Pharmacogenetics of irinotecan and oxaliplatin in advanced colorectal cancer

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GENERAL DISCUSSION AND CONCLUSION

Colorectal cancer (CRC) is a global health problem, accounting for 677,000 deaths on a yearly basis[1]. In The Netherlands, 12% of cancer-related deaths are due to this disease[2]. Although potentially curable in case of locoregional disease, many patients initially present with distant metastases, or develop distant metastases during follow-up. Currently, patients with metastatic disease are treated with palliative systemic treatment, consisting of a fluoropyrimidine (intravenous 5-fluorouracil or its oral derivatives capecitabine and UFT) with or without oxaliplatin or irinotecan, and the targeted agents bevacizumab or cetuximab. The aim of palliative systemic treatment is to prolong life expectancy, maintain or improve quality of life, and reduce tumor burden (in order to make metastases potentially resectable or to alleviate symptoms).

The earliest form of chemotherapy in metastatic CRC patients consisted of 5-fluorouracil (plus leucovorin) monotherapy, resulting in a median overall survival of approximately 12 months [3]. With modern treatments, the overall survival has almost doubled. Although this success is largely due to the currently available drugs, it may also be caused by the fact that systemic chemotherapy is nowadays being administered at earlier, asymptomatic stages because of its wider acceptance and more intensive screening procedures. Since there is no outright preference for a specific regimen, medical oncologists have various options to choose from. Several large clinical trials that address this issue have been launched. One of these, the CAIRO (**ca**pecitabine – **ir**inotecan – **o**xaliplatin) study of the Dutch Colorectal Cancer Group (DCCG), tested the sequential versus combined administration of capecitabine and irinotecan, followed by a regimen of capecitabine plus oxaliplatin [4]. Since initial combination treatment did not result in an improved overall survival, the results of this study indicate that the sequential administration of individual cytotoxics is a valid alternative to the more commonly used combination treatment. As expected, the progression-free survival during first-line treatment was longer in patients receiving combination treatment. The results of the CAIRO study were confirmed by the British FOCUS study, although the latter study tested this concept only for two of the three cytotoxics (5-fluorouracil with either irinotecan or oxaliplatin) [5]. However, many patients will not benefit from systemic treatment, and the toxicity of these treatments remains largely unpredictable. Therefore, in order to individualize treatment for patients with metastatic CRC, predictive markers for efficacy and toxicity are needed.

This thesis is subdivided into 3 parts, with each part addressing a different topic. In the first part, we show that it is possible to perform the simultaneous detection of two SNPs (single nucleotide polymorphisms) in the *XRCC1* gene. Furthermore, we investigated if it is possible to use paraffin-embedded, formalin-fixed colorectal tumor samples for the detection of germline SNPs in drug metabolism pathways. This is a relevant subject, since concordance of germline SNPs in peripheral blood and paraffin-embedded tumor samples has not been investigated before.

In the second part, we present an overview of the clinical parameters and pharmacogenetic factors that are associated with the toxicity of irinotecan. Next, we investigate if the *UGT1A1**28 variation may predict irinotecan antitumor efficacy, febrile neutropenia, or the need for dose reduction. We also studied pharmacogenetic parameters in correlation with the efficacy of irinotecan, in terms of progression-free survival. It has been suggested that elevated GSTP1 expression decreases irinotecan cytotoxicity. Therefore, we hypothesized that the *GSTP1* codon 105 SNP is associated with the clinical efficacy of irinotecan.

In the final part of this thesis, we focus on oxaliplatin (cyto)toxicity. First, we present an overview of the pharmacology of oxaliplatin and its association with pharmacogenetic factors. Next, we investigated the functional effects of the silent codon 118 SNP in *ERCC1* with regard to differences in DNA repair or survival after oxaliplatin exposure. In addition, we show that the cumulative neurotoxicity of oxaliplatin is not associated with the *GSTP1* codon 105 substitution. Finally, we present the results of our efforts to identify novel candidate genes associated with oxaliplatin efficacy or toxicity. In the following sections of the General Discussion we will summarize our results and discuss the implications of our findings for future research and clinical practice.

DNA FROM FORMALIN FIXED PARAFFIN-EMBEDDED TUMORS IS A VALID ALTERNATIVE TO EDTA-BLOOD, AND *VICE VERSA*

In many previously conducted trials of CRC, tumor biopsies were archived as formalin fixed, paraffin-embedded (FFPE) tissues. DNA is still preserved in these samples, but depending on the duration of fixation, it is more or less fragmented. Fragmentation can cause technical problems during genotyping, with lower success rates compared to DNA derived from peripheral blood. It is essential to choose primers which bind close to the region of interest to overcome this problem, but some genetic variations (repetitive elements such as in the *UGT1A1**28 and *TS* 28bp repeat genotypes) remain difficult to determine in DNA derived from FFPE. Therefore, most pharmacogenetic trials predominantly rely on peripheral blood samples for DNA isolation. However, one may argue that the use of tumor DNA is more informative when studying treatment efficacy. For these reasons, we studied a set of 11 pharmacological candidate genes in peripheral blood and tumor FFPE samples obtained from 149 advanced CRC patients.

We found that for *ABCB1* (rs1128503 and 1045642), *ABCG2* (rs2231142), *ERCC1* (rs11615), *MTHFR* (rs1801133), *RFC* (rs1051266), *ERCC1* (rs1799793 and 13181), *p53* (rs1042522) and *XRCC1* (rs25487), there were no statistically significant differences between FFPE tumor and peripheral blood DNA. For these genotypes, the use of FFPE is a valid alternative to peripheral blood, and it can be used in pharmacogenetic studies of CRC. Also, the findings validate the use of peripheral blood in CRC treatment efficacy studies, at least for the genetic variants mentioned. For the *GSTP1* rs1695 SNP, there was a small discrepancy (3.0%) that may be attributed to the effects of multiple testing. Indeed, when we applied the Bonferroni correction, the difference became nonsignificant. On the other hand, the difference may also be the result of loss of heterozygosity (LOH), although LOH at this chromosome is unusual [6]. We describe that for GSTP1, 6% of the tumors of heterozygote patients were genotyped as homozygote wild-type. If we assume that this is not the result of genotyping errors, this finding indicates that the tumor GSTP1 wild-type genotype may be related to survival benefit for tumor cells. One of the options for future mechanistic studies is therefore to compare cell growth of *GSTP1* knock-down cells containing plasmids that carry either the wild-type or the mutant sequence. These cells may also be transplanted into mouse models to compare

xenograft growth characteristics. Finally, large retrospective studies may be carried out that compare *GSTP1* genotype (determined in FFPE) with clinical outcome. This may shed some light on whether *GSTP1* confers a survival benefit to tumor cells, whether it is a prognostic marker and if so, by which mechanism this is accomplished.

*UGT1A1**28 PREDICTS FEBRILE NEUTROPENIA, BUT NOT IRINOTECAN EFFICACY OR DOSAGE

There are numerous studies investigating the association between irinotecan toxicity and the *UGT1A1* TA-repeat polymorphism. This genetic variation includes a 5-, 6-, 7- or 8-TA repeat in the UGT1A1 promotor, of which 6 or 7 repeats are most common in caucasians. The UGT1A1 enzyme catalyzes the glucuronidation of endogenous and exogenous compounds, the latter of which includes SN38 (the active metabolite of irinotecan) [7]. Patients who are homozygous for the 7 TA repeat sequence (alternatively called *28) have lower UGT1A1 production and, hence, lower metabolism of SN38 into its inactive glucuronide [8-11]. As a result, patients carrying the *28 allele may experience more benefit (in terms of anti-tumor efficacy) and/or toxicity from irinotecan treatment. Previous studies have indeed shown that neutropenia is more common in UGT1A1 *28 homozygotes [12-14]. For toxicity reasons, it is recommended that these patients receive an initial dose reduction to 75% of standard irinotecan dosage. However, it is unknown whether this dose reduction will result in a decreased toxicity risk, and at the same time preserve the anti-tumor efficacy.

In our study, we demonstrate that *febrile* neutropenia is strongly associated with the UGT1A1 *28 homozygote genotype. This is an important addition to current data, because febrile neutropenia may have clinical implications such as hospital admission and prescription of antibiotics. We also show that, when advanced CRC patients receive standard irinotecan doses, tumor response rates are similar among all *UGT1A1* genotypes. More specifically, the UGT1A1 *28 homozygotes did not experience more anti-tumor response or stable disease compared to the other genotypes. With respect to irinotecan dosage, we found that the number of dose reductions and treatment discontinuations were comparable among all genotypes. Severe diarrhea was the most frequently observed toxicity in our patients and was the main reason for treatment discontinuation or dose reductions.

Genotyping of the *UGT1A1**28 genetic variant may help to identify those subjects who have an increased risk of developing febrile neutropenia, which may allow preventive measures such as decreasing the dose or the use of growth factors. Dose reduction may be effective in preventing severe toxicity in all UGT1A1 *28 patients (although this has never been formally studied), but the remainder 90% of patients with other *UGT1A1* genotypes are unaffected by this measure and may still develop toxicity. As a result, if 100 patients are genotyped, toxicity may be avoided in 5 of them (because 50% of *28 homozygotes experienced grades 3-4 toxicity in our study), and toxicity such as severe diarrhea may still occur in about 30 other patients. Although there is a strong association between genotype and febrile neutropenia, the number needed to genotype (NNTG) to avoid one case of febrile neutropenia in *28 homozygotes is quite large in caucasian patients (about 50). Moreover, severe adverse events that occur in homozygote wild-type and heterozygote patients are not prevented by this measure (about 85% of all irinotecan-related toxicities).

Future studies should therefore focus on several issues: 1) How effective is dose reduction in preventing toxicity in UGT1A1 *28 homozygotes? 2) Can we predict severe diarrhoea more accurately, because this appears to be the major reason for dose reduction or discontinuation in most patients receiving irinotecan plus capecitabine? 3) Is there a role for growth factors in UGT1A1*28 homozygotes?

These questions will be addressed in the near future, while at this moment, efforts are undertaken to set up a consortium of collaborative irinotecan investigators. This consortium aims to combine the results of the largest pharmacogenetic studies involving irinotecan and *UGT1A1*. It is our aim, with the help of this consortium, to investigate combined toxicity that requires dose reduction (diarrhoea and febrile neutropenia), and to develop an algorithm that predicts which patients are at increased risk. The algorithm should include not only pharmacogenetic variables, but also those patient characteristics that are associated with toxicity, such as performance status and age. We aim to develop an algorithm that can be used by clinicians, in order to identify patients who are in need of clinical measures preventing irinotecan toxicity. After such a prediction model has been developed, it needs to be validated in an independent cohort of patients and tested in a prospective study.

GSTP1 ILE105VAL IS ASSOCIATED WITH IRINOTECAN EFFICACY

GSTP1 is an enzyme involved in the conjugation of various compounds to glutathione. It also has an important function in cell cycle regulation and apoptosis, through the JNK pathway [15]. Binding of GSTP1 to the JNK protein prevents activation of this pathway. A nonsynonymous codon 105 SNP in the *GSTP1* gene not only results in modification of a substrate binding site (resulting in lower enzymatic capacity of GSTP1 enzyme) [16], but one of the JNK binding sites is also affected [17]. It is not known whether irinotecan and SN38 are substrates of GSTP1, but *in vitro* data indicate that an elevated *GSTP1* expression in the nucleus protects a cell from irinotecan induced cytotoxicity [18]. We therefore hypothesized that the codon 105 SNP is associated with increased treatment efficacy in CRC patients receiving irinotecan.

Indeed, we found that patients carrying one or two variant alleles had longer progression-free survival (PFS) compared to homozygous wild-type patients receiving irinotecan and capecitabine. Also, patients had a longer median PFS when receiving irinotecan plus capecitabine instead of capecitabine monotherapy; except for patients who were homozygous wild-type. These patients had a comparable PFS, irrespective of treatment regimen. The addition of irinotecan to first-line therapy did not result in additional treatment efficacy for those patients, at least not in terms of PFS. On the other hand, they suffered similar incidence of grades 3 and 4 toxicity compared to the other patients. If confirmed, these findings may have important implications for the future selection of patients that are eligible for irinotecan treatment. One of our goals for future research therefore includes to set up a replication study to confirm these findings. This may also be done in cooperation with the other members of the aforementioned irinotecan consortium. Other research may involve functional studies of both *GSTP1* codon 105 variants with respect to the JNK pathway, and studies of irinotecan metabolism through the GSTP1 enzyme.

OXALIPLATIN DNA ADDUCTS ARE REPAIRED EQUALLY BY BOTH *ERCC1* C118 AND 118T GENETIC VARIANTS

Oxaliplatin causes formation of DNA adducts with platinum, which results in deregulation of cellular processes, inhibition of DNA replication and ultimately leads to cell death. Excision repair cross-complementing group 1 (ERCC1) enzyme is essential for the removal of platinum DNA adducts, and associations of SNPs in the *ERCC1* gene with the therapeutic efficacy of platinum analogues have therefore been studied in solid tumors [19-21]. One of these studies includes advanced CRC patients treated with second- or third-line oxaliplatin [22]. In this study, the *ERCC1* codon 118 CC genotype patients showed a favorable overall survival (OS) compared to the other patients. Another study demonstrated that *ERCC1* mRNA expression in colorectal tumors is inversely related to the therapeutic effect of oxaliplatin with respect to survival [19]. This is remarkable since the codon 118 SNP is synonymous and does not result in an amino acid change. However, this SNP is linked to the non-synonymous C8092A SNP that is also located in the 3' adjacent gene *CAST* (CD3ε-associated signal transducer). CAST is an RNA polymerase I-specific subunit and has a role in the activation of transcription [23]. In addition, it has been hypothesized that polymerase I may play a role in sensing for DNA damage, indicating a role for CAST in DNA repair [24]. It is hypothesized that the codon 118 SNP influences oxaliplatin efficacy either due to linkage disequilibrium, or due to translational differences resulting from the unusual AAT codon [25-27]. Interestingly, elevated *ERCC1* mRNA expression results in elevated ERCC1 protein levels only if its cofactor, *ERCC4*, is also overexpressed [28;29]. Therefore, instead of mRNA expression, we studied the more informative ERCC1 protein levels by an immunohistochemical method. We found no association of the C8092A or C118T SNPs with ERCC1 protein expression in colorectal tumors. Furthermore, functional studies revealed for the first time that *ERCC1*-deficient cell lines, transfected with plasmids containing the *ERCC1* gene harboring either 118C or 118T, were equally effective in the removal of oxaliplatin-induced DNA cross-links. Similarly, PFS in advanced CRC patients receiving second- or third-line oxaliplatin was not associated with the C8092A or C118T genotypes.

Our study aims to provide an overview of basic science (transfection experiments) to protein expression and clinical data analysis in a single patient population, rather than an isolated data presentation. However, our study does not support earlier reports that described survival benefits depending on *ERCC1* genotype; nor does it show functional relevance of the genotypes in *in vitro* or *in vivo* parameters. Therefore, there are a number of issues that need to be considered in future research regarding this topic. First, rather than studying the individual *ERCC1* genotypes, we could focus on the codon 118 and 3'-UTR SNPs as a haplotype, since both genetic variants are inherited jointly and may exert their effects (if any) together. This haplotype-based approach is currently under investigation with regard to the clinical data. Second, functional assays regarding the 3'-UTR (or *CAST*) SNP need to be conducted in suitable cell lines, i.e. *CAST*-knockdown cells. A possible mechanism to create such knockdown cells is through the use of siRNA directed against *CAST* mRNA. Apart from the mentioned C8092A SNP, there may be other SNPs in *CAST* that are in linkage disequilibrium with this genetic variation; these SNPs should be included in haplotype analysis and functional studies as well.

GSTP1 ILE105VAL IS NOT ASSOCIATED WITH OXALIPLATIN EFFICACY OR NEUROTOXICITY

The metabolism of oxaliplatin is complex. One of the biotransformation routes includes conjugation to glutathione by the GSTP1 enzyme. A number of studies have investigated a possible association of oxaliplatin treatment efficacy (in terms of survival) or toxicity with the *GSTP1* codon 105 SNP Ile>Val. However, results are contradicting with respect to survival (longer overall survival (OS) in Val/Val patients [30] or no association with OS [31;32]), as well as toxicity (Val/Val patients experience either more [32] or less neurotoxicity [33]). With regard to toxicity, it seems important to select only those patients who have received cumulative oxaliplatin dosages of ≥500 mg/m^2, because dosage is strongly correlated with neurotoxicity [34]. Nevertheless, we did not confirm the association of this SNP with neurotoxicity as has been described in the literature. This may, at least partly, be due to the use of a different toxicity scale compared to the previous report.

Our study also does not confirm the association between the *GSTP1* genotype and OS (or PFS) in patients receiving second-line oxaliplatin plus capecitabine for advanced CRC. However, when we included patients in our analysis who had received oxaliplatin for third-line treatment, we found that Val/Val patients had even shorter OS compared to the other genotypes. This was due to the fact that third-line patients were overrepresented in the Val/Val genotype group. This shows that patient selection is a very important issue when studying previously treated patients, because imbalances between genotypes in patients receiving second- and third line therapies may potentially confound the outcomes of a study. In our opinion this is an important source of bias, and therefore when patients receiving various regimens are combined, pharmacogenetic studies should correct for this type of confounding.

IDENTIFICATION OF NOVEL CANDIDATE GENES RELATED TO OXALIPLATIN EFFICACY AND TOXICITY

In general, there are three ways of studying associations between drug effects and genetic variations; 1) the candidate gene method, 2) the whole genome approach and 3) the pathway gene method. This last method combines the advantages of relatively hypothesis-free testing and an acceptable number of comparisons (and hence, a lower risk of false-positive findings). We carried out a 100 SNP array that includes SNPs located in genes of various DNA repair pathways, and performed association analysis with regard to PFS, OS and toxicity in patients receiving oxaliplatin. Some of the SNPs in the array have been studied before by ourselves or by others, using the candidate gene approach. This includes SNPs in ERCC1 (Chapter 8,[20-22;32;35-37]), ERCC2 [22;31;32;38-41], XRCC1 [39;42] and ERCC5 [40;43]. Except for the ERCC5 SNP rs1047768, we were unable to replicate previously reported associations with treatment efficacy, survival or toxicity. The inability to replicate may reflect differences in patient selection, publication bias, chance or low correlation of a marker with the outcome measure [44]; however, in this case it may also be caused by relatively low statistical power. The risk of false-negative findings can only be minimized by increasing sample size, which, in turn, is not always feasible due to limited access to clinical data and patient samples. Nonetheless, we identified a promising new candidate SNP in the ATM (ataxia telangiectasia mutated) gene, rs1801516, that has not

yet been studied in association with PFS in CRC patients. The results of the current study, although explorative in nature, may serve as a basis for new candidate SNP studies of genes located in the various DNA repair pathways, especially ATM.

FINAL REMARKS

Pharmacogenetics is a relatively new science: we are just beginning to understand the complexity of genetic variations, and their impact on and association with the complex trait drug response. The ultimate goal of pharmacogenetics is to provide clinicians with tools that include both genotypes and other clinical patient variables, in order to help them choose the optimal treatment for an individual patient. This is especially important in the chemotherapeutic treatment of (colorectal) cancer, in which there is a delicate balance between severe adverse events and anti-tumor efficacy. In the field of medical oncology, the choice of a chemotherapeutic agent and its optimal dose may vary considerably among patients. Adverse events may be severe and even life-threatening, whereas under-dosing results in tumor progression and treatment failure. Currently, pharmacogenetic factors can predict up to 5% of all variation in drug response and toxicity. Researchers should therefore aim to develop sophisticated algorithms that incorporate both novel variables, such as genotypes, and classical clinical parameters such as age, gender and organ function, in order to distinguish between patients with an average risk of severe toxicity and those with unacceptable risk. For this last category of patients, strategies can then be undertaken by the clinician to deal with this risk, for example a different choice of therapy, lower dosage or preventive use of supportive measures such as growth factors. However, we need to stress that these algorithms as well as the preventive strategies need to be prospectively validated. It is also very important to determine a level of unacceptable risk for a type of toxicity and to use this as a starting point when developing an algorithm. Furthermore, algorithms need to be applicable to the general patient population and not only to a highly selected patient group in a specific situation. For development and validation of such algorithms, collaboration between research groups is therefore essential. The current thesis is an example of this type of collaboration, because these studies would not have been possible without the outstanding work done by the Dutch Colorectal Cancer Group.

The possibilities of pharmacogenetics in CRC are many, and new insights are emerging every day. Critics may argue that the individual patient may be indefinitely variable with regard to drug response, and that pharmacogenetics may not be able to predict every single (adverse) effect in each separate patient. However, the combination of genetic information and clinical patient variables into predictive algorithms holds a great promise for the future.

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