom H, Guchelaar HJ. Therapeutic modulation of KRAS signaling in colorectal cancer. Drug Discov Today. 2010 Jul;15(13-14):502-16.

Not related to this thesis

Baas JM, **Krens LL**, Guchelaar HJ, Ouwerkerk J, de Jong FA, Lavrijsen AP, Gelderblom H. Recommendations on management of EGFR inhibitor-induced skin toxicity: a systematic review. Cancer Treat Rev. 2012 Aug;38(5):505-14.

Baas JM, **Krens LL**, Guchelaar HJ, Morreau H, Gelderblom H. Concordance of predictive markers for EGFR inhibitors in primary tumors and metastases in colorectal cancer: a review. Oncologist. 2011;16(9):1239-49.