Pharmacogenetics of capecitabine and oxaliplatin in treatment of advanced colorectal cancer

Lieke van Huis – Tanja

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Introduction to this thesis

Colorectal cancer

Colorectal cancer is one of the most prevalent forms of cancer and the third leading cause of cancer related death worldwide.[1;2] Disease stage is the most important prognostic factor, with a 5-year survival of only ten percent for patients with stage IV metastatic disease.[3] At present, approximately 20 percent of patients present with distant metastases[4;5], and another 26 percent will develop metastases in the first 5 years after surgery.[6]

Survival for metastasized colorectal cancer (mCRC) has increased substantially over the last four decades. This increase in survival is due to the improvement of surgical techniques, including the introduction of hepatic resection for liver-only metastases, but it is also largely the result of the implementation of new multidrug systemic therapies.[5;7]

Whereas 5-fluorouracil (5-FU) was the only systemic treatment available until the turn of the century, the treatment arsenal has since then been expanded and now also includes other fluoropyrimidine analogues (such as capecitabine), oxaliplatin, irinotecan, and drugs targeted against angiogenesis (bevacizumab) or the endothelial growth factor receptor (EGFR; cetuximab and panitimumab). During this time, overall survival for mCRC patients has risen from 5.9 months on 5-FU monotherapy, to 23 months for patients treated with modern-day combination treatment.[4] At the same time, a larger proportion of mCRC patients are now prescribed chemotherapy, increasing from 23 to 64 percent between 1989 and 2006 in the Netherlands.[5]

Despite encouraging results, not all patients benefit equally from these developments. There is great inter-patient variation in efficacy of treatment, and adverse events do not affect all patients to the same extent. Pretreatment predictors of efficacy and toxicity could safeguard patients from unnecessary adverse events, as well as reduce health care costs by preventing pointless treatments in patients who will not respond.

Genetic variation between patients and between their tumors is responsible for at least some of the inter-patient difference in treatment response. Tumor DNA harbors somatic mutations, on top of germline genetic characteristics, which can interfere with treatment efficacy. As an example, mutations in *Ras* oncogenes restrict activity of EGFR-inhibiting treatment, reducing its efficacy to almost zero percent.[8-11]

In contrast, pharmacogenetics focus on variation in germline DNA, present in all nucleated cells. Genetic alterations in a myriad of genes are thought to induce phenotypic changes in pharmacokinetics and dynamics for cytostatic drugs. The information obtained from genotyping in healthy DNA can therefore be of aid in the choice and dosage of anti-cancer treatment. Whereas treatment efficacy is mostly affected by tumor characteristics, toxicity associated with anti-cancer treatment is determined by its effect on healthy tissues. Therefore, it is likely that adverse events are far better predicted by germline genetic variation than by somatic changes in tumor DNA.

Scope of this thesis

The aim of this thesis is the search for germline genetic markers to pre-emptively predict treatment efficacy and adverse events of capecitabine and oxaliplatin, prescribed to patients with advanced colorectal cancer. For this thesis, patient data and DNA were obtained from patients included in the CAIRO and the CAIRO2 study by the Dutch Colorectal Cancer Group (DCCG). The CAIRO trial was a multicenter open label randomized phase III trial, comparing sequential versus combination chemotherapy with capecitabine, irinotecan, and oxaliplatin in a total of 803 patients with mCRC.[12] The phase III CAIRO2 trial was conducted in 755 mCRC patients, and randomized between first-line treatment with capecitabine, oxaliplatin and bevacizumab (CAPOX-B) versus CAPOX-B plus cetuximab.[13]

The studies described in this thesis address different aspects of pharmacogenetic research in colorectal cancer. **Chapter 2** gives an overview of the current knowledge on the effect of genetic variation on chemosensitivity for most common anti-cancer drugs prescribed in colorectal cancer treatment. New studies are reported every week and knowledge is constantly increasing. As a result, at the moment this thesis is published, more recent and more elaborate information will certainly be available.

Germline DNA for pharmacogenetic research is derived from peripheral blood leukocytes or other healthy tissue in most studies. However, all early pharmacogenetic studies in colorectal cancer treatment, as well as several more recent publications, have used archived paraffin-embedded surgical resection samples as the primary source of DNA for their analyses. For reliable comparison between studies, it is essential to know if the source of DNA affects genotype for the markers under investigation. **Chapter 3** addresses a basic element of pharmacogenetics research, whether there is sufficient concordance between tumor and germline DNA, to exclude the source of DNA as a confounding factor in the interpretation of data from different studies.

In the following section of this thesis, focus is on pharmacogenetics of capecitabine. Fluoropyrimidines have been the mainstay of systemic treatment for metastatic colorectal cancer, since their first development in 1957. The oral pro-drug capecitabine is equal to 5-FU in terms of efficacy, although pharmacokinetics differ.[14] The cytotoxic effect of fluoropyrimidines is thought to derive from interference with the thymidylate synthase (TS), an essential enzyme for the formation of DNA precursors, as well as direct incorporation into DNA and RNA. Methylenetetrahydrofolate, which is under the direct influence of methylene tetrahydrofolate reductase (MTHFR), is an essential component for binding of 5-FU to TS. *MTHFR* polymorphisms could therefore influence 5-FU chemosensitivity. **Chapter 4** focuses on the effect of *MTHFR* polymorphisms on adverse effects of capecitabine-based chemotherapy. In **chapter 5**, additional single nucleotide polymorphisms (SNPs) are investigated for their association with capecitabine efficacy. Some of these SNPs were selected based on their genome wide significance in an *in vitro* study using lymphoblastoid cell lines. Additionally, SNPs were selected based on their location in the gene encoding for methylene synthase reductase (MTRR), which, like

MTHFR, is involved in the folate pathway.

Next, focus is shifted to oxaliplatin. Organic cation transporters (OCTs) play an important role in the uptake of oxaliplatin into the cells.[15] Several types of OCTs have been associated with adverse effects of oxaliplatin, specifically platinum-induced neurotoxicity.[16] **Chapter 6** focusses on genetic variation in the genes encoding for three OCTs and the correlation with oxaliplatin-induced neurotoxicity.

The cytotoxic effect of oxaliplatin depends on the formation of DNA interstrand crosslinks. Repair of this DNA damage by the nucleotide excision repair (NER) system is likely to affect cellular chemosensitivity to oxaliplatin. Excision Repair Cross-Complementation group 1 (ERCC1) is a major component of the NER system. In **chapter 7**, the effect of a common variation in this gene, *ERCC1* C118T, on oxaliplatin response is addressed, combining *in vitro* studies and clinical association analysis.

In addition to the candidate-gene approach that was adopted in the previous chapters, in **chapter 8** a genome wide search for predictors of efficacy of combination chemotherapy is presented. Progression free survival of patients treated with first-line chemotherapy consisting of capecitabine-oxaliplatin-bevacizumab (CAPOX-B), either with or without cetuximab, is explored.

Finally, **chapter 9** gives a summary and general discussion on the results presented in this thesis.

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Pharmacogenetics in chemotherapy of colorectal cancer

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Abstract

Although in recent years, chemotherapeutic options for colorectal carcinoma have expanded, overall response rates are still too low, with high rates of toxicity. Pharmacogenetics aim at predicting both treatment response and adverse effects in individual patients.

This review describes the current knowledge of pharmacogenetic markers in the systemic treatment of colorectal cancer. *UGT1A1*28* leads to reduced conjugation of SN-38, the active metabolite of irinotecan, resulting in an increased rate of adverse effects, especially neutropenia. To a lesser extent, increased 5-FU toxicity is predicted by *DPYD*2A*. A variable number of tandem repeats polymorphism in the thymidylate synthase enhancer region, in combination with a single nucleotide polymorphism C>G, may predict poorer response to 5-FU. Efficacy of oxaliplatin is influenced by polymorphisms in components of DNA repair systems, such as *ERCC1* and *XRCC1*. Polymorphic changes in the endothelial growth factor receptor probably predict cetuximab efficacy. Furthermore, the antibody-depended cell-mediated cytotoxic effect of cetuximab may be reduced by polymorphisms in the immunoglobin G fragment C receptors. Bevacizumab efficacy is suspected to be influenced by polymorphisms in the *VEGF* gene and the hypoxia inducible factor 1α gene. Although the interpretation of pharmacogenetic studies is complicated, results imply a promising way of pretreatment prediction of chemotherapy efficacy and toxicity.

Introduction

In the last decades, important developments in the chemotherapeutic treatment of colorectal cancers have taken place. Since its discovery in 1957, 5-fluorouracil (5-FU) has played an important role in the therapy of colorectal carcinoma, both in adjuvant treatment combined with oxaliplatin, as well as in metastatic disease, where it is also combined with irinotecan. Recently, the anti-vascular endothelial growth factor (anti-VEGF) monoclonal antibody bevacizumab and the human epidermal growth factor receptor (EGFR)-targeted monoclonal antibodies cetuximab and panitimumab have been included in the treatment for advanced colorectal carcinoma. The addition of either of these compounds to conventional chemotherapy has led to a significant increase in progression free survival (PFS), although including both substances in first line treatment does not further increase PFS.[1]

However, the prognosis for patients with metastatic colorectal cancer patients is still limited. Moreover, many patients suffer from severe toxic side effects of chemotherapy. It would be useful to identify patients who are most likely to benefit from a specific chemotherapeutic regimen, as well as those who may experience severe adverse reactions. Clinical parameters alone have proven to be inadequate in predicting chemosensitivity. Pharmacogenetics aims at developing germline genetic markers to be used for predicting pharmacological response in the individual patient.[2] This review presents recent developments in pharmacogenetic studies in chemotherapy of colorectal cancer.

5-Fluorouracil (5-FU)

The fluoropyrimidine derivative 5-fluorouracil (5-FU) is thought to have two major mechanisms of action to explain its cytotoxic effect. Most importantly, the active metabolite of 5-FU (5-FdUMP) prevents DNA synthesis by forming a complex with thymidylate synthase (TS) that is stabilized by 5,10-methylenetetrahydrofolate (5,10-MTHF), thereby inhibiting the conversion of 2'-deoxyuridine-5'-monophosphate (dUMP) to 2'-deoxythymidine-5'-monophosphate (dTMP), the latter of which is an essential precursor for DNA-synthesis. (Figure 1) Furthermore, incorporation of 5-FU nucleotides into DNA and RNA leads to altered RNA processing and DNA damage.

Thymidylate synthase (TS)

The gene encoding for TS contains a unique tandemly repeated 28bp sequence in the enhancer region (TSER) in the 5'-untranslated region (5'-UTR), that was shown to be polymorphic with regard to the number of repeats (variable number of tandem repeats, VNTR). Although alleles containing up to 9 repeats have been described, two (2R) and three (3R) repeat copies are the most prevalent alleles in all ethnic populations.[3;4] The 3R-allele leads to increased tumoral TS expression, due to either enhanced mRNA translation efficiency[5] or increased *TS* mRNA

levels.[6] In addition to this VNTR polymorphism, a single nucleotide polymorphism (SNP) G>C at bp12 of the second repeat in 3R individuals has been described, leading to a three-allelic locus (2R, 3RC, 3RG). The 3RC allele leads to a reduced transcriptional activity comparable to that of the 2R allele, by disrupting an area critical to *TS* promoter activation.[4]

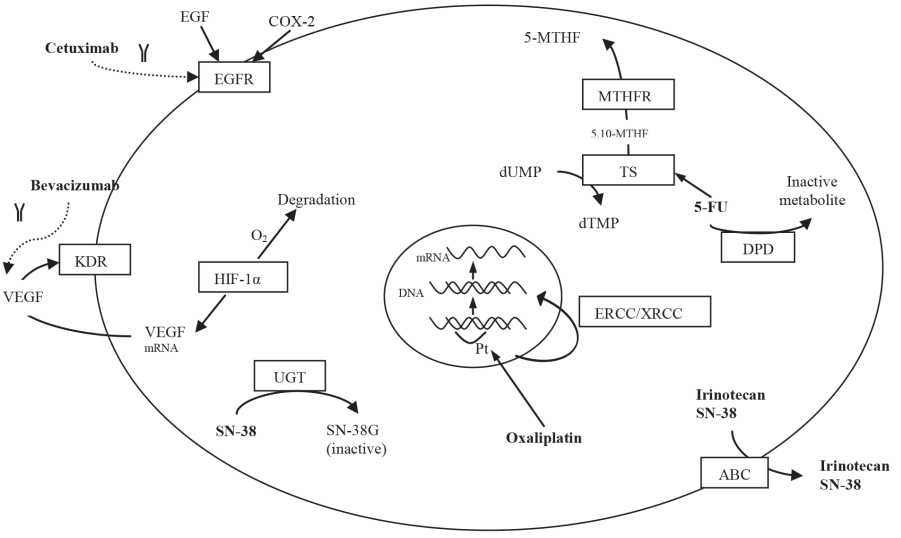

Figure 1. Schematic simplified overview of enzymes involved in the cellular response and metabolism of chemotherapeutic compounds in colorectal cancer

5-FU: 5-fluorouracil; ABC: ATP-binding cassette; DPD: Dihydropyrimidine dehydrogenase; dTMP: deoxythymidine monophosphate; dUMP: deoxyuridine monophosphate; EGFR: endothelial growth factor receptor; ERCC1: Excision repair cross complementation group 1; HIF-1α: hypoxia inducible factor 1α ; KDR: Kinase domain receptor; MTHF Methylene hydrofolate; MTHFR: Methylene hydrofolate reductase; Pt: Platinum; SN-38: active metabolite of irinotecan; SN-38G: SN-38 glucoronide; TS: Thymidylate synthase; UGT: UDP-glucuronosyl transferase; VEGF: vascular endothelial growth factor; XRCC1: X-ray repair cross complementation group 1.

In several studies, carriers of the 3R allele showed a poorer response to 5-FU chemotherapy[6-9], as well as decreased rates of grade 3-4 overall toxicity[3;6] and diarrhea[10], as would be expected from the higher TS levels associated with this allele.(Table 1) Conversely, two independent studies found a significantly better response rate and survival for 3R3R homozygotes, compared with individuals carrying at least one 2R allele.[11;12] The C>G SNP has frequently been used to explain the discrepancies in studies addressing only the VNTR polymorphism. The 3RG genotype was most often associated with either shorter response duration[13;14], shorter OS[14;15] or reduced overall response[14] to 5-FU, when compared to 2R or 3RC genotypes. However, although a lower rate of toxicity for 3RG3RG individuals was found in some studies[3;11], another study found a trend towards more toxicity during the first cycle of capecitabine treatment.[13]

Another explanation for these discrepant findings may be the ethnic diversity in relative allele frequencies, with only 3-4% 2R homozygotes in most Asian populations, compared to 17-24% in Caucasians.[3;6]

In addition to the TSER polymorphism another polymorphic locus is found in the *TS* 3'-UTR, consisting of a 6bp deletion at position 1494.[16] The del6 allele has been associated with better response rate[17;18] and reduced risk of death[19], although in another study ins6 homozygotes showed significantly better response to capecitabine or raltitrexed.[9] Regarding adverse reactions, although several studies showed the 3'-UTR polymorphism had no influence on toxicity[3;13;20], one study found a higher incidence of toxicity in del6 homozygotes.[18]

Methylenetetrahydrofolate reductase (MTHFR)

Methylenetetrahydrofolate reductase (MTHFR) catalyzes the irreversible conversion MTHF to 5-methyltetrahydrofolate. The former is essential in stabilizing the complex formed by TS and 5-FdUMP. (Figure 1)

The *MTHFR* gene is subject to several polymorphisms. Most common are two SNPs, 677C>T (Ala222Val, *MTHFR**4) and 1298A>C (Glu428Ala, *MTHFR**6), that are in linkage disequilibrium.[3;13;21] These polymorphisms lead to decreased MTHFR enzyme activity, and may thereby induce more effective stabilization of the FdUMP-TS ternary complex. In vivo studies showed a significantly better response rate for genotypes with at least one *MTHFR* 677T allele.[12;22](Table 1) Conversely, a study in 142 patients with primary rectal adenocarcinoma showed increased tumor regression for *MTHFR* 677CC homozygotes after preoperative 5-FU based chemoradiation therapy.[21] However, most studies found that both the *MTHFR* 677C>T and *MTHFR* 1298A>T polymorphisms were not predictive of objective response or survival in patients treated with 5-FU chemotherapy.[12;20;23;24] Enhanced stability of the ternary complex could also be expected to increase 5-FU toxicity. However, a recent study unexpectedly found lower rates of toxicity for the *MTHFR* 677TT and 1298AA genotypes.[20] Most studies found both *MTHFR* variants were not associated with altered toxicity rates in diverse patient groups treated with 5-FU derivatives.[13;22;23]

Dihydropyrimidine dehydrogenase (DPD)

5-FU depends on the activity of dihydropyrimidine dehydrogenase (DPD) for 80% of its catabolism.[25] Deficiency of DPD-activity leads to prolongation of the plasma half-time with resulting accumulation of 5-FU and has been associated with severe, mainly hematological toxicity and even death after administration of 5-FU.[26] DPD deficiency is present in approximately 3% of all cancer patients[27] but accounts for approximately 50% of patients with unexpected severe 5-FU toxicity.[28]

To date, over 30 polymorphisms in the *DPYD* gene (encoding for DPD) have been identified, many of which were found to be common variants with no apparent effect on DPD-activity.[29;30] However, a G>A mutation of the invariant splice site in exon 14 (IVS14+1G>A, *DPYD**2A) leads to skipping of exon 14 and formation of a truncated protein with no apparent residual

activity. Homozygosity for *DPYD**2A can lead to complete DPD-deficiency.[26] Although the incidence of this allele is rare, with a population frequency of 0,9-1,8% heterozygotes[9;26;31], it is estimated to be responsible for about 25% of all cases of unexpected severe 5-FU-toxicity.[10;28;31] However, it has also been described in individuals with normal DPD-activity.[28] There are profound ethnic differences in the incidence of DPD gene mutations. Although *DPYD**2A is the most common polymorphism in Caucasians, it is rare in Asians.[29]

Irinotecan

Irinotecan (CPT-11), a potent inhibitor of topoisomerase I, is widely used in the therapy for various solid tumors, including advanced colorectal carcinoma. Irinotecan undergoes several metabolic steps, both anabolic and catabolic.(Figure 1) It undergoes biotransformation by CYP3A-mediated oxidation to form APC, a substance which shows little cytotoxic activity. By another route, it is converted by liver carboxylesterases to its active metabolite (SN-38). This compound is then further conjugated by several UDP-glucuronyltransferases (UGTs) to yield the inactive SN-38G, which is mainly excreted with bile and urine. To enable excretion, SN-38 and irinotecan are actively transported out of the cell by an ATP-dependent efflux pump (ABC-binding cassette B1, ABCB1). After biliary excretion, SN-38G can be converted to active SN-38 by bacterial β-glucuronidase, which can lead to gastro-intestinal toxicity. Finally, SN-38 is subject to enterohepatic recirculation, leading to an unexpected peak in plasma after gall bladder emptying.

UDP-glucuronosyltransferase (UDP)

Reduced glucuronidation of SN-38 has been shown to significantly increase gastro-intestinal toxicity of irinotecan.[32] The principle UGT involved in the conjugation of SN-38 is UGT-1A1.[33] Up to 25 polymorphisms have been described for *UGT1A1*[34], of which a polymorphism in the promoter region, consisting of seven instead of six TA-repeats (-53$(TA)_{6>7}$, *UGT1A1**28) is the most common. The higher number of TA-repeats is associated with reduced transcriptional activity of *UGT1A1*, leading to various degrees of impaired glucuronidation.[35-39] This allele is also associated with Gilbert's syndrome.[40] The presence of the *UGT1A1**28 allele in either heterozygote or homozygote form, was shown to be a significant predictor for severe toxicity after the admission of irinotecan[41], with up to seven times increased risk of severe neutropenia[37;39;42;43] or diarrhea[36;42;43], and a severely increased risk of febrile neutropenia.[44] This increase in toxicity, however, may only be present in the first cycle of chemotherapy.[39] Although most studies have shown no significant association between *UGT1A1*-genotype and objective response[42-44], a study in 250 white patients with advanced colorectal cancer showed a higher incidence of partial or complete response for *UGT1A1**28 patients.[39]

Although, with a population frequency of 43% heterozygotes, the *UGT1A1**28 polymorphism is the most frequent mutation in Caucasians[36;38], it is significantly less frequent in

Asians.[45;46] Contrary, in the Asian population, the most common polymorphism is a SNP in the 3'-UTR (211G>A, *UGT1A1**6)[45], which is also associated with significantly reduced enzyme activity.[47] One study showed a higher incidence of grade 4 neutropenia after administration of irinotecan for *UGT1A1**6 homozygotes, combined with a significantly lower response rate and shorter PFS.[45] Conversely, most studies showed no increased risk of toxicity for the *UGT1A1**6 allele.[42]

Two other UGT isoenzymes are thought to be involved in the glucuronidation of SN-38: the hepatic UGT1A9 and the extra-hepatic UGT1A7.[48] The -118 in/del polymorphism (-118(T)$_{9>10}$, *UGT1A9**22), showing higher *UGT1A9* mRNA expression for the -118(T)9 allele, might explain part of the phenotypic variability in SN-38 glucuronidation that is not explained by *UGT1A1**28.[46]

Oxaliplatin

Platinum-containing drugs exert their cytotoxic effect by forming bulky interstrand and intrastrand DNA-adducts, resulting in DNA replication inhibition and apoptosis. The major pathway for removal of these adducts is nucleotide excision repair (NER).(Figure 1) During NER, damaged DNA is recognised and DNA helixes are unwound by the action of several factors, including xeroderma pigmentosum complementation group D protein (XPD, also known as ERCC2), XPC and XPA. The DNA strands are separated and a DNA residue containing the adducts is removed. Cleaving of the damaged strand is performed by the nucleases XPG and excision repair cross-complementing group 1 protein (ERCC1) on the 3' and 5' side, respectively. Suboptimal repair mechanisms may lead to increased sensitivity to platinum containing chemotherapy.[49] Higher tumoral *ERCC1* mRNA levels have been associated with significantly worse outcome in gastric cancer[50] and advanced colorectal cancer[51] treated with cisplatin or oxaliplatin based chemotherapy. Polymorphisms have been described for many of the constituents of NER. However, to date, only few have proven clinically significant.

Xeroderma pigmentosum complementation group D (XPD)

The synonymous *XPD* Arg156Arg (C>A) polymorphism was associated with a higher response rate and longer time to progression for C/A or A/A genotypes in gastric cancer patients treated with oxaliplatin based chemotherapy.[17] A trend towards higher response and longer median survival rate for these genotypes was also seen in metastasized colorectal cancer patients.[52](Table 1) Another polymorphism, *XPD* Lys751Gln (A>C), was shown to reduce DNA repair capacity for the 751Gln/Gln genotype in normal cells of lung cancer patients.[53] Conversely, a reduced capacity for DNA repair for 751Lys/Lys genotype was found in another report, possibly because of methodological differences.[54] The *XPD* Lys571Gln polymorphism did not show significant survival difference according to genotype in gastro-oesophageal cancer[14;55] and colorectal cancer[56;57] in response to various platinum based chemotherapy regimens.

Excision repair cross complementing group 1 (ERCC1)

A synonymous C>T (Arg118Arg) polymorphism in the *ERCC1* gene has been described. Although the mechanism by which this substitution affects ERCC1 activity is unknown, it has been suggested that replacement of the common codon AAC by the infrequently used codon AAT affects translation efficiency, with a 50% decrease for the variant allele.[58] Advanced colorectal cancer patients carrying the -118TT genotype experienced higher response rates to oxaliplatin treatment[59] and longer progression free survival[60] in two studies.(Table 1) However, in another two studies survival was most favorable for patients who carried the *ERCC* -118CC genotype.[23;61] Two studies in gastric cancer patients found no predictive effect of this polymorphism.[14;17]

X-ray cross complementing group 1 (XRCC1)

In addition to NER, the basepair excision repair pathway (BER) is also involved in chemosensitivity to platinum agents. An important player in BER is X-ray repair cross complementing group 1 (XRCC1). A common polymorphism, *XRCC1* Arg399Gln (G>A), has been suggested to produce significant conformational changes at a domain important for XRCC1 interaction with other components of BER.[62] The germline wildtype allele has been associated with significant survival benefit in gastric cancer patients[55] and lung cancer patients[63] in response to platinum compounds.(Table 1) Expression of the wildtype allele in colorectal tumoral tissue was also associated with better survival and response to oxaliplatin.[24] However, in recent studies in advanced colorectal cancer and gastric cancer patients, *XRCC1* genotype did not predict outcome after oxaliplatin treatment.[23;56;61]

Cetuximab

Cetuximab, a chimeric immunoglobin G1 monoclonal antibody, exerts is action by binding to the extracellular domain of the epidermal growth factor receptor (EGFR) with a higher affinity than epidermal growth factor (EGF), thereby blocking ligand-induced phosphorylation of EGFR.(Figure 1) So far, only clinical parameters have been used to predict cetuximab efficacy, of which the grade of skin toxicity is the most important.[64] However, pretreatment markers for selecting patients who may benefit from therapy are currently lacking. EGFR-staining intensity in tumor tissue is not associated with response, survival or toxicity.[65-67] Pharmacogenetics may prove a possible way of optimizing monoclonal antibody therapy.[68]

Epithelial growth factor receptor (EGFR)

A polymorphic $(CA)_n$-repeat variant in *EGFR* intron-1 has been described with 16 up to 26 repeats and *EGFR* gene transcription declines with increasing number of (CA)-repeats.[69] In a study in 110 heavily pretreated patients with advanced colorectal carcinoma, the homozygous *EGFR* intron-1 short allele was associated not only with favorable survival, but also with a higher grade of skin toxicity.[64](Table 1) In an earlier study, however, no relation between this

polymorphism and colorectal cancer survival was detected.[67] Another polymorphic locus in the *EGFR* gene has been described, consisting of a SNP G>A leading to substitution of arginine by lysine at codon 497 (also denominated 521). This polymorphism was shown to be predictive of cetuximab efficacy, with a better response rate as well as longer PFS and OS for advanced colorectal cancer patients carrying at least one A allele.[70;71] Earlier studies, however, showed no influence of this and another (*EGFR* -216G>T) polymorphism on cetuximab efficacy.[64;67]

Induction of downstream pathways of EGFR leads to synthesis of various ligands, such as Cyclin-D1 (CCND1). Therefore, genetic variation in the cyclin-D1 gene might affect cetuximab efficacy, and germline polymorphisms have been described for *CCND1*. In metastatic colorectal cancer patients receiving cetuximab single-agent therapy, harboring the SNP (*CCND1* 870 A>G) is associated with longer OS for the G allele.[67](Table 1) Conversely, in another study in advanced colorectal patients treated cetuximab and irinotecan combination therapy, this polymorphism was not associated with PFS or OS.[64] In addition, Cox-2 acts as an upstream regulator of EGFR. A frequent *Cox2* -756G>C polymorphism has been described, leading to decreased COX-2 expression.[72] Until recently, this polymorphisms had not been related to response or outcome in patients treated with cetuximab[67], but in a recent study *Cox-2* -756CC individuals showed longer PFS.[71]

A difference in the expression of the natural ligand for the EGFR might also influence cetuximab efficacy. A SNP is found in the *EGF* gene 61G>A, which leads to upregulated EGF levels for the transcriptionally more active G allele. Although higher circulating EGF levels have been associated with higher tumor aggressiveness, the *EFG* 61GG genotype was associated with a more favorable overall survival in patients treated with cetuximab and irinotecan.[64;71] However, in another study AA homozygosity tended to associate with longer overall survival.[67]

Antibody-dependent cell-mediated cytotoxicity

Cetuximab may also exert an indirect anti-tumor activity by attracting cytotoxic host effector cells, like monocytes and natural killer cells. The effect of this antibody-dependent cell-mediated cytotoxicity (ADCC) may depend on the degree of activation of effector cells after engagement of immunoglobin G fragment C receptors (FcγR) IIa and IIIa. Polymorphic alleles have been described for *FcγR-IIIa* (559T>G, Val158Phe) and *FcγR-IIa* (535G>A, His131Arg), that were shown to negatively affect receptors' affinities for the fragment C of antibodies, and probably ADCC efficiency.[73] *FcγR-IIIa* 158VV genotype was associated with a higher affinity of natural killer cells for the chimeric IgG1 monoclonal antibody rituximab in vitro[74], and with a higher response rate and longer progression free survival in breast cancer patients treated with the humanized anti-Her2/neu immunoglobin G monoclonal antibody trastuzumab.[75] (Table 1) A study in 39 mCRC patients treated with cetuximab monotherapy unexpectedly showed a significantly shorter PFS for *FcγR-IIa* 131A and *FcγR-IIIa* 158V homozygotes.[76] However, another study in advanced colorectal patients treated with irinotecan an cetuximab, showed neither polymorphism was associated with PFS or OS.[64]

Bevacizumab

Bevacizumab is a humanized recombinant monoclonal IgG type antibody, directed against all VEGF-A isoforms.(Figure 1) VEGF is an important regulator of angiogenesis and its inhibition by bevacizumab not only reduces tumor volume, but also large vessel density in a colorectal tumor model in mice.[77] Hypoxia is a potent stimulus for VEGF expression and one of the regulating elements in this mechanism is hypoxia-inducible factor 1α (HIF-1α). This factor binds to a 28-bp enhancer in the 5' upstream region of the *VEGF*-gene, thereby stimulating transcription. Under normoxic cellular conditions, HIF-1α rapidly degrades. However, it may be strikingly induced under hypoxic conditions, which are often found in tumor mass. In addition to HIF-1α, other regulating elements for VEGF expression are located in the 3'-UTR.

Vascular endothelial growth factor

Several polymorphisms have been described for the *VEGF* promoter region, 5'-UTR and 3'-UTR[78;79], but only few have shown functional implications.[80] Two SNP's (*VEGF* +936C>T and *VEGF* -1154G>A) lead to decreased VEGF expression for the variant allele.[78;81-84] A third SNP (*VEGF*-2578C>A), that is in complete linkage disequilibrium with an 18bp insertion at bp -2549, has also been associated with higher VEGF expression and serum levels for the wildtype -2578C/-2549del allele.[78;79] However, lower VEGF expression for the *VEGF*-2578CC genotype was found in one study.[78;81] Another common polymorphism (*VEGF* -634G>C, also denominated +405G>C) was most commonly reported to induce lower VEGF expression and serum protein levels for wildtype homozygotes[81;83;85], although one study conversely found decreased VEGF expression for the variant allele.[86] In addition, recent studies found no association between these *VEGF* polymorphisms and tumoral VEGF protein expression or circulating VEGF levels.[87;88] Methodological differences may have contributed herein, since one report found increased *VEGF* mRNA expression in colorectal carcinoma tissue, but not in adjacent healthy tissue.[89]

So far, only one study on the pharmacogenetic interaction between bevacizumab and *VEGF* polymorphisms has been published. A recent study in 363 breast cancer patients found improved median survival time for patients with the *VEGF* -2578AA and -1154AA genotypes when treated with paclitaxel combined with bevacizumab.[90] Instead, most studies have focused on prognostic, rather than predictive effects. *VEGF* -634CC genotype was associated with higher tumor stage and grade in one study in breast cancer patients[91], but with increased OS in another.[92] In colorectal cancer patients, this polymorphism was not associated with tumor differentiation[93] or time to tumor recurrence.[94] In early stage gastric carcinoma the *VEGF*-460CC genotype was associated with a better DFS and OS[87], but the same genotype was associated with reduced OS in Chinese breast cancer patients.[92] Stage III colorectal cancer patients with the *VEGF* +936CC homozygote genotype had significantly shorter time to tumor recurrence compared to patients with at least one T allele.[94] *VEGF* -2578CC homozygotic patients with renal cell carcinoma showed significantly lower cancer specific survival, compared

to patients with at least one variant allele.[95] Another polymorphism *VEGF* -1498T>C showed poorer differentiation of colorectal carcinomas for the CC_genotype.[93] These conflicting and sometimes unexpected results concur with our current lack of full understanding of these polymorphisms on VEGF expression and function.

Hypoxia-inducible factor 1α

Three polymorphisms have been described in exon 12 of the *HIF-1α* gene, *HIF-1α* Pro582Ser (1722C>T), *HIF-1α* Ala588Thr (1790G>A), *HIF-1α* Pro564Ala, and one in exon 13, which is a GT-repeat polymorphism.[96;97] Genotypes coding for variant proteins showed higher transcription capacity in vitro, both under normoxic and hypoxic conditions, compared to wildtype.[97;98] Expression of a Pro582Ser variant allele was associated with a significantly increased risk of ulcerative disease in colorectal cancer, although vascularization was not increased.[99] Whereas both Pro582Ser and Ala588Thr were not associated with tumor grade or stage in transitional cell carcinoma, patients with at least one variant allele showed significantly worse disease-free and overall survival.[100]

Discussion

So far, pharmacogenetic studies hold the promise of becoming a useful way of predicting results for chemotherapeutic treatment in colorectal carcinoma. Results from earlier trials have even lead to a label change for irinotecan, now advising dose reduction for *UGT1A1*28* homozygotic individuals. There are, however, some difficulties in interpreting study results.

Pharmacogenetic studies aim at understanding the influence of germline polymorphisms. This does not account for the potential bias of somatic mutations or loss of heterozygosity in the tumor, which is a frequent phenomenon with regard to the *TYMS* polymorphisms(26-54%).[3;19] Patients who have a 2R3R genotype, with tumoral 3R-loss might obtain significant benefit from 5-FU based chemotherapy, with a lower risk of toxicity. In addition, the frequent presence of linkage disequilibrium between variant alleles makes it difficult to ascertain which allele is essential in predicting chemosensitivity. Haplotype analysis may eventually overcome this problem.

Furthermore, ethnic differences in relative allele frequencies may want for different strategies for the respective populations. Substantial interethnic differences have been found for the allele frequencies of *ERCC1* 118, *XRCC1* 399 and *XPD* 751 polymorphisms, with African Americans carrying the wildtype allele more often than Americans of European descent.[101] Asian populations show only very limited expression of the beneficial *TYMS* 2R2R genotype, and a different spectrum of *UGT1A1* polymorphisms, compared to Caucasians.[102;103]

Another problem regarding pharmacogenetic results lies in the fact that some polymorphisms are not only predictive of chemotherapeutic efficacy, but also of general prognosis in cancer patients[104] and may even have contributed to the development of colorectal

carcinoma[105]. This is especially true for components of DNA repair mechanisms. By impairing DNA repair polymorphic changes may predispose to carcinogenesis, whereas they may also improve response to chemotherapy when cancer has developed.

In developing predictive pretreatment models for colorectal cancer therapy genetic and non-genetic factors with proven effect on outcome, such as performance status and tumor stage will need to be combined. This has proven effective in other fields, such as predicting MTX treatment efficacy rheumatoid arthritis.[106]

Conclusion

In conclusion, pharmacogenetic studies in colorectal cancer therapy show promising results with regard to prediction of tumor response, survival and toxicity. Although further research is warranted, predictive models including genotypic testing will influence the choice for a chemotherapy regimen in the future.

First Author	Ethnicity	Cancer type	Study design	Gene studied	Polymorphism	Effect on toxcity	Effect on outcome	Reference
Pullarkat S.T. 2001	Mixed	mCRC	Retrospective	TS	TSER	3R/3R decreased grade 3 toxicity (27% vs 63%)	3R/3R decreased RR (9% vs 50%)	6
Salgado J.2007	NS (Spanish)	mCRC	Prospective	TS	TSER	NA	3R/3R decreased RR (7/33 vs 13/15)	9
					Ins/del 6bp	NA	Del6/del6 decreased RR (0/11 vs 24/34)	
Park D.J. 2002	20/24 Caucasian	mCRC	Prospective	TS	TSER	3R/3R decreased grade 3 toxicity (37.5% vs 100%, p 0.17)	3R/3R decreased RR (25% vs 75%)	8
Lecomte T. 2004	NS (French)	CRC	Retrospective	TS	TSER, C>G	3R (C or G) decreaed grade 3-4 toxicity (43% vs 4%)	No effect of polymorphism	3
					Ins/del 6bp	No effect of polymorphism	No effect of polymorphism	
Marsh S. 2001	Caucasian	CRC	?	TS	TSER	NA	3R/3R decreased RR + MST (HR 2.33, 12 vs 16 months)	7
Schwab M. 2008	NS (German)	Mixed	Prospective	TS	TSER	3R/3R decreased risk of diarrhoea (OR 0.36)	NA	10
Jakobsen A. 2005	NS (Danish)	mCRC	Retrospective	TS	TSER	NA	3R/3R increased RR (52% vs 24%)	12
Hitre E. 2005	NS (Hungary)	CRC	Prospective	TS	TSER	3R/3R decreased toxicity (22% vs 30%)	3R/3R increased OS (HR 3.79)	11
Largillier R. 2006	NS (French)	Breast cancer	Prospective	TS	TSER, C>G	3G/3G increased grade 3-4 toxicity 1st cycle (p 0.064)	3G/3G longer response duration	13
					Ins/del 6bp	No effect of polymorphism	No effect of polymorphism	
Ruzzo A. 2006	NS (Italian, Japanese)	AGC	Prospective	TS	TSER, C>G	NA	3G allele decreased OS and PFS (RR 2.21 and 2.16)	14
Marcuello E. 2004	NS (Spanish)	mCRC	Prospective	TS	TSER, C>G	NA	3G allele decreased OS and RR (HR 2.4 and OR 2.9)	15

First Author	Ethnicity	Cancer type	Study design	Gene studied	Polymorphism	Effect on toxcity	Effect on outcome	Reference
Lu J.W. 2006	Chinese	AGC	Prospective	TS	Ins/del 6bp	Del6/del6 increased grade 3-4 toxicity	Del6 allele increased RR (37% vs 0%)	18
Keam B. 2008	Korean	AGC	Prospective	TS	Ins/del 6bp	No effect of polymorphism	Del6/del6 increased RR (55% vs 30,3%)	17
Dotor E. 2006	NS (Spanish)	CRC	Prospective	TS	Ins/del 6bp	NA	Del6 increased OS (HR 0.47)	19
Sharma R. 2008	NS (Australia)	mCRC	Prospective	TS	Ins/del 6bp	No effect of polymorphism	No effect of polymorphism	20
Jakobsen A. 2005	NS (Danish)	mCRC	Retrospective	MTHFR	677C>T	NA	T allele increaed RR (67% vs 34%)	12
					1298A>C	NA	No effect of polymorphism	
Largillier R. 2006	NS (French)	Breast cancer	Prospective	MTHFR	677C>T	No effect of polymorphism	No effect of polymorphism	13
					1298A>C	No effect of polymorphism	No effect of polymorphism	
Sharma R. 2008	NS (Australia)	mCRC	Prospective	MTHFR	677C>T	T/T decreased grade 2-3 toxicity (OR 0.1)	No effect of polymorphism	20
					1298A>C	A/A decreased grade 2-3 toxicity (32% vs 72%)	No effect of polymorphism	
Terrazino S. 2006	NS (Italian)	Rectal cancer	Prospective	MTHFR	677C>T	NA	C/C increased RR (57% vs 34%, OR 0.32)	21
Cohen V. 2003	38/43 Caucasian	mCRC	Prospective	MTHFR	677C>T	No effect of polymorphism	T allele increased RR (OR 2.86)	22
Ruzzo A. 2007	NS (Italian)	mCRC	Prospective	MTHFR	677C>T	No effect of polymorphism	No effect of polymorphism	23
					1298A>C	No effect of polymorphism	No effect of polymorphism	
Suh K.W. 2006	Korean	mCRC	Retrospective	MTHFR	677C>T	NA	No effect of polymorphism	24
Innocenti F. 2004	50/66 Caucasian	mCRC	Prospective	UGT1A1	53$(TA)_{6>7}$	TA_7 increased grade 4 neutropenia (3/6 vs 0/29)	NA	37
Toffoli G. 2006	NS (Italian)	mCRC	Prospective	UGT1A1	53$(TA)_{6>7}$	TA_7 increased grade 3-4 neutropenia (OR 8.63)	TA7 decreased risk of progression (OR 0.19)	39

First Author	Ethnicity	Cancer type	Study design	Gene studied	Polymorphism	Effect on toxcity	Effect on outcome	Reference
De Jong F.A. 2006	NS (Dutch)	CRC and GC	Prospective	UGT1A1	$53(TA)_{6>7}$	TA_7 increased grade 2-3 diarrhoea (69.2% vs 34.6%)	NA	36
Kweekel D.M. 2008	NS (Dutch)	mCRC	Prospective	UGT1A1	$53(TA)_{6>7}$	TA_7 increased febrile neutropenia (OR 14.2)	No effect of polymorphism	41
Marcuello E. 2004	NS (Spanish)	mCRC	Prospective	UGT1A1	$53(TA)_{6>7}$	TA_7 increased diarrhoea (7/10 vs 7/40)	No effect of polymorphism	43
Ando Y. 2000	Japanese	Mixed	Retrospective	UGT1A1	$53(TA)_{6>7}$	TA_7 increased grade 4 toxicity (4/7 vs 14/93)	NA	42
					211G>A	No effect of polymorphism	NA	
Han J.Y. 2006	Korean	NSCLC	Prospective	UGT1A1	211G>A	211A increased grade 4 neutropenia (4/6 vs 18/75)	211A lower RR (0/5 vs 36/72)	45
Minami H. 2006	Japanese	NS	Prospective	UGT1A1	$53(TA)_{6>7}$ /211G>A	TA_7/211A increased neutropenia (4/5 vs 3/21)	NA	107
Stoehlmacher J. 2004	75/106 Caucasian	mCRC	Retrospective	XPD	Lys751Gln	NA	Gln/Gln increased risk of dying (OR 2.44)	61
Keam B. 2008	Korean	AGC	Prospective	XPD	Arg156Arg	No effect of polymorphism	A/A increased RR (52% vs 26,1%)	17
Ruzzo A. 2007	NS (Italian)	mCRC	Prospective	XPD	Lys751Gln	No effect of polymorphism	Gln/Gln decreased PFS (HR 2.21)	23
					Asp312Asn	No effect of polymorphism	No effect of polymorphism	
Park D.J. 2001	55/73 Caucasian	mCRC	Prospective	XPD	Lys751Gln	NA	Gln/Gln decreased MST (17,4 vs 3,3 months)	52
					Asp312Asn	NA	No effect of polymorphism	
					Arg156Arg	NA	A/A decreased RR (24 vs. 13%, p 0.69)	
Gurubhagavatula S. 2004	NS (US)	NSCLC	Prospective	XPD	Asp312Asn	NA	Asn/Asn decreased OS (HR 2.18)	63
Liu B. 2007	Chinese	Gastric cancer	Prospective	XPD	Lys751Gln	NA	No effect of polymorphism	55

First Author	Ethnicity	Cancer type	Study design	Gene studied	Polymorphism	Effect on toxcity	Effect on outcome	Reference
Ruzzo A. 2006	NS (Italian, Japanese)	AGC	Prospective	XPD	Lys751Gln	NA	No effect of polymorphism	14
Monzo M. 2007	NS (Spanish)	mCRC	Prospective	XPG	Lys751Gln	No effect of polymorphism	No effect of polymorphism	57
					Hys46Hys	No effect of polymorphism	C/C increased RR (CR+PR 74% vs 34%)	
Braun M.S. 2008	NS (UK)	mCRC	Prospective	XPD	Lys751Gln	NA	No effect of polymorphism	56
Ruzzo A. 2006	NS (Italian, Japanese)	AGC	Prospective	ERCC1	Asn118Asn	NA	No effect of polymorphism	14
Keam B. 2008	Korean	AGC	Prospective	ERCC1	Asn118Asn	No effect of polymorphism	No effect of polymorphism	17
Ruzzo A. 2007	NS (Italian)	mCRC	Prospective	ERCC1	Asn118Asn	No effect of polymorphism	T/T decreased PFS (HR 2.34)	23
Stoehlmacher J. 2004	75/106 Caucasian	mCRC	Retrospective	ERCC1	Asn118Asn	NA	T/T increased risk of dying (OR 1.86)	61
Viguier J. 2005	NS (French)	mCRC	Prospective	ERCC1	Asn118Asn	NA	T/T increased response (OR 3.03)	59
Martinez-Balibrea E. 2008	Caucasian	mCRC	Prospective	ERCC1	Asn118Asn	NA	C allele decreased PFS (HR 1.96, p 0.0501)	60
Ruzzo A. 2007	NS (Italian)	mCRC	Prospective	XRCC1	Arg399Gln	No effect of polymorphism	No effect of polymorphism	23
Gurubhagavatula S. 2004	NS (US)	NSCLC	Prospective	XRCC1	Arg399Gln	NA	Gln/Gln decreased OS (HR 2.88)	63
Liu B. 2007	Chinese	Gastric cancer	Prospective	XRCC1	Arg399Gln	NA	Gln/Gln decreased MST (212 vs 347 days)	55
Braun M.S. 2008	NS (UK)	mCRC	Prospective	XRCC1	Arg399Gln	NA	No effect of polymorphism	56
Stoehlmacher J. 2004	75/106 Caucasian	mCRC	Retrospective	XRCC1	Arg399Gln	NA	No effect of polymorphism	61
Suh K.W. 2006	Korean	mCRC	Retrospective	XRCC1	Arg399Gln	NA	Gln/Gln decreased MST (12.8 vs 30 months)	24
Graziano F. 2008	NS	mCRC	Prospective	EGFR	$(CA)_n$-repeat	$(CA)_{\leq 17}$ increased grade 2-3 skintoxicity	$(CA)_{\leq 17}$ increased OS (HR 0.54)	64
					497G>A	No effect of polymorphism	No effect of polymorphism	
					216G>T	No effect of polymorphism	No effect of polymorphism	

First Author	Ethnicity	Cancer type	Study design	Gene studied	Polymorphism	Effect on toxcity	Effect on outcome	Reference
Zhang W. 2006	31/39 Caucasian	mCRC	Prospective	EGFR	$(CA)_n$-repeat	No effect of polymorphism	No effect of polymorphism	67
Nagashima F. 2007	NS (US)	mCRC	Prospective	EGFR	497G>A	NA	GA increased PFS (1.8 vs 1.2 months)	71
Goncalves A. 2008	NS (French)	mCRC	Retrospective	EGFR	497G>A	NA	A allele increased PFS and OS (5.7/20.1 vs 3.1/13.8 months)	70
Graziano F. 2008	NS	mCRC	Prospective	CCND1	870A>G	No effect of polymorphism	No effect of polymorphism	64
Zhang W. 2006	31/39 Caucasian	mCRC	Prospective	CCND1	870 A>G	No effect of polymorphism	A/A decreased OS (2.3 vs 7.8 months)	67
Zhang W. 2006	31/39 Caucasian	mCRC	Prospective	EGF	61G>A	No effect of polymorphism	AA increased PFS (15.0 vs 2.3 months, *p* 0.07)	67
Nagashima F. 2007	NS (US)	mCRC	Prospective	EGF	61G>A	NA	G/G increased PFS (1.4 vs 1.2 months)	71
Graziano F. 2008	NS	mCRC	Prospective	EGF	61A>G	No effect of polymorphism	EGF 61GG increased OS (HR 0.55)	64
Zhang W. 2006	31/39 Caucasian	mCRC	Prospective	COX-2	-756G>C	No effect of polymorphism	No effect of polymorphism	67
Nagashima F. 2007	NS (US)	mCRC	Prospective	COX-2	-756G>C	NA	C/C increased PFS (6.9 vs 1.3 months)	71
Lurje G. 2008	70/125 Caucasian	CRC	Prospective	VEGF	936C>T	NA	T/T increased time to recurrence (11 vs 2.6 years)	94
				IL-8	-251T>A	NA	A/A decreased time to re-currence (2.4 vs 5.7 years)	
Graziano F. 2008	NS	mCRC	Prospective	FCGR IIa	His131Arg	No effect of polymorphism	No effect of polymorphism	64
				FCGR IIIa	Val158Phe	No effect of polymorphism	No effect of polymorphism	
Musolino A. 2008	NS (Italian)	Breast cancer	Prospective	FCGR IIa	His131Arg	NA	His/His increased RR (70% vs 40%)	75
				FCGR IIIa	Val158Phe	NA	Val/Val increased RR (82% vs 35%)	
Zhang W. 2007	31/39 Caucasian	mCRC	Prospective	FCGR IIa	His131Arg	NA	Arg/Arg decreased PFS (1.1 vs 2.4 months)	67

First Author	Ethnicity	Cancer type	Study design	Gene studied	Polymorphism	Effect on toxcity	Effect on outcome	Reference
				FCGR IIIa	Val158Phe	NA	Phe/Phe increased PFS (2.3 vs 1.1 months)	
Kim J.G. 2007	Korean	GC	Prospective	VEGF	-460T>C	NA	C allele increased DFS (HR 4.87)	87
Jin Q. 2005	NS (Europe)	Breast cancer	Prospective	VEGF	-634G>C	NA	-634CC higher tumor stage/grade	91
Lu H. 2005	Chinese	Breast cancer	Prospective	VEGF	-460T>C	NA	C/C decreased OS (HR 1.5)	92
Yamamori M. 2008	Japanese	CRC	In vitro	VEGF	-1498T>C	NA	C/C poorer tumor differentiation	89
					-634G>C	NA	No effect of polymorphism	
Kawai Y. 2007	Japanese	Renal cell carcinoma	Prospective	VEGF	-2578C>A	NA	A allele increased CSS (HR 0.71)	95
Schneider B. 2008	NS (Canada)	Breast cancer	Retrospective	VEGF	-2578C>A	No effect of polymorphism	A/A increased MST (HR 1.70)	90
					-1154 G>A	No effect of polymorphism	A/A increased MST (HR 2.69)	
					-1498T>C	T/T decreased grade 3-4 hypertension (8% vs 23%)	No effect of polymorphism	
					-634G>C	C/C decreased grade 3-4 hypertension (0% vs 19%)	No effect of polymorphism	

Table 1. Pharmacogenetic studies in drugs used for colorectal cancer

Differences between genotypes are for homozygote wildtype versus homozygote variant genotype, unless indicated otherwise.
(A)GC: (advanced) gastric cancer; CSS: cancer specific survival; ERCC1: excision repair cross complementation group1; FCGR: Fragment C immunoglobin C receptor; HIF-1α: Hypoxia inducible factor 1α; HNSCC: head and neck squamous cell carcinoma; (m)CRC: (metastasized) colorectal cancer; MST: median survival time; MTHFR: Methylenetetrahydrofolate reductase; NA: not assessed; NS: not specified; NSCLC: non small cell lung carcinoma; OS: overall survival; RR: response rate; TS: thymidylate synthase: TSER: Thymidylate synthase enhancer region; TTP: time to progression; UGT1A1: UTP-glucuronosyltransferase 1A1; VEGF: vascular endothelial growth factor; XPD: xeroderma pigmentosum group D; XPG: xeroderma pigmentosum group G; XRCC1: X-ray cross complementation group1.

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3

Concordance of genotype for polymorphisms in DNA isolated from peripheral blood and colorectal cancer tumor samples

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Abstract

Background & aim: Results from different pharmacogenetic association studies in colorectal cancer are often conflicting. Both peripheral blood and formalin-fixed, paraffin-embedded (FFPE) tissue are routinely used as DNA source. This could cause bias due to somatic alterations in tumor tissue, such as loss of heterozygosity. We therefore compared genotypes in DNA from peripheral blood and FFPE colorectal tumor samples for SNPs with putative influence on the cytotoxicity of chemotherapy.

Materials & methods: Eleven SNPs in nine genes involved in anticancer drug metabolism or efficacy were determined in matched samples from blood and FFPE tissue of colorectal tumors by pyrosequencing and TaqMan® techniques. The k-statistic was calculated to assess concordance.

Results: A total of 149 paired FFPE tissue and EDTA blood DNA samples were available for comparison. Overall, 20 out of 1418 genotypes were discordant (1.4%); in ten cases, loss of heterozygosity could not be ruled out. Only *GSTP1* showed significant discordance between FFPE tissue and blood genotype (k = 0.947; 95% CI: 0.896–0.998).

Conclusion: FFPE tissue-derived DNA can be used as a valid proxy for germline DNA for a selection of SNPs in (retrospective) pharmacogenetic association studies in colorectal cancer. However, for future studies, genotyping of blood-derived DNA is preferred.

Introduction

The field of pharmacogenetics has developed rapidly over the last decade[1;2], as have techniques for DNA isolation and SNP genotyping. Pharmacogenetic association studies have shown inconsistent results for candidate genes in many pharmacologic pathways. Among other variables, the source of DNA could be a confounding factor explaining these inconsistencies. The majority of studies use whole blood as a source of DNA, since it supplies genomic DNA and is easy to obtain in a clinical setting. Alternative sample types include buccal swabs or saliva, both of which are ideal for use in nonclinical settings and for shipping by mail.[1;3]

Tumor tissue as a source of DNA is of particular interest, as tumor biopsies and resection specimens of large patient populations have been archived and are potentially useful for retrospective pharmacogenetic studies.[4;5] However, the quality of DNA isolated from formalin-fixed, paraffin-embedded (FFPE) material is often inferior to DNA from peripheral blood leukocytes.[5;6] As a result of chromosomal damage, DNA amplification and primer recognition may be hampered. Indeed, unpublished pilot experiments showed that genetic variations in the *TYMS* 28-bp repeat and *UGT1A1**28, both spanning a larger number of base pairs, could not be determined in most of our FFPE samples due to low DNA quality.

Although these arguments make peripheral blood the preferred DNA source for most pharmacogenetic studies, blood samples are not always available, especially in retrospective studies. Indeed, many pharmacogenetic association studies in colorectal cancer (CRC) have used FFPE tissue specimens as the primary source of DNA.[7–19] However, the use of FFPE tissue may lead to genotyping results that differ from the germline genotype, because tumor DNA harbors somatic alterations that are associated with carcinogenesis on top of germline characteristics. These may involve point mutations, copy number variations or, more frequently, loss of heterozygosity (LOH).

Concordance of germline drug metabolism pathway polymorphisms in DNA from peripheral blood and FFPE tissue has not been extensively investigated in colorectal carcinomas. It is therefore unknown whether results from studies using archived tumor samples as the primary source of DNA can be assessed alongside those from studies using blood-derived DNA. Therefore, the aim of the present study was to compare the genotypes of a large set of paired blood and FFPE samples in CRC patients for a selection of SNPs with putative influence on the cytotoxicity of commonly used chemotherapeutic drugs for CRC.[20]

Materials and methods

DNA was obtained from previously untreated metastatic CRC patients participating in the multicenter CAIRO trial of the Dutch Colorectal Cancer Group. The inclusion criteria and clinical results of this study have been published elsewhere.[21] All included patients gave written informed consent before inclusion. EDTA–blood was stored at -20°C before DNA

isolation. FFPE colorectal tumor resection samples were collected from multiple pathology laboratories in The Netherlands and stored under standard conditions. Procedures for tissue collection and fixation are uniform across Dutch hospitals. Germline DNA was isolated from EDTA–blood with the MagNA Pure LC® (Roche Diagnostics, The Netherlands) according to the manufacturer's instructions. Tissue DNA was obtained by macrodissection from FFPE samples of areas optically containing predominantly tumor tissue with QIAamp® DNA Mini Kit columns (Qiagen, The Netherlands).

SNP selection and genotyping assays

We selected polymorphisms in genes with putative influence on the pharmacokinetics or pharmacodynamics of fluoropyrimidines, oxaliplatin and irinotecan. SNPs in the following genes were included in our analyses: *ABCB1*, *ABCG2*, *MTHFR*, *SLC91A1*, *ERCC1*, *ERCC2*, *XRCC1*, *GSTP1* and *TP53*. In addition to *MTHFR*, other genes have been associated with fluoropyrimidine cytotoxicity, including the genes *TYMS*, *DPYD* and *TYMP*. However, polymorphisms in these genes were not included in the present study. The *TSER* polymorphisms (rs34743033 and rs11540151) were not included because, in pilot experiments, genotyping for these SNPs failed in the majority of FFPE samples, presumably due to the extended length of the polymorphism (data not shown). *DPYD**2A (rs3918290) was also excluded, because the very low minor allele frequency and expected low number of heterozygotes in our population (1–2%[9;22]) would preclude statistical analyses. Although *TYMP* expression has been linked to fluoropyrimidine cytotoxicity, very few studies have addressed the effect of genetic variation in this gene. It was therefore not included in this study. Regarding irinotecan pharmacogenetics, it would have been interesting to test UDP-glucuronosyltransferase polymorphisms, particularly *UGT1A1**28. Unfortunately, genotyping was not successful in most FFPE samples, which led us to exclude this polymorphism from further analyses.

In order to maintain statistical power, we chose to limit the number of polymorphisms to be studied per gene. Because our intention was to validate results from earlier publications, we aimed to include the polymorphism that was cited most frequently in publications to date for each gene. Therefore, in these cases, we searched PubMed for the respective polymorphisms and their rs numbers and included the SNP with the highest number of citations.

The following SNPs were determined using the TaqMan® 7500 real-time PCR system (Applied Biosystems, The Netherlands) according to the manufacturer's protocol: *ABCB1* rs1128503 and rs1045642; *ABCG2* rs2231142; *ERCC1* rs11615; *MTHFR* rs1801133; *SLC19A1* rs1051266; and *ERCC2* rs1799793 and rs13181. To genotype *ABCB1*, *ABCG2* and *SLC19A1*, we used custom-designed assays. To genotype *ERCC1*, *MTHFR* and *ERCC2*, we used predesigned assays. Additionally, *GSTP1* rs1695, *TP53* rs1042522 and *XRCC1* rs25487 were determined using the Pyrosequencer 96MA™ (Isogen, The Netherlands). The pyrosequencing reactions were performed according to the manufacturer's protocol. PCR primers, target sequences and the calculated dispensation orders for each SNP are listed in Table 1. Note that the lowercase nucleotides in the dispensation order are negative controls, which would not be incorporated

into the target DNA and consequently should not appear in the pyrogram.

The technicians performing the analyses were blinded with respect to sample identity. As a quality control in the pyrosequencing and TaqMan assays, at least 5% of samples were genotyped in duplicate, and water was used as a negative control. No inconsistencies were observed. In case of discrepancies between genotypes in tumor and blood DNA, the discordant pair was reanalyzed in one run. In this run, we included five randomly selected samples and water as controls.

SNP	Target#	Sequence 5'-3'	Modification
GSTP1 rs1695	PCR-f	AGGACCTCCGCTGCAAATAC	Biotin
	PCR-r	CTGGTGCAGATGCTCACATAGTT	
	Sequence primer-f	CTCCGCTGCAAATAC	
	Target Sequence	A/GTCTCCCTCAT	
	Dispensation order	cAGaTCTCT	
TP53 rs1042522	PCR-f	GAAGACCCAGGTCCAGATGAAG	Biotin
	PCR-r	CCGGTGTAGGAGCTGCTGG	
	Sequence primer-r	GGTGCAGGGGCCACG	
	Target Sequence	C/GGGGGAGCAGCCT	
	Dispensation order	tGCGcAGCAG	
***XRCC1* rs25487**	PCR-f	TAAGGAGTGGGTGCTGGACTGTC	Biotin
	PCR-r	CAGGGTTGGCGTGTGAGG	
	Sequence primer-r	CGTGTGAGGCCTTACC	
	Target Sequence	TCC/TGGGAGGGCA	
	Dispensation order	gTCTcGAGC	

Table 1. Primers and probes for pyrosequence analysis and fragment length analysis
f = forward orientated, r = reverse orientated

Statistical analysis

Concordance of genotypes determined in DNA from EDTA–blood and FFPE samples was tested using the k-statistic, which tests the agreement between two paired results. A k-value larger than 0.95 was considered good agreement. We calculated 95% confidence intervals (95% CI) of the k-statistic. The null hypothesis was that there is no difference in genotyping results due to source of DNA, corresponding to a k of 1.00. The two-sided significance level was set at $p < 0.05$. All statistical analyses were performed using SPPS software, version 20.0 (SPSS, Inc., IL, USA).

Gene	Genetic variation	rs No.	Chrom	% success in blood	Genotype distribution in blood (%)	MAF in blood	% success in FFPE	Genotype distribution in FFPE (%)	MAF in FFPE
ABCB1	C1236T, Gly412Gly	1128503	7q1	95.3%	17.6-50.0-32.4	0.426	91.9%	18.2-48.9-32.8	0.427
ABCB1	T3435C, Ile1145Ile	1045642	7q1	96.0%	28.0-48.3-23.8	0.479	96.0%	28.7-46.2-25.2	0.483
ABCG2	C421A, Lys141Gln	2231142	4q21	94.6%	66.7-32.6-0.7	0.170	95.3%	69.7-30.3-0.0	0.154
ERCC1	T496C, Asn118Asn	11615	19q13.3	91.9%	39.4-48.9-11.7	0.361	96.6%	40.3-47.9-11.8	0.358
ERCC2	A2251C, Lys751Gln	13181	19q13.3	97.3%	35.9-49.7-14.5	0.393	85.9%	41.4-41.4-17.2	0.379
ERCC2	G965A, Asp312Asn	1799793	19q13.3	91.9%	41.6-48.2-10.2	0.343	91.3%	44.1-42.6-13.2	0.346
GSTP1	G342A, Ile105Val	1695	11q13	100%	38.3-53.0-8.7	0.352	89.9%	44.0-48.5-7.5	0.317
MTHFR	C677T, Val222Ala	1801133	1p36.3	87.2%	42.3-50.8-6.9	0.382	91.9%	43.1-47.4-9.5	0.332
TP53	G466C, Arg72 Pro	1042522	17p13	100%	45.6-48.3-6.0	0.302	77.2%	49.6-44.3-6.1	0.283
SLC19A1*	G80A, Arg27His	1051266	21q22	99.3%	33.8-50.0-16.2	0.412	85.2%	31.5-51.2-17.3	0.429
XRCC1	G1301A, Arg399Gln	25487	19q13.3	100%	34.2-55.7-10.1	0.379	94.6%	35.5-54.6-9.9	0.372

Table 2. Genotype results

* Also denominated reduced folate carrier (RFC). MAF, minor allele frequency; FFPE, formalin-fixed paraffin embedded tissue.
Genotype distributions are shown as: homozygous wildtype-heterozygous-homozygous mutant.

Results

Genotyping results

Both EDTA–blood and FFPE tissue samples were available for 149 patients with metastatic CRC who participated in the CAIRO trial. Paraffin tissue and peripheral blood genotypes were determined for a total of 11 SNPs from nine chromosomal regions. Depending on genotype, results were obtained in 87–100% of peripheral blood samples; FFPE tissue-derived genotypes were obtained in 77–97% of samples. Paired results for FFPE tissue and peripheral blood genotype were obtained for 77–95% of patients, depending on the studied SNP. Genotype distributions were in accordance with previously published results.[9;23–25](Table 2) All genotypes were in Hardy–Weinberg equilibrium, with the exception of *XRCC1* (both in EDTA–blood and FFPE tissue; $\chi^2 = 4.999$, p = 0.025 and $\chi^2 = 3.997$, p = 0.046, respectively), *GSTP1* (EDTA–blood only; $\chi^2 = 3.896$, p = 0.048) and *ABCG2* (FFPE tissue only; $\chi^2 = 4.520$, p = 0.034).

Gene	rs No	Number of evaluable pairs (% of 149 pairs)	No of discordant pairs (%[#])	κ statistic	95% Confidence interval[$]
ABCB1	1128503	131 (88%)	3 (2.3%)	0.963	0.921-1.00
ABCB1	1045642	137 (92%)	0 (0%)	1.00	-
ABCG2	2231142	134 (90%)	0 (0%)	1.00	-
ERCC1	11615	132 (89%)	2 (1.5%)	0.975	0.940-1.00
ERCC2	13181	124 (83%)	2 (1.6%)	0.974	0.914-1.00
ERCC2	1799793	124 (83%)	3 (2.4%)	0.960	0.915-1.00
GSTP1	1695	134 (90%)	4 (3.0%)	0.947	0.896-0.998
MTHFR	1801133	120 (81%)	2 (1.7%)	0.971	0.932-1.00
TP53	1042522	115 (77%)	2 (1.7%)	0.969	0.926-1.00
SLC19A1*	1051266	126 (85%)	1 (0.8%)	0.987	0.961-1.00
XRCC1	25487	141 (95%)	1 (0.7%)	0.987	0.961-1.00

Table 3. Concordance of genotypes between peripheral blood and formalin-fixed paraffin-embedded tissue

* Also denominated: Reduced folate carrier (RFC). [#] Percentage of evaluable pairs that is discordant.
[$] Ninety-five percent confidence interval for κ statistic.

Concordance of EDTA–blood & FFPE genotype

We found an excellent agreement between the peripheral blood genotypes and the genotypes determined in corresponding FFPE samples.(Table 3) With the exception of *GTSP1* rs1695 (95% CI for k-statistic: 0.896–0.998), all SNPs showed an agreement between samples that was not significantly different from 100%. The 95% confidence levels of genotype concordance ranged from 0.961–1.000 (*XRCC1* rs25487) to 0.914–1.000 (*ERCC2* rs1799793).

Description of discordant results

In total, 20 out of 1418 (1.4%) genotype pairs in 18 patients were discordant in our sample set.

For each individual SNP, no more than 3.0% of pairs showed dissimilar results. Table 4 shows the genotypes found in peripheral blood and FFPE tissue for all discordant pairs. A potential source of discordance is LOH. We assessed LOH using heterozygous genotypes from adjacent loci. Because LOH involves larger stretches of DNA, loss of an allele for one SNP should be accompanied by loss of an allele for neighboring loci.[26] Using this approach, the number of mismatches potentially due to LOH was reduced to ten, including four sample pairs for *GSTP1* in which LOH could not be ruled out.

SNP	Subject No.	Genotype blood	Genotype FFPE	FFPE genotype in adjacent locus		Difference caused by LOH?
				Adjacent locus		
***ABCB1* rs1128503**	1	CC	TT	-	-	No
	2	CC	CT	-	-	No
	3	CT	CC	*ABCB1* rs1045642	missing	Possible
***ERCC1* rs11615**	4	TC	TT	*XRCC1* rs5487	heterozygous	No
	5	TC	CC	*ERCC2* rs13181	heterozygous	No
***ERCC2* rs13181**	6	AC	AA	*ERCC2* rs1799793	missing	Possible
	7	AC	AA	*XRCC1* rs5487	heterozygous	No
***ERCC2* rs1799793**	8	GA	GG	*ERCC2* rs13181	missing	Possible
	5	GA	AA	*ERCC2* rs13181	heterozygous	No
	9	GA	AA	*ERCC2* rs13181	heterozygous	No
***GSTP1* rs1695**	10	AG	AA	-	-	Possible
	11	AG	AA	-	-	Possible
	12	AG	AA	-	-	Possible
	13	AG	AA	-	-	Possible
***MTHFR* rs1801133**	2	CT	CC	-	-	Unlikely*
	14	CT	CC	-	-	Possible
***TP53* rs1042522**	15	GC	CC	-	-	Possible
	16	CC	GC	-	-	No
***SLC19A1* rs1051266**	17	AA	GA	-	-	No
***XRCC1* rs25487**	18	GA	AA	*ERCC2* rs13181	heterozygous	No

Table 4. Exploration of discordant pairs on the basis of genotype in adjacent loci

* Mix-up of samples is likely to have happened, given the genotyping results for ABCB1 rs1128503 for this patient.

LOH: loss of heterozygosity; Possible: loss of heterozygosity cannot be determined due to missing genotypes and/or no adjacent loci were genotyped. Other explanations for discordant pairs include patient mix-up, sample mix-up, genotype errors.

Discussion

Although concordance of genotypes between colorectal tumor and adjacent normal mucosa has been studied by others, this is the first study in which the results of an extended set of genotypes were compared between peripheral blood DNA and archived FFPE tissue DNA from patients with metastatic CRC. The results of our study show that genotyping using material from FFPE tissue and EDTA–blood yields highly concordant results. Consequently, this implies that the findings from retrospective trials using archived FFPE tissue can be reliably compared with studies using peripheral blood leukocytes as the DNA source for a considerable number of SNPs.

Although for most SNPs no significant discordance between DNA derived from EDTA–blood and FFPE tissue was found, a small discrepancy was found for *GSTP1*. This is of particular interest because of the possible role of *GSTP1* in carcinogenesis. Knockdown experiments with a CRC cell line showed that GSTP1 function is essential for *in vivo* growth of xenografts[27] and GSTP protein levels are frequently increased in colon cancer tissue.[28] The enzyme is part of the JNK pathway and as such is involved in cell cycle regulation and apoptosis.[29;30] The *GSTP1* rs1695 SNP leads to an amino acid substitution (Ile105Val) that lies within a JNK protein binding site, and may therefore have functional implications in carcinogenesis.[31] Despite the high concordance rate between blood-derived and FFPE tissue-derived DNA, we cannot exclude the possibility that LOH occurs for this gene. Therefore, use of FFPE tissue from resection specimens for genotyping this SNP in pharmacogenetic association studies is possibly not advisable. Additionally, genotyping for *TP53* failed in 23% of our FPPE tissue samples, thereby indicating that this SNP is also less suitable for genotyping in archived tumor tissue.

Only a few studies have compared genetic variations in malignant and normal tissues, and most did not include CRC patients. Marsh and coworkers compared genotypes of tumor and adjacent normal tissue in fresh-frozen tumor samples of 44 CRC patients for 28 polymorphisms in 13 genes.[32] Overall, 13 out of 1139 genotypes (1.1%) showed discordant results, similar to our findings. By contrast, Le Morvan and colleagues found considerable LOH in FFPE colorectal tumor samples for *GSTP1* (five out of 25 samples) and *ERCC2* (14 out of 32 samples).[16] These authors did not elaborate on the methods used for detecting LOH in their samples. A third study compared genotypes in EDTA–blood and FFPE rectal tumor samples for seven genes, including *GSTP1*, and found extensive discordance, with only 14 out of 65 sample pairs showing no discrepancies.[33] Explanations for these contrasting results include different rates of genotyping success and different tumor characteristics in colon and rectal tumors.

In a recent debate, the concordance between germline and tumor genotype has been questioned when using tumor samples in the determination of *CYP2D6* genotype for breast cancer patients in large association studies of tamoxifen efficacy.[34–37] Significant deviation from the Hardy–Weinberg equilibrium in tumor material was occasionally found for *CYP2D6* genotypes, possibly due to hemizygous chromosomal deletions. According to some authors,

this should preclude studies with pharmacodynamic end points using tumor material as a source of DNA.[34]

Indeed, LOH may explain discordance between genotype in DNA from EDTA–blood and FFPE tissue samples. However, in our study, we excluded LOH in ten out of 20 discordant cases, using heterozygous alleles in adjacent loci as a marker for chromosomal loss. This technique does not exclude hemizygous loss of very short chromosomal regions. Nevertheless, we believe that this is a valid method for evaluating the presence of LOH in our sample set, because LOH in colorectal carcinogenesis is thought to involve large stretches of chromosomal material in most cases.[38] Minimal presence of LOH was also expected, since most of these SNPs have not been convincingly associated with colorectal carcinogenesis and therefore selection of somatic mutations in these genes is unlikely.

For cases in which LOH could not be excluded, copy number gain of one allele could also explain discordant results, as the strong signal from the amplified allele would obscure the signal from the other allele. However, copy number amplification for the selected genes is not a common event in colorectal carcinogenesis, and is therefore a less likely cause of discordance. Alternative explanations for discordance include patient mix-up, sample mix-up or repeated genotyping errors. Reanalyzing all discordant pairs reduced the chance that disagreements were induced by genotyping errors or sample mix-up, but does not rule out patient mix-up or technical problems in earlier stages of sample preparation. However, assuming that only one result in every discordant pair is incorrect, we observed a maximum error rate of 0.7% for all samples (20 out of 2836 assays). This is likely to reflect the actual practice in clinical trials, which, in our opinion, is an acceptable level of inaccuracy.

The almost complete concordance of genotypes between blood-derived and FFPE tissue-derived DNA most likely reflects the actual absence of LOH in our tumor samples. However, our method may be hampered by the presence of stromal cells in our FFPE samples. In the presence of large amounts of non-cancerous stromal cells, FFPE tissue-derived genotypes may actually reflect germline genotypes, rather than tumor genotypes. Techniques for DNA extraction from FFPE tissues have been optimized in recent years and the use of microdissection instead of macrodissection would presumably have reduced the amount of stromal contamination in our samples. By contrast, most previously published articles have used macrodissection as the technique for DNA extraction.[7–19] It was our aim to confirm that results from these studies can be reliably compared with those from studies using blood-derived DNA for genotyping. Whether the concordance when using macrodissection is a reflection of a large stromal component or of actual agreement between the germline and tumor genotype is therefore only of theoretical importance.

We showed excellent concordance for all studied genetic variations. Unfortunately, it is unknown whether this concordance can be extrapolated to other genes or genetic variations of interest, or other types of cancer. The current study does not account for other genetic variations, such as amplification, methylation and copy number variation, which could be pharmacogenetically relevant. Taking these limitations into consideration, our results show

that FFPE tissue-derived DNA can be used as a valid proxy for germline DNA in CRC when blood samples are not available. However, even with the expanding possibilities of DNA collection from archived material, we believe peripheral blood should be the preferential source of DNA for future prospective pharmacogenetic studies.

Future perspectives

The field of pharmacogenomics is rapidly evolving. Most oncological clinical trials now include pharmacogenetic side-studies, for which peripheral blood samples are routinely collected. The use of archived FFPE tumor tissue will therefore be limited to retrospective studies and pharmacodynamic endpoints.

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4

MTHFR polymorphisms and capecitabine-induced toxicity in patients with metastatic colorectal cancer

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Abstract

Objective: The availability of current chemotherapeutic options for metastatic colorectal cancer (mCRC) has increased survival, but it is also accompanied by considerable morbidity. Fluoropyrimidines are the mainstay of systemic therapy. Germline pharmacogenetic markers involved in 5-fluorouracil pharmacodynamics could provide individualized pretreatment tools for predicting toxicity. Research on methylenetetrahydrofolate reductase (*MTHFR*) gene polymorphisms and fluoropyrimidine treatment outcome has focused on intravenous 5-fluorouracil and has yielded inconclusive results. The literature on pharmacogenetics in capecitabine-based chemotherapy is scarce. Therefore, we analyzed the association of *MTHFR* gene polymorphisms and the occurrence of serious toxicity of first-line capecitabine monotherapy and combination therapy.

Methods: One hundred and twenty-seven patients treated with first-line monotherapy capecitabine and 141 patients on capecitabine–irinotecan combination therapy were recruited from the CAIRO trial, an open-label phase III randomized trial, comparing sequential versus combination chemotherapy with capecitabine, irinotecan and oxaliplatin in mCRC. All patients were genotyped for *MTHFR* 1298A > C and 677C > T polymorphisms and analyzed in both cohorts separately for the association between the *MTHFR* genotype and incidence of grade 3–4 overall toxicity and specific adverse events, as well as efficacy parameters.

Results: *MTHFR* 1298A > C and 677C > T genotypes were not associated with grade 3–4 overall toxicity, febrile neutropenia or hand–foot syndrome. *MTHFR* 1298CC homozygotes showed a borderline significantly higher incidence of grade 3–4 diarrhea compared with *MTHFR* 1298AC or AA individuals (25 vs. 5%, P = 0.041) in the monotherapy cohort. No significant association was found between the *MTHFR* genotypes and efficacy parameters in either treatment cohort.

Conclusion: *MTHFR* polymorphisms are not associated with toxicity or efficacy in mCRC patients treated with capecitabine-based chemotherapy.

Introduction

In recent years, chemotherapeutic options for the treatment of metastatic colorectal cancer (mCRC) have expanded and have improved overall survival (OS) considerably.[1;2] Fluoropyrimidines, such as 5-fluorouracil (5-FU) and the oral pro-drug capecitabine, are still the mainstay of systemic treatment. However, despite the significant progress made with systemic therapy, the prognosis for mCRC remains relatively poor, with a median OS time of 19–22 months after diagnosis.[3;4] At the same time, chemotherapeutic regimens used in mCRC may result in toxicity, causing morbidity and occasionally even mortality, and frequently necessitating dose reductions. Unfortunately, predictors for adverse drug events in mCRC are scarce. In addition to clinical parameters, such as age and sex[5], germline pharmacogenetic markers could provide pretreatment information on the risk of toxicity.[6;7]

Several pharmacogenetic studies examining genetic variants related to 5-FU therapy, such as methylenetetrahydrofolate reductase (MTHFR) and thymidylate synthase (TS), have been published, but without conclusive results. Although polymorphisms in the gene coding for dihydropyrimidine dehydrogenase, the main catabolic enzyme of 5-FU, are linked to increased toxicity, the low allele frequency of the most common polymorphism in this gene limits its clinical usefulness. In addition, the role of pharmacogenetic biomarkers in predicting the toxicity and efficacy of capecitabine is as yet largely unexplored.

Fluoropyrimidines act in two different ways.[8] First, 5-FU is incorporated into RNA, precluding protein synthesis, the preferential mode of action for 5-FU bolus infusion. In addition, when administered as a continuous infusion, 5-FU binds to TS. This prevents the conversion of 2'-deoxyuridine-5'-monophosphate into 2'-deoxythymidine-5'-monophosphate, the latter of which is an essential precursor for DNA synthesis. In forming this ternary complex, 5,10-methylenetetrahydrofolate (5,10-MTHF) is required as an essential cofactor. MTHFR catalyzes the irreversible conversion of 5,10-MTHF into 5-methyltetrahydrofolate, thereby reducing the amount of 5,10-MTHF available for binding to TS.

Although over 60 germline polymorphisms in the *MTHFR* gene have been described, only two have shown functional effects on enzyme activity.[9] A non-synonymous single nucleotide polymorphism (SNP) at base pair 677 (C>T, Ala222Val) and a second SNP at base pair 1298 (A>C, Glu428Ala) both reduce *MTHFR* enzyme activity.[10;11]

Functional polymorphisms have also been described for TS, including a variable number of tandem repeats (VNTR) polymorphism in the enhancer region (TSER) in the 5'-untranslated region and an SNP G > C at bp12 of the second repeat of this VNTR.[12;13] In addition, a polymorphic locus is found in the TS 3'-untranslated region, consisting of a 6 bp deletion at position 1494.[12;14]

It is hypothesized that by reducing enzyme activity, *MTHFR* polymorphisms enhance the stable formation of the TS/fluorodeoxyuridine monophosphate complex, thereby resulting in both greater effect and toxicity of fluoropyrimidines. Higher intratumoral TS-levels are considered to hinder cytotoxicity. These assumptions have been studied extensively for

intravenous 5-FU therapy, but with contradictory results.(Table 1) In both the adjuvant and the metastatic setting of CRC, capecitabine is often replacing 5-FU, both in monotherapy and in combination therapy. A schematic overview of the capecitabine pharmacodynamics is presented in Figure 1. To our knowledge, only one previous pharmacogenetic study of capecitabine monotherapy in mCRC patients has been published.[16]

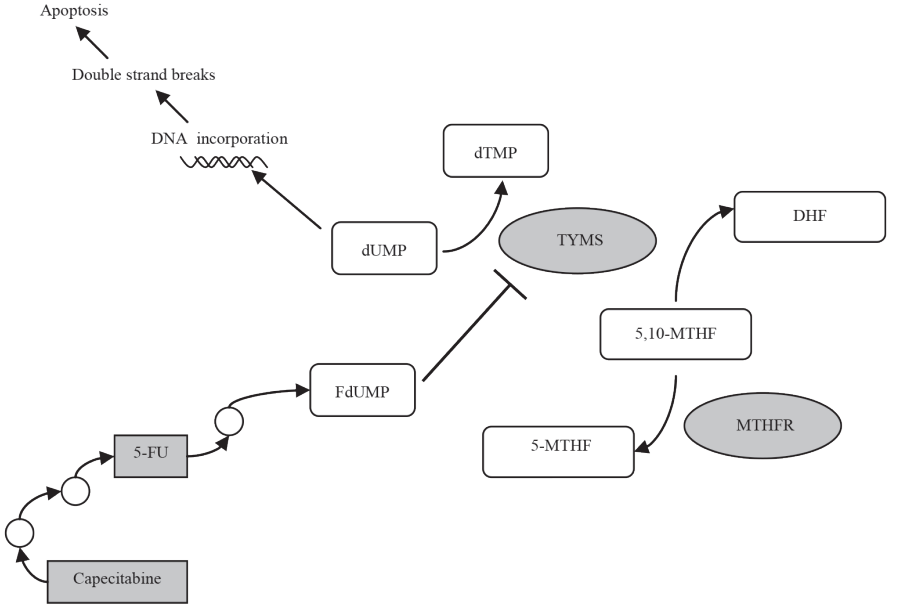

Figure 1. Schematic overview of enzymes involved in the cellular response of capecitabine

5-FU: 5-fluoro-uracil; DHF: dihydrofolate; dTMP: deoxythymidine monophosphate; dUMP: deoxyuridine monophosphate; FdUMP: fluoro- deoxyuridine monophosphate; MTHF: methylene hydrofolate; MTHFR: methylene tetrahydrofolate reductase; TYMS: thymidylate synthase. Figure based on: Thorn C.F., et al.[15]

Therefore, in this multicenter study, we aimed to determine the effect of the germline polymorphisms *MTHFR* 677C > T and *MTHFR* 1298A > C on the toxicity and efficacy profile of capecitabine in patients with mCRC who started first-line palliative chemotherapy. Patients treated with two frequently used treatment schedules were studied: a cohort of patients treated with capecitabine monotherapy and a second cohort of patients treated with a combination therapy of capecitabine and irinotecan. Genetic variants in the gene encoding for TS were included as covariates.

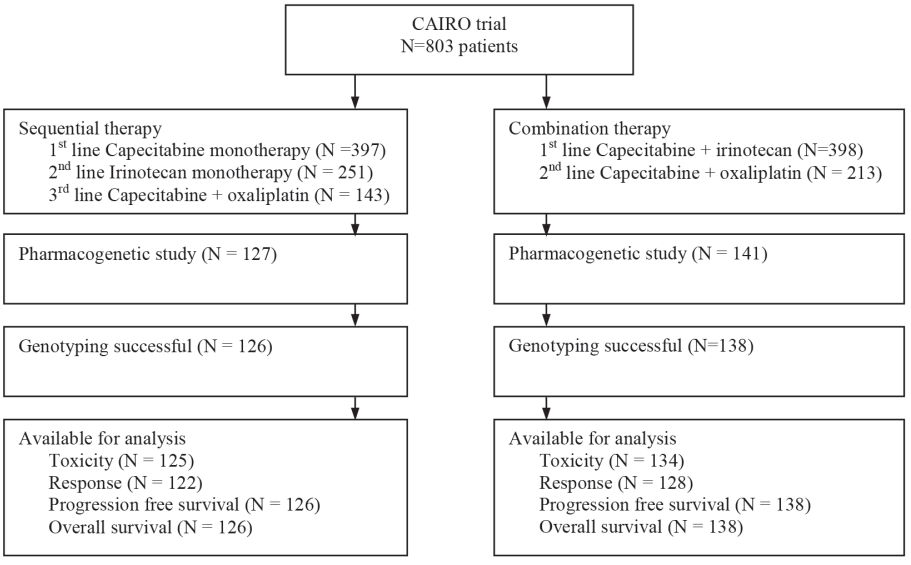

Figure 2. Study flowchart of the CAIRO study

Author, year (Ref.)	No of patients	Patient population	Treatment	Endpoint		Confirming hypothesis	Disproving hypothesis	
			5-FU bolus + LV		1298A>C	677C>T	1298A>C	677C>T
Afzal et al, 2009[1]	331	Stage II-III colon carcinoma (adjuvant)	5-FU bolus + LV	Toxicity			No effect of polymorphism	677CC more overall toxicity; and diarrhea
Afzal et al, 2011[2]	161 + 340	Stage II-III colon carcinoma (adjuvant) + mCRC	5-FU bolus + LV	Toxicity Efficacy			No effect of polymorphism*	No effect of polymorphism*
Castillo et al, 2010[3]	34	mCRC	5-FU bolus + LV	Efficacy	NA	TT longer TTP TT longer OS		
Derwinger et al, 2009[4]	142	Stage III colon carcinoma	5-FU bolus + LV (5% + oxali)	Toxicity Efficacy	NA	CT/TT more toxicity		CC better OS
Gusella et al, 2009[5]	130	Stage II-III colon carcinoma (adjuvant)	5-FU bolus + LV	Toxicity Efficacy		TT increased grade 3-4 toxicity	No effect of polymorphism	
Huang et al, 2008[6]	201	Stage II-III colon carcinoma (adjuvant)	5-FU bolus + LV	Efficacy			NA	No effect of polymorphism
Jakobsen et al, 2005[7]	88	mCRC	5-FU bolus + LV	Efficacy		TT higher RR	No effect of polymorphism	

Table 1. Overview of literature: MTHFR polymorphisms and cytotoxic effects of fluoropyrimidine-based chemotherapy in colorectal cancer, according to mode of administration

mCRC metastasized colorectal carcinoma; RR response rate; LV leucovorin; NA not assessed in this trial; pCR pathologic complete response; PFS progression free survival; DSS disease specific survival; OS overall survival; TTP time to progression; ACUP Adenocarcinoma of unknown primary.

*Compound heterozygotes or *MTHFR* 1298CC or *MTHFR* 677TT: less gastro-intestinal toxicity.

Only if associated with *TYMS* 3R3R

$ Other drugs included: platinum derivatives, gefitinib and irinotecan

Author, year (Ref.)	No of patients	Patient population	Treatment	Endpoint		Hypothesis confirming	Hypothesis disproving	
			Monotherapy fluoropyrimidine					
Balboa et al, 2010[8]	65	Pre-operative chemoradiation rectal carcinoma	5-FU bolus or cont. or capecitabine	Efficacy			No effect of polymorphism	No effect of polymorphism
Braun et al. 2009[9]	688	mCRC	5FU bolus, followed by cont.	Toxicity			No effect of polymorphism	No effect of polymorphism
Capitain et al, 2008[10]	76	mCRC	LV5FU2 or 5FU bolus + LV	Toxicity Efficacy	AA shorter OS#, CC more grade 3-4 toxicity	CC shorter OS#		
Cohen et al, 2003[11]	43	mCRC	Fluoro-pyrimidine	Toxicity Efficacy	NA	T allele higher RR		
Etienne et al, 2004[12]	98	mCRC	5-FU bolus or cont. + LV	Efficacy		TT higher RR	CC trend towards worse DSS	
Fernandez-Peralta et al, 2010[13]	143	Sporadic colorectal carcinoma	5-FU based chemotherapy	Efficacy		CT/TT better OS	AC/CC reduced OS	
Lamas et al. 2011[14]	93	Pre-operative chemoradiation rectal carcinoma	5-FU cont.	Efficacy			No effect of polymorphism	No effect of polymorphism
Pardini et al, 2011[15]	273	Colon carcinoma (adjuvant)	5-FU bolus + LV ± followed by cont.	Efficacy	AC/CC increased PFS			No effect of polymorphism
Schwab et al, 2008[16]	683	Colon cancer, breast cancer, other GI cancer, ACUP	5-FU bolus or cont. ± LV	Toxicity			No effect of polymorphism	No effect of polymorphism
Sharma et al, 2008[17]	54	mCRC	Capecitabine fixed-dose	Toxicity Efficacy	AA less toxicity		TT less toxicity	

Table 1. Continued

Author, year (Ref.)	No of patients	Patient population	Treatment	Endpoint		Hypothesis confirming	Hypothesis disproving	
			Combination therapy		1298A>C	677C>T	1298A>C	677C>T
Agostini et al, 2008[18]	42	Pre-operative chemoradiation rectal carcinoma (aged > 65y)	5-FU cont. or capecitabine ± oxaliplatin	Toxicity			No effect of polymorphism	No effect of polymorphism
Boigé et al, 2010[19]	349	mCRC	LV5FU2, FOLFIRI, FOLFOX	Toxicity Efficacy	CC higher RR only in FOLFOX			No effect of polymorphism
Budai et al, 2011[20]	85	mCRC	FOLFIRI + bevacizumab	Toxicity Efficacy			NA	No effect of polymorphism
Cecchin et al, 2011[21]	238	Pre-operative chemoradiation rectal carcinoma	Fluoro-pyrimidine ± other drug[$]	Efficacy			No effect of polymorphism	CT/TT lower RR
Chua et al, 2009[22]	118	mCRC	FOLFOX	Toxicity Efficacy		TT more grade 3-4 diarrhea	NA	
Etienne-Grimaldi et al, 2010[23]	117	mCRC	FOLFOX	Toxicity Efficacy	AC/CC higher RR	CT/TT higher RR		
Garcia-Aguilar et al, 2011[24]	132	Pre-operative chemoradiation rectal carcinoma	5-FU cont. ± followed by FOLFOX	Efficacy			NA	TT lower pCR
Glimelius et al, 2010[25]	140	mCRC	FLIRI or LV5FU2-IRI	Toxicity Efficacy			No effect of polymorphism	CT less early toxicity
Huang et al, 2010[26]	157	mCRC	FOLFOX	Efficacy			NA	No effect of polymorphism
Kristensen et al, 2010[27]	68	Stage II-II coloncarcinoma and mCRC	5-FU bolus / cont. or capecitabine ± oxaliplatin	Toxicity	CC increased toxicity			CT increased toxicity

Table 1. Continued

Author, year (Ref.)	No of patients	Patient population	Treatment	Endpoint		Hypothesis confirming	Hypothesis disproving	
			Combination therapy		1298A>C	677C>T	1298A>C	677C>T
Lamas et al, 2011[28]	72	mCRC	FOLFOX	Efficacy Toxicity			No effect of polymorphism	No effect of polymorphism
Lurje et al, 2008[29]	197	Stage II-III colon carcinoma (adjuvant)	5-FU bolus or cont.	Efficacy			No effect of polymorphism	No effect of polymorphism
Marcuello et al, 2006 [30]	94	mCRC	5-FU cont. + irinotecan or oxaliplatin/LV	Efficacy			No effect of polymorphism	No effect of polymorphism
Ruzzo et al, 2007[31]	166	mCRC	FOLFOX	Toxicity Efficacy			No effect of polymorphism	No effect of polymorphism
Ruzzo et al, 2008[32]	146	mCRC	FOLFIRI	Toxicity Efficacy			No effect of polymorphism	No effect of polymorphism
Suh et al, 2006[33]	54	mCRC	FOLFOX	Efficacy			NA	No effect of polymorphism
Terrazino et al, 2006[34]	125	Pre-operative chemoradiation rectal carcinoma	5-FU ± other drugs	Efficacy		CC higher RR	No effect of polymorphism	
Thomas et al, 2011[35]	96 + 35	Pre-operative chemoradiation rectal carcinoma	5-FU cont./ 5-FU cont. + irinotecan	Toxicity Efficacy			AA more mucositis/ diarrhea	No effect of polymorphism
Zhang et al, 2007[36]	318	mCRC	5-FU ± irinotecan ± oxaliplatin	Efficacy			♂: no effect ♂: AA longer OS	No effect of polymorphism

Table 1. Continued

Methods

Patients and treatment

In total, 127 patients who were treated with capecitabine monotherapy as the first-line treatment for mCRC were prospectively included in the study. In addition, a second cohort including 141 patients treated with first-line capecitabine–irinotecan combination therapy was studied. Patients were recruited from the CAIRO study, a multicenter open-label randomized phase III trial, comparing sequential versus combination chemotherapy with capecitabine, irinotecan and oxaliplatin in a total of 803 patients with mCRC.[17]

Patients with mCRC were enrolled in the CAIRO study between January 2003 and December 2004 by the Dutch Colorectal Cancer Group in 74 hospitals in the Netherlands. The study flowchart and number of patients available for analysis are shown in Figure 2. As this pharmacogenetic substudy was initiated later than the CAIRO clinical trial and not all study centers participated, the number of patients included in the pharmacogenetic analyses is limited to a total of 268 patients. The inclusion criteria were a WHO performance score of 0–2 and adequate renal, hepatic and bone marrow function. A history of previous adjuvant chemotherapy was allowed only if the last administration was 6 months before randomization. The main exclusion criteria were as follows: serious concomitant disease preventing the safe administration of chemotherapy and other malignancies in the past 5 years. Capecitabine (1250 mg/m^2, twice daily) was administered on days 1–14 in the monotherapy group every 3 weeks. In the combination therapy group, capecitabine (1000 mg/m^2, twice daily) was administered on days 1–14, and irinotecan (250 mg/m^2) on day 1, in a 3-weekly cycle. Tumor response was assessed by computed tomography scan, every 9 weeks, using the response evaluation criteria for solid tumors (version 1.0). Toxicity during first-line therapy was assessed at each visit by determining the patient's history, physical examination and hematology and biochemical laboratory tests. Toxic effects were classified following the US National Cancer Institute Common Toxicity Criteria, version 2.0. The CAIRO study protocol provided guidelines for dose modification in case of serious toxicity.

The study was approved both by the Central Committee on Research involving Human Subjects (CCMO) and by the local ethics committees of all participating centers. All patients included provided written informed consent before inclusion in the main study and the pharmacogenetic side study.

Genotyping data

Peripheral EDTA-blood samples were collected and stored at – 20°C before DNA isolation. Germline DNA was extracted with the Magnapure LC (Roche Diagnostics, Almere, the Netherlands) according to the manufacturer's instructions. *MTHFR* rs1801133 (677C > T) and *MTHFR* rs1801131 (1298A > C) genotypes were determined with TaqMan 7500 (Applied Biosystems, Nieuwerkerk aan de Ijssel, the Netherlands) according to the manufacturer's protocol using a predesigned assay. The call rate for the *MTHFR* genotypes was greater than

98%. Five per cent of samples were analyzed in duplicate, with 100% concordance. In addition, negative controls using water were included. To exclude confounding by other known pharmacogenetic determinants in 5-FU-based chemotherapy, we also assessed the *TYMS* genotype. The VNTR polymorphism in the *TYMS* 5'-untranslated region (TSER, rs34743033), including the additional G/C SNP in the second base pair for 3-repeat individuals (rs11540151), was determined by direct sequencing. The TYMS 1494del6bp polymorphism in the 3'-untranslated region (rs11280056) was determined with TaqMan 7500 (Applied Biosystems) according to the prescribed protocol. *DPYD**2A (IVS14 + 1G > A) was not included because of low expected population allele frequency.

Data and statistical analysis

The primary endpoints of this study were overall toxicity (i.e. grade 3 or higher) on first-line therapy, and specific adverse events, including hand–foot syndrome (HFS), diarrhea and febrile neutropenia. Toxicity was assessed for all patients who started treatment. The secondary endpoints were progression-free survival (PFS), OS and response rate. PFS was calculated from the date of randomization until the first observation of disease progression or death from any cause. OS was calculated as the interval from randomization until death from any cause or until the date of last follow-up. Survival data have been updated since the publication of the original CAIRO trial. Best response was assessed in all patients who completed at least three cycles of treatment. Clinical benefit was defined as either a complete or a partial response, or stable disease.

Genotype distributions were tested for agreement with those expected under Hardy–Weinberg equilibrium using the χ^2-test. The association between genotype or diplotype and the presence of overall toxicity of at least grade 3, specific toxicity parameters of at least grade 3 and clinical benefit were determined using a χ^2-test. Kaplan– Meier survival analysis and the log-rank test were used to test the relationship between genotype and OS/PFS. For *TYMS* polymorphisms, patients were further subdivided into three groups according to the expected level of TS expression: low (2R/2R or 2R/3RC or 3RC/3RC), intermediate (2R/3RG or 3RC/3RG) or high (3RG/3RG). *MTHFR* genotypes were grouped as follows: wild-type homozygote versus all other genotypes or variant-type homozygote versus all other genotypes. The association of genotype and OS or PFS was determined using the Mann–Whitney test. Fisher's exact test was used to determine the effect of grouped genotype on toxicity and clinical benefit. Multivariate analysis was carried out with *TYMS* genotype and sex. Because of the linkage disequilibrium between both *MTHFR* genotypes, we did not carry out multivariate analyses including both polymorphisms as independent covariates, but rather carried out separate analyses for *MTHFR* 1298A > C and 677C > T genotypes. On the basis of reports finding a difference in the effect of *MTHFR* genotype on 5-FU efficacy according to sex, subgroup analysis was carried out. Subgroup analysis was also carried out by subdividing patients according to previous adjuvant therapy. Patients with missing genotyping data were excluded from analysis. A two-sided significance level of P less than 0.01 was accepted for all analyses to compensate for multiple

testing. Analyses were carried out using the SPSS version 17.0 software (SPSS Inc., Chicago, Illinois, USA).

Results

Clinical data

The baseline characteristics of the studied patients are listed in Table 2. The median age at randomization for all patients was 61 years, ranging from 27 to 81 years. Patients were predominantly men (61%) and most patients had not received previous adjuvant chemotherapy (88%).

	Total N= 264	**Capecitabine monotherapy N= 126**	**Combination therapy N= 138**
Age			
Age at randomization, median (range)	61 years (27-81)	61 years (27-78)	62 years (37-81)
Sex			
Male	163 (62%)	77 (61%)	86 (62%)
Female	101 (38%)	49 (39%)	52 (38%)
Performance status			
PS 0	152 (58%)	77 (61%)	75 (54%)
PS 1	96 (36%)	42 (33%)	54 (40%)
PS 2	16 (6%)	7 (6%)	9 (6%)
LDH at randomization			
Normal	173 (66%)	82 (65%)	91 (66%)
>Upper limit of normal	91 (34%)	44 (35%)	47 (34%)
Site of primary tumor			
Colon	146 (55%)	73 (58%)	73 (53%)
Rectosigmoid	96 (37%)	40 (32%)	56 (41%)
Rectum	20 (8%)	11 (9%)	9 (6%)
Multiple tumors	1 (<1%)	1 (<1%)	0
Unknown	1 (<1%)	1 (<1%)	0
Predominant localization of metastases			
Liver	179 (68%)	83 (66%)	96 (70%)
Extra-hepatic	81 (31%)	39 (31%)	42 (30%)
Unknown	4 (1%)	4 (3%)	0
Previous adjuvant therapy			
Yes	31 (12%)	13 (10%)	18 (13%)
No	233 (88%)	113 (90%)	120 (87%)

Table 2. Baseline characteristics.

LDH, lactate dehydrogenase

Genotype frequencies

Genotype frequencies are listed in Table 3. Genotyping for *MTHFR* polymorphisms was successful for 126 of the 127 (99%) patients in the capecitabine monotherapy group. For the *MTHFR* 677C > T locus, we found that 51 (41%) patients were homozygote wild type, 14 (11%) were homozygote variant type and 61 (48%) were heterozygote. For *MTHFR* 1298A > C, 58 (46%) patients were homozygote wild type, 12 (10%) were homozygote variant type and 56 (44%) patients were heterozygote.

Genotyping was successful for 138 of the 141 (98%) patients in the combination therapy group. For *MTHFR* 677C > T, in this group 55 (40%) patients were homozygote wild type, 13 (9%) were homozygote variant type and 70 (51%) heterozygote. For *MTHFR* 1298A > C, 57 (42%) patients were homozygote wild type, 13 (9%) were homozygote variant type and 68 (49%) were heterozygote.

Allele frequencies for *MTHFR* 677C > T and for *MTHFR* 1298A > C in both groups were consistent with Hardy–Weinberg equilibrium (χ^2-test: $P > 0.05$). Genotype frequencies are similar to those reported by other authors.[18–20] No patients were found to be homozygous for both loci, consistent with the linkage disequilibrium between both polymorphisms described elsewhere.[11]

Genotyping for *TYMS* was successful for 112 (88%) patients in the monotherapy group and for 120 (85%) patients in the combination therapy group. In the capecitabine monotherapy group, *TSER*-genotype frequencies were 26 (23%) 2R2R, 31 (28%) 3R3R and 55 (49%) 2R/3R. A predicted low TS-expression genotype was present in 70 (62%) patients, an intermediate-expression genotype in 33 (30%) and a high-expression genotype in nine (8%) patients (see the Methods section for definition of expression level in individual genotypes). For the *TYMS* 1494del6bp genotype, 13 (12%) patients had the del/del genotype, 40 (36%) had the del/ins genotype and 59 (52%) had the ins/ins genotype.

In the combination therapy group, *TSER* genotypes were as follows: 28 (23%) 2R2R, 37 (31%) 3R3R and 55 (46%) heterozygote. TS expression genotypes were predicted to be low in 65 (54%) patients, intermediate in 46 (38%) and high in nine (8%) patients. The *TYMS* 1494 del/del genotype was found in 11 (9%) patients; 57 (48%) patients were heterozygote and 52 (43%) were ins/ins homozygote. The *TSER* genotype for patients in the monotherapy group was not consistent with the Hardy–Weinberg equilibrium ($\chi^2 = 12.2$, $P < 0.001$). However, because no deviation from the equilibrium was found (χ^2-test: $P > 0.05$) in the total population and in the combination therapy group, it is likely that this inconsistency was derived by chance. All other genotype frequencies were as expected under the Hardy– Weinberg equilibrium.

Genotype	Total	Capecitabine monotherapy	CapIri combination therapy
N=	264	126	138
***MTHFR* 677C>T**			
677CC	106 (40%)	51 (41%)	55 (40%)
677CT	131 (50%)	61 (48%)	70 (51%)
677TT	27 (10%)	14 (11%)	13 (9%)
***MTHFR* 1298A>C**			
1298AA	115 (44%)	58 (46%)	57 (42%)
1298AC	124 (47%)	56 (44%)	68 (49%)
1298CC	25 (9%)	12 (10%)	13 (9%)
Diplotype			
1298AA/677CC	26 (10%)	15 (12%)	11 (8%)
1298AA/677CT	64 (24%)	31 (25%)	33 (24%)
1298AA/677TT	25 (10%)	12 (10%)	13 (9%)
1298AC/677CC	57 (22%)	26 (21%)	31 (23%)
1298AC/677CT	65 (25%)	28 (22%)	37 (27%)
1298AC/677TT	2 (<1%)	2 (2%)	0
1298CC/677CC	23 (9%)	10 (8%)	13 (9%)
1298CC/677CT	2 (<1%)	2 (2%)	0
1298CC/677TT	0	0	0

Table 3. MTHFR genotype and diplotype frequencies

Correlation between MTHFR and TYMS genotypes/ diplotypes and toxicity

The results for toxicity analyses are listed in Table 3. No correlation was found between genotype and overall toxicity of at least grade 3 for the *MTHFR* 677C > T genotype and 1298A>C in patients treated with capecitabine monotherapy. Grouping genotypes according to the presence or absence of variant alleles did not show any statistically significant association with the incidence of severe overall toxicity. Diplotype analysis was carried out grouping patients according to the number of variant alleles (gene score). This did not result in a significant association between any gene score and overall toxicity of at least grade 3 (P = 0.838).

In addition, we carried out association analyses for the *MTHFR* genotype and specific adverse events. No significant association was found for the *MTHFR* 1298A > C genotype, the *MTHFR* 677C > T genotype or the *MTHFR* diplotype and the incidence of HFS of at least grade 3 or febrile neutropenia of at least grade 3. However, a trend towards a higher incidence of diarrhea of at least grade 3 was observed for *MTHFR* 1298CC homozygotes (AA and AC vs. CC: 5 vs. 25%, P = 0.041). No episodes of febrile neutropenia of at least grade 3 were observed in the capecitabine monotherapy group.

Next, all analyses were repeated for patients in the combination treatment arm. No associations were found between *MTHFR* 677C > T and 1298A > C genotypes and severe overall toxicity. These results remained similar after grouping genotypes according to the presence or absence of variant alleles. In terms of the effect of *MTHFR* polymorphisms on specific adverse events, we found no association for diarrhea or febrile neutropenia. *MTHFR*

1298CC individuals experienced a significantly higher incidence of HFS in the combination therapy group (*MTHFR* 1298AA 7% vs. AC 5% vs. CC 31%, P = 0.006; 1298AA and AC vs. CC: 6 vs. 31%, P = 0.011). No toxic deaths were observed in either treatment cohort.

The *TSER* genotype was found not to be associated with the incidence of overall toxicity of at least grade 3, diarrhea of at least grade 3 or febrile neutropenia of at least grade 3 in either treatment cohort (data not shown). However, a trend towards a protective effect of the *TSER* 2R allele on the incidence of HFS of at least grade 3 was found in the capecitabine monotherapy group (2R/2R 8% vs. 2R/3R 11% vs. 3R/3R 29%, P = 0.041; 2R2R and 2R/ 3R vs. 3R/3R: 10 vs. 29%, P = 0.019). This association was not found in the combination therapy group (2R/2R and 2R/3R vs. 3R/3R: 11 vs. 5%, P = 0.499). In both groups, no effect was found of the G > C SNP or the TYMS 1494 del6bp genotype on the incidence of overall or specific toxicity (data not shown).

TSER and *TYMS* 1494 del6bp genotypes were not associated with a difference in PFS or OS, and no significant interaction was found after combining *TSER* genotypes according to the expected level of TS expression on the basis of the G > C SNP. In addition, no association was found between clinical benefit and the *TYMS* 1494 del6bp or *TSER* (including G > C SNP) polymorphisms (data not shown). These results were found in both treatment groups. Only in the monotherapy group was there a non-significant trend towards a longer PFS for high TS-expression (3RG/3RG) individuals (3RG/3RG vs. all other genotypes, 10.2 vs. 6.2 months, P = 0.022), as well as for *TYMS* 1494del/del individuals (1494del/del vs. del/ins and ins/ins: 10.2 vs. 6.1 months, P = 0.017).

Subgroup and multivariate analysis

Multivariate analyses including the *MTHFR* genotype, *TYMS* genotype and sex as covariates did not yield any significant results. No patients with the *MTHFR* 1298CC genotype in first-line capecitabine monotherapy had received previous adjuvant chemotherapy. This is probably because of chance (χ^2-test: P = 0.453). In either treatment group, very few *MTHFR* 677TT individuals had received previous chemotherapy. Therefore, multivariate analysis for the association of *MTHFR* genotype with efficacy and toxicity parameters according to previous adjuvant treatment could not be carried out.

Subdividing our population according to sex showed no significant association between the incidence of adverse events and any *MTHFR* genotype or diplotype (data not shown). Male patients with the *MTHFR* 1298AA genotype had slightly, but non-significant, shorter PFS than patients with at least one variant allele (median PFS 5.7 vs. 6.9 months, P = 0.043).

	Capecitabine	*p*-value		*p*-value		*p*-value		*p*-value
	Overall toxicity ≥ grade 3		**Diarrhea ≥ grade 3**		**HFS ≥ grade 3**		**Febrile neutropenia**	
Overall	37%		7%		16%		0%	
MTHFR 677C>T								
MTHFR 677CC	40%		12%		29%		-	
MTHFR 677CT	33%		5%		7%		-	
MTHFR 677TT	43%	*0.649*	0%	*0.193*	24%	*0.018*	-	*n.a.*
MTHFR 677CC	40%		12%		24%		-	
MTHFR 677CT + 677TT	35%	*0.574*	4%	*0.154*	11%	*0.079*	-	*n.a.*
MTHFR 677CT + 677CC	36%		8%		14%		-	
MTHFR 677TT	43%	*0.770*	0%	*0.596*	29%	*0.237*	-	*n.a.*
MTHFR 1298A>C								
MTHFR 1298AA	37%		7%		19%		-	
MTHFR 1298AC	34%		4%		11%		-	
MTHFR 1298CC	50%	*0.578*	25%	*0.033*	25%	*0.309*	-	*n.a.*
MTHFR1298AA	37%		7%		19%		-	
MTHFR 1298AC + 1298CC	37%	*1.000*	7%	*1.000*	13%	*0.464*	-	*n.a.*
MTHFR 1298AC + 1298AA	35%		5%		15%		-	
MTHFR1298CC	50%	*0.355*	25%	*0.041*	25%	*0.406*	-	*n.a.*
MTHFR diplotype*								
No variant alleles	43%		14%		36%		-	
One variant allele	33%		5%		12%		-	
Two variant alleles	38%		8%		14%		-	
Three variant alleles	50%	*0.838*	0%	*0.630*	25%	*0.170*	-	*n.a.*

Table 4. MTHFR genotype and adverse events of first-line chemotherapy

HFS, hand-foot syndrome

*No variant alleles, 1298AA/677CC; one variant allele, 1298AC/677CC or 1298AA/677CT; two variant alleles, 1298CC/677CC or 1298AA/677TT or 1298AC/677CT; three variant alleles, 1298CC/677CT or 1298AC/67TT.

	Combination therapy	***p*-value**		***p*-value**		***p*-value**		***p*-value**
	Overall toxicity ≥ grade 3		**Diarrhea ≥ grade 3**		**HFS ≥ grade 3**		**Febrile neutropenia**	
Overall	50%		23%		8%		6%	
MTHFR 677C>T								
MTHFR 677CC	47%		22%		11%		4%	
MTHFR 677CT	51%		27%		6%		7%	
MTHFR 677TT	54%	*0.862*	8%	*0.297*	8%	*0.581*	8%	*0.665*
MTHFR 677CC	47%		22%		6%		4%	
MTHFR 677CT + 677TT	52%	*0.728*	24%	*0.838*	11%	*0.348*	7%	*0.475*
MTHFR 677CT + 677CC	50%		25%		8%		6%	
MTHFR 677TT	54%	*1.000*	8%	*0.298*	8%	*1.000*	8%	*0.560*
MTHFR 1298A>C								
MTHFR 1298AA	54%		16%		7%		7%	
MTHFR 1298AC	44%		29%		5%		6%	
MTHFR 1298CC	62%	*0.355*	23%	*0.199*	31%	*0.006*	0%	*0.621*
MTHFR1298AA	54%		16%		7%		7%	
MTHFR 1298AC + 1298CC	47%	*0.489*	28%	*0.103*	9%	*0.762*	5%	*0.719*
MTHFR 1298AC + 1298AA	49%		23%		6%		7%	
MTHFR1298CC	62%	*0.562*	23%	*1.000*	31%	*0.011*	0%	*1.000*
MTHFR diplotype*								
No variant alleles	36%		0%		9%		0%	
One variant allele	53%		27%		5%		8%	
Two variant alleles	49%		24%		11%		5%	
Three variant alleles	-	*0.582*	-	*0.154*	-	*0.391*	-	*0.536*

Table 4. Continued

	Capecitabine monotherapy	*p*-value		*p*-value		*p*-value
	PFS in months (95%CI)		OS in months (95%CI)		Clinical benefit	
	median		median			
Overall	6.3 (5.8-6.9)		19.5 (16.7-22.2)		85%	
MTHFR 677C>T						
MTHFR 677CC	6.6 (5.1-8.2)		19.5 (15.6-23.3)		90%	
MTHFR 677CT	6.1 (5.1-7.1)		18.1 (12.8-23.4)		78%	
MTHFR 677TT	6.6 (5.5-7.7)	*0.148*	24.0 (19.9-28.1)	*0.857*	100%	*0.067*
MTHFR 677CC	6.6 (5.1-8.2)		19.5 (15.6-23.3)		90%	
MTHFR 677CT + 677TT	6.1 (5.3-6.9)	*0.190*	19.5 (13.1-25.8)	*0.609*	82%	*0.310*
MTHFR 677CT + 677CC	6.3 (5.7-6.8)		19.2 (16.3-22.0)		83%	
MTHFR 677TT	6.6 (5.5-7.7)	*0.807*	24.0 (19.9-28.1)	*0.270*	100%	*0.127*
MTHFR 1298A>C						
MTHFR 1298AA	6.1 (5.7-6.5)		19.4 (11.4-27.4)		93%	
MTHFR 1298AC	6.9 (5.0-8.8)		19.8 (12.1-27.4)		78%	
MTHFR 1298CC	6.5 (4.2-8.8)	*0.610*	18.7 (17.6-19.8)	*0.534*	83%	*0.097*
MTHFR1298AA	6.1 (5.7-6.5)		19.4 (11.4-27.4)		93%	
MTHFR 1298AC + 1298CC	6.7 (5.1-8.4)	*0.582*	19.5 (15.4-23.5)	*0.957*	79%	*0.042*
MTHFR 1298AC + 1298AA	6.3 (5.8-6.8)		18.7 (17.6-19.8)		86%	
MTHFR1298CC	6.5 (4.2-8.8)	*0.904*	19.5 (13.5-25.4)	*0.758*	83%	*0.691*
MTHFR diplotype*						
No variant alleles	6.3 (5.8-6.8)		21.0 (13.5-28.6)		100%	
One variant allele	6.7 (4.7-8.6)		19.4 (10.9-27.9)		87%	
Two variant alleles	6.0 (4.5-7.5)		19.1 (17.2-21.1)		78%	
Three variant alleles	8.3 (6.2-10.5)	*0.470*	21.4 (12.6-30.2)	*0.942*	100%	*0.157*

Table 5. MTHFR genotype and efficacy of first-line chemotherapy

CI, confidence interval; OS, overall survival; PFS, progression-free survival.
*No variant alleles, 1298AA/677CC; one variant allele, 1298AC/677CC or 1298AA/677CT; two variant alleles, 1298CC/677CC or 1298AA/677TT or 1298AC/677CT; three variant alleles, 1298CC/677CT or 1298AC/67TT.

	Combination therapy	*p*-value		*p*-value		*p*-value
	PFS in months (95%CI)		OS in months (95%CI)		Clinical benefit	
	median		median			
Overall	8.3 (7.6-9.1)		20.0 (16.9-23.1)		93%	
MTHFR 677C>T						
MTHFR 677CC	9.2 (7.9-10.5)		19.8 (14.1-25.4)		94%	
MTHFR 677CT	7.8 (6.6-8.9)		20.0 (16.0-24.0)		93%	
MTHFR 677TT	6.3 (3.5-9.1)	*0.047*	23.1 (12.4-33.8)	*0.923*	92%	*0.964*
MTHFR 677CC	9.2 (7.9-10.5)		19.8 (14.1-25.4)		94%	
MTHFR 677CT + 677TT	7.8 (6.5-9.0)	*0.052*	20.5 (16.7-24.3)	*0.888*	93%	*1.000*
MTHFR 677CT + 677CC	8.3 (7.5-9.1)		20.0 (16.6-23.4)		93%	
MTHFR 677TT	6.3 (3.5-9.1)	*0.625*	23.1(12.4-33.8)	*0.994*	92%	*1.000*
MTHFR 1298A>C						
MTHFR 1298AA	7.1 (5.8-8.4)		17.9 (15.0-20.9)		94%	
MTHFR 1298AC	8.8 (7.9-9.7)		22.3 (18.8-25.9)		92%	
MTHFR 1298CC	9.4 (5.8-13.0)	*0.107*	22.4 (14.9-29.9)	*0.529*	90%	*0.848*
MTHFR1298AA	7.1 (5.8-8.4)		17.9 (15.0-20.9)		94%	
MTHFR 1298AC + 1298CC	9.0 (8.1-10.0)	*0.033*	22.4 (18.9-25.8)	*0.148*	92%	*0.735*
MTHFR 1298AC + 1298AA	8.3 (7.7-8.9)		20.0 (16.6-23.4)		93%	
MTHFR1298CC	9.4 (5.8-13.0)	*0.425*	22.4 (14.9-29.9)	*0.916*	90%	*0.531*
MTHFR diplotype*						
No variant alleles	6.5 (5.7-7.3)		14.4 (6.9-21.9)		90%	
One variant allele	8.4 (7.7-9.2)		19.8 (15.3-24.2)		97%	
Two variant alleles	8.3 (7.1-9.6)		22.3 (18.9-25.8)		90%	
Three variant alleles	-	*0.216*	-	*0.165*	-	*0.353*

Table 5. Continued

Discussion

The present study is the second and the largest to address *MTHFR* pharmacogenetics of capecitabine-based therapy in mCRC. No significant association was found between the *MTHFR* 677C > T or the 1298A > C genotype or diplotype and the incidence of severe chemotherapy-induced adverse events for either monotherapy or combination therapy with capecitabine. *MTHFR* 1298CC homozygotes showed a non-significant increase in grade 3–4 diarrhea when treated with capecitabine monotherapy, in accordance with our hypothesis. No effect was found of these genotypes on clinical response or survival statistics in our populations.

Because of the prospective accrual of patients in the CAIRO study, there is homogeneity in the treatment protocol for all patients, thereby obviating the risk of confounding by dosage or mode of 5-FU administration. Publications to date, including many clinical trials[3;18;19;21–39] and two recent meta-analyses[40;41], could not show a convincing effect of *MTHFR* polymorphisms on fluoropyrimidine-induced toxicity or treatment benefit in mCRC. However, whereas all these studies have focused on intravenous 5-FU therapy, capecitabine is increasingly being incorporated into the first-line treatment for mCRC, making the existing literature on 5-FU pharmacogenetics less relevant. Although capecitabine has comparable efficacy to 5-FU as monotherapy or in combination therapy[1], toxicity profiles differ. Capecitabine leads to a higher incidence of HFS than a 5-FU bolus injection.[42] This side effect is also found more frequently in 5-FU continuous infusion and suggests a difference in 5-FU pharmacodynamics depending on the mode of administration.[5] As a result, polymorphisms involved in the folate pathway may have a different effect on efficacy and toxicity according to the treatment schedule and mode of administration, and pharmacogenetic studies with 5-FU cannot be extrapolated to capecitabine. Only one previous small clinical trial studying pharmacogenetics of capecitabine monotherapy in mCRC patients has been published.[16] In this study, *MTHFR* 677TTand *MTHFR* 1298AA genotypes were associated with a lower incidence of grade 2–3 toxicity. Although the results for *MTHFR* 1298AA individuals confirm the hypothesis that *MTHFR* polymorphisms enhance capecitabine cytotoxicity, the results for *MTHFR* 677TT are contrary to what was expected. As there is no obvious pharmacological explanation for this incongruence, the results may have been affected by the small sample size. By choosing a stricter significance level, we reduced the risk of false-positive results because of multiple testing and found no association of the *MTHFR* genotype and capecitabine-induced adverse events. Furthermore, we focused only on the occurrence of severe toxicity (i.e. grade 3 or 4) because the goal of pretreatment testing is the prevention of serious adverse events. Indeed, an increase in grade 2 toxicity will not lead to pre-emptive dose reduction and therefore may not be considered clinically relevant.

As capecitabine cytotoxicity is the result of many interdependent enzymatic reactions, not only including *MTHFR* but also *TS*, the effects of one aberrant enzyme may be obscured by those of another. Therefore, addressing only polymorphisms in one gene may be an oversimplification of reality and this was the motivation to also include genetic variants in *TYMS* and *TSER*. The importance of the variants is supported by two studies, showing that patients with *TYMS*

3R/3R and either the *MTHFR* 1298CC or the 677TT genotype had a higher response rate to 5-FU-based chemotherapy, and longer OS or time to progression.[18;34] We univariately and multivariately evaluated the contributing effect of *TYMS* polymorphisms, without any effect on study outcome. We then identified the patients in our cohort carrying the *TYMS* 3R/3R genotype and a homozygote variant genotype for at least one of the *MTHFR* polymorphisms, in an attempt to replicate the two above-mentioned studies. In our population, however, only a few patients carried the *TYMS* 3R/3R-*MTHFR* 677TT or *TYMS* 3R/3R-*MTHFR* 1298CC genotype (five and four patients in the monotherapy group, respectively). Although we found no significant associations of these genotypes and the clinical outcome parameters, analyses are limited by the small number of affected patients (data not shown).

In early phase I trials, capecitabine was combined with oral leucovorin. This addition showed no effect on capecitabine pharmacokinetics, but appeared to reduce the maximum tolerable dose.[43;44] Currently, capecitabine therapy is not combined with leucovorin, in contrast to intravenous 5-FU therapy and tegafur. It can be hypothesized that in case of high serum levels of active folate, either by diet or by administration of leucovorin, the effects of *MTHFR* polymorphisms are masked. Folate intake and serum folate levels differ according to the geographical location of the population.[45;46] The folate levels in a Dutch population are on average lower than those for other European populations.[45] Therefore, in our population, folate level does not seem to explain the lack of effect of *MTHFR* polymorphisms.

In many modern chemotherapy regimens, capecitabine is combined with other chemotherapeutic agents, such as irinotecan or oxaliplatin. In combination therapy, toxicity caused by one of the agents may lead to dose reduction, and this may affect the possible pharmacogenetic associations of the other drugs. Our study is unique in the fact that it studies *MTHFR* pharmacogenetics in both capecitabine monotherapy and combined therapy. In the combination therapy group, however, the results may have been biased by the known pharmacogenetic effects of *UGT1A1*. Indeed, in Caucasians, the incidence of diarrhea and specifically febrile neutropenia because of irinotecan have been shown to be influenced by *UGT1A1**28 genotype.[6;31] We found no significant association between *MTHFR* genotypes and the incidence of febrile neutropenia or diarrhea in the combination treatment cohort. Therefore, inclusion of the *UGT1A1* genotype in multivariate analysis was not deemed contributory. To fully exclude an interference of the *UGT1A1* genotype, we carried out toxicity analyses for all patients in the combination therapy cohort responding to the *UGT1A1* homozygote wild-type genotype. In this subgroup of 57 patients, we found a preventive effect of the *MTHFR* 677TT genotype on the occurrence of severe diarrhea. None of eight patients with the *MTHFR* 677TT genotype developed grade 3 or 4 diarrhea, versus 11 out of 49 patients with the *MTHFR* 677CT or TT genotype (0 vs 22%, P = 0.009, data not shown). As no statistically significant effect was found in the monotherapy group, these results would suggest an effect of *MTHFR* 677C > T on irinotecan toxicity, for which there is no obvious pharmacologic explanation. Furthermore, introducing additional subgroup analysis should lead to the acceptance of an even stricter significance level, thereby making the outcome statistically non-significant.

Therefore, we conclude that this remarkable result was because of multiple testing, rather than a pharmacogenetic effect.

In a recent study involving neoadjuvant chemoradiation treatment for rectal carcinoma, *MTHFR* polymorphisms were predictive of grade 3–4 diarrhea and mucositis in patients receiving 5-FU monotherapy, but not in patients receiving 5-FU in combination with irinotecan.[20] However, because patients were prospectively assigned to either 5-FU monotherapy or 5-FU/irinotecan on the basis of the *TYMS* genotype, it cannot be excluded that the difference between the two groups was caused by the *TYMS* genotype, rather than the addition of irinotecan.

It has been proposed that the conflicting results of pharmacogenetic studies with 5-FU in mCRC are related to sex differences. Zhang et al.[39] reported a better OS for the *MTHFR* 1298AA genotype only for women in a heavily pre-treated cohort of mCRC patients. Another study found that *MTHFR* 1298AC heterozygote women had a shorter OS.[47] However, a sex-specific effect could not be confirmed by others.[21;25–27;48] Our data show a slightly, albeit non-significant, shorter PFS for male patients with the *MTHFR* 1298AA genotype. In our opinion, these conflicting data suggest that the difference between sexes may be the result of multiple testing in increasingly small groups.

Interestingly, epigenetic changes may act in concert with genetic variations. Cancer cell lines expressing the *MTHFR* 1298CC homozygous genotype show a higher number of methylated genes compared with heterozygotes or wild-type homozygotes.[49] The *MTHFR* 1298C allele was associated with a longer doubling time in cancer xenografts, with the shortest doubling time for 1298AA homozygotes, independent of 5-FU.[50]

Conversely, colorectal cancer cells and xenografts transfected with variant *MTHFR* 677T showed an accelerated growth rate compared with non-transfected cells, but were also inhibited more effectively by 5-FU plus leucovorin.[51] *MTHFR* polymorphisms may therefore be a prognostic, rather than a predictive marker. Indeed, Fernandez-Peralta et al.[29] found that the *MTHFR* 1298C variant allele was associated with shorter OS in sporadic colorectal cancer patients, even in the absence of 5-FU-containing chemotherapy.

Although we studied a larger group of patients on capecitabine monotherapy than any previous study, our sample size remains relatively small. The small number of homozygote variant individuals in our population limits the statistical power of this study to detect small effects of pharmacogenetics on clinical outcome. However, if *MTHFR* genotypes were strongly associated with toxicity or efficacy parameters, these effects would have been found even in a relatively small cohort. Other polymorphisms have been described for *MTHFR*. It cannot be excluded that a full haplotype analysis, including all known *MTHFR* polymorphisms, would show an effect on fluoropyrimidine toxicity. However, as *MTHFR* 677C > T and *MTHFR* 1298A > C are the polymorphisms showing functional importance, we believe that the chances of finding an effect on 5-FU or capecitabine toxicity are small. Therefore, we conclude that *MTHFR* 677C > T and 1298A > C polymorphisms are not related to the occurrence of severe toxicity (and efficacy) of capecitabine-based chemotherapy in mCRC.

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Clinical validation study of genetic markers for capecitabine efficacy in metastatic colorectal cancer patients

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Abstract

Background & aim: Pharmacogenetic studies continue to search for pretreatment predictors of chemotherapeutic efficacy and toxicity in metastatic colorectal cancer (mCRC). Both genome wide association (GWA) studies and candidate gene studies have yielded potential genetic markers for chemosensitivity. We conducted a clinical association study, validating the effect of specific genetic markers cited in recently published papers on the efficacy of the oral 5-FU pro-drug capecitabine.

Patients & methods: Germline DNA was collected for 268 mCRC patients from the CAIRO trial, a multicenter phase III trial, randomizing between combined or sequential first-line treatment with capecitabine, irinotecan and oxaliplatin. Genotyping was performed for eight single nucleotide polymorphisms (SNPs), using high resolution melting curves. Four SNPs are located in the *MTRR* gene, and another four SNPs showed significant association with 5-FU cytotoxicity in a recent *in vitro* GWA study. Primary endpoint was progression free survival (PFS); secondary endpoints were objective response and overall survival (OS).

Results: In patients receiving capecitabine monotherapy, rs4702484, located in *ADCY2* and close to *MTRR*, was associated with slightly reduced PFS for homozygous wildtype patients (CC 6.2 vs. CT 8.0 months, P = *0.018*). For the other selected genetic markers, we found no association with PFS, OS or radiologic response upon treatment with capecitabine, either in the total study population, or the capecitabine monotherapy subgroup.

Conclusion: With the exception of rs4702484, we found no evidence of an effect on capecitabine chemosensitivity of any of the studied SNPs. More specifically, variants in MTRR are not likely associated with capecitabine efficacy.

Background

Colorectal cancer is the third leading cause of cancer death worldwide.[1] Survival is strongly dependent on disease stage.[2] For patients presenting with distant irresectable metastases, systemic therapy is indicated with the objective of prolongation of survival and sometimes cure if downsizing permits secondary resection of metastases. Fluoropyrimidines, including the oral pro-drug capecitabine, remain the cornerstone of chemotherapeutic treatment, although treatment options have expanded and now include oxaliplatin and irinotecan, as well as the monoclonal vascular endothelial growth factor (VEGF) inhibitor bevacizumab and the endothelial growth factor receptor (EGFR) blockers panitimumab and cetuximab.[3] Despite the fact that systemic therapy significantly improves median survival, a substantial portion of patients do not benefit from this. Chemotherapy is sometimes accompanied by severe adverse events, which can delay or even abrogate further treatment. There is an urgent need to preemptively identify patients who will both tolerate and benefit from a specific chemotherapeutic schedule. Up until now, no germline molecular markers have been identified that may predict for the efficacy of chemotherapy.[4] Pharmacogenetics may provide such a tool, by identifying genetic predictors for both efficacy and toxicity.[5]

Up to now, most studies searching for pretreatment genetic markers in colorectal cancer have used a pathway-based approach. This has led to the identification of *UGT1A1**28 as a risk factor for increased toxicity (specifically neutropenia) after treatment with irinotecan[6], and *DPYD**2A as a risk factor for severe and sometimes lethal toxicity in response to fluoropyrimidine therapy.[7] However, this candidate gene approach is limited by our a priori knowledge of the genes involved in the pathway, and is therefore unable to identify novel markers in genes not previously associated with the drug under investigation. Recently, a genome wide association (GWA) study applying a hypothesis free approach was published, identifying single nucleotide polymorphisms (SNPs) with putative influence on cytotoxicity of capecitabine in human lymphoblastoid cell lines (LCL).[8] The most significant marker was located near the gene encoding for 5-methyltetrahydrofolate-homocysteine methyltransferase reductase (methionine synthase reductase, MTRR). As part of the methionine-folate pathway, MTRR is involved in fluoropyrimidine cytotoxicity. (Figure 1) Furthermore, variation in this gene has been implicated in colorectal carcinogenesis.[9]

The number of genes and polymorphisms that are being implicated as pretreatment biomarkers has expanded rapidly, necessitating validation of reported results.

In this study, we tested eight single nucleotide polymorphisms (SNPs), selected for their location within the *MTRR*-gene or their significance in the recent GWA paper by O'Donnell and co-workers[8], for their association with progression free survival (PFS) in a clinical trial population of 268 metastatic colorectal cancer (mCRC) patients who were treated with first-line capecitabine-based chemotherapy.

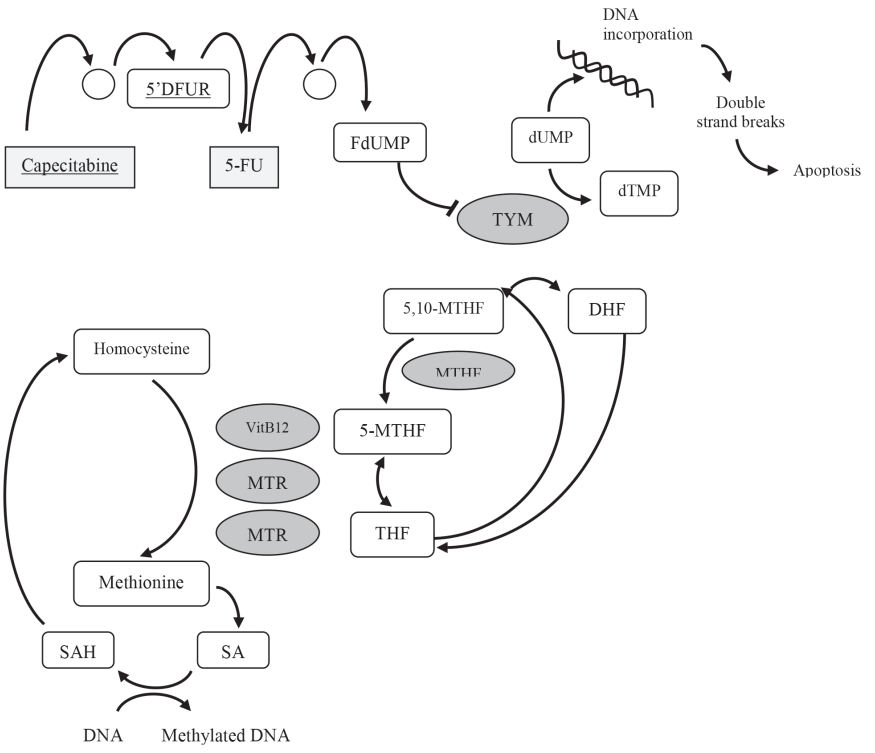

Figure 1. Schematic overview of pathways involved in cellular response of fluoropyrimidines

5-FU, 5-fluoro-uracil; DHF, dihydrofolate; dTMP, deoxythymidine monophosphate; dUMP, deoxyuridine monophosphate; FdUMP, fluoro-deoxyuridine monophosphate; MTHF, methylene tetrahydrofolate; MTHFR, methylene tetrahydrofolate reductase; MTR, methionine synthase; MTRR, methionine synthase reductase; SAH, S-adenosyl homocysteine; SAM S-adenosyl methionine; THF, tetrahydrofolate. Figure based on: M. Whirl-Carrillo, et al.[31]

Patients and methods

Clinical association study

Patients were recruited from the CAIRO trial, a multicenter open label randomized phase III clinical trial, comparing sequential versus combination chemotherapy with capecitabine, irinotecan, and oxaliplatin in a total of 803 mCRC patients.[10] A total of 268 patients were included in this pharmacogenetic study, of whom 127 received first-line capecitabine monotherapy, and 141 patients received first-line capecitabine plus irinotecan combination therapy. Patients with mCRC were enrolled in the CAIRO study between January 2003 and December 2004, by the Dutch Colorectal Cancer Group (DCCG) in 74 hospitals in The Netherlands. Inclusion criteria were a WHO performance score of 0-2 and adequate renal, hepatic and bone marrow function. A history of previous adjuvant chemotherapy was allowed, only if the last administration was

given six months prior to randomization. Main exclusion criteria were: serious concomitant disease preventing the safe administration of chemotherapy and other malignancies in the past five years. Capecitabine (1250 mg/m2, bid) was administered on day 1-14 in the monotherapy group, every three weeks. In the combination therapy group, capecitabine (1000 mg/m2, bid) was given on day 1-14, and irinotecan (250 mg/m2) on day 1, in a three weekly cycle. Tumor response was assessed by computed tomography (CT)-scan, every nine weeks, using Response Evaluation Criteria for Solid Tumors (RECIST, version 1.0).

The CAIRO study was approved both by the Central Committee on Research involving Human Subjects (CCMO) and by the local ethics committees of all participating centers. As sample collection for this pharmacogenetic substudy was initiated later than the CAIRO clinical trial and not all study centers participated, the number of patients included in the pharmacogenetic analyses is limited to 268 patients. All included patients gave written informed consent before inclusion for the main study and the pharmacogenetic side study.

SNP selection and genotyping

Four SNPs were selected from the results of a recently published *in vitro* GWA study[8] (rs4702484, rs8101143, rs576523 and rs11722476), based on their genome-wide significance levels in meta-analysis. A fifth SNP (rs361433) showed near genome-wide significance in this study. Unfortunately, no primers could be designed for this marker and it was therefore not included in our analyses. The GWA study suggested involvement of *MTRR* in capecitabine cytotoxicity. Although the *MTRR* gene has been suggested to be involved in colorectal carcinogenesis[11], current knowledge on the effect of *MTRR* polymorphisms on efficacy of fluoropyrimidine treatment in colorectal carcinoma is limited to one publication. In that study, no association of *MTRR* genotype with PFS was found.[12] To further investigate the predictive effect of *MTRR* polymorphisms in colorectal cancer treatment, four additional SNPs were selected based on their location in the this gene and citation in recent pharmacogenetic papers (rs1801394, rs10380, rs162036 and rs1532268). Variants in *MTHFR* (rs1801133 and rs1801131) and *TYMS* (rs34743033, rs11540151 and rs11280056) were also included as covariates, because an effect on capecitabine efficacy has been suggested for these polymorphisms.[11;13;14] *DPYD*2A* (IVS14+1G>A) was not included as a covariate in the model, because of the low estimated population frequency (minor allele frequency 0.003[16]). Furthermore, it was previously shown that individual SNPs in *DPYD,* including *DPYD*2A,* did not influence treatment efficacy in our patient group.[7]

Peripheral EDTA-blood samples were collected and stored at -20°C before DNA isolation. Germline DNA was extracted with the Magnapure LC (Roche Diagnostics, Almere, The Netherlands) according to manufacturer's instructions.

A short amplicon high resolution melting (HRM) assay was designed for each SNP and genotype allocations were confirmed by conventional Sanger sequencing. Genotype calls were made using the Call-IT 2.0 software. Oligonucleotide sequences and annealing temperatures are available on request. As quality control, all HRM assays were validated on a panel of DNA

from 18 healthy individuals. In addition, negative controls (water) were included in each run. Samples failing initial genotyping were repeated and in this run samples with confirmed genotypes were included as positive controls. By repeating HRM and sequencing samples, more than 5% of samples were genotyped in duplicate. *MTHFR* rs1801133 (677C>T) and *MTHFR* rs1801131 (1298 A>C) genotypes were determined using commercially available Taqman genotyping assays and analyzed on 7500 realtime PCR system (Lifetechnologies, Bleiswijk, The Netherlands) according to manufacturer's protocol. The VNTR polymorphism in the *TYMS* 5'-untranslated region (TSER, rs34743033), including the additional G/C SNP in the second base pair for 3-repeat individuals (rs11540151), was determined by direct sequencing. The *TYMS* 1494del6bp polymorphism in the 3'-untranslated region (rs11280056) was also determined using a pre-designed Taqman genotyping assay.

Data and statistical analysis

The primary endpoint of this study was progression free survival (PFS), which was calculated from the date of randomization until the first observation of disease progression or death from any cause. Secondary endpoints were overall survival (OS), objective response and clinical benefit. OS was calculated as the interval from randomization until death from any cause or until the date of last follow-up. Response to chemotherapy was assessed in all patients who completed at least 3 cycles of treatment. Objective response was determined as either complete or partial response. Clinical benefit was determined as stable disease, complete or partial response.

We chose not to include analyses for SNP effects on treatment toxicity. *In vitro* experiments, such as performed by O'Donnell and co-workers[8], are useful in examining cytotoxic effects of chemotherapeutic drugs, but do not take into account the multitude of patient-related factors that influence adverse events in clinical practice. We therefore believe that these *in vitro* results cannot be extrapolated to predict fluoropyrimidine-induced toxicity.

Differences in PFS and OS according to genotype were determined by Kaplan-Meier survival curves and log-rank testing. Multiple regression analysis was performed assessing the effect of genotype on PFS and OS by Cox regression analysis, treating gender, age, treatment arm and LDH at baseline as covariates. Variants in *MTHFR* (rs1801133 and rs1801131) and *TYMS* (rs34743033, rs11540151 and rs11280056) were also included as covariates, because these polymorphisms have been associated with efficacy of fluoropyrimidine therapy by others.[11;13;14] Although we previously showed that these SNPs did not affect treatment efficacy in our patient group[15], they were nonetheless included to minimize bias. Data are expressed as medians and 95 percent confidence intervals (95% CI). Additionally, the ten percent of all patients showing the longest PFS times and the ten percent of patients showing the shortest PFS times were selected and genotype distributions were compared between these groups by the Chi-squared test. The association of objective response and clinical benefit with genotypes was determined by cross tabulation and the Chi-squared test. All analyses were performed for the treatment population as a whole and for patients in the capecitabine monotherapy group separately. Conservative Bonferroni-correction for multiple testing would lead to the

adoption of a significance level of $\alpha = 0.05/8 = 0{\cdot}00625$, if all genetic markers are assumed to be unrelated. Earlier research has shown that there is a moderate amount of linkage disequilibrium between MTRR polymorphisms[11], and these SNPs are therefore not completely independent. We confirmed the presence of linkage disequilibrium between these polymorphisms in our population, using Haploview. (Figure 2A and B) A more lenient correction was therefore adopted, with a significance level of $\alpha = 0.05/5 = 0{\cdot}01$.

Genotype distributions were tested for agreement with those expected under Hardy-Weinberg equilibrium using the Chi-squared test, with a statistical cut-off value of $\chi^2 \geq 3.84$.

All statistical analyses were performed using SPSS software, version 20.0 (IBM Corp., Armonk, New York, USA).

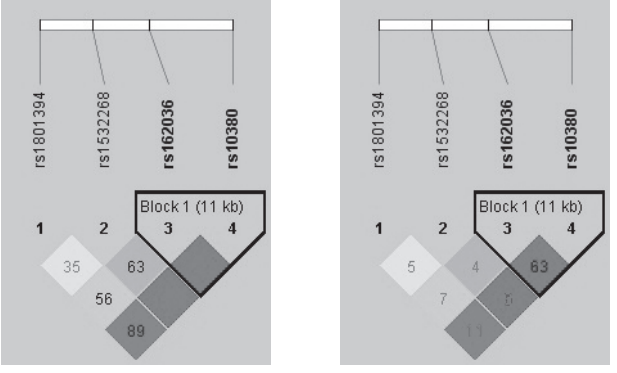

Figure 2. Linkage disequilibrium analyses for polymorphisms located in the gene encoding for 5-methyltetrahydrofolate-homocysteine methyltransferase reductase (MTRR)

A. Numbers in squares represent D' values between the respective SNPs. D' for rs10380 and rs162036, as well as for rs10380 and rs1532268 are 1. **B.** Numbers in squares represent hundredfold R-square values.

	Total N= 265	Capecitabine monotherapy N= 126	Combination therapy N= 139	P-value*
Age				*0.60*
Age at randomisation, median (range)	62 (27-81)	61 (27-78)	62 (37-81)	
Sex				*0.90*
Male	161 (61%)	76 (60%)	85 (61%)	
Female	104 (39%)	50 (40%)	54 (39%)	
Performance status				*0.57*
PS 0	153 (58%)	77 (61%)	76 (55%)	
PS 1	96 (36%)	42 (33%)	54 (39%)	
PS 2	16 (6%)	7 (6%)	9 (6%)	
LDH at randomisation				*0.89*
Normal	174 (66%)	82 (65%)	92 (66%)	
>Upper limit of normal	91 (34%)	44 (35%)	47 (34%)	
Previous adjuvant therapy				*0.70*
Yes	235 (89%)	113 (90%)	122 (88%)	
No	30 (11%)	13 (10%)	17 (12%)	

Table 1. Baseline characteristics

LDH, Lactate dehydrogenase
* Significance level for the difference in distribution between "Capecitabine monotherapy" and "Combination therapy".

Results

Clinical data

Baseline characteristics are shown in Table 1. Baseline characteristics were not significantly different between both treatment groups. The majority of patients (61%) were male and median age at randomization was 62 years (range 27-81 years). Baseline characteristics were evaluated for their relationship with SNP-genotypes, and no associations were found (data not shown).

Genotyping data

Genotyping was successful for all SNPs in 248 of 268 patients (93%). Three samples failed genotyping for three or more SNPs, and these were excluded from the statistical analysis. For individual SNPs, genotyping results ranged from 96% for rs1081394 to 100% for rs1532268.

Genotype frequencies are shown in Table 2. Genotype distributions were consistent with Hardy Weinberg equilibrium (HWE), except for rs11722476 ($\chi^2 = 4.38$) and rs4702484 ($\chi^2 = 4.86$). However, allele frequencies are consistent with those reported by others[16] and HWE would have been met in both cases with the addition of even one homozygous variant-type sample.

SNP	Chrom	Position	Gene	Mutation	Observed / expected MAF*	Genotype frequencies#
rs576523	1q23.3	159012700	*intergenic*	A>G	0.01 / 0.07	98.9 – 1.1 – 0
rs11722476	4q22.3	95389862	*SMARCAD1*	G>A	0.40 / 0.45	33.0 – 54.2 – 12.9
rs4702484	5p15.31	7702860	*ADCY2*	C>T	0.12 / 0.15	76.1 – 23.9 – 0
rs1801394	5p15.31	7923973	*MTRR*	G>A	0.42 / 0.38	34.8 – 46.8 – 18.4
rs1532268	5p15.31	7931179	*MTRR*	G>A	0.38 / 0.26	37.7 – 48.0 – 14.3
rs162036	5p15.31	7938959	*MTRR*	A>G	0.14 / 0.23	74.8 – 21.8 – 3.4
rs10380	5p15.31	7950191	*MTRR*	C>T	0.09 / 0.19	81.7 – 18.3 – 0
rs8101143	19p12	21747976	*intergenic*	A>G	0.29 / 0.18	51.5 – 38.6 – 9.9

Table 2. Genotype frequencies

ADCY2, adenylate cyclase type 2; MAF, minor allele frequency; MTRR, 5-methyltetrahydrofolate-homocysteine methyltransferase reductase; SMARCAD1, SWI/SNF-related, matrix-associated actin-dependent regulator of chromatin, subfamily A, containing DEAD/H box 1; SNP, single nucleotide polymorphism.

* Observed minor allele frequencies in our population. Expected minor allele frequencies, based on those reported in: Database of Single Nucleotide Polymorphisms (dbSNP). Bethesda (MD): National Center for Biotechnology Information, National Library of Medicine.(dbSNP Build ID: 36.3). http://www.ncbi.nlm.nih.gov/SNP/.

Genotype frequencies are displayed as percentages homozygous wildtype – heterozygous – homozygous variant type.

Association with capecitabine efficacy

Association with PFS

Updated PFS data were available for all but three patients. Updated OS data were available for 243 patients. In the remaining cases censored data were used for the analyses. Results for the association analyses are shown in Table 3.

Considering the total study population, we found no difference in PFS according to genotype for any of the SNPs. Also, when comparing genotypes between patients with the longest and those with the shortest PFS times, no significant differences in genotype distributions were found for any of the SNPs (P = *0.183* for rs1801394, to P = *1.000* for rs576523, data not shown).

For rs4702484, PFS for homozygous wildtype patients was 7.5 months (95% CI: 6.4-8.5 months), versus 7.8 months (95% CI: 5.9-9.6 months) for heterozygous patients (P = *0.351*), with no patients carrying the rs4702484 homozygous variant genotype. However, when patients in the capecitabine monotherapy group were considered separately, a borderline significant effect of rs4702484 genotype was seen. (Table 4 and Figure 3A) PFS for patients with the homozygous wildtype genotype was 6.2 months (95% CI: 5.6-6.7), versus 8.0 months (95% CI: 6.2-9.8) for heterozygous patients (P univariate = *0.018*). This result did not remain statistically significant in multivariate analysis (P multivariate = *0.029*).

Variant		Objective respons	P-value	Clinical benefit	P-value	PFS, median (95% CI)	HR (95% CI)	P-value univariate/ multivariate	OS median (95% CI)	HR (95% CI)	P-value univariate/ multivariate
rs576523	AA	34%		89%		7.6 (6.8-8.4)	*ref*		19.6 (17.5-21.8)	*ref*	
(intergenic)	AG	67%		67%		8.9 (0-19.6)	2.04 (0.66-3.46)		14.8 (0.0-34.1)	1.19 (0.00-2.63)	
	GG	NA	*0.275*	NA	*0.292*	NA	-	*0.206/0.327*	NA		*0.604/0.812*
rs11722476	GG	37%		89%		6.8 (5.4-8.3)	ref		17.7 (14.3-21.1)	*ref*	
(SMARCAD1)	GA	31%		90%		7.8 (7.0-8.6)	1.26 (0.82-1.70)		21.4 (18.3-24.5)	1.29 (0.83-1.74)	
	AA	44%	*0.317*	88%	*0.949*	6.7 (4.2-9.3)	1.16 (0.74-1.58)	*0.953/0.578*	20.0 (17.0-23.1)	1.29 (0.79-1.77)	*0.870/0.494*
rs4702484	CC	36%		89%		7.5 (6.4-8.5)	*ref*		19.5 (17.1-21.8)	*ref*	
(ADCY2)	CT	31%		90%		7.8 (5.9-9.6)	1.2 (0.89-1.55)		19.8 (14.0-25.5)	1.22 (0.87-1.57)	
	TT	NA	*0.539*	NA	*1.000*	NA	NA	*0.351/0.229*	NA		*0.759/0.271*
rs1801394	GG	36%		90%		8.1 (6.8-9.4)	ref		21.6 (17.6-25.5)	*ref*	
(MTRR)	GA	34%		91%		6.8 (5.9-7.8)	1.12 (0.71-1.53)		19.4 (17.2-21.7)	0.86 (0.42-1.30)	
	AA	35%	*0.953*	81%	*0.223*	8.3 (6.7-9.9)	0.96 (0.57-1.34)	*0.617/0.614*	21.0 (16.7-25.3)	0.90 (0.50-1.30)	*0.799/0.794*
rs1532268	GG	37%		84%		6.8 (5.5-8.1)	*ref*		19.8 (16.6-22.9)	*ref*	
(MTRR)	GA	31%		91%		8.0 (6.8-9.2)	0.85 (0.44-1.27)		18.9 (17.4-20.4)	1.17 (0.72-1.62)	
	AA	39%	*0.537*	97%	*0.054*	7.0 (4.7-9.2)	0.78 (0.38-1.19)	*0.134/0.493*	21.6 (16.4-26.8)	1.35 (0.92-1.78)	*0.961/0.352*
rs162036	AA	35%		91%		7.9 (7.1-8.7)	ref		19.5 (17.0-22.0)	ref	
(MTRR)	AG	32%		85%		6.5 (5.5-7.5)	1.21 (0.45-1.97)		18.7 (14.9-22.4)	1.20 (0.41-1.99)	
	GG	33%	*0.934*	67%	*0.039*	6.6 (6.3-7.0)	1.51 (0.71-2.32)	*0.498/0.380*	26.0 (0.0-66.5)	1.39 (0.55-2.23)	*0.653/0.619*
rs10380	CC	36%		90%		7.8 (7.0-8.5)	*ref*		19.5 (17.3-21.6)	*ref*	
(MTRR)	CT	28%		85%		6.2 (5.6-6.9)	0.85 (0.48-1.22)		21.0 (15.9-26.2)	0.88 (0.51-1,26)	
	TT	NA	*0.391*	NA	*0.300*	NA	NA	*0.339/0.402*	NA	NA	*0.837/0.532*
rs8101143	AA	33%		89%		7.0 (5.9-8.1)	*ref*		21.9 (18.2-25.6)	*ref*	
(intergenic)	AG	32%		88%		8.0 (6.7-9.3)	1.35 (0.87-1.84)		19.5 (18.2-20.7)	1.23 (0.69-1.77)	
	GG	50%	*0.242*	92%	*0.886*	8.3 (5.7-11.0)	1.18 (0.68-1.67)	*0.597/0.379*	21.2 (2.7-39.8)	1.46 (0.91-2.01)	*0.412/0.308*

Table 3. Association between genetic variants and efficacy of capecitabine in the total population

CI, confidence interval; HR, Hazard ratio; NA, no patients carried this genotype; PFS, progression free survival; OS, overall survival.

Variant		Objective response	P-value	Clinical benefit	P-value	PFS, median (95% CI)	HR (95% CI)	P-value univariate/ multivariate	OS. median (95% CI)	HR (95% CI)	P-value univariate/ multivariate
rs576523	AA	20%		85%		6.3 (5.8-6.8)	*ref*		19.5 (16.7-22.2)	*ref*	
(intergenic)	AG	100%		100%		8.9 (-)	2.98 (0.96-5.00)		14.8 (-)	2.19 (0.14-4.24)	
	GG	NA	0.044	NA	*1.000*	NA	NA	*0.083/0.290*	NA	NA	*0.201/0.454*
rs11722476	GG	23%		86%		6.1 (5.5-6.8)	*ref*		14.7 (7.8-21.5)	*ref*	
(SMARCAD1)	GA	18%		85%		6.6 (3.7-9.5)	1.13 (0.51-1.76)		19.4 (15.0-23.8)	0.91 (0.22-1.59)	
	AA	29%	0.578	86%	*0.991*	6.3 (5.7-6.9)	0.98 (0.39-1.56)	*0.889/0.812*	21.6 (17.6-25.6)	1.02 (0.40-1.65)	*0.881/0.893*
rs4702484	CC	20%		84%		6.2 (5.6-6.7)	*ref*		19.2 (15.1-23.2)	*ref*	
(ADCY2)	CT	24%		90%		8.0 (6.2-9.8)	1.75 (1.25-2.25)		22.1 (15.6-28.6)	1.56 (1.04-2.08)	
	TT	NA	0.795	NA	*0.559*	NA	NA	*0.018/0.029*	NA	NA	*0.457/0.096*
rs1801394	GG	22%		88%		6.6 (4.5-8.7)	*ref*		19.5 (11.9-27.0)	*ref*	
(MTRR)	GA	17%		85%		6.1 (5.2-7.1)	1.20 (0.58-1.82)		18.9 (15.6-22.1)	1.42 (0.78-2.07)	
	AA	33%	0.259	80%	*0.642*	8.3 (7.9-8.7)	1.00 (0.44-1.56)	*0.616/0.729*	22.1 (14.3-29.9)	1.14 (0.56-1.71)	*0.816/0.516*
rs1532268	GG	27%		82%		6.6 (4.7-8.5)	*ref*		21.4 (17.2-25.5)	*ref*	
(MTRR)	GA	18%		86%		6.1 (5.4-6.8)	0.91 (0.21-1.61)		18.1 (13.7-22.5)	1.26 (0.51-2.02)	
	AA	21%	0.515	93%	*0.612*	6.1 (4.8-7.4)	0.97 (0.32-1.62)	*0.915/0.948*	19.8 (4.9-35.7)	1.56 (0.86-2.27)	*0.808/0.396*
rs162036	AA	17%		86%		6.3 (5.4-7.1)	*ref*		18.1 (13.9-22.3)	ref	
(MTRR)	AG	27%		85%		6.2 (5.4-6.9)	1.97 (0.87-3.08)		24.1 (18.1-30.1)	1.46 (0.39-2.53)	
	GG	40%	0.253	80%	*0.943*	6.6 (6.4-6.9)	2.59 (1.44-3.73)	*0.440/0.227*	26.0 (0-55.8)	1.56 (0.43-2.68)	*0.543/0.744*
rs10380	CC	21%		85%		6.3 (5.7-7.0)	*ref*		18.9 (15.0-22.7)	*ref*	
(MTRR)	CT	23%		87%		6.2 (5.5-6.9)	0.77 (0.25-1.29)		24.1 (18.3-30.0)	0.90 (0.36-1.43)	
	TT	NA	0.805	NA	*1.000*	NA	NA	*0.876/0.330*	NA	NA	*0.720/0.681*
rs8101143	AA	20%		86%		6.1 (5.0-7.1)	*ref*		18.1 (10.5-25.6)	*ref*	
(intergenic)	AG	21%		81%		6.6 (4.5-8.7)	1.38 (0.61-2.15)		19.5 (16.7-22.2)	1.00 (0.15-1.86)	
	GG	33%	0.582	92%	*0.630S*	6.8 (3.5-10.2)	1.23 (0.47-2.00)	*0.560/0.690*	29.0 (15.2-42.8)	1.17 (0.33-2.02)	*0.649/0.799*

Table 4. Association between genetic variants and efficacy of capecitabine in patients treated with capecitabine monotherapy

CI, confidence interval; HR, Hazard Ratio; NA, no patients carried this genotype; PFS, progression free survival; OS, overall survival.

Association with OS

None of the genetic markers showed interaction with OS in our data set. OS for rs4702484 was 19.5 months for homozygote wildtype (95% CI: 17.1-21.8 months) and 19.8 months (95% CI: 14.0-25.5 months) for heterozygotes (P = *0.759*) in the total study population; and 19.2 (95% CI: 11.9-27.0) versus 22.1 months (95% CI: 15.6-28.6) for the capecitabine monotherapy group (P univariate = *0.457*, P multivariate = *0.096*; Figure 3B).

When the capecitabine monotherapy group was evaluated separately, results for PFS and OS remained similar.(Table 4)

Association with radiologic response

Regarding radiologic response to capecitabine, no association with genotype was found for any of the selected SNPs. No effect of genotype was present, whether objective response was used as the outcome measure, or clinical benefit.(Table 3)

A trend toward significant results was found for the association with clinical benefit of rs1533268 (GG vs. GA vs. AA: 84% vs. 91% vs. 97%; P = *0.054*) and of rs162036 (AA vs. AG vs. GG: 91% vs. 85% vs. 67%; P = *0.039*). When both treatment arms were evaluated separately, results remained statistically significant for patients receiving capecitabine-irinotecan combination treatment (rs1533268: GG vs. GA vs. AA: 85% vs. 97% vs. 100%; P = *0.023*; and rs162036: AA vs. AG vs. GG: 96% vs. 85% vs. 50%; P = *0.001,* data not shown), but not for patients receiving capecitabine monotherapy (rs1533268: GG vs. GA vs. AA: 82% vs. 86% vs. 93%; P = *0.612*; and rs162036: AA vs. AG vs. GG: 86% vs. 85% vs. 80%; P = *0.943*).

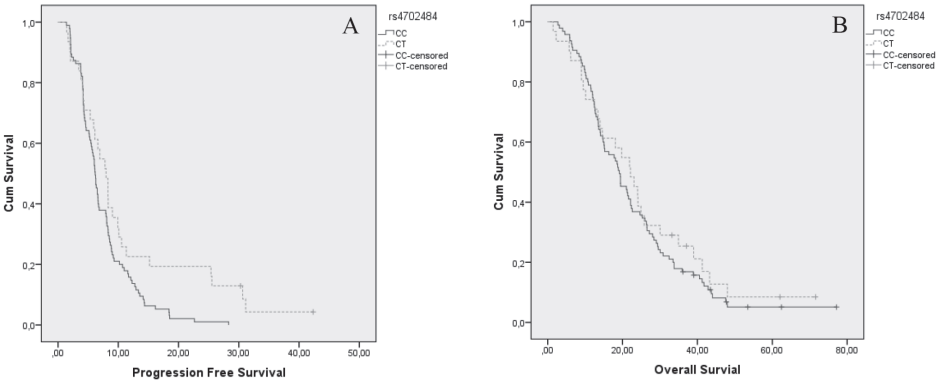

Figure 3. Kaplan-Meijer survival curves for patients receiving capecitabine first-line monotherapy, according to genotype for rs4702484

A. Progression free survival; **B.** Overall survival.

Discussion

We designed this clinical pharmacogenetic association study to validate if a specific selection of SNPs implicated in recent pharmacogenetic papers was associated with efficacy of capecitabine in a large cohort of mCRC patients, treated with first-line capecitabine-based chemotherapy. The genetic markers were carefully chosen based on reports from previous studies.[8;11] In our evaluation of eight selected SNPs, we found a small, borderline significant effect of rs4702484 on PFS in a subgroup of patients treated with capecitabine monotherapy. However, none of the other genetic variants showed significant association with capecitabine efficacy, neither in the total study population, nor in patients receiving capecitabine monotherapy.

Rs4702484, located *intronic* in the gene encoding for adenylate cyclase type 2 (*ADCY2*) and 200kbp upstream of *MTRR*, was first implicated in capecitabine chemosensitivity in a recent GWA report by O'Donnell and co-workers.[8] Consistent with their results, we found PFS for patients carrying the rs4702484 heterozygous genotype was marginally better than for patients carrying the homozygous wildtype genotype, but only if they were treated with capecitabine monotherapy. As this did not translate, however, into a statistically significant overall survival benefit, the clinical implications of these findings remain uncertain.

Despite the positive result for rs4702484, we could not confirm an effect on capecitabine chemosensitivity for the other SNPs from the GWA study in our patient population. Replication of GWA results is subject to statistical difficulty. Genome wide studies tend to overestimate the effect of the associated SNPs and these extreme results will be closer to the average when replicated in a second measurement.[17] To replicate these inflated results, large population sizes are necessary. Our patient sample is relatively small and lack of power may explain our inability to replicate the results found by O'Donnell et al.[8] For most SNPs, however, median values and confidence intervals are almost identical between genotype groups, without a trend towards an effect for the genetic markers. We therefore believe that increasing population size would not have led to substantially different results.

Furthermore, although the use of cell lines allows for analyses that would be unethical or infeasible in humans, it has certain disadvantages. Many pharmacokinetic influences, both genetic and non-genetic, are excluded. To partly circumvent this problem, the capecitabine metabolite 5'-deoxy-5-fluorouridine (5'DFUR) was used for the cited *in vitro* GWA study.[8] Although the impact of genetic variation in carboxylesterase (CES) and cytidine deaminase (CDD), both involved in the conversion of capecitabine to 5'DFUR *in vivo*, is still unclear[18;19], this may also partly explain the lack of replication in our patient group. In addition, tissue specific and tumor specific characteristics are eliminated when non-malignant cell lines, such as LCLs, are used.

We also studied four SNPs within *MTRR*. This gene has been implicated in the development of colorectal cancer[11] and is located in proximity to rs4720484. In our population of mCRC patients, the selection of four *MTRR* polymorphisms was not associated with capecitabine efficacy, which is consistent with results of another study showing no relation to efficacy of

adjuvant treatment with 5-FU in colorectal cancer patients.[20] Although a trend towards a significant effect was found for the association of rs1532268 and rs162036 with clinical benefit of capecitabine in combination therapy, this is probably due to statistical error associated with the small number of patients carrying the minor allele and with bias induced by multiple testing. Furthermore, since the effect is only present in patients treated with capecitabine-irinotecan combination therapy, this would imply an effect of this SNP on irinotecan, rather than capecitabine efficacy.

In designing this study, we hypothesized that *MTRR* could be important in capecitabine efficacy for two reasons. Firstly, MTRR as part of the folate pathway is involved in fluoropyrimidine pharmacodynamics (see also Figure 1). However, the relationship of fluoropyrimidine sensitivity to genetic variation in other components of this pathway, such as methylene tetrahydrofolate reductase (*MTHFR*), has not been confirmed[12;15;21], making an effect of *MTRR* genetic variation questionable. Secondly, polymorphisms in *MTRR* have been associated with colorectal carcinogenesis and as such may also influence disease prognosis. Whereas some authors described that cancer susceptibility genes show prognostic or predictive value in colorectal cancer patients[22;23], most studies found no correlation between these genes and survival of colorectal cancer patients, whether they were treated with chemotherapy[24] or not.[25;26] Furthermore, although two *MTRR* variants (*MTRR A66G*, rs1801394; and *MTRR C1793T*, rs10380) were associated with increased colorectal cancer risk in a case control study[11], these results were not replicated consistently in meta-analyses.[9;27] Based on these considerations, we believe our results should be seen as evidence that variation in *MTRR* is not essential in capecitabine chemosensitivity.

Genetic effects on drug metabolism have been recognized for decades.[28] Nevertheless, in today's practice, only few genetic markers have been integrated in algorithms for therapeutic control. The Pharmacogenetics Working Group of the Royal Dutch Pharmacists Association has provided pharmacogenetics-based therapeutic recommendations for 53 drugs, related to eleven genes.[29;30] Before implementation into clinical practice, extensive validation of genetic markers in varied patient groups is warranted. Validation studies like the present contribute to the conscientious transfer of basic research results into clinical practice. Although we believe the selected markers are not useful as pretreatment biomarkers of capecitabine efficacy in colorectal cancer, we cannot exclude an effect of some of these SNPs in other types of cancer and with other 5-FU derivatives. Further research therefore remains necessary.

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6

Effects of genetic variation in organic cation transporters (OCT) on oxaliplatin-induced neurotoxicity

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Abstract

Background: Although the addition of oxaliplatin to fluoropyrimidine treatment leads to increased progression free survival in metastatic colorectal cancer (mCRC), its use is hampered by the frequent occurrence of peripheral sensory neuropathy. Based on pre-clinical studies, it has been assumed that organic cation transports (OCTs) are involved in the neurotoxic effects of oxaliplatin. We hypothesized that polymorphisms in the genes encoding for OCT1 and OCT2, as well as for the human multidrug and compound extrusion protein 1 (hMATE1), influence the incidence and severity of oxaliplatin-induced neurotoxicity.

Methods: Patients with mCRC were recruited from the CAIRO2 study, a multicenter trial randomizing between first-line treatment with capecitabine-oxaliplatin-bevacizumab (CAPOX-B) versus CAPOX-B plus cetuximab. Patients were divided into three phenotype groups, dependent on the extent of neurotoxicity. A total of nine SNPs in *SLC22A1, SLC22A2* and *SLC47A1,* encoding for OCT1, OCT2 and hMATE1, respectively, were selected based on literature search. Genotyping was performed on germline DNA, derived from EDTA-blood samples collected at baseline.

Results: 419 patients who completed at least 4 cycles of oxaliplatin and had available clinical and genotyping data were included in the analyses. We found *SLC22A1* Arg61Cys univariately and multivariately associated with neurotoxicity phenotype, with a protective effect for homozygote variant genotype carriers. None of the other selected markers showed an association with neurotoxicity in our patient group.

Conclusion: *SLC22A1* Arg61Cys is a potential predictive marker for decreased risk of oxaliplatin-induced neurotoxicity in mCRC patients. This marker may select a group of patients capable of tolerating a higher dose of oxaliplatin, or with a lower risk of neurotoxicity at standard dose.

Introduction

First-line treatment for metastatic colorectal cancer (mCRC) often consists of a fluoropyrimidine and oxaliplatin, combined with bevacizumab. In the adjuvant setting, oxaliplatin was introduced for the combination with 5-FU, after publication of the MOSAIC-trial.[1] Oxaliplatin was shown to have synergistic effects when combined with 5-FU[2], and its addition to fluoropyrimidine-therapy in standard first-line treatment of mCRC has resulted in an improvement of progression free survival.[3] However, treatment is hampered by the development of peripheral sensory neuropathy, which can persist for months to years after the last dose of oxaliplatin or even throughout life.[4] This can have a substantial effect on quality of life with persistent handicaps, including pain and loss of sensory or motor nerve function. Phase I studies of oxaliplatin showed that neurotoxicity is the main dose-limiting side effect.[5] Many strategies have been applied in an attempt to reduce its incidence, including altered infusion schedules[6], administration of carbamazepine[7], and calcium/magnesium infusions.[8] However, chronic neurotoxicity remains an important cause of dose-modifications and treatment discontinuation in patients treated with oxaliplatin.

In current practice, it cannot be reliably predicted which patients will experience severe neuropathic side-effects. Clinical patient characteristics have been associated with increased neurotoxicity, such as anemia, hypoalbuminemia and alcohol consumption.[9] The interpatient variation may also be explained by differences in metabolism, cellular uptake and excretion of oxaliplatin, or by a different effect on neuronal cellular components. Genetic variants in genes encoding for oxaliplatin metabolizing enzymes (such as *GSTP1*[10;11] and *AGXT*[12]), for membrane efflux proteins (such as *ABCC2*[12]) and for voltage-gated sodium channels (such as *SCN4A* and *SCN10A*[13]) have been evaluated for their effect on oxaliplatin-induced neurotoxicity, with varying results.

Organic cation transporters type 1 and 2 (OCT 1 and OCT2, respectively) are involved in the cellular uptake of platinum-compounds. OCT1/OCT2 negative cells show impaired uptake of oxaliplatin, as well as increased cell survival upon incubation with oxaliplatin, compared to OCT transfected cells.[14] Pre-clinical studies showed that the accumulation of platinum is higher in dorsal root ganglia than most other tissues.[15;16] OCTs have been detected in dorsal root ganglia, and are therefore potential determinants of oxaliplatin-induced neurotoxicity.[17] In addition, the human multidrug and compound extrusion protein 1 (hMATE1) appears to play a functional role in cellular uptake of oxaliplatin.[18]

Genetic variation in the genes encoding for these cellular transporters may interfere with oxaliplatin uptake, and therefore influence its cytotoxic effects. Multiple single nucleotide polymorphisms (SNPs) have been described for each of these genes. In this clinical association study, we evaluated the effect of SNPs in the genes encoding for OCT1 (*SLC22A1*), OCT2 (*SLC22A2*) and hMATE1 (*SLC47A1*) on the incidence and severity of oxaliplatin-induced neurotoxicity in mCRC patients treated with oxaliplatin-containing chemotherapy.

Patients and methods

Patients for this clinical association study were recruited from the CAIRO2 trial[19], a multicenter phase III trial of the Dutch Colorectal Cancer Group (DCCG), which randomized between first-line treatment for mCRC with capecitabine-oxaliplatin-bevacizumab (CAPOX-B) versus CAPOX-B plus cetuximab. Patients were enrolled between June 2005 and December 2006 in 79 hospitals across the Netherlands. All patients received capecitabine 1000mg/m^2 b.i.d. orally on days 1-14 in a three weekly cycle. Oxaliplatin 130mg/m2 was administered intravenously on day 1 of each treatment cycle, with a maximum of 6 treatment cycles. Bevacizumab 7.5mg/m^2 was administered intravenously on day 1 of each cycle. For patients randomized to the CAPOX-B plus cetuximab group, cetuximab was administered intravenously at a loading dose of 400mg/m^2 on the first treatment day, followed by 250mg/m^2 once weekly thereafter. Treatment was continued until disease progression, unacceptable toxicity or death, whichever occurred first.

Patient eligibility criteria and guidelines for response assessment in the CAIRO2 trial have been described in detail elsewhere.[19] The trial protocol provided guidelines for dose-modifications in case of serious toxicity. In case of persistent paresthesia, temporary painful paresthesia or functional impairment, a 25% dose reduction of oxaliplatin was ordered. If painful paresthesia or functional impairment persisted for more than two weeks, oxaliplatin was omitted until recovery and was then initiated again at a reduced dose of 50% of the initial dose. If despite 50% dose reduction neurotoxicity recurred, patients went off-study. In case of dose reduction, dose delay, or discontinuation of treatment, the reason for the adjustment was noted in the patient file.

All included patients gave written informed consent before inclusion for the main study and the pharmacogenetic side study.

SNP selection and genotyping

A Pubmed literature search was performed to find relevant citations of SNPs in *SLC22A1*, *SLC22A2*, and *SLC47A1*. Keywords used were: (oxaliplatin, or platinum); ("organic cation transporter", or OCT; OCT1, or SLC22A1); (OCT2, or SLC22A2); (MATE1, or hMATE1, or "multidrug and toxin extrusion", or SLC47A1); (pharmacogenetics, or pharmacogenomics, or polymorphism, or SNP, or mutation).

SNPs with a minimum of three citations in Pubmed, of which at least one had to report on functional effects, were evaluated for selection. This criterion was adopted to limit the SNP selection to established markers. Only polymorphisms with a minor allele frequency (MAF) in Caucasians of 0.04 or higher were considered for inclusion. This led to the selection of four SNPs in *SLC22A1* (rs34059508, rs12208357, rs35167514, rs628031), two SNPs in *SLC22A2* (rs316019, rs145405955), and one SNP in *SLC47A1* (rs2289669).

In addition, three SNPs in *SLC47A1* (rs77630697, rs76645859, rs35395280) were selected. Although these SNPs did not fulfill the pre-set criteria, they were nonetheless included, because

they were specifically reported to influence cellular oxaliplatin uptake in a recent report.[18]

Peripheral EDTA-blood samples were collected at baseline and germline DNA was extracted with the Magnapure LC (Roche Diagnostics, Almere, The Netherlands) according to manufacturer's instructions. An Open Array technique (Lifetechnologies) was applied for genotyping of all SNPs, using predesigned assays for rs34059508 (*SLC22A1* Gly465Arg), rs12208357 (*SLC22A1* Arg61Cys), rs628031 (*SLC22A1* Met408Val), rs316019 (*SLC22A2* Ala270Ser), rs2289669 (*SLC47A1intronic*), and rs3595280 (*SLC47A1* Cys497Phe). Custom assays were designed for the genotyping of rs145450955 (*SLC22A2* Thr201Met), rs76645859 (*SLC47A1* Val480Met), rs77630697 (*SLC47A1* Gly64Asp). Unfortunately, no assay could be designed for rs35167514 (*SLC22A1* Met420del) for this technique, and this marker was therefore not included in the analyses. Negative controls (water), as well as positive controls with known genotypes were included in all runs. For further quality control, a proportion of the samples was analysed in duplicate. Because of technical difficulties, more samples were re-analysed than initially planned. In total, 280 analyses were performed in duplicate, with no inconsistencies. Samples in which five or more SNPs failed to genotype were excluded from the analyses.

Statistical analyses

Toxicity was assessed at each visit by taking the patients history, physical examination, and hematology and biochemical laboratory tests. Toxic effects were classified following the US National Cancer Institute Common Toxicity Criteria (CTC), version 2.0. Neurotoxicity was scored based on patient report only.

Symptoms of neurotoxicity were only included if developed during the first 6 courses of chemotherapy (i.e. during administration of oxaliplatin). For statistical reasons, the extent of neuropathic symptoms was categorized in three distinct phenotype groups: no neurotoxicity (patients who did not experience peripheral sensory neuropathy during any of the first 6 treatment cycles), severe neurotoxicity (patients who either experienced grade 3 neuropathy, or who had oxaliplatin dose reduction or treatment discontinuation because of neuropathy) and intermediate phenotype (all other patients). The development of oxaliplatin-induced neurotoxicity is thought to be dependent on cumulative dosage.[20] Therefore, patients with early treatment discontinuation (i.e. during the first three cycles), whether due to adverse events, disease progression or other factors, were excluded from the analyses.

Association of genotype with neurotoxicity phenotype group was then assessed by crosstabulation and the Chi-squared test. An additive effect of variant alleles was assumed. A significance threshold of $P<0.05$ was adopted for this exploratory study. Multivariate linear regression analysis was performed for the association of genotype with neurotoxicity phenotype, including cumulative dose of oxaliplatin per square meter, treatment arm and age at inclusion as covariates.

Genotype distributions were tested for agreement with those expected under Hardy-Weinberg equilibrium (HWE) using the Chi-squared test, with a threshold of $P<0.05$. Because

the selection of patients who completed a minimum of four cycles of oxaliplatin may have inadvertently led to the selection of patients with a specific genotype, adherence to HWE was calculated in the total population of patients for whom genotyping was successful for at least half of the SNPs.

All analyses were performed using SPSS version 20 software (IBM Corp., Armonk, New York, USA).

Results

Patient characteristics

In the CAIRO2 study, in total 755 patients were randomized between first-line treatment with CAPOX-B or CAPOX-B with cetuximab. Four hundred and nineteen patients who completed a minimum of four cycles of oxaliplatin and had successful genotyping for at least five of the selected SNPs were included in the analyses.(Figure 1)

Baseline characteristics for the included patients are shown in Table 1. Distributions across baseline characteristics were similar for the patients included in the pharmacogenetics analyses, compared to the total CAIRO2 population.[19]

	PGx analyses population N = 419	**CAIRO2 population[19]**
Age in years median (range)	61.3 (27.6-83.6)	62 (27-84)
Gender		
Male	249 (59.4%)	59.5%
Female	170 (40.6%)	40.5%
Treatment arm		
CAPOX-B	214 (51.1%)	50.0%
CAPOX-B plus C	205 (48.9%)	50.0%
LDH at baseline		
Normal	234 (55.8%)	56.7%
Above ULN	185 (44.2%)	43.3%
PFS		
0	263 (62.8%)	62.5%
1	150 (35.8)	37.5%
unknown	6 (1.4%)	
Prior chemotherapy		
No	361 (86.2%)	84.9%
Yes	58 (13.8%)	15.1%

Table 1. Baseline characteristics

CAPOX-B, capecitabine, oxaliplatin and bevacizumab; CAPOX-B plus C, capecitabine, oxaliplatin and bevacizumab plus cetuximab; LDH lactate dehydrogenase; PFS, performance score; PGx, pharmacogenetics; ULN, upper limit of normal.

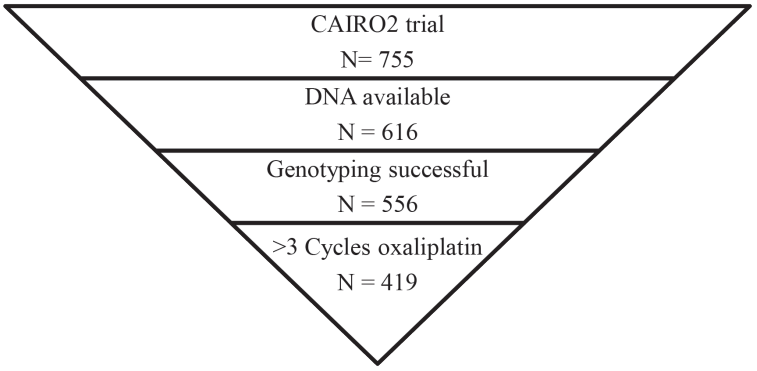

Figure 1. Schematic presentation of patient numbers included in pharmacogenetic analyses

Association of clinical parameters with neurotoxicity

Patients included in the pharmacogenetic analyses were separated into three phenotype groups, as described in the methods section. We found 47 (11.2%) patients experienced no neurotoxicity during the first six courses of chemotherapy, 66 (15.8%) patients had severe neurotoxicity, and the remaining 306 (73.0%) patients had an intermediate phenotype.

Cumulative doses of oxaliplatin, expressed as milligrams per square meter of body surface area (BSA) were calculated for all included patients and rounded to the nearest ten. Mean cumulative doses of oxaliplatin were significantly lower for patients with severe neurotoxicity (688 mg/m^2, 95% confidence interval [95% CI] 644-691mg/m^2), than for patients without neurotoxicity (727 mg/m^2, 95% CI 701-754mg/m^2), and for those with the intermediate phenotype (739 mg/m^2, 95% CI 730-749mg/m^2) (Kruskal-Wallis test, P = *0.000*). Consistent with these results, a significantly higher number of patients with the severe neurotoxicity phenotype had a dose reduction of oxaliplatin in any cycle (50/66, 92%), compared to patients with either no neurotoxicity (6/47, 13%) or the intermediate phenotype (54/306, 18%) (Chi-square test, P = *0.000*). However, there were no differences between the phenotype groups in the administered number of cycles of oxaliplatin per patient, or in delays in oxaliplatin-administration (data not shown).

Patients with severe neurotoxicity had a slightly higher mean age (63.8 years, 95% CI 61.5-66.1 years), than patients without neurotoxicity (60.7 years, 95% CI 57.9-63.4 years) or those with the intermediate phenotype (61.0 years, 95% CI 60.0-62.1 years), but the difference was non-significant (one way ANOVA, P = *0.088*). The extent of neurotoxicity was not associated with patient gender (P = *0.255*), LDH at baseline (P = *0.322*), WHO performance score (P = *0.709*) or prior adjuvant chemotherapy (P = *0.724*).

There was an overrepresentation of patients treated with CAPOX-B plus cetuximab in the "no neurotoxicity" phenotype group. In other words, patients who were treated with CAPOX-B alone had a slightly higher chance of at least intermediate phenotype neurotoxicity, compared to patients who were treated with CAPOX-B plus cetuximab (P = *0.052*).

Genotyping results

Genotyping was successful for all nine polymorphisms in 274 of the 419 included patients (65.4%). Call rates for individual polymorphisms varied from 77.1% (323/419 patients) for *SLC47A1* Cys497Phe to 99.3% (416/419 patients) for *SLC47A1* Gly46Asp.

Genotype distributions followed Hardy-Weinberg equilibrium for all selected SNPs, accept for *SLC22A1* Arg61Cys ($\chi^2 = 9.77$) and *SLC22A1* Met408Val ($\chi^2 = 6.08$), but allele frequencies are consistent with those reported in literature.[21] Four of the selected SNPs were monoallelic in our population (*SLC22A2* Thr201Met, *SLC47A1* Val480Met, *SLC47A1* Gly64Asp, and *SLC47A1* Cys497Phe). Genotype distributions for all patients in whom genotyping was successful and for those included in the pharmacogenetic analyses are described in Table 2.

Association of genotype with neurotoxicity

Polymorphic SNPs (*SLC22A1* Gly465Arg, *SLC22A1* Arg61Cys, *SLC22A1* Met408Val, *SLC22A2* Ala270Ser, and *SLC47A1* non-coding) were univariately assessed for their association with neurotoxicity phenotype. Results for these analyses are shown in Table 3.

SLC22A1 Arg61Cys (rs12208357) showed a statistically significant association with neurotoxicity phenotype. None of the patients carrying the homozygote variant genotype experienced severe neurotoxicity, compared to 8.5% of heterozygote patients and 17.8% of patients carrying the homozygote wildtype genotype (P = *0.011*).

The association of genotype with neurotoxicity phenotype was then explored in multivariate analysis, treating age, treatment arm and cumulative oxaliplatin dose per BSA as covariates. The association of *SLC22A1* Arg61Cys genotype with the extent of neurotoxicity was preserved in multivariate analysis (P multivariate = *0.015*). This effect was also seen when patients who discontinued oxaliplatin treatment during the first 3 courses were included in the analysis (P = *0.045,* data not shown). None of the other polymorphisms were associated with neurotoxicity phenotype in univariate or multivariate analysis.

	SNP number	Variation	Call rate	Total population*	PGx analyses population*
				N = 556	N = 419
SLC22A1 Gly465Arg	rs34059508	1393G>A	94.5%	0.97-0.03-0.00	0.96-0.04-0.00
SLC22A1 Arg61Cys	rs12208357	181C>T	85.2%	0.85-0.13-0.02	0.85-0.13-0.02
SLC22A1 Met408Val	rs628031	1222G>A	85.4%	0.35-0.44-0.21	0.35-0.43-0.22
SLC22A2 Ala270Ser	rs316019	808C>A	87.8%	0.80-0.18-0.02	0.80-0.18-0.02
SLC22A2 Thr201Met	rs145450955	602C>T	98.3%	1.00-0.00-0.00	1.00-0.00-0.00
SLC47A1 non-coding	rs2289669	G>A	96.4%	0.33-0.51-0.16	0.34-0.50-0.16
SLC47A1 Gly64Asp	rs77630697	191G>A	99.3%	1.00-0.00-0.00	1.00-0.00-0.00
SLC47A1 Val480Met	rs76645859	1438G>A	99.0%	1.00-0.00-0.00	1.00-0.00-0.00
SLC47A1 Cys497Phe	rs35395280	1490G>C	77.1%	1.00-0.00-0.00	1.00-0.00-0.00

Table 2. Genotype distributions

SNP, Single nucleotide polymorphism; PGx, pharmacogenetics.
* Genotype distributions are shown as: homozygote wildtype – heterozygote – homozygote variant type.

		Neurotoxicity phenotype				
		No neurotoxicity	Intermediate phenotype	Severe neurotoxicity	P-value univariate	P-value multivariate
SLC22A1 Gly465Arg	GG	11.0%	73.0%	16.0%	*0.365*	*0.627*
	GA	21.4%	57.1%	21.4%		
SLC22A1 Arg61Cys	CC	9.5%	72.8%	17.8%		
	CT	12.8%	78.7%	8.5%	*0.011*	*0.015*
	TT	50.0%	50.0%	0.0%		
SLC22A1 Met408Val	GG	9.7%	77.4%	12.9%		
	GA	9.0%	75.0%	16.0%	*0.728*	*0.331*
	AA	11.5%	69.2%	19.2%		
SLC22A2 Ala270Ser	CC	12.2%	72.1%	15.6%		
	CA	3.0%	80.6%	16.4%	*0.280*	*0.321*
	AA	14.3%	71.4%	14.3%		
SLC47A1 intron	GG	12.6%	70.4%	17.0%		
	GA	10.3%	73.4%	16.3%	*0.843*	*0.851*
	AA	10.6%	77.3%	12.1%		

Table 3. Univariate analyses for the association of genotype with extent of oxaliplatin-induced neurotoxicity

Discussion

Organic cation transporters are proposed determinants of oxaliplatin-induced neurotoxicity. We investigated the effect of germline genetic variation in genes encoding for OCT1 (*SLC22A1*), OCT2 (*SLC22A2*), and hMATE1 (*SLC47A1*) on the incidence and severity of neurotoxicity in 419 mCRC patients treated with oxaliplatin-containing chemotherapy. We found that *SLC22A1* Arg61Cys was significantly associated with the extent of oxaliplatin-induced peripheral sensory neuropathy, with a protective effect for carriers of the homozygote variant genotype. None of the other selected polymorphisms showed an association with neurotoxicity in our study population.

To our knowledge, this is the first study to address the effect of polymorphisms in these genes on oxaliplatin-induced neurotoxicity. Pharmacogenetic research so far has led to the identification of other potential predictors for neurotoxicity. Polymorphisms in the gene encoding for the metabolic enzyme alanine glyoxylate transferase (*AGXT)*[12], as well as variation in *SCN4A* and *SCN10A*, encoding for voltage gated sodium channels[13], have been associated with the incidence of oxaliplatin-induced neuropathy. Earlier, germline genetic variation in the detoxifying enzyme glutathione S-transferase π1 (*GSTP1*), was identified as a possible predictor of neurotoxicity caused by oxaliplatin[22], but this result could not be replicated by our own studygroup.[10] Additionally, a genome wide association study in Korean colorectal cancer patients identified five SNPs that were not previously associated with oxaliplatin-induced neurotoxicity.[23] However, all of these results have not been sufficiently validated, and cannot be incorporated into clinical practice at present.

Based on the assumptions that peripheral neuropathy is an important complication of oxaliplatin-treatment[5;20], that platinum-uptake is increased in dorsal root ganglia[15;16], and that OCT2 is present as a transporter in dorsal root ganglia[17], we proposed that the influence of OCT-function on oxaliplatin-induced neurotoxicity should be further evaluated. Indeed, OCT1 and -2, as well as hMATE1, are involved in transportation of oxaliplatin into cells expressing these transporters[24], and OCT-transfected cells are over 20 times more sensitive to oxaliplatin than empty-vector cells.[14] Likewise, cells completely lacking hMATE1 are more chemoresistent to oxaliplatin than hMATE1 positive cells.[18] Noteworthy, colorectal cancer cells express a high level of organic cation transporter type 3 (OCT3)[25], but not OCT2[17], which may explain the lack of correlation between anti-cancer effect and the development of neuropathy on oxaliplatin-based chemotherapy.

In vivo experiments showed that, after a single administration of oxaliplatin, OCT1/2 positive mice experienced increased sensitivity to cold and mechanical stimulation, compared to OCT1/2 knock-out mice[17]. Concurrent administration of cimetidine, a known competitive inhibitor of OCT1 and -2[14], resulted in complete protection from cold-sensitivity in OCT1/2 positive mice.[17] These preclinical results confirm the relationship between OCT1/2 function and oxaliplatin-induced neuropathy.

A multitude of germline genetic polymorphisms have been described for OCT1, OCT2 and hMATE1, and selected variants have shown functional effects on compound transport.[26-29] We hypothesized that functional polymorphisms in *SLC22A1*, *SLC22A2* and *SLC47A1* are associated with the incidence and severity of oxaliplatin-induced neurotoxicity. Indeed, *SLC22A1* Arg61Cys is a missense variant located in exon 1, on the first large loop of the protein, and the residue change results in reduced mRNA expression and loss of OCT protein function.[21;30-36] Our results, showing a protective effect of the Cys/Cys homozygote variant genotype on neuropathic complaints after oxaliplatin-administration, are in line with the aforementioned data. This SNP may therefore be a predictive marker for a decreased risk of oxaliplatin-induced neurotoxicity. Since neurotoxicity is the main dose-limiting event in oxaliplatin-treatment, patients carrying the protective genotype may actually be able to endure a higher dose of oxaliplatin than determined in phase I trials.

Despite this promising result, our study is subject to several difficulties, both in clinical and technical aspects. Oxaliplatin-induced neurotoxicity appears in two distinct forms: acute and chronic neuropathy. Acute neuropathy occurs in the vast majority of patients, during hours or days after infusion of oxaliplatin.[12] It is characterized by dysesthesias and paresthesias in the oropharyngeal region and extremities, which are induced or aggravated by cold.[37] These sensory symptoms are presumably caused by neuromyotonic discharges, consistent with peripheral nerve hyperexcitability.[7] In contrast, chronic neurotoxicity develops in the course of treatment. It was originally thought to occur in less than a quarter of patients[12], and only after a cumulative dosage of 500mg/m2 oxaliplatin.[20] However, newer studies have shown that almost 85 percent of patients treated with oxaliplatin experience some degree of chronic neurotoxicity.[38] This phenomenon is caused by progressive loss of sensory fibers, thereby

inducing sensory axonal neuropathy.[39] There appears to be a correlation between the amount of acute neuropathic symptoms and the severity of chronic oxaliplatin-induced neuropathy.[40] Different scoring systems are used for assessing the intensity of oxaliplatin-induced neuropathy. Most studies use the National Cancer Institute-Common Toxicity Criteria (NCI-CTC). An oxaliplatin-specific scale has been described by Levi et al[41], which takes into account the duration of symptoms, as well as the intensity. In our patient population, no distinction was made between acute and chronic neuropathy.

As described above, it is suggested that there is a difference in the etiology of chronic versus acute oxaliplatin-induced neurotoxicity.[7;39] Pre-clinical studies so far have only shown an effect of OCT2 on acute neurotoxicity in mice.[17] No data are available for OCT effects on chronic oxaliplatin-induced neurotoxicity. Our results may therefore have been clouded by the inclusion of patients in whom neurotoxic symptoms, whether acute or chronic, were mediated by systems other than OCT.

Another clinical pitfall is our lack of complete information on other determinants of neurotoxicity, such as alcohol consumption or co-medication. For instance, uptake efficacy of hMATE1 is influenced by many other drugs, such as omeprazole and antibiotics.[42] Interestingly, its transport capacity may also be inhibited by irinotecan.[43] If any of the co-administered medications impair oxaliplatin uptake by OCT1, OCT2 or hMATE1, they may have lowered the incidence of neurotoxicity in patients taking these drugs independent of the investigated genotypes, thereby obscuring our results.

Technical difficulties may also weaken the validity of our results. Genotyping failed for five or more SNPs in 60 patients, almost 10% of our population. Even after elimination of these samples, genotyping was successful for all markers in less than half of the patients. The exclusion of samples based on missing genotypes may have induced bias in our analyses. We also excluded patients who completed only 1 to 3 cycles of oxaliplatin. However, baseline-characteristics in the selected population are similar to those for the total CAIRO2 population (Table 1), and allele frequencies are comparable between the selected population and all genotyped patients.(Table 2) We therefore believe this clinical selection did not confound the analyses.

We found that four out of nine SNPs were monoallelic in our population. Frequency data on these SNPs in all ethnicities are scarce and minor allele frequencies may differ between populations.[21] In addition, *SLC22A1* Arg61Cys is not in strong linkage disequilibrium with the other selected SNPs in Caucasians, but a haplotype block including this SNP and *SLC22A1* Met408Val was identified in Asian subjects.[26;44] It is therefore not certain that results for *SLC22A1* Arg61Cys can be extrapolated to other populations, or that this is the best predicting SNP in the chromosomal region.

Although the evidence supporting our hypothesis seems solid, there are some inconsistencies. Whereas one study found both OCT1 and OCT2 are involved in cellular uptake and cytotoxicity of oxaliplatin[14], others found that OCT2, but not OCT1 was essential for oxaliplatin transport into the cell.[17;24;45] Furthermore, contrasting results have been published

for the effect of the selected polymorphisms on OCT function. Whereas some studies report on the cellular uptake of different substances[46-48], others have used more elaborate endpoints, such as drug efficacy or renal clearance.[49-53] This may in part explain the contradicting results.

In conclusion, in our population of mCRC patients treated with CAPOX-B either with or without cetuximab, a SNP in the gene encoding for OCT1 (*SLC22A1* Arg61Cys) associated with the absence of severe oxaliplatin-induced neurotoxicity. This result needs to be validated in independent patient cohorts. In these validation studies, neurotoxicity should be assessed by an oxaliplatin-specific scale, or physical diagnostic tests, to better grade the extent of sensory neuropathy and to distinguish acute from chronic neurotoxicity. Because of the potential interaction with other drugs, treatment regimen should ideally be uniform across all patients and co-medication meticulously monitored.

Because peripheral sensory neuropathy is the main dose-limiting toxicity in oxaliplatin-based chemotherapy, this SNP may select a group of patients capable of tolerating a higher than average dose of oxaliplatin, perhaps leading to an increment in treatment efficacy.

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7

Excision Repair Cross-Complementation group 1 (ERCC1) C118T SNP does not affect cellular response to oxaliplatin

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Abstract

Aims: ERCC1 is involved in the repair of oxaliplatin-induced DNA damage. Studies for the association of the C118T SNP with clinical response to treatment with platinum drugs have rendered inconsistent results. We investigated the *ERCC1* C118T SNP with respect to overall and progression-free survival in patients with advanced colorectal cancer (ACC) treated with oxaliplatin and in vitro DNA repair capacity after oxaliplatin exposure. In addition we discuss discrepancies from other studies concerning *ERCC1* C118T.

Materials & methods: Progression-free survival was determined in 145 ACC patients treated with oxaliplatin-based chemotherapy in a phase 3 trial. For the in vitro studies regarding *ERCC1* functionality, we transfected an *ERCC1* negative cell line with 118C or 118T *ERCC1*. Cellular sensitivity and DNA repair capacity after exposure to oxaliplatin was examined by Sulphorodamine B growth inhibition assay, COMET assay and Rad51 foci staining.

Results: We found no association between *ERCC1* C118T and progression-free or overall survival. In addition, transfection of either 118C or 118T restores DNA-repair capacity of UV20 cells to the same level and chemosensitivity to oxaliplatin was similar in *ERCC1* 118C and 118T transfected cells.

Conclusion: This study shows that the *ERCC1* C118T variants are not associated with survival in ACC patients treated with oxaliplatin or the in vitro sensitivity and DNA-repair capacity in 118C and 118T transfected cell lines. Therefore, *ERCC1* C118T genotyping seems of no value in individualizing oxaliplatin based chemotherapy in ACC.

Introduction

The third-generation platinum analogue oxaliplatin is a widely used chemotherapeutic agent, especially in the treatment of colorectal cancer. The antitumor effect of oxaliplatin results from intercalation of diaminocyclohexane (DACH)-platinum (Pt) in the DNA helix, causing Pt-DNA cross-links, and ultimately leading to programmed cell death. However, several cellular DNA repair mechanisms are capable of repairing damage from Pt-DNA adducts, such as the Nucleotide Excision Repair (NER) system.[1] Within the NER system, ERCC1 is involved in the excision of DNA adducts, which are then replaced by a new piece of DNA strand that is synthesized in situ. As a result, NER and ERCC1 function may influence cellular sensitivity towards platinum analogues.

Absolute ERCC1 defects in humans are rare[2] and were found lethal in mice.[3] Most information about ERCC1 protein function is therefore based on observations in cell lines with NER defects, such as the UV-light sensitive CHO mutant UV20 cell line.[4;5] Removal of cisplatin-induced Pt-adducts in ERCC1 deficient UV20 CHO cells appeared to be low compared to its wild type counterpart AA8 cell line[6], stressing the importance of the NER system in cellular platinum sensitivity. The overall rate and efficiency of the NER process was found comparable for cisplatin and oxaliplatin[7], and lack of ERCC1 function is thought to influence oxaliplatin sensitivity as well.[8]

A common single nucleotide polymorphism (SNP) in the *ERCC1* gene (C118T, rs11615) changes the common AAC codon into the infrequently expressed AAT, both coding for asparagine (Asn).[9] The consequences of this synonymous substitution are not fully understood, but there is evidence that the 118T allele may be associated with lower *ERCC1* expression caused by a difference in translational efficiency for this codon.[10;11] Differences in translation kinetics may also give rise to conformational changes of the ERCC1 protein, thereby causing a change in function.[12] Consequently, it has been hypothesized that this SNP influences clinical response to oxaliplatin chemotherapy.

Although many authors have studied the association between *ERCC1* C118T genotype and treatment outcome of oxaliplatin-based chemotherapy in colorectal cancer, only one previous study tried to determine if this SNP is causally involved in oxaliplatin resistance.[8] It was shown that the polymorphism does not alter cellular sensitivity to platinum treatment, but no attempt was made to clarify its influence on DNA repair capacity. Because of the contradictory results of clinical association studies for this SNP, we investigated the potential association of *ERCC1* C118T with overall and progression free survival in advanced colorectal cancer (ACC) patients treated with oxaliplatin based chemotherapy in the phase 3 CAIRO trial of the Dutch Colorectal Cancer Group (DCCG).[13] In addition, we performed functional in vitro tests using *ERCC1* transfected cells, to study the involvement of these genetic variants not only in oxaliplatin sensitivity, but also on cellular DNA repair mechanisms.

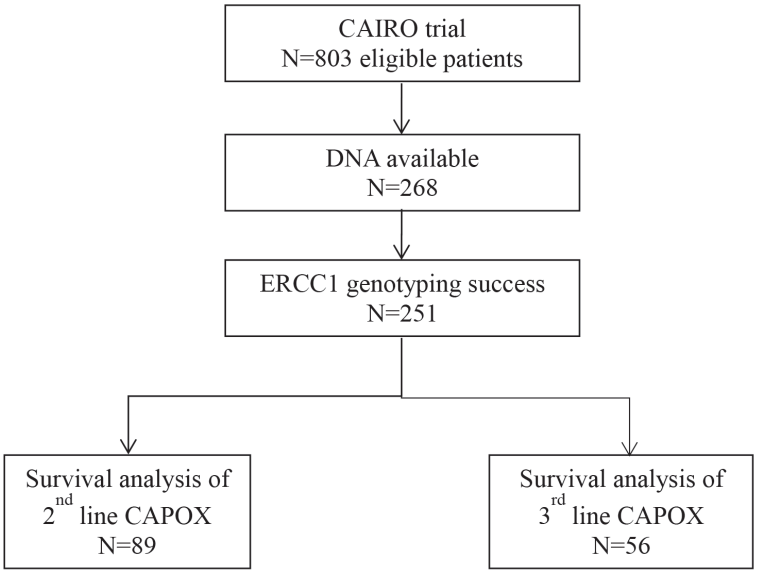


Figure 1. CAIRO study flowchart
CAPOX, capecitabine-oxaliplatin.

Patients and methods

Patients

Patient DNA for the clinical association study was isolated from venous EDTA blood, collected as part of the CAIRO study (previously described in detail).[13;14] The study flowchart and number of patients available for analysis are shown in Figure 1. As this pharmacogenetic substudy was initiated later than the CAIRO clinical trial and not all study centers participated, the number of patients included in the pharmacogenetic analyses is limited to a total of 268 patients. Baseline characteristics and stratification parameters were not different between our subset of patients and the total CAIRO population (data not shown). Tumor response to treatment was assessed every 9 weeks by computed tomography (CT) scanning using Response Evaluation Criteria In Solid Tumors (RECIST 1.0).[15] All included patients gave written informed consent before inclusion for the main study and the pharmacogenetic side study.

Genotyping ERCC1 C118T

DNA was isolated from whole blood with the total MagnaPure Total Nucleic Acid Isolation Kit I on the MagnaPure LC (Roche Diagnostics, Mannheim, Germany). Chromosomal DNA was quantified using the Nanodrop (Isogen, IJsselstein, The Netherlands) and diluted to a concentration of 10 ng/ l. TaqMan assay was purchased from Applied Biosystems (Nieuwerkerk

aan den IJssel, The Netherlands). The *ERCC1* C118T SNP was determined on Realtime PCR system 7500 (Applied Biosys-tems, Nieuwerkerk aan den IJssel, The Netherlands) according to their instructions. PCR primer sequences are available on request.

Cell lines/plasmids

Wildtype CHO (AA8) and *ERCC1* negative CHO (UV20) cells[4] were used for in vitro transfection experiments. All cells were grown in DMEM supplemented with 5% FCS and G418 when required, at 37°C and 5% CO_2 conditions. All reagents were obtained from Invitrogen (Breda, The Netherlands).

Transfection of ERCC1 C118T in UV20-cells

Bicistronic plasmids expressing the open reading frame of wild type or mutant *ERCC1* allele at codon 118, and expressing Green Fluorescent Protein (GFP) for selection of transfected cells, were created by Gateway technology (Invitrogen, Breda, The Netherlands). Briefly, the *ERCC1* gene was PCR amplified from human cDNA, which was genotyped as heterozygous for *ERCC1* C118T, with attB-flanked primers p121 and p122.(Table 1) The PCR product was recombined in pDONR201. The PCR product in the resulted entry vector was sequenced to confirm genotype. Second, the entry vector was recombined with destination vector pExp-IRES-GFP (Clontech, Oxford, UK) that had been made gateway compatible using the Gateway Conversion system (Invitrogen, Breda, The Netherlands). The resulting plasmids were designated pTS401 and pTS402 and were used to transfect UV20 cells with GeneJuice (VWR, Amsterdam, The Netherlands). Transfected cells were selected by cellsorting, and plasmid expression was maintained by growth under selection by G418 (Sigma-Aldrich, Zwijndrecht, The Netherlands). The derived cell lines were designated UV20+118C (transfected with pTS401) and UV20+118T (transfected with pTS402). UV20 cells were also transfected with blank plasmid, and designated UV20-GFP.

Cell lines	**Characteristics**	**Origin/Reference**
AA8	wildtype CHO-cells	ATCC
UV20	ERCC1 deficient CHO- cells	Thompson (1980)
UV20+118C	UV20 expressing ERCC1-118C and GFP	this study
UV20+118T	UV20 expressing ERCC1-118T and GFP	this study
UV20-GFP	UV20 expressing GFP	this study
Plasmids		
pDONR201	donor vector	Invitrogen
pEXP-IRES-GFP	destiny vector	Clontech
TS401	pERCC1:118C-ires-GFP	this study
TS402	pERCC1:118T-ires-GFP	this study

Table 1. Plasmids and cell lines used in the ERCC1 transfection experiment.

Sulphorodamine B growth inhibition assay

Oxaliplatin cytotoxicity was assessed using the Sulphorodamine B (SRB) growth inhibition assay described by Skehan et al.[16] A total of 1.2×10^3 cells (AA8, UV20, UV20-GFP, UV20+118C, UV20+118T) were seeded into 96-well plates in a volume of 150 µl and incubated at 37°C overnight. Oxaliplatin concentrations were prepared in culture medium immediately before use. To the cells, 50 µl of drug-medium mixture was added. Triplicates were used for each drug concentration. Plates were incubated for 24 h at 37°C. Following drug treatment, the medium was replaced with 200 µl of fresh complete medium, and the plates were incubated for 3 days at 37°C. The growth medium in the wells was removed, and 50 µl of ice-cold 50% (w/v) trichloroacetic acid was added to fix the cells for 1 h at 4°C. Then, cells were washed six times with water, and stained with 50 µl of 0.4% (w/v) SRB-1% acetic acid for 20 min at room temperature. Unbound dye was removed by washing six times with 1% acetic acid, and plates were dried. The dye was solubilized by the addition of 150 µl of 10 mM Tris-base into each well. Plates were left at room temperature for 20 min, and the optical density (OD) at 570 nm was measured.

Clonogenic assay

The clonogenic assay was performed as described by Franken et al.[17] Briefly, a total of 50 exponentially growing cells (AA8, UV20, and transfected cells UV20+118C, UV20+118T and UV20-GFP) were plated in triplicate in 6-wells plates and incubated for 24 h to allow for cellular attachment. Attached single cells were treated with different concentrations of oxaliplatin (195 nM, 781 nM and 3125 nM) for 24 h at 37°C in medium. Cells were washed and incubated for 7–9 days until visible colonies of more than 50 cells were obtained. Cells were then stained with 1% Giemsa and the number of colonies in each dish was counted.

Modified COMET assay

DNA damage was studied by the modified COMET assay.[18;19] Exponentially growing cells were exposed to oxaliplatin 195 nM or 3125 nM for 1 h. The cells were washed and incubated overnight at 37°C and 5% CO_2. Next, the cells were trypsinized and resuspended in 1 ml culture medium. To induce DNA damage, the cells were treated with 30 M H_2O_2 (peroxide) for 10 min at 4°C. After spinning down, the degraded DNA was resuspended in 1% low melting point agarose (Promega, Leiden, The Netherlands) put on a microscope slide. The slides were soaked in lysisbuffer (100 mM EDTA, 10 mM Tris, 2.5 M NaCl, 1% Triton and 10% DMSO) for at least 90 min. In order to denature the DNA, the slides were air-dried and incubated in electrophoresis buffer (1 M EDTA, 300 mM NaOH pH 13) for 40 min. Next, the DNA was electrophoresed at 25 V and 250 mA for 30 min. After electrophoresis, the slides were washed at least three times with 0.4 M Tris pH 7.5 prior to staining with ethidiumbromide (20 g/l) (Promega, Leiden, The Netherlands) for 5 min.

Comet tails were visualized using FITC filtered fluorescent microscopy and visually scored as described elsewhere.[20] In these experiments, cells with high amounts of oxaliplatin-

induced cross-links have shorter tails compared to cells in which cross-links have been repaired effectively. This is explained by the fact that unrepaired platinum-induced DNA cross-links will stay together after DNA degradation by H_2O_2, resulting in larger DNA fragments. Mean difference in tail olives moment (and tail areas) between treated and untreated cells indicates the presence of DNA damage due to exposure to oxaliplatin. The damage level is expressed according to the formula: (unexposed cells – oxaliplatin exposed cells) tail olives moment/tail olives moment of unexposed cells.

Rad51 foci staining

Rad51 foci, indicative of DNA damage, were shown as follows.[21] Exponentially growing cells (AA8, UV20, and transfected cells UV20+118C, UV20+118T and UV20-GFP) were exposed to oxaliplatin and incubated as described in section 2.5. Next day, cells were washed with PBS and fixated with 2% formaldehyde for 15 min at room temperature. To enhance permeability, cells were incubated in 0.1% Triton for 10 min. To prevent non-specific binding of Rad51 antibody, the cells were washed with PBS+ (PBS containing 0.15% glycine (Biorad, Veenendaal, The Netherlands) and 0.5% BSA (Sigma–Aldrich, MO, USA)). Rabbit-anti-Rad51 antibody (Sigma–Aldrich, MO, USA) diluted in PBS+ was added to the cells and incubated at room temperature for 90 min under dark and humid conditions. Alexa488 conjugated goat-anti-rabbit antibody (Molecular Probes, Leiden, The Netherlands) was used to visualize Rad51 foci using FITC filtered fluorescent microscopy. Rad51 expression was quantified by counting the number of cells that have more than 5 foci. A representative image of the Rad51 foci staining used for analysis is shown in Figure 2.

Statistical methods

DNA from venous blood and tumor samples was obtained from a subset of patients participating in the previously described CAIRO study.[13]

One-way ANOVA with LSD-t test was used to compare the results of the SRB assay. A Cox proportional hazard model was used to investigate the association between *ERCC1* status and death and/or progression. PFS for second-line treatment was defined as the time from randomization until first progression reported after the start of second-line treatment and, if a patient did not start second-line treatment, the date of first progression reported after randomization or death or last follow-up. Likewise, PFS for third-line treatment was defined as the time from randomization until first progression, death, or last follow-up after the start of third-line treatment. OS was calculated as the interval from the date of randomization until death from any cause or until the date of last follow-up. Overall and progression-free survival curves were estimated with the Kaplan–Meier method and compared with the Log-Rank test. Association between survival and *ERCC1* status were analyzed by a univariate Cox proportional hazards model.

All tests were two-sided and p values of less than 0.05 were considered statistically significant. All data received before February 2008, with a median follow-up of 40.6 months

(range 0.3; 53 months), are included in this report.

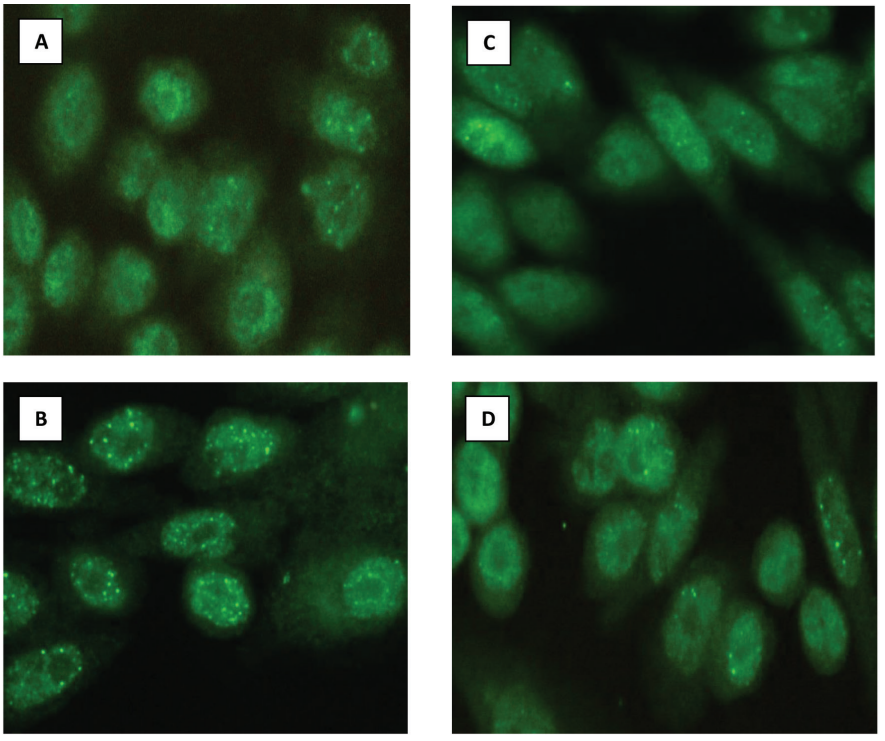


Figure 2. Representative picture used for assessment of Rad51 foci of oxaliplatin treated cells.
All cells have been incubated with 3125nM oxaliplatin, during 24 hours.
A: AA8 (wildtype CHO cells); B: UV20 (*ERCC1*-/- CHO cells); C: UV20+118C (UV20 cells transfected with *ERCC1*-118C allele); D: UV20+118T (UV20 cells transfected with *ERCC1*-118T allele).

Results

ERCC1 C118T is not associated with survival in ACC patients treated with oxaliplatin

Genotyping was successful in 251 out of 268 patients, and genotype frequencies were 118TT 92 (37%), 118TC 129 (51%), 118CC 30(12%). The genotype frequencies were in Hardy–Weinberg equilibrium (HWE) and comparable with those reported in Caucasians.[22;23]

A total of 145 patients treated with capecitabine and oxaliplatin were evaluated for analysis of PFS and OS. The C118T genotype was not correlated with PFS ($p = 0.145$ and $p = 0.614$ for second-and third-line treatment, respectively) or with OS (second-line therapy: $p = 0.121$; third-line therapy: $p = 0.331$). Similar results were obtained when patients of both regimens receiving second-and third line treatment were analyzed together. (Table 2)

C118T genotype		TT n=59	CT n=72	CC n=14	Total n=145	Log rank p value
Overall survival (months)	median	10.0	12.1	10.8	11.0	p= 0.186
	95% CI	(8.7-11.8)	(10.3-14.4)	(6.9-13.7)	(9.8-12.1)	
PFS (months)	median	4.2	4.2	4.5	4.2	p= 0.190
	95% CI	(3.1-4.9)	(3.4-6.1)	(1.9-5.2)	(3.8-4.9)	
Oxaliplatin 2nd line Number of patients		**31**	**48**	**10**	**89**	
Overall survival (months)	median	9.6	13.2	12.5	11.6	p= 0.121
	95% CI	(7.4-12.0)	(10.6-15.3)	(8.2-18.9)	(9.6-13.6)	
PFS (months)	median	4.1	4.1	4.5	4.1	p= 0.145
Oxaliplatin 3rd line Number of patients		**28**	**24**	**4**	**56**	
Overall survival (months)	median	10.7	10.4	7.6	10.3	p= 0.331
	95% CI	(8.1-13.0)	(6.4-12.7)	(1.5-13.6)	(8.3-12.1)	
PFS survival (months)	median	4.3	4.5	3.2	4.4	p= 0.614
	95% CI	(2.8-5.5)	(2.1-5.9)	(0.0-7.4)	(2.8-5.4)	
	95% CI	(2.1-5.8)	(3.7-6.7)	(1.9-5.2)	(3.8-5.1)	

Table 2. ERCC1 C118T genotypes and clinical outcome after treatment with CAPOX

CAPOX, capecitabine-oxaliplatin; CI, confidence interval; ERCC1, Excision Repair Cross-Complementation group 1 (ERCC1); OS, overall survival; PFS, progression free survival.

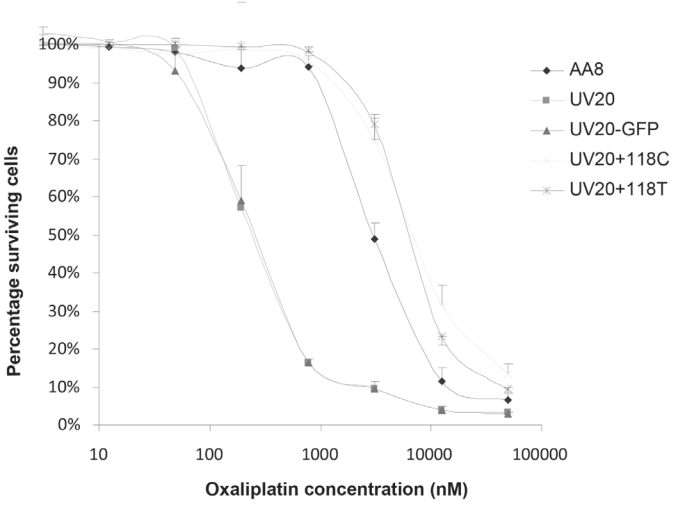


Figure 3. Sulphorodamine B (SRB) assay determined oxaliplatin sensitivity in parental AA8, UV20 (ERCC1-/-), and transfected cells UV20+118C, UV20+118T and UV20-GFP.

Growth inhibition was determined using the SRB assay and the percentage of surviving cells was calculated as described. All results are the mean of at least three independent experiments and error bars show the standard deviation of the mean.

ERCC1, but not C118T genotype, influences cellular sensitivity to oxaliplatin in vitro

To test whether C118T genetic variants are causally related to cellular sensitivity to oxaliplatin, we conducted transfection experiments with *ERCC1* negative CHO cells. Figure 3 shows the sensitivity to oxaliplatin of AA8 (wildtype CHO), *ERCC1* defective CHO mutant UV20 and the three transfected cell lines (UV20+118C, UV20+118T and UV20-GFP). The drug concentrations to inhibit cell growth by 50% (IC_{50} values) in the *ERCC1*-defective cells (UV20 and UV20-GFP) are approximately 16-fold lower than for their parental cell line (AA8). UV20 and UV20-GFP showed similar IC_{50} values, indicating that the transfection of GFP had no effect on cell growth. In contrast, the *ERCC1* overexpressing cells, UV20+118C and UV20+118T, show substantial resistance to oxaliplatin. IC_{50} values for these cells were approximately 32-fold higher compared to the UV20 cell line (p-value < 0.005, paired Student's t-test). No difference was found in IC_{50} values between UV20+118C and UV20+118T. These results indicate that *ERCC1* influences cellular sensitivity to oxaliplatin, and that chemosensitivity is not different between *ERCC1* 118C and 118T expressing variants.

We repeated these experiments using the clonogenic assay. Consistent with the results from the SRB assay, all *ERCC1* expressing cells (AA8, UV20+118C and UV20+118T) showed significant numbers of surviving colonies after treatment with oxaliplatin. However, *ERCC1* negative UV20 cells and cells transfected with the empty vector (UV20-GFP) showed no colony formation after treatment with oxaliplatin. A schematic representation of these results is shown in Figure 4.

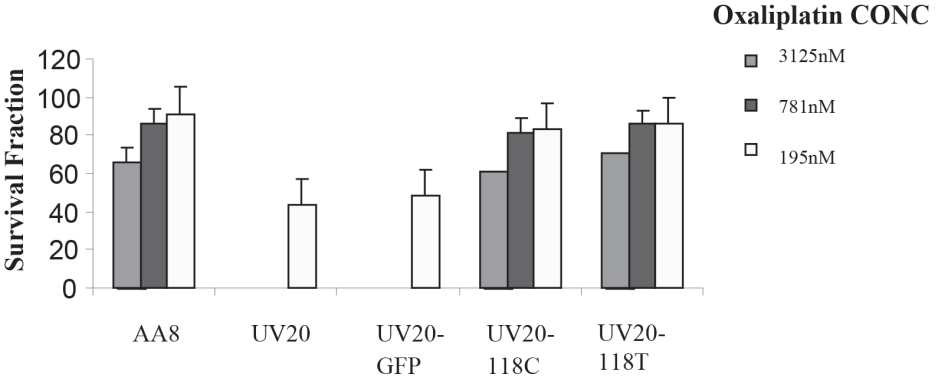


Figure 4. Clonogenic assay determined oxaliplatin sensitivity in parental AA8, UV20 (ERCC1-/-), and transfected cells UV20+118C, UV20+118T and UV20-GFP.

Number of visible colonies of more than 50 exponentially growing cells, after 24h treatment with different concentrations of oxaliplatin (195nM, 781nM and 3125nM) and subsequent incubation during 7-9 days

ERCC1, regardless of C118T genotype, is essential for oxaliplatin-induced DNA-damage in vitro

To validate whether the high susceptibility of UV20 cells to oxaliplatin is caused by a defect in DNA repair, we evaluated oxaliplatin-induced DNA damage using the COMET assay, in wild-type AA8 cells, *ERCC1* defective UV20, and the transfected variants UV20+118C and UV20+118T. As expected, tails of the *ERCC1* deficient UV20 cell line are smaller than AA8 tails. The comet tails in UV20+118C and UV20+118T are comparable with AA8. These results indicate that DNA repair activity in *ERCC1* deficient UV20 is fully restored by introducing *ERCC1* and that this is equally the case for *ERCC1* 118C and 118T transfected cells.(Figure 5) A representative image of the modified COMET assay used for data analysis is shown in Figure 6.

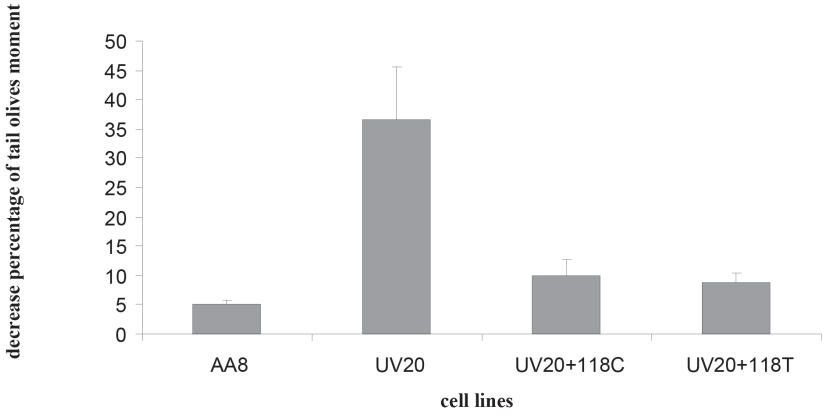
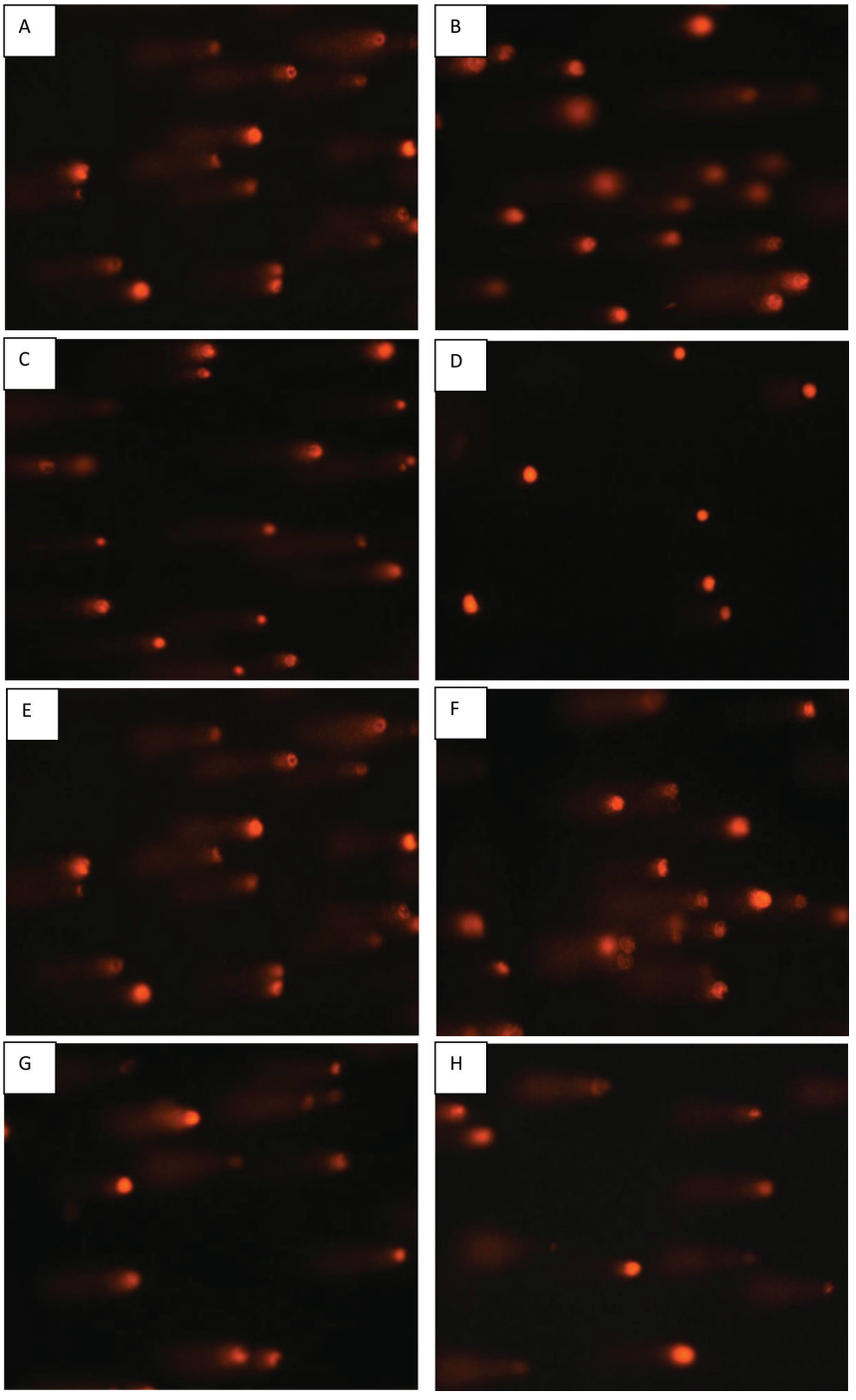


Figure 5. Difference in comet tail length after exposure to oxaliplatin of parental AA8, UV20 (ERCC1-/-), and transfected cells UV20+118C and UV20+118T.

A large decrease in comet-tail olives moment is indicative of higher amounts of residual DNA-cross links caused by oxaliplatin. Results are the mean of 100 comets and error bars show the standard deviation of the mean.

Because *ERCC1* is also considered to be involved in homologous recombinational repair (HRR)[24;25], we stained for Rad51 foci in oxaliplatin treated cells. Rad51 is important for recognition of DNA damage in HRR[26] and impaired *ERCC1* function leads to constant expression of Rad51 at the site of the lesion. Results for these analyses are shown in Figure 7. The data shown are the means of at least three experiments. After 24 h, the amount of foci per cell was more abundant in UV20 compared to AA8 (Figures 2B and A, respectively) or *ERCC1* complemented cells.(Figures 2C and D) In UV20 *ERCC1* negative cells, on average 61% of cells showed more than five Rad51 foci, versus only 27% for AA8 wildtype cells. In the *ERCC1* overexpressing cells (UV20+118C, UV20+118T) very few Rad51 foci positive cells were present, irrespective of their codon 118 genotype (13% versus 11% for 118C and 118T, respectively). These results indicate that HRR pathway is less active in the *ERCC1* deficient cell line and can be restored to wildtype activity by ectopic expression of *ERCC1* 118C, as well as 118T.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(Opposite page) Figure 6. Representative image of the modified COMET assay used for interpretation of remaining DNA damage after treatment with oxaliplatin.

A: AAV8, not treated with oxaliplatin; B: AAV8 treated with oxaliplatin 3125nM; C: UV20, not treated with oxaliplatin; D: UV20, treated with oxaliplatin 3125nM; E: UV20-118C, not treated with oxaliplatin; F: UV20-118C, treated with oxaliplatin 3125nM; G: UV20-118T, not treated with oxaliplatin; H: UV20-118T, treated with oxaliplatin 3125nM.

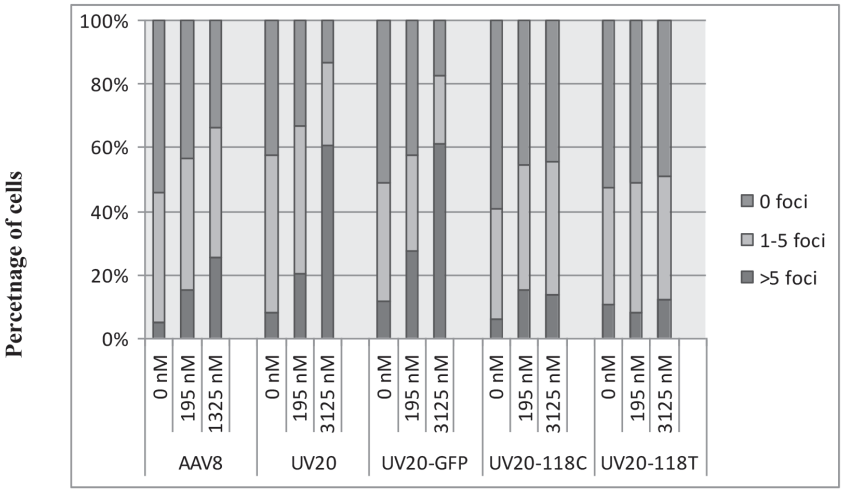

Figure 7. Change is Rad51 foci staining after treatment with increasing oxaliplatin concentrations, for parental AA8, UV20 (ERCC1-/-), and transfected cells UV20+118C and UV20+118T.

Results are means of at least three experiments.

Discussion

Despite numerous publications in recent years, there has been ongoing debate on the role of *ERCC1* and the common SNP at codon 118 of this gene regarding chemosensitivity to oxaliplatin in colorectal carcinoma. This is the most comprehensive study of *ERCC1* C118T functionality in the repair of oxaliplatin-induced DNA-damage to date. We investigated the association of *ERCC1* genotype with PFS and OS after oxaliplatin-containing chemotherapy in ACC and found no effect of the C118T SNP. We did, however, confirm *ERCC1* functionality in the repair of oxaliplatin-induced DNA-damage, using a modified COMET assay and staining of Rad51. Most importantly, we excluded an effect of the *ERCC1* C118T SNP on cellular sensitivity to oxaliplatin and on DNA-repair efficacy in oxaliplatin-induced DNA damage, by using cellular transfection experiments.

Publications regarding the effect of *ERCC1* C118T in platinum therapy have been both extensive and contradictory. Consistent with our results, several authors reported no effect of the SNP on clinical efficacy of oxaliplatin-based chemotherapy in colorectal carcinoma patients.[23;27–30] Other studies, however, found shorter survival times for the *ERCC1* 118T allele in ACC patients treated with oxaliplatin-containing schedules.[10;11;31–33] Contrarily, a smaller number of authors found an adverse effect of the C allele.[34–36] It should be noted that our study population, as most other clinical pharmacogenetic association studies, lacks statistical power to definitively rule out an effect of the *ERCC1* C118T polymorphism on oxaliplatin efficacy. However, our results concur with those from a meta-analysis published in 2011, showing that PFS following oxaliplatin-based chemotherapy for colorectal cancer patients carrying the *ERCC1* 118C allele was not influenced by rs11615 genotype in Caucasian populations.[37]

Based on the results of our clinical association study, we questioned the functional relevance of the *ERCC1* SNP C118T. By in vitro experiments, transfecting UV20 cells with either 118C or 118T, we showed that the SNP does not have functional consequences for either in vitro sensitivity to oxaliplatin, DNA-repair capacity, or HRR. Previously, Gao et al. followed a similar approach and also found no differences in viability upon platinum treatment between *ERCC1* 118C and 118T expressing cells.[8] Seetharam et al. found that *ERCC1* mRNA and protein expression were upregulated upon oxaliplatin in resistant CRC cell lines, but not in oxaliplatin sensitive cell lines. In addition, silencing of *ERCC1* by siRNA led to an increase in chemosensitivity in previously oxaliplatin-resistant cell lines.[38]

Given our results and those by Gao and co-workers, it remains striking that many authors found an effect of the *ERCC1* C118T polymorphism on oxaliplatin efficacy. Several explanations can be offered for this discrepancy. Firstly, it should be noted that many of the previous pharmacogenetic association studies did not correct adequately for multiple testing and positive results may therefore have arisen by chance. Secondly, differences in genotype frequencies between populations may have caused accidental statistical associations, especially in populations with mixed ethnicities. Whereas the 118C allele is the minor allele in Caucasians, it is the common variant found in people from Asian descent.[22] Notably, a positive effect of the *ERCC1* 118C allele was pre-dominantly found in Asian populations.[11;31;32] An alternative hypothesis is that the C118T SNP is indirectly functional due to its linkage disequilibrium with another SNP in the *ERCC1* gene. The C118T SNP was found to be in a tight haplotype block with other SNPs in both coding and non-coding regions of the *ERCC1* gene.[27;34;39;40] Consistent with this hypothesis, a haplotype containing both *ERCC1* C118T and a SNP in the 3'-UTR of *ERCC1* (C8092A, rs3212986) was recently shown to associate with decreased DNA damage repair capacity for patients carrying *ERCC1* 8092A.[20;41] In addition, with the redefinition of the gene concept, cis- or trans-acting elements and genetic variations could also be located distantly in the genome.[42;43]

It can be argued that our analyses did not include other loci with putative influence on oxaliplatin efficacy. Although some authors found an effect of glucuronyl-S-transferase pi (GSTP1) on oxaliplatin toxicity[44;45], we previously showed that this polymorphism did not

influence chemosensitivity in our CAIRO population.[46] However, it cannot be excluded that results for the *ERCC1* polymorphism were confounded by the effect of another coinciding polymorphism or haplotype.

Although the use of *in vitro* data and transfection experiments offers important information on the molecular physiology of oxaliplatin DNA damage repair, it is subject to limitations. Protein and mRNA expression in transfected cells are plasmid-derived and are no longer regulated by epigenetic systems, leading to supernatural levels of protein. This limits functional analyses in these transfected cells in general and impairs extrapolation of in vitro results to epidemiologic populations.[47] However, in the case of *ERCC1*, protein functionality and, consequentially, the level of DNA repair are dependent on the concomitant expression of ERCC4.[48;49] Higher ERCC1 protein levels due to unregulated plasmid derived transcription will therefore not lead to more effective DNA repair. Therefore, we believe our transfection model provides a valid proxy for the analysis of the in vivo effect of the *ERCC1* C118T polymorphism on DNA repair after treatment with oxaliplatin.

Overall, our results show that *ERCC1* activity is essential in response or resistance to oxaliplatin in colorectal cancer. However, it remains unclear if inter-individual variations in *ERCC1* activity influence chemosensitivity. More importantly, it is not known how *ERCC1* activity can reliably be measured in a clinical setting. Many studies have used *ERCC1* staining in correlation to oxaliplatin efficacy.[38;50;51] However, a recently published article highly questions the validity of ERCC1 staining, stressing that only one ERCC1 isoform is functionally active, whereas staining methods cannot differentiate between functional and non-functional iso-forms.[52] Others have used *ERCC1* mRNA expression, but results have been equally diverse.[38;53–57] Nonetheless, pretreatment indicators of *ERCC1* activity are warranted, before its use as a biomarker is possible.

Conclusion

In summary, we showed that ERCC1 function is essential in repair of oxaliplatin-induced DNA damage and thereby influences oxaliplatin cytotoxicity *in vitro*. However, we found no evidence for functional differences of *ERCC1* C118T variants in the cellular response to oxaliplatin exposure. This coincides with our findings that this genetic variant is not associated with clinical outcome in ACC patients treated with oxaliplatin. Further research should focus on elucidating the optimal method for assessing ERCC1 activity and the search for genetic variation in other loci of *ERCC1*, or epigenetic regulation affecting the gene, since it is evident that *ERCC1* expression is indispensable for DNA repair in response to platinum treatment.

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8

Genome wide association study for predictors of progression free survival in patients on capecitabine, oxaliplatin, bevacizumab and cetuximab in first-line therapy of metastatic colorectal cancer

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Abstract

Purpose: Despite expanding options for systemic treatment, survival for metastatic colorectal cancer (mCRC) remains limited and individual response is difficult to predict. In search of pretreatment predictors, pharmacogenetic research has mainly used a candidate gene approach. Genome wide association (GWA) studies offer the benefit of simultaneously analyzing a large number of SNPs, in both known and still unidentified functional regions. Using a GWA approach, we searched for genetic markers affecting progression free survival (PFS) in mCRC patients treated with first-line capecitabine, oxaliplatin and bevacizumab (CAPOX-B), with or without cetuximab.

Patients & methods: 755 patients were included in the CAIRO2-trial, a multicenter phase III trial, randomizing between first-line treatment with CAPOX-B versus CAPOX-B plus cetuximab. Germline DNA and complete clinical information was available from 553 patients and genome wide genotyping was performed, using Illumina's OmniExpress beadchip arrays, with 647,550 markers passing all quality checks. Another 2,202,473 markers were imputated by using HapMap2. Association with PFS was analyzed using a Cox proportional hazards model.

Results: One marker, rs885036, associated significantly with PFS ($P = 2.17x10^{-8}$) showing opposite effects on PFS depending on treatment arm. The minor allele was associated with increased PFS in patients receiving cetuximab. A cluster of markers located on chromosome 8 associated with PFS, irrespective of treatment arm (P-values of $2.30x10^{-7}$ to $1.04x10^{-6}$).

Conclusion: This is the first GWA study to find SNPs affecting PFS in mCRC patients treated with CAPOX-B, either with or without cetuximab. Rs885036 is a potential predictive marker for cetuximab efficacy. These markers need to be validated in independent treatment cohorts.

Background

Colorectal cancer is among the most prevalent forms of cancer worldwide, with the estimated number of new diagnoses in the United States for 2013 exceeding 142,000 cases.[1] Combination chemotherapy including a fluoropyrimidine plus the monoclonal agent bevacizumab and either oxaliplatin or irinotecan is generally recommended for first-line treatment of metastatic colorectal carcinoma (mCRC).[2] Unfortunately, even with these modern-day treatment options, median survival is limited and variation in response is largely unpredictable. Optimal selection of patients who will benefit from these extensive treatment schedules is warranted, both from the individual aspect of preventing needless burden of toxicity, and from a population-based aspect of optimal cost-effectiveness.

Germline genetic variation has been shown to predict differences in response to many chemotherapeutic drugs.[3] Until now, most research has focused on genetic alterations in genes encoding for known target or metabolic enzymes. A disadvantage of this candidate-gene approach is the limited knowledge of the exact mechanism of action for many drugs. Considering the immense amount of single nucleotide polymorphisms (SNPs) the human genome harbors, it is likely that many SNPs with potential effect on drug efficacy have not yet been detected. Circumventing the limitations of a candidate gene approach, genome wide association (GWA) studies offer the possibility of simultaneously analyzing a large number of SNPs, even in regions that have not previously been associated with the drug under investigation. This type of study has been applied to identify risk factors for multiple types of cancers, including colorectal carcinoma.[4;5] Moreover, in pharmacogenetic GWA studies, an increasing number of SNPs associated with treatment response in cancer are identified. A recent GWA study in colorectal cancer patients identified SNPs that were associated with adverse drug reactions in response to treatment with 5-FU and oxaliplatin.[6]

Here, we present the results of the first clinical GWA study to find SNPs that are associated with progression free survival (PFS) of first-line combination chemotherapy for mCRC with capecitabine, oxaliplatin and bevacizumab (CAPOX-B), either with or without cetuximab.

Patients and methods

Germline DNA was obtained from 553 of 755 previously untreated mCRC patients, who were recruited from the CAIRO2 trial, a multicenter phase III trial of the Dutch Colorectal Cancer Group (DCCG), which randomized between first-line treatment with CAPOX-B versus CAPOX-B plus cetuximab.[7] All patients received three-weekly cycles of capecitabine 1000mg/m^2 b.i.d. orally on days 1-14 plus oxaliplatin 130mg/m^2 (maximum of 6 cycles) and bevacizumab 7.5mg/m2, both intravenously on day 1 of each treatment cycle. From cycle 7 the capecitabine dosage was increased to 1250mg/m^2. For patients randomized to treatment with CAPOX-B plus cetuximab, cetuximab was administered intravenously at a loading dose of

400mg/m^2 on the first treatment day, followed by 250mg/m^2 once weekly thereafter. Treatment was continued until disease progression, unacceptable toxicity or death, whichever occurred first. Patient eligibility criteria and guidelines for response assessment have been described in detail elsewhere.[7] The study was approved by the Committee on Research involving Human Subjects Arnhem-Nijmegen and by all local institutional ethics boards. All subjects gave written informed consent.

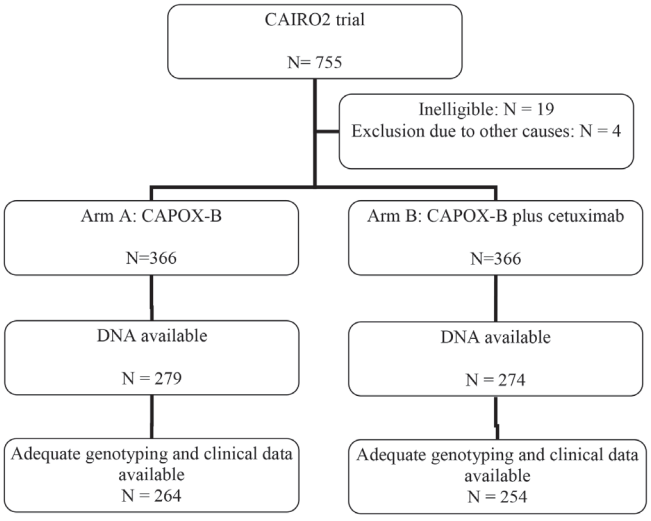

Figure 1. Cairo2 study flow-chart

Genotyping

Whole blood was collected at baseline and germline DNA was isolated from peripheral leukocytes using MagnaPure Compact (Roche diagnostics, Almere, The Netherlands). Genotyping was performed on Human OmniExpress v12 BeadChip arrays containing 733,202 markers (Illumina, San Diego, CA, USA). Genotype calls were set using GenomeStudio software (Illumina). All products were used according to manufacturer's prescriptions. The following cut-off values were used to filter out incorrectly called genotypes: GenCall ≥ 0.85; ClusterSep ≥ 0.3; CallFreq >0.85; AB T-mean 0.2-0.8, resulting in the exclusion of 3172 markers (0.43%).

Statistical analysis

Quality control

Quality control was performed using R, version 3.0.1 (http://www.r-project.org/), and *plink*.[8] Markers were excluded based on a minor allele frequency (MAF) threshold of 0.01 (1636 excluded markers; 0.3%) and missingness threshold of 0.05 (no excluded markers). Hardy-Weinberg equilibrium (HWE) was evaluated per marker, using a χ^2 goodness-of-fit statistic with a cut-off P-value of $\leq 10^{-7}$ (36 excluded markers). After these quality checks, 647,550 markers remained in the analysis.

Individuals were excluded based on missingness > 2% (no excluded individuals).

Multidimensional scaling (MDS) was used to investigate possible stratification and outliers. After removal of outliers MDS did not show clustering. Association analysis was performed with and without the first four MDS coordinates as covariates. Ranking and P-values of the top 30 SNPs were almost identical and only analyses without MDS coordinates are shown. In total 33 patients were excluded due to low genotyping quality. Complete information, including genotype, was available for 520 patients. The study flowchart is shown in Figure 1.

Imputation

Additionally, non-measured genotypes were imputated using HapMap2, to further enhance SNP density. A minimum imputation accuracy of R^2>0.40 was applied to select reliably imputed SNPs. In total 2,202,473 imputed markers were used in this analysis.

Association model

For each marker, the association with PFS was calculated with a Cox-proportional hazards model using *R* package *survival*. Age, gender, treatment arm were included as covariates. A first model contained each SNP as variable of interest (marginal model) and a second model tested the interaction SNP*arm as variable of interest while controlling for the main SNP effect (interaction model). Markers were evaluated using an additive genetic model in all analyses. The inflation factor for association models was calculated based on the χ^2 -quantiles for the P-values of the evaluated models. Formal significance for a marker was assumed for a two-sided $P < 5x10^{-8}$ to correct for multiple testing, as has been described by others.[9–11]

Kaplan-Meier curves were estimated for the marker with the lowest P-value using *R*.

	All patients (n = 520)	**CAPOX-B (n = 264)**	**CAPOX-B plus cetuximab (n = 256)**
Age in years Median (range)	63.2 (27.6-83.6)	62.9 (27.6-83.6)	63.9 (33.2-80.0)
Sex – no (%)			
Male	316 (60.8)	144 (54.5)	172 (67.2)
Female	204 (39.2)	120 (45.5)	84 (32.8)
LDH at baseline – no (%)*			
Normal	307 (59.0)	152 (57.6)	155 (60.5)
Above upper limit of normal	213 (41.0)	112 (42.4)	101 (39.5)
Previous adjuvant chemotherapy – no (%)			
Yes	443 (85.2)	223 (84.5)	220 (86.6)
No	71 (13.7)	37 (14.0)	34 (13.4)
Unknown	6 (1.2)	4 (1.5)	2 (0.8)
Progression free survival Median (range)	10.3 (0.1 – 44.7)	10.6 (0.4 – 44.7)	10.0 (0.1 – 40.8)

Table 1. Baseline patient characteristics

* According to cut-off values of each individual center
CAPOX-B, capecitabine, oxaliplatin and bevacizumab; LDH, lactate dehydrogenase.

Results

Base-line characteristics

Baseline patient characteristics are described in Table 1. Median PFS was 10.3 months for all patients included in the analyses (range 0.1 – 44.7 months). At the time of analysis the primary endpoint PFS was reached by 487 of 520 patients (93.7%).

Genome-wide association analysis including effect of treatment arm

Initially, analyses were performed including the interaction term of genetic markers with treatment arm as a covariate. The Manhattan plot for these analyses is shown in Figure 2 and details for the ten most significant markers are provided in Table 2. Markers with a differential effect according to treatment arm may actually reflect predictive markers for cetuximab efficacy. One marker (rs885036, position 98671226) on chromosome 2q12 showed a highly significant interaction with treatment arm ($P = 2.17x10^{-8}$). In a stratified analysis, this SNP proved to have a contrasting effect in both treatment arms. Kaplan-Meyer survival curves for PFS according to genotype are shown in Figure 3. For patients treated with CAPOX-B, the G allele associated with shorter PFS (AA 13.47 months [95%CI 12.16-16.43]; AG 12.22 months [95%CI 9.17-14.32]; GG 9.00 months [95%CI 7.56-10.68]). For patients treated with CAPOX-B plus cetuximab, the G allele associated with increased PFS (AA 7.31 months [95%CI 6.53-10.05]; AG 10.05 months [95%CI 8.83-12.16]; GG 12.35 months [95%CI 10.35-15.44]).

Marker	Chr	Position	Gene	A0	A1	Allele frequency	$R^{2\#}$	Allele HR* (95% CI)	P-value
rs885036	2	98671226	*GnT- IVa*	A	G	0.533	1.000	0.47 (0.36-0.61)	$2.17x10^{-8}$
rs3769689	2	98660129	*GnT- IVa*	A	G	0.327	0.975	0.49 (0.37-0.65)	$6.99x10^{-7}$
rs3769688	2	98660242	*GnT- IVa*	A	G	0.329	0.980	0.50 (0.38-0.66)	$1.02x10^{-6}$
rs17448420	2	98664315	*GnT- IVa*	C	T	0.329	0.983	0.50 (0.38-0.66)	$1.06x10^{-6}$
rs17448190	2	98656831	*GnT- IVa*	C	T	0.325	0.972	0.50 (0.38-0.66)	$1.06x10^{-6}$
rs17448211	2	98657628	*GnT- IVa*	A	T	0.325	0.973	0.50 (0.38-0.66)	$1.07x10^{-6}$
rs17448085	2	98653118	*GnT- IVa*	C	T	0.328	0.974	0.50 (0.38-0.67)	$1.49x10^{-6}$
rs17514013	2	98653226	*GnT- IVa*	A	G	0.672	0.974	1.99 (1.50-2.64)	$1.49x10^{-6}$
rs10199926	2	98658908	*GnT- IVa*	C	T	0.656	0.982	1.96 (1.49-2.60)	$1.87x10^{-6}$
rs2309434	2	98672936	*GnT- IVa*	C	T	0.340	0.987	0.51 (0.39-0.68)	$2.46x10^{-6}$

Table 2. Ten SNPs with lowest P-values for association with PFS

Abbreviations: HR, Hazard ratio; Chr, chromosome; CI, confidence interval; GnT-IVa: mannosyl (alpha-1,3)-glycoprotein beta-1,4-N-acetylglucosaminyltransferase isozyme A; R2: imputation accuracy. A0: reference allele according to hapmap2, A1: alternate allele.

* HR assuming an additive effect depending on dosage of the alternate alleles, obtained with multivariate Cox-proportional hazards model, including age, gender, treatment arm, and the interaction treatment arm* marker as covariates.

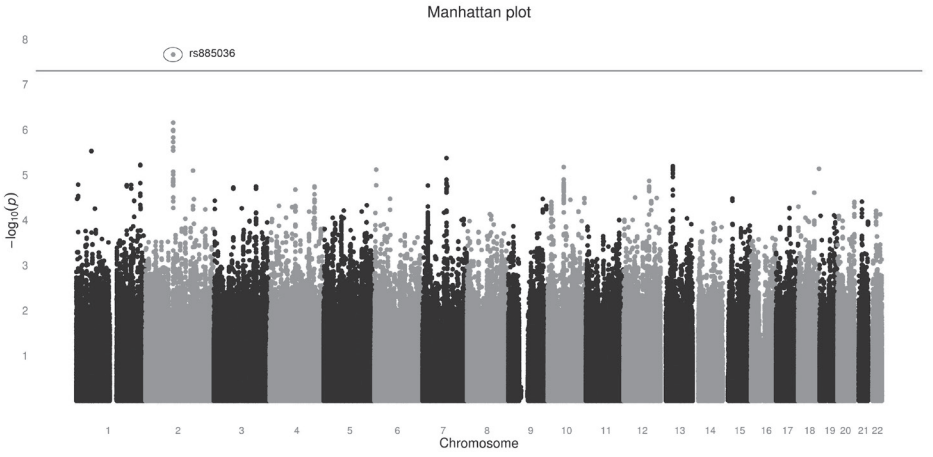

Figure 2. Manhattan plot of –log$_{10}$ (P-value) of the Cox-proportional hazards model
Adjusted for age, gender and treatment arm and the interaction of marker with treatment arm.
The horizontal line represents the formal genome-wide significance level of 5x10^{-8}.

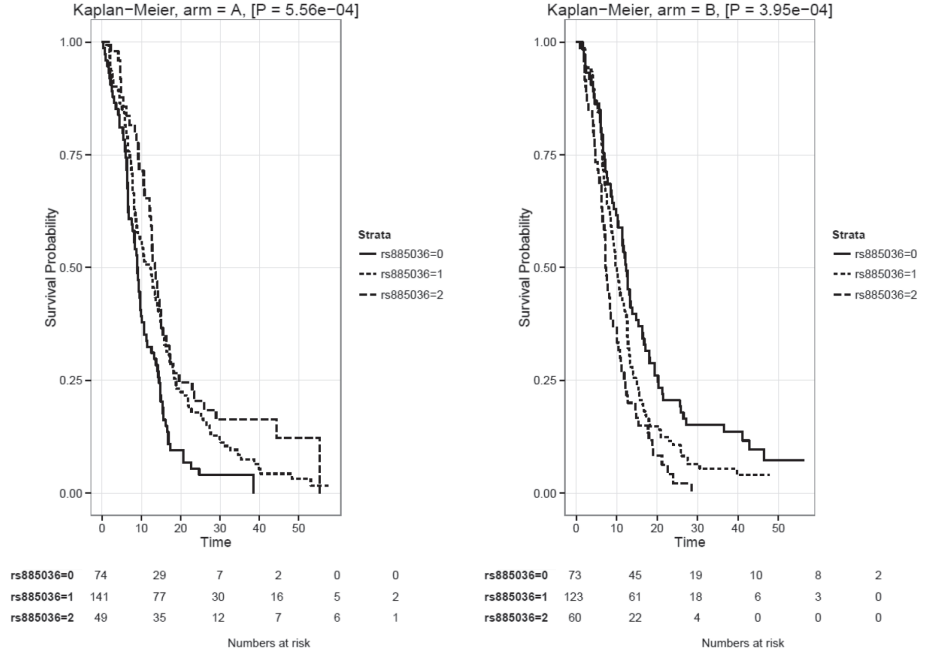

Figure 3. Kaplan-Meyer survival curves according to rs885036 genotype
A. Survival curves for patients in arm A, treated with CAPOX-B in first-line chemotherapy. **B.** Survival curves for patients in arm B, treated with CAPOX-B plus cetuximab in first-line chemotherapy.

Overall genome-wide association analysis results

The Manhattan plot for the overall GWA analysis is shown in Figure 4. No marker reached the pre-determined formal significance level. A cluster of markers, located on chromosome 8, showed the lowest P-values ($P = 2.30x10^{-7}$ to $P = 1.04x10^{-6}$, respectively). The inflation factor for this analysis was modest (λ 1.02), indicating there was no evidence of population stratification or other bias in the analysis. The ten most significant markers are described in Table 3.

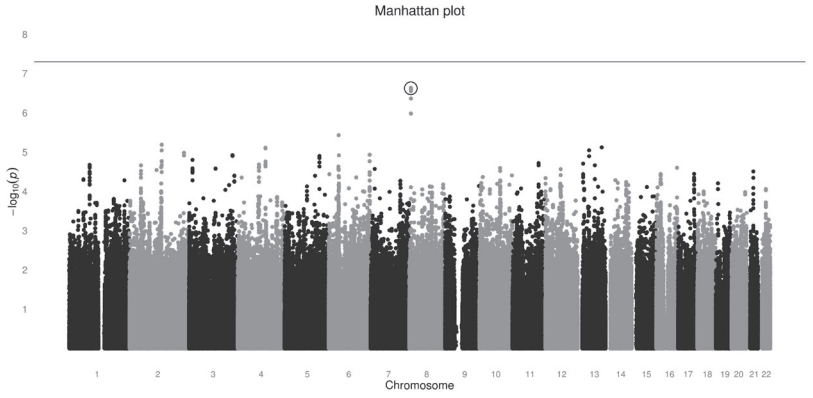

Figure 4. Manhattan plot of –log10 (P-value) of the Cox-proprotional hazards model

Adjusted for age, gender and treatment arm. The horizontal line represents the formal genome-wide significance level of $5x10^{-8}$. The topmost significant markers are circled, i.e. rs2936519, rs2928608, rs2928609, rs2912024, rs2978926, rs2928607.

Marker	Chr	Position	Gene	A0	A1	Allele frequency	R^{2*}	Allele HR# (95% CI)	P-value
rs2936519	8	6626650	*n.a.*	A	G	0.90	1.00	0.53 (0.43-0.67)	$2.30x10^{-7}$
rs2928608	8	6626434	*n.a.*	C	T	0.90	0.99	0.54 (0.43-0.67)	$2.33x10^{-7}$
rs2928609	8	6626528	*n.a.*	C	T	0.90	0.99	0.54 (0.43-0.67)	$2.36x10^{-7}$
rs2912024	8	6626309	*n.a.*	C	T	0.10	1.00	1.86 (1.49-2.32)	$2.60x10^{-7}$
rs2978926	8	6626835	*n.a.*	A	G	0.10	0.98	1.86 (1.49-2.33)	$2.67x10^{-7}$
rs2928607	8	6626120	*n.a.*	C	G	0.10	0.97	1.86 (1.49-2.33)	$2.79x10^{-7}$
rs11997869	8	6625874	*n.a.*	C	G	0.10	0.97	1.84 (1.47-2.30)	$4.32x10^{-7}$
rs2978931	8	6625491	*n.a.*	A	C	0.91	1.00	0.55 (0.44-0.69)	$1.04x10^{-6}$
rs2073016	6	41128900	*n.a.*	C	T	0.83	0.72	1.62 (1.31-2.00)	$3.70x10^{-6}$
rs4377367	2	131441375	*ARHGEF4*	C	T	0.86	0.99	0.63 (0.52-0.76)	$6.47x10^{-6}$

Table 3. Ten SNPs with lowest P-values for overall association with PFS

Abbreviations: HR, Hazard ratio; Chr, chromosome; CI, confidence interval; *n.a.,* marker is not localized within a gene. ARHGEF4: Rho guanine nucleotide exchange factor 4.

* R2: imputation accuracy. A0: reference allele according to hapmap2, A1: alternate allele.

HR assuming an additive effect depending on dosage of the alternate alleles, obtained with multivariate Cox-proportional hazards model, including age, gender, treatment arm, and the interaction treatment arm* marker as covariates.

Discussion

This is the first prospective GWA study to find SNPs that predict efficacy of first-line chemotherapy with CAPOX-B, with or without cetuximab, in mCRC patients in a clinical trial setting. One marker on chromosome 2, rs885036, was significantly associated with PFS, with a contrasting effect in both treatment arms. This SNP may therefore be a potential predictive marker for efficacy of cetuximab containing chemotherapy. Additionally, a cluster of SNPs on chromosome 8 influenced PFS with a similar effect in both treatment arms, almost reaching genome wide significance.

A genome wide significant effect on PFS was found for rs885036, when treatment arm was taken into consideration. Interestingly, we found opposite effects of genotype on PFS in both treatment arms. This suggests that this polymorphism may have a deleterious prognostic effect on survival per se, but this effect is no longer seen after the addition of cetuximab to standard treatment with CAPOX-B.

Rs885036 is located in the gene encoding for mannosyl (alpha-1,3)-glycoprotein beta-1,4-N-acetylglucosaminyltransferase isozyme A, *GnT-IVa* (also denominated MGAT4A), as are all ten most significant markers from these analyses. The top region of most significant markers exhibits long ranging linkage disequilibrium (LD), extending >50kb in both directions (HapMap3 data, data not shown), which also covers the candidate gene *GnT-IVa*. Of note, rs885036 is a directly measured SNP.

GnT-IV encodes for a glycosyltransferase, which is involved in the biosynthesis of oligosaccharides and has previously been associated with tumor behavior. In one study, *GnT-IV* mRNA was upregulated in metastatic colorectal tumors, as opposed to non-metastatic primary colorectal tumors.[12] Strong expression of *GnT-IVa* mRNA and enzyme activity was also found in drug-resistant hepatocellular carcinoma cells.[13]

The rs885036 SNP constitutes a C>T nucleotide change in the second intron of *GnT-IVa*, and is located within a predicted binding site for the highly conserved microRNA-34A (miR-34a).[14;15] MicroRNAs play an important role in post-transcriptional silencing of genes, as their interference with RNA leads to repressed translation or cleavage of RNA.[16] Expression of miR-34a itself is regulated by p53 expression.[17] Cytotoxic stress induced by chemotherapy or irradiation markedly increases miR-34a levels in the presence of p53[18], emphasizing its importance in response to anti-cancer treatment.

We believe that the C>T change leads to altered binding of miR-34a, resulting in differential translation efficacy of *GnT-IVa*. Although our analyses cannot clarify any further functional correlations, we speculate that the altered GnT-IVa protein level may then influence EGFR glycosylation, and thereby form a possible determinant of cetuximab efficacy.

Although no SNP in the overall analyses reached formal genome wide significance, a cluster of SNPs on chromosome 8p23.1 showed very low P-values for the association with PFS. Within the cluster, are two directly genotyped SPNs, including the most significant SNP (rs2936519).

These markers are not localized within any known gene, but the gene encoding for 1-acylglycerol-3-phosphate O-acyltransferase 5 (*APGAT5*), is found approximately 20 kbp upstream.[19] Again, the topmost significant SNPs are in long ranging LD, which covers this candidate gene (HapMap3 data, data not shown). AGPAT5 comes from a family of highly conserved enzymes that catalyze the acylation of lysophosphatidic acid (LPA) to phosphatidic acid, the second step in the *de novo* formation of glycerophospholipids.[20;21] Although the function of most AGPAT isoforms is largely unknown, multiple AGPAT isoforms have been linked to cancer. AGPAT9 and 11 are overexpressed in colon cancer tissue, as well as in other cancer types.[20;22] AGPAT2 inhibition was shown to induce *in vitro* growth arrest and cell death in different tumor types, including several colon cancer cell lines.[23]

To our knowledge, this is the first prospective GWA study investigating systemic treatment efficacy in Caucasian mCRC patients. Only two previous similar studies have been published worldwide, and none of the markers identified by those experiments overlap with our results.[24;25] These studies, both by the same Korean research group, followed a three step design, validating results from GWA analysis in both an independent patient cohort and transfected colorectal cancer cell lines. They found two SNPs with putative influence on efficacy of cetuximab-containing regimens for colorectal carcinoma[25], as well as one SNP influencing disease recurrence after adjuvant chemotherapy with 5-FU for stage II or III colorectal carcinoma.[24] In both studies, the selection of markers to be carried forward to the validation studies was based on the minor allele frequency and location in a haplotype block in the Asian population, and only non-synonymous SNPs were included. Considerable differences in allele frequencies exist between Asian and European populations for the relevant genetic markers from both our and Kim's analyses, which in part may explain the lack of overlap between our results.[19] Also, considerably larger treatment heterogeneity was present in their population, thereby possibly diluting the effect of germline genetic markers on treatment efficacy.

Our study is subject to several limitations. Interpretation of GWA studies in general is hampered by the need for large patient populations, to ensure sufficient power to detect genotype effects. With 547 patients, our cohort used for this GWA study is larger than many other pharmacogenetic study populations. Non-significant results could therefore lead to the conclusion that indeed none of the tested SNPs are associated with PFS upon CAPOX-B treatment in mCRC. However, the power to detect associations depends not only on population size, but also on the size of a SNP-effect and on genotype frequency. Despite a relatively large sample size, false negative results could arise for SNPs with low minor allele frequencies. In fact, minor allele frequencies of the three topmost significant SNPs from the overall analyses are only 0.10 in our population, which may well have led the results to be non-significant, despite an actual effect on PFS.

Obviously, it can be argued that rare variants or markers with a small effect on treatment efficacy are not clinically relevant, irrespective of their statistical significance level. However, genetic predisposition is unlikely to depend on only a few SNPs for most drug effects. Rather, multiple genetic markers could attribute in small amount and interact with each other.[26;27]

Their combined effect, in conjunction with clinical and pathologic parameters, could be of value in predictive models. Such a model has recently been proposed for colorectal cancer risk assessment.[28] In future, similar models should be constructed for predicting treatment response as well as adverse events for diverse systemic treatment regimens. In this scenario, any SNP influencing the complex trait 'drug efficacy' is of value.

Another drawback of GWA analyses is that these studies only focus on the influence of genetic variation, without taking into consideration most patient and tumor characteristics. Whereas in the time the CAIRO2 trial was performed cetuximab was prescribed to unselected patients, regardless of tumor characteristics, it was shown shortly thereafter that patients carrying a somatic mutation in *Kras* codon 12 or 13 are resistant to treatment with cetuximab.[29–32] More recently, patients with mutations in *Nras* were also shown not to benefit from treatment with anti-epidermal growth factor antibodies.[33] More somatic mutations precluding EGFR inhibitor efficacy may be discovered.

At the time the study was conducted, EGFR inhibitor therapy was not restricted to *Kras* wild type tumors only. However, *Kras* mutation status has been analyzed restrospectively in those patients for whom tumor DNA was available. For this subgroup, we performed additional analyses, taking *Kras* codon 12 and codon 13 alleles into consideration. We found that rs885036 remained an independent predictor for PFS, with only a small change in HR (HR 0.466 without *Kras*, HR 0.472 with *Kras* as a covariate). Recently, it was found that patient survival in colorectal cancer is influenced by different molecular subtypes, including not only *Kras* mutation status, but several other genetic and epigenetic factors.[34] Because molecular subtype was not determined in the CAIRO2 population, we cannot completely exclude an effect of molecular subtype on the outcome of our analyses. However, there is no evidence in literature for an association between *GnT-IVa* genotype and colorectal cancer molecular subtype.

Contrary to practice in clinical trials, serum LDH level at baseline, although strongly correlated to PFS[7], was not included in our multivariate model. Confounding by the prognostic effect of LDH cannot be excluded, since there was a slight overrepresentation of patients with increased LDH levels in several genotype groups with shorter PFS (data not shown). LDH level in itself may be a predictive as well as a prognostic factor, and not completely independent of germline genotype. Serum LDH-levels have been associated with intratumoral gene expression of vascular endothelial growth factor type A (*VEGFA*) and vascular endothelial growth factor receptor type 1 (*VEGFR1*).[35] Both phenomena are thought to be the result of stimulation of hypoxia-inducible factor 1α (HIF-1α), due to the intratumoral aerobic glycolysis known as the Warburg effect.[36] Results of a recent study suggest that, although high serum LDH levels correlate with reduced survival in colorectal cancer patients, treatment with bevacizumab can improve PFS for patients with high pretreatment LDH levels to that of patients with normal baseline LDH levels.[37] Because an interaction of LDH with genotype cannot be excluded, and inclusion of LDH in the experimental model could possibly have obscured genotype effects, we chose not to correct for the effect of LDH level at baseline in this study.

Conclusion

In this GWA study, investigating the effect of genetic variation on PFS on first line CAPOX-B with or without cetuximab in mCRC, we found one marker with a significant effect on treatment efficacy, with opposing results in each treatment arm.

This SNP, rs885036, may be of predictive importance for patients treated with cetuximab-containing regimens, and should be further validated in patients with *Ras* wildtype tumors. Patients in our sample carrying the rs885036 GG genotype seem to respond better to CAPOX-B plus cetuximab, whereas patients with the AA genotype benefit more from CAPOX-B alone. Therefore, it is possible a subgroup of patients carrying the GG genotype may benefit from the addition of cetuximab to CAPOX-B, contrary to overall findings for the general study population of CAIRO2 and study with comparable design.[7;38] Whether this marker also predicts PFS in patients treated with cetuximab monotherapy should be subject to further research. We formulated a hypothesis for the pathophysiological basis of this effect, which we are now testing in preclinical studies.

In addition, a cluster of SNPs on chromosome 8 with very low P-values was found, which may also have functional significance. For these SNPs, we cannot differentiate between a prognostic effect of these polymorphisms on PFS and a predictive effect in relation to chemotherapy. These results should also be validated in other populations.

Acknowledgements

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9

General discussion and future research

General discussion

Over the last decades, colorectal cancer incidence has risen worldwide.[1] In the Netherlands, 9,301 patients were diagnosed with colorectal cancer in 2001, increasing to 13,408 new diagnoses in 2012.[2] Although with the initiation of population-based screening programs a reduction in both colorectal cancer incidence and mortality is anticipated, based on earlier studies a considerate number of patients is still expected to present with interval tumors[3], underscoring the importance of continuing research for optimal treatment strategies.

In the struggle to reduce colorectal cancer mortality, it is not only important to develop new therapeutic strategies, but it is equally essential to optimize the use of currently available treatment modalities. Large costs are associated with anti-cancer treatment, both in relation to quality of life and in financial terms. Patients frequently experience severe and debilitating side-effects, even without any beneficial treatment effect. On the other hand, health care costs for colorectal cancer treatment are exceedingly high, especially for patients with advanced stage disease.[4] Therefore, pretreatment predictors are required, to identify patients with the best likelihood of treatment response, as well as those who are most susceptible to toxicity.

Pharmacogenetics, studying the effect of heritable germline genetic variation on response to drug treatment, may provide such a tool. For this thesis, we studied the association of germline polymorphisms with effects of capecitabine and oxaliplatin in patients with advanced colorectal cancer (ACC).

General pharmacogenetic considerations

The term "pharmacogenetics" entered medical literature as early as 1961.[5] Since then, this research field has evolved rapidly and, at the completion of this thesis, more than 12,000 articles are indexed in Pubmed for the word "pharmacogenetics". **Chapter 2** summarizes the literature regarding pharmacogenetic studies in colorectal cancer available at the start of the research leading up to this thesis. It provides an overview of the understanding of genetic variation in pathways involved in anti-cancer drug effects, not only for capecitabine and oxaliplatin, but also for irinotecan and the newer targeted drugs bevacizumab and cetuximab. Since the writing of the article, many newer studies have been published, and our knowledge on the effects of germline genetic variation on treatment outcome in colorectal cancer is continuously growing.

Pharmacogenetic studies aim at understanding the influence of variation in germline DNA on inter-patient differences in drug effects. Preferably, DNA for these studies should be derived from healthy tissues, such as peripheral blood leucocytes or buccal swabs. However, many of the early pharmacogenetic studies in colorectal cancer have used tumor tissue as the primary source of DNA for their analyses. This approach does not account for the potential bias of somatic mutations or loss of heterozygosity (LOH) in the tumor, and differences between tumor and germline genotype could potentially explain the often contradicting results between pharmacogenetic publications. Studies for the association of *CYP2D6* genotype

with efficacy of tamoxifen in breast cancer have occasionally found significant deviation from Hardy-Weinberg equilibrium (HWE) in tumor material, possibly due to hemizygous chromosomal deletions in this gene.[6-9] This has led some authors to conclude that studies with pharmacodynamic endpoints should not use tumor material as a source of DNA.[6] In **chapter 3** we addressed this possible cause of confounding, by comparing genotyping results in DNA isolated in peripheral blood leukocytes, with results in DNA extracted from archived colorectal cancer tumor samples in the same patients. Analyses were restricted to a defined set of genetic markers that have been frequently selected for pharmacogenetic studies in colorectal cancer, and could successfully be genotyped in all samples.

We found that only 1.4 percent of all blood-tumor pairs showed discordant results. We then evaluated if these discrepancies could be the result of LOH, using heterozygous loci adjacent to the SNP of interest as a marker for chromosomal loss. In this way, we showed that only half of the mismatches could have been induced by LOH.

Of note, we used macro-dissection for the collection of DNA from colorectal tumor tissue, which may have unintentionally led to the inclusion of significant amounts of germline DNA from healthy stromal tissue in samples designated as tumor DNA. This may alternatively explain the high level of agreement between both sample types. Although this mixture of DNA types could have been prevented by the use of micro-dissection for DNA collection from tumor samples, this is not the method used by most previous studies. Furthermore, it was not our goal to rule out any somatic variation in tumor DNA for these polymorphisms. Regardless if the concordance is a reflection of a large stromal component or of actual agreement between germline and tumor genotype, our analyses showed that results from previous pharmacogenetic studies using DNA from macro-dissected tumor tissue, can reliably be compared with those from newer studies using blood-derived DNA. However, the inferior DNA quality in tumor-derived DNA often leads to lower call-rates. Therefore, for future pharmacogenetic research, peripheral blood should be the preferred source of DNA for genotyping.

Pathway-based approach

Capecitabine

Cytotoxicity for fluoropyrimidines is exerted, at least in part, by binding of the active metabolite, fluorodeoxyuridine monophosphate (FdUMP), to thymidylate synthase (TS). This prevents the formation of 20-deoxythymidine-50-monophosphate, an essential precursor for DNA synthesis. For the binding of FdUMP to TS, 5,10-methylenetetrahydrofolate (5,10-MTHF) is required as an essential cofactor. The amount of 5,10-MTHF available for binding to TS is under the direct influence of methylene hydrofolate reductase (MTHFR).

In **chapter 4**, we hypothesized that polymorphisms in the gene encoding for MTHFR may affect capecitabine cytotoxicity in colorectal cancer patients, by increasing the availability of 5,10-MTHF and thereby the complex formation with TS. Two common, functional polymorphisms in *MTHFR* (*MTHFR* 677C>T and *MTHFR* 1298A>C) were evaluated for their association with capecitabine-induced toxicity. We found no effect of these polymorphisms on

the incidence of severe adverse events in our analyses. Whether this absence of association is due to the overshadowing effect of other genetic and non-genetic influences, or whether there is indeed no effect of these polymorphisms on capecitabine cytotoxicity, cannot conclusively be answered by our research. However, correction for common polymorphisms in the gene encoding for TS, which have been suggested to influence fluoropyrimidine cytotoxicity, did not alter our results. It should be noted, patients who experienced severe toxicity during adjuvant treatment with 5-FU or capecitabine, may not have entered the CAIRO study and this selection could also confound our pharmacogenetic analyses. Nevertheless, if this selection had been due to a specific *MTHFR* allele, the genotype distribution at baseline would not have adhered to Hardy-Weinberg equilibrium. Therefore, we advocate that *MTHFR* SNPs are not useful in the pretreatment prediction of capecitabine-induced adverse effects (or treatment efficacy) in ACC patients.

In addition to *MTHFR* genotype, other polymorphisms haven been suggested to explain the variation in individual response to capecitabine. Recently, a genome wide association study (GWAS) in lymfoblastoid cell lines identified new possible markers for capecitabine cytotoxicity.[10] The most noticeable result was for a SNP, rs4702484, located near the gene encoding for 5-methyltetrahydrofolate-homocysteine methyltransferase reductase (*MTRR*), which has been studied previously for its effect on fluoropyrimidine-induced cytotoxicity, and for its potential role in colorectal carcinogenesis.[11;12] In **chapter 5** we attempted to confirm the influence of the most common *MTRR* polymorphisms, as well as four other promising SNPs from the aforementioned GWAS, on efficacy of capecitabine in our population of ACC patients from the Dutch CAIRO trial.[13] Although rs4702484 showed a borderline significant association with increased progression free survival (PFS) for carriers of the variant allele, this effect was only present in univariate analysis in patients treated with capecitabine monotherapy. Thus, even if this marker affects capecitabine cytotoxicity, its effect is overshadowed by the influence of clinical patient characteristics and it is lost in combination therapy. This also illustrates that results from GWAS, especially *in vitro* studies, are difficult to replicate in clinical practice. Our relatively small cohort size may have compromised the power to detect genotype effects. However, for almost all analyses, no trend could be discovered for a genotype effect on treatment efficacy and it is unlikely that increasing sample size would have led to significant results. We therefore conclude that none of the tested genetic markers are helpful in the pre-treatment prediction of efficacy of capecitabine in ACC patients.

Oxaliplatin

The addition of oxaliplatin to fluoropyrimidine treatment has considerably increased survival for patients with metastatic colorectal cancer.[14] However, treatment with oxaliplatin is often hindered by the occurrence of neurotoxicity, leading to dose reductions or treatment discontinuation. Preclinical data suggest that there is a relationship between the presence of specific organic cation transporters (OCTs) in dorsal ganglia, responsible for cellular uptake of oxaliplatin, and oxaliplatin-induced peripheral neuropathy. For instance, the competitive

OCT2 inhibitor cimetidine not only impairs cellular uptake of oxaliplatin *in vitro*[15], but also reduces neuropathic symptoms in oxaliplatin-exposed mice.[16] However, results from *in vitro* and *in vivo* studies are not in complete agreement on which cation transporter is most likely to be involved in oxaliplatin uptake in dorsal ganglia.[15;16] We theorized that SNPs in the genes encoding for three cellular transporters (*SLC22A1*, *SLC22A2* and *SLC47A1*) could explain the divergent expression of neurotoxicity between patients treated with oxaliplatin. This hypothesis was tested in **chapter 6**. A functional, non-synonymous SNP in OCT1, *SLC22A1* Arg61Cys, was associated with a low risk of severe oxaliplatin-induced neurotoxicity for patients carrying two variant alleles, even after correction for cumulative oxaliplatin dose. Unfortunately, the low population frequency of only 2 percent homozygote variant carriers, in combination with a small effect size, impairs the implications of this marker for clinical practice. Moreover, because our study was hindered by a low genotyping call rate, as well as a suboptimal clinical scoring system for neurotoxicity, this result needs to be validated in independent treatment cohorts and mechanistic and functional studies before implementation into clinical practice.

The anti-tumor effect of oxaliplatin stems from binding of diaminocyclohexane (DACH)-platinum (Pt) to the DNA helix, causing Pt-DNA cross-links, and ultimately leading to programmed cell death. This process is inhibited by the action of cellular DNA repair mechanisms, such as the Excision Repair Cross Complementation type I (ERCC1). **Chapter 7** focuses on the hypothesis that a common synonymous SNP in *ERCC1 (ERCC1* C118T) alters oxaliplatin cytotoxicity depending on genotype, through an effect on DNA repair capacity. Transfection experiments were performed, assessing the effect of *ERCC1* C118T on DNA repair capacity *in vitro.* In addition, we performed a clinical association study for the effect of this SNP on survival upon oxaliplatin-based chemotherapy in ACC patients from the CAIRO study.[13] We showed that both *in vitro* cell survival after oxaliplatin-administration, and DNA repair-capacity of the transfected cells, depend on the presence of a functional *ERCC1* gene. However, we found that *ERCC1* C118T neither changes the *in vitro* capacity for DNA repair, nor affects survival of ACC patients receiving treatment with oxaliplatin. Strictly speaking, the population size for the clinical association study was not sufficiently powered to definitely rule out any effect of *ERCC1* genotype on patient survival. However, our results concur with a meta-analysis published in 2011, showing that PFS following oxaliplatin-based chemotherapy for colorectal cancer patients was not influenced by *ERCC1* C118T genotype in Caucasian populations.[17] Although transfection experiments do not account for the normal cellular regulation of gene expression, *in vivo* ERCC1 functionality depends on the co-expression of ERCC4. Any super-natural ERCC1 protein levels due to unregulated plasmid derived transcription would therefore not have resulted in more effective DNA repair in our experiments. We therefore believe both the clinical and the transfection model provide valid evidence that *ERCC1* C118T does not alter cellular or clinical response to oxaliplatin.

Genome wide approach

In chapters 4 to 7, a pathway-based, candidate gene approach was used to identify predictive markers for efficacy and toxicity of capecitabine and oxaliplatin. However, this approach is restricted by the limited knowledge of the pathways and genes involved in individual drug response. Therefore, as described in **chapter 8**, we performed a GWAS to identify novel markers for the prediction of PFS on treatment with a multidrug schedule for ACC. For this study, patients were accrued from the CAIRO2 trial, which compared treatment with capecitabine, oxaliplatin and bevacizumab (CAPOX-B) with CAPOX-B plus cetuximab.[18] One marker on chromosome 2 showed a significant effect on PFS, that was opposite in patients treated with CAPOX-B, compared to those treated with cetuximab in addition to CAPOX-B. This marker could therefore be a potential marker for cetuximab efficacy in ACC. This SNP is located in *GnT-IVa*, in an intronic region that is a predicted binding site for microRNA-34A. We presented a pathophysiological hypothesis based on this remarkable finding, which will be further evaluated in pre-clinical studies. Even if a functional effect of this marker is validated in laboratory experiments, these results should also be replicated in clinical patient cohorts. However, since cetuximab is no longer prescribed in combination with bevacizumab, and it is often included in second- or third-line treatment only, finding a patient cohort similar to ours is extremely difficult.

Future research

Colorectal cancer survival is highly dependent on tumor stage. For patients with stage I-III disease, treatment is aimed at curation by surgical removal of the tumor, mostly followed by adjuvant systemic treatment for patients with stage III or high risk stage II disease.[19;20] Over time, the boundaries of what is considered to be curable disease have broadened. In current practice, not only liver metastases, but also solitary lung metastases are often treated with localized therapies in hope of curation.[19] However, for most patients with metastatic disease, treatment still consists of systemic therapy with palliative intent.

Unfortunately, there is great disparity in individual response to chemotherapy, both in terms of efficacy and toxic events. Because of the poor prognosis of ACC, and the increasing emphasis on quality (rather than prolongation) of life, it can be questioned whether this disease should be treated by a one-size-fits-all regime. If the goal is to offer the most effective, least toxic therapy to each individual upfront, we need a form of personalized medicine.

Ideally, in the near future, the choice for a specific treatment will become tailor-made, taking into account a multitude of biomarkers, as well as clinical factors, such as age, renal function, co-medication and patient preference. Pharmacogenetics provides valuable pre-treatment markers of efficacy and toxicity, and is slowly beginning to enter clinical practice. It is now generally accepted that carriers of the rare *DPYD**2A allele are prone to severe fluoropyrimidine-induced toxicity, and should be treated with reduced dose or alternative

treatment.[21;22] Patients carrying the variant *UGT1A1*28* allele are at increased risk of febrile neutropenia when treated with irinotecan, and dose reductions are advised.[21-23] A genotype-based dosing system has recently been proposed according to *UGT1A1*28* genotype, which is a further step toward genotype-guided, personalized cancer-care.[24]

In addition to its merits for clinical practice, pharmacogenetic research could also aid in the development of new drugs. Currently, new agents are still tested in genetically heterogeneous populations, without recognition of genotype-phenotype interactions. In case of insufficient survival benefit or intolerable toxicity for the total study population, the drug will not enter clinical practice. However, the treatment under investigation could be a safe and effective option for a genetically distinct subset of patients, and its development could be continued for this specific group. In fact, it has been shown that such stratification markers improve the success rate of drug development programs.[25]

Our GWAS results, described in **chapter 8**, also provide support for this assumption. Although the addition of cetuximab to CAPOX-B in first-line treatment of ACC has a negative effect on survival in unselected, genetically heterogeneous populations[18;26], we found that carriers of the variant allele of the common SNP rs885036, conversely, may benefit from the addition of cetuximab. Although these data are preliminary and need to be further validated, they illustrate that analysis of germline genetic variation could indeed identify groups of patients who differ significantly in their response to a specific drug regimen.

As our knowledge of the genome is increasing, so are the technological possibilities for genotyping. Whereas the original dogma was that our genome was made up of protein-encoding genes surrounded by non-functional DNA[27], it was later discovered that it harbors a vast amount of non-coding RNA isoforms, involved in regulation of transcription.[28] New technologies for genotyping have been developed, allowing us to include these former "gene deserts" into our analyses. GWAS address between 500,000 and 1,000,000 SNPs across the genome, although analyses are usually restricted to polymorphisms with a population frequency of >0.05. In addition, next generation sequencing (NGS) offers the potential of genotyping all coding regions, or even the whole genome.[29]

Even if the proof of principle has been delivered, cost-effectiveness and clinical utility of pharmacogenetics continue to be questioned.[30] Cost-effectiveness is determined by many different aspects, including drug price and cost of genotyping. Costs for whole genome genotyping with NGS have decreased from $95.000.000 in 2001, to $4.000 in 2014.[31] For various clinical purposes, a multitude of SNPs are tested at the same time, with complete arrays at less than €500 per patient.[32] Both these arrays and NGS yield information on a myriad of polymorphisms, important for drugs currently prescribed to the patient, but also for potential future prescriptions. This reduces cost per genotyped SNP to only a few cents. Independent of financial cost, clinical utility is dependent on prevalence and penetrance of the allele in question, test specificity and sensitivity, cost of an alternative drug, and on spendings saved by increased survival or better quality of life.[33] Therefore, clinical applicability is not the same for all markers, but dependent on the characteristics of the SNP, the population in which it is

tested, the drug and the disease under investigation. With reducing genotyping cost per SNP associated with NGS, and with increasing drug cost, the break-even point will be met more easily in future.

Despite pharmacogenetics finding its way into clinical practice, we do not know yet which amount of inter-individual variation in drug response can ultimately be explained by genetic variation, and how many different loci influence each drug effect. Although past studies showed that heritability explained around 97-99 percent of the variation in elimination of number of non-cancer drugs[34-36], an *in vitro* study using lymphoblastoid cell lines found that heritability of 5-FU cytotoxicity is only 26-65 percent, depending on the administered dose.[37] In contrast, *DPYD**2A alone predicts 50 percent of all cases of grade IV febrile neutropenia in patients treated with standard dose 5-FU.[38] The degree to which germline pharmacogenetics explains drug behavior is likely to depend not only on the drug and gene at hand, but also on the administered dose, the method of administration and whether the endpoint is efficacy or toxicity. Furthermore, epigenetic regulation may even lead to day-to-day variations in genetic influences on drug behavior.

The influence of genetic variation on drug behavior is best analyzed in extreme phenotype populations, because of the large effect size. Patients experiencing severe toxicity on chemotherapeutic treatment form such a population. On the one hand, extreme phenotypes could be explained by a small number of rare polymorphisms, each individually evoking the phenotype in a proportion of patients, through various mechanisms. Although rare variants often embody protein-changing mutations, and are therefore predisposed to causing extreme phenotypes, half of all variants at a minor allele frequency of 0.5 percent are found in only one single ethnic population, restricting the world-wide implementations in pharmacogenetic guidelines.[39] The effect of *DPYD**2A, with a population frequency of only 1.8 percent heterozygotes in Western populations and a large effect on 5-FU induced toxicity, fits this rare variant hypothesis.[40] Also consistent with the hypothesis, this SNP is not found in Asian populations and therefore cannot explain fluoropyrimidine-induced toxicity in Asian cancer patients.[41]

On the other hand, extreme phenotypes may be explained by a multitude of different polymorphisms with a higher population frequency, all causing a very small fraction of the variation in drug response in each affected individual. Chemotherapy-induced toxicity is not an ordinal endpoint, but rather a continuous scale, ranging from minor complaints to severe and life-threatening events. Although the array of possible outcomes could be explained by the effect of many different rare variants with an equal number of different effect sizes, it is better explained by this common variant hypothesis. Because we are far from understanding all processes and gene products involved in individual drug behavior, we need a hypothesis-free approach to unravel all of these contributing variants. It is only because of modern technologies such as GWAS or NGS, that we are now able to perform such broad searches. However, because of the relatively small effect sizes, very large patient populations are needed to identify and validate these markers, before incorporation into clinical practice. This necessitates the inclusion of pharmacogenetic research into all clinical trials involving systemic anti-cancer

treatment. For definite conclusions on these small effect genetic markers, the formation of consortia and the conduction of meta-analyses from observational studies is indispensable. A dedicated, randomized controlled trial, withholding genotyping in half of the patients, may be regarded unethical if retrospective evidence for the genotype-phenotype interaction is overwhelming. Consequently, focus should also be shifted from clinical validation to gathering functional proof by laboratory studies.

In conclusion, results of pharmacogenetic research are already being incorporated into clinical practice of anti-cancer therapy. In a survey in 2012, more than two thirds of oncologists in the United States reported using a pharmacogenetic test in the previous six months.[42] In future, new technological possibilities, increasing availability and decreasing financial costs of genotyping will further increase the applicability of pre-emptive pharmacogenetic testing for clinical practice. Current knowledge on genes, and molecular and clinical effects is now integrated into pathways and registered online, in the Pharmacogenomics Knowledge Base (PharmGKB).[43] Implications for clinical practice are being formulated by consortia[21;44], and the applicability for clinical practice is under current investigation.[32]

For patients with advanced colorectal cancer, tailoring therapy is of great importance, because of the small window of opportunity for effective treatment, and the burden of adverse effects associated with anti-cancer drugs. Through its incorporation into drug development programs and clinical trials, and through collaborating efforts for the introduction into clinical practice, pharmacogenetics will help maximize the chances of efficacy and minimize the risks of adverse reactions for all patients.

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Summary

This thesis describes the results of pharmacogenetic studies for the association of germline genetic polymorphisms with drug effects of capecitabine and oxaliplatin in patients with advanced colorectal cancer (ACC) from the CAIRO and CAIRO2 study.

The first section of the thesis focuses on general pharmacogenetic considerations.

Chapter 2 gives an overview of information from pharmacogenetic publications available until the time when the research leading up to this thesis was initiated. In addition to pharmacogenetic considerations for capecitabine and oxaliplatin, irinotecan and the targeted drugs bevacizumab and cetuximab are also addressed.

In the analyses described in **chapter 3**, we compared genotyping results in DNA samples extracted from peripheral blood derived leukocytes, with results in DNA extracted from archived colorectal tumor samples in the same patients. We found an almost complete concordance between both sample types for the selected polymorphisms, with only 1.4% of sample pairs showing unequal genotypes. Conflicting results were shown to result from logistic errors, rather than somatic mutations or loss of heterozygosity in at least half of all discordant sample pairs.

In the second section of this thesis, four candidate-gene studies are described. Capecitabine pharmacogenetics is addressed first, followed by two studies focusing on the pharmacogenetics of oxaliplatin.

In **chapter 4**, we investigated the effect of two common polymorphisms in the gene encoding for methylene tetrahydrofolate reductase (*MTHFR* 677C>T and *MTHFR* 1298 A>C) on capecitabine-induced adverse events. We found that neither polymorphism was associated with the incidence of severe (grade 3 or higher) toxicity, regardless whether expressed as overall toxicity, or specific adverse events, including hand-foot syndrome, diarrhea and febrile neutropenia. Also, no effect of these polymorphisms on patient survival upon capecitabine treatment for ACC was found.

Chapter 5 describes a study for the effect of eight germline polymorphisms on efficacy of capecitabine in ACC patients. Four single nucleotide polymorphisms were selected based on their location in the methionine synthase reductase (*MTRR*) gene, and another four markers were included because of their significance in a recently published *in vitro* genome wide association study (GWAS). Our results showed, however, that none of the selected markers are useful predictors of capecitabine efficacy in ACC.

In **chapter 6**, results are presented for our study investigating the association of variation in the genes encoding for organic cation transporter 1 (OCT1, *SLC22A1*), OCT2 (*SLC22A2*) and the human multidrug and compound extrusion protein 1 (hMATE1, *SLC47A1*) on the incidence and severity of oxaliplatin-induced neurotoxicity. We found that homozygote carriers of the rare allele of *SLC22A1* Arg61Cys had a reduced risk of severe neurotoxicity,

regardless of the cumulative dose of oxaliplatin. None of the other selected polymorphisms were associated with oxaliplatin-induced neurotoxicity.

Chapter 7 focuses on the effect of the common, synonymous SNP *ERCC1* C118T on DNA repair capacity after administration of oxaliplatin. Using *in vitro* transfection experiments, we showed that this SNP does not affect cellular DNA repair, or cell survival upon oxaliplatin administration. In addition, our clinical association analysis in ACC patients found no effect of *ERCC1* C118T genotype on patient survival upon second-line or third-line treatment with oxaliplatin.

After the pathway-based analyses in the previous chapters, the last section of this thesis describes a study applying a genome wide association approach.

Chapter 8 describes a GWAS searching for germline genetic markers for the prediction of progression free survival (PFS) of ACC patients treated with either capecitabine-oxaliplatin-bevacizumab (CAPOX-B), or CAPOX-B plus cetuximab. We found that a cluster of SNPs on chromosome 8 that was associated with PFS, with almost genome wide significance. More importantly, a marker on chromosome 2 showed a significant effect on PFS, that was opposite in both treatment arms. The minor allele was associated with increased PFS in patients receiving CAPOX-B plus cetuximab, but a reduced PFS in patients treated only with CAPOX-B.

Samenvatting

Dit proefschrift beschrijft de resultaten van een aantal farmacogenetische studies naar de associatie van kiembaanmutaties met het effect van capecitabine en oxaliplatin bij patiënten met gevorderd colorectaal carcinoom, uit de CAIRO en CAIRO2 studies.

Het eerste gedeelte van het proefschrift richt zich op een aantal algemene farmacogenetische beschouwingen.

Hoofdstuk 2 geeft een overzicht van de beschikbare informatie uit farmacogenetische publicaties, op het moment dat het onderzoek uitmondend in dit proefschrift werd gestart. Naast farmacogenetische overwegingen ten aanzien van capecitabine en oxaliplatin, wordt daarin ook aandacht besteed aan irinotecan en de zogenoemde doelgerichte of "targeted" middelen bevacizumab en cetuximab.

Bij de analyses die in **hoofdstuk 3** worden beschreven, vergeleken we resultaten van genotypering in DNA geïsoleerd uit leukocyten in perifeer bloed, met de genotyperingsresultaten in DNA uit gearchiveerde tumormonsters van dezelfde patiënten. We vonden een vrijwel complete overeenkomst tussen beide typen monsters, waarbij slechts 1,4% van de monsterparen een verschil in genotype lieten zien. Van de tegenstrijdige resultaten kon worden aangetoond dat deze voor minimaal de helft op logistieke onvolkomenheden berustten in plaats van op somatische mutaties of verlies van heterozygotie in de tumor.

In het tweede gedeelte van dit proefschrift, worden vier kandidaat-gen studies beschreven. Eerst wordt aandacht besteed aan de farmacogenetica van capecitabine, daarna volgen twee studies die zich richten op de farmacogenetica van oxaliplatin.

In **hoofdstuk 4** onderzochten we de associatie van twee veelvoorkomende polymorfismen in het gen dat codeert voor methyleentetrahydrofolate reductase (*MTHFR* 677C>T en *MTHFR* 1298A>C) met bijwerkingen ten gevolge van capecitabine. Geen van beide polymorfismen was gerelateerd aan de incidentie van ernstige (graad 3 of hoger) toxiciteit; noch voor totale toxiciteit, noch voor specifieke bijwerkingen, waaronder hand-voet syndroom, diarree en febriele neutropenie, werd een associatie gevonden. Ook werd geen effect gezien van deze polymorfismen op de overleving na behandeling met capecitabine bij gevorderd colorectaal carcinoom.

Hoofdstuk 5 beschrijft een studie naar het effect van acht kiembaanpolymorfismen op de effectiviteit van capecitabine bij patiënten met gevorderd colorectaal carcinoom. Vier van deze polymorfismen werden geselecteerd op basis van hun ligging in het gen dat codeert voor methylsynthase reductase (*MTRR*). Vier andere werden geïncludeerd, vanwege hun significantieniveau in een recent gepubliceerde *in vitro* genoomwijde associatie studie (GWAS). Onze resultaten lieten echter zien dat geen van deze polymorfismen een zinvolle voorspeller is voor de effectiviteit van capecitabine bij het gevorderd colorectaal carcinoom.

In **hoofdstuk 6** worden de resultaten gepresenteerd van onze studie naar de associatie van variatie in de genen coderend voor organische cation transporter 1 (OCT1, *SLC22A1*),

OCT2 (SLC22A2) en de human multidrug and compound extrusion protein 1 (hMATE1, *SLC47A1*), met de incidentie en ernst van oxaliplatin-geïnduceerde neurotoxiciteit. Hierbij vonden we dat homozygote dragers van het zeldzame variant allel van *SLC22A1* Arg61Cys een verminderd risico hebben op het ontwikkelen van ernstige neurotoxiciteit, onafhankelijk van de toegediende cumulatieve dosering oxaliplatin.

Hoofdstuk 7 richt zich op het effect van de veelvoorkomende, synonieme SNP *ERCC1* C118T op de capaciteit tot DNA herstel na toediening van oxaliplatin. Onze *in vitro* transfectie experimenten lieten zien, dat deze SNP geen invloed heeft op het cellulaire DNA herstel, of op de cellulaire overleving na toediening van oxaliplatin. Daarnaast toonde onze klinische associatiestudie aan, dat *ERCC1* C118T genotype geen effect heeft op de overleving van patiënten met gevorderd colorectaal carcinoom, wanneer zij worden behandeld met tweede- of derdelijns therapie met oxaliplatin.

Na de kandidaat-gen studies in de vorige hoofdstukken, beschrijft het laatste gedeelte van dit proefschrift een studie waarin een genoomwijde benadering werd gebruikt.

Hoofdstuk 8 beschrijft een GWAS, waarin gezocht werd naar kiembaan genetische voorspellers voor de progressievrije overleving van patiënten met gevorderd colorectaal carcinoom, die behandeld werden met capecitabine-oxaplatin-bevacizumab (CAPOX-B), danwel met cetuximab in aanvulling op CAPOX-B. We vonden een cluster van SNPs op chromosoom 8 dat geassocieerd was met progressievrije overleving, waarbij het vooraf gestelde genoomwijde significantieniveau bijna werd bereikt. Tevens vonden we een SNP op chromosoom 2 dat significant geassocieerd was met progressievrije overleving, met een tegengesteld effect in de beide behandelarmen. Bij patiënten die behandeld werden met CAPOX-B en cetuximab was het variant allel geassocieerd met een verlengde progressievrije overleving, maar in patiënten die behandeld werden met alleen CAPOX-B juist met een verkorte overlevingsduur.

Curriculum vitae

Lieke van Huis – Tanja werd geboren op 12 november 1979 in Heerlen. Haar middelbare schooltijd doorliep zij op het Stedelijk Gymnasium in Leeuwarden, waar zij in 1998 haar eindexamen behaalde. In datzelfde jaar startte zij met haar studie Geneeskunde aan de Rijksuniversiteit in Groningen. Op de afdeling kinderhematologie van het Wilhelmina Kinderziekenhuis in Utrecht verrichtte zij haar afstudeeronderzoek (begeleider: dr. M.B. Bierings), waarna zij in september 2005 gelijktijdig haar doctoraalexamen en artsexamen behaalde.

Nadat zij ervaring opgedaan had als arts-assistent niet in opleiding tot specialist (ANIOS) op de afdeling interne geneeskunde van het Groene Hart Ziekenhuis in Gouda, startte zij in januari 2008 haar opleiding tot internist in ditzelfde ziekenhuis (opleider: dr. J.J.M. van der Heijden). In april van dat jaar begon zij in het Leids Universitair Medisch Centrum (LUMC) aan het onderzoek dat geresulteerd heeft in dit proefschrift. Zij werd hierbij begeleid door prof. dr. H.J. Guchelaar (afdeling Klinische Farmacie en Toxicologie), prof. dr. A.J. Gelderblom (afdeling Klinische Oncologie) en dr. D.M. Kweekel (afdeling Klinische Farmacie en Toxicologie).

Vanaf januari 2010 zette zij haar opleiding tot internist voort in het LUMC (opleiders: prof. dr. J.A. Romijn; prof.dr. J.W.A. Smit; prof.dr. J.H. Bolk; prof.dr. J.T. van Dissel; prof.dr. J.W. de Fijter), waar zij vanaf 2011 bezighield met haar aandachtsgebied Medische Oncologie (opleiders: prof. dr. J.W.R. Nortier; prof. dr. A.J. Gelderblom). Sinds het afronden van haar specialisatie in juni 2014 is zij werkzaam als internist-oncoloog in het Diakonessenziekenhuis te Utrecht.

Lieke is getrouwd met Bram van Huis; zij zijn de trotse ouders van Merel.

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