Pharmacogenetics of advanced colorectal cancer treatment

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Pharmacogenetics of advanced colorectal cancer treatment

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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 bunch 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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Outline of the thesis
Colorectal cancer is one of the leading causes of cancer related deaths. Surgery with curative intent is indicated for patients without distant metastases and in a subset of patients with resectable distant metastases. 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) and the epidermal growth factor receptor (EGFR; cetuximab and panitumumab). 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. With the currently available regimens, the median overall survival of metastatic colorectal cancer patients is approximately two years. Despite the improvement of prognosis of metastatic colorectal cancer patients from roughly 12 to 24 months in the past fifteen years, 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 and KRAS mutation testing for the selection of metastatic colorectal cancer patients for cetuximab or panitumumab treatment. 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.
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). 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 study, an exploratory study was performed with candidate polymorphisms in DNA repair genes. 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. 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. To investigate epistasis in relation to drug response, novel methods such as 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 study (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).
References

Cetuximab
Pharmacogenetics of EGFR and VEGF inhibition

Jan Pander • Hans Gelderblom • Henk-Jan Guchelaar

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.

Epidermal growth factor (EGF) pathway

Cetuximab is a chimeric mouse/human IgG\(_1\) type MAb, whereas panitumumab is a fully human IgG\(_2\) 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 (TGFα).

The small G protein k-ras, the protein kinase b-raf, and phosphoinositide 3-kinase (encoded by \(\text{KRAS}\), \(\text{BRAF}\) and \(\text{PIK3CA}\) respectively) play a central role as intracellular mediators of EGFR signalling\(^5\), ultimately leading to induced transcription of several factors including interleukin-8 (IL8), VEGF and cyclin D1 (CD1, coded by \(\text{CCND1}\)). Cyclooxygenase-2 (COX2, encoded by \(\text{PTGS2}\)) is an upstream mediator of EGFR activity, presumably through the effect of prostaglandin E\(_2\) (see Figure 1).
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. There is good to complete (93-100%) similarity of this polymorphism between normal and tumour tissue, which is reassuring since the EGFR gene is highly sensitive to somatic alteration through loss of heterozygosity, mutations or copy number.
alterations. A higher number of CA-repeats is associated with decreased expression of EGFR on both mRNA and protein level in vitro, but this association was not consistently found in vivo. Two single nucleotide polymorphisms (SNPs; see glossary box) in the promoter (-216G>T and -191C>A) are both associated with increased expression of EGFR. 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 factors in vitro. A nonsynonymous SNP in exon 2 (2607G>C) whose function remains unclear. A SNP in the 5'-UTR of the IL8 gene (-251T>A) has been associated with increased IL8 protein expression in vitro and in vivo. The 61GG genotype was associated with decreased risk of myocardial infarction and stroke for the -765C allele. Illustrative of its function is the strong association with decreased risk of myocardial infarction and stroke for the -765C allele. Recently, an association for increased PFS for the -765C genotype was reported in mCRC patients treated with single agent cetuximab.

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

**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, though not unequivocally. 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. The IL8 receptor alpha, IL8RA (encoded by the gene CXCR1) contains a nonsynonymous SNP in exon 2 (2607G>C), whose function remains unclear. A SNP in the CCND1 gene (870G>A) has been associated with higher expression of CD1.

Zhang et al. investigated whether there was an association for the polymorphisms in the CCND1 (870A>G), PTGS2 (765G>C), EGF (61A>G), EGRF (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. Homozygotes for the CCND1 870A allele had a shorter OS compared with carriers of the 870G allele. 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. 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 possibility of false positive findings.
between the HIF1A SNPs and VEGF mRNA levels has been demonstrated, whereas no relationship with VEGF protein expression was found.\textsuperscript{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.\textsuperscript{48}

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 IgG, 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 \textit{FCGRT}).\textsuperscript{48}

\textbf{Vascular endothelial growth factor (VEGF) pathway}

Bevacizumab is a humanized IgG\textsubscript{1} type MAb directed against soluble VEGF, one of the key moderators in angiogenesis, which is thought to be important for tumour growth and invasiveness.\textsuperscript{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).\textsuperscript{30-32} The variant alleles of the -1154G＞A and +936C＞T SNPs are associated with lower VEGF production\textsuperscript{34,35}, whereas the variant allele of the -460C＞T SNP results in increased promoter activity.\textsuperscript{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.\textsuperscript{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).\textsuperscript{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)\textsubscript{11-14}) has been determined.\textsuperscript{42} The 11-repeat polymorphism results in higher promoter activity in vitro.\textsuperscript{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.\textsuperscript{43-45} The enzyme coded by the variant allele is also less sensitive to hydroxlation dependent degradation\textsuperscript{46}, which results in further increased protein levels. Another nonsynonymous SNP (1790G＞A) in the HIF1A gene has also been associated with increased HIF1α expression.\textsuperscript{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 between the HIF1A SNPs and VEGF mRNA levels has been demonstrated, whereas no relationship with VEGF protein expression was found.\textsuperscript{46,47}

\textbf{Table 2} Overview of polymorphisms in genes that code for enzymes involved in the VEGF pathway

<table>
<thead>
<tr>
<th>Enzyme (gene)</th>
<th>Polymorphism</th>
<th>Phenotype</th>
<th>Function</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF (VEGF)</td>
<td>-2578C＞A</td>
<td>lower or higher VEGF</td>
<td>24,31</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-1154G＞A</td>
<td>lower VEGF</td>
<td>24,31</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-460C＞T</td>
<td>increased promoter activity</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+405G＞C</td>
<td>lower or higher VEGF</td>
<td>31,34,37</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+936C＞T</td>
<td>lower VEGF</td>
<td>31,34,37</td>
<td></td>
</tr>
<tr>
<td>KDR (KDR)</td>
<td>+4422(AC)\textsubscript{11-14}</td>
<td>11 CA repeats higher promoter activity than 12 CA repeats</td>
<td>42</td>
<td></td>
</tr>
<tr>
<td>HIF1α (HIF1A)</td>
<td>1772C＞T</td>
<td>Pro582Ser</td>
<td>higher HIF1α</td>
<td>43,46</td>
</tr>
<tr>
<td></td>
<td>1790G＞A</td>
<td>Ala588Thr</td>
<td>higher HIF1α</td>
<td>43</td>
</tr>
</tbody>
</table>

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 IgG, 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 \textit{FCGRT}).\textsuperscript{48}
Sachs et al. recently described a variable number of tandem repeats (VNTR) within the promoter of the \textit{FCGRT} gene consisting of five different alleles with one to five repeats (VNTR 1-5). 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. 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 \textit{FCGR3A} and \textit{FCGR2A}) are polymorphic (see table 3).

A SNP in the \textit{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. Also, the affinity of the IgG1-type MAb against CD20 expressing B cells, rituximab, was highest for the 158Val allele. It is therefore not surprising that response and PFS to rituximab in follicular lymphoma was higher for homoyzgotes of the 158Val allele, compared with carriers of the 158Phe allele. 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. Even though the function of a SNP in the \textit{FCGR2A} gene (535A>G; His131Arg) with regard to IgG, has not been established, an association with worse response and PFS to rituximab in follicular lymphoma was found. However, this association was not confirmed by another study. Very recently, Zhang et al. showed that mCRC patients treated with cetuximab monotherapy, who were homozygous for either the \textit{FCGR2A} 131Arg or \textit{FCGR3A} 158Val allele had shorter PFS and decreased response. The reason for this result, which for \textit{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 \textit{FCGR2A} 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. 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

<table>
<thead>
<tr>
<th>Enzyme (gene)</th>
<th>Polymorphism</th>
<th>Function</th>
<th>Ref</th>
<th>Pharmacogenetic association</th>
</tr>
</thead>
<tbody>
<tr>
<td>FcRn (FCGRT)</td>
<td>VNTR1-5</td>
<td>higher FcRn expression</td>
<td>49</td>
<td>higher response and lower PFS</td>
</tr>
<tr>
<td>FcγR2A</td>
<td>535A&gt;C</td>
<td>unknown</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FcγR3A</td>
<td>559T&gt;G</td>
<td>higher affinity for IgG1</td>
<td>50,51</td>
<td>lower response and lower PFS</td>
</tr>
<tr>
<td>FcγR3A</td>
<td>559T&gt;G</td>
<td>higher affinity for IgG1</td>
<td>53-57</td>
<td>lower response and lower PFS</td>
</tr>
<tr>
<td>FcγR3A</td>
<td>535A&gt;C</td>
<td>unknown</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FcγR3A</td>
<td>559T&gt;G</td>
<td>higher affinity for IgG1</td>
<td>53-57</td>
<td>lower response and lower PFS</td>
</tr>
<tr>
<td>FcγR3A</td>
<td>535A&gt;C</td>
<td>unknown</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Even though ADCC does not play a role for bevacizumab, it is likely that its effect is modified by similar mechanisms.
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.\(^\text{58}\) 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\(^\text{59,60}\), an open-access online database has been developed in which structural variations of the human genome are assembled\(^\text{60,61}\) (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\(^\text{22,24,62}\). However, these studies do not provide sufficient evidence to routinely genotype patients before applying these therapies. The associations need to be confirmed in future studies, and other, more specific assays 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.\(^\text{45}\) 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.\(^\text{66}\) 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 pathways will most likely find its way to daily clinical practice, provided that the above suggestions for future research have been met.
References


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

Jan Pander • Hans Gelderblom • Ninja F. Antonini • Jolien Tol
Johan H.J.M. van Krieken • Tahar van der Straaten • Cornelis J.A. Punt
Henk-Jan Guchelaar

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) CA14-22, 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=1.06-2.19, 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 EGF61G-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. Cetuximab is effective in patients with chemotherapy-refractory metastatic colorectal cancer (mCRC). A modest clinical benefit was shown for cetuximab when added to first-line chemotherapy. Recently, it has been demonstrated that the efficacy of cetuximab is limited to patients with wild-type KRAS tumors. 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. The severity of acneiform skin rash is also associated with the efficacy of cetuximab, 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. 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, 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 or in combination with irinotecan. However, these findings have been investigated in relation to KRAS mutation status in only one small study. 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 capcitabine, 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 capcitabine, oxaliplatin and bevacizumab or the same regimen plus...
cetuximab, the CAIRO2 study of the Dutch Colorectal Cancer Group (DCCG).\textsuperscript{11,15} Patient eligibility criteria are described in detail elsewhere.\textsuperscript{19} 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/m\(^2\) on the first day, followed by 250 mg/m\(^2\) 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.\textsuperscript{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 \textit{EGFR} c.61A>G (rs44444903), \textit{CCND1} c.932G>A (rs9344; also referred to as 870G>A), \textit{FCGR3A} c.535A>G (rs1801274; resulting in amino-acid change of histidine to arginine at position 131) and \textit{FCGR3A} c.818A>C (rs396999; 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 \textit{FCGR3A} polymorphism was also analyzed by Pyrosequencing for 15% of the samples, which confirmed the Taqman results.

The \textit{EGFR} (CA) polymorphism was analyzed by fragment analysis. Briefly, 10 ng of DNA was PCR amplified using primers FAM-5’-CCAATAATTTAATCACTGTTCT3’ and S’-AACACGTAGCAGCAATCC3’. PCR products were run on an ABI PRISM® 3730x Analyzer and analyzed with Genemapper v3.5 software (Applied Biosystems). Plasmids with an \textit{EGFR} insert containing 14 to 21 CA-repeats were used as a control.\textsuperscript{11} For the purpose of this analysis, the \textit{EGFR} CA-repeat polymorphism was dichotomized according to the criterion applied by Zhang and colleagues.\textsuperscript{11} Patients with two alleles containing less than 20 CA-repeats were designated ‘\textit{EGFR}<20’, whereas patients with either one or two alleles with 20 CA-repeats or more were designated as ‘\textit{EGFR}≥20’. All genotype frequencies were in Hardy-Weinberg equilibrium.

The \textit{KRAS} mutation status was determined in patients from whom primary tumor tissue was available. Tumor DNA was extracted and \textit{KRAS} mutation status was analyzed using a commercially available real-time PCR-based assay (DxS, Manchester, UK) and by direct sequencing.\textsuperscript{8}

**Statistical analysis**

The primary objective was to assess the association of the \textit{EGFR}, \textit{EGF}, \textit{CCND1}, \textit{FCGR2A} and \textit{FCGR3A} polymorphisms with PFS according to \textit{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%CIs) 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 polymorphism,\textsuperscript{19} 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.\textsuperscript{11} For the analysis of \textit{KRAS} wild-type and mutant combined, \textit{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%CIs 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.\textsuperscript{20,21} Factors that were significantly associated (p<0.05) with PFS in a multivariate analysis including treatment arm were 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). Additionally, the interaction terms of treatment arm and KRAS mutation status and the polymorphisms in CCND1, EGFR, FCGRA2 and FCGRA4 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. 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 91 (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 FCGRA3 C-allele (AC and CC genotypes combined) had a significantly decreased PFS compared with patients with the FCGRA3 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 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.

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) (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).

<table>
<thead>
<tr>
<th>Baseline characteristics</th>
<th>Patients included in the pharmacogenetic analysis</th>
<th>Patients with no DNA available</th>
<th>Patients with no DNA available</th>
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<td>Arm A (n=282)</td>
<td>Arm B (n=282)</td>
<td>P value</td>
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<td>Female</td>
<td>123 (44%)</td>
<td>102 (36%)</td>
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</tr>
<tr>
<td>Gender</td>
<td>159 (56%)</td>
<td>180 (64%)</td>
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</tr>
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<td>KRAS mutation status – no. (%)</td>
<td>119 (42%)</td>
<td>127 (45%)</td>
<td>0.23</td>
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<td>Progression-free survival – months</td>
<td>Median</td>
<td>10.8</td>
<td>0.00</td>
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<td>Overall survival – months</td>
<td>Median</td>
<td>17.8</td>
<td>0.00</td>
</tr>
<tr>
<td>Overall worst grade cetuximab-related skin toxicity – no. (%)</td>
<td>grade 0 – 2</td>
<td>280 (99%)</td>
<td>0.30</td>
</tr>
<tr>
<td></td>
<td>grade 3</td>
<td>21 (7%)</td>
<td>0.94</td>
</tr>
</tbody>
</table>

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

Chapter 3
Pharmacogenetics of cetuximab in advanced colorectal cancer

3

43
### Table 2: Analysis of progression-free survival in KRAS wild-type patients

<table>
<thead>
<tr>
<th>Gene</th>
<th>SNP</th>
<th>Genotype</th>
<th>Arm A</th>
<th>n</th>
<th>Median PFS</th>
<th>HR</th>
<th>95% CI</th>
<th>P</th>
<th>Arm B</th>
<th>n</th>
<th>Median PFS</th>
<th>HR</th>
<th>95% CI</th>
<th>P</th>
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<td>FCGR3A</td>
<td>818A&gt;C</td>
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<td>0.69-1.58</td>
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<td></td>
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<td>AC or CC</td>
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<td>1.05</td>
<td>0.69-1.58</td>
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<td>FCGR2A</td>
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<td>1.00</td>
<td>0.44-1.09</td>
<td>1.11</td>
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<td>0.44-1.09</td>
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<td>CA-repeat</td>
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<td>10.7</td>
<td>1.00</td>
<td>0.73-1.67</td>
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<td>72</td>
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<td></td>
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<td>EGFR≥20</td>
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<td>0.73-1.67</td>
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<td>GA</td>
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<td>9.7</td>
<td>0.93</td>
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<td>0.72</td>
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<td>0.96-2.36</td>
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</table>

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

<table>
<thead>
<tr>
<th>Gene</th>
<th>SNP</th>
<th>Genotype</th>
<th>Arm A</th>
<th>n</th>
<th>Median PFS</th>
<th>HR</th>
<th>95% CI</th>
<th>P</th>
<th>Arm B</th>
<th>n</th>
<th>Median PFS</th>
<th>HR</th>
<th>95% CI</th>
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<td>40</td>
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</table>

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
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

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

1 Odds 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-allele, 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 agent; though this was not confirmed in an extended analysis of this study with more patients. 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 not find a significant association between the FCGR3A polymorphism and the efficacy of cetuximab. A possible mechanism for the opposite association of the FCGR3A polymorphism could be that the high affinity C-allele results in increased activation of tumor associated macrophages (TAMs) by cetuximab through cross-linking of the Fc gamma receptor, 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). In our study, patients who 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, which could have altered the infiltration of cells of the myeloid lineage, such as TAMs. However, it must be noted that the FCGR3A C-allele was associated with increased efficacy of the IgG-type monoclonal antibodies rituximab in lymphoma and trastuzumab in advanced breast cancer. 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 colleagues, 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 patients. 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-repeats. Although EGFR expression, as measured by immunohistochemistry, is not a predictor of the efficacy of cetuximab, the number of EGFR gene copies is associated with the response to cetuximab treatment. 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. Skin toxicity is a major side effect of cetuximab treatment and the severity of skin toxicity is associated with the response to cetuximab, but the underlying mechanism is not yet unraveled. Since we demonstrate a relationship between the FCGR3A polymorphism and the efficacy of cetuximab, a possible role of the polymorphism in the incidence of skin toxicity during anti-EGFR therapy cannot be excluded. 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, as well as normal tissue or tumor tissue, this should not have influenced the results, because there is an almost perfect degree of concordance between germline genotype in tumor and normal tissue. 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. 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 group, 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.\(^{44,45}\)

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.

References

2. Cunningham D, Humblet Y, Siena S, et al. Cetuximab monotherapy and cetuximab plus irinotecan in
Activation of tumor-promoting type 2 macrophages by the EGFR-targeting antibody cetuximab

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Renée Baak-Pablo • Hans Gelderblom • Hans Morreau • Sjoerd van der Burg
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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 Fcy receptors (FcyRs) 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), which was especially significant for patients carrying the high affinity valine allele.6 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.6 Recent literature even implies an active involvement of FcyRs and myeloid cells in carcinoma development.7 We therefore hypothesized that therapeutic antibodies, such as cetuximab, might locally cross-link FcyRs 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

Formaline fixed and paraffin embedded tumors from ten untreated stage III colorectal cancer patients were selected form the pathology archive of the Leiden University Medical Center. Staining for NK cells and M2 macrophages was described before.13
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 (Erbitux®, 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). Fcy 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. 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.

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 FcγRII genotype encoding the valine residue. 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 2](image-url)

**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.
Known to actively contribute to tumor growth via angiogenesis and immune suppression. 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 cetuximab, however, staining for NK cells in 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 known to actively contribute to tumor growth via angiogenesis and immune suppression. 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 cetuximab, 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 cells, 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 unanticipated, since the combination of cetuximab and anti-VEGF therapy appeared effective in mouse models. 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. The fact that M2 macrophages encoding the high affinity FcγRIIA (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. 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. Also, expression of EGFR on tumor cells could be up-regulated by chemotherapy such as fluoropyrimidines and irinotecan.

In conclusion, clinical testing of engineered MAbs with Fc-regions with increased affinity to FcγRs should be performed very carefully, 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.

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**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.
References


Capecitabine, oxaliplatin and bevacizumab
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

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 though 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. 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...
Pharmacogenetics 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. 1,2 TPMT activity is inversely associated with exposure to the cytotoxic metabolite of 6MP, 6-thioguanine (6TGN), in red blood cells 3 and in ALL blasts. 4 Because of severe haematological
tumours is 5-Fluorouracil (5FU). Over 80% of 5FU is inactivated in the liver by the Dihydropyrimidine dehydrogenase (DPD) and by UGT1A7 in the upper gastrointestinal tract. 38 Irinotecan, is predominantly inactivated by the isoforms UGT1A1 and UGT1A9 in the liver and by UGT1A7 in the upper gastrointestinal tract. 37 A TA insert polymorphism in the TATA box of the promoter region of the UGT1A 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 glucuronidation 39 and has also been associated with decreased SN-38 glucuronidation in vitro 40 and in vivo. 41 The allele frequency of UGT1A1*28 is higher in Caucasians (22-39%) 42-44 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 a DPD deficient patient experiencing severe haematological toxicity to 5FU was described in 1988 46 many mutations in the DPDY 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 47, 48, a SNP in the 5’ invariant splice donor sequence in intron 14 of the DPDY gene (IVS14+1G>A; deletion of exon 14, DPDY*2A) seems to be one of the key mutations resulting in low DPD activity and increased incidence of 5FU toxicity. 49 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) [20]) to 5FU containing chemotherapy, the frequency of the DPDY*2A allele was 15%, which was significantly higher than 0.91% in the control population (P=0.001). 50 Also, 50% of patients who experienced NCI-CTC grade 4 neutropenia carried the DPDY*2A allele. 51 The DPDY*2A allele has not been detected in Asian populations. 52-54 Other variants of the DPDY gene that have been linked to 5FU toxicity include the DPDY*4 (1601G>A; Ser534Asn), DPDY*10 (1003G>T; Val335Leu), DPDY*12 (62G>A; Arg21Gln + 1156G>T; Glu386Ter) and DPDY*23 (1679T>G; Ile560Ser) alleles. 55 On the other hand, methylation of the promoter region of the DPDY gene also seems to reduce DPD activity due to a decrease of transcription. 56 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. 57-58

**Uridine diphosphatase 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 gastrointestinal 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 glucuronidation 39 and has also been associated with decreased SN-38 glucuronidation in vitro 40 and in vivo. 41 The allele frequency of UGT1A1*28 is higher in Caucasians (22-39%) 42-44 than in Asians (7-17%) 44-46, 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))\(^{31}\) and NCI-CTC grade 3 or 4 neutropenia\(^{62,63}\) 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 patients\(^{34}\) and higher incidence of grade 3 or 4 diarrhoea and/or grade 4 leukaemia (according to criteria of the Japan Society for Cancer Therapy\(^{53}\)) in patients with solid tumours\(^{47}\) 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.\(^{14}\) 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 leukaemia and grade 3 or 4 diarrhoea; Japanese criteria) in Japanese cancer patients treated with irinotecan.\(^{51}\) However, the UGT1A1*60 variant allele was linked to the UGT1A1*28 variant.\(^{52}\)

Several SNPs in various regions of the UGT1A1, UGT1A7 and UGT1A9 genes have been found to be in linkage disequilibrium (LD).\(^{44,46,47}\) The functional and clinical relevance of these haplotypes has not yet been established. In 2005, the FDA approved the Invader\textsuperscript{®} UGT1A1 molecular assay, a test for the UGT1A1*28 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 \(\pi\), GST \(\mu\) and GST \(\theta\), which are products of distinct loci in the genome. Among substrates for GSTs are cyclophosphamide, etoposide, doxorubicin, cisplatin, carboplatin and oxalipatin 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 \(\pi\) (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 \(\pi\) activity.\(^{37}\) 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 oxalipatin.\(^{61}\) Survival was 24.9, 13.3 and 7.9 months for Val/Val, Val/Ile and Ile/Ile genotypes respectively (\(P=0.001\)).\(^{46}\) 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 \(\mu\) (GSTM1) and GST \(\theta\) (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 patients\(^{65}\), nor in a cohort of colorectal cancer (CRC) patients.\(^{66}\) Survival of ovarian cancer patients treated with platinum containing chemotherapy was also better for GSTM1 null patients.\(^{55,66}\)

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

\(\text{ABC}B1\)

The ATP-binding cassette (ABC) B1 gene (\(\text{ABC}B1\)), formerly known as multi-drug resistance (\(\text{MDR}1\)) 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 (\(\text{MDR}1\)*2), with a frequency of 27%, 31-49% and 6.5% in Caucasians, Asians and Blacks respectively.\(^{67-69}\) The \(\text{MDR}1\)*2 haplotype was associated with lower irinotecan and SN-38 clearance\(^{70}\) and with lower \(C_{\text{max}}\) for glucuronidated SN-38 (SN-38G).\(^{71,72}\)

Cancer patients homozygous for the 1236C>T variant allele had higher
exposure to both irinotecan and SN-38. In 58 patients with solid tumors, all patients homozygous for the 3435T variant allele experienced grade 3-4 neutropenia (specified as neutrophil count between 0.5 and 1.0 x 10^9/L and less than 0.5 x 10^9/L respectively) to docetaxel, compared with 77% and 54% for heterozygote and wild-type individuals respectively. 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. A SNP in exon 5 (421C>A; Gln141Lys) has an allele frequency of 34%, 12% and 1-5% in Han Chinese, Caucasians and Blacks respectively, and results in lower BCRP expression and higher SN-38 and topotecan accumulation. As exposure to docetaxel is increased in carriers of the variant allele, one would expect that the SNP leading to reduced ERCC1 expression and hence to increased ERCC1 mRNA expression guided phase III study is ongoing. A silent SNP has been identified in exon 4 at codon 118 in the X-ray repair cross complementing group 1 (XRCC1) enzyme is involved in repair of DNA containing compounds such as cisplatin, carboplatin and oxaliplatin. Increased ERCC1 expression has been shown to lead to cisplatin resistance in vitro and to lower response in cisplatin treated bladder cancer patients in vivo. A prospective ERCC1 mRNA expression guided phase III study is ongoing. 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. Even though encoding the same amino acid, the variant codon is believed to occur less commonly, therefore resulting in reduced ERCC1 expression. 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. This same association was found in another cohort of NSCLC stage IIIb-IV patients treated with cisplatin and docetaxel. Homozygote wild-type mCRC patients were also found to have longer survival compared to carriers of the variant allele when treated with oxaliplatin and 5FU. In melanoma patients (stage IV) treated with cisplatin containing chemotherapy, the wild-type genotype was associated with worse response and shorter overall survival. Ovarian cancer patients who carried the variant allele had reduced risk of platinum resistance, but survival was not affected by genotype.

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 (XPD) 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. The allele frequency of the XPD-321 variant allele was 0.32 in a general Western population. The allele frequency of the XPD-751 variant allele is 0.44 in Caucasians, 0.16 in Blacks and 0.09 in Asians. 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. The two SNPs in the ERCC2 gene were not associated with response and survival in cisplatin treated NSCLC patients, but the wild-type ERCC2-751 allele was associated with increased incidence of WHO grade 2 or higher neutropenia. 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.

**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 Asians) has been associated with worse response to oxaliplatin and 5FU in mCRC patients, but there was no difference in time to progression or survival. In stage III NSCLC patients, survival was shorter for individuals homozygote for the variant allele compared with carriers of the wild-type allele. 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. In contrast, in patients with squamous cell cancer of the head and neck (SCC) 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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{100-105} Other alleles resulting in lower in gene deletion and thus in absent CYP2D6 activity and has a frequency of 15-20% in the general population in Western countries.\textsuperscript{100-105} Other alleles resulting in lower CYP2D6 activity are CYP2D6*3 (Δ2549A), CYP2D6*5 (deletion of entire CYP2D6 gene) and CYP2D6*6 (Δ1707T). The CYP2D6*4 allele has been linked to reduced conversion of tamoxifen into 4-hydroxy-tamoxifen\textsuperscript{100} and the CYP2D6*3-6 genotypes have been related to lower endoxifen formation.\textsuperscript{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\textsuperscript{101} patients did not experience moderate to severe flashes, which is a side effect of (the active metabolite of) tamoxifen.\textsuperscript{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\textsuperscript{101} genotype was higher in women who developed breast cancer during follow up than in women free of cancer.\textsuperscript{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.\textsuperscript{100} Selection bias in this study may have influenced the outcome of this study.\textsuperscript{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 subtype S10-methylene tetrahydrofolate (S10-MTHF) facilitates the inhibition of thymidylate synthase (TS) by SFU, 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\textsuperscript{105-104} MTHFR activity is 70% and 35% for heterozygote and homozygote individuals respectively.\textsuperscript{103} In vitro assays showed that cell lines with the variant allele are more sensitive to SFU and less sensitive to MTX.\textsuperscript{103} Of six patients who experienced severe toxicity to adjuvant CMF (cyclophosphamide, MTX, SFU) for breast cancer, five were homozygous for the 677T allele.\textsuperscript{106} In a cohort of cancer patients who were treated with the SFU analogue raltitrexed, patients with 677TT genotype had significant more therapy related toxicity.\textsuperscript{107} Leukaemia patients homozygous for the 677T allele experienced more MTX related toxicity compared with patients who carried at least one wild-type allele.\textsuperscript{108} 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.\textsuperscript{102}

In mCRC patients, the 677T allele has been associated with improved response to SFU based chemotherapy in several studies\textsuperscript{109,126,132}, whereas another study did not find a significant association.\textsuperscript{122} No association was found in a cohort of advanced gastric cancer patients treated with SFU and cisplatin.\textsuperscript{62}

**Thymidylate synthase (TS)**

TS is the central enzyme in the de-novo thymidine synthesis. In vitro resistance to SFU is associated with increased TS activity, which is also induced by SFU itself.\textsuperscript{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.\textsuperscript{124,125} The allele frequency of the TSER*2 allele is 0.40-0.46 in Caucasians and Blacks\textsuperscript{126,127}, compared to 0.18-0.21 in Asian populations.\textsuperscript{126,128,130}

The TSER*3 allele was associated with increased response in CRC patients treated with SFU.\textsuperscript{112} On the other hand, the TSER*2 allele was associated with improved response to capecitabine in CRC patients.\textsuperscript{111}

These conflicting results could in part be explained by a G>C SNP in the 12th base pair of the TSER*3 allele\textsuperscript{32} that results in TS activity similar to that of the TSER*2 allele.\textsuperscript{113} This TSER*3C allele is found in 29%-57% of all TSER*3 alleles.\textsuperscript{124,125} Carriers of the TSER*3C allele had significantly worse response, disease free survival and overall survival in a cohort of mCRC patients treated with SFU.\textsuperscript{136} The TSER*3G allele was also associated with worse survival in advanced gastric cancer patients who were treated with SFU.\textsuperscript{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. The -6bp mutation is in linkage disequilibrium (LD) with the TSER*3 allele, and the +6bp allele is in LD with TSER*2. The -6bp variant allele is associated with decreased survival in mCRC patients treated with 5FU and oxaliplatin and with decreased response to 5FU based chemotherapy in advanced gastric cancer patients.

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 to the other genotypes. 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. 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.

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 GSTP1-105Val 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. 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 ≥ two favourable genotypes (P<0.001). 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. 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.

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.

Chapter 5

Pharmacogenetics of anti-cancer drugs
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.

References

Pharmacogenetics of anti-cancer drugs


Pharmacogenetics of anti-cancer drugs

5

Chapter 5


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

Jan Pander • Hans Gelderblom • Tahar van der Straaten • Cornelis J.A. Punt
Henk-Jan Guchelaar

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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). 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. 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. 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 results 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
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. 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, 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.

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

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Median PFS in months (95%CI)</th>
<th>Univariate HR (95%CI)</th>
<th>P*</th>
<th>Multivariate HR (95%CI)**</th>
<th>P**</th>
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<tr>
<td>ATM rs1801516</td>
<td></td>
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<tr>
<td>Wild-type</td>
<td>371</td>
<td>9.1 (8.3-10.4)</td>
<td>1</td>
<td>-</td>
<td>1</td>
<td>-</td>
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<tr>
<td>Heterozygote</td>
<td>127</td>
<td>12.4 (9.6-13.5)</td>
<td>0.88 (0.70-1.09)</td>
<td>.245</td>
<td>0.93 (0.75-1.17)</td>
<td>.543</td>
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<td>Homozygote mutant</td>
<td>8</td>
<td>11.8 (7.2-15)</td>
<td>0.61 (0.27-1.36)</td>
<td>.225</td>
<td>0.94 (0.42-2.12)</td>
<td>.881</td>
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<tr>
<td>ERCC5 rs1047768</td>
<td></td>
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<tr>
<td>Wild-type</td>
<td>180</td>
<td>10.6 (9.1-12.5)</td>
<td>1</td>
<td>-</td>
<td>1</td>
<td>-</td>
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<tr>
<td>Heterozygote</td>
<td>267</td>
<td>9.2 (8.2-10.6)</td>
<td>1.13 (0.93-1.39)</td>
<td>.227</td>
<td>1.15 (0.93-1.42)</td>
<td>.194</td>
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<tr>
<td>Homozygote mutant</td>
<td>77</td>
<td>10.1 (8.5-12.2)</td>
<td>0.96 (0.72-1.29)</td>
<td>.797</td>
<td>0.94 (0.69-1.28)</td>
<td>.689</td>
</tr>
</tbody>
</table>

* 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

References

Pharmacogenetics of tomorrow: the 1+1=3 principle

Jan Pander • Judith A.M. Wessels • Ron H.J. Mathijssen • Hans Gelderblom
Henk-Jan Guchelaar

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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 is 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 disappointing,2-4 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 lead 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)20,21 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), VEGFR1-92G>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=0.08 obtained by permutation)23,24 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
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. 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 $\chi^2$ 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.
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. 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 \( \text{FLT3}_{738C} \)-allele and not for carriers of the \( \text{FLT3}_{738T} \) 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.

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**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.
References


Websites

Freely available MDR software (accessed February, 2010)

Freely available permutation testing software for MDR (accessed February, 2010)

Chapter 7
Pharmacogenetic interaction analysis for the efficacy of systemic treatment in metastatic colorectal cancer

Jan Pander • Judith A.M. Wessels • Hans Gelderblom • Tahar van der Straaten • Cornelis J.A. Punt • Henk-Jan Guchelaar

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 \( \text{TYMS} \) 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 \( \text{TYMS} \) 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). 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. As a result, none of these polymorphisms are currently used in general practice to identify patients with an increased chance of response.

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). Capecitabine 1000 mg/m\(^2\) (increased to 1250 mg/m\(^2\) from cycle 7) was administered orally twice daily on days 1 to 14 of each 3-week treatment cycle. Oxaliplatin 130 mg/m\(^2\) (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,
Pharmacogenetic interaction analysis of the efficacy of CAPOX-B

Table 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>rs-number</th>
<th>genetic alteration</th>
<th>effect of polymorphism on the protein level</th>
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<tbody>
<tr>
<td><strong>Capecitabine</strong>&lt;sup&gt;8-10&lt;/sup&gt;</td>
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<tr>
<td>MTHFR</td>
<td>-180113</td>
<td>677C&gt;T</td>
<td>Ala222Val; reduced MTHFR activity for the 222Val-allele&lt;sup&gt;11&lt;/sup&gt;</td>
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<td>MTHFR</td>
<td>-180131</td>
<td>1298A&gt;C</td>
<td>Gln429Ala; decreased MTHFR activity for the 429Ala-allele&lt;sup&gt;12&lt;/sup&gt;</td>
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<td>TYMS</td>
<td>-1494 +/- 6bp (3'-UTR)</td>
<td>Reduced TYMS expression for the 6bp deletion allele&lt;sup&gt;7&lt;/sup&gt;</td>
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<td>TYMS</td>
<td>-232/3G (TSER)</td>
<td>Increased TYMS expression for the 3G allele&lt;sup&gt;13&lt;/sup&gt;</td>
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<td><strong>Oxaliplatin</strong>&lt;sup&gt;18-29&lt;/sup&gt;</td>
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<td>ERCC1</td>
<td>-11615</td>
<td>496C&gt;T</td>
<td>Asn118Asn; decreased ERCC1 expression for the 496T allele&lt;sup&gt;8&lt;/sup&gt;</td>
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<td>ERCC2</td>
<td>-2868</td>
<td>499C&gt;A</td>
<td>Arg156Glu; supposed reduced DNA-repair capacity for 499A-allele&lt;sup&gt;9&lt;/sup&gt;</td>
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<td>ERCC2</td>
<td>-13181</td>
<td>2231A&gt;C</td>
<td>Lys751Gln; reduced DNA-repair capacity for 751Gln-allele&lt;sup&gt;10&lt;/sup&gt;</td>
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<td>XRC1</td>
<td>-25687</td>
<td>1301G&gt;A</td>
<td>Arg399Gln; deficient DNA-repair for 399A-allele&lt;sup&gt;11&lt;/sup&gt;</td>
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<td>GSTP1</td>
<td>-1695</td>
<td>313A&gt;G</td>
<td>Ile105Val; decreased GSTπ activity for 105Val-allele&lt;sup&gt;10,11&lt;/sup&gt;</td>
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<td><strong>Bevacizumab</strong>&lt;sup&gt;30,31&lt;/sup&gt;</td>
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<td>KDR</td>
<td>-187377</td>
<td>1719A&gt;T</td>
<td>Glu472His; amino acid substitution located in fifth NH2-terminal Ig-like domain within the extracellular ligand binding region&lt;sup&gt;22&lt;/sup&gt;</td>
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<td>KDR</td>
<td>-2071559</td>
<td>-604T&gt;C (promoter)</td>
<td>Altered the binding site for transcriptional factor E2F&lt;sup&gt;22&lt;/sup&gt;</td>
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<td>VEGF</td>
<td>-3190</td>
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<td>Decreased VEGF production and expression for -1154A allele&lt;sup&gt;12&lt;/sup&gt;</td>
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<td>VEGF</td>
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<td>Decreased VEGF levels for the 936T allele&lt;sup&gt;13&lt;/sup&gt;</td>
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<td>-833061</td>
<td>-405C&gt;G</td>
<td>Increased VEGF expression for -405G allele&lt;sup&gt;13&lt;/sup&gt;</td>
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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; VNTR, variable number of tandem repeats; UTR, untranslated region; XRCC1, X-ray cross-complementing group 1.

Statistical analysis

The studied genetic polymorphisms are shown in Table 1. These polymorphisms were selected primarily on the basis of the pharmacokinetics and pharmacodynamics of the study agents, including serum LDH, age and gender as covariates. Polymorphisms significantly associated with drug response were included for further analysis, especially those that deviated Hardy-Weinberg equilibrium (P>0.01). Each study was conducted with 10% duplicates, with water as negative control. The genotype was expressed as non-carriers 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 result in increased TYMS activity). 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 result in increased TYMS activity). 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 result in increased TYMS activity). 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 result in increased TYMS activity). 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 result in increased TYMS activity). 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 result in increased TYMS activity). 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 result in increased TYMS activity). 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 result in increased TYMS activity). 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 result in increased TYMS activity). 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 result in increased TYMS activity). 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 result in increased TYMS activity). 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 result in increased TYMS activity). 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 result in increased TYMS activity). 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 result in increased TYMS activity). 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 result in increased TYMS activity). 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 result in increased TYMS activity). 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 result in increased TYMS activity). 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 result in increased TYMS activity). 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 result in increased TYMS activity). 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 result in increased TYMS activity). 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 result in increased TYMS activity). 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 result in increased TYMS activity).
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 ≥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) 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, χ² 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

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>allelic HR*</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTHFR 677C&gt;T</td>
<td>1.00</td>
<td>0.81-1.23</td>
<td>0.991</td>
</tr>
<tr>
<td>MTHFR 1298A&gt;C</td>
<td>0.91</td>
<td>0.74-1.13</td>
<td>0.393</td>
</tr>
<tr>
<td>TMY5 1494 +/- 6bp</td>
<td>1.10</td>
<td>0.87-1.40</td>
<td>0.410</td>
</tr>
<tr>
<td>TMY5 VNTR 2/3C/3G</td>
<td>1.02</td>
<td>0.77-1.36</td>
<td>0.884</td>
</tr>
<tr>
<td>ERCC1 496C&gt;T</td>
<td>1.12</td>
<td>0.92-1.37</td>
<td>0.243</td>
</tr>
<tr>
<td>ERCC2 499C&gt;A</td>
<td>1.15</td>
<td>0.94-1.40</td>
<td>0.185</td>
</tr>
<tr>
<td>ERCC2 2251A&gt;C</td>
<td>1.00</td>
<td>0.82-1.21</td>
<td>0.968</td>
</tr>
<tr>
<td>ERCC2 965G&gt;A</td>
<td>0.80</td>
<td>0.63-1.01</td>
<td>0.058</td>
</tr>
<tr>
<td>XRCC1 1301G&gt;A</td>
<td>0.98</td>
<td>0.81-1.18</td>
<td>0.811</td>
</tr>
<tr>
<td>GSTP1 313A&gt;G</td>
<td>0.98</td>
<td>0.81-1.19</td>
<td>0.837</td>
</tr>
<tr>
<td>KDR 1719A&gt;T</td>
<td>1.08</td>
<td>0.88-1.33</td>
<td>0.465</td>
</tr>
<tr>
<td>KDR -604T&gt;C</td>
<td>1.03</td>
<td>0.86-1.24</td>
<td>0.738</td>
</tr>
<tr>
<td>VEGF -1154G&gt;A</td>
<td>1.09</td>
<td>0.90-1.33</td>
<td>0.381</td>
</tr>
<tr>
<td>VEGF 405G&gt;C</td>
<td>0.97</td>
<td>0.81-1.18</td>
<td>0.785</td>
</tr>
<tr>
<td>VEGF 938C&gt;T</td>
<td>0.98</td>
<td>0.74-1.29</td>
<td>0.889</td>
</tr>
<tr>
<td>VEGF -2578C&gt;A</td>
<td>1.03</td>
<td>0.86-1.23</td>
<td>0.763</td>
</tr>
<tr>
<td>VEGF -600G&gt;T</td>
<td>1.00</td>
<td>0.84-1.20</td>
<td>0.990</td>
</tr>
</tbody>
</table>

* 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; TMY5, 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).

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**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 TMY5-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 TMY5-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).
the TYMS-TSER polymorphism and the VEGF +405G>C polymorphisms are dependent of each other in their impact on PFS. 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 monoclonal blood cells with the VEGF +405G-allele 40, 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 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 –

The frequency of the unfavorable profile decreases for each quartile for PFS (P<0.001, χ² 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 –
et al. that the TYMS-TSER 3G allele is associated with decreased efficacy of SFU-based chemotherapy in mCRC patients, is therefore only present for VEGF -4405C-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. 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 data, 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.

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. 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 -4405C>G 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.

Reference list

Pharmacogenetic interaction analysis of the efficacy of CAPOX-B


32. Pander J, Gelderblom H, Brenchley PE. Identification of polymorphisms within the vascular endothelial growth factor-2 genetic polymorphisms with outcome in a trial of paclitaxel (mCRC) patients (pts). Eur J Cancer Supplements, 7[2], 357. 2009. (Abstract)


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

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Yavuz Ariyurek • Jeanine Houwing-Duistermaat • Hans Gelderblom
Cornelis Punt • Henk-Jan Guchelaar

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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). 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%.

Heritable genetic variation has proven to predict variation in response to many therapeutics drugs. 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. Regarding outcome of systemic therapy, genome-wide association studies have identified SNPs associated with muscoskeletal adverse reactions to aromatase inhibitors, treatment response for childhood acute lymphoblastic lymphoma and pharmacokinetics of methotrexate. All of these studies are based upon a case-control design with $\chi^2$-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). Capecitabine 1000 mg/m$^2$ (increased to 1250 mg/m$^2$ from cycle 7) was administered orally twice daily on days 1–14 of each 3-week treatment cycle. Oxaliplatin 130 mg/m$^2$ (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. 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 OmniExpress 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; CaliFreq ≥ 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 allelic 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 χ² 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 ≤ 5.0 x 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 \((\text{median}(T_1,…,T_n)^{10} / 0.675)^2\) with \(T_1,…,T_n\) being the square roots of the χ² 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

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Age - year</th>
<th>Sex - no (%)</th>
<th>Arm - no (%)</th>
<th>Serum lactate dehydrogenase level - no (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>median</td>
<td>range</td>
<td>male</td>
<td>female</td>
</tr>
<tr>
<td></td>
<td>63.1</td>
<td>27.6 - 83.6</td>
<td>316 (60.7%)</td>
<td>205 (39.3%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CAPOX-B</td>
<td>264 (50.7%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CAPOX-B plus cetuximab</td>
<td>257 (49.3%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>正常*</td>
<td>307 (58.9%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>正常*</td>
<td>213 (40.9%)</td>
</tr>
</tbody>
</table>

* 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 \(P = 6.75 \times 10^{-7}\)).
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. 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 $5 \times 10^{-8}$ 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 included 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

<table>
<thead>
<tr>
<th>marker</th>
<th>chr position</th>
<th>gene</th>
<th>allele frequency</th>
<th>P-value</th>
<th>allelic HR (95%CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs2936519</td>
<td>8</td>
<td>6626650</td>
<td>n.a.</td>
<td>0.104</td>
<td>1.24 x 10^{-3}</td>
</tr>
<tr>
<td>rs2912024</td>
<td>8</td>
<td>6626309</td>
<td>n.a.</td>
<td>0.105</td>
<td>1.38 x 10^{-3}</td>
</tr>
<tr>
<td>rs2978931</td>
<td>8</td>
<td>6625491</td>
<td>n.a.</td>
<td>0.101</td>
<td>6.75 x 10^{-7}</td>
</tr>
<tr>
<td>rs4850159</td>
<td>2</td>
<td>131442241</td>
<td>ARHGEF4</td>
<td>0.136</td>
<td>2.58 x 10^{-4}</td>
</tr>
<tr>
<td>rs6734725</td>
<td>2</td>
<td>46751074</td>
<td>n.a.</td>
<td>0.350</td>
<td>2.99 x 10^{-4}</td>
</tr>
<tr>
<td>rs17688362</td>
<td>18</td>
<td>39999678</td>
<td>n.a.</td>
<td>0.185</td>
<td>4.68 x 10^{-4}</td>
</tr>
<tr>
<td>rs17448629</td>
<td>4</td>
<td>113593423</td>
<td>n.a.</td>
<td>0.133</td>
<td>5.49 x 10^{-10}</td>
</tr>
<tr>
<td>rs11730442</td>
<td>4</td>
<td>113581912</td>
<td>ALPK1</td>
<td>0.132</td>
<td>6.62 x 10^{-4}</td>
</tr>
<tr>
<td>rs1009490</td>
<td>8</td>
<td>92317629</td>
<td>n.a.</td>
<td>0.061</td>
<td>7.20 x 10^{-12}</td>
</tr>
<tr>
<td>rs1795016</td>
<td>4</td>
<td>86945264</td>
<td>ARHGEF4</td>
<td>0.402</td>
<td>7.61 x 10^{-4}</td>
</tr>
</tbody>
</table>

Abbreviations: ALPK1, α-kinase 1; ARHGEF4, Rho GTPase activating protein 4; 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).

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. 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 $5 \times 10^{-8}$ 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 included in the analysis.
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.
References

1. CBD guideline management of colorectal cancer (http://www.cbo.nl/product/richtlijnen/folder2002-1023121843/l_colonc_08.pdf)
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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. In both cases, SNPs cause decreased functionality of these key detoxifying enzymes, 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 biomarkers, 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. 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 study\(^\)\(^10\)). 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)\(^2\), 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.\(^13,14\) 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.\(^15,16\) 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. Current studies include many polymorphisms in many potentially relevant genes. 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 repair, 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. 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. 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.

Pharmacodynamics
In most cases, the principal mechanism of action of a drug is relatively well studied. For capcitabine 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). However, at least both the pyrimidine and folate metabolism routes are involved, in which many enzymes play a role. 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 capcitabine. However, most pharmacogenetic studies on capcitabine and other fluoropyrimidines – including our study presented in chapter 8 – include only polymorphisms in TYMS, DPYD 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 (TGFα), by blocking their receptor EGFR. 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. 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. 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. 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-angiogenic – 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, as patients in the CAIRO2 study received bevacizumab, capcitabine and oxaliplatin combined with cetuximab, whereas the other studies included cetuximab as monotherapy or in combination with irinotecan. 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.

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. 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. 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. 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.

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. 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. 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 x 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. 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. 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.

References

Summary
Nederlandse samenvatting
Dankwoord
List of publications
Curriculum vitae
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-FcγR 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 VEGF enhancer region and VEGF +405G>C polymorphisms was significantly associated with PFS. Median PFS was 15.3 (95%CI, 11.4 to 15.3) and 9.7 (95%CI, 76 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 VEGF 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 x 10^{-8}. Three single nucleotide polymorphisms located at 8p23.1 showed a trend toward significance for association with PFS (rs3996619, P = 1.24 x 10^{-5}; rs2912024, P = 1.38 x 10^{-5} and rs2978931, P = 6.75 x 10^{-6}). These SNPs are 20 kbp downstream of the AGPAT5 gene, which encodes a protein that converts lysophosphaticid acid (a mitogen that has been linked to cancer in different ways) to phosphatic 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 routing 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
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.
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 patienten 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 patienten 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.

Hoofdstuk 3 beschrijven we de correlatie tussen deze polymorfismen en progressie-vrije overleving van 576 gemetastaseerde dikke darm kanker patienten 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 behandelmee k 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 patienten 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 patienten 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 patienten die niet werden behandeld met cetuximab. In de patienten met een KRAS gemuteerde tumor was het EGF 61-allel geassocieerd met een kortere progressie-vrije overleving in patienten 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 concluden 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 allele niet was geassocieerd met kortere progressie-vrije overleving (zoals beschreven in hoofdstuk 3), hebben we in vitro experimenten gedaan om het onderriggende 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 IL1β, IL8 en VEGF. In een experiment 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 IL1β, IL8 en VEGF. In een experiment waarbij tumorcellen, cetuximab en type 2 macrofagen gezamenlijk werden geincubeerd, zagen wij een cetuximab concentratie afhankelijke activatie van de type 2 macrofagen. Deze activatie was sterk voor type 2 macrofagen met het FCGR3A 158-valine allele (P=0,048). Hieruit concluderen we dat tumorgroei bevorderende macrofagen geactiveerd kunnen worden door cetuximab.

Cetuximabe, 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-efffectiviteit 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 patienten 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 patienten. 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 illustreer 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 geïntegreerd in de analyse in 279 patienten 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 patienten uit de CAIRO2 studie gebruikt. Het genotypieren werd gedaan met OmniExpress arrays van Illumina, waar meer dan 700.000 polymorfismen gelijktijdig kunnen worden bepaald. Drie polymorfismen op de genomische locatie 8p23.1 werden herkend. 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.
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 geneesmiddel-werking 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 patienten 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 patienten is noodzakelijk, en prospectief onderzoek is nodig voordat een farmacogenetische test in de dagelijkse klinische praktijk kan worden toegepast.

Nederlandse samenvatting

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Mijn 3 schatjes aan het thuisfront.
List of publications


Curriculum Vitae


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.