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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.

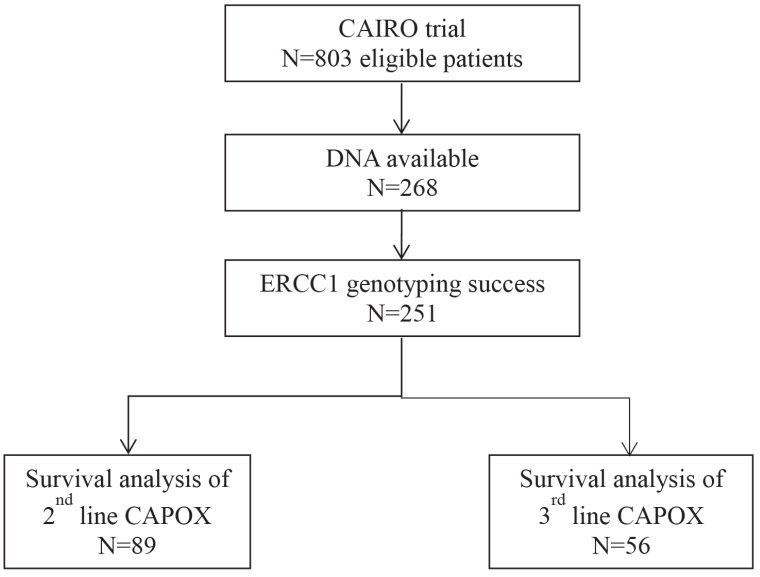


Figure 1. CAIRO study flowchart
CAPOX, capecitabine-oxaliplatin.

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.

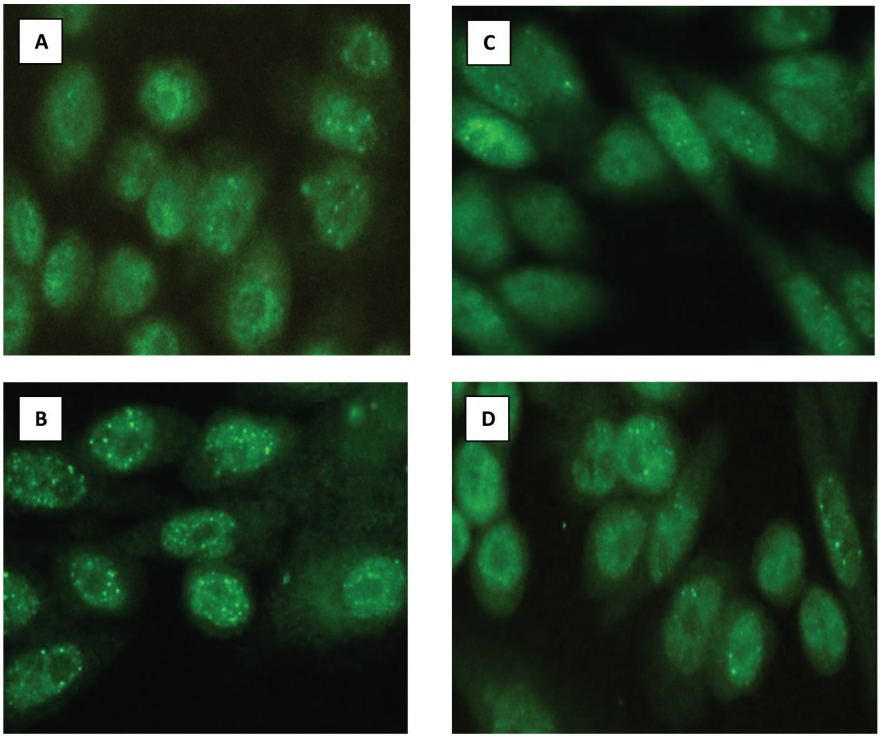


Figure 2. Representative picture used for assessment of Rad51 foci of oxaliplatin treated cells.
All cells have been incubated with 3125nM oxaliplatin, during 24 hours.
A: AA8 (wildtype CHO cells); B: UV20 (*ERCC1*-/- CHO cells); C: UV20+118C (UV20 cells transfected with *ERCC1*-118C allele); D: UV20+118T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

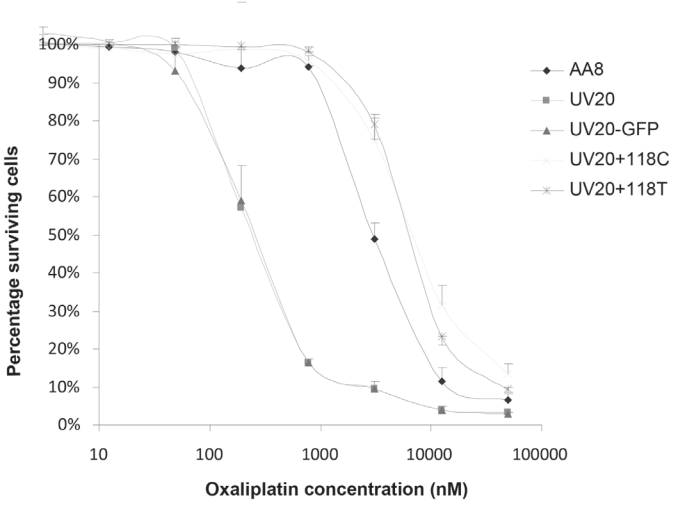


Figure 3. Sulphorodamine B (SRB) assay determined oxaliplatin sensitivity in parental AA8, UV20 (ERCC1-/-), and transfected cells UV20+118C, UV20+118T and UV20-GFP.

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.

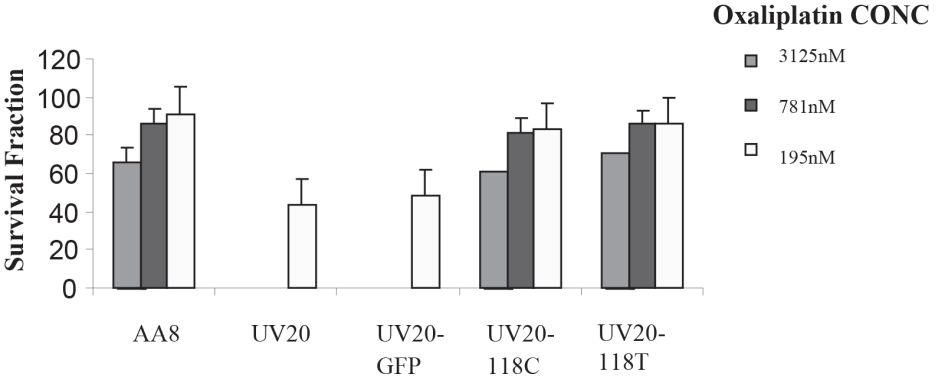


Figure 4. Clonogenic assay determined oxaliplatin sensitivity in parental AA8, UV20 (ERCC1-/-), and transfected cells UV20+118C, UV20+118T and UV20-GFP.

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.

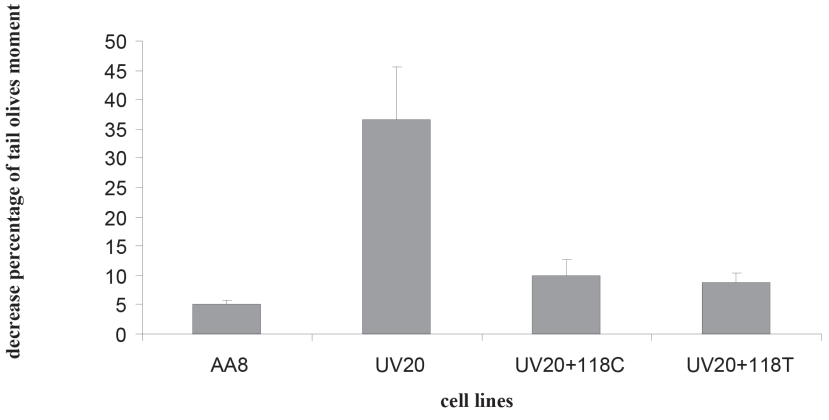
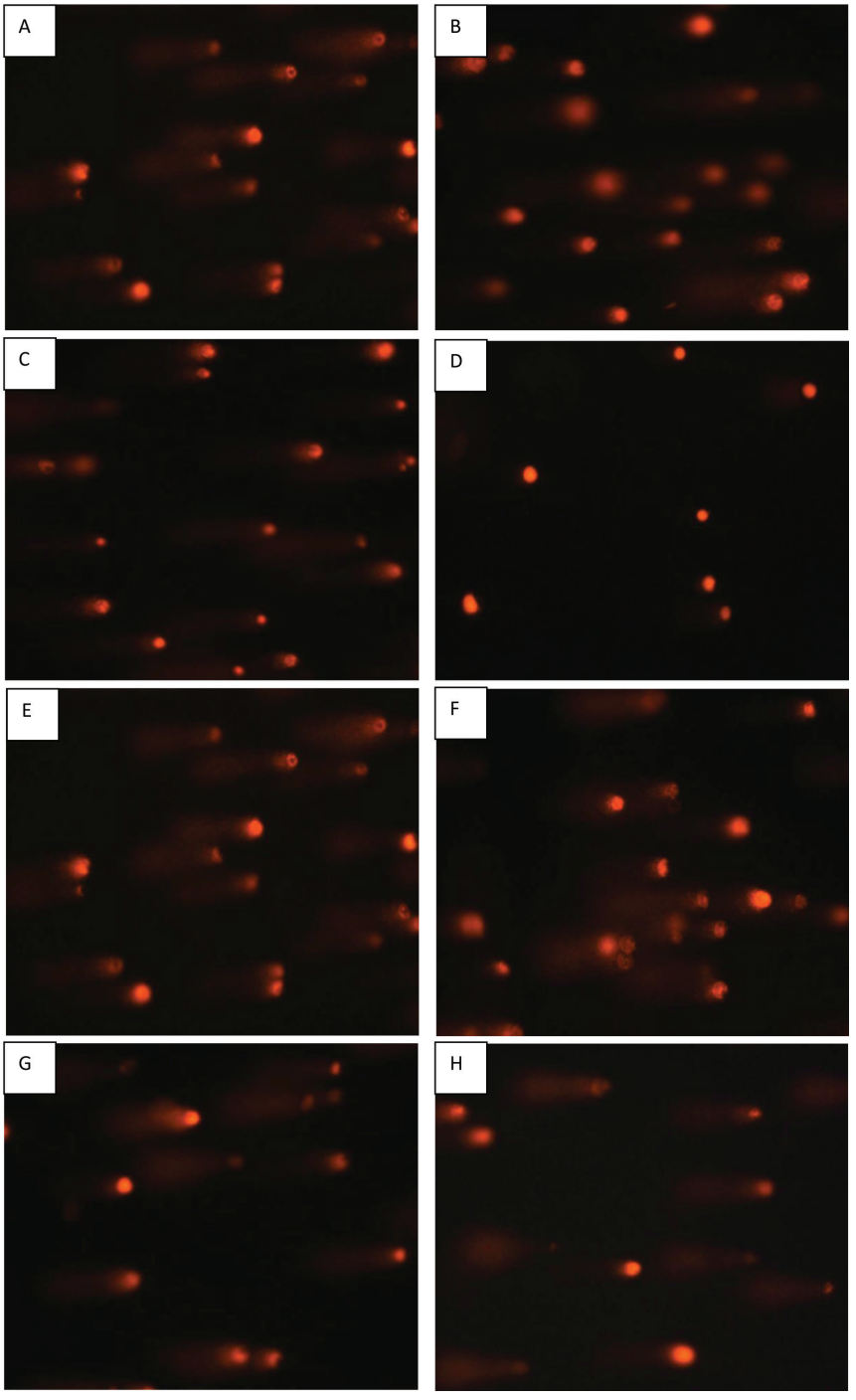


Figure 5. Difference in comet tail length after exposure to oxaliplatin of parental AA8, UV20 (ERCC1-/-), and transfected cells UV20+118C and UV20+118T.

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.

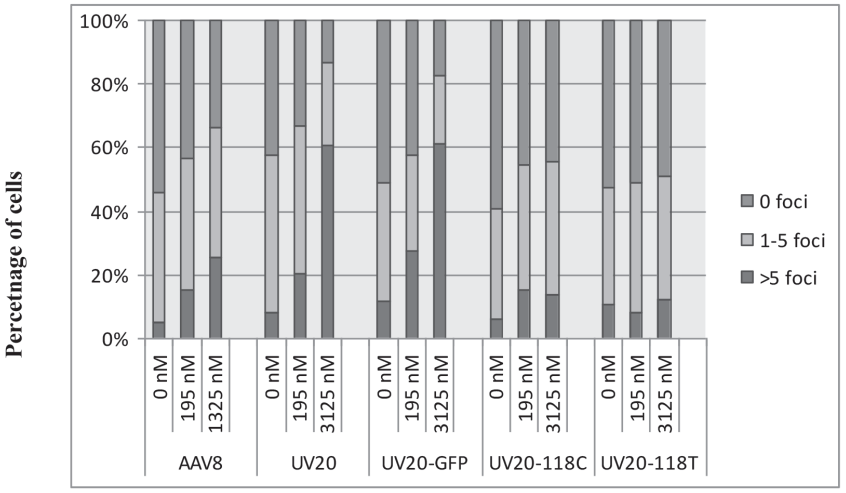

Figure 7. Change is Rad51 foci staining after treatment with increasing oxaliplatin concentrations, for parental AA8, UV20 (ERCC1-/-), and transfected cells UV20+118C and UV20+118T.

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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References

1. D.M. Kweekel, H. Gelderblom, H.J. Guchelaar, Pharmacology of oxaliplatin and the use of pharmacogenomics to individualize therapy, Cancer Treat. Rev. 31 (2005) 90–105.
2. N.G. Jaspers, A. Raams, M.C. Silengo, et al., First reported patient with human ERCC1 deficiency has cerebro-oculo-facio-skeletal syndrome with a mild defect in nucleotide excision repair and severe developmental failure, Am. J. Hum. Genet. 80 (2007) 457–466.
3. J. McWhir, J. Selfridge, D.J. Harrison, et al., Mice with DNA repair gene (ERCC- 1) deficiency have elevated levels of p53, liver nuclear abnormalities and die before weaning, Nat. Genet. 5 (1993) 217–224.
4. L.H. Thompson, J.S. Rubin, J.E. Cleaver, et al., A screening method for isolat- ing DNA repair -deficient mutants of CHO cells, Somatic Cell Genetics 6 (1980) 391–405.
5. L.H. Thompson, C.L. Mooney, K. Burkhart-Schultz, et al., Correction of a nucleotide -excision-repair mutation by human chromosome 19 in hamster- human hybrid cells, Somat. Cell Mol. Genet. 11 (1985) 87–92.
6. R.E. Meyn, S.F. Jenkins, L.H. Thompson, Defective removal of DNA cross-links in a repair -deficient mutant of Chinese hamster cells, Cancer Res. 42 (1982) 3106–3110.
7. A.M. Di Francesco, A. Ruggiero, R. Riccardi, Cellular and molecular aspects of drugs of the future: oxaliplatin, Cell. Mol. Life Sci. 59 (2002) 1914–1927.
8. R. Gao, K. Reece, T. Sissung, et al., The ERCC1 N118N polymorphism does not change cellular ERCC1 protein expression or platinum sensitivity, Mutat. Res. 708 (2011) 21–27.
9. J.J. Yu, C. Mu, K.B. Lee, et al., A nucleotide polymorphism in ERCC1 in human ovarian cancer cell lines and tumor tissues, Mutat. Res. 382 (1997) 13–20.
10. D.J. Park, W. Zhang, J. Stoehlmacher, et al., ERCC1 gene polymorphism as a pre- dictor for clinical outcome in advanced colorectal cancer patients treated with platinum -based chemotherapy, Clin. Adv. Hematol. Oncol. 1 (2003) 162–166.
11. P.M. Chang, C.H. Tzeng, P.M. Chen, et al., ERCC1 codon 118 C→T polymor- phism associated with ERCC1 expression and outcome of FOLFOX-4 treatment in Asian patients with metastatic colorectal carcinoma, Cancer Sci. 100 (2009) 278–283.
12. A.A. Komar, Genetics SNPs, silent but not invisible, Science 315 (2007) 466–467.
13. M. Koopman, N.F. Antonini, J. Douma, et al., Sequential versus combination chemotherapy with capecitabine, irinotecan, and oxaliplatin in advanced colo- rectal cancer (CAIRO): a phase III randomised controlled trial, Lancet 370 (2007) 135–142.
14. D.M. Kweekel, H. Gelderblom, T. Van der Straaten, et al., UGT1A1*28 genotype and irinotecan dosage in patients with metastatic colorectal cancer: a Dutch Colorectal Cancer Group study, Br. J. Cancer 99 (2008) 275–282.
15. P. Therasse, S.G. Arbuck, E.A. Eisenhauer, et al., New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada, J. Natl. Cancer Inst. 92 (2000) 205–216.
16. P. Skehan, R. Storeng, D. Scudiero, et al., New colorimetric cytotoxicity assay for anticancer -drug screening, J. Natl. Cancer Inst. 82 (1990) 1107–1112.
17. N.A. Franken, H.M. Rodermond, J. Stap, et al., Clonogenic assay of cells in vitro, Nat. Protoc. 1 (2006) 2315–2319.
18. I.U. De Silva, P.J. McHugh, P.H. Clingen, et al., Defining the roles of nucleotide excision repair and recombination in the repair of DNA interstrand cross-links in mammalian cells, Mol. Cell. Biol. 20 (2000) 7980–7990.
19. J.M. Hartley, V.J. Spanswick, M. Gander, et al., Measurement of DNA cross -linking in patients on ifosfamide therapy using the single cell gel elec- trophoresis (comet) assay, Clin. Cancer Res. 5 (1999) 507–512.
20. X. Lu, Y. Liu, T. Yu, et al., ERCC1 and ERCC2 haplotype modulates induced BPDE- DNA adducts in primary cultured lymphocytes, PLoS ONE 8 (2013) Apr 4;8(4): e60006.
21. T. Haaf, E.I. Golub, G. Reddy, et al., Nuclear foci of mammalian Rad51 recom- bination protein in somatic cells after DNA damage and its localization in synaptonemal complexes, Proc. Natl. Acad. Sci. U.S.A. 92 (1995) 2298–2302.
22. Database of Single Nucleotide Polymorphisms (dbSNP), National Center for Biotechnology Information, National Library of Medicine (dbSNP Build ID: 36.3), Bethesda, MD, http://www.ncbi.nlm.nih.gov/SNP/
23. K.L. Spindler, R.F. Andersen, L.H. Jensen, et al., EGF61A>G polymorphism as predictive marker of clinical outcome to first-line capecitabine and oxaliplatin in metastatic colorectal cancer, Ann. Oncol. 21 (2010) 535–539.
24. G.M. Adair, R.L. Rolig, D. Moore-Faver, et al., Role of ERCC1 in removal of long non -homologous tails during targeted homologous recombination, EMBO J. 19 (2000) 5552–5561.
25. L.J. Niedernhofer, J. Essers, G. Weeda, et al., The structure -specific endonuclease Ercc1 -Xpf is required for targeted gene replacement in embryonic stem cells, EMBO J. 20 (2001) 6540–6549.
26. P. Baumann, F.E. Benson, S.C. West, Human Rad51 protein promotes ATP- dependent homologous pairing and strand transfer reactions in vitro, Cell 87 (1996) 757–766.

27. V. Boige, J. Mendiboure, J.P. Pignon, et al., Pharmacogenetic assessment of toxicity and outcome in patients with metastatic colorectal cancer treated with LV5FU2, FOLFOX, and FOLFIRI: FFCD 2000–05, J. Clin. Oncol. 28 (2010) 2556–2564.
28. M.C. Etienne-Grimaldi, G. Milano, F. Maindrault-Goebel, et al., Methylenetetra- hydrofolate reductase (MTHFR) gene polymorphisms and FOLFOX response in colorectal cancer patients, Br. J. Clin. Pharmacol. 69 (2010) 58–66.
29. M.J. Lamas, G. Duran, E. Balboa, et al., Use of a comprehensive panel of biomarkers to predict response to a fluorouracil -oxaliplatin regimen in patients with metastatic colorectal cancer, Pharmacogenomics 12 (2011) 433–442.
30. M.J. Lamas, G. Duran, A. Gomez, et al., X-ray cross -complementing group 1 and thymidylate synthase polymorphisms might predict response to chemoradio- therapy in rectal cancer patients, Int. J. Radiat. Oncol. Biol. Phys. 82 (2012) 138–144.
31. H.Y. Li, X. Ge, G.M. Huang, et al., GSTP1, ERCC1 and ERCC2 polymorphisms, expression and clinical outcome of oxaliplatin -based adjuvant chemotherapy in colorectal cancer in Chinese population, Asian Pacific J. Cancer Prev. 13 (2012) 3465–3469.
32. J. Liang, T. Jiang, R.Y. Yao, et al., The combination of ERCC1 and XRCC1 gene polymorphisms better predicts clinical outcome to oxaliplatin -based chemotherapy in metastatic colorectal cancer, Cancer Chemother. Pharmacol. 66 (2010) 493–500.
33. A. Ruzzo, F. Graziano, F. Loupakis, et al., Pharmacogenetic profiling in patients with advanced colorectal cancer treated with first-line FOLFOX-4 chemother- apy, J. Clin. Oncol. 25 (2007) 1247–1254.
34. L. Pare, E. Marcuello, A. Altes, et al., Pharmacogenetic prediction of clinical outcome in advanced colorectal cancer patients receiving oxaliplatin/5- fluorouracil as first-line chemotherapy, Br. J. Cancer 99 (2008) 1050–1055.
35. V. Moreno, F. Gemignani, S. Landi, et al., Polymorphisms in genes of nucleotide and base excision repair: risk and prognosis of colorectal cancer, Clin. Cancer Res. 12 (2006) 2101–2108.
36. J. Viguier, V. Boige, C. Miquel, et al., ERCC1 codon 118 polymorphism is a pre- dictive factor for the tumor response to oxaliplatin/5 -fluorouracil combination chemotherapy in patients with advanced colorectal cancer, Clin. Cancer Res. 11 (2005) 6212–6217.
37. M. Yin, J. Yan, E. Martinez-Balibrea, et al., ERCC1 and ERCC2 polymorphisms predict clinical outcomes of oxaliplatin -based chemotherapies in gastric and colorectal cancer: a systemic review and meta -analysis, Clin. Cancer Res. 17 (2011) 1632–1640.
38. R.N. Seetharam, A. Sood, A. Basu-Mallick, et al., Oxaliplatin resistance induced by ERCC1 up -regulation is abrogated by siRNA-mediated gene silencing in human colorectal cancer cells, Anticancer Res. 30 (2010) 2531–2538.
39. J. Yin, U. Vogel, C. Wang, et al., Hapmap -based evaluation of ERCC2, PPP1R13L, and ERCC1 and lung cancer risk in a Chinese population, Environ. Mol. Mutagen. 53 (2012) 239–245.
40. W. Zhou, S. Gurubhagavatula, G. Liu, et al., Excision repair cross- complementation group 1 polymorphism predicts overall survival in advanced non-small cell lung cancer patients treated with platinum -based chemother- apy, Clin. Cancer Res. 10 (2004) 4939–4943.
41. T. Yu, Y. Liu, X. Lu, et al., Excision repair of BPDE-adducts in human lym- phocytes: diminished capacity associated with ERCC1 C8092A (rs3212986) polymorphism, Arch. Toxicol. 87 (2013) 699–709.
42. S. Djebali, C.A. Davis, A. Merkel, et al., Landscape of transcription in human cells, Nature 489 (2012) 101–108.
43. J.T. Lee, Epigenetic regulation by long noncoding RNAs, Science 338 (2012) 1435–1439.
44. M.S. Braun, S.D. Richman, L. Thompson, et al., Association of molecular markers with toxicity outcomes in a randomized trial of chemotherapy for advanced colorectal cancer: the FOCUS trial, J. Clin. Oncol. 27 (2009) 5519–5528.
45. H.L. McLeod, D.J. Sargent, S. Marsh, et al., Pharmacogenetic predictors of adverse events and response to chemotherapy in metastatic colorectal cancer: results from North American Gastrointestinal Intergroup Trial N9741, J. Clin. Oncol. 28 (2010) 3227–3233.
46. D.M. Kweekel, H. Gelderblom, N.F. Antonini, et al., Glutathione-S-transferase pi (GSTP1) codon 105 polymorphism is not associated with oxaliplatin efficacy or toxicity in advanced colorectal cancer patients, Eur. J. Cancer 45 (2009) 572–578.
47. J.P. Ioannidis, F.K. Kavvoura, Concordance of functional in vitro data and epi- demiological associations in complex disease genetics, Genet. Med. 8 (2006) 583–593.
48. M. Biggerstaff, D.E. Szymkowski, R.D. Wood, Co-correction of the ERCC1, ERCC4 and xeroderma pigmentosum group F DNA repair defects in vitro, EMBO J. 12 (1993) 3685–3692.
49. A.J. van Vuuren, E. Appeldoorn, H. Odijk, et al., Evidence for a repair enzyme complex involving ERCC1 and complementing activities of ERCC4, ERCC11 and xeroderma pigmentosum group F, EMBO J. 12 (1993) 3693–3701.
50. S.H. Kim, H.C. Kwon, S.Y. Oh, et al., Prognostic value of ERCC1, thymidylate synthase, and glutathione S-transferase pi for 5 -FU/oxaliplatin chemotherapy in advanced colorectal cancer, Am. J. Clin. Oncol. 32 (2009) 38–43.
51. M. Koopman, S. Venderbosch, H. van Tinteren, et al., Predictive and prognostic markers for the outcome of chemotherapy in advanced colorectal

cancer, a retrospective analysis of the phase III randomised CAIRO study, Eur. J. Cancer 45 (2009) 1999–2006.
52. L. Friboulet, K.A. Olaussen, J.P. Pignon, et al., ERCC1 isoform expression and DNA repair in non-small-cell lung cancer, N. Engl. J. Med. 368 (2013) 1101–1110.
53. H. Baba, M. Watanabe, H. Okabe, et al., Upregulation of ERCC1 and DPD expres- sions after oxaliplatin -based first-line chemotherapy for metastatic colorectal cancer, Br. J. Cancer 107 (2012) 1950–1955.
54. K. Kumamoto, K. Kuwabara, Y. Tajima, et al., Thymidylate synthase and thymi- dine phosphorylase mRNA expression in primary lesions using laser capture microdissection is useful for prediction of the efficacy of FOLFOX treatment in colorectal cancer patients with liver metastasis, Oncol. Lett. 3 (2012) 983–989.
55. S.K. Maithel, M. Gonen, H. Ito, et al., Improving the clinical risk score: an analysis of molecular biomarkers in the era of modern chemotherapy for resectable hepatic colorectal cancer metastases, Surgery 151 (2012) 162–170.
56. Y. Shirota, J. Stoehlmacher, J. Brabender, et al., ERCC1 and thymidylate synthase mRNA levels predict survival for colorectal cancer patients receiving com- bination oxaliplatin and fluorouracil chemotherapy, J. Clin. Oncol. 19 (2001) 4298–4304.
57. K. Uchida, P.V. Danenberg, K.D. Danenberg, et al., Thymidylate synthase, dihy- dropyrimidine dehydrogenase, ERCC1, and thymidine phosphorylase gene expression in primary and metastatic gastrointestinal adenocarcinoma tissue in patients treated on a phase I trial of oxaliplatin and capecitabine, BMC Cancer 8 (2008) 386.