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Pharmacogenetics of advanced colorectal cancer treatment

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Citation

Pander, J. (2011, June 29). *Pharmacogenetics of advanced colorectal cancer treatment*. Retrieved from <https://hdl.handle.net/1887/17746>

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Pharmacogenetics of advanced colorectal cancer treatment

Jan Pander

Pharmacogenetics of advanced colorectal cancer treatment

Proefschrift

ter verkrijging van

de graad van Doctor aan de Universiteit Leiden,
op gezag van Rector Magnificus prof. mr. P.F. van der Heijden,
volgens besluit van het College voor Promoties
te verdedigen op woensdag 29 juni 2011
klokke 15:00 uur

door

Jan Pander

geboren te Amsterdam
in 1979

The research presented in this thesis was performed at the Department of Clinical
Pharmacy & Toxicology of the Leiden University Medical Center, The Netherlands

The printing of this thesis was financially supported by AZL Onderzoeks- en
Ontwikkelingskrediet (OOK) Apotheek

Cover design and lay-out by In Zicht Grafisch Ontwerp, Arnhem
Printed by Ipskamp drukkers, Enschede

ISBN 978-90-90261126

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“For the first time I felt the truth that the sky begins a quarter of an inch from the ground. In the mornings the bush smelled like the best underarm deodorant you ever smelled, and I quickly got used to the mysterious movements of the trees, which heaved rhythmically like a man chloroformed. From time to time the night sky seemed uneven, closer in points, then smoothed out, like a tablecloth bunched up then suddenly pulled taut. I'd wake up to see low-lying clouds balanced precariously on the tops of trees. Sometimes the wind was so gentle it seemed to come from a child's nostril, while other times it was so strong all the trees seemed held tenuously to the earth by roots as weak as doubled-over sticky tape.”

From: A fraction of the whole. Steve Toltz

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1

Outline of the thesis

Colorectal cancer is one of the leading causes of cancer related deaths.¹ Surgery with curative intent is indicated for patients without distant metastases and in a subset of patients with resectable distant metastases.² For irresectable metastatic colorectal cancer, only palliative treatment options remain. Current standard treatment consists of chemotherapeutic drugs (the fluoropyrimidines, oxaliplatin and irinotecan) and antibodies against vascular endothelial growth factor (VEGF; bevacizumab)³ and the epidermal growth factor receptor (EGFR; cetuximab and panitumumab).⁴⁻⁶ Even though the optimal use of these agents has not been defined, the most commonly applied first-line treatment consists of a fluoropyrimidine as monotherapy, or combined with oxaliplatin or irinotecan, plus bevacizumab, while the other drugs are used as salvage treatments.^{7,8} With the currently available regimens, the median overall survival of metastatic colorectal cancer patients is approximately two years.² Despite the improvement of prognosis of metastatic colorectal cancer patients from roughly 12 to 24 months in the past fifteen years², the efficacy of these expensive and potentially toxic treatments remains limited and unpredictable. It is therefore desirable to develop predictive markers to aid better selecting patients for these treatments.

In order to select patients for treatment, germline genetic variation between patients, as well as somatic mutations in their tumors can be used. As anti-cancer treatment exerts its effect in the tumor, it is reasonable to correlate the genetic mutations in the tumor to the anti-tumor response. Indeed, some of these mutations are used in routine clinical practice, such as *EGFR* mutation testing for the selection of non-small cell lung cancer patients for treatment with the small-molecule tyrosine kinase inhibitors against EGFR gefitinib and erlotinib^{9,10} and *KRAS* mutation testing for the selection of metastatic colorectal cancer patients for cetuximab or panitumumab treatment.¹¹ A disadvantage of the use of somatic mutations, is that the tumor is genetically unstable, resulting in different genetic composition over time. Moreover, discordance in mutational status may be present between the primary tumor and corresponding metastatic lesions for some genetic variants, as well as discordance within one tumor sample.

Heritable germline variation in DNA derived from peripheral blood or other normal tissue is studied in the field of pharmacogenetics. Genetic polymorphisms may be present in drug target proteins, or in enzymes involved in the pharmacokinetics of the drug of interest. The presence of a genetic polymorphism in a gene can result in increased or decreased expression, or altered function of the protein. As a result, drug response – either efficacy or toxicity – may be altered. Advantages over tumor-derived genetic variation are that germline genotypes remain constant over time, and that the collection of blood or saliva is only mildly invasive. Moreover, the germline genetic variation is the same as in tumor tissue, but not vice-versa: somatic mutations that originate in tumor tissues cannot be detected in germline material.¹²

The aim of this thesis is to identify germline pharmacogenetic markers for predicting the response to palliative treatment of metastatic colorectal cancer.

The first part of the thesis focuses on predictive germline markers for the efficacy of cetuximab. A review of pharmacogenetic studies for EGFR and VEGF targeted therapy is given in **chapter 2**. Germline DNA was obtained from patients in the CAIRO2 trial of the Dutch Colorectal Cancer Group (DCCG). In this randomized phase III study, patients with previously untreated metastatic colorectal cancer were treated with capecitabine, oxaliplatin and bevacizumab or the same regimen plus cetuximab. Surprisingly, the addition of cetuximab resulted in decreased median progression-free survival (PFS).¹³ The influence of five different germline polymorphisms on the efficacy of cetuximab was investigated in patients of the CAIRO2 study (**chapter 3**). To further explore the mechanism underlying the results of this pharmacogenetic analysis, *in vitro* research on the influence of the *FCGR3A* Phe158Val polymorphism was performed. As a model for tumor-associated macrophages, type 2 macrophages were cultured from monocytes of healthy donors harboring the different *FCGR3A* genotypes. The activation of these type 2 macrophages under the influence of cetuximab was studied (**chapter 4**).

In the second part of the thesis, predictive germline variation for the efficacy of capecitabine, oxaliplatin and bevacizumab – the treatment in the control arm of the CAIRO2 study – was studied. The literature on pharmacogenetics of cytotoxic therapy is reviewed in **chapter 5**.

In the previous CAIRO study⁷, an exploratory study was performed with candidate polymorphisms in DNA repair genes.¹⁴ Polymorphisms in the *ATM* and *ERCC5* genes were associated with the efficacy of an oxaliplatin-based regimen. To confirm these preliminary findings, the effects of these polymorphisms on treatment response were investigated in the control arm of the CAIRO2 study (**chapter 6**).

In classic pharmacogenetic studies, each polymorphism is correlated with the clinical end-point. A limitation to this method is that the complexity underlying drug response is not fully taken into account. It is therefore not surprising that inconsistent results have been published for most pharmacogenetic markers.^{15,16} Since drug response involves many different proteins – such as therapeutic targets, molecules in the signaling pathway, metabolic enzymes or drug transporters – it is likely that the impact of polymorphisms in the corresponding genes exert their influence only in the presence of other polymorphisms. This concept is known as non-linear interaction, or epistasis.¹⁷

To investigate epistasis in relation to drug response, novel methods such the multifactor dimensionality reduction (MDR) and classification and regression tree (CART) techniques can be applied. The technical aspects of these techniques are

described and illustrated using sunitinib induced toxicity data from a previous study¹⁸ (**chapter 7**). The MDR method was applied to explore the association and interaction of 17 frequently studied polymorphisms in different candidate genes in the control arm of the CAIRO2 study (**chapter 8**).

Currently, most pharmacogenetic studies include polymorphisms in so-called candidate genes. A limitation of this approach is that only mechanistically related genes and polymorphisms are studied, which is by definition restricted by our current understanding of the mechanism of action of the drugs of interest. To identify novel polymorphisms – and genes – that are associated with response to capecitabine, oxaliplatin and bevacizumab, a hypothesis-free genome wide association study was performed with an array including more than 700,000 polymorphisms (**chapter 9**).

The results from these studies are summarized (**chapter 10**) and put into perspective in the general discussion (**chapter 11**).

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Cetuximab



2

Pharmacogenetics of EGFR and VEGF inhibition

Jan Pander • Hans Gelderblom • Henk-Jan Guchelaar

Drug Discovery Today 2007 Dec;12(23-24):1054-60

Abstract

Even though treatment of several types of solid tumours has improved in the past few years with the introduction of the monoclonal antibodies against epidermal growth factor receptor (EGFR) and vascular endothelial growth factor (VEGF), response rates to these targeted therapies are modest. Pharmacogenetic factors have the potential to select patients with higher chance of response to agents that target these pathways. This review provides an overview over germ-line variations in genes that are potentially involved in the pharmacodynamics of the monoclonal antibodies cetuximab, panitumumab and bevacizumab, and which may underlie variable anti-tumour response.

The treatment of solid tumours has changed in the past five years with the introduction of monoclonal antibody (MAB) drugs targeting growth factor pathways that are critical for tumour growth and invasiveness. The epidermal growth factor receptor (EGFR) targeting MABs cetuximab and panitumumab and the vascular endothelial growth factor (VEGF) targeting MAB bevacizumab are approved for the treatment of metastasized colorectal cancer (mCRC). Cetuximab and bevacizumab are also approved for the treatment of advanced squamous cell carcinoma of the head and neck (SCCHN) and advanced non-squamous, non-small cell lung cancer (NSCLC) respectively. These MABs are commonly administered in combination with first-line chemotherapy, whereas monotherapy is also applied in subsequent lines of therapy. Despite overall improving cancer treatment, the addition of these MABs to chemotherapy increases response rates by only 10-20%¹⁻³ and adverse events such as moderate to severe rash for the EGFR inhibitors and gastro-intestinal perforations and hypertension for bevacizumab are relatively common.¹⁻³ Moreover, the introduction of these MABs has almost doubled cost of treatment.⁴

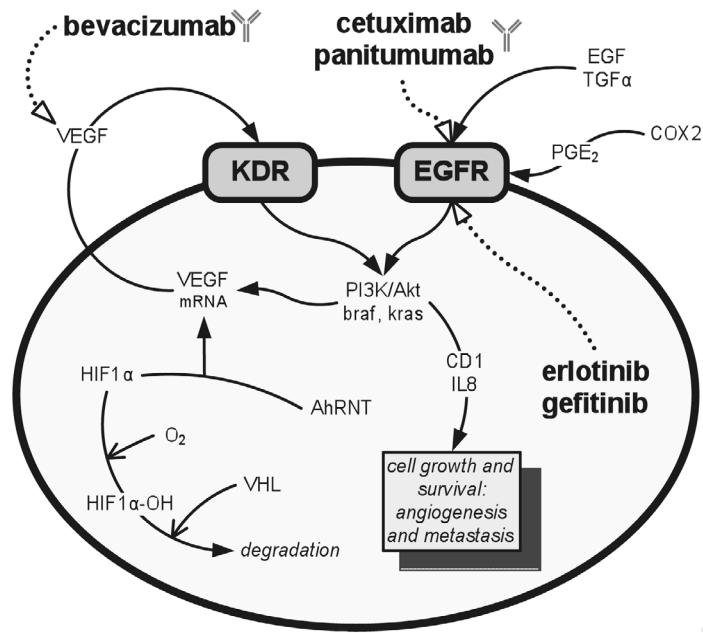
Therefore, selection of patients for treatment based on predictive factors for response, survival and/or toxicity could improve treatment success as well as cost-effectiveness. Pharmacogenetics is aimed at understanding and predicting an individual's drug response based upon genetic variation. Whereas somatic mutations occur only in the affected organ or disease locus (tumour) and result in a different genetic composition of a tumour compared with other tissues in the body, germ-line polymorphisms have an ancestral origin and are heritable. In this review, we give an overview of heritable genetic factors that might predict drug induced anti-tumour response and toxicity of EGFR and VEGF targeting MABs, based upon candidate genes for these pathways. Also, we give an overview of pharmacogenetic studies with drugs that target these pathways.

Epidermal growth factor (EGF) pathway

Cetuximab is a chimeric mouse/human IgG₁ type MAB, whereas panitumumab is a fully human IgG₂ type MAB. Both MABs bind specifically to the extracellular domain of the EGFR and are competitive inhibitors of the natural ligands EGF and transforming growth factor- α (TGF α).

The small G protein k-ras, the protein kinase b-raf, and phosphoinositide 3-kinase (encoded by *KRAS*, *BRAF* and *PIK3CA* respectively) play a central role as intracellular mediators of EGFR signalling⁵, ultimately leading to induced transcription of several factors including interleukin-8 (IL8), VEGF and cyclin D1 (CD1, coded by *CCND1*). Cyclooxygenase-2 (COX2, encoded by *PTGS2*) is an upstream mediator of EGFR activity, presumably through the effect of prostaglandin E₂ (see Figure 1).

Figure 1 Simplified overview of the EGF and VEGF pathways



Abbreviations: ARNT: aryl hydrocarbon receptor nuclear translocator; braf: protein kinase b-raf; CD1: cyclin D1; COX2: cyclooxygenase 2; EGF: epidermal growth factor; EGFR: epidermal growth factor receptor; HIF1α: hypoxia inducible factor 1α; HIF1α-OH: hydroxylated hypoxia inducible factor 1α; IL8: interleukin 8; IL8RA: interleukin 8 receptor A; KDR: kinase domain receptor; kras: small G protein k-ras; PGE₂: prostaglandin E₂; PI3K/Akt: phosphoinositide 3-kinase/akt protein kinase; TGFα: transforming growth factor α; VEGF: vascular endothelial growth factor; VHL: von Hippel-Landau tumor suppressor

Heritable genetic variants in genes in the EGF pathway will be discussed below (see Table 1).

Epidermal Growth Factor Receptor (EGFR)

The first intron of the *EGFR* gene has an important regulatory function and contains a heritable polymorphic microsatellite sequence of 9 to 23 CA repeats. Most common alleles are the 16-repeat allele in Caucasians and Afro-Americans, and the 20-repeat in Asians.^{6,7} There is good to complete (93-100%) similarity of this polymorphism between normal and tumour tissue⁸⁻¹⁰, which is reassuring since the *EGFR* gene is highly sensitive to somatic alteration through loss of heterozygosity, mutations or copy number

Table 1 Overview of polymorphisms in genes that code for enzymes involved in the EGFR pathway

Enzyme (gene)	Polymorphism	Phenotype	Function	Ref	Pharmacogenetic association	Ref
EGFR (<i>EGFR</i>)	-191C>A		higher EGFR	7,13		
	-216G>T (CA) _{9,23}		higher EGFR	7,13	gefitinib (NSCLC): carriage of -216T allele → longer PFS	15
			higher EGFR for lower amount of CA-repeats	10,11	gefitinib (NSCLC): low amount of CA-repeats → higher response and TTP and PFS	9,15
EGF (<i>EGF</i>)	1808G>A	Arg497Lys	unknown		monotherapy cetuximab (mCRC): heterozygotes → longer PFS	18
	61A>G		higher EGF	16,17	monotherapy cetuximab (mCRC): simultaneous carriage of 61A and <i>CCND1</i> 870G allele → longer OS	18,28
COX2 (<i>PTGS2</i>)	-765G>C		lower COX2	19,20	monotherapy cetuximab (mCRC): 61GG homozygotes → longer PFS	18
IL8 (<i>IL8</i>)	-251T>A		higher IL8	25		
IL8RA (<i>CXCR1</i>)	2607G>C	Ser276Thr	unknown			
CD1 (<i>CCND1</i>)	870G>A		higher CD1	27	monotherapy cetuximab (mCRC): carriage of 870G allele → longer OS	28

alterations. A higher number of CA-repeats is associated with decreased expression of EGFR on both mRNA and protein level *in vitro*^{10,11}, but this association was not consistently found *in vivo*.^{9,12}

Two single nucleotide polymorphisms (SNPs; see glossary box) in the promoter (-216G>T and -191C>A) are both associated with increased expression of EGFR.^{7,13} A nonsynonymous SNP (1808G>A) in the extracellular domain of EGFR results in lower binding-affinity of EGF and TGF- α and attenuated growth response to these growth factors *in vitro*.¹⁴

Glossary box

- SNP (Single Nucleotide Polymorphism): a change of a single base of germ-line DNA, as compared with wild-type, which occurs in $\geq 1\%$ of the population. Because any individual carries two alleles, the SNP can be present in both alleles (homozygote), on only one allele (heterozygote) or not at all (wild-type).
- CNP (Copy Number Polymorphism): in contrast to a SNP, a CNP encompasses ≥ 1000 base pairs or more. Regarding heritability and population frequency, the definitions are the same.
- Mutation: a change in DNA that occurs either very infrequently ($\leq 1\%$ in the population), or only in an affected organ. In the latter case, the mutation is not inherited.
- Haplotype Block: SNPs are naturally inherited in neighboring clusters, which are called haplotype blocks.
- ADCC (antibody dependent cell-mediated cytotoxicity): the recognition by natural killer cells of the Fc region of an antibody after binding to the antigen, followed by killing of the antigen presenting cell.
- Prognostic factor: a marker for prognosis of a disease, not related to treatment
- Predictive factor: a marker for response to a certain treatment

Recently, two pharmacogenetic studies were published on the sensitivity of NSCLC patients to the EGFR tyrosine kinase inhibitor (TKI) gefitinib. These studies are important examples of the utility of pharmacogenetics for EGFR inhibitors. Han et al. reported an increased response and time to progression (TTP) for patients with ≤ 37 CA-repeats in Korean NSCLC patients, regardless of the presence of somatic mutations.⁹

Similarly, NSCLC patients of predominantly Caucasian origin, who were homozygous for two short CA-repeat alleles (defined as ≤ 16 CA-repeats per allele) had better progression free survival (PFS) compared with carriers of at least one allele with >17 CA-repeats. Also, patients who carried the -216T allele had longer PFS. Patients who

were homozygous for the short CA-repeat allele and simultaneously carrier of the -216T allele, had improved PFS and overall survival (OS).¹⁵

In line with these findings, Amador et al. reported higher sensitivity to another EGFR inhibiting TKI, erlotinib, in cell lines with ≤ 35 CA-repeats compared with cell lines with >35 repeats. Also, the authors found increased incidence of skin toxicity in gefitinib treated CRC patients with ≤ 35 CA-repeats.¹⁰

Upstream regulators of EGFR

A SNP in the 5'-UTR of the *EGF* gene (61A>G) has been associated with higher EGF protein expression *in vitro*¹⁶ and *in vivo*.¹⁷ The 61GG genotype was associated with increased PFS in mCRC patients who were treated with cetuximab monotherapy.¹⁸ A functional SNP in the promoter region of the *PTGS2* gene (-765G>C) has been associated with lower promoter activity *in vitro*¹⁹ and with lower expression of the *PTGS2* gene product, COX2 *in vivo*.²⁰ Illustrative of its function is the strong association with decreased risk of myocardial infarction and stroke for the -765C allele.²¹ Recently, an association for increased PFS for the -765CC genotype was reported in mCRC patients treated with single agent cetuximab.¹⁸

Downstream signalling

The presence of somatic mutations in *KRAS*, but not in *BRAF* and *PIK3CA*, has been associated with decreased effect of cetuximab in CRC patients^{22,23}, though not unequivocally.²⁴ However, no reports are available on heritable polymorphisms in these genes.

A SNP in the 5'-UTR of the *IL8* gene (-251T>A) has been associated with increased IL8 production.²⁵ The IL8 receptor alpha, IL8RA (encoded by the gene *CXCR1*) contains a nonsynonymous SNP in exon 2 (2607G>C)²⁶, whose function remains unclear. A SNP in the *CCND1* gene (870G>A) has been associated with higher expression of CD1.²⁷

Zhang et al. investigated whether there was an association for the polymorphisms in the *CCND1* (870A>G), *PTGS2* (-765G>C), *EGF* (61A>G), *EGFR* (1808G>A and CA-repeats), *IL8* (-251T>A) and *VEGF* (+936C>T) genes with the effect of cetuximab given as a single agent in advanced CRC patients.²⁸ Homozygotes for the *CCND1* 870A allele had a shorter OS compared with carriers of the 870G allele.²⁸ In combined analysis, patients who carried both a *CCND1* 870G allele and an *EGF* 61A allele had longer OS, whereas the other polymorphisms were not associated with survival.²⁸ These results, though valuable, need to be interpreted with care, as this was an exploratory study. The fact that seven different polymorphisms were analyzed in a small population raises the

probability of false positive associations. However, together with the other association studies of the EGF pathway, these findings provide an important starting point for adequately powered confirmation studies.

Vascular endothelial growth factor (VEGF) pathway

Bevacizumab is a humanized IgG₁ type MAb directed against soluble VEGF, one of the key moderators in angiogenesis, which is thought to be important for tumour growth and invasiveness.²⁹ VEGF exerts its pro-angiogenic effect via VEGF receptor-2, a tyrosine kinase receptor that is also referred to as kinase insert domain receptor (KDR). Transcription of VEGF is regulated by hypoxia inducible factor-1α (HIF1α) (see Figure 1).

To date, five functional SNPs in the 5' and 3' regions of the *VEGF* gene have been described (Table 2).³⁰⁻³²

The variant alleles of the -1154G>A and +936C>T SNPs are associated with lower VEGF production³²⁻³⁵, whereas the variant allele of the -460C>T SNP results in increased promoter activity.³⁶ There is less agreement on the functionality of the -2578C>A and +405G>C SNPs, as both increased as decreased VEGF production have been reported.^{31,34,35,37}

It must be noted though that the above mentioned SNPs are inherited in clusters in so called haplotype blocks (see glossary box).^{31,35,36,38-41} It is likely that only one SNP is truly functional with regard to VEGF expression, whereas the others are merely proxies for this one. This truly causal SNP, however, has so far not been identified.

There are several nonsynonymous SNPs in the coding region of the *KDR* gene (see: <http://www.ncbi.nlm.nih.gov/projects/SNP>). Nonetheless, only functionality of a CA-repeat polymorphism in intron 2 of the *KDR* gene (+4422(AC)₁₁₋₁₄) has been determined. The 11-repeat polymorphism results in higher promoter activity *in vitro*.⁴² Even though the 11- and 12-repeat alleles were most common in the Japanese population, the allele frequencies in other populations are unknown.

The nonsynonymous SNP 1772C>T in the gene encoding HIF1α (*HIF1A*) has been associated with increased expression of HIF1α.⁴³⁻⁴⁵ The enzyme coded by the variant allele is also less sensitive to hydroxylation dependent degradation⁴⁶, which results in further increased protein levels. Another nonsynonymous SNP (1790G>A) in the *HIF1A* gene has also been associated with increased HIF1α expression.⁴³

As these SNPs result in increased abundance of the HIF1α protein, it is expected that the SNPs ultimately result in increased VEGF expression (see Figure 1). The relationship

Table 2 Overview of polymorphisms in genes that code for enzymes involved in the VEGF pathway

Enzyme (gene)	Polymorphism	Phenotype	Function	Ref
VEGF (<i>VEGF</i>)	-2578C>A		lower or higher VEGF	34,35
	-1154G>A		lower VEGF	34,35
	-460C>T		increased promoter activity	36
	+405G>C		lower or higher VEGF	31,34,37
	+936C>T		lower VEGF	32,33
KDR (<i>KDR</i>)	+4422(AC) ₁₁₋₁₄		11 CA repeats higher promoter activity than 12 CA repeats	42
HIF1α (<i>HIF1A</i>)	1772C>T	Pro582Ser	higher HIF1α	43-46
	1790G>A	Ala588Thr	higher HIF1α	43

between the *HIF1A* SNPs and VEGF mRNA levels has been demonstrated, whereas no relationship with VEGF protein expression was found.^{45,47}

ARNT is most commonly described as a subunit of aryl hydrocarbon receptor (AHR), which induces transcription of the cytochrome P450 isozyme CYP1A1 in response to exogenous stimuli such as cyclic aromatic hydrocarbons from cigarette smoke. As part of a dimer with HIF1α, ARNT induces VEGF transcription. Genetic variation of the ARNT gene may therefore be of importance for VEGF production. However, to date no functional polymorphisms in the *ARNT* gene have been described.

Up to now, no pharmacogenetic studies have been published for agents that target the VEGF pathway. The publication of these studies is eagerly awaited, as they will provide a foundation for further, hypothesis testing research for this pathway.

Polymorphisms for MABs in general

The plasma half-life of MABs is generally relatively long: the half life of bevacizumab (20 days) is similar to that of endogenous IgG₁, whereas the half life of cetuximab and panitumumab is 70-100 hours and 7.5 days respectively. The shorter half life of the latter MABs can in part be explained by internalization and degradation of the receptor-MAB complex after binding. It is postulated that antibodies of the IgG type, such as bevacizumab, are protected from degradation by the neonatal Fc receptor (FcRn, coded by *FCGRT*).⁴⁸

Sachs et al. recently described a variable number of tandem repeats (VNTR) within the promoter of the *FCGRT* gene consisting of five different alleles with one to five repeats (VNTR 1-5).⁴⁹ The allele frequencies of VNTR2 and VNTR3 were 0.075 and 0.92 respectively in Caucasians. The VNTR3 allele was associated with higher FcRn expression both *in vitro* and *in vivo*. Also, binding of IgG was higher among VNTR3 homozygotes compared with VNTR2/VNTR3 heterozygotes.⁴⁹ Possibly, individuals carrying the VNTR3 allele have prolonged plasma half-life of bevacizumab, and even increased response.

Cetuximab is a competitive inhibitor of EGFR, which results in decreased utilization of this pathway. As cetuximab is of the IgG₁ type, it is likely that antibody dependent cell-mediated cytotoxicity (ADCC) also plays a role in its mechanism of action. The Fc region of the antibody can be recognized by Fcγ-receptors on cytotoxic immune effector cells such as natural killer cells and macrophages. Two activating Fcγ-receptors are CD16A and CD32A (encoded by respectively *FCGR3A* and *FCGR2A*) are polymorphic (see table 3).

A SNP in the *FCGR3A* gene (559T>G; Phe158Val) has been studied since the early 1990's. IgG₁ binding is higher for the 158Val allele, which results in increased activation of ADCC but not in altered expression.^{50,51} Also, the affinity of the IgG₁-type MAb against CD20 expressing B cells, rituximab, was highest for the 158Val allele.⁵² It is therefore not surprising that response and PFS to rituximab in follicular lymphoma was higher for homozygotes of the 158Val allele, compared with carriers of the 158Phe allele.^{53,54} This SNP was not associated with clinical response to another IgG₁ MAb against TNFα, infliximab, in Crohn's disease, but increased biological response (decrease in C-reactive protein) was associated with the 158Val allele.^{55,56}

Even though the function of a SNP in the *FCGR2A* gene (535A>G; His131Arg) with regard to IgG₁ has not been established, an association with worse response and PFS to rituximab in follicular lymphoma was found.⁵⁴ However, this association was not confirmed by another study.⁵³

Very recently, Zhang et al. showed that mCRC patients treated with cetuximab monotherapy, who were homozygous for either the *FCGR2A* 131Arg or *FCGR3A* 158Val allele had shorter PFS and decreased response.⁵⁷ The reason for this result, which for *FCGR3A* is opposite to what would be expected, is not known. A possible explanation is that copy number polymorphism (CNP) (see next paragraph) at the locus of *FCGR3A* plays a role. This, however, has not been investigated. It is also probable that this finding is a false positive discovery, since not only these two genotypes have been investigated, but also seven others in a previous analysis of the data.²⁸

Even though ADCC does not play a role for bevacizumab, it is likely that its effect is modified by similar mechanisms.

Table 3 Overview of polymorphisms in genes that code for enzymes involved in the mechanism of action of IgG type monoclonal antibodies

Enzyme (gene)	Polymorphism	Phenotype	Function	Ref	Pharmacogenetic association	Ref
FcRn (<i>FCGRT</i>)	VNTR1-5		VNTR3 higher FcRn than VNTR2	49		
FcγR2A (<i>FCGR2A</i>)	535A>C	His131Arg	unknown		rituximab (lymphoma): carriage of 131Arg allele → lower response and PFS cetuximab (mCRC): 131Arg homozygote → lower response and PFS	54,57
FcγR3A (<i>FCGR3A</i>)	559T>G	Phe158Val	higher affinity for IgG ₁	50,51	rituximab (lymphoma): carriage of 158Phe allele → lower response and PFS infliximab (mCrohn): 158Val allele → increased biological response cetuximab (mCRC): 158Val homozygote → lower response and PFS	53,57

Copy number polymorphisms

An interesting novel field of pharmacogenetic research includes heritable variation of copy number of DNA segments of 1 kb or larger of the genome.⁵⁸ Analogous to the definition of a SNP, a CNP is a structural variant that occurs at a frequency of >1% in the population. Since the first whole genome array studies of this phenomenon were published in 2004^{59,60}, an open-access online database has been developed in which structural variations of the human genome are assembled^{60,61} (see: <http://projects.tcag.ca/variation>).

Several studies have demonstrated that increased intratumoral EGFR copy number in advanced CRC patients is associated with effectiveness of cetuximab.^{22,24,62} However, it must be noted that this is a somatic phenomenon, which is probably involved in the aetiology of the tumour. In the Database of Genomic Variants, there are no CNPs on the EGFR locus. Also, no CNPs are reported at the loci that cover the *TGFA*, *IL8*, *CXCR1*, *BRAF*, *KRAS*, *PIK3CA*, *PTGS2*, *VEGF*, *KDR*, *HIF1A* or *ARNT* genes. There is an infrequent CNP in the *EGF* gene (one reference to loss of the locus in 36 subjects), but CNPs on the locus that covers the *CCND1* gene occurs in 6 of 95 subjects. Also, the locus that contains the *FCGR2A* gene shows heritable loss at a frequency of approximately 0.26. There is also considerable CNP covering the *FCGR2A* and *FCGR3A* genes, with equal amount of gain and loss of this locus. Illustrative of the influence of copy number variation was recently published, showing that copy number variation of the *FCGR2A* and *FCGR3A* containing region (that also contains the *FCGR3B* gene encoding CD16B) was associated with susceptibility to systemic autoimmune diseases.⁶³

Discussion

It is accepted that germ-line polymorphisms (both SNPs and CNPs) have the potential to predict outcome of therapy. Predicting outcome of therapy with cetuximab, panitumumab and bevacizumab is especially warranted, as response rates are moderate, with possible serious adverse events, at high financial cost. In this review, we give an overview of studies on polymorphisms in candidate genes in the EGF and VEGF pathways.

To date, only few small studies have shown an association of genetic polymorphisms in genes of the EGF pathway with response to EGFR targeting therapies (see Table 1), whereas studies for the VEGF pathway are thus far lacking.

However, these studies do not provide sufficient evidence to routinely genotype patients before applying these therapies. The associations need to be confirmed in one or more sufficiently powered prospective studies first. A requirement for these studies is the presence of a control group without the treatment of interest. Only then

a distinction between predictive and prognostic factors (see glossary box) can be established. This latter point is of great importance, because numerous studies have shown an association of polymorphisms within genes in the EGF and VEGF pathways with the risk and progression of several types of cancer.⁶⁴

Based upon these considerations and available studies, the predictive value of *FCGR2A*, *FCGR3A*, *EGF* and *CCND1* genotyping should be investigated prospectively for cetuximab in cases and controls. For bevacizumab and panitumumab, hypothesis generating association studies, based upon the candidate genes in this review, are required for further research.

In any pharmacogenetic association study, confounders must be carefully corrected for, in order to find independent predictive factors. Factors that need to be taken into account are gender and race, as these can impact on the response to therapy. Moreover, allele frequencies are usually different among populations. Also, care should be taken to reduce the chance of false positive associations when testing multiple genotypes. This can be accomplished by adjusting the level of significance based upon the number of genotypes tested, for example with the Bonferroni correction.

Interestingly, there appears to be major interplay between these two pathways. For example, higher intratumoral VEGF levels were associated with resistance to single agent cetuximab in mCRC patients.⁶⁵ Moreover, the combination of cetuximab and irinotecan in mCRC patients reduced circulating VEGF levels, and of these patients with the most prominent decrease of VEGF responded better as indicated by TTP and OS compared with patients who showed only a modest reduction in VEGF levels.⁶⁶ Therefore, it makes sense to look at multiple SNPs and CNPs within both pathways simultaneously.

Finally, true usefulness of a predictive marker can only be assessed with the application of a validated predictive test in a prospective setting. The test should allocate different treatment options for patients with the genotype of interest, and solid endpoints should be investigated.

In conclusion, pharmacogenetics (including germ-line SNPs and CNPs) of EGFR and VEGF inhibitors will most likely find its way to daily clinical practice, provided that the above suggestions for future research have been met.

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3

Correlation between germline polymorphisms and the efficacy of cetuximab in metastatic colorectal cancer

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Adapted from: *European Journal of Cancer* 2010 Jul;46(10):1829-34

Abstract

Background

Previous studies indicated that germline polymorphisms in specific genes may predict efficacy and toxicity of cetuximab in metastatic colorectal cancer (mCRC) patients.

Methods

Germline DNA was isolated from 576 mCRC patients who were treated in the phase III CAIRO2 study with chemotherapy and bevacizumab alone or with cetuximab. Associations of epidermal growth factor (*EGF*) 61A>G, EGF receptor (*EGFR*) CA_{14-22'} cyclin D1 (*CCND1*) 932G>A, fragment-C gamma receptor (*FCGR*) 2A 535A>G and *FCGR3A* 818A>C polymorphisms with progression-free survival (PFS) were studied with regard to *KRAS* status.

Results

In the cetuximab arm, the *FCGR3A*818C-allele was associated with decreased PFS, both overall and in the *KRAS* wild-type subgroup (HR=1.56, 95%CI=1.14-2.15 and HR=1.57, 95%CI=1.06-2.34, respectively) and decreased incidence of grade 2-3 skin toxicity (OR=0.48, 95%CI=0.24-0.94). The *EGFR*≥20 genotype was associated with decreased PFS, both overall and in the *KRAS* wild-type subgroup (HR=1.60, 95%CI=1.17-2.19 and HR=1.58, 95%CI=1.06-2.35, respectively). The *FCGR3A* and *EGFR* polymorphisms were not associated with PFS in the no-cetuximab arm. In *KRAS* mutated patients, the *EGF*61G-allele was associated with decreased PFS in the cetuximab arm, and increased PFS in the no-cetuximab arm (HR=2.22, 95%CI=1.24-3.96 and HR=0.59, 95%CI=0.36-0.98, respectively).

Conclusion

EGFR, *FCGR3A* and *EGF* polymorphisms are associated with PFS in mCRC patients treated with cetuximab, bevacizumab and chemotherapy. Confirmation is needed before these markers could be applied clinically.

Introduction

Cetuximab is an IgG₁-type chimeric monoclonal antibody that targets the epidermal growth factor receptor (EGFR). Its principal mechanism of action is the inhibition of ligand induced EGFR activation, resulting in reduced cell proliferation, cell survival and angiogenesis. Also, cetuximab may induce antibody-dependent cell-mediated cytotoxicity (ADCC) by recruitment of immune effector cells.¹

Cetuximab is effective in patients with chemotherapy-refractory metastatic colorectal cancer (mCRC).^{2,3} A modest clinical benefit was shown for cetuximab when added to first-line chemotherapy.⁴⁻⁶ Recently, it has been demonstrated that the efficacy of cetuximab is limited to patients with wild-type *KRAS* tumors.^{7,8} However, the *KRAS* mutation status does not completely predict the response to cetuximab and other tumor characteristics such as *BRAF* mutation status have been investigated.^{9,10} The severity of acneiform skin rash is also associated with the efficacy of cetuximab^{2,3}, but as this adverse event occurs after therapy has started, it cannot be used to predict response before start of treatment. Therefore, additional predictive markers are needed to better identify patients who will benefit from cetuximab.

Germline polymorphisms in genes involved in the mechanism of action of cetuximab have been investigated previously.¹¹⁻¹⁴ A CA-repeat polymorphism in intron 1 of the *EGFR* gene and the single nucleotide polymorphisms (SNPs) *EGF* c.61A>G, cyclin D1 (*CCND1*) c.932G>A and fragment-C gamma receptors 2A (*FCGR2A*) c.535A>G and 3A (*FCGR3A*) c.818A>C have previously been associated with the efficacy of cetuximab in chemotherapy-refractory mCRC patients who were treated with cetuximab either as monotherapy^{11,12} or in combination with irinotecan.^{13,14} However, these findings have been investigated in relation to *KRAS* mutation status in only one small study.¹⁴ Furthermore, these former studies were hypothesis generating, and lacked a control group.

To provide more robust data, we investigated the associations of these germline polymorphisms in combination with *KRAS* mutation status with the efficacy of cetuximab in a large cohort of mCRC patients who were treated in first-line with capecitabine, oxaliplatin, bevacizumab and cetuximab and included a control group treated with the same regimen but without cetuximab.

Materials and methods

Study population

Blood samples were collected from 576 of 755 previously untreated mCRC patients who participated in a multicenter prospective, randomized phase III study and were treated with capecitabine, oxaliplatin and bevacizumab or the same regimen plus

cetuximab, the CAIRO2 study of the Dutch Colorectal Cancer Group (DCCG).^{15,16} Patient eligibility criteria are described in detail elsewhere.¹⁵ Patients were stratified according to prior adjuvant chemotherapy, serum LDH, number of affected organs and per institution. Membrane expression of EGFR in the tumor was not required.

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.

The collection of a peripheral blood sample for pharmacogenetic research was pre-specified in the study protocol and required additional written informed consent. The protocol was approved by the local institutional review boards of all participating centers.

Clinical evaluation and toxicity criteria

Progression-free survival (PFS) was calculated using tumor response assessments every three cycles by CT scan according to RECIST 1.0 criteria.¹⁵ PFS was defined as the interval from the date of randomization to the date of disease progression, death, or last follow-up, whichever occurred first. Toxicity was scored according to the National Cancer Institute Common Toxicity Criteria version 3.0. Cetuximab-related skin toxicity was defined as any skin toxicity with the exception of hand-foot syndrome.

Analysis of genetic variants

Germline DNA was isolated from peripheral white blood cells by the standard manual salting-out method. Genotyping was performed on a TaqMan 7500 (Applied Biosystems, Foster City, CA, USA) with pre-designed assays for *EGF* c.61A>G (rs4444903), *CCND1* c.932G>A (rs9344; also referred to as 870G>A), *FCGR2A* c.535A>G (rs1801274; resulting in amino-acid change of histidine to arginine at position 131) and *FCGR3A* c.818A>C (rs396991; resulting in amino-acid change of phenylalanine to valine at position 158), according to the manufacturer's protocol. Negative controls (water) were included. In addition, genotypes were confirmed on the Biomark (Fluidigm, South San Francisco, CA, USA) according to the protocol provided by the manufacturer using the same TaqMan assays. The *FCGR3A* polymorphism was also analyzed by Pyrosequencing for 15% of the samples, which confirmed the Taqman results.

The *EGFR* (CA)_n polymorphism was analyzed by fragment analysis. Briefly, 10 ng of DNA was PCR amplified using primers FAM-5'-CCAAAATATTAACCTGTCTT-3' and 5'-AACCAGGGACAGCAATCC-3'. PCR products were run on an ABI PRISM® 3730xl Analyzer and analyzed with Genemapper v3.5 software (Applied Biosystems). Plasmids with an *EGFR* insert containing 14 to 21 CA-repeats were used as a control.¹⁷ For the purpose of this analysis, the *EGFR* CA-repeat polymorphism was dichotomized

according to the criterion applied by Zhang and colleagues.¹¹ Patients with two alleles containing less than 20 CA-repeats were designated 'EGFR<20', whereas patients with either one or two alleles with 20 CA-repeats or more were designated as 'EGFR≥20'. All genotype frequencies were in Hardy-Weinberg equilibrium.

The *KRAS* mutation status was determined in patients from whom primary tumor tissue was available. Tumor DNA was extracted and *KRAS* mutation status was analyzed using a commercially available real-time PCR-based assay (DxS, Manchester, UK) and by direct sequencing.¹⁸

Statistical analysis

The primary objective was to assess the association of the *EGFR*, *EGF*, *CCND1*, *FCGR2A* and *FCGR3A* polymorphisms with PFS according to *KRAS* mutation status in mCRC patients treated with cetuximab added to chemotherapy and bevacizumab. The secondary objective was to assess the association between these polymorphisms and cetuximab-related skin toxicity (grade 0-1 versus 2-3).

The PFS of each polymorphism was analyzed per treatment arm. Survival curves were estimated using the Kaplan-Meier method. The hazard ratios and 95% confidence intervals (95%CI) were estimated using a multivariate Cox proportional hazards model per treatment arm, using the most appropriate of a dominant or recessive model. The effects of the genotypes were assessed with the wild-type genotype as the reference, as this is the most frequent and therefore 'normal' genotype. Since age (<65 versus ≥65 years) and gender potentially affect the influence of a genetic polymorphism¹⁹, these factors were included in the multivariate analysis in addition to serum LDH (normal versus abnormal), which was an independent prognostic factor in the CAIRO2 study.¹⁵ For the analysis of *KRAS* wild-type and mutant combined, *KRAS* mutation status was added to the multivariate model (wild-type versus mutant).

For patients in cetuximab arm, the association between the genotype and cetuximab-related skin toxicity (grades 0-1 versus 2-3) was analyzed and odds ratios (ORs) and 95%CI were estimated using a univariate logistic regression model.

A Predictive Score for PFS was generated by assessing the interaction between treatment arm and previously published baseline prognostic variables for mCRC in a multivariate Cox proportional hazards model. Baseline prognostic factors for PFS were identified from a Medline search for original articles on clinical trials of mCRC patients who were treated with first-line chemotherapy.²⁰⁻²⁵ Factors that were significantly associated (p<0.05) with PFS in a multivariate analysis including treatment arm were considered prognostic factor, and the cut-off values from these studies were used subsequently. Prognostic factors for OS were not included because these could also be related to subsequent lines of treatment. The resulting baseline prognostic variables were gender, age (<65 vs. ≥65 years), performance status (0 vs. 1), number of organs involved (1 vs. >1), LDH (normal vs. above normal), alkaline phosphatase

(normal vs. above normal), prior adjuvant chemotherapy (yes vs. no), white blood cell count (<8 000 vs. ≥8 000 cells per μL), hemoglobin (<11 vs. ≥ 11 g/dL) and total bilirubin (normal vs. above normal).²⁰⁻²⁵ Additionally, the interaction terms of treatment arm and *KRAS* mutation status and the polymorphisms in *CCND1*, *EGFR*, *EGF*, *FCGR2A* and *FCGR3A* were included. Using the resulting Cox proportional hazards model, the regression coefficients of the significant interaction terms were converted into a Partial Score analogous to the method used by Chow and colleagues.²⁶ By using the regression coefficients of the interaction term instead of the regression coefficient of the variable itself, correction took place for cetuximab-unrelated prognostic value of the variable. A Predictive Score for a given patient was obtained by the sum of the Partial Scores.

All statistical analyses were performed using the Statistical Analysis Software version 9.1 (SAS Inc. Bethesda, Maryland, USA).

Results

Study population

Germline DNA was obtained from 576 included patients, of which 564 received the allocated treatment (282 in each arm). The baseline clinical characteristics, *KRAS* mutation status, median PFS and OS, and the incidence of cetuximab-related skin toxicity of these patients were not statistically significant different from the 172 patients of whom no blood sample was available (Table 1).

Association with outcome in the cetuximab arm

Progression free survival

KRAS wild-type patients

In the cetuximab arm, patients who were carriers of the *FCGR3A* C-allele (AC and CC genotypes combined) had a significantly decreased PFS compared with patients with the *FCGR3A* AA genotype (median PFS, 8.2 versus 12.8 months, respectively; HR 1.57; 95%CI 1.06 to 2.34; P=.025, table 2). Patients in the cetuximab arm with the *EGFR*<20 genotype had significantly decreased PFS compared with patients with the *EGFR*≥20 genotype (median PFS, 7.6 versus 12.4 months, respectively; HR 1.58; 95%CI 1.06 to 2.35; P=.024, table 2). The other polymorphisms were not significantly associated with PFS.

KRAS mutant patients

In the cetuximab arm, patients who were carriers of the *EGF* G-allele (AG and GG genotypes combined) had a significantly decreased PFS compared with patients with the *EGF* AA genotype (median PFS, 7.4 versus 13.3 months, respectively; HR 2.22; 95%CI 1.24 to 3.96; P=.007, table 3). The other polymorphisms were not significantly associated with PFS.

Table 1 Baseline clinical and demographic characteristics and clinical outcome of metastatic colorectal cancer patients treated with first-line capecitabine, oxaliplatin and bevacizumab only (arm A) or with the addition of cetuximab (arm B)

	Patients included in the pharmacogenetic analysis		Patients with no DNA available		P value ¹	
	Arm A (n = 282)	Arm B (n = 282)	Arm A (n = 86)	Arm B (n = 86)		
Baseline characteristics						
Age – yr	Median Range	62 31–83	63 33–80	60.5 27–77	60.0 33–78	0.19
Gender – no. (%)	Male	159 (56%)	180 (64%)	46 (53%)	53 (62%)	0.55
	Female	123 (44%)	102 (36%)	40 (47%)	33 (38%)	
KRAS mutation status – no. (%)	KRAS wild-type	119 (42%)	127 (45%)	36 (42%)	32 (37%)	0.23
	KRAS mutant	83 (29%)	69 (24%)	25 (29%)	29 (34%)	
	Missing	80 (28%)	86 (30%)	25 (29%)	25 (29%)	
Outcome parameters						
Progression-free survival – months	Median	10.8	10.1	9.9	7.9	0.09
	95% CI	9.7–12.5	8.6–11.0	8.4–13.4	6.7–10.5	
Overall survival – months	Median	20.4	20.5	17.9	15.6	0.08
	95% CI	17.8–24.9	18.7–22.1	15.6–31.1	11.7–20.3	
Overall worst grade cetuximab-related skin toxicity – no. (%)	grade 0 – 2	280 (99%)	207 (73%)	86 (100%)	67 (78%)	0.39
	grade 3	2 (1%)	75 (27%)	0 (0%)	19 (22%)	

¹ Comparison between patients included in the pharmacogenetic analysis versus patients not included in the pharmacogenetic analysis.

Table 2 Analysis of progression-free survival in KRAS wild-type patients

	Arm A ¹				Arm B ²			
	n	median PFS ³	HR ⁴	95% CI	P	n	median PFS ³	P
FCGR3A 818A>C								
AA	55	11.7	1.00			57	12.8	1.00
AC or CC	63	10.1	1.05	0.69-1.58	.832	65	8.2	1.57
								1.06-2.34
								.025
FCGR2A 535A>G								
AA	33	9.4	1.00			34	12.6	1.00
AG or GG	84	12.7	0.69	0.44-1.09	.111	92	10.0	1.50
								0.96-2.36
								.076
EGFR CA-repeat								
EGFR<20	54	10.7	1.00			72	12.4	1.00
EGFR≥20	63	11.7	1.10	0.73-1.67	.649	51	7.6	1.58
								1.06-2.35
								.024
EGF 61A>G								
AA	51	10.1	1.00			50	10.8	1.00
AG or GG	66	12.5	0.67	0.44-1.03	.067	70	10.3	1.00
								0.67-1.49
								.999
CCND1 870G>A								
GG	39	9.6	1.00			33	12.7	1.00
GA	54	9.7	0.93	0.57-1.50	.752	58	9.6	1.14
AA	26	15.1	0.67	0.36-1.23	.193	34	9.9	1.53
								0.91-2.57
								.112

¹ arm A: capecitabine, oxaliplatin and bevacizumab
² arm B: capecitabine, oxaliplatin, bevacizumab and cetuximab
³ median progression free survival (PFS) in months
⁴ Covariates included in the multivariate model were age, gender and serum LDH. Hazard ratios (HR), 95% confidence intervals (95% CI) and P values were calculated from the Cox proportional hazards model, with the wild-type genotype as the reference.
Abbreviations: CCND1, cyclin-D1; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; FCGR, immunoglobulin-G fragment C receptor.

Table 3 Analysis of progression-free survival in KRAS mutant patients

	Arm A ¹				Arm B ²			
	n	median PFS ³	HR ⁴	95% CI	P	n	median PFS ³	P
FCGR3A 818A>C								
AA	29	11.5	1.00			25	10.4	1.00
AC or CC	52	12.5	0.77	0.46-1.28	.311	42	7.9	1.61
								0.92-2.82
								.097
FCGR2A 535A>G								
AA	22	12.9	1.00			23	8.1	1.00
AG or GG	60	12.5	0.99	0.57-1.73	.985	46	9.7	1.32
								0.74-2.33
								.346
EGFR CA-repeat								
EGFR<20	42	10.8	1.00			29	12.1	1.00
EGFR≥20	40	12.7	0.63	0.38-1.03	.068	38	7.4	1.71
								0.98-2.98
								.060
EGF 61A>G								
AA	35	10.6	1.00			29	13.3	1.00
AG or GG	46	13.6	0.59	0.36-0.98	.041	39	7.4	2.22
								1.24-3.96
								.007
CCND1 870G>A								
GG	23	12.1	1.00			21	7.4	1.00
GA	40	11.5	0.93	0.52-1.68	.816	36	9.5	1.07
AA	20	13.1	0.76	0.40-1.46	.413	12	7.1	1.33
								0.58-1.99
								.821
								0.54-3.27
								.528

¹ arm A: capecitabine, oxaliplatin and bevacizumab
² arm B: capecitabine, oxaliplatin, bevacizumab and cetuximab
³ median progression free survival (PFS) in months
⁴ Covariates included in the multivariate model were age, gender and serum LDH. Hazard ratios (HR), 95% confidence intervals (95% CI) and P values were calculated from the Cox proportional hazards model, with the wild-type genotype as the reference.
Abbreviations: CCND1, cyclin-D1; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; FCGR, immunoglobulin-G fragment C receptor.

KRAS wild-type and mutant combined

When the associations were assessed in the entire cohort without subdivision by *KRAS* mutation status but with *KRAS* mutation status as a covariate, patients who were carriers of the *FCGR3A* C-allele had a significantly decreased PFS compared with patients with the *FCGR3A* AA genotype (median PFS, 7.8 versus 12.1 months, respectively; HR 1.56; 95%CI 1.14 to 2.15; P=.006, table 4). Also, patients with the *EGFR*<20 genotype had a significantly decreased PFS compared with patients with the *EGFR*≥20 genotype (median PFS, 8.8 versus 10.8 months, respectively; HR 1.60; 95%CI 1.17 to 2.19; P=.003, table 4). The other polymorphisms were not significantly associated with PFS. *KRAS* mutation status was not significantly associated with PFS in the multivariate analyses. There was significant interaction between treatment arm and the *FCGR3A* and *EGFR* polymorphisms (P=.015 and P=.009, respectively). In figure 1A and 1B, the PFS curves for the cetuximab arm are shown for *KRAS* mutation status combined with the *EGFR* and *FCGR3A* polymorphisms, respectively.

Cetuximab-related skin toxicity

In the overall cetuximab arm (i.e. not subdivided by *KRAS* mutation status), patients who were carriers of the *FCGR3A* C-allele had significantly decreased incidence of grade 2-3 cetuximab related skin toxicity compared with patients with the *FCGR3A* AA genotype (OR, 0.46; 95%CI 0.27 to 0.78; table 5). In the multivariate analysis including age, gender, *KRAS* mutation, and serum LDH, the *FCGR3A* polymorphism remained associated with the incidence of grade 2-3 skin toxicity (OR, 0.48; 95%CI 0.24 to 0.94). The other polymorphisms were not significantly associated with cetuximab related skin toxicity.

Association with outcome in the no-cetuximab arm

In the no-cetuximab arm, *KRAS* mutant patients who were carriers of the *EGF* G-allele had significantly increased PFS compared with patients with the *EGF* AA genotype (median PFS, 13.6 versus 10.6 months, respectively; HR 0.59; 95%CI 0.36 to 0.98; P=.041, table 3). The other polymorphisms were not significantly associated with PFS.

Predictive Score for PFS

The variables that showed significant interaction with treatment for the prediction of PFS were: gender (regression coefficient, 0.56), white blood cell count (WBC <8 000 vs. ≥8 000 cells per µL; regression coefficient, 0.44) and the *FCGR3A* polymorphism (AA genotype versus C-allele carriers; regression coefficient, 0.58). *KRAS* mutation status showed no significant interaction with treatment arm (regression coefficient, 0.06). A score of one point was awarded to each of the following parameters: females, *FCGR3A* C-allele carriers and patients with ≥8 000 WBCs per µL. By summarizing the Partial Scores, a Predictive Score per patient was derived, which ranged from 0 to 3. The 32

Table 4 Analysis of progression-free survival in all patients, regardless of KRAS mutation status

Table 1. Clinical outcomes in patients with EGFR exon 19 deletion or exon 21 L858R mutation, stratified by treatment group and biomarker status.											
Treatment Group	Arm A ¹				Arm B ²				P	95% CI	P
	n	median PFS ³	HR ⁴	95% CI	n	median PFS ³	HR ⁴	95% CI			
FCGR3A 818A>C	AA	119	10.7	1.00		112	12.1	1.00			
	AC or CC	157	10.8	0.92	0.67-1.27	158	7.8	1.56	1.14-2.15	.006	
FCGR2A 535A>G	AA	73	10.8	1.00		84	9.8	1.00			
	AG or GG	204	11.3	0.81	0.57-1.14	193	10.2	1.40	0.98-1.98	.062	
EGFR CA-repeat	EGFR<20	143	10.7	1.00		141	10.8	1.00			
	EGFR≥20	134	11.7	0.88	0.65-1.21	129	8.8	1.60	1.17-2.19	.003	
EGF 61A>G	AA	117	10.1	1.00		111	10.2	1.00			
	AG or GG	161	12.5	0.65	0.47-0.89	160	10.1	1.26	0.91-1.74	.158	
CCND1 870G>A	GG	83	9.7	1.00		80	10.4	1.00			
	GA	137	10.6	0.91	0.63-1.31	138	9.7	1.14	0.79-1.66	.485	
	AA	62	13.5	0.69	0.44-1.06	62	9.7	1.52	0.99-2.33	.056	

¹ arm A: capecitabine, oxaliplatin and bevacizumab
² arm B: capecitabine, oxaliplatin, bevacizumab and cetuximab
³ median progression free survival (PFS) in months
⁴ Covariates included in the multivariate model were age, gender, serum LDH and *KRAS* mutation status. Hazard ratios (HR), 95% confidence intervals (95% CI) and P values were calculated from the Cox proportional hazards model, with the wild-type genotype as the reference.
Abbreviations: CCND1, cyclin-D1; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; FCGR, immunoglobulin-G fragment C receptor.

Figure 1A Progression-free survival for the *EGFR* CA-repeat polymorphism and *KRAS* mutation status for patients with metastatic colorectal cancer treated with first-line capecitabine, oxaliplatin, bevacizumab and cetuximab

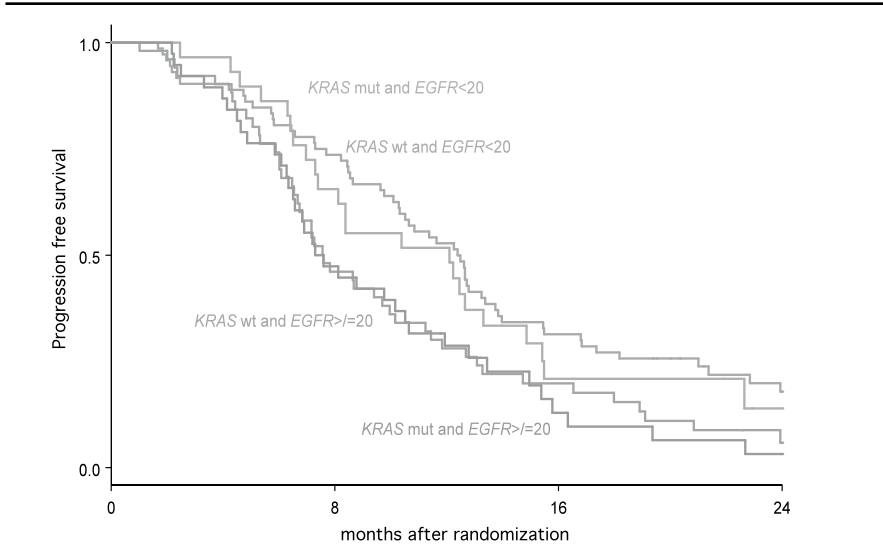
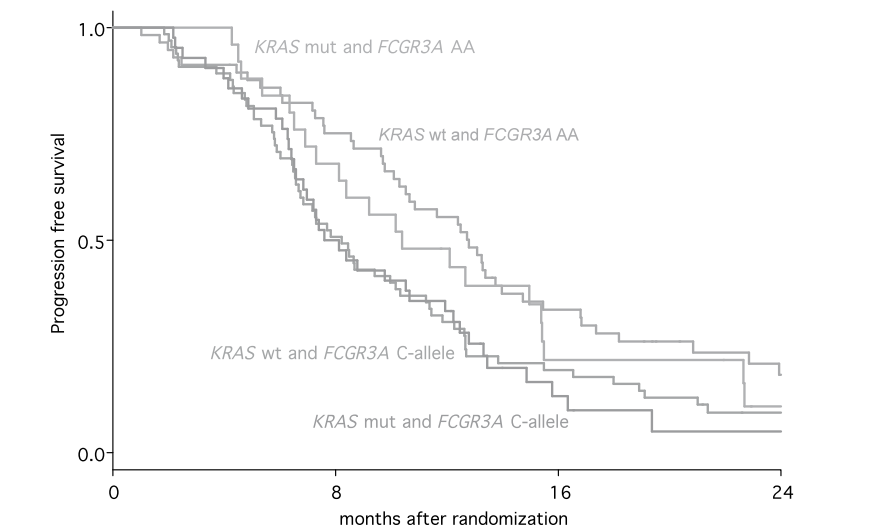


Figure 1B Progression-free survival for the *FCGR3A* 818A>C polymorphism and *KRAS* mutation status for patients with metastatic colorectal cancer treated with first-line capecitabine, oxaliplatin, bevacizumab and cetuximab



patients with a Predictive Score of 0 in the cetuximab arm had significantly improved PFS compared with all patients in the no-cetuximab arm (median PFS 15.4 versus 10.8 months, respectively; HR 0.61; 95%CI 0.39 to 0.95). Grouping of patients with a Predictive Score of 0 and 1 (a total of 142 patients) led to a non-significant improvement of PFS for the cetuximab arm compared with the no-cetuximab arm (HR 0.83; 95%CI 0.66 to 1.05).

Table 5 Analysis of the incidence of grade 2-3 cetuximab-related skin toxicity for patients with metastatic colorectal cancer treated with first-line capecitabine, oxaliplatin, bevacizumab and cetuximab

	OR ¹	95%CI	P
<i>FCGR3A</i> 818A>C			
AA	1.00		
AC or CC	0.46	0.27 to 0.78	.005
<i>FCGR2A</i> 535A>G			
AA	1.00		
AG or GG	1.66	0.97 to 2.84	.062
<i>EGFR</i> CA-repeat			
<i>EGFR</i> <20	1.00		
<i>EGFR</i> ≥20	1.11	0.67 to 1.84	.693
<i>EGF</i> 61A>G			
AA	1.00		
AG or GG	1.28	0.76 to 2.13	.351
<i>CCND1</i> 870G>A			
GG	1.00		
GA	1.21	0.67 to 2.17	.535
AA	0.86	0.43 to 1.73	.679

¹Odds ratios (OR), 95% confidence intervals (95% CI) and P values were calculated from the logistic regression model, with the wild-type genotype as the reference.
Abbreviations: CCND1, cyclin-D1; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; FCGR, immunoglobulin-G fragment C receptor

Discussion

We demonstrate that the *FCGR3A* 818C-allele and the *EGFR*≥20 genotype were associated with a decreased PFS in a large group of *KRAS* wild-type mCRC patients treated with cetuximab, bevacizumab and chemotherapy in a randomized trial, compared with patients with the *FCGR3A* 818AA or *EGFR*<20 genotype, respectively. Moreover, the predictive role of these polymorphisms appears to be independent of *KRAS* mutation status. *KRAS* mutant patients who carried the *EGF* 61G-allele had shorter PFS when treated with cetuximab, bevacizumab and chemotherapy, and longer PFS when treated with bevacizumab and chemotherapy alone, compared with patients with the *EGF* 61AA genotype. Patients who carried the *FCGR3A* 818C-allele had decreased risk of cetuximab related skin toxicity, compared with patients with the *FCGR3A* 818AA genotype.

Bibeau and colleagues recently also reported that the *FCGR3A* polymorphism is independent of *KRAS* mutation status. However, in their study patients who were homozygous for the C-allele had longer PFS compared with carriers of the A-allele¹⁴, which is not in agreement with our data. In one other previous study, the *FCGR3A* C-allele was also associated with decreased PFS in previously pretreated mCRC patients who were treated with cetuximab as a single agent¹², though this was not confirmed in an extended analysis of this study with more patients.²⁷ This indicates that the earlier association could have been a false positive finding, making it not suitable for comparison with our study. Another study with 110 patients who received cetuximab monotherapy as salvage treatment for mCRC did also not find a significant association between the *FCGR3A* polymorphism and the efficacy of cetuximab.¹³

A possible mechanism for the opposite association of the *FCGR3A* polymorphism could be that the high affinity C-allele²⁸⁻³⁰ results in increased activation of tumor associated macrophages (TAMs) by cetuximab through cross-linking of the Fc gamma receptor³¹, instead of increasing ADCC in our study. As a result of TAM activation, pro-angiogenic mediators are released in the tumor microenvironment, such as VEGF and matrix metalloproteinases (MMPs).^{32,33} In our study, patients had not received palliative chemotherapy before, whereas patients in the other studies had been exposed to irinotecan and/or other lines of chemotherapy prior to cetuximab^{12-14,27}, which could have altered the infiltration of cells of the myeloid lineage, such as TAMs.³⁴ However, it must be noted that the *FCGR3A* C-allele was associated with increased efficacy of the IgG₁-type monoclonal antibodies rituximab in lymphoma^{35,36} and trastuzumab in advanced breast cancer.³⁷ Therefore, fundamental research should be performed to support our highly speculative hypothesis. However, because this is an extracellular mechanism, and therefore independent of intracellular *KRAS* signaling, it explains why the effect of the *FCGR3A* polymorphism was independent of *KRAS* mutation status.

Our finding that patients with a lower number of CA-repeats for the *EGFR* polymorphism experience longer PFS is in line with the study by Graziano and colleagues¹³, even though the categorization of genotypes was different. However, another study did not find a significant association between this *EGFR* polymorphism and PFS in cetuximab treated mCRC patients²⁷.

The biological mechanism for the association of the *EGFR* polymorphism is concordant with the finding that patients with the *EGFR*≥20 genotype had shorter PFS. Transcription of the *EGFR* gene is lower for increased number of CA-repeats³⁸. Although *EGFR* expression, as measured by immunohistochemistry, is not a predictor of the efficacy of cetuximab,^{39,40} the number of *EGFR* gene copies is associated with the response to cetuximab treatment⁴¹.

It would be expected that the *EGFR* CA-repeat polymorphism is only associated with PFS in *KRAS* wild-type patients, because *EGFR* is upstream of *KRAS*. Since VEGF expression is regulated by the *EGFR* pathway, a role of the *EGFR* polymorphism in the response to cetuximab in combination with bevacizumab cannot be excluded.

In our study, patients who carried the *EGF* G-allele had increased PFS. In two other studies, the G-allele was associated with decreased PFS in advanced colorectal cancer patients treated with cetuximab^{13,27}.

Skin toxicity is a major side effect of cetuximab treatment and the severity of skin toxicity is associated with the response to cetuximab,^{5,42} but the underlying mechanism is not yet unraveled. Since we demonstrate a relationship between the *FCGR3A* polymorphism with the incidence of grade 2-3 skin toxicity, the involvement of immune effector cells is likely. Unexpectedly, we did not confirm previous findings that a lower number of CA-repeats is associated with increased incidence of skin toxicity during anti-*EGFR* therapy.^{13,43} However, previous findings could have been biased by the correlation between the response to anti-*EGFR* therapy and the incidence of skin toxicity.

Even though the previous pharmacogenetic studies on cetuximab have used peripheral blood^{12,13}, normal tissue¹⁴ or tumor tissue²⁷, this should not have influenced the results, because there is an almost perfect degree of concordance between germline genotype in tumor and normal tissue.⁴⁴

Importantly, the polymorphisms in *FCGR3A* and *EGFR* are only predictive for the efficacy of cetuximab and do not influence the PFS in patients not treated with cetuximab.

Biomarker- and genetic association studies are hampered by divergent and inconsistent results.⁴⁵ Retrospective pharmacogenetic studies must therefore be interpreted as hypothesis generating that require confirmation in an independent cohort.

Although our large study was set up to confirm previously published associations and included a control group¹¹⁻¹⁴, the results are conflicting and therefore remain inconclusive. It is likely that heterogeneity among the different studies, such as the

stage and nature of the disease, previous treatment and concomitant medication may explain the discordance. These variables should therefore be carefully considered in retrospective biomarker studies, as these factors probably have large influence on the results.

Apart from rare examples such as *KRAS*, a biomarker usually identifies subsets of patients with relatively higher or lower risk of response. Therefore, a predictive model should be developed with genetic and other predictive factors, such as the model we present. Such models can be clinically applied only after confirmation in a prospective trial.^{45,46}

In conclusion, we demonstrate that germline polymorphisms in *FCGR3A*, *EGFR* and *EGF* are associated with the efficacy of cetuximab. Due to inconsistent results among studies, our results require confirmation before they can be applied in clinical practice.

Acknowledgement

We thank S.H. van der Burg and T. van Hall for their contribution to the discussion.

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4

Activation of tumor-promoting type 2 macrophages by the EGFR-targeting antibody cetuximab

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Submitted

Abstract

In a recent randomized phase III clinical trial in metastatic colorectal cancer patients, the addition of the anti-epidermal growth factor receptor (EGFR) monoclonal antibody (MAB) cetuximab to bevacizumab and chemotherapy resulted in decreased efficacy. In order to explain this unexpected clinical observation, we undertook the current hypothesis driven study. Antibody-dependent cell-mediated cytotoxicity (ADCC) by natural killer (NK) cells is generally proposed as one of the anti-tumor mechanisms of MABs. However, we found that CD163-positive, type 2 macrophages (M2s) are much more abundant in colorectal carcinomas. *In vitro* analysis of M2 macrophages revealed high levels of Fc-gamma receptors (FcγRs) and PD-L1, and production of IL-10 and VEGF, but not IL-12. These anti-inflammatory and tumor-promoting mediators were released upon co-culture with EGFR-positive tumor cells that were pulsed with low concentrations of cetuximab. Macrophage activation depended on EGFR expression on the tumor cells, FcγRs, target specificity of the MAB and mobility of antibody complexes. Cetuximab-mediated macrophage responses seemed more pronounced for *FCGR3A* 158-valine carriers, which is the high affinity variant. These results suggest that tumor-promoting type 2 macrophages are activated by this therapeutic MAB in the local tumor microenvironment and argue that this immune mechanism should be taken into account for the application of therapeutic antibodies.

Introduction

Monoclonal antibodies (MABs) have become important agents for the treatment of many types of malignancies. Generally, their principal mechanism of action is blocking growth factor pathways that are essential for tumor growth and progression. So far, all clinically applied MABs contain the Fc region of human IgG, which efficiently mediates activation through Fcγ receptors (FcγRs) on several types of immune cells. These receptors actually contribute to the clinical effect of MABs, in addition to their direct inhibition on tumor growth.¹ The role of immune activation is corroborated by several studies describing an association between the rs396991 polymorphism (*FCGR3A* 158Phe→Val) in the gene encoding FcγRIIIA (also known as CD16), and clinical outcome after treatment with the therapeutic MABs rituximab (anti-CD20), trastuzumab (anti-HER2/*neu*) and cetuximab (anti-epidermal growth factor receptor, EGFR).²⁻⁴ The high affinity valine allele has been associated with increased clinical response and survival in these studies, which is in line with *in vitro* studies indicating that antibody-dependent cellular cytotoxicity (ADCC) is more extensive for this allele.⁵

Recently, the addition of cetuximab to bevacizumab plus chemotherapy resulted in decreased progression-free survival in a large clinical trial in metastatic colorectal cancer (CAIRO2 study)⁶, which was especially significant for patients carrying the high affinity valine allele.⁷ Possibly, the detrimental effect of cetuximab is a consequence of activation of tumor promoting immune cells, rather than triggering tumoricidal ADCC. Tumor-associated macrophages are characterized as M2-type cells and are known to possess anti-inflammatory, pro-angiogenic and tumor-promoting properties.⁸ Recent literature even implies an active involvement of FcγRs and myeloid cells in carcinoma development.⁹ We therefore hypothesized that therapeutic antibodies, such as cetuximab, might locally cross-link FcγRs on intratumoral M2-type macrophages by EGFR-binding on tumor cells, resulting in activation and release of tumor-promoting mediators. These molecular interactions might explain the negative effect of the addition of cetuximab to bevacizumab and chemotherapy in the CAIRO2 trial.

Materials and methods

Immunohistochemical stainings

Formalin fixed and paraffin embedded tumors from ten untreated stage III colorectal cancer patients were selected from the pathology archive of the Leiden University Medical Center. Staining for NK cells and M2 macrophages was described before.¹⁰

Cell-cultures

Colorectal adenocarcinoma cell lines LoVo and HCT-15 were kindly provided by Dr. van Wezel (Leiden University Medical Center, the Netherlands) and epidermoid skin cancer line A431 was obtained from ATCC (Manassas, VA, USA). M2-type macrophages and dendritic cells (DCs) were differentiated from purified CD14⁺ monocytes (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany) and differentiated as previously described¹⁰, using M-CSF (R&D, Minneapolis, USA) or GM-CSF (Invitrogen) with IL-4 (Invitrogen). Cells were activated by 250 ng/ml LPS (Sigma-Aldrich, St. Louis, USA) or tumor cells with MAbs cetuximab (Erbix[®], Merck, Darmstadt, Germany), rituximab or bevacizumab (Mabthera[®] and Avastin[®], respectively; Roche, Welwyn Garden City, UK).

Experimental conditions

At day 6 of the monocyte differentiation cultures, tumor cell lines were plated in a density of 50.000 cells per well in 48-wells plates. After 2 h, 250 ng/ml LPS or MAbs were added together with M2 macrophages in a density of 100.000 cells per well. After 24 h, supernatants were collected and analyzed for IL-10 (Sanquin, Amsterdam, Netherlands), IL-8, VEGF (eBioscience, Vienna, Austria) and IL-12p70 (BD-Biosciences, Minneapolis, MN, USA) production. Macrophages were removed from the culture plates by scraping and stained with monoclonal antibodies (all purchased from BD-Biosciences, except for anti- PD1-L from eBioscience). Samples were recorded using a FACS Calibur with Cellquest software (BD-Biosciences). Data were analyzed with FlowJo software (Tree star, Ashland, USA). Macrophages were separated from tumor cell-lines by gating for HLA-DR.

Genotyping

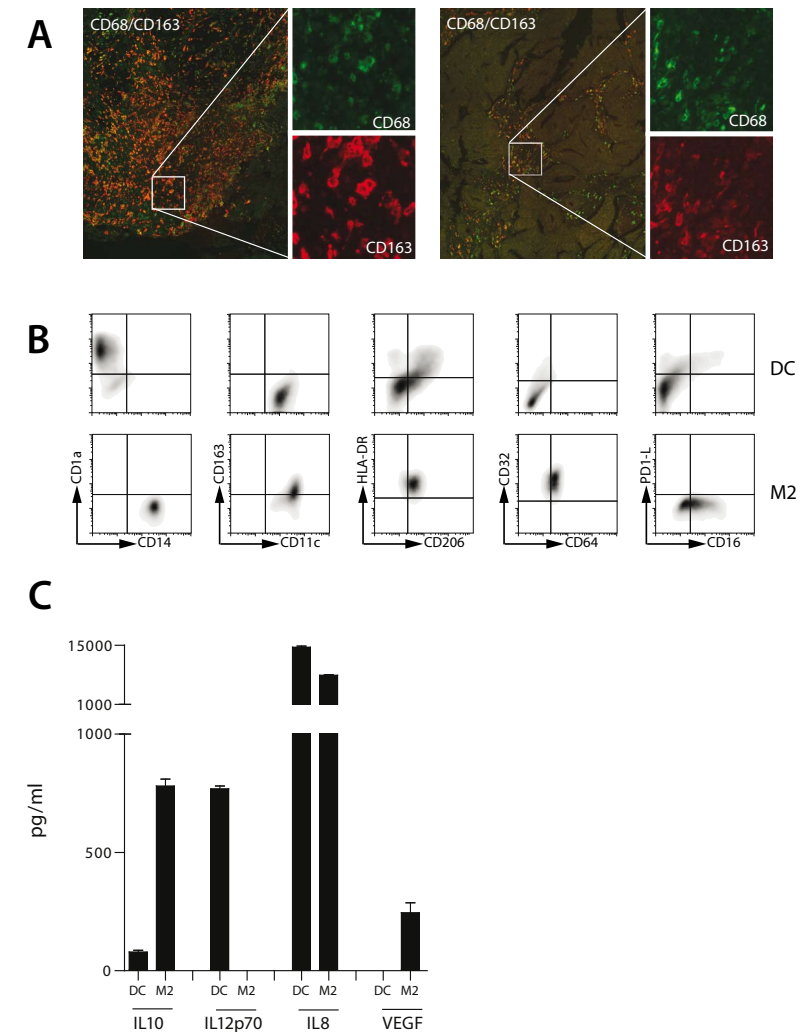
Genomic DNA was isolated from monocytes with MagnaPure Compact (Roche, Almere, Netherlands) and genotyping for *FCGR3A* c.818A>C (C_25815666_10; rs396991) was performed as previously described.⁷

Results

Colon carcinomas are heavily infiltrated with type 2 macrophages, but not with NK cells

To investigate immune cell infiltration of primary stage III colorectal cancers, we stained ten tumors for the common macrophage marker CD68, and the scavenger receptor CD163, which is typically expressed by M2-type cells. All colorectal tumors were extensively infiltrated with this type of macrophages (figure 1a). By contrast, hardly any NK cells were observed using the NK-lineage specific receptor NKp46. We thus envisage that cetuximab treatment might impact on these infiltrating macrophages, and that local ADCC via NK cells plays a minor role.

Figure 1 Detection and characterization of M2 macrophages in colorectal cancer



A, Two representative examples of two color immunofluorescent staining of stage III colorectal cancers with high (left panel) and low (right panel) infiltration of CD68+ (green) macrophages expressing the typical type 2 marker CD163+ (red). **B**, Flow cytometry analysis of M2 macrophages and monocyte-derived dendritic cells (mDC). Fcγ receptors are indicated by the CD nomenclature: FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16). **C**, Production of IL-10, IL-12p70, IL-8 and VEGF by mDCs and M2 macrophages after overnight stimulation with LPS

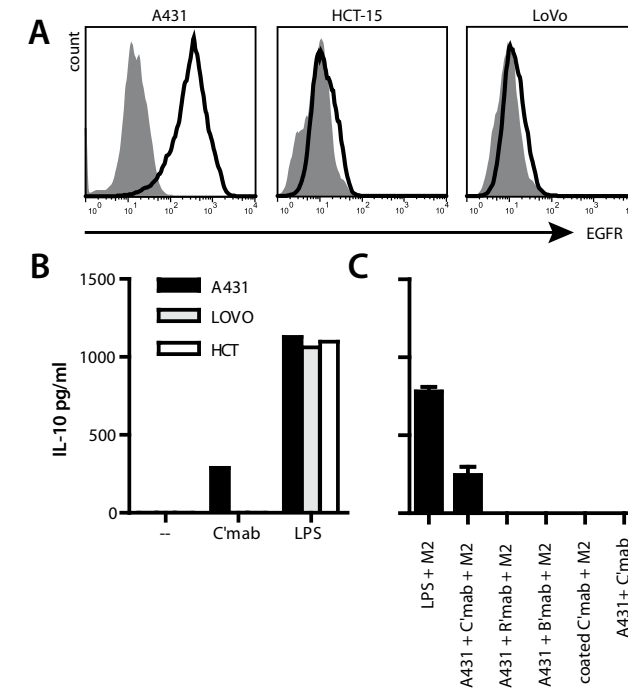
The influence of cetuximab on macrophages was studied on freshly isolated monocytes that were differentiated *in vitro* into CD1a⁺CD14⁺CD163⁺ macrophages.¹⁰ The expression of Fcγ receptors FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) and release of cytokines after activation by the strong TLR stimulus LPS was examined (figure 1b, c). M2 macrophages strongly displayed all three Fc-binding receptors and produced high amounts of the anti-inflammatory IL-10, as well as IL-8 and the pro-angiogenic VEGF, but not the immunostimulatory IL-12. Control monocyte-derived DCs displayed an opposite profile, which is in line with their T-cell stimulating function. These data strongly suggested that M2-type macrophages could potentially be stimulated by MAbs to exert an anti-inflammatory and pro-angiogenic role in the tumor microenvironment.

Cetuximab induces activation of type 2 macrophages

M2 macrophages were then activated by cetuximab in the presence of tumor cells. Three tumor lines were used (A431, LoVo and HCT-15) and flow cytometry analysis showed that A431 highly expressed EGFR, whereas EGFR staining of LoVo and HCT-15 was much lower (figure 2a). Importantly, co-culture of macrophages with cetuximab-opsonized A431 tumor cells resulted in production of IL-10 and IL-8, whereas EGFR-low tumors LoVo and HCT-15 did not activate macrophages (figure 2b, figure S1). Notably, the release of IL-8 upon cetuximab treatment exceeded that of the positive control LPS (figure S1). The A431 tumor cells spontaneously produced VEGF, so this mediator could not be used in succeeding experiments to determine macrophage activation. To corroborate the notion that macrophage activation was the result of cross-linking Fcγ receptors, we incubated tumor cells with MAbs specific for the non-expressed CD20 (rituximab), or the soluble VEGF (bevacizumab), both containing the same IgG₁ isotype. This did not lead to IL-10 production (figure 2c). Furthermore, competition of Fc-binding by high concentrations of rituximab resulted in a dose-dependent decrease of IL-10 production (figure S2). Interestingly, IL-10 was also not detected when cetuximab was coated on culture plates (figure 2c), suggesting that the molecular interaction of EGFR-cetuximab-FcγR required the flexibility of fluid membranes for proper cross-linking.

The cetuximab mediated activation of M2 macrophages was dose-dependent (figure 3) and concentrations as low as 10 ng/ml were sufficient to down-regulate cell surface levels of CD16 and to up-regulate the inhibitory molecule PD1-L (figure 3a) and to release IL-10 and IL-8 (figure 3b, figure S1). These data showed that very low concentrations cetuximab induced the release of anti-inflammatory mediators from M2 macrophages through cross-linking of Fcγ receptors.

Figure 2 FcγR cross-linking by cetuximab activates M2 macrophages

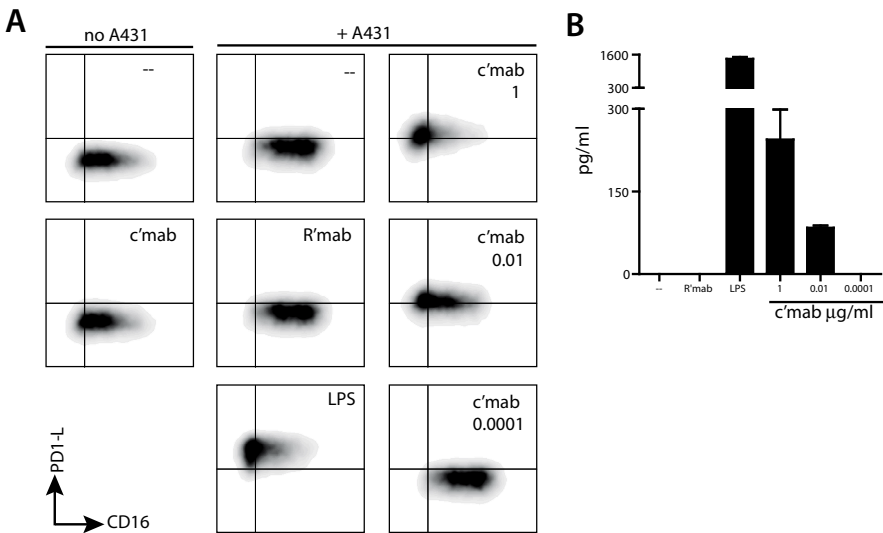


A, Flow cytometry analysis of EGFR expression on tumor cell lines A431, HCT-15 and LoVo. Filled histograms represent isotype control antibody, solid lines indicate staining with anti-EGFR antibody. **B**, IL-10 production by M2 macrophages upon co-culture with tumor cells and 1 µg/ml cetuximab (c'mab). Addition of LPS (250 ng/ml) served as positive control. **C**, IL-10 production by macrophages depends on interaction of macrophages and cetuximab-pulsed A431 tumor cells. Plate-bound cetuximab (10 µg/ml) ('coated') could not replace the EGFR-expressing tumor cells and control antibodies rituximab (r'mab, 1 µg/ml) or bevacizumab (b'mab, 1 µg/ml) could not replace cetuximab.

Effect of FCGR3A polymorphism

Addition of cetuximab to bevacizumab and chemotherapy in the CAIRO2 trial decreased the progression-free survival of metastatic colorectal cancer patients, especially for those with high affinity FcγRIII genotype encoding the valine residue.^{6,7} We examined the influence of this polymorphism on the degree of M2 macrophage activation by cetuximab using 22 healthy donors, consisting of 12 homozygous 158-Phe and ten 158-Val carriers (figure 4). Analysis of IL-10 release and CD16 down-

Figure 3 Degree of M2 macrophage activation depends on cetuximab concentration



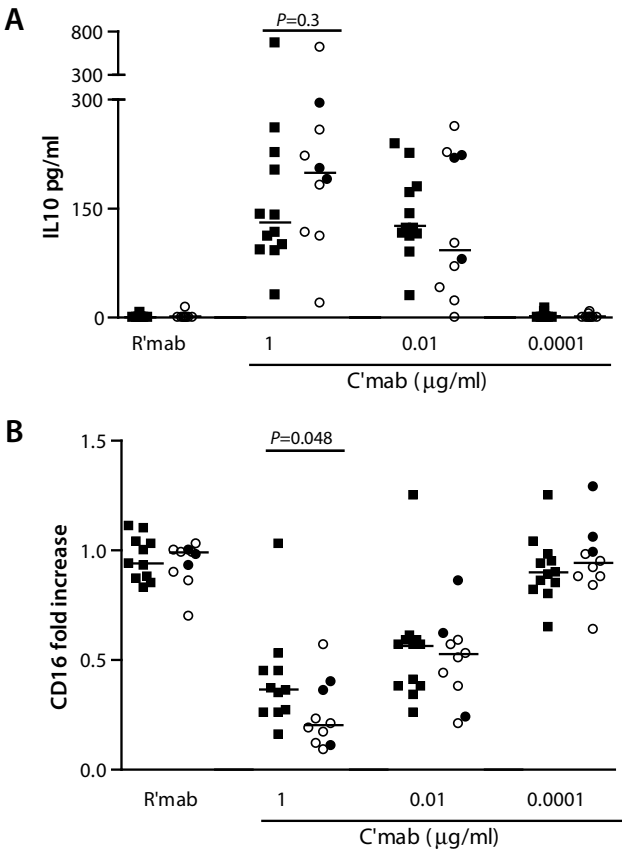
A, Flow cytometry analysis of PD1-L up-regulation and CD16 down-regulation on M2 macrophages upon co-culture with EGFR-positive A431 tumor cells and different cetuximab (c'mab) concentrations. Rituximab (r'mab) served as negative control, LPS as positive control. **B**, Macrophage-derived IL-10 was measured in overnight supernatants.

regulation on M2 macrophages showed an apparent stronger activation of cells with the high affinity valine allele (figure 4). These differences did not reach statistical significance for cytokine release, most likely due to high variation within the groups and very high production (figure 4b and figure S1, respectively). In conclusion, our data show that cetuximab can induce the release of anti-inflammatory mediators from M2 macrophages and that this effect might explain the negative clinical effect of this MAb in the recent CAIRO2 study.

Discussion

Our data show that type 2 macrophages are abundantly present in colon carcinoma and are activated by cetuximab-opsonized tumor cells, resulting in anti-inflammatory and tumor promoting mediators, including IL-10 and VEGF. M2 macrophages are

Figure 4 Activation difference of *FCGR3A* (CD16)-typed M2 macrophages



M2 macrophages of twelve homozygous 158-Phe (solid squares) and ten 158-Valine allele carriers (open circles are heterozygous and solid circles are homozygous) were activated by cetuximab (c'mab) and EGFR-positive A431 tumor cells. **A**, CD16 down-regulation and **B**, IL-10 release were measured. Lines represent median values and differences between the two groups at 1 µg/ml cetuximab were calculated by Mann Whitney test.

known to actively contribute to tumor growth via angiogenesis and immune suppression.⁸ Previous research on the immune mechanisms of therapeutic MAbs has focused on anti-tumor effects such as ADCC or phagocytosis. ADCC mediated by NK cells or PBMCs has been described for cetuximab⁵, however, staining for NK cells in

stage III colorectal carcinoma revealed that these cells are rare in colorectal cancers. Fcγ receptors are also expressed by macrophages and these cells were abundantly present in this tumor type (figure 1). M2 macrophages are efficient in phagocytosis of rituximab-opsonized B cells¹¹, but we question the relevance of this FcγR-mediated process for solid tumors like colorectal carcinoma. We here report that cetuximab-mediated cross-linking of Fcγ receptors leads to release of tumor-promoting mediators.

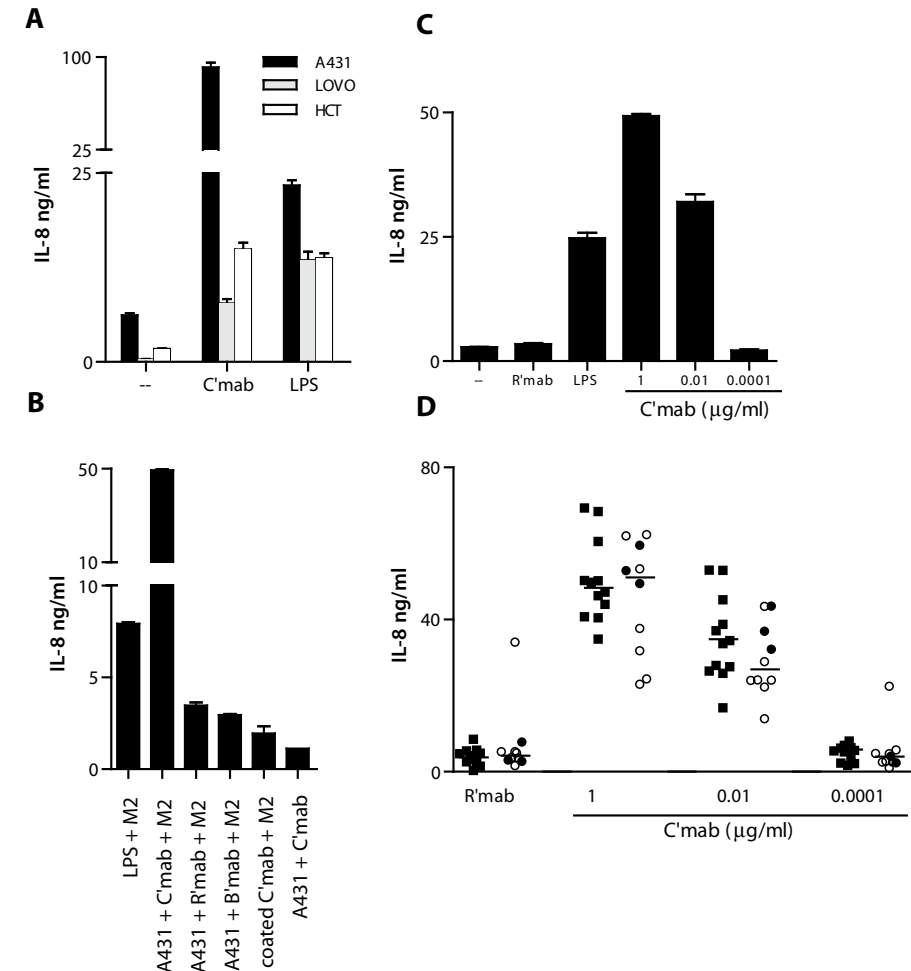
The detrimental effect of cetuximab addition in the CAIRO2 trial was unanticipated⁶, since the combination of cetuximab and anti-VEGF therapy appeared effective in mouse models.¹²⁻¹⁴ However, the Fcγ receptor-mediated effects by cetuximab could not be evaluated in these models, as the human Fc-region of cetuximab does not interact with the murine FcγRs. Our results indicate that the release of multiple anti-inflammatory and pro-angiogenic mediators by M2 macrophages could account for the decreased therapy efficacy for those patients that were treated with the combination of cetuximab, the anti-VEGF MAb bevacizumab and chemotherapy.⁶ The fact that M2 macrophages encoding the high affinity FcγRIIIA (valine-carriers) displayed a more pronounced activation (figure 4) corroborated the finding that patients with this high affinity receptor had an even worse progression-free survival than those with 158-Phe homozygosity.⁷ Notably, bevacizumab binds soluble VEGF and therefore does not cross-link FcγRs. Moreover, the use of previous or concomitant chemotherapy could influence the infiltration of myeloid cells because of bone marrow suppression.¹⁵ Also, expression of EGFR on tumor cells could be up-regulated by chemotherapy such as fluoropyrimidines and irinotecan.¹⁶

In conclusion, clinical testing of engineered MABs with Fc-regions with increased affinity to FcγRs should be performed very carefully^{1,17}, because tumor-promoting effects by intratumoral M2 macrophages could lead to tumor promotion instead of tumor repression.

Acknowledgements

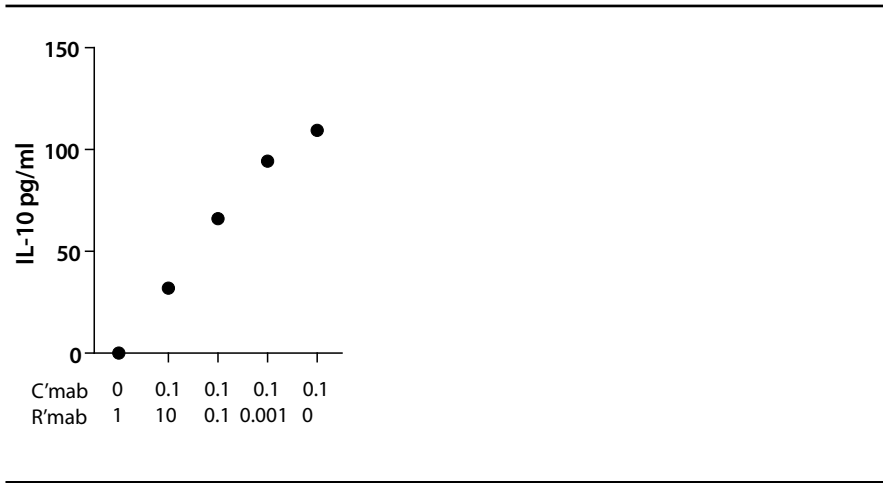
We thank R. Goedemans for technical support and M.H. Lampen and M. Meyering for providing CD14-monocytes.

Figure S1 IL-8 production upon Fc-mediated M2 activation



A, IL-8 production after 24 h co-culture of M2 macrophages with cetuximab (c'mab, 1 µg/ml) opsonized A431 tumor cells (white bars), LoVo cells (light grey bars) and HCT-15 cells (dark grey bars). Addition of LPS (250 ng/ml) served as positive control. **B**, IL-8 production by macrophages depends on interaction of macrophages and cetuximab opsonized tumor cells. Plate-bound cetuximab (10 µg/ml) ('coated') could not replace the EGFR-expressing tumor cells and control antibody rituximab (r'mab, 1 µg/ml) or bevacizumab (b'mab 1 µg/ml) could not replace cetuximab. **C**, Overnight IL-8 production by M2 macrophages is dependent on the concentration of cetuximab. **D**, Both the phenylalanine homozygote donors (solid squares, n = 12) and valine allele carriers (Phe/Val [open circles] and Val/Val [solid circles] donors combined; n = 10) were activated by cetuximab (c'mab) and EGFR-positive A431 tumor cells. IL-8 release was measured and lines represent median values.

Figure S2 Competition for Fc-binding by rituximab



A431 tumor cells were loaded without cetuximab (c'mab 0) or with a sub-optimal dose of 0.1 µg/ml cetuximab (c'mab 0.1) Rituximab (r'mab) was added in higher (10 ug/ml), equal (0.1 ug/ml) or lower concentration and M2 macrophages were added. IL10 release was measured after 24 hr by ELISA.

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Capecitabine, oxaliplatin
and bevacizumab



5

Current insights in the role of heritable genetic variation in the pharmacokinetics and pharmacodynamics of anti-cancer drugs

Jan Pander • Hans Gelderblom • Henk-Jan Guchelaar

Expert Opinion on Pharmacotherapy 2007 Jun;8(9):1197-210

Abstract

Pharmacogenetics in oncology ideally will allow oncologists to individualise therapy based upon a genetic test result. Severe toxicity and clinically significant under-dosing may be avoided, whereas predicted non-responders can be offered alternative therapy.

This manuscript gives an overview of heritable variants in the genes of nine enzymes or pathways that have been studied most extensively in anti-cancer chemotherapy. Even though many pharmacogenetic association studies have been published, there is need for more research. In particular, there is need for replication of data and development of predictive models. Prospective trials are required to establish clinical value and cost-effectiveness of pharmacogenetic testing in oncology.

Introduction

Pharmacogenetics studies the association between heritable functional variants in DNA (genotype) with outcome of therapy (phenotype). In the recent years, pharmacogenetics in oncology has become an increasing field of research. Ideally, pharmacogenetic testing will allow oncologists to individualise therapy, with respect to the choice of a drug and the dose of the drug administered, based upon a genetic test result. Severe toxicity may be avoided, whereas predicted non-responders can be offered alternative therapy.

A polymorphism is an inheritable variant that occurs within at least 1% of the population. Moreover, a polymorphism is a neutral variant: the variant may have functional consequences on the protein level, without influencing existence of the individual. Variants in DNA can be single nucleotide polymorphisms (SNPs), deletions or insertions of a number of base pairs (bp) or variable number of tandem repeats resulting in changes in exons, introns or in untranslated regions (UTR), such as the promoter region of the gene. When DNA is transcribed into mRNA, some of these variants may result in altered mRNA stability. Some variants result in different amino acid composition of proteins or truncated proteins which may lead to altered enzyme activity and thus functionality (non-synonymous variants), whereas other variants do not result in amino acid change (synonymous or silent variants). Finally, variants in a UTR of a gene can alter the transcriptional activity of a gene and thus change the expression of an enzyme.

As specific regions in DNA are conserved through generations, variants are often inherited as so called haplotypes, which can be measured by assessing linkage disequilibrium (LD).

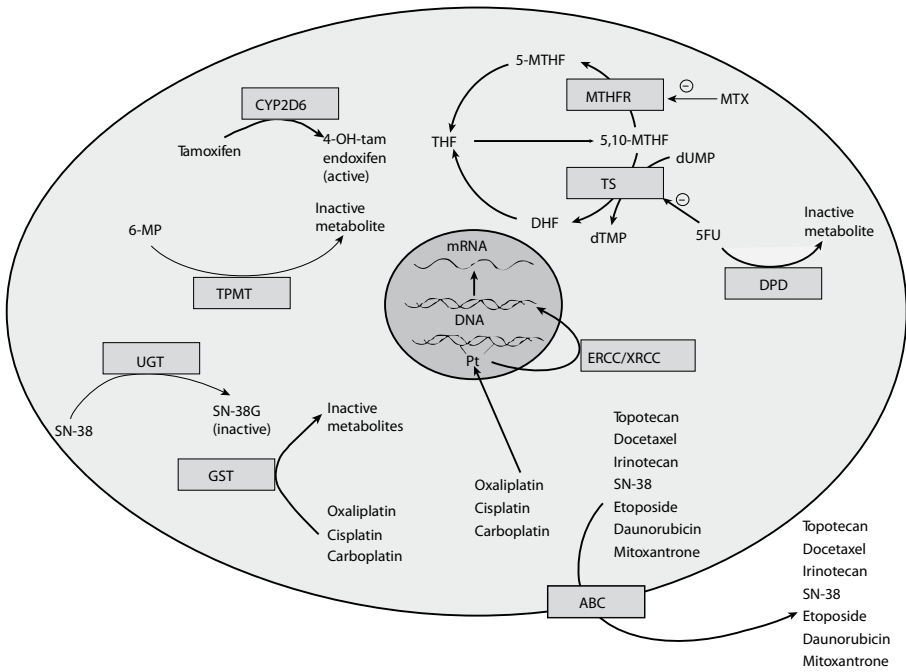
In contrast to somatic variants, heritable (germ-line) variants in DNA are inherited from parents, and the presence of a variant can be either heterozygous (carrier of one normal and one variant allele) or homozygous (carrier of two variant alleles) as compared to wild-type (two normal alleles). The determination of germ-line variants in, for example DNA isolated from peripheral blood is much more feasible than determination of variants in DNA isolated from tumour samples. Interestingly, in a recent paper it was shown that there is a high degree of concordance between germ-line and somatic variants for a number of SNPs.¹ However, genetic mutations related to the origin of the malignant phenotype are by definition in discordance to the germ-line phenotype.

In this manuscript, we give an overview of heritable variants in the genes encoding nine enzymes or pathways that have been studied most extensively in patients

treated with anti-cancer drugs (Figure 1). The variants in these genes are summarised in Table 1.

Medline was systematically searched (from July 1st to September 30th 2006) with the following set of keywords: pharmacogenetics, pharmacogenomics, polymorphism, SNP, genotype, phenotype, antineoplastic (protocols), chemotherapy, combined with the names of genes and enzymes, limiting results to human research published in English.

Figure 1 Schematic overview of enzymes involved in cellular response and metabolism to anti-cancer drugs



Abbreviations: CYP2D6: cytochrome P450 2D6; 4-OH-tam: 4-hydroxy-tamoxifen; DPD: dihydropyrimidine dehydrogenase; 5FU: 5-fluorouracil; MTHF: methylene tetrahydrofolate; MTHFR: methylene tetrahydrofolate reductase; MTX: methotrexate; THF: tetrahydrofolate; 6-MP: 6-mercaptopurine; dUMP: deoxyuridine monophosphate; TPMT: thiopurine S-methyltransferase; TS: thymidylate synthase; DHF: dihydrofolate; dTMP: deoxythymidine monophosphate; UGT: uridine diphosphate glucuronosyl transferase; ERCC: excision repair cross complementing; XRCC: X-ray repair cross complementing; SN-38: active metabolite of irinotecan; SN-38G: SN-38 glucuronide; GST: glutathione S-transferase; ABC: ATP-binding cassette

Table 5 Enzymes involved in response to anti-cancer drugs and their common polymorphisms

enzyme	variant allele	polymorphism	phenotype
TPMT	TPMT*2	238G>C	Ala80Pro
	TPMT*3A	460G>A + 719A>G	Ala154Thr + Tyr240Cys
DPD	DPYD*2A	IVS14+1G>A	Δ exon 14
UGT	UGT1A1*28	(TA) ₇ TAA	reduced enzyme activity
GST π	GSTP1-105	313A>G	Ile105Val
GST μ	GSTM1-null		deletion of gene
GST θ	GSTT1-null		deletion of gene
P-glycoprotein	ABCB1 = MDR1	1236C>T	silent
		3435C>T	silent
		2677G>T/A	Ala893Ser/Thr
BCRP	ABCG2-421	421C>A	Gln141Lys
ERCC1	ERCC1-118	496C>T	Asn118Asn
XPD	ERCC2-321	965G>A	Asp321Asn
		2251A>C	Lys751Gln
XRCC1	XRCC1-399	1301G>A	Arg399Gln
CYP2D6	CYP2D6*4	1846G>A	null enzyme activity
MTHFR	MTHFR-677	677C>T	Ala222Val
TS	TYMS TSER	28 bp insert in TSER	increased expression
	TYMS	G>C at bp 12 in TSER-3	restored enzyme activity
	TYMS 3' UTR	1494 6bp indel	decreased expression

TPMT: thiopurine S-methyltransferase; DPD/DPYD: dihydropyrimidine dehydrogenase; UGT: uridine diphosphate glucuronosyl transferase; GST: glutathione S-transferase; ABCB1: ATP-binding cassette B1; MDR1: multi drug resistance 1; BCRP: breast cancer resistance protein; ABCG2: ATP-binding cassette G2; ERCC1: excision repair cross complementing group 1; XPD: xeroderma pigmentosum group 1; ERCC2: excision repair cross complementing group 2; XRCC1: X-ray repair cross complementing group 1; CYP2D6: cytochrome P450 2D6; MTHFR: methylene tetrahydrofolate dehydrogenase; TS/TYMS: thymidylate synthase; TSER: thymidylate synthase enhancer region; UTR: untranslated region; bp: base pair; indel: insertion/deletion

Pharmacogenetic association studies

Thiopurine S-methyltransferase

An alkylating agent commonly used in maintenance treatment of acute lymphoblastic leukaemia (ALL) 6-mercaptopurine (6MP), which is deactivated by the enzyme thiopurine S-methyltransferase (TPMT). Approximately 0.3% and 10% of the population has undetectable and intermediate TPMT enzyme activity respectively.^{2,3} TPMT activity is inversely associated with exposure to the cytotoxic metabolite of 6MP, 6-thioguanine (6TGN), in red blood cells⁴ and in ALL blasts.⁵ Because of severe haematological

toxicity, 6MP dose must be reduced⁶, with as much as 90% and 50-66% for the respective phenotypes.⁷ Because dose intensity proved to be a prognostic marker for outcome in ALL patients treated with 6MP, it is important to administer the right dose with regard to toxicity⁸ and efficacy.⁴

Therefore, TPMT activity is a determinant for predicting the occurrence of toxicity. However, it must be noticed that TPMT activity is influenced by several common factors in ALL, such as methotrexate (MTX)/trimethoprim treatment⁹ or administration of red blood cells transfusions.¹⁰

The molecular basis of decreased TPMT activity was found in 1995. A 238G>C SNP resulting in amino acid change of alanine to proline in codon 80 (Ala80Pro), and in 100 fold decrease in enzyme activity was found in a patient who experienced severe toxicity to 6MP.¹¹ This allele is referred to as *TPMT*2*.

The variant allele *TPMT*3A* was found a year later (460G>A; Ala154Thr + 719A>G; Tyr240Cys) in a patient with almost absent TPMT activity.¹² To date, at least 25 variant alleles have been found, and their functional significance has been described.^{13,14} However, approximately 85-95% of all variant alleles in Caucasians is *TPMT*2*, *TPMT*3A* and *TPMT*3C*.^{15,16} The *TPMT*3A* has an allele frequency of 4%¹⁷ but is absent in African and Asian populations.^{16,18-21} In these populations, the *TPMT*3C* allele (719A>G; Tyr240Cys) is the most frequent variant allele^{16,18-21} and its functional impact has been demonstrated in Japanese children with ALL.²²

A strong relationship between genotype and phenotype has been demonstrated, resulting in 90% sensitivity and 99% specificity (variants *TPMT*2*-**18*).³ However, in another report no *TPMT*2*, *TPMT*3A* and *TPMT*3C* allele was detected in 5 of 9 patients with intermediate TPMT activity.⁷

Even though cost effectiveness models of TPMT genotyping have been reported recently²³, and the Food and Drug Administration (FDA) has included more information on inherited TPMT deficiency in the 6MP label [201], only few institutions commonly genotype patients prior to 6MP treatment.²⁴

Interestingly, exposure to the cytotoxic metabolite 6TGN is not only related to toxicity, but also to efficacy of 6MP therapy as shown by Stanulla *et al.* They found a 2.9 fold lower occurrence of residual disease in ALL patients who were heterozygous for any *TPMT*2*, *TPMT*3A*, *TPMT*3C* or *TPMT*9* (356A>C; Lys119Thr) allele and treated with a similar 6MP dose. They did not find a difference in the occurrence of toxicity.²⁵

Dihydropyrimidine dehydrogenase

An important anti-metabolite used for a vast number of different types of solid tumours is 5-Fluorouracil (5FU). Over 80% of 5FU is inactivated in the liver by the enzyme dihydropyrimidine dehydrogenase (DPD), encoded by the gene *DPYD*. Since

a DPD deficient patient experiencing severe haematological toxicity to 5FU was described in 1988²⁶ many mutations in the *DPYD* gene that result in decreased DPD activity have been identified. Apart from association studies between DPD enzyme activity and 5FU toxicity, genetic associations have also been described. It must be noted that not all 5FU related toxicity can be attributed to decreased DPD activity though.

Despite having an allele frequency of <1% in Caucasians^{27,28}, a SNP in the 5' invariant splice donor sequence in intron 14 of the *DPYD* gene (IVS14+1G>A; deletion of exon 14; *DPYD*2A*) seems to be one of the key mutations resulting in low DPD activity and increased incidence of 5FU toxicity.²⁹ Two studies have shown considerable effect of this polymorphism on the incidence of 5FU toxicity. In 60 cancer patients who experienced grade 3-4 toxicity (according to the National Cancer Institute Common Toxicity Criteria (NCI-CTC) [202]) to 5FU containing chemotherapy, the frequency of the *DPYD*2A* allele was 15%, which was significantly higher than 0.91% in the control population (P=0.001).³⁰ Also, 50% of patients who experienced NCI-CTC grade 4 neutropenia carried the *DPYD*2A* allele.³¹ The *DPYD*2A* allele has not been detected in Asian populations.^{32,33}

Other variants of the *DPYD* gene that have been linked to 5FU toxicity include the *DPYD*4* (1601G>A; Ser534Asn), *DPYD*11* (1003G>T; Val335Leu), *DPYD*12* (62G>A; Arg21Gln + 1156G>T; Glu386Ter) and *DPYD*13* (1679T>G; Ile560Ser) alleles.^{34,35} On the other hand, methylation of the promoter region of the *DPYD* gene also seems to reduce DPD activity due to a decrease of transcription.³⁶

Homozygous patients with two low DPD activity alleles are rare, but multiple cases have been described of lethal outcome to 5FU treatment in these patients.^{27,28}

Uridine diphosphate glucuronosyl transferase

Uridine diphosphate glucuronosyl transferase (UGT) is a phase II metabolic enzyme responsible for glucuronidation of several endogenous (such as bilirubin) and exogenous compounds. SN-38, the active metabolite of the topo-isomerase I inhibitor irinotecan, is predominantly inactivated by the isoforms UGT1A1 and UGT1A9 in the liver and by UGT1A7 in the upper gastro-intestinal tract.³⁷

A TA insert polymorphism in the TATA box of the promoter region of the *UGT1A1* gene has been studied extensively. The *UGT1A1*28* allele has 7 TA repeats, whereas wild-type has 6 TA repeats. This polymorphism is associated with Gilbert's syndrome, a condition of reduced bilirubin glucuronidation³⁸ and has also been associated with decreased SN-38 glucuronidation *in vitro*³⁹ and *in vivo*.^{40,41} The allele frequency of *UGT1A1*28* is higher in Caucasians (22-39%)⁴¹⁻⁴⁴ than in Asians (7-17%)⁴⁴⁻⁴⁹, and even higher in Blacks (~45%).^{45,46}

Patients with metastatic colorectal cancer (mCRC) or other solid tumours who were treated with irinotecan, and who were homozygous for the *UGT1A1*28* allele had

significant higher occurrence of grade 3 or 4 diarrhoea (according to criteria of the World Health Organization (WHO)⁵⁰)⁵¹ and NCI-CTC grade 3 or 4 neutropenia^{40,43} compared with patients who carried at least one wild-type allele. Other studies have shown higher incidence of NCI-CTC grade 3 or 4 diarrhoea in mCRC patients⁵² and higher incidence of grade 3 or 4 diarrhoea and/or grade 4 leukopenia (according to criteria of the Japan Society for Cancer Therapy⁵³) in patients with solid tumours⁴⁷ when carriers of the variant allele were compared with homozygote wild-type patients. In a recent report of 250 mCRC patients, the odds ratio for the incidence of NCI-CTC grade 3 or 4 haematological toxicity was 8.63 for patients homozygous for the variant allele compared with patients who were homozygote wild-type. However, this was only significant after the first cycle of treatment.⁵⁴ Interestingly, response to irinotecan therapy was also improved for homozygote individuals for the variant allele compared with homozygote individuals for the wild-type allele because of increased SN-38 exposure.⁵⁴

From these studies it is clear that the *UGT1A1**28 allele is associated with increased risk for neutropenia in patients receiving irinotecan. Due to increased exposure to SN-38, the active metabolite of irinotecan, it may also be expected that carriers of this allele experience increased efficacy but this has not yet been proven.

A SNP in the phenobarbital responsive enhancer module (PBREM) of the *UGT1A1* gene (-3279T>G; *UGT1A1**60) has been associated with severe toxicity (grade 4 leukopenia and grade 3 or 4 diarrhoea; Japanese criteria) in Japanese cancer patients treated with irinotecan.⁵⁵ However, the *UGT1A1**60 variant allele was linked to the *UGT1A1**28 variant.³⁹

Several SNPs in various regions of the *UGT1A1*, *UGT1A7* and *UGT1A9* genes have been found to be in linkage disequilibrium (LD).^{44,48,49,56} The functional and clinical relevance of these haplotypes has not yet been established.

In 2005, the FDA approved the Invader® *UGT1A1* molecular assay, a test for the *UGT1A1**28 variant allele [203]. Also, the package insert of irinotecan was modified in 2005 by the FDA, to include information on *UGT1A1* variability [204]. Unfortunately, because no studies have determined the optimal dose per genotype, no advice for dose adjustment is made.

Glutathione S-transferase

Glutathione S-transferases (GSTs) make up a family of phase II enzymes that catalyze the conjugation of reduced glutathione to toxic substances. Members of this family are GST π , GST μ and GST θ , which are products of distinct loci in the genome. Among substrates for GSTs are cyclophosphamide, etoposide, doxorubicin, cisplatin, carboplatin and oxaliplatin and their metabolites. Theoretically, reduced activity of these enzymes would result in increased exposure to these drugs, possibly resulting in increased efficacy and toxicity.

The gene for GST π , *GSTP1*, is known to be polymorphic. One polymorphism resulting in a non-synonymous SNP at codon 105 (313A>G; Ile105Val) in exon 5 causes decreased GST π activity.⁵⁷ The allele frequency is approximately 20%, 30% and 40% in Asian, Caucasian and African American populations respectively.⁵⁷⁻⁶⁰

A significant association toward better survival after cyclophosphamide containing chemotherapy was found for breast cancer patients carrying the variant 105Val allele.^{58,59} The variant allele was also associated with increased survival in 107 mCRC patients who were treated with a combination of 5FU and oxaliplatin.⁶¹ Survival was 24.9, 13.3 and 7.9 months for Val/Val, Val/Ile and Ile/Ile genotypes respectively ($P=0.001$).⁶¹ The variant allele was also associated with better response and longer survival in gastric cancer patients who were treated with 5FU and cisplatin.⁶² Colorectal, gastric and pancreatic cancer patients carrying at least one *GSTP1*-105Val allele experienced less toxicity to oxaliplatin containing chemotherapy.⁶³

The genes for subclasses GST μ (*GSTM1*) and GST θ (*GSTT1*) both have 'null' polymorphisms, where the total gene is deleted on both alleles. Both null genotypes were associated with increased survival among breast cancer patients, irrespective of treatment (either chemotherapy or radiation).⁶⁴ However, this association was not found in another cohort of breast cancer patients⁵⁹, nor in a cohort of colorectal cancer (CRC) patients.⁶¹ Survival of ovarian cancer patients treated with platinum containing chemotherapy was also better for *GSTM1* null patients.^{65,66}

These studies demonstrate that, because of decreased inactivation of the respective anti-cancer agents, carriers of the less active variant GST alleles have increased response and survival to chemotherapy.

Drug transporters

ABCB1

The ATP-binding cassette (ABC) B1 gene (*ABCB1*), formerly known as multi-drug resistance (*MDR1*) gene, encodes the P-glycoprotein (PGP), an ATP-dependent efflux pump that exports exogenous substances across the cell membrane. Through this mechanism, substances such as cytostatics are unable to retain sufficient intracellular concentrations to exert their anti-tumour activity. Two synonymous SNPs in exons 12 and 26 (1236C>T and 3435C>T respectively) and a non-synonymous SNP in exon 21 (2677G>T/A; Ala893Ser/Thr) have been studied extensively. These variant alleles occur together in a common haplotype (*MDR1**2), with a frequency of 27%, 31-49% and 6.5% in Caucasians, Asians and Blacks respectively.⁶⁷⁻⁶⁹ The *MDR1**2 haplotype was associated with lower irinotecan and SN-38 clearance⁶⁹ and with lower C_{max} for glucuronidated SN-38 (SN-38G).⁷⁰

The individual polymorphisms have been associated with decreased PGP function *in vivo*.^{71,72} Cancer patients homozygous for the 1236C>T variant allele had higher

exposure to both irinotecan and SN-38.⁷³ In 58 patients with solid tumours, all patients homozygous for the 3435T variant allele experienced grade 3-4 neutropenia (specified as neutrophil count between 0.5 and $1.0 \times 10^9/L$ and less than $0.5 \times 10^9/L$ respectively) to docetaxel, compared with 77% and 54% for heterozygote and wild-type individuals respectively.⁷⁴ As exposure to docetaxel is increased in carriers of the variant allele, this finding would be expected.

ABCG2

Another ATP binding cassette, formerly known as the breast cancer resistance protein (BCRP) is coded by the gene *ABCG2*. Overexpression of this enzyme is related to the occurrence of resistance to several anticancer agents such as SN-38, mitoxantrone, topotecan, daunorubicin and etoposide.⁷⁵ A SNP in exon 5 (421C>A; Gln141Lys) has an allele frequency of 34%, 12% and 1-5% in Han Chinese, Caucasians and Blacks respectively⁷⁶, and results in lower BCRP expression and higher SN-38 and topotecan sensitivity *in vitro*.⁷⁷ However, this SNP has found not to be associated with pharmacokinetic parameters of irinotecan and its metabolites in a cohort of cancer patients.⁷⁶

DNA repair

Excision repair cross complementing group 1

As part of the nucleotide excision (NER) pathway, excision repair cross complementing group 1 (ERCC1) is involved in DNA damage repair caused by platinum containing compounds such as cisplatin, carboplatin and oxaliplatin.^{78,79} Increased ERCC1 expression has been shown to lead to cisplatin resistance *in vitro*⁸⁰ and to lower response in cisplatin treated bladder cancer patients *in vivo*.⁸¹ A prospective ERCC1 mRNA expression guided phase III study is ongoing.⁸² A silent SNP has been identified in exon 4 at codon 118 in the *ERCC1* gene (496C>T; Asn118Asn). The allele frequency of the T allele is 0.58 in Caucasians, 0.24-0.36 in Asians and 0 in Blacks.^{83,84}

Even though encoding the same amino acid, the variant codon is believed to occur less commonly, therefore resulting in reduced ERCC1 expression.^{85,86}

Homozygote *ERCC1* wild-type patients with stage IIIB-IV non-small cell lung cancer (NSCLC) who were treated with cisplatin containing chemotherapy had longer survival than patients carrying the variant allele.⁸⁷ This same association was found in another cohort of NSCLC stage IIIB-IV patients treated with cisplatin and docetaxel.⁸⁸

Homozygote wild-type mCRC patients were also found to have longer survival compared to carriers of the variant allele when treated with oxaliplatin and 5FU⁸³, whereas in another cohort of mCRC patients, the variant genotype was associated with better response to oxaliplatin and 5FU.⁸⁹

In melanoma patients (stage IV) treated with cisplatin containing chemotherapy, the wild-type genotype was associated with worse response and shorter overall survival.⁹⁰

Ovarian cancer patients who carried the variant allele had reduced risk of platinum resistance, but survival was not affected by genotype.⁸⁴

One would expect that the SNP leading to reduced ERCC1 expression and hence to decreased DNA repair of DNA-platinum adducts, would result in increased platinum sensitivity and consequently to increased response and survival. However, most studies that are presented show the opposite result. The occurrence of linkage disequilibrium of the evaluated variant with other variants with opposing effects on enzyme function as well as that of other enzymes and pathways with a role in the drug's pharmacokinetics and clinical variables could be of importance and explain this discrepancy.

Excision repair cross complementing group 2

The enzyme xeroderma pigmentosum group D (XPB) is coded by the excision repair cross complementing group 2 (*ERCC2*) gene, and is also involved in the NER pathway. Two SNPs in this gene (965G>A, Asp321Asn and 2251A>C, Lys751Gln) are associated with reduced DNA repair capacity.⁹¹ The allele frequency of the XPB-321 variant allele was 0.32 in a general Western population.⁹² The allele frequency of the XPB-751 variant allele is 0.44 in Caucasians, 0.16 in Blacks and 0.09 in Asians.⁸³

Patients with mCRC who were homozygous for the variant *ERCC2-751* allele and who were treated with oxaliplatin based chemotherapy had higher mortality compared with patients carrying the wild-type allele.^{83,93} The two SNPs in the *ERCC2* gene were not associated with response and survival in cisplatin treated NSCLC patients^{87,88}, but the wild-type *ERCC2-751* allele was associated with increased incidence of WHO grade 2 or higher neutropenia.⁸⁸ In stage III-IV NSCLC patients treated with platinum containing chemotherapy, homozygote individuals for the *ERCC2-321* variant had worse survival compared to carriers of a wild-type allele.⁹²

X-ray repair cross complementing group 1

The X-ray repair cross complementing group 1 (XRCC1) enzyme is involved in repair of single-strand breaks in DNA. A SNP in the *XRCC1* gene (1301G>A, Arg399Gln, allele frequency of 0.35 in Caucasians, 0.36 in Blacks and 0.22 in Asians⁸³) has been associated with worse response to oxaliplatin and 5FU in mCRC patients⁹⁴, but there was no difference in time to progression or survival.⁸³ In stage III NSCLC patients, survival was shorter for individuals homozygote for the variant allele compared with carriers of the wild-type allele.⁹²

An increased number of variant alleles of the *ERCC2* and *XRCC1* genes have been associated with worse survival in NSCLC patients who were treated with platinum containing chemotherapy.⁹² In contrast, in patients with squamous cell cancer of the head and neck (SCCHN) treated with cisplatin containing therapy, an increased number of variant alleles of *ERCC2-321*, *ERCC2-751*, *XRCC1-399* and *ERCC1* (8092C>A in

the 3'UTR) was associated with increased survival.⁹⁵ Moreover, cisplatin treated patients with muscle invasive bladder cancer who carried one or more variant *ERCC2-751* or *XRCC1-399* allele had better survival compared to wild-type patients.⁹⁶ These inconsistent and non-intuitive findings could in part be explained by the reasons that are given for conflicting results for *ERCC1*.

CYP2D6

Tamoxifen is a widely used agent in treatment of breast cancer and is hydroxylated into the 100 times more active metabolites 4-hydroxy-tamoxifen and endoxifen by the cytochrome P450 iso-enzyme CYP2D6.⁹⁷⁻⁹⁹ The *CYP2D6*4* (1846G>A) allele results in gene deletion and thus in absent CYP2D6 activity and has a frequency of 15-20% in the general population in Western countries.¹⁰⁰⁻¹⁰⁵ Other alleles resulting in lower CYP2D6 activity are *CYP2D6*3* (Δ 2549A), *CYP2D6*5* (deletion of entire *CYP2D6* gene) and *CYP2D6*6* (Δ 1707T). The *CYP2D6*4* allele has been linked to reduced conversion of tamoxifen into 4-hydroxy-tamoxifen¹⁰⁶ and the *CYP2D6*3-6* genotypes have been related to lower endoxifen formation.^{103,104}

As expected, breast cancer patients treated with adjuvant tamoxifen who were homozygous for the *CYP2D6*4* allele had significant worse relapse-free time and shorter disease free survival compared with carriers of the wild-type allele. However, significance was not retained in multivariate analysis. Homozygote *CYP2D6*4* patients did not experience moderate to severe flashes, which is a side effect of (the active metabolite of) tamoxifen.¹⁰² A similar finding was reported in a case control study for prevention of breast cancer with tamoxifen in hysterectomised women. The frequency of the *CYP2D6*4/*4* genotype was higher in women who developed breast cancer during follow up than in women free of cancer.¹⁰⁷

Contradictory to this, another study found that oestrogen receptor positive (ER+) breast cancer patients who carried the *CYP2D6*4* allele had significant longer recurrence free survival when treated with adjuvant tamoxifen compared with *CYP2D6*4* carriers who were not treated with tamoxifen. This difference was not observed for wild-type patients.¹⁰⁰ Selection bias in this study may have influenced the outcome of this study.¹⁰⁸

Methylene tetrahydrofolate reductase (MTHFR)

The enzyme MTHFR is one of the key enzymes in the folate pathway (see figure 1). Reduced MTHFR expression results in reduced sensitivity to the MTHFR inhibitor MTX. On the other hand, abundance of the MTHFR substrate 5,10-methylene tetrahydrofolate (5,10-MTHF) facilitates the inhibition of thymidylate synthase (TS) by 5FU, therefore increasing sensitivity to this agent.

A SNP in the *MTHFR* gene, 677C>T results in an amino-acid change of alanine to valine at codon 222 (Ala222Val). The allele frequency in all populations is 0.27-0.57.^{62,109-114}

MTHFR activity is 70% and 35% for heterozygote and homozygote individuals respectively.¹⁰⁹ *In vitro* assays showed that cell lines with the variant allele are more sensitive to 5FU and less sensitive to MTX.¹¹⁵

Of six patients who experienced severe toxicity to adjuvant CMF (cyclophosphamide, MTX, 5FU) for breast cancer, five were homozygous for the 677T allele.¹¹⁶ In a cohort of cancer patients who were treated with the 5FU analogue raltitrexed, patients with 677TT genotype had significant more therapy related toxicity.¹¹⁷ Leukaemia patients homozygous for the 677T allele experienced more MTX related toxicity compared with patients who carried at least one wild-type allele.¹¹⁸ Also, ovarian cancer patients who were treated with MTX and were homozygous for the 677T allele experienced significant more WHO grade 3/4 side effects.¹¹²

In mCRC patients, the 677T allele has been associated with improved response to 5FU based chemotherapy in several studies¹¹⁹⁻¹²¹, whereas another study did not find a significant association.¹²² No association was found in a cohort of advanced gastric cancer patients treated with 5FU and cisplatin.⁶²

Thymidylate synthase (TS)

TS is the central enzyme in the de-novo thymidine synthesis. *In vitro* resistance to 5FU is associated with increased TS activity, which is also induced by 5FU itself.¹²³ The TS promoter enhancer region (TSER) of the gene encoding TS (*TYMS*) has been shown to contain either two or three tandem repeats designated as TSER*2 and TSER*3 respectively. The TSER*3 genotype results in increased TS expression, either through higher mRNA levels or increase in efficiency of mRNA translation.^{124,125} The allele frequency of the TSER*2 allele is 0.40-0.46 in Caucasians and Blacks^{126,127}, compared to 0.18-0.21 in Asian populations.^{126,128-130}

The TSER*3 allele was associated with increased response in CRC patients treated with 5FU.¹²¹ On the other hand, the TSER*2 allele was associated with improved response to capecitabine in CRC patients.¹³¹

These conflicting results could in part be explained by a G>C SNP in the 12th base pair of the TSER*3 allele¹³² that results in TS activity similar to that of the TSER*2 allele.¹³³ This TSER*3C allele is found in 29%-57% of all TSER*3 alleles.^{132,134,135}

Carriers of the TSER*3G allele had significantly worse response, disease free survival and overall survival in a cohort of mCRC patients treated with 5FU.¹³⁵ The TSER*3G allele was also associated with worse survival in advanced gastric cancer patients who were treated with 5FU.⁶²

As expected, most studies show that the TSER*3 allele, and especially the TSER*3G allele, is associated with lower response and survival to fluoropyrimidine therapy. Conflicting results could be explained by the G>C SNP in the TSER*3 allele.

A six base pair deletion (-6bp) in the 3' UTR of the *TYMS* gene results in decreased mRNA stability and lower TS expression.¹³⁶ The -6bp mutation is in linkage disequilibrium (LD) with the TSER*3 allele, and the +6bp allele is in LD with TSER*2.¹³⁷ The -6bp variant allele is associated with decreased survival in mCRC patients treated with 5FU and oxaliplatin⁸³ and with decreased response to 5FU based chemotherapy in advanced gastric cancer patients.¹³⁸

CRC patients treated with 5FU who were either homozygous for the TSER*3 allele (regardless of 3' UTR genotype) or heterozygous for the TSER allele combined with homozygous for the +6bp genotype had significant better disease free survival (DFS) and overall survival (OS) compared with the other genotypes.¹³⁹ In a cohort of gastric cancer patients, non-carriers of the TSER*3G allele together with one or two -6bp alleles had significant better DFS and OS compared to carriers of TSER*3G and two copies of +6bp.¹⁴⁰ The haplotype TSER*3C and -6bp was associated with significant better OS compared with the haplotype TSER*2 and +6bp in CRC patients treated with 5FU.¹⁴¹

Multiple gene studies

Drug response is a complex phenotype, especially in anti-cancer therapy, where multiple drug regimens are often applied. Only few studies have explored the influence of polymorphisms of multiple genes that are involved in the pathway of the drug.

The role of polymorphisms in genes involved in response to 5FU (*TYMS*) and metabolism of cisplatin (*GSTP1*) was investigated by Ruzzo *et al.* Advanced gastric cancer patients treated with 5FU and cisplatin who were both homozygous for the *GSTP1*-105Ile allele and carrier of the TSER*3G allele had significant shorter progression free survival and over all survival compared with patients who were carriers of the *GSTP1*-105Val allele or patients who did not carry the TSER*3G allele.⁶²

Stoehlmacher *et al.* looked at genes involved in response and metabolism of oxaliplatin (*ERCC1*, *ERCC2* and *GSTP1*) and 5FU (*TYMS*). Favourable genotypes in mCRC patients were *ERCC2*-751 Lys/Lys, *ERCC1*-496 C/C, *GSTP1*-105Val/Val and *TYMS*-3'UTR +6bp/+6bp. Patients who carried none of these genotypes had median survival of 5.4 months, compared with 10.2 and 17.4 months for patients with one or $\geq$ two favourable genotypes ($P < 0.001$).⁸³

When multiple variants in genes or variants in multiple genes are surveyed for example in combination chemotherapy regimens, both sample size and power are of great importance since opposite effects of different genetic variants can obliterate each other in small samples, whereas multiple testing may reveal false-positive associations.

Conclusion

There is ample evidence that pharmacogenetic traits are able to predict pharmacodynamics of several anti-cancer drugs. Polymorphisms that result in decreased metabolic enzyme levels or activity have shown to result in either increased toxicity or increased efficacy or both. Other polymorphisms lead to increased exposure to chemotherapy through decreased expression of membrane efflux pumps, whereas others lead to decreased capability to repair DNA damage caused by chemotherapy. Variants in genes that code for enzymes involved in the mode of action of anti-cancer drugs give altered response to chemotherapy. Despite emerging evidence, pharmacogenetic testing has not yet found its way to routine patient care.

Expert Opinion

Many pharmacogenetic studies that point towards association of heritable genetic variants and cytotoxic drug response have been presented in this paper. These genes and variations have been studied most extensively until now. This does not necessarily imply that these genes hold most promise for implementation in the standard of oncology care in the near future. Possibly, other genes and variations may emerge as potential predictors of response or toxicity.

Consequently, there is a need for additional, but also for other types of research in pharmacogenetics to find its way to routine patient care. Obviously, there is need for replication of apparent conflicting findings, such as for the TSER polymorphism in the *TYMS* gene, or polymorphisms in the DNA repair genes, in larger cohorts in routine patient care environment.¹⁴²

Also, cost-effectiveness of testing needs to be determined for pharmacogenetic tests. In this light, it is important to develop tests that are sensitive and specific, as well as simple and cheap.

Genetic variability is only one of the determinants of drug response. Therefore, another type of research that holds promise for the future is the development of prediction models that not only include pharmacogenetic data, but also non-genetic traits such as WHO performance status and organ function. Such models are only starting being developed, for instance regarding MTX response in rheumatoid arthritis.¹⁴³

Until now, genes are selected mainly through the candidate pathway gene approach. Obviously, this mechanistic approach seems logical. However, the disadvantage of this approach is that it is limited by current knowledge of pathophysiology and the mechanism of action of a drug. Therefore, future research will use hypothesis-free whole genome approach such as SNP arrays.¹⁴⁴

Finally, it is important to perform prospective studies on applying pharmacogenetics in patient care and to assess optimal dose and drug per genotype upon a predictive model including a pharmacogenetic test result. After all it is not possible to predict necessary dose adjustment based upon current knowledge. This is illustrated by the phrase in the irinotecan label that has recently been modified by the FDA: "A reduced initial dose should be considered for patients known to be homozygous for the *UGT1A1**28 allele" [204]. Therefore, studies are necessary that prospectively investigate an adjusted dose for a certain genotype compared with normal dose for wild-type patients.

The above mentioned future pharmacogenetic research will enable oncologists to implement pharmacogenetics and to optimize individual cancer treatment.

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6

**Letter to the editor regarding:
“Explorative study to identify novel candidate
genes related to oxaliplatin efficacy and
toxicity using a DNA repair array”**

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British Journal of Cancer 2010 Jun 8;102(12):1791-2

Sir,

We earlier reported in this journal results from an explorative pharmacogenetic study for the efficacy of second-line treatment of oxaliplatin combined with capecitabine of advanced colorectal cancer (ACC).¹ These results were obtained using a DNA repair array (Asper Biotech, Tartu, Estonia) to identify novel single nucleotide polymorphisms (SNPs) that are associated with progression-free survival (PFS) for oxaliplatin/capecitabine combination therapy.² After correction for multiple testing for five DNA repair pathways investigated, SNPs in the genes encoding ataxia telangiectasia mutated (*ATM* rs1801516) and excision repair cross-complementing group 5 (*ERCC5* rs1047768) were significantly associated with PFS in the final multivariate analysis.

Owing to the explorative nature of the study, we concluded that confirmation was required in a separate cohort of oxaliplatin/capecitabine-treated patients. We, therefore, tested the associations of the same SNPs in the *ATM* and *ERCC5* genes with PFS in patients treated in another cohort – the CAIRO2 study. Blood samples were available of 560 patients who were treated with oxaliplatin combined with capecitabine and bevacizumab, with or without cetuximab, as first-line treatment of ACC.³ Germline DNA was isolated from peripheral white blood cells by the standard manual salting-out method. We genotyped the *ATM* and *ERCC5* polymorphisms using a Taqman 7500 (Applied Biosystems, Foster City, CA, USA) with pre-designed assays according to the manufacturer's protocol. Negative controls (water) were included. The collection of blood samples for pharmacogenetic research was approved by the local institutional review boards of all participating centers, and all patients gave written informed consent.

The genotype frequencies in the CAIRO2 patients were not significantly different from the earlier study ($P=0.38$ and $P=0.68$ for *ATM* and *ERCC5*, respectively), and were in Hardy-Weinberg equilibrium. However, the frequency of *ATM* homozygote mutants was 1.6% in the CAIRO2 patients vs 4.4% in patients in the earlier study.

The results for the associations with PFS are shown in table 1. As opposed to our initial observation, the *ATM* and *ERCC5* polymorphisms were not significantly associated with PFS in the CAIRO2 patients.

Several reasons could underlie the lack of replication of association. First, our initial results¹ may have been false positive findings. Even though we had corrected for multiple testing, this approach may have been ineffective to correct for false positives. On the other hand, the frequency of *ATM* homozygote mutant patients in the CAIRO2 was lower than in the earlier study, which could have impacted the power to detect the association. However, the HR for PFS was 4.25 (95%CI 1.45 to 12.44; homozygote mutants vs wild-type) in our initial study, whereas it was 0.90 (95%CI, 0.37 to 2.18) in the CAIRO2 patients, indicating lack of association regardless of genotype frequency. Second, our initial findings were derived from patients receiving second-line therapy of oxaliplatin combined with capecitabine, while CAIRO2 concerns data from first-line

Table 1 Associations of *ATM* (rs1801516) and *ERCC5* (rs1047768) polymorphisms with PFS

	n	median PFS in months (95%CI)	Univariate HR (95%CI) [#]	P [#]	Multivariate HR (95%CI) ^{#*}	P ^{**}
<i>ATM</i> rs1801516						
Wild-type	371	9.1 (8.3-10.4)	1	-	1	-
Heterozygote	127	12.4 (9.6-13.5)	0.88 (0.70-1.09)	.245	0.93 (0.75-1.17)	.543
Homozygote mutant	8	11.8 (7.2-∞) [†]	0.61 (0.27-1.36)	.225	0.94 (0.42-2.12)	.881
<i>ERCC5</i> rs1047768						
Wild-type	180	10.6 (9.1-12.5)	1	-	1	-
Heterozygote	267	9.2 (8.2-10.6)	1.13 (0.93-1.39)	.227	1.15 (0.93-1.42)	.194
Homozygote mutant	77	10.1 (8.5-12.2)	0.96 (0.72-1.29)	.797	0.94 (0.69-1.28)	.689

[#] Hazard ratios (HR), 95% confidence intervals (95%CI) and P-values computed using a Cox proportional hazards model with the wild-type as reference

^{*} Covariates included in the multivariate model: age, gender, serum LDH (normal vs above normal) and treatment arm (oxaliplatin, capecitabine and bevacizumab vs oxaliplatin, capecitabine, bevacizumab and cetuximab)

[†] The upper limit of the 95%CI for PFS of the ATM homozygote mutants could not be estimated because of the low number of patients

therapy with the addition of bevacizumab and cetuximab also. We also recently also reported an opposite association of the *FCGR3A* Phe158Val polymorphism with PFS for cetuximab in the first-line setting for ACC compared with results from third-line settings.⁴ As the DNA repair array should theoretically be applicable to any platinum-containing regimen, this explanation is less likely for the present finding. Finally, it is possible that the addition of cetuximab could have negatively influenced the efficacy of oxaliplatin in the cetuximab-arm in the CAIRO2 study^{5,6}, which may have obscured the associations when both treatment arms were combined for analysis. However, the outcome of our analysis did not change when we restricted this to patients treated without cetuximab in the CAIRO2 study (data not shown). We, therefore, conclude that the *ATM* and *ERCC5* SNPs have no relevant impact on the PFS of oxaliplatin-based therapy for ACC. The negative result of this study underlines the importance of validating and reporting the findings from retrospective explorative studies.⁷

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7

Pharmacogenetics of tomorrow: the 1+1=3 principle

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Pharmacogenomics 2010 Jul;11(7):1011-7

Summary

Disappointing results from replicating pharmacogenetic association studies have prompted the search for novel statistical techniques to analyze the data, while taking into account the biological complexity underlying drug response. Two of these techniques – multifactor dimensionality reduction and classification and regression tree – will probably be applied in increasing numbers of future pharmacogenetic studies. In this article, we describe the concepts underlying both techniques and illustrate their application in a recent pharmacogenetic study.

Pharmacogenetic studies aim at predicting drug response. These studies commonly test associations between single candidate genetic polymorphisms and the efficacy or toxicity of drugs. Often, genetic polymorphisms in genes that have a putative impact on the function of the corresponding protein are selected. For their part, these proteins are assumed to have an impact on drug response, being enzymes involved in the pharmacokinetics or pharmacodynamic targets of the drug of interest. Each polymorphism is then separately associated with drug efficacy or toxicity.

Unfortunately, initial results from these candidate gene approach studies are often not replicated in subsequent studies.¹ This is clearly illustrated in large pharmacogenetic studies across different diseases.²⁻⁴ Even when a study is successfully replicated, the effect of a polymorphism on drug response is often lower than initially described.⁵ This is one of the reasons that only a handful of pharmacogenetic markers are actually useful to individualize treatment in clinical practice.⁶

An explanation for the disappointing results could be that the classic candidate gene approach does not take into account the full complexity underlying drug response. Drug response is likely to be influenced simultaneously by different biochemical components, such as pharmacokinetic enzymes and molecular targets within a biochemical pathway. Furthermore, it is recognized that the interplay between these different molecular components is extensive and complex. From a biological point of view, it seems therefore not only appropriate to study polymorphisms in candidate genes collectively – the so called candidate pathway approach⁷ – but also to assess the interaction between the polymorphic genes. This interaction means that the impact attributed to one genetic polymorphism depends on one or more others.⁸ In some cases, haplotype analysis can reveal relevant but simple interactions between polymorphisms, such as combined analysis of *CYP3A4* and *CYP3A5* variation for docetaxel pharmacokinetics.⁹ However, for genes that are located on different chromosomes, haplotype analysis is usually not possible.

Genetic interaction studies have already been published investigating susceptibility to several complex diseases; thus, the concept itself not new.¹⁰⁻¹⁵ However, the application of this concept in pharmacogenetic studies is scarce.¹⁶⁻¹⁸

Since results from the candidate gene approach have been disappointing²⁻⁴, and because the biologic rationale supports studying gene-gene interactions, we anticipate that novel techniques for analysis will be applied to pharmacogenetic studies in the near future.

To determine which interactions are most important for drug response, statistical techniques must be used. The most widely used technique in genetics is (logistic) regression analysis with interaction. The advantage of this technique is its availability in common statistical packages, and that covariate adjustments can be made in the same analysis. However, assumptions on the genetic model must be made beforehand, which may not be accurate in complex interaction analysis. Moreover, (logistic)

regression analysis is of only limited application with increasing numbers of polymorphisms, as the number of possible interactions increases substantially with increasing numbers of polymorphisms. For instance, the total number of possible two-, three- and four-way interactions for ten polymorphisms is 375, whereas it is more than 4,000,000 for 100 polymorphisms. This illustrates the complexity of the interaction analysis, and has led to the application and development of more advanced techniques for interaction analysis.

These techniques rely on algorithms that reduce the number of dimensions – that is, possible combinations of polymorphisms – in order to establish a genetic classifier to predict drug response. An important aspect of these genetic classifiers is that the combination of different polymorphisms results in information gain (the 1+1=3 principle). This concept of synergy illustrates the impact of interaction most intuitively, but it must be noted that other types of interaction exist, as reviewed by Perez-Perez et al.¹⁹

Two of these advanced techniques will be described: ‘multifactor dimensionality reduction’ (MDR)^{20,101} and ‘classification and regression tree’ (CART) analysis, for their application in pharmacogenetics. Since these methods use different and unique approaches, we have no explicit preference. To illustrate the application of both techniques, genetic classifiers were created to predict the incidence of leukopenia (grade 0 versus grade ≥1 according to the National Cancer Institute Common Toxicity Criteria) in patients treated with single-agent sunitinib, using data from a candidate gene analysis for sunitinib induced toxicity.²¹ In this cohort of 198 Dutch patients (predominantly with metastatic renal cell carcinoma and gastrointestinal stromal tumors) who were assessable for leukopenia, 31 polymorphisms were analyzed in 12 genes that encode enzymes in the pharmacokinetic and pharmacodynamic pathways of sunitinib. Genotyping was performed on the Biomark™ 48.48 Dynamic Array (Fluidigm, San Francisco, CA, USA) using Taqman® assays (Applied Biosystems, Nieuwekerk aan den IJssel, the Netherlands) according to the manufacturer’s protocol as previously described.²¹ There are more than 36,000 possible two-, three- and four-way interactions possible for these 31 polymorphisms, emphasizing the complexity of the problem and the need for advanced statistical techniques.

Multifactor dimensionality reduction

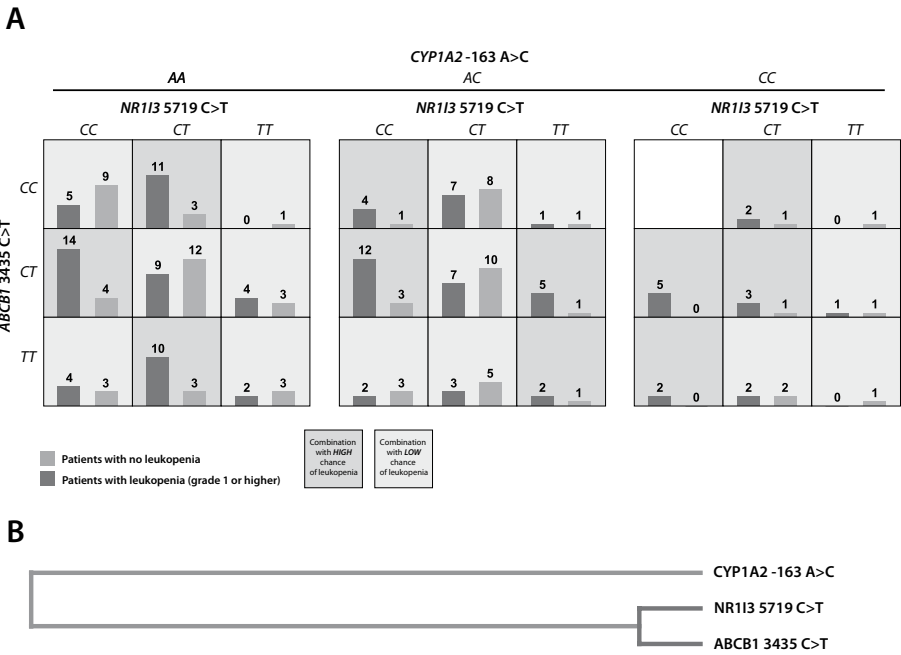
The theoretical application of the MDR analysis software to pharmacogenetic studies has previously been reviewed by Ritchie et al.²² The essence of the MDR analysis is that all possible combinations of genotypes are evaluated to predict drug response. Each combination of genotypes contains a ratio of responders to non-responders, which is used to classify patients. In this way, the complexity of genotype combinations

– dimensions – is reduced into a straightforward high-/low-risk factor. From all the possible combinations of genotypes, the MDR method presents the combination – or genetic classifier – that predicts drug response or toxicity the best.

The MDR method selects the best genetic classifier based upon accuracy – or lowest classification error. Genotypes that are individually associated with drug response contribute to a higher accuracy, and will therefore usually end up in the best genetic classifier, while it is uncertain whether they provide substantial information gain. However, it is possible that a polymorphism with a main effect also contributes substantially to the interaction model. Therefore, excluding polymorphisms could be disadvantageous. Currently, the MDR software is being updated, so that it is possible to adjust for main effects of individual polymorphisms. However, as the method of covariate adjustment has not proved its value, we excluded individually associated polymorphisms from the MDR analysis. Furthermore, haplotypes disturb the selection of the best genetic classifier because of over-fitting due to the increased number of genotype groups based upon haplotype combinations. Therefore, preferably only single nucleotide polymorphisms should be included in the analysis. In the example of our previous sunitinib analysis, polymorphisms in *NR1I3* (7738A>C and 7837T>G), *VEGFR* (-92G>A and 1718T>A), *CYP1A1* (2455A>G) and *FLT3* (738T>C) had to be excluded from the analysis because of their individual associations. Next, every possible combination of genotypes is evaluated, and the software computes how well the best genetic classifier predicts drug response. The analysis is performed across tenfold cross-validation samples to correct for over-fitting, and the combination with the highest accuracy in the cross-validation is considered the best genetic classifier. In our example, the combination containing three polymorphisms, *NR1I3* 5719C>T, *ABCB1* 3435C>T and *CYP1A2* -163A>C, showed the highest accuracy of 61.8% ($P=0.008$ obtained by permutation^{23,102}) which means that the average classification error in the prediction sets from cross-validation is 38.2%. Other combinations of polymorphisms resulted in lower accuracies. The distribution of patients with and without leukopenia across the three polymorphisms is shown in figure 1A. The interaction dendrogram for this genetic classifier is shown in figure 1B. The orange and red lines indicate a synergistic interaction between the polymorphisms. The short red lines between the *ABCB1* and *NR1I3* polymorphisms indicate that the interaction between these polymorphisms is the strongest in this model.

The results can be used to create a genetic classifier of response. This classifier can then be used in regular statistical analysis to compute an odds ratio (OR), and to perform a multivariate analysis. In the sunitinib example, the multivariate logistic regression analysis was performed including age, gender, WHO performance status, the genetic classifier, and the polymorphisms that were individually associated with leukopenia. The genetic classifier obtained by MDR has a corrected OR of 4.06 (95% confidence interval (CI), 1.99 to 8.31), whereas only the polymorphisms in *CYP1A1* and

Figure 1 Multifactor dimensionality reduction analysis of sunitinib induced leukopenia



FLT3 remained statistically significant ($P=.043$ and $P=.010$, respectively) in the multivariate analysis.

When a logistic regression analysis was performed with the three-way interaction between the *NR1I3* 5719C>T, *ABCB1* 3435C>T and *CYP1A2* -163A>C polymorphisms, the interaction term was not significantly associated with leukopenia. Each polymorphism was included as an ordinal factor, whereas the MDR method did not rely on this *a priori* assumption. This underlines the fundamental difference between

these two methods, besides the fact that every possible interaction could not be assessed using logistic regression.

When the polymorphisms with a main effect were also included in the MDR analysis, all top models contained at least either the *NR1I3* 7738A>C or *FLT3* 738T>C polymorphism.

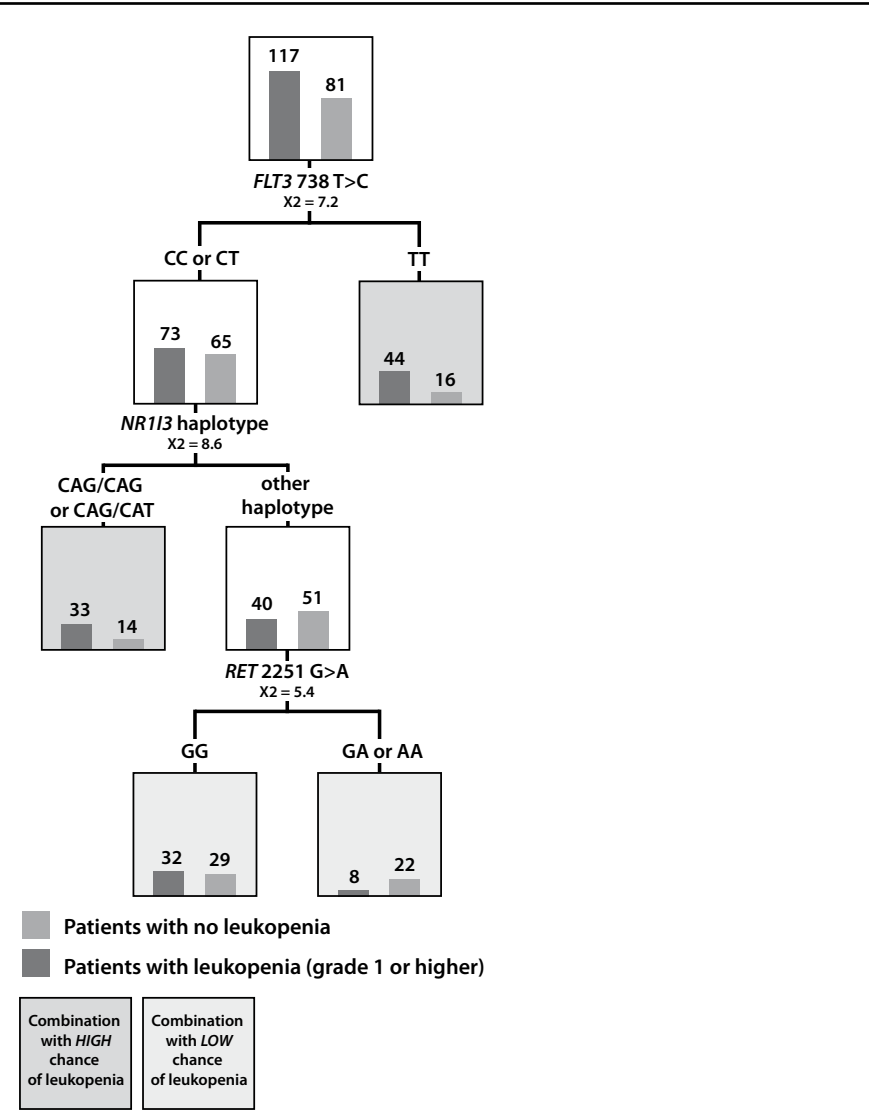
Classification and regression tree

The essence of the CART analysis is that patients are divided into groups with a unique genotype combination that predicts drug response. During the CART analysis, patients are subdivided successively, in such a way that a so-called 'classification tree' is grown. Each subgroup is split by the most discriminating polymorphism, which could be a different polymorphism for each subgroup. This procedure is continued until the pre-specified maximum tree depth is reached, or when each subgroup reaches a pre-specified minimum number of patients. Each terminal subgroup of the tree contains a ratio of responders to non-responders, which can be used to classify patients. Since each subgroup can be split by a different polymorphism, interaction can be detected, meaning that the influence of each polymorphism depends on the polymorphisms that split the subgroup in a previous level of the tree.

For the CART analysis, polymorphisms that are associated individually with drug response can be included, as well as haplotypes. In the sunitinib example, all polymorphisms and haplotypes were included as previously reported.²¹ The maximum tree depth was set to three levels, and no subgroup was allowed to contain less than 25 patients. Each subgroup was split based upon the highest χ^2 value. In figure 2, the classification tree is shown. The tree contained the polymorphism in *FLT3* (738T>C, step 1), the haplotype in *NR1I3* (step 2) and the polymorphism in *RET* (2251G>A, step 3). Each terminal group of the tree can be seen as a unique combination of genotypes – a genetic classifier. As in the MDR analysis, the genetic classifier was used in a regular statistical analysis to compute the OR. In a logistic regression analysis including WHO performance status, age and gender, the OR for the genetic classifier was 3.36 (95%CI, 1.84 to 6.15).

When a logistic regression analysis was performed with the three-way interaction between the *FLT3* 738T>C and *RET* 2251G>A polymorphisms and the *NR1I3* haplotype, the interaction term was not significantly associated with leukopenia, again emphasizing the fundamental difference between these two methods.

Figure 2 Classification and regression tree analysis of sunitinib-induced leukopenia



Each branch of the tree is divided by the polymorphism or haplotype with the highest χ^2 value. Terminal groups are shaded light grey or dark grey for relatively low and high risk of sunitinib-induced leukopenia, respectively. Since 59.1% of the patients experienced leukopenia (grade 1 or higher), a genetic classifier is considered high chance of leukopenia when the percentage of patients experiencing leukopenia exceeds 59.1%.

Interpretation and validation of the genetic classifier

Both MDR and CART analyses result in genetic classifiers that are associated with drug efficacy or toxicity. Since these methods rely on different ways to create this genetic classifier, they result in different classifiers that do not necessarily contain the same polymorphisms. In the MDR analysis, a genetic classifier is created in addition to polymorphisms that were individually associated with drug response, so that the genetic contribution to drug response is further explored. In the CART analysis, the genetic contribution to drug response is analyzed taking into account that a polymorphism may only have impact on drug response under the condition that another polymorphism is present. The similarity between the methods is that combinations of genotypes are investigated, rather than individual polymorphisms. This is more plausible from a biological point of view, because drug response is a complex trait and involves many proteins. Importantly, the CART and MDR methods detect statistical interaction, and the models do not necessarily contain polymorphisms in genes encoding enzymes that interact biologically. The interpretation of the genetic classifiers from a biological point of view is therefore not straightforward. The genetic classifiers contain polymorphisms that only exert their influence under the condition that other polymorphisms are present. In the MDR analysis of our sunitinib example, three polymorphisms in metabolic enzymes were included in the genetic classifier. From a biological point, it is likely that metabolic routes compete, and that the effect of one polymorphism on the metabolic capacity can be altered by others. When interpreting the genetic classifier obtained by CART in our sunitinib example, it appears that genetic variation in the metabolic enzyme NR1I3 is only relevant for carriers of the *FLT3* 738C-allele and not for carriers of the *FLT3* 738TT genotype, possibly because the latter are more sensitive to sunitinib-induced leukopenia regardless of the plasma levels of sunitinib.

Critical choices have to be made before these techniques can be applied, such as the number and selection of patients, the selected polymorphisms and the settings of the software. Importantly, when large numbers of polymorphisms are included in the interaction analysis, the number of possible interactions becomes enormous. In the current era of whole-genome profiling of more than a million polymorphisms, intelligent filtering of polymorphisms must be performed before interaction analysis, due to the computational requirement of such analysis.²⁴ Furthermore, both MDR and CART may result in genetic classifiers that predict drug response in the original patients better than in new patients because of potential over-fitting. The ORs for the genetic classifiers in our examples are therefore likely to be biased, and the true OR has to be obtained in an independent validation cohort. For these genetic classifiers to be applied in clinical practice, the genetic classifier should therefore be confirmed in independent cohorts.²⁵ Before the effort of external validation is undertaken,

internal validation can be performed to correct for over-fitting using for instance cross-validation.

Conclusion

Statistical techniques to analyze high-order interactions between polymorphisms, such as the MDR and CART techniques, create genetic classifiers that predict drug response. They have the major advantage over classic pharmacogenetic association studies that the complexity underlying drug response is studied and may therefore be more likely to be successfully replicated. When validated, these genetic classifiers can provide a novel and more rational approach to individualizing drug treatment.

Future perspective

We believe that complex interaction between polymorphisms will increasingly be studied in the near future, since the results from traditional pharmacogenetic association studies have been disappointing. The MDR and CART methods will probably be the most widely used, as they are widely available and relatively easy to apply. However, for the resulting genetic classifiers to reach the clinic, thorough validation must be performed using independent patient populations. Only when validation has been successful can the genetic classifiers be used to guide individualized therapy.

Executive summary

Background

- Recent pharmacogenetic association studies on frequently studied polymorphisms failed to replicate initial findings.
- Drug response is a complex phenomenon, and involves many different biochemical components, such as pharmacokinetic enzymes and molecular targets within a biochemical pathway.
- Traditional statistical analytical methods, such as (logistic) regression, are not suitable for detecting complex gene-gene interactions.

Multifactor dimensionality reduction and classification and regression tree

- Statistical analysis testing for gene-gene interactions can be performed using multifactor dimensionality reduction (MDR) or classification and regression tree (CART) analysis.
- The MDR and CART techniques have been applied successfully to identify genetic classifiers of sunitinib-induced toxicity.

Interpretation and validation of the results

- The MDR and CART techniques both result in genetic classifiers that predict drug response.
- These genetic classifiers must be validated in new patients before they can be used to individualize treatment.

Conclusion and future perspective

- The MDR and CART methods are more rational approaches to individualizing drug treatment when compared with traditional methods.

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102. <https://sourceforge.net/projects/mdr/files/mdrpt/>
Freely available permutation testing software for MDR (accessed February, 2010)



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**Pharmacogenetic interaction analysis
for the efficacy of systemic treatment in
metastatic colorectal cancer**

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Annals of Oncology 2010 doi:10.1093/annonc/mdq572

Abstract

Background

Pharmacogenetic markers related to drug metabolism and mechanisms of action could help to better select patients with metastatic colorectal cancer (mCRC) for treatment. Genetic interaction analysis is used as a rational tool to study the contribution of polygenic variation in relation to drug response.

Patients and methods

A selection of 17 polymorphisms in genes encoding drug targets, pathway molecules and detoxification enzymes was analyzed in 279 previously untreated mCRC patients treated with capecitabine, oxaliplatin and bevacizumab (CAPOX-B). Multifactor dimensionality reduction analysis was used to identify a genetic interaction profile for progression-free survival (PFS).

Results

Median PFS was 10.9 (95%CI, 9.4 to 12.4) months. A genetic interaction profile consisting of the *TYMS* enhancer region and *VEGF* +405G>C polymorphisms was significantly associated with PFS. Median PFS was 13.3 (95%CI, 11.4 to 15.3) and 9.7 (95%CI, 7.6 to 11.8) months for the beneficial and unfavorable genetic profiles, respectively, corresponding to a hazards ratio for PFS of 1.58 (95%CI, 1.14 to 2.19). None of the studied polymorphisms were individually associated with PFS.

Conclusions

Our results support a genetic interaction between the *TYMS* enhancer region and *VEGF* +405G>C polymorphisms as a predictor of the efficacy of CAPOX-B in mCRC patients.

Introduction

The combination of a fluoropyrimidine, such as 5-fluorouracil (5-FU) or capecitabine, oxaliplatin and the vascular endothelial growth factor (VEGF) blocking antibody bevacizumab (CAPOX-B) is a frequently used standard first-line treatment strategy for metastatic colorectal cancer (mCRC).^{1,2} However, since not all patients respond to this regimen, better criteria to select patients for this treatment are warranted. For this purpose, pharmacogenetic studies have been carried out with germline polymorphisms in genes that encode metabolic enzymes and drug targets (Table 1). However, the findings from these studies are not consistent.³ As a result, none of these polymorphisms are currently used in general practice to identify patients with an increased chance of response.

An explanation for these results could be that current analytical methods ignore or underestimate the complexity underlying drug response. Drug response involves many different proteins, such as therapeutic targets, molecules in the signaling pathway, metabolic enzymes or drug transporters. It may therefore be likely that the impact of polymorphisms in the corresponding genes exert their influence only in the presence of other polymorphisms. This concept is known as non-linear interaction, or epistasis.⁴ Studying the interaction between polymorphisms could therefore provide more reliable information compared with separate analyses of associations between individual polymorphisms and response.⁵ The resulting information can be transformed into genetic profiles that may have a prognostic and/or predictive value for mCRC patients.

The multifactor dimensionality reduction (MDR) methodology has been developed to study non-linear patterns of interactions between genetic profiles and drug response.⁶ In this study, we applied genetic interaction analysis using the MDR method to evaluate interaction between candidate polymorphisms in relation to the efficacy of CAPOX-B as first-line treatment in mCRC patients.

Materials and methods

Study population

Blood samples were collected from 279 of 368 previously untreated mCRC patients who were treated with CAPOX-B in the control arm of the multicenter prospective randomized phase III CAIRO2 study of the Dutch Colorectal Cancer Group (DCCG).¹ Capecitabine 1000 mg/m² (increased to 1250 mg/m² from cycle 7) was administered orally twice daily on days 1 to 14 of each 3-week treatment cycle. Oxaliplatin 130 mg/m² (maximum of six cycles) and bevacizumab 7.5 mg/kg were administered i.v. on day 1 of each treatment cycle. Treatment was continued until disease progression,

Table 1 Selected polymorphisms in drug targets, pathway molecules, metabolic enzymes and detoxification enzymes in mCRC patients treated with capecitabine, oxaliplatin and bevacizumab as first-line therapy

Gene	rs-number	genetic alteration	effect of polymorphism on the protein level
Capecitabine^{8,10,20}			
MTHFR	rs1801133	677C>T	Ala222Val; reduced MTHFR activity for the 222Val-allele ⁴⁵
MTHFR	rs1801131	1298A>C	Gln429Ala; decreased MTHFR activity for the 429Ala-allele ⁴⁶
TYMS	-	1494 +/- 6bp (3'-UTR)	Reduced TYMS expression for the 6bp deletion allele ⁴⁷
TYMS	-	VNTR 2/3C/3G (TSER)	Increased TYMS expression for the 3G allele ^{33,34}
Oxaliplatin^{18,29}			
ERCC1	rs11615	496C>T	Asn118Asn; decreased ERCC1 expression for the 496T allele ⁴⁸
ERCC2	rs238406	499C>A	Arg156Arg; supposed reduced DNA-repair capacity for 499A-allele ⁴⁹
ERCC2	rs13181	2251A>C	Lys751Gln; reduced DNA-repair capacity for 751Gln-allele ⁵⁰
ERCC2	rs1799793	965G>A	Asp321Asn; reduced DNA-repair capacity for 321Asn-allele ⁵⁰
XRCC1	rs25487	1301G>A	Arg399Gln; deficient DNA-repair for 399Gln-allele
GSTP1	rs1695	313A>G	Ile105Val; decreased GSTP1 activity for 105Val-allele ⁵¹
Bevacizumab^{30,31}			
KDR	rs1870377	1719A>T	Gln472His; amino acid substitution located in fifth NH2-terminal Ig-like domain within the extracellular ligand binding region ⁵²
KDR	rs2071559	-604T>C (promoter)	alteration of the binding site for transcriptional factor E2F ⁵²
VEGF	rs1570360	-1154G>A (promoter)	decreased VEGF production and expression for -1154A allele ^{53,54}
VEGF	rs2010963	+405G>C (5'-UTR)	decreased VEGF release for +405C allele ⁴⁰ and increased serum VEGF for +405C allele ⁴¹
VEGF	rs3025039	936C>T (3'-UTR)	decreased VEGF levels for the 936T allele ^{55,56}
VEGF	rs699947	-2578C>A (promoter)	decreased VEGF production for -2578A allele ⁵⁴ and increased VEGF expression for -2578A allele ⁵³
VEGF	rs833061	-460C>T (promoter)	increased promoter activity for -460T allele ⁵⁷

Abbreviations: 5FU, 5-fluorouracil; ERCC1, excision repair cross-complementing group 1; ERCC2, excision repair cross-complementing group 2; GSTP1, glutathione s-transferase pi 1; KDR, kinase domain receptor (=vascular endothelial growth factor receptor 2); MTHFR, methylene tetrahydrofolate reductase; TSER, thymidylate synthase enhancer region; TYMS, thymidylate synthase; VEGF, vascular endothelial growth factor A; VNTR, variable number of tandem repeats; UTR, untranslated region; XRCC1, X-ray cross-complementing group 1.

death or unacceptable toxicity, whichever occurred first. Patient eligibility criteria and further details of the study have been previously described.¹ The collection of a peripheral blood sample for pharmacogenetic research was pre-specified in the study protocol and required additional written informed consent. The protocol was approved by the local institutional review boards of all participating centers. Patients in the experimental cetuximab-containing study arm of the CAIRO2 study were not included in this pharmacogenetic study since the addition of cetuximab resulted in a decreased progression-free survival (PFS), the primary endpoint of the study.¹

Genotyping

The studied genetic polymorphisms are shown in Table 1. These polymorphisms were selected primarily on the basis of the pharmacokinetics and pharmacodynamics of capecitabine, oxaliplatin and bevacizumab and on the known functional effects at the protein level.⁷ Moreover, these polymorphisms have been included in previous pharmacogenetic association studies of 5-FU, capecitabine or oxaliplatin in mCRC.⁸⁻²⁹ Since results of only two pharmacogenetic studies for bevacizumab have been reported^{30,31}, polymorphisms in VEGF and its receptor (kinase domain receptor, KDR) were selected.³² Germline DNA was isolated from peripheral white blood cells by the standard manual salting-out method. Genotyping was carried out on a Biomark system (Fluidigm, South San Francisco, CA, USA) according to the protocol provided by the manufacturer using pre-designed TaqMan assays (Applied Biosystems, Foster City, CA, USA). The polymorphisms in the thymidylate synthase enhancer region (TSER) in the promoter of the *TYMS* gene (two or three 28-bp repeats including the C>G polymorphism in the third repeat; *TYMS*-TSER) were analyzed by direct sequencing. The genotype was expressed as non-carriage of the 3G-allele (2/2, 2/3C and 3C/3C genotypes) versus carriage of the 3G-allele (2/3G, 3C/3G and 3G/3G), since the 3G allele results in increased TYMS activity.^{33,34} The 6-bp insertion/deletion (*TYMS* +/-6bp) polymorphism in the 3' untranslated region was determined using fragment analysis. Each assay was conducted with 10% duplicates, with water as negative control. The overall call rate was 0.948 (0.803 to 0.989) and none of the polymorphisms significantly deviated Hardy-Weinberg equilibrium (P>0.01).

Statistical analysis

Genotypes that are individually associated with drug response will usually end up in the best genetic profile in the genetic interaction analysis without providing substantial information gain. Therefore, the association between each individual polymorphism (treated as an ordinal variable, representing an additive model) and PFS as dependent variable was tested using a Cox proportional hazards model including serum LDH, age and gender as covariates. Polymorphisms significantly

associated with PFS ($P < 0.05$) were excluded from the subsequent interaction analysis, but would be introduced in the final multivariate analysis (see below). Also, haplotypes disturb the selection of the best genetic profile because of over fitting the data due to the number of possible haplotype combinations and were therefore also not used in the interaction analysis. No haplotype was individually associated with PFS in our study (data not shown).

To study interaction between the polymorphisms in relation to response, the MDR software was used (version 2.0 beta 6; available on <http://sourceforge.net/projects/mdr/>).⁶ The software requires a complete dataset with no missing data. Therefore, missing data for polymorphisms with $\leq 5\%$ missing data were imputed by genotypes based upon the genotype frequency of the polymorphism, taking the distribution of other polymorphisms in the same gene into account. Missing data for polymorphisms with $> 5\%$ missing data (*TYMS*-TSER, *TYMS* +/-6bp, *VEGF* -1154G>A and *VEGF* +936C>T) were considered a separate 'missing genotype group' in the genetic interaction analysis. If the genetic interaction analysis resulted in a combination consisting of a genotype with a 'missing genotype group', the procedure was repeated without this group and results were compared with the initial results.

Our study is designed to identify a subgroup of patients with increased PFS. The median PFS in our study population was 10.9 months. However, it is assumed that the patients with beneficial genetic profiles have a PFS much longer than the median, whereas patients with unfavorable genetic profiles have PFS much shorter than the median. We therefore included patients in the shortest and longest quartiles for PFS in the genetic interaction analysis, in order to increase discriminating power.³⁵⁻³⁷

The entire cohort was used in the final analysis of the genetic profile (see below).

Sensitivity analysis showed that when the patients with censored data before the 75% quartile cut-off point were included in the longest quartile, or when tertiles were used instead of quartiles, results remained unchanged (data not shown), indicating that our choices regarding censoring and enrichment do not influence the results of the study.

In the genetic interaction analysis, the ratio between patients in the shortest quartile to patients in the longest quartile for each genotype combination is evaluated. Combinations with more patients in shortest quartile than in the longest quartile are considered high chance of short PFS, and vice versa. This procedure was carried out across 10-fold cross-validation samples to avoid over fitting, and was repeated for all possible combinations of two up to four polymorphisms. The genotype combination with the highest accuracy (fraction of correctly classified patients) in the validation sample was considered the combination that best predicts PFS, and was selected for further analysis. A P-value for the statistical significance of the accuracy was obtained using 1000-fold permutation testing (software available on <https://sourceforge.net/projects/mdr/files/mdrpt/>).

The genotype combination with the highest accuracy in the validation sample was recoded into a genetic profile predictive for PFS. This genetic profile was subsequently used for all 279 patients in the CAIRO2 study from whom a blood sample was available, including the patients from the intermediate PFS group, to estimate survival curves using the Kaplan-Meier method. The difference in PFS from the beneficial genetic profile versus the unfavorable genetic profile was estimated using the log-rank test. A Cox proportional hazards model including the genetic profile, age, gender, prior adjuvant chemotherapy (yes versus no), number of affected organs (1 versus > 1), serum LDH and any polymorphisms that were individually associated with PFS was used to compute the adjusted hazards ratio (HR) and 95% confidence interval (95%CI). Given the exploratory nature of this study, no adjustment for multiple testing was carried out, and a P value of < 0.05 was considered significant. The Kaplan-Meier and Cox proportional hazards analyses were carried out using SPSS version 17.0 (SPSS, Chicago, IL, USA).

Results

At the time of analysis, the primary end point of PFS was reached in 225 of 279 eligible patients (80.6%). Median PFS was 10.9 months (95%CI, 9.4 to 12.4 months). Two patients were censored in the shortest quartile, and were excluded from the genetic interaction analysis, since the actual PFS of these patients was unknown. Censored events in the longest quartile were not excluded, since PFS for these patients was at least longer than the 75% quartile cut-off point. The shortest and longest quartiles for PFS were below 6.7 and above 15.5 months, respectively, each consisting of 70 patients.

None of the genetic polymorphisms were individually associated with PFS in the Cox proportional hazards analysis (Table 2). Therefore, all polymorphisms were included in the genetic interaction analysis with PFS.

The combination of the *TYMS*-TSER and *VEGF* +405G>C had the highest accuracy of 0.650 ($P = 0.027$, 1000-fold permutation testing; 0.624 after exclusion of missing data), meaning that 65% of the patients were correctly classified according to the genetic profile (Figure 1a). The distribution of patients in the shortest and longest PFS quartiles for the combination of *TYMS*-TSER and *VEGF* +405G>C genotypes is shown in Figure 1b. All other combinations of two, three and four polymorphisms each resulted in lower accuracies in the genetic interaction analysis, and were therefore not considered for further evaluation.

When all 246 patients with complete genotype data were used, 137 and 109 patients were in the beneficial and unfavorable profiles for PFS, respectively. In Figure 2, the frequency distribution of the genetic profile across the four quartiles for PFS is shown. Interestingly, the frequency of the unfavorable profile decreases for every quartile, even for the two middle quartiles ($P < 0.001$, χ^2 test for trend).

Table 2 Individual associations of polymorphisms with progression free survival in mCRC patients treated with capecitabine, oxaliplatin and bevacizumab as first-line therapy

Polymorphism	allelic HR*	95% CI	P
MTHFR 677C>T	1.00	0.81-1.23	0.991
MTHFR 1298A>C	0.91	0.74-1.13	0.393
TYMS 1494 +/- 6bp	1.10	0.87-1.40	0.410
TYMS VNTR 2/3C/3G	1.02	0.77-1.36	0.884
ERCC1 496C>T	1.12	0.92-1.37	0.243
ERCC2 499C>A	1.15	0.94-1.40	0.185
ERCC2 2251A>C	1.00	0.82-1.21	0.968
ERCC2 965G>A	0.80	0.63-1.01	0.058
XRCC1 1301G>A	0.98	0.81-1.18	0.811
GSTP1 313A>G	0.98	0.81-1.19	0.837
KDR 1719A>T	1.08	0.88-1.33	0.465
KDR -604T>C	1.03	0.86-1.24	0.738
VEGF -1154G>A	1.09	0.90-1.33	0.381
VEGF 405G>C	0.97	0.81-1.18	0.785
VEGF 936C>T	0.98	0.74-1.29	0.889
VEGF -2578C>A	1.03	0.86-1.23	0.763
VEGF -460C>T	1.00	0.84-1.20	0.990

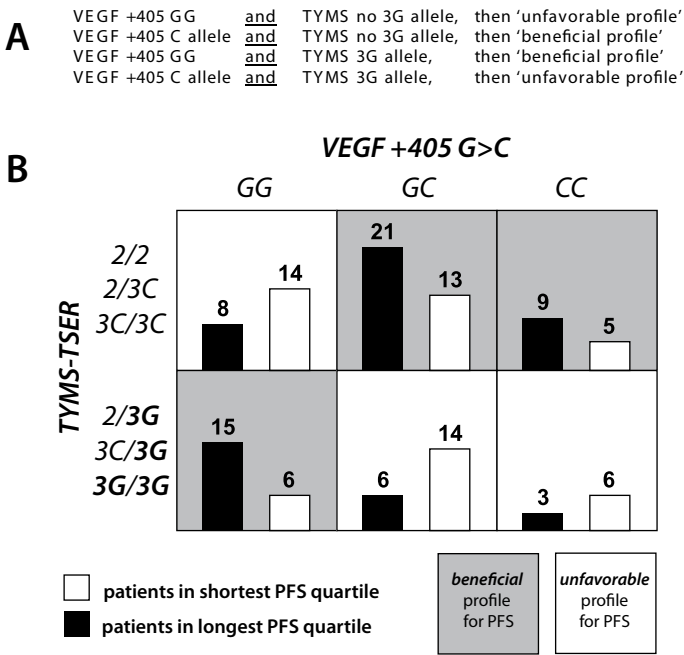
* Hazard ratios (HR), 95% confidence intervals (95%CI) and P-values were calculated for each polymorphism using a Cox proportional hazards model with age, gender and serum LDH as covariates.

Abbreviations: ERCC1, excision repair cross-complementing group 1; ERCC2, excision repair cross-complementing group 2; GSTP1, glutathione s-transferase pi 1; KDR, kinase domain receptor (=vascular endothelial growth factor receptor 2); MTHFR, methylene tetrahydrofolate reductase; TYMS, thymidylate synthase; VEGF, vascular endothelial growth factor A; VNTR, variable number of tandem repeats; XRCC1, X-ray cross-complementing group 1.

The PFS curves for the genetic profile for all patients are shown in Figure 3. The median PFS was 13.3 (95%CI, 11.4 to 15.3) and 9.7 (95%CI, 7.6 to 11.8) months for the beneficial and unfavorable profiles, respectively (P<0.001, log-rank test).

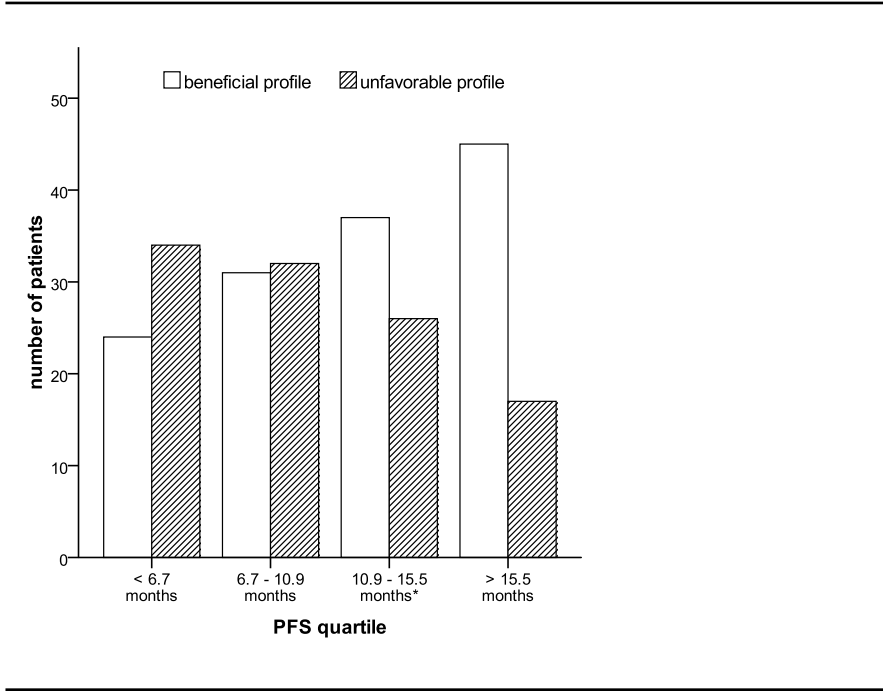
In the multivariate Cox proportional hazards model including age, gender, prior adjuvant chemotherapy, number of affected organs and serum LDH, the HR for the genetic profile for PFS was 1.58 (95%CI, 1.14 to 2.19; P=0.006).

Figure 1 Genetic interaction profile for CAPOX-B



A: Algorithm based upon the results of the genetic interaction analysis to translate the genotype-combinations of the *TYMS*-*TSER* and *VEGF* +405G>C polymorphisms into a risk factor – or genetic profile – for PFS. **B:** Distribution of patients in the short (white bars) and long PFS quartiles (black bars) across the different genotype combinations of *TYMS*-*TSER* and *VEGF* +405G>C. Combinations with more patients in the short quartile are shaded white (unfavorable profile), whereas combinations with more patients in the long quartile are shaded grey (beneficial profile).

Figure 2 Distribution of the genetic profile across the four PFS quartiles



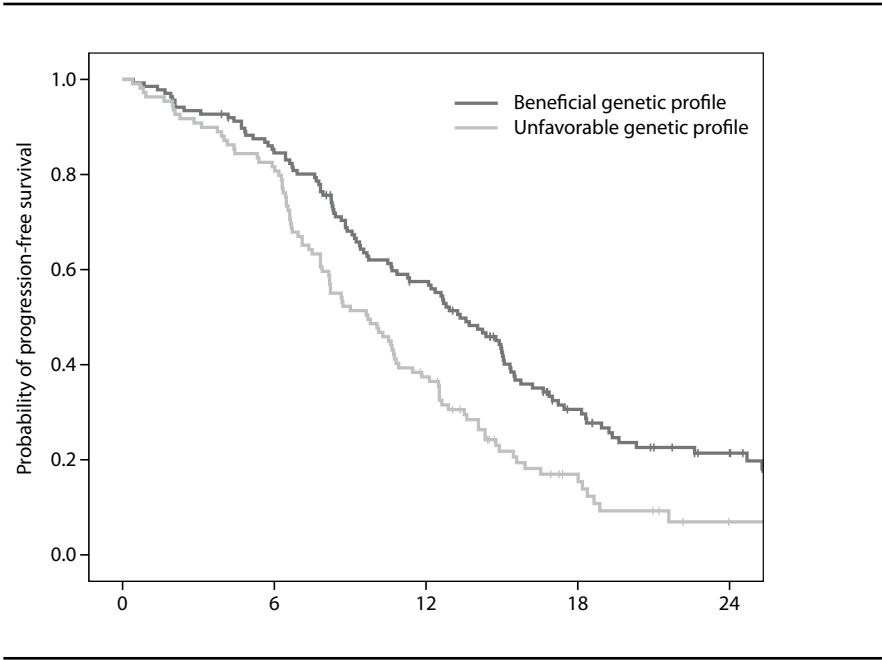
The frequency of the unfavorable profile decreases for each quartile for PFS ($P<0.001$, χ^2 test for trend).
* Patients who were censored before the fourth quartile were included in the third quartile.

Discussion

We showed that a genetic interaction profile consisting of the *VEGF* +405G>C and *TYMS*-TSER polymorphisms correlates with PFS in mCRC patients treated with CAPOX-B. This approach provides a novel way to use pharmacogenetic variation to individualize treatment since individual polymorphisms were not associated with PFS.

To exclude profound individual associations with PFS that could interfere with the genetic interaction analysis, we first tested for associations of the individual polymorphisms with PFS. No significant associations were detected, analogous to the absence of associations for other individual molecular markers in mCRC.³⁸ The genetic interaction analysis takes the complexity of interacting polymorphisms in genes encoding drug targets, metabolic enzymes and detoxification enzymes into account. Our study shows that – in mCRC patients treated with first-line CAPOX-B –

Figure 3 Kaplan Meier curves for the genetic profile for all mCRC patients treated with capecitabine, oxaliplatin and bevacizumab as first-line treatment



137 and 109 patients were in the unfavorable and beneficial profile groups, respectively. The median PFS was 13.3 (95%CI, 11.4 to 15.3) and 9.7 (95%CI, 7.6 to 11.8) months for the beneficial and unfavorable profiles, respectively ($P<0.001$, log rank test).

the *TYMS*-TSER polymorphism and the *VEGF* +405G>C polymorphisms are dependent of each other in their impact on PFS. VEGF is the natural ligand for the VEGF receptor, through which it induces angiogenesis. Bevacizumab neutralizes VEGF, resulting in decreased tumor angiogenesis, which in turn affects intratumoral hypoxia, nutrition status and/or disposition of concurrent chemotherapy.³⁹ The functional consequence of the *VEGF* +405G>C polymorphism remains to be elucidated. One *in vitro* study reported increased VEGF release by lipopolysaccharide-stimulated peripheral mononuclear blood cells with the *VEGF* +405G-allele⁴⁰, but another study showed that VEGF serum levels were highest for healthy volunteers with the *VEGF* +405CC genotype.⁴¹ The fluorodeoxyuridine monophosphate (FdUMP) metabolite of capecitabine inhibits the *TYMS* enzyme, and thereby induces DNA damage.⁴² Previous *in vitro* experiments indicated that the *TYMS*-TSER 3G allele results in higher expression of *TYMS*.^{33,34} The finding by Marcuello

et al. that the *TYMS*-T581 3G allele is associated with decreased efficacy of 5FU-based chemotherapy in mCRC patients¹⁵, is therefore only present for *VEGF* +405C-allele carriers in our study. However, further fundamental research should be undertaken to understand the exact biological mechanism of the genetic profile with regard to the efficacy of CAPOX-B.

For the genetic interaction analysis, the PFS endpoint initially was converted into a binary outcome. We assumed that the patients with beneficial genetic profiles have a PFS much longer than the median, whereas those with unfavorable genetic profiles have PFS much shorter than the median. By using the shortest and longest quartiles for PFS, we anticipated sufficient discriminating power, while keeping the groups reasonably large. This concept of enrichment is an accepted method in genetics.³⁵⁻³⁷ Even though our choice of cut-off remains arbitrary, our sensitivity analysis showed similar results for the genetic profile, indicating that the results were not significantly influenced by the choice of quartiles for PFS.

There are some limitations to our findings. First, the genetic interaction analysis relies on data mining to identify the best model – or genetic profile – to fit the data⁶, potentially leading to over fitting, with optimal results only in the initial test cohort. Although we used cross-validation to correct for over fitting, the genetic profile should be validated in an independent cohort to confirm our present finding and to assess its clinical utility.⁴³

Finally, without an untreated control group it remains unclear whether the genetic profile is predictive for response to CAPOX-B in mCRC patients, or prognostic for mCRC outcome regardless of treatment.⁴⁴ Also, given the many available salvage treatments which were not part of the study protocol and were therefore not controlled, the assessment of a potential prognostic role was not feasible. However, the fact that both polymorphisms of the genetic profile are in the targets of two of the drugs suggests that the profile is predictive rather than prognostic.

In conclusion, we demonstrated a significant correlation between a genetic profile consisting of the *TYMS*-enhancer region and *VEGF* +405G>C polymorphisms and improved PFS. This genetic profile is a novel marker that may identify a subgroup of mCRC patients with increased probability of benefit to CAPOX-B. To our knowledge, this is the first study to explore the interaction between polymorphisms in relation to the efficacy of cancer chemotherapy. Testing for the interaction between polymorphisms is probably more rational than testing of each individual polymorphism, since drug response is a complex phenomenon. If confirmed in independent studies, our results provide a novel tool to better select cancer patients for potentially toxic and expensive treatments.

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9

Genome-wide association study of the efficacy of capecitabine, oxaliplatin and bevacizumab in metastatic colorectal cancer

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This study was financially supported by a grant from the Dutch Cancer Society UL2009-4528

Abstract

Background

A more optimal selection of patients that will benefit from the frequently used first-line treatment of advanced colorectal cancer (ACC) consisting of the combination of capecitabine, oxaliplatin and bevacizumab (CAPOX-B) is warranted. We used a genome-wide association study to find single nucleotide polymorphisms (SNPs) that are associated with the efficacy of CAPOX-B.

Methods

Germline DNA was obtained from 547 previously untreated ACC patients in the randomized phase III CAIRO2 trial, in which patients were randomized between CAPOX-B or CAPOX-B plus cetuximab. Whole-genome genotyping was performed using 700 k Illumina OmniExpress BeadChip arrays. Associations between SNPs and progression-free survival (PFS) were tested using Cox-proportional hazard models. Associations were considered significant when $P < 5 \times 10^{-8}$.

Results

Three SNPs located at 8p23.1 showed a trend toward significance for association with PFS (rs292936519, $P = 1.24 \times 10^{-7}$; rs2912024, $P = 1.38 \times 10^{-7}$ and rs2978931, $P = 6.75 \times 10^{-7}$). These SNPs are 20 kbp downstream of the *AGPAT5* gene, which encodes a protein that is involved in phospholipid biosynthesis.

Conclusion

Even though these results possibly identify a novel genetic predictor for the efficacy of CAPOX-B, further analyses are required before definitive conclusions can be made based upon these data.

Background

A frequently used first-line treatment of advanced colorectal cancer (ACC) consists of the combination of capecitabine, oxaliplatin and bevacizumab (CAPOX-B).¹ Even though this combination results in a prolongation of survival compared with no treatment, the one year progression-free survival (PFS) rate is below 50%.^{2,3} In order to reduce toxicity and costs, a more optimal selection of patients that will benefit from modern systemic treatment is warranted.

Heritable genetic variation has proven to predict variation in response to many therapeutics drugs.⁴ The basis of such research is currently limited to genetic variation in target or metabolic enzymes that have been selected using the candidate gene approach. The disadvantage of this approach is that it is limited to current knowledge of the mechanism of action of the investigated drugs. Since it is estimated that there are more than 10,000,000 single nucleotide polymorphisms (SNPs) in the human genome, it is very likely that many of these SNPs are not detected in the current approach of pathway based research.

Genome-wide association studies, in which the entire genome is characterized for SNPs, have been applied in the past years to identify risk factors for several types of cancers in large case-control series.⁵ Regarding outcome of systemic therapy, genome-wide association studies have identified SNPs associated with musculoskeletal adverse reactions to aromatase inhibitors⁶, treatment response for childhood acute lymphoblastic lymphoma⁷ and pharmacokinetics of methotrexate⁸. All of these studies are based upon a case-control design with χ^2 -tests to test for associations, but survival could also be applied as an endpoint using Cox-proportional hazards models to test for associations.

Here we present the first results of a genome-wide association study to find SNPs that are associated with the efficacy of first-line CAPOX-B for ACC in a clinical trial setting with PFS as the primary endpoint.

Patients and Methods

Patients

Germline DNA was obtained from 547 of 736 previously untreated ACC patients who were randomized between treatment with CAPOX-B or CAPOX-B plus cetuximab in the multicenter randomized phase III CAIRO2 trial of the Dutch Colorectal Cancer Group (DCCG).⁹ Capecitabine 1000 mg/m² (increased to 1250 mg/m² from cycle 7) was administered orally twice daily on days 1–14 of each 3-week treatment cycle. Oxaliplatin 130 mg/m² (maximum of six cycles) and bevacizumab 7.5 mg/kg were administered intravenously on day 1 of each treatment cycle. For patients randomized to the

CAPOX-B plus cetuximab arm, cetuximab was administered intravenously at a dose of 400 mg/m² on the first day, followed by 250 mg/m² weekly thereafter. Treatment was continued until disease progression, death or unacceptable toxicity, whichever occurred first. Patient eligibility criteria are described in detail elsewhere.⁹ The collection of a peripheral blood sample for pharmacogenetic research was pre-specified in the study protocol and required additional written informed consent. The protocol was approved by the local institutional review boards of all participating centers.

Genotyping and quality control

Germline DNA was isolated from peripheral white blood cells by the standard manual salting-out method. Genotyping was performed on Human OnmiExpress v12 BeadChip arrays containing 733,202 markers (Illumina, San Diego, CA, USA) using technical facilities at the Leiden Genome Technology Center (LGTC, Leiden, The Netherlands). Genotype calls were set using GenomeStudio software (Illumina). Patients with a call-rate of < 0.98 were excluded from further analysis. The following cut-off values were used to filter out incorrectly called genotypes: GenCall ≥ 0.85; ClusterSep ≥ 0.3; CallFreq > 0.85; AB T-mean 0.2 – 0.8, resulting in the exclusion of 3172 markers (0.43%). Further quality control of the data was performed using R (<http://www.r-project.org/>). Ten patients (1.83%) were excluded based upon the sex check, and 16 patients (2.92%) were excluded based upon 4-dimensional multi-dimensional scaling (MDS) analysis to detect possible population stratification. In total, 26 patients were excluded, resulting in 521 evaluable patients. For the markers, a minimum allele frequency for this analysis was set at 0.05, resulting in excluding 125,800 markers (17.2%). The call-rate cut-off per marker was set at 0.98, resulting in excluding 16,981 markers (2.3%). The distribution of the marker-missingness per chromosome showed no unexpected pattern. Hardy-Weinberg equilibrium (HWE) was evaluated per marker using a χ^2 goodness-of-fit statistic. Based upon the QQ-plot of observed P-values against expected P-values for HWE, 1168 markers (0.2%) were excluded with a HWE P-value of $\leq 5.0 \times 10^{-7}$. After these quality checks, 589,274 markers remained for the statistical analysis.

Statistical analysis

For each marker, a Cox proportional hazards model was calculated using R, which included age, gender and treatment arm as covariates. Since it is not known whether the effects of the markers are dominant, recessive or multiplicative, each marker was included in a multiplicative model (i.e. AA = 0, AB = 1 and BB = 2). Observed P-values were plotted against theoretical P-values (QQ-plot), and the inflation factor was calculated by $(\text{median}(T_{p_1}, \dots, T_{p_n})/0.675)^2$ with $T_{p_1}, \dots, T_{p_n}$ being the square roots of the χ^2

quantiles for the P-values of the markers. Formal significance for a marker was assumed for $P < 5 \times 10^{-8}$. To check for effects that could be ascribed to the treatment arm, interaction between the marker and treatment arm was included in the model. The association was tested only in the CAPOX-B arm if the P-value of the marker*arm interaction term was < 0.001. Kaplan-Meier curves were estimated for the marker with the lowest P-value using SPSS version 17.0 (SPSS, Chicago, IL, USA).

Results

Patients

At the time of the analysis (December 2010), the primary endpoint PFS was reached in 459 patients (88.1%). Median PFS was 10.6 months (95% confidence interval [95%CI], 9.5 to 11.6 months). In the CAPOX-B and the CAPOX-B plus cetuximab arms, median PFS was 10.8 months (95%CI, 9.0 to 12.5 months) and 10.1 months (95%CI, 9.0 to 11.3 months), respectively. Baseline patient characteristics are described in Table 1.

Table 1 Baseline patient characteristics

Characteristic	
Age - year	
median	63.1
range	27.6 - 83.6
Sex - no (%)	
male	316 (60.7%)
female	205 (39.3%)
Arm - no (%)	
CAPOX-B	264 (50.7%)
CAPOX-B plus cetuximab	257 (49.3%)
Serum lactate dehydrogenase level - no (%)	
normal*	307 (58.9%)
above normal*	213 (40.9%)

* according to the cutoff values of each individual center

Genotype results

Three SNPs (rs2936519, rs2912024 and rs2978931) located on chromosome 8, cytogenic band 8p23.1, showed the lowest P-values ($P = 1.24 \times 10^{-7}$, $P = 1.38 \times 10^{-7}$ and $P = 6.75 \times$

10⁻⁷, respectively; Table 2 and Figure 1), but did not reach the formal significance level of $P < 5 \times 10^{-8}$. These three SNPs were in linkage. None of ten most significant SNPs showed a significant interaction with treatment arm. The inflation factor for the analysis was 0.98, indicating that there was no population stratification or other bias in the analysis.

Table 2 Top 10 SNPs with lowest P-values for association with PFS in a Cox-proportional hazards model with age, gender and treatment arm as covariates

marker	chr	position	gene	allele frequency	P-value	allelic HR (95%CI)
rs2936519	8	6626650	n.a.	0.104	1.24×10^{-7}	0.545 (0.435 – 0.682)
rs2912024	8	6626309	n.a.	0.105	1.38×10^{-7}	0.547 (0.437 – 0.685)
rs2978931	8	6625491	n.a.	0.101	6.75×10^{-7}	0.561 (0.447 – 0.705)
rs4850159	2	131442241	ARHGEF4	0.136	2.58×10^{-6}	0.627 (0.516 – 0.762)
rs6734725	2	46751074	n.a.	0.350	2.99×10^{-6}	0.713 (0.619 – 0.822)
rs17688362	18	39999678	n.a.	0.185	4.68×10^{-6}	0.657 (0.548 – 0.786)
rs17444829	4	113593423	n.a.	0.133	5.49×10^{-6}	1.556 (1.285 – 1.884)
rs11730442	4	113581912	ALPK1	0.132	6.62×10^{-6}	1.554 (1.282 – 1.884)
rs10089490	8	92317629	n.a.	0.061	7.20×10^{-6}	1.849 (1.413 – 2.420)
rs17395916	4	86945264	ARHGAP24	0.402	7.61×10^{-6}	0.739 (0.647 – 0.844)

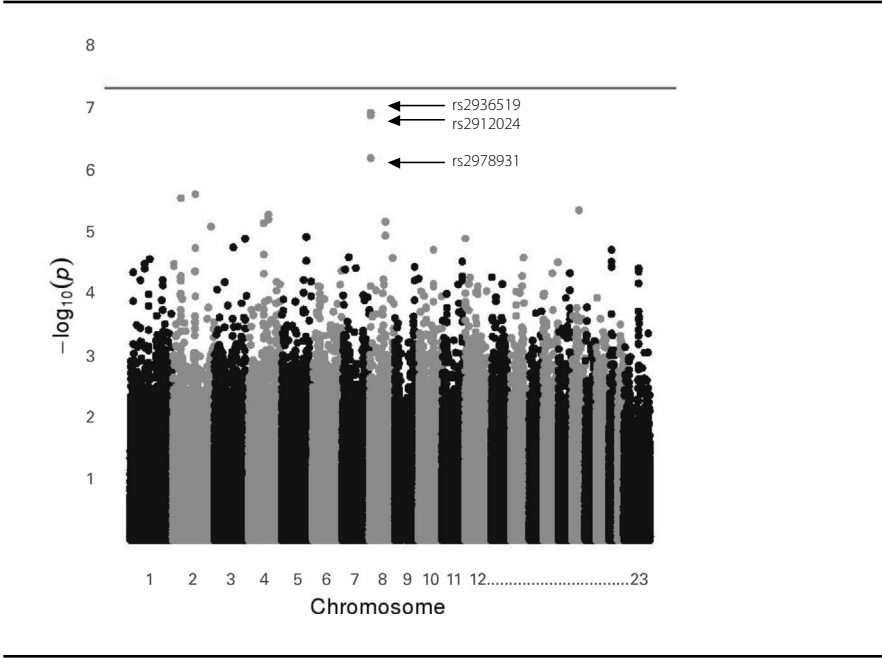
Abbreviations: ALPK1, α-kinase 1; ARHGAP24, Rho GTPase activating protein 24; ARHGEF4, Rho guanine nucleotide exchange factor 4; chr, chromosome; 95%CI, 95% confidence interval; HR, hazards ratio; n.a., marker is not located within a gene

In Figure 2, the Kaplan-Meier curves are shown for the most significant SNP, rs2936519. Median PFS was 8.1 months (95%CI, 6.6 to 9.7 months) and 11.4 (95%CI, 10.4 to 12.4 months) for C/T and C/C genotypes, respectively. Only one patient was homozygous for the T-allele (this patient did contribute to the P-value, but is not shown in figure 2).

Discussion

In this first analysis, three SNPs – that are in linkage – located at 8p23.1 showed a trend toward significance for association with PFS in ACC patients treated with CAPOX-B. The top three most significant SNPs are not located within a known gene, but are approximately 20 kbp downstream of the *AGPAT5* gene (1-acylglycerol-3-phosphate O-acyltransferase 5, also known as lysophosphatidic acid acyltransferase, epsilon).

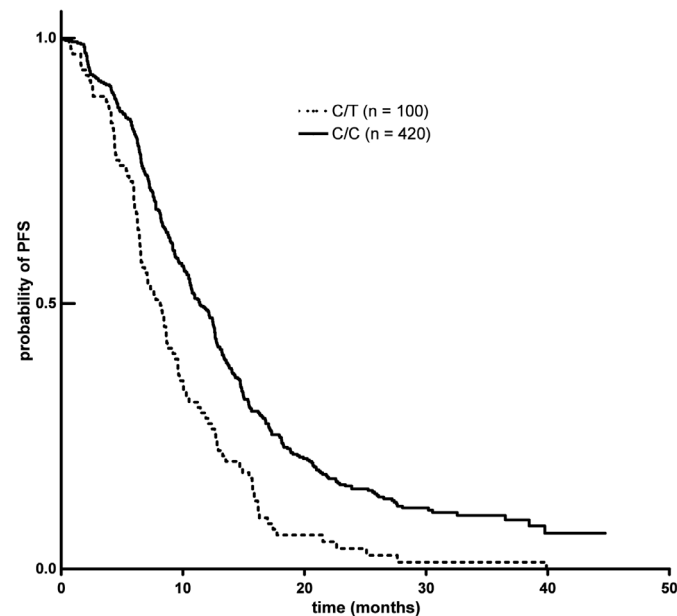
Figure 1 Manhattan plot of $-\log_{10}(P\text{-values})$ from the Cox-proportional hazards model adjusted for age, gender and treatment arm



The horizontal line represents the formal genome-wide significance level of 5×10^{-8}

This gene encodes an integral membrane protein that converts lysophosphatidic acid (LPA) to phosphatidic acid (PA), the second step in de novo phospholipid biosynthesis, the major constituent of the cell-membrane (<http://www.ncbi.nlm.nih.gov>). Additionally, LPA is a potent mitogen that has been linked to the development and progression of breast cancer.¹⁰ When these markers have been fully evaluated (i.e. functional analysis on gene-function or gene-expression level) and their associations have been confirmed in an independent cohort, they could be used to optimize selection of ACC patients for CAPOX-B treatment.

This is the first pharmacogenomic genome-wide study on the efficacy of palliative therapy for ACC. Unfortunately, the associations between the SNPs and PFS did not reach formal statistical significance at the 5×10^{-8} level, but a trend toward significance was found for 3 SNPs. This could be the result of insufficient power, possibly in combination with very stringent correction for multiple testing. Otherwise, the results may simply be false positive findings based upon the large number of statistical tests. There are 47 more patients that have to be genotyped, and were therefore not

Figure 2 Kaplan Meier curves of PFS for the rs2936519 SNP

included in this analysis. Possibly, including these patients would increase power enough for the results to become significant.

On the other hand, it is unlikely that a complex phenotype such as drug response depends on only a handful single SNPs. As with other complex traits, a series of polymorphisms could contribute to the phenotype. A predictive model can be built to assess the combined contribution of SNPs to the phenotype – which will be further evaluated in our study. That this could be a feasible approach is illustrated by a recent example on human height, in which it initially seemed that only a few SNPs were associated with this phenotype. However, the explained variability was only ~5%.^{11–13} When other genetic information from the same genome-wide studies was included in a predictive model for human height, 45% of the variability could be explained. This strategy has also been applied for the risk of schizophrenia and bipolar disorder.¹⁴ Validation of such a predictive model in an independent cohort is very important because of the possibility of false positive findings due to the huge number of polymorphisms that are included, even though internal cross-validation can be used while developing the predictive model.¹⁵

Since all patients in our study were treated with CAPOX-B, no distinction could be made between prognostic (i.e. not related to treatment) and predictive (i.e. related to treatment) effects. If true significant effects would be found, the effects of the markers could therefore also be unrelated to therapy. However, it would be difficult to test whether the associations are predictive or prognostic, since a no-treatment control arm in the first-line treatment of ACC would be unethical. When the same associations would be found in a cohort of ACC patients that are treated with other agents as first-line therapy, the markers could then be regarded as prognostic rather than predictive. However, such a cohort is not feasible with fluoropyrimidines currently being the backbone of first-line ACC treatment.

The top three SNPs are in linkage, and are located near the *AGPAT5* gene, which encodes a protein that converts LPA into PA, and is involved in phospholipid biosynthesis. It has to be elucidated whether these SNPs have an effect on the expression or function of this gene, or whether these SNPs are in linkage with a functional SNP in this gene. Possibly, fine-mapping or imputation in the region around the three significant SNPs could help finding the true causative SNP.

LPA has been linked to development and progression of breast cancer. Downstream of LPA receptor activation, the GTPase rho is activated.¹⁶ Two other genes with SNPs that are in the top 10 of most significant SNPs are possibly also involved in this signaling route (*ARHGEF4* and *ARHGAP24*), suggesting that the LPA signaling pathway could be important for CAPOX-B efficacy or prognosis of ACC. Moreover, as phospholipids make up an essential component of the cell-membrane, altered biosynthesis of phospholipids could have an effect on (tumor) cell division and therefore also efficacy of chemotherapy. However, such reasonings remain highly speculative, and the mechanism underlying the associations found in this study requires fundamental research.

Two SNPs in the top 10 are located in or near the gene encoding α -kinase 1 (*ALPK1*), which has been implicated in epithelial cell polarity and exocytic vesicular transport towards the apical plasma membrane.¹⁷ It is not clear how ALPK1 could be linked to (colorectal) cancer or the mechanism of action of CAPOX-B. Also, the consequence of the other SNPs in the top 10 is unknown.

For this study, we included patients who were treated with CAPOX-B as well as patients who were treated with CAPOX-B plus cetuximab. In our analysis, treatment allocation was included as a covariate. It is unlikely that the effects of the SNPs are linked to the efficacy of cetuximab, since none of the polymorphisms showed significant interaction with the treatment arm.

In conclusion, even though these results possibly identify a novel region that is associated with the efficacy of CAPOX-B, further analyses are required before firm conclusions can be made. A prediction model using the data from this study will probably better discriminate patients with long from short PFS than individual SNPs.

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Appendix



10

General discussion

It is evident that drug response varies among individual cancer patients, but the mechanisms underlying this variability are not fully understood. However, since many differences between people have a genetic background, it is likely that drug response also has a genetic – and therefore heritable – component. Over fifty years ago, the term pharmacogenetics was introduced when describing hemolytic anemia during treatment with primaquine for subjects with deficiency of the G6PD enzyme. Another classic example is the prolonged muscular relaxation following the administration of suxamethonium to patients who are deficient for the butyrylcholinesterase enzyme. In the early nineties, the molecular basis of variation in drug response in oncology was unraveled by the discovery of single nucleotide polymorphisms (SNPs) in genes encoding detoxifying enzymes. Severe toxicities of 6-mercaptopurine (6MP) and 5-fluorouracil (5FU) were related to rare variants in the genes encoding thiopurine-S-methyltransferase (TPMT) and dihydropyrimidine dehydrogenase (DPD), respectively.¹⁻⁴ In both cases, SNPs cause decreased functionality of these key detoxifying enzymes⁴⁻⁶, resulting in exposure to these drugs exceeding their toxic thresholds, leading to the development of severe toxicities.

Many other pharmacogenetic markers are now identified that are correlated with drug response – being either toxicity or efficacy. However, unlike somatic molecular biomarkers⁷, and beside germline polymorphisms in the *TPMT* gene, no other germline polymorphism is applied in routine clinical practice to guide optimal use of anti-cancer drugs.⁸ The question is: why? In this discussion, the following fundamental points are evaluated:

- What are the results of pharmacogenetic studies when placed in perspective?
- Are pharmacogenetic studies properly designed in relation to the expected outcome?
- Are the results of pharmacogenetic studies ‘ready to use’?
- And finally: what would be the ideal pharmacogenetic study?

The results of pharmacogenetic studies in perspective

Publication of results

Many studies have been published describing associations between genetic polymorphisms and drug response. In **chapter 2 and 5**, an overview is given for VEGF and EGFR targeting drugs, and for chemotherapeutic agents, respectively. These chapters describe the positive – or statistically significant – results of these studies, which therefore may be used as a tool for selecting promising predictive polymorphisms for future research.

However, in many pharmacogenetic studies, an important aspect is frequently overlooked. Many studies start with selecting candidate polymorphisms for the study,

in the same way as we selected five polymorphisms for our analysis for **chapter 3**. Regularly, ten or even more polymorphisms are selected for analysis in pharmacogenetic studies. The most striking results are subsequently highlighted in the results and discussion sections – usually being the significant findings – whereas the non-significant results are often not shown nor discussed, apart from the comment that they were not significant.

There are two problems that result from highlighting significant results and not showing non-significant results. Firstly, the lack of confirmation of association between a polymorphism and drug response (that is: results from study A are not found in study B) is easily overlooked because of the attention paid to the other, significant, findings. As a result, the initial publication describing the significant association (study A) remains apparently undisputed, whereas doubts should have been placed based upon lack of confirmation in the second study (study B). Secondly, ‘absence of evidence’ does not necessarily imply ‘evidence of absence’.⁹ In an underpowered study, a lack of significant association could mean anything ranging from an actual lack of association to falsely missing a true association. A table with all results including the effect sizes would be very useful for gathering all available information to assess the quality of the evidence for a given polymorphism.

Confirmation

Successful replication of initial findings is a requirement before solid conclusion regarding a polymorphism can be made, as these initial findings could have been false positives. With this in mind, we investigated the initial results from a previous hypothesis generating study in metastatic colorectal cancer patients who were treated with oxaliplatin based therapy (the CAIRO study¹⁰). In this study, 81 polymorphisms in genes that encode DNA repair enzymes were studied.¹¹ Polymorphisms in the *ATM* and *ERCC5* genes were significantly associated with PFS. In **chapter 6**, we show that the initial results could not be replicated in patients participating in another study (CAIRO2)¹², from which we concluded that the initial significant results were probably false-positive findings. As a critical note, it could be possible that some of the non-significant results from the initial study were actually associated with response, but were missed because of lack of power. Another strategy of replication – such as replicating all polymorphisms instead of only the significant polymorphisms – could have revealed whether any of the initial 81 polymorphisms were associated with response. These, on their turn would have required confirmation in another cohort, indicating the complexity of the problem.

For **chapter 8**, we selected 17 polymorphisms that had previously been associated with the response to fluoropyrimidines, oxaliplatin or bevacizumab. Surprisingly, none of these polymorphisms were significantly associated with progression-free survival.

The hazard ratios for these polymorphisms were all very close to 1, indicating that the lack of association was not merely a question of (lack of) power.

Even more striking was our finding that the high affinity Valine-allele of the *FCGR3A* polymorphism was associated with decreased progression-free survival (PFS) in metastatic colorectal cancer patients treated with cetuximab, bevacizumab and chemotherapy (**chapter 3**). This result was opposite of the expected outcome, as previous studies showed beneficial effect of the monoclonal antibodies cetuximab, rituximab and trastuzumab for the Valine allele compared with the Phenylalanine allele.¹³⁻¹⁶ An explanation for this opposite result could be our incomplete understanding of the mechanism of action of cetuximab.

As a side note, the association of the *FCGR3A* polymorphism in the CAIRO2 study could be less unexpected than considered at first glance: The effect of cetuximab in the CAIRO2 was unintentionally decreasing PFS. The Valine allele was indeed associated with increased ‘efficacy’ – being decreased PFS – and therefore in line with the original hypothesis.

In general terms, careful selection of an optimal replication cohort and confirmation of initial findings is crucial before solid conclusions can be drawn. Factors that vary almost by definition between cohorts, such as previous treatment, concomitant treatment and other clinical variables such as disease state, should be considered carefully. Other factors, such as gender, age, prognostic variables and other (somatic) genetic variation, should be used in covariate analyses to correct for confounding. Simply attempting to confirm an association between one polymorphism and one drug without considering other factors is inappropriate.

The design of pharmacogenetic studies

Choice of polymorphisms

Most pharmacogenetic studies to date include a selection of candidate polymorphisms in so-called candidate genes. These genes are selected based upon knowledge of the pharmacokinetics and pharmacodynamics of the drug. The candidate polymorphisms generally have either impact on the function or the expression of the enzyme encoded by the gene.¹⁷ A more advanced method is to select tagging polymorphisms in candidate genes, in order to cover as much genetic variation with an optimal number of polymorphisms. To date, however, only single nucleotide polymorphism (SNPs), some short repeat polymorphisms and some insertion/deletion polymorphisms are studied in pharmacogenetic studies. Other heritable genetic variation, such as copy number alterations, could also play an important role in variability of drug response.¹⁸

The first published pharmacogenetic studies reported associations between one single polymorphism in one single gene and drug response.^{2,3} Current studies include many polymorphisms in many potentially relevant genes.¹⁹⁻²¹ This candidate gene procedure can be taken one step further with the candidate pathway method, in which candidate polymorphisms in candidate genes are selected that encode enzymes involved in the entire – known – pathway for a (class of) drug. This approach was applied in the previous CAIRO study using polymorphisms in genes that encode enzymes involved in DNA repair¹¹, but the results could not be confirmed (**chapter 6**). The weakness of the candidate polymorphism, candidate gene and candidate pathway methods, is that these depend on mechanistic knowledge and understanding of drug response.

Pharmacokinetics

For a drug of which the rate-limiting detoxifying step is determined by a single enzyme, an alteration in function or expression of that enzyme may have direct impact on plasma drug levels. However, many drugs are absorbed in the gastro-intestinal tract, distributed over tissues and cells, metabolized and excreted by many different enzymes and transporters. Also, metabolism at steady-state may differ from metabolism at first exposure, due to reduction and/or induction of enzymatic activity after some time.²² Since the relevant pharmacokinetic enzymes are usually determined in *in vitro* models or during the first phase clinical studies, our knowledge of which enzymes are important in the daily clinical setting may be inadequate for the candidate gene method. However, the most promising germline variants in oncology still are *CYP2D6* polymorphisms for tamoxifen efficacy, *UGT1A1* polymorphisms for irinotecan toxicity, *CYP3A4* polymorphisms for dasatinib efficacy, *TPMT* polymorphisms for 6-mercaptopurine toxicity and *DPYD* polymorphisms for fluoropyrimidine toxicity – all genes encoding pharmacokinetic enzymes.⁸ Moreover, guidelines have been presented for the implementation of pharmacogenetics in daily routine – and these guidelines also include only polymorphisms in the pharmacokinetic enzymes *CYP2D6*, *CYP2C9*, *CYP2C19* and *UGT1A1*.²³

Pharmacodynamics

In most cases, the principal mechanism of action of a drug is relatively well studied. For capecitabine for instance, the efficacy relies on RNA or DNA damage caused by the incorporation of fluorinated uracil residues in RNA and DNA, and by the inhibition of the enzyme thymidylate synthase (TYMS).²⁴ However, at least both the pyrimidine and folate metabolism routes are involved, in which many enzymes play a role.²⁵ The pyrimidine and folate metabolism are crucial for cell proliferation, and it is likely that endogenous feedback loops may influence these pathways, and therefore the efficacy of capecitabine. However, most pharmacogenetic studies on capecitabine

and other fluoropyrimidines – including our study presented in **chapter 8** – include only polymorphisms in *TYMS*, *DPD* and *MTHFR*, thereby ignoring many other potentially relevant enzymes.

In some cases, the mechanism of drug action is not completely understood. In this thesis, the pharmacogenetics of the monoclonal antibody cetuximab has been studied. Cetuximab was originally developed to inhibit the signaling of the growth factors EGF and transforming growth factor α (TGF α), by blocking their receptor EGFR.²⁶ Both these growth factors are crucial for cell proliferation, and therefore also tumor growth. Since cetuximab is a monoclonal antibody of the IgG1 type, immune responses such as antibody-dependent cell-mediated cytotoxicity (ADCC) or activation of immune cells may also be triggered. Indeed, *in vitro* research models showed that cetuximab and the therapeutic monoclonal antibody against CD20, rituximab, trigger ADCC by NK cells, which was most pronounced for the *FCGR3A* Valine allele.^{27,28}

However, the clinical relevance of these findings relies on the assumption that ADCC plays a role in the mechanism of action of cetuximab, ignoring other possibilities in other settings. For instance, we and others showed that NK cells are not present in colorectal cancer tissue, whereas these tumors are infiltrated by macrophages.²⁹ It has been described that tumors are infiltrated by a specific type of macrophages, tumor associated macrophages, which have anti-inflammatory and tumor-promoting properties.^{30,31} In experiments that we undertook with a model system for tumor associated macrophages, we show that cetuximab stimulates the release of anti-inflammatory – and possibly also pro-angiogenic³² – cytokines by type 2 macrophages (**chapter 4**). We hypothesized that the effect of these tumor associated macrophages in the CAIRO2 study was different from the previously published studies with cetuximab used in other settings^{13,33,34}, as patients in the CAIRO2 study received bevacizumab, capecitabine and oxaliplatin combined with cetuximab, whereas the other studies included cetuximab as monotherapy or in combination with irinotecan.^{13,33,34}

The results described in **chapter 4** – that cetuximab mediates production of anti-inflammatory cytokines by macrophages – indicates that the exact mechanism of action of cetuximab is not fully understood. Importantly, the development and application of therapeutic antibodies with increased affinity for the FCGR2A and FCGR3A receptors could be impacted by this finding.^{35,36}

In summary, when our knowledge of the pharmacokinetics and pharmacodynamics is incomplete, it is even more challenging to select polymorphisms that are involved in drug response. Selecting only known polymorphisms in known genes could easily lead to an underestimation of the genetic impact on drug response.

Method of statistical analysis

Currently, most pharmacogenetic studies apply simple statistical analyses for the associations between the polymorphisms and drug response. Usually, Chi square and Kaplan Meier tests are applied for univariate analyses. For multivariate analyses with possible confounders such as age and gender, logistic or Cox proportional hazard regression models are used. These types of analyses have in common that only the effect of one single polymorphism on drug response is studied.

As described above, many biological molecules – such as metabolic enzymes, drug transporters or drug targets – contribute to drug response. It is likely that alterations in these enzymes have only impact on drug response under the condition that other alterations are also present. As a hypothetical example: for a drug that is metabolized by two enzymes, decreased activity of one of these enzymes may have no impact on metabolic activity, as the other enzyme may take over. Only when both enzymes have decreased activity, total metabolism could be impacted, resulting in increased plasma levels and potentially increased toxicity or efficacy.

This concept is known as gene-gene interaction or epistasis, and can be analyzed using statistical interaction – not to be confused with biological interaction between enzymes.³⁷ In the example described above with only two enzymes, the interaction can be detected relatively easily by including an interaction in a regression analysis. When the number of polymorphisms studied increases, the possible number of interactions increases exponentially, making it not feasible to use parametric statistical analyses such as regression. Other non-parametric methods exist, such as classification and regression analysis (CART) or multifactor dimensionality reduction (MDR) analysis.³⁸ The underlying concepts of these methods were described and illustrated in **chapter 7**.

In **chapter 8**, the MDR method was applied to the CAIRO2 study for the association between candidate polymorphisms in candidate genes for the efficacy of capecitabine, oxaliplatin and bevacizumab. Even though this study may not have been optimal in terms of selection of polymorphisms – by selecting 17 polymorphisms, other potentially relevant polymorphisms may have been overlooked, as described above – an interaction between the *VEGF* +405G>C and *TYMS* TSER polymorphisms was found. The exact underlying biological mechanism of this interaction – which was detected using a statistical method – is not exactly understood, but it is apparent that the impact of either of these polymorphisms depends on the presence of the other, and *vice versa*.

Studying interaction seems more rational compared with the ‘classical’ method of testing one polymorphism at the time, since it takes the complexity underlying drug response into account. As a consequence, the results from such studies may provide more robust results that have more chance of successful replication. Unfortunately, the interaction analyses become very complex with increasing numbers of genotypes studied,

possibly leading to spurious findings. Internal validation – such as cross-validation – and confirmation in another cohort is required before definite conclusions can be drawn.

The applicability of the results

From retrospective analyses, it was concluded that cetuximab and panitumumab are ineffective for metastatic colorectal cancer patients with a somatic activating *KRAS* mutation.³⁹ This even led to the alteration of the drug labels of both agents. The success of the implementation of the *KRAS* mutation to guide therapy is due to the fact that very few patients with *KRAS* mutations responded to either cetuximab or panitumumab. A *KRAS* mutation is therefore highly predictive for non-responders, and therefore readily applicable to exclude patients from cetuximab or panitumumab. Even though testing for *KRAS* mutations is widely accepted based upon retrospective studies, a recent analysis showed that cetuximab is effective in patients with the *KRAS* G13D mutation – which was previously considered as one of the activating mutations.⁴⁰ This finding demonstrates that patients with the *KRAS* mutated tumors are less homogeneous with respect to their response to anti-EGFR therapy than initially thought. It is also a lesson that it takes more to validate a biomarker than only retrospective analyses of several randomized studies, even when each of these included several hundreds of patients.

The association between *TPMT* and *DPYD* polymorphisms and the incidence of severe toxicities of 6-MP and fluoropyrimidines, respectively, is relatively strong. However, the genetic variants involved are very rare (1% or less), and more importantly, no prospective studies have determined what the alternative should be for patients at risk of toxicity. Based upon retrospective analysis, 50% and 90% lower dosing of 6MP is suggested for *TPMT* heterozygotes and homozygotes, respectively.⁴¹ In the most recent pharmacogenetics guide of the WINAp, a 50% dose reduction is suggested for heterozygotes for *DPYD* polymorphisms treated with 5FU, UFT and capecitabine, whereas alternative therapy is advised for homozygotes.

For most other pharmacogenetic markers, the effect on drug response is usually expressed as an odds-ratio (OR) or hazards ratio (HR). The effect sizes are relatively small - frequently in the order of 1.5 to 2.0, meaning that the chance of response for patients with one genotype is 1.5 to 2.0 higher compared with patients with the other genotype. Even though such results may be statistically significant, they are not readily applicable. As an example, there are many studies that report that the efficacy of gefitinib for NSCLC is increased for patients harboring a low number of CA-repeats in the *EGFR* gene.⁴² Even though all currently available studies show the same results, this polymorphism is not used to optimize gefitinib treatment.

It would be helpful if the response rate or survival of patients with an unfavorable genotype could be compared with the response rate of untreated patients. In that way, the absolute efficacy of the therapy for each genotype could be assessed and compared with no treatment.

Predictive models including genetic and non-genetic information could help to better discriminate responders from non-responders. In **chapter 3**, we describe the development of a predictive model for cetuximab efficacy. As this model was developed using the CAIRO2 study in which patients were randomized to receive cetuximab or no cetuximab added to CAPOX-B, predictive variables for cetuximab (related to the outcome of therapy) could be discriminated from prognostic variables (variables that are related to survival regardless of the therapy). Surprisingly, *KRAS* mutation status was no predictor in this model, whereas the *FCGR3A* Phe158Val polymorphism, gender and white blood cell count were included in the model. This predictive model cannot be generalized for cetuximab therapy, since treatment with a combination of cetuximab and bevacizumab, as in the CAIRO2 study, will not be used in the clinic given the inferior outcome.

In **chapter 8**, we describe the results from a genetic interaction analysis for the efficacy of capecitabine, oxaliplatin and bevacizumab. A genetic profile consisting of two polymorphisms, *VEGF* +405G>C and *TYMS* TSER, was associated with PFS. This profile was developed in the standard treatment arm of the CAIRO2 study. Since a no-treatment control arm would be unethical in the first-line treatment of metastatic colorectal cancer, no conclusion could be drawn whether the genetic profile was predictive for the efficacy of capecitabine, oxaliplatin and bevacizumab, or also prognostic (i.e. associated with outcome regardless of treatment). Because the polymorphisms in the genetic profile are in the pathway of the mechanism of action of bevacizumab (the *VEGF* polymorphism) and capecitabine (the *TYMS* polymorphism), a control group that was treated with other agents would also be possible. However, as long as a fluoropyrimidine is the cornerstone of first-line metastatic colorectal cancer treatment, this will not be possible.

Apart from the question whether the genetic profile is predictive or prognostic, not only confirmation in another cohort would be required for the genetic profile before it could be applied in clinical practice to select patients for treatment. Prospective studies would also be needed to show that genotype guided treatment is better than standard care. A parallel could be drawn with drug development, in which it is not uncommon that compounds – even though these had been rationally designed and showed clinical efficacy in non-controlled studies – fail to demonstrate efficacy when tested in a prospective and controlled fashion.

The ideal pharmacogenetic study

Before a genetic test could be applied to adequately predict response to treatment, prospective testing would be required, as described above. The question remains: what would be the ideal study to find the optimal pharmacogenetic marker(s)?

In this thesis, different approaches have been described for detecting potentially relevant pharmacogenetic markers: attempting to validate previous findings (**chapter 6**), developing a predictive model (**chapter 3**) and developing a genetic interaction model (**chapter 8**). For these chapters, the candidate polymorphism method was applied. As described earlier, this method is not optimal.

In **chapter 9**, we describe another approach – a genome wide association study (GWAS). In this study, patients from the CAIRO2 study were genotyped for more than 700.000 polymorphisms across the entire genome. For the statistical analysis, each polymorphism was initially individually tested for correlation with PFS. No formal significant results (i.e. below the threshold of $P = 5 \times 10^{-8}$) were found, but three SNPs located on chromosome 8, down-stream of the *AGPAT5* gene, had P-values close to this threshold. Further research is required before it can be concluded that this is indeed a true association, and that these SNPs affect the function or expression of this gene.

However, as described above, considering each polymorphism as a single variable could underestimate the impact on drug response. Therefore, a predictive model will be developed, for which the top 100 SNPs with the lowest P-values will be included. Such an approach of developing a predictive model using GWAS data has recently been applied to explain variability in human height. Initial results from GWAS studies, being the associations between individual polymorphisms and human height, could explain only ~5% of the variation in human height.⁴³⁻⁴⁵ In contrast, a model that included also non-significant SNPs could explain 45% of the variation, indicating that the use of other non-significant SNPs to explain variability between patients is a feasible strategy.⁴⁶

As previously described, this predictive model requires confirmation and preferably also prospective testing – such as described previously for the genetic profile – before it could be applied in clinical practice for selecting treatment for metastatic colorectal cancer patients.

A design such as applied in **chapter 9** seems the most promising type of pharmacogenetic study: using a GWAS instead of candidate polymorphisms, and developing a predictive model including interaction. With the rapid development of whole genome genotyping platforms, it is likely that the costs of whole genome genotyping will drop within the next decade, making it increasingly affordable. Even whole genome sequencing might become feasible in the future, so that the entire genome of patients becomes available instead of a (large) selection of SNPs on a chip. For such studies

with enormous amounts of genotyping data, adequate quality and appropriate size of the cohort remains paramount: preferably patients included in large prospective trials should be included.

Conclusion

In this thesis, different pharmacogenetic studies are presented for the efficacy of systemic treatment of metastatic colorectal cancer. The results that were obtained for cetuximab prompted the search for a mechanistic explanation, which could be the activation of tumor promoting macrophages mediated by cetuximab.

Different approaches were used to find pharmacogenetic predictors for the efficacy of CAPOX-B, such as genetic interaction and a GWAS. A combination of a GWAS and development of a predictive model including interaction seems the most promising approach for a successful pharmacogenetic study. It remains important to confirm initial findings in a separate cohort. For a pharmacogenetic test to be implemented in routine clinical practice, prospective testing of the test is necessary.

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Summary

Nederlandse samenvatting

Dankwoord

List of publications

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Summary

Cetuximab

Even though treatment of several types of solid tumors has improved in the past few years with the introduction of the monoclonal antibodies against the epidermal growth factor receptor (EGFR) and vascular endothelial growth factor (VEGF), the clinical benefit of these targeted therapies is modest. Pharmacogenetics has the potential to select patients with higher chance of response to agents that target these pathways. **Chapter 2** provides an overview of germ-line variations in genes that are involved in the pharmacodynamics of the anti-EGFR monoclonal antibodies cetuximab and panitumumab and the anti-VEGF monoclonal antibody bevacizumab, and which may underlie variable anti-tumor response. Based upon this review, we suggest further investigations on the prognostic or predictive role of the following polymorphisms for cetuximab: *FCGR2A* His131Arg, *FCGR3A* Phe158Val, *EGF* 61A>G, *EGFR* CA₁₄₋₂₂ and *CCND1* 870G>A.

In **chapter 3**, we describe the association between these polymorphisms and progression-free survival (PFS) in 576 advanced colorectal cancer patients who were treated in the phase III CAIRO2 study of the Dutch Colorectal Cancer Group with capecitabine, oxaliplatin and bevacizumab or the same regimen plus cetuximab. The analysis was done with regard to *KRAS* mutation status, since this is a strong predictor for cetuximab efficacy. In the cetuximab arm, the *FCGR3A* 158 valine-allele was associated with a decreased PFS, both in the overall population and in the subgroup with *KRAS* wild-type tumors (HR=1.56, 95%CI=1.14-2.15 and HR=1.57, 95%CI=1.06-2.34, respectively) and with a decreased incidence of grade 2-3 skin toxicity (OR=0.48, 95%CI=0.24-0.94). This association was not in agreement with previous research, in which the valine-allele was associated with increased benefit from monoclonal antibody therapy.

The *EGFR* ≥20 genotype was associated with a decreased PFS, both in the overall population and in the subgroup with *KRAS* wild-type tumors (HR=1.60, 95%CI=1.17-2.19 and HR=1.58, 95%CI=1.06-2.35, respectively). The *FCGR3A* and *EGFR* polymorphisms were not associated with PFS in patients treated without cetuximab. In patients with *KRAS* mutated tumors, the *EGF* 61G-allele was associated with decreased PFS in the cetuximab arm, and increased PFS in the no-cetuximab arm (HR=2.22, 95%CI=1.24-3.96 and HR=0.59, 95%CI=0.36-0.98, respectively). We conclude that *EGFR*, *FCGR3A* and *EGF* polymorphisms are associated with PFS in advanced colorectal cancer patients treated with cetuximab, bevacizumab and chemotherapy, and that these results should be confirmed before these markers are used in general practice.

Since the association between the *FCGR3A* Phe158Val polymorphism was opposite from expected, as described in **chapter 3**, we performed *in vitro* experiments to better understand the underlying mechanism, which we describe in **chapter 4**.

Therapeutic monoclonal antibodies, such as cetuximab, may trigger *in vitro* immune responses such as antibody-dependent cell-mediated cytotoxicity (ADCC) by natural killer (NK) cells through binding of their Fc region to Fc-gamma receptors. As solid tumors may be infiltrated by tumor-promoting tumor associated macrophages, monoclonal antibodies could also mediate tumor-promoting effects through the Fc region-FCGR interaction. In 10 tumor samples of previously untreated stage III colorectal cancer patients, we indeed observed infiltration of tumor-associated macrophages (CD68+CD163+ cells), but no NK cells. In a laboratory model for tumor-associated macrophages, we cultured type 2 macrophages (MF2s) from monocytes derived from healthy donors. LPS activated MF2s produced IL10, IL8 and VEGF, but no IL12p70. Activation of MF2s showed a cetuximab concentration dependent effect in an A431/MF2/cetuximab co-culture, whereas no activation was observed for the A431/MF2/rituximab co-culture. There was lower down-regulation of CD16 for *FCGR3A* 158-valine carriers compared with *FCGR3A* 158-phenylalanine homozygotes ($P=0.048$). These results indicate that tumor-promoting macrophages can be activated by therapeutic monoclonal antibodies. This could implicate the clinical development of engineered monoclonal antibodies with increased affinity for Fc-gamma receptors.

Capecitabine, oxaliplatin and bevacizumab

Chapter 5 gives an overview of germline polymorphisms genes that have been studied most extensively in anti-cancer chemotherapy. Even though many pharmacogenetic association studies have been published, we conclude that there is need for more research. In particular, there is need for replication of results and development of predictive models. Prospective trials are required to establish the clinical value and cost-effectiveness of pharmacogenetic tests in oncology.

A previous study indicated that SNPs in the genes encoding ataxia telangiectasia mutated (*ATM* rs1801516) and excision repair cross-complementing group 5 (*ERCC5* rs1047768) were significantly associated with PFS in advanced colorectal cancer patients treated with second-line oxaliplatin combined with capecitabine. We were not able to validate the results of these SNPs in the CAIRO2 study, as described in **chapter 6**. We conclude that these SNPs have no relevant impact on the PFS of oxaliplatin-based therapy for advanced colorectal cancer patients, and that the negative result of this study underlines the importance of validating and reporting the findings from retrospective explorative studies.

Disappointing results from replicating pharmacogenetic association studies have prompted the search for novel statistical techniques to analyze the data, while taking into account the biological complexity underlying drug response. In **chapter 7**, we describe two of these techniques – multifactor dimensionality reduction and classification and regression tree analysis. In addition to describing the concepts

underlying both techniques, we also illustrate their application in a recent pharmacogenetic study on pharmacogenetic determinants on sunitinib induced toxicity.

In order to study the contribution of polygenic variation in relation to response to capecitabine, oxaliplatin and bevacizumab in advanced colorectal cancer patients, we applied the multifactor dimensionality reduction analysis in **chapter 8**. Based upon the data presented in chapter 2 and chapter 5, a selection of 17 polymorphisms in genes encoding drug targets, pathway molecules and detoxification enzymes was analyzed in 279 advanced colorectal cancer patients treated with capecitabine, oxaliplatin and bevacizumab in the CAIRO2 study. Multifactor dimensionality reduction analysis was used to identify a genetic interaction profile for PFS. A genetic interaction profile consisting of the *TYMS* enhancer region and *VEGF* +405G>C polymorphisms was significantly associated with PFS. Median PFS was 13.3 (95%CI, 11.4 to 15.3) and 9.7 (95%CI, 7.6 to 11.8) months for the beneficial and unfavorable genetic profiles, respectively, corresponding to a hazards ratio for PFS of 1.58 (95%CI, 1.14 to 2.19). None of the studied polymorphisms were individually associated with PFS. We conclude that these results support a genetic interaction between the *TYMS* enhancer region and *VEGF* +405G>C polymorphisms as a predictor of the efficacy of capecitabine, oxaliplatin and bevacizumab in advanced colorectal cancer patients.

In **chapter 9**, we describe the preliminary results from a genome-wide association study to find single nucleotide polymorphisms that are associated with the efficacy of capecitabine, oxaliplatin and bevacizumab. We used germline DNA from 547 patients participating in the CAIRO2 study. Whole-genome genotyping was performed using 700k Illumina OmniExpress BeadChip arrays. Associations between single nucleotide polymorphisms and PFS were tested using Cox-proportional hazard models. Associations were considered significant when $P < 5 \times 10^{-8}$. Three single nucleotide polymorphisms located at 8p23.1 showed a trend toward significance for association with PFS (rs2936519, $P = 1.24 \times 10^{-7}$; rs2912024, $P = 1.38 \times 10^{-7}$ and rs2978931, $P = 6.75 \times 10^{-7}$). These SNPs are 20 kbp downstream of the *AGPAT5* gene, which encodes a protein that converts lysophosphatidic acid (a mitogen that has been linked to cancer in different ways) to phosphatidic acid, which is also involved in phospholipid biosynthesis. We conclude that these results possibly identify a novel genetic predictor for the efficacy of capecitabine, oxaliplatin and bevacizumab. However, further analyses are required before definitive conclusions can be made based upon these data.

General discussion

In **chapter 10**, the results presented in this thesis, as well as the question why hardly any pharmacogenetic test is currently applied in routine patient care, are discussed. Many pharmacogenetic studies have been published, and the significant results get most attention. Results from such studies are usually not validated in a systematic

way. Successful replication of results is important, since initial results could have been false positive findings based upon the large number of statistical tests that are frequently applied.

When a pharmacogenetic study is set up, decisions have to be made regarding the selection of polymorphisms. Usually, candidate polymorphisms in candidate genes are selected, which is based upon our current understanding of the mechanism of drug action, and this may not be optimal. Genome-wide genotyping has the advantage that no prior selection of polymorphisms is required.

Apart from selecting the polymorphisms, selecting a proper statistical technique could also impact the results. In most pharmacogenetic studies, every single polymorphism is individually tested for association with drug response. Using statistical techniques that take gene-gene interactions into account is more rational and could provide more robust results.

Still most pharmacogenetic studies identify risk factors, which are probabilistic in nature, and cannot easily be applied to select individual patients for treatment. Development of pharmacogenetic predictive tests may better discriminate responders from non-responders and prospective testing is warranted to show that genotype-guided therapy is better than standard care.

The ideal pharmacogenetic study would therefore be a genome-wide study in combination with the development of a predictive model. Confirmation of the results in a separate cohort remains important. For a pharmacogenetic test to be implemented in routine clinical practice, prospective testing of the test is essential.

Nederlandse samenvatting

Cetuximab

In de afgelopen jaren zijn monoclonale antilichamen bevacizumab (gericht tegen de vasculaire endotheliale groei factor, VEGF), cetuximab en panitumumab (gericht tegen de epidermale groei factor receptor, EGFR) beschikbaar gekomen voor de behandeling van verschillende soorten solide tumoren. Hoewel met deze middelen de behandeling is verbeterd, hebben niet alle patiënten even veel baat bij de therapie.

Farmacogenetica – waarbij gebruik wordt gemaakt van kiembaan polymorfismen om het effect van geneesmiddelen te voorspellen of te verklaren – zou ingezet kunnen worden om patiënten op te sporen met een hogere kans op goede effectiviteit van dit soort middelen. **Hoofdstuk 2** geeft een overzicht van kiembaan polymorfismen in genen die betrokken zijn bij de farmacodynamiek en farmacokinetiek van bevacizumab, cetuximab en panitumumab. Deze polymorfismen zouden kunnen bijdragen aan de variabele effectiviteit van deze middelen. Op basis van dit hoofdstuk concluderen we dat de volgende polymorfismen onderzocht zouden moeten worden in relatie tot de effectiviteit van cetuximab: *FCGR2A* His131Arg, *FCGR3A* Phe158Val, *EGF* 61A>G, *EGFR* CA₁₄₋₂₂ en *CCND1* 870G>A.

In **hoofdstuk 3** beschrijven we de correlatie tussen deze polymorfismen en progressie-vrije overleving van 576 gemetastaseerde dikke darm kanker patiënten die behandeld werden met capecitabine, oxaliplatin en bevacizumab, of met dezelfde combinatie plus cetuximab in de gerandomiseerde fase III CAIRO2 studie. Omdat *KRAS* mutatie status een sterke voorspeller is voor de effectiviteit van cetuximab, werden dit meegenomen in de analyses. In de behandelarm met cetuximab was het *FCGR3A* 158-valine allel geassocieerd met een kortere progressie-vrije overleving, zowel in de totale populatie als in de subgroep van patiënten met een *KRAS* wild-type tumor (HR=1,56, 95%CI=1,14-2,15 en HR=1,57, 95%CI=1,06-2,34, respectievelijk) en met een lagere incidentie van graad 2-3 huidtoxiciteit (OR=0,48, 95%CI=0,24-0,94). Deze resultaten waren niet in overeenstemming met eerdere studies, waaruit bleek dat het valine allel met betere effectiviteit van monoclonale antilichamen geassocieerd was. Het *EGFR* ≥ 20 polymorfisme was geassocieerd met een kortere progressie-vrije overleving, zowel in de totale populatie als in de subgroep van patiënten met een *KRAS* wild-type tumor (HR=1,60, 95%CI=1,17-2,19 en HR=1,58, 95%CI=1,06-2,35, respectievelijk). De *FCGR3A* en *EGFR* polymorfismen waren niet geassocieerd met progressie-vrije overleving in de patiënten die niet werden behandeld met cetuximab. In de patiënten met een *KRAS* gemuteerde tumor was het *EGF* 61-allel geassocieerd met een kortere progressie-vrije overleving. In patiënten in de behandel-arm met cetuximab, en met een langere progressie-vrije overleving in de behandel-arm zonder cetuximab (HR=2,22, 95%CI=1,24-3,96 en HR=0,59, 95%CI=0,36-0,98, respectievelijk).

We concluderen dat polymorfismen in *FCGR3A*, *EGFR* en *EGF* zijn geassocieerd met de effectiviteit van cetuximab in combinatie met bevacizumab en chemotherapie voor de behandeling van gemetastaseerde dikke darm kanker. Voordat deze polymorfismen kunnen worden gebruikt in de dagelijkse klinische praktijk, is bevestiging van de resultaten vereist.

Omdat het *FCGR3A* 158-valine allel onverwacht was geassocieerd met kortere progressie-vrije overleving (zoals beschreven in **hoofdstuk 3**), hebben we *in vitro* experimenten gedaan om het onderliggende mechanisme op te helderen. Deze experimenten zijn beschreven in **hoofdstuk 4**. Monoclonale antilichamen zoals cetuximab kunnen immuun reacties opwekken door binding van hun Fc-staart aan Fc-receptoren op immuuncellen zoals natural killer (NK) cellen en macrofagen. Eerdere onderzoeken waren gericht op immuun effecten van NK cellen, waardoor de tumor werd bestreden. Wij tonen echter aan dat dikke darm kanker tumoren zogenaamde tumor geassocieerde, type 2 macrofagen bevatten, maar geen NK cellen. Deze macrofagen zijn bekend om hun tumorgroei bevorderende eigenschappen. In een laboratoriumsetting toonden we aan dat activatie (met de bekende activator LPS) van deze macrofagen leidde tot uitscheiding van IL10, IL8 en VEGF. In een experiment waarbij tumorcellen, cetuximab en type 2 macrofagen gezamenlijk werden geïncubeerd, zagen wij een cetuximab concentratie afhankelijke activatie van de type 2 macrofagen. Deze activatie was sterker voor type 2 macrofagen met het *FCGR3A* 158-valine allel ($P=0,048$). Hieruit concluderen we dat tumorgroei bevorderende macrofagen geactiveerd kunnen worden door cetuximab.

Capecitabine, oxaliplatin en bevacizumab

Hoofdstuk 5 geeft een overzicht van kiembaan polymorfismen die uitvoerig bestudeerd zijn voor verschillende soorten chemotherapie. Hoewel er veel studies over dit onderwerp zijn gepubliceerd, concluderen we dat er meer onderzoek nodig is. Vooral replicatie van de resultaten en ontwikkelen van voorspellende modellen is nodig. Prospectieve studies zijn nodig om de klinische toepasbaarheid en kosten-effectiviteit van farmacogenetische tests aan te tonen.

In een eerder onderzoek bleek dat twee polymorfismen waren geassocieerd met progressie-vrije overleving van gemetastaseerde dikke darm kanker patiënten die werden behandeld met oxaliplatin bevattende therapie: *ATM* rs1801516 en *ERCC5* rs1047768. Deze associaties konden we echter niet bevestigen in de CAIRO2 studie, zoals beschreven in **hoofdstuk 6**. Hieruit concluderen we dat deze polymorfismen geen relevante invloed hebben op de progressie-vrije overleving van oxaliplatin bevattende therapie van gemetastaseerde dikke darm kanker patiënten. Dit resultaat toont verder aan dat het belangrijk is om eerdere resultaten van exploratieve onderzoeken te valideren.

Omdat de resultaten van replicatie studies in de farmacogenetica tegen vallen, wordt er gezocht naar nieuwe statistische methoden om de data te analyseren, waarbij rekening wordt gehouden met de complexiteit die aan de geneesmiddelenwerking ten grondslag ligt. In **hoofdstuk 7** beschrijven we twee technieken waarbij gen-gen interactie wordt meegenomen in de analyse: multifactor dimensionality reduction (MDR) en classification and regression tree (CART) analyse. Daarnaast illustreren we deze toepassing van deze technieken met data van een recente studie naar toxiciteit van sunitinib.

De MDR analysetechniek werd toegepast om de bijdrage van verschillende polymorfismen op de effectiviteit van capecitabine, oxaliplatin en bevacizumab te bestuderen, zoals beschreven in **hoofdstuk 8**. Op basis van de gegevens beschreven in hoofdstukken 2 en 5, werden in totaal 17 verschillende polymorfismen meegenomen in de analyse in 279 patiënten uit de CAIRO2 studie, die werden behandeld met capecitabine, oxaliplatin en bevacizumab. We vonden een genetisch interactieprofiel tussen de *VEGF* +405G>C en *TYMS*-TSER polymorfismen, welke was geassocieerd met progressie-vrije overleving. De mediane progressie-vrije overleving was 13,3 (95%CI=11,4-15,3) en 9,7 (95%CI=7,6-11,8) maanden voor het gunstige en ongunstige profiel, respectievelijk. De bijbehorende HR voor progressie-vrije overleving was 1,58 (95%CI=1,14-2,19). Opvallend was dat geen van de 17 polymorfismen afzonderlijk met progressie-vrije overleving was geassocieerd. We concluderen dat een genetisch interactieprofiel tussen de *VEGF* +405G>C en *TYMS*-TSER polymorfismen de effectiviteit van capecitabine, oxaliplatin en bevacizumab in gemetastaseerde dikke darm kanker kan voorspellen.

In **hoofdstuk 9** presenteren we de eerste resultaten van een genome-wide onderzoek om polymorfismen op te sporen die geassocieerd zijn met de effectiviteit van capecitabine, oxaliplatin en bevacizumab. Hiervoor werd DNA van 541 patiënten uit de CAIRO2 studie gebruikt. Het genotyperen werd gedaan met OmniExpress arrays van Illumina, waar meer dan 700.000 polymorfismen gelijktijdig kunnen worden bepaald. Drie polymorfismen op de genomische lokatie 8p23.1 lieten een trend zien voor de associatie met progressie-vrije overleving (rs2936519, $P = 1,24 \times 10^{-7}$; rs2912024, $P = 1,38 \times 10^{-7}$ en rs2978931, $P = 6,75 \times 10^{-7}$). Deze polymorfismen liggen in de buurt van het *AGPAT5* gen, dat een enzym codeert welke betrokken is bij de omzetting van lysofosfatidezuur naar fosfatidezuur. Deze stoffen zijn betrokken bij de fosfolipide synthese en zijn eerder onderzocht bij kanker. Hieruit concluderen we dat deze resultaten mogelijk leiden tot een nieuwe genetische voorspeller voor de effectiviteit van capecitabine, oxaliplatin en bevacizumab bij de behandeling van gemetastaseerde dikke darm kanker. Nader onderzoek is echter nodig voordat definitieve conclusies kunnen worden getrokken.

Algemene discussie

In **hoofdstuk 10** worden de resultaten van de onderzoeken in dit proefschrift bediscussieerd. Daarnaast wordt de vraag gesteld waarom vrijwel geen farmacogenetische test routinematig wordt ingezet om anti-kanker behandeling te optimaliseren. Er wordt weliswaar veel onderzoek gedaan naar farmacogenetica, maar de significante resultaten krijgen in het algemeen de meeste aandacht, en initiele resultaten worden niet systematisch gevalideerd. Validatie is van belang omdat de kans aanwezig is dat initiele significante resultaten vals positief zijn door het grote aantal statistische tests dat vaak wordt toegepast.

Bij het ontwerpen van een farmacogenetisch onderzoek wordt meestal een selectie gemaakt van (bekende) polymorfismen in genen waarvan bekend is dat zij betrokken zijn bij de werking van het geneesmiddel. Hiervoor is gedetailleerde kennis over de werking van het geneesmiddel vereist, wat niet altijd optimaal is. Genome-wide genotyperen heeft als voordeel dat er vooraf geen selectie van polymorfismen nodig is. Naast het selecteren van de polymorfismen, is het van belang welke statistische analysemethode wordt gekozen. In de meeste farmacogenetische studies wordt elk polymorfisme afzonderlijk getest of het geassocieerd is met de werking van het geneesmiddel. Omdat er complexe biologische mechanismen aan geneesmiddelwerking ten grondslag liggen, is het rationeel om dit mee te nemen in de statistische analyse. Hiervoor kunnen technieken worden gebruikt die gen-gen interactie meenemen in de analyse.

In de meeste gevallen is de uitkomst van een farmacogenetische studie dat een genotype leidt tot verhoogde kans op goede werking van een geneesmiddel. Het vertalen van een dergelijke risicofactor naar dagelijkse praktijk is niet eenvoudig. Voorspellende testen op basis van farmacogenetische informatie zou beter onderscheid kunnen maken tussen patiënten met goede of minder goede effectiviteit van een geneesmiddel. Prospectief onderzoek is nodig om aan te tonen dat behandeling op geleide van een genetische test is beter dan standaard behandeling.

De ideale farmacogenetische studie zou daarom als volgt zijn: een genome-wide onderzoek in combinatie met een voorspellende test. Bevestiging van de resultaten in een aparte groep patiënten is noodzakelijk, en prospectief onderzoek is nodig voordat een farmacogenetische test in de dagelijkse klinische praktijk kan worden toegepast.

Met dank aan

mijn promotoren Henk-Jan, Hans en Kees

al mijn collega's van de afdeling KFT – in het bijzonder de ondersteuning van de farmacogenetici Judith en Tahar, de technische hulp van Renée, Judith en Marco, en de gezelligheid van mijn (oud) kamergenoten Nielka, Marloes, Rogier, Jesse, Lisanne, Dirk Jan, Els en Wouter

de collega onderzoekers van het UMC St. Radboud: Jolien, Miriam, Linda en Lieke

de mensen van het lab van de klinische oncologie; met een speciaal woord van dank voor Moniek, Thorbald, Sjoerd en Renske

de hulp op het LGTC van Yavuz en Sophie

de statistische ondersteuning door Stefan, Jeanine, Ninja, Steven en Andrew

het beschikbaar stellen van de data door de 'SUTOX groep'

mijn paranimfen Nielka en Matthijs

de patiënten die deelnamen aan het CAIRO2 onderzoek

de artsen die patiënten in de CAIRO2 studie hebben geïnccludeerd en ervoor hebben gezorgd dat er bloedmonsters waren voor mijn onderzoek:

C. Smorenburg, R. Hoekstra, C. Rodenburg, J. van der Hoeven, A. Cats, M. Geenen, C. van Groeningen, D. Richel, B. de Valk, N. Weijl, J. Douma, P. Nieboer, F. Valster, R. Rietbroek, A. Ten Tije, O. Loosveld, D. Kehrer, M. Bos, Z. Erjavec, H. Sinnige, C. Knibbeler, W. Van Deijk, F. Jeurissen, H. Sleeboom, A. Imholz, E. Muller, J. van den Bosch, S. Hovenga, E. Balk, G. Creemers, M. Dercksen, M. Legdeur, A. Smals, H. van Halteren, M. van Hennik, A. van der Torren, G. Hospers, R. de Jong, G. de Klerk, P. Zoon, J. Wals, V. Derleyn, H. Dankbaar, S. Luyckx, C. de Swart, J. Haasjes, W. Meijer, M. Polee, M. Tesselaar, H. Oosterkamp, J. Bollen, R. Jansen, P. van der Velden, P. Slee, C. Punt, C. Mandigers, A. Vos, M. den Boer, D. de Gooyer, A. Planting, A. vander Gaast, J. Pegels, T. Kok, F. de Jongh, J. Braun, F. Erdkamp, G. Veldhuis, A. van Reisen, C. Kruijtzer, H. Roerdink, J. van Riel, D. ten Bokkel Huinink, E. Voest, M. van Diemen, G. Vreugdenhil, M. Werter, L. Kerkhofs, P. Schiphorst, A. van Bochove, A. Ogilvie, A. Honkoop.

Mijn 3 schatjes aan het thuisfront.

List of publications

Pander J, Wessels JA, Gelderblom H, *et al.* Pharmacogenetic interaction analysis for the efficacy of systemic treatment in metastatic colorectal cancer. *Ann Oncol* 2010;doi: 10.1093/annonc/mdq572.

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Curriculum Vitae

Jan Pander is op 20 juli 1979 in Amsterdam geboren, en groeide op in Leeuwarden. Na het behalen van zijn diploma aan het Stedelijk Gymnasium te Leeuwarden, begon hij in 1997 met de opleiding Farmacie aan de Rijksuniversiteit Groningen. Tijdens zijn doctoraalfase deed hij onderzoek bij de vakgroep Farmacokinetiek en Drug Delivery naar de farmacokinetiek van een captopril dimeer (onder begeleiding van dr. F. Moolenaar). In 2003 rondde hij de doctoraalfase af, waarna hij in 2004 het apothekersdiploma behaalde.

Hierna startte Jan als trialapotheker bij de afdeling Klinische Farmacie & Toxicologie (KFT) van het Leids Universitair Medisch Centrum (LUMC). Hij begon in 2007 met het promotieonderzoek naar genetische voorspellers voor de effectiviteit van de behandeling van gemetastaseerde dikke darm kanker. Dit onderzoek werd begeleid door Prof. Dr. H.-J. Guchelaar (afdeling KFT, LUMC), Prof. Dr. H. Gelderblom (afdeling Klinische Oncologie, LUMC) en Prof. Dr. C.J.A. Punt (afdeling Medische Oncologie, Universitair Medisch Centrum St. Radboud, Nijmegen). In 2009 ontving hij een subsidie van de KWF Kankerbestrijding voor het doen van een genome-wide studie.

Vanaf februari 2011 is Jan werkzaam als Medical Scientific Liaison bij MSD, Haarlem.

