World Journal of Gastroenterology

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DOI: 10.3748/wjg.v20.i29.9775

World J Gastroenterol 2014 August 7; 20(29): 9775-9827
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (5): Colorectal cancer

Pharmacogenetics research on chemotherapy resistance in colorectal cancer over the last 20 years

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Received: September 28, 2013 Revised: January 17, 2014
Accepted: April 21, 2014
Published online: August 7, 2014

Abstract

During the past two decades the first sequencing of the human genome was performed showing its high degree of inter-individual differentiation, as a result of large international research projects (Human Genome Project, the 1000 Genomes Project International HapMap Project, and Programs for Genomic Applications NHLBI-PGA). This period was also a time of intensive development of molecular biology techniques and enormous knowledge growth in the biology of cancer. For clinical use in the treatment of patients with colorectal cancer (CRC), in addition to fluoropyrimidines, another two new cytostatic drugs were allowed: irinotecan and oxaliplatin. Intensive research into new treatment regimens and a new generation of drugs used in targeted therapy has also been conducted. The last 20 years was a time of numerous *in vitro* and *in vivo* studies on the molecular basis of drug resistance. One of the most important factors limiting the effectiveness of chemotherapy is the primary and secondary resistance of cancer cells. Understanding the genetic factors and mechanisms that contribute to the lack of or low sensitivity of tumour tissue to cytostatics is a key element in the currently developing trend of personalized medicine. Scientists hope to increase the percentage of positive treatment response in CRC patients due to practical applications of pharmacogenetics/pharmacogenomics. Over the past 20 years the clinical usability of different predictive markers has been tested among which only a few have been confirmed to have high application potential. This review is a synthetic presentation of drug resistance in the context of CRC patient chemotherapy. The multifactorial nature and volume of the issues involved do not allow the author to present a comprehensive study on this subject in one review.

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Key words: Pharmacogenetics; Pharmacogenomics; Drug resistance; Colorectal cancer; Chemoresistance; Individualized medicine

Core tip: Insufficient effectiveness of chemotherapy is still the most important factor limiting the successful treatment of patients with colorectal cancer (CRC). Drug resistance in anticancer therapy has been recognized virtually from the very beginning, as cytostatic drugs were first used in oncology practice. Intensive research on the causes of low sensitivity in colorectal cancer cells to such drugs as fluoropyrimidines, irinotecan and oxaliplatin, has resulted in evidence on the importance of genetic factors in phenotype conditioning of drug resistance. This review is a synthetic presentation of drug resistance in the context of its role in chemotherapy, and the potential clinical use of different biomarkers in individualization of CRC patient treatment.

Panczyk M. Pharmacogenetics research on chemotherapy resistance in colorectal cancer over the last 20 years. *World J Gastroenterol* 2014; 20(29): 9775-9827 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i29/9775.htm DOI:

http://dx.doi.org/10.3748/wjg.v20.i29.9775

RESEARCH ON THE EFFECTIVENESS OF CYTOTOXIC ANTINEOPLASTIC DRUGS FOR THE TREATMENT OF COLORECTAL CANCER

Since the beginning of the 21st century, very rapid development of high-throughput research techniques described by the term "omics" (genomics, transcriptomics, proteomics and metabolomics) has been observed. Pharmacogenomics uses advanced research techniques "omics", which allow researchers to identify the genetic basis of inter-individual differences in the pharmacodynamics and pharmacokinetics of drugs[1,2]. An important objective of this research is to identify biomarkers for predicting treatment outcomes, as well as avoiding the toxic effects arising during the course of pharmacotherapy (prognostic and predictive markers)[3]. The terms pharmacogenetics and pharmacogenomics are closely related and are often used interchangeably, although there are some historical differences between them. Today, pharmacogenomics is commonly used synonymously with "individualized" or "personalized" medicine, although the latter term is often understood to stratify medical treatment by the use of genomic biomarkers rather than to treat an individual. Accordingly, the Personalized Medicine Coalition defined personalized medicine as "the application of genomic and molecular data to better target the delivery of health care, facilitate the discovery and clinical testing of new products, and help determine a person's predisposition to a particular disease or condition"[4,5].

Environmental factors such as age, sex or health condition of the patient are the classic factors which affect treatment outcomes and have been studied for decades. The influence of genetic factors on response variability is far greater than sex, age, or interactions with other drugs. Therefore, it seems advisable to determine the basis of all abnormal body reactions in relation to the treatment used. It should also be noted that the distribution frequency of correct responses to drug usage in a population is far from a normal distribution, which means that the presence of treatment non-responders and over-responders (increased toxicity) is much more common than has been assumed so far[6]. The first studies on pharmacogenomics and colorectal cancer (CRC) outcome were conducted and published approximately 20 years ago[7]. Since then, hundreds of possible biodeterminants have been studied with many expectations. The technology, and its spread, has improved incredibly, and the importance with which this subject is regarded by many research groups throughout the world has grown relentlessly. The reproducibility of some results was, initially, promising, as were some confirmatory clues derived from deeper biological studies, but the final step of clinical validation has remained an unmet objective for almost all putative biomarkers[8].

Treatment options in CRC have systematically advanced over the last several years with the introduction of effective chemotherapeutic and targeted drugs. However, providing individual treatment with low toxicity and significant benefit is still an unsolved problem[9]. This part of the review focuses on pharmacogenomic knowledge of substances routinely administered in patients with CRC: fluoropyrimidines, irinotecan (CPT-11), and oxaliplatin (OX).

5-FLUOROURACIL AND FLUOROPYRIMIDINES

In 1957 Heidelberger *et al*[10] reported the antitumour activity of 5-fluorouracil (5-FU). Charles Heidelberger synthesized 5-FU as a result of experiments which showed the ability of tumour cells to acquire uracil for DNA synthesis[11]. Fifty years after the first synthesis of 5-FU it is still a standard component of adjuvant and palliative therapy having a proven impact on survival time in patients with CRC[12]. Experimental studies have shown that 5-FU is converted to an active metabolite, FdUMP (fluorodeoxyuridine monophosphate), which is a potent inhibitor of DNA synthesis (Figure 1). FdUMP forms a ternary complex together with thymidylate synthase enzyme (TS) and 5,10-methylenetetrahydrofolate (CH_2THF) cofactor, responsible for the catalytic conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP). dTMP is a substrate for deoxythymidine triphosphate (dTTP) necessary for the process of DNA synthesis (Figure 2). Furthermore, on the basis of fundamental and clinical research it has been proven that the addition to an exogenous therapy a source of folic acid, such as leucovorin (LV) increases the degree of inhibition of TS supporting the formation of active complexes of 5-FU with the enzyme[13]. 5-FU/LV combination therapy in patients with diagnosed CRC is much more effective than monotherapy with 5-FU[14].

The purpose of individualized therapy is to choose the most effective treatment and the optimal dosage for each patient, while minimizing toxicity and side effects of the therapy. This objective is particularly important in the case of the new generation of anticancer drugs which include expensive targeted therapies such as the antibodies cetuximab and bevacizumab. The much cheaper 5-FU therapy can also be individualized in a selection of CRC patients with potentially best response to the administration of 5-FU which appears to be justified medically and financially. Despite significant progress in understanding the 5-FU activity mechanisms, the identification of molecular markers potentially clinically useful in predicting 5-FU treatment efficacy is still the subject of research.

TS

TS is an important enzyme involved in the metabolism of folic acid and catalyzes dUMP methylation to dTMP,

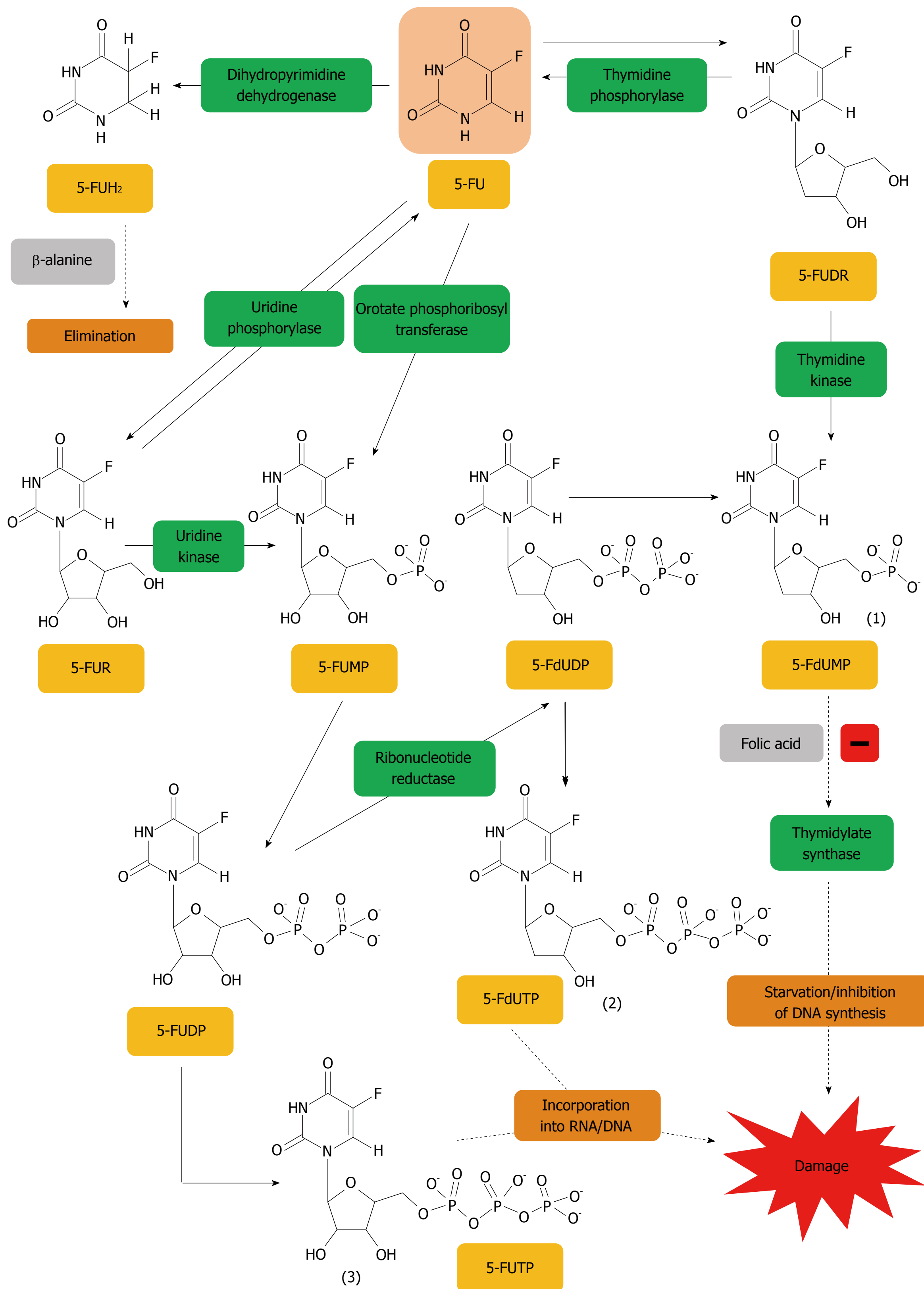

Figure 1 5-fluorouracil is converted to three major active metabolites. (1) fluorodeoxyuridine monophosphate (FdUMP); (2) fluorodeoxyuridine triphosphate (FdUTP); and (3) fluorouridine triphosphate (FUTP). The main mechanism of 5-fluorouracil (5-FU) activation is conversion to fluorouridine monophosphate (FUMP) either directly by orotate phosphoribosyl transferase (OPRT), or indirectly *via* fluorouridine (FUR) through the sequential action of uridine phosphorylase and uridine kinase. FUMP is then phosphorylated to fluorouridine diphosphate (FUDP), which can be either further phosphorylated to the active metabolite fluorouridine triphosphate (FUTP), or converted to fluorodeoxyuridine diphosphate (FdUDP) by ribonucleotide reductase. In turn, FdUDP can either be phosphorylated or dephosphorylated to generate the active metabolites FdUTP and FdUMP, respectively. An alternative activation pathway involves the thymidine phosphorylase catalyzed conversion of 5-FU to 5-fluoro-2'-deoxyuridine (5-FUDR), which is then phosphorylated by thymidine kinase to the thymidylate synthase inhibitor, FdUMP. Dihydropyrimidine dehydrogenase (DPD)-mediated conversion of 5-FU to dihydrofluorouracil (DHFU) is the rate-limiting step of 5-FU catabolism in normal and tumour cells[401].

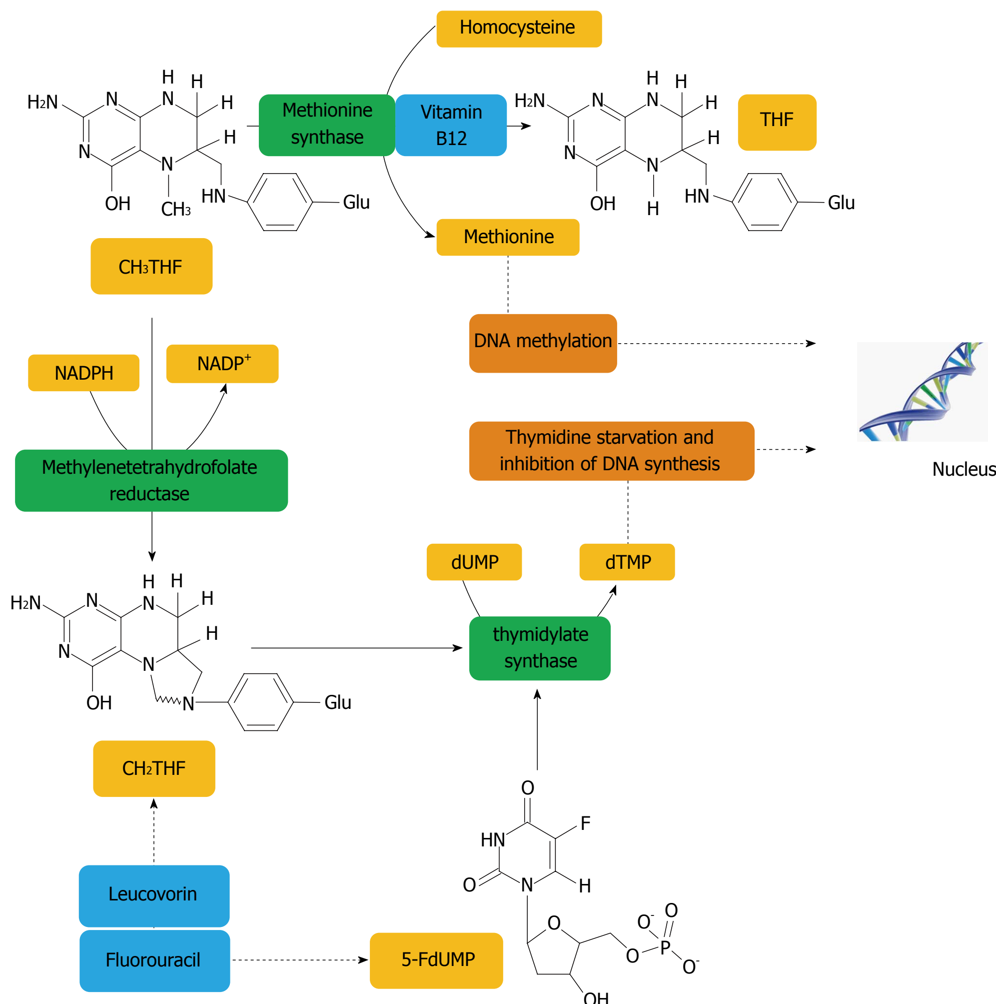

Figure 2 Methylentetrahydrofolate reductase plays an important role in the action of 5-fluorouracil, an inhibitor of thymidylate synthase. Methylentetrahydrofolate reductase (MTHFR) catalyses a unidirectional reaction that lowers the levels of 5,10-methylenetetrahydrofolate (CH_2THF) by increasing levels of 5-methyltetrahydrofolate (CH_3THF) which is used for biological methylation. Other factors, such as vitamin B12 and homocysteine, are involved in biological methylation processes. The addition of folinic acid (leucovorin) to 5-FU improves the response rates and survival of CRC patients. Thymidylate synthase (TS) catalyses the reductive methylation of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) with the reduced folate, CH_2THF, as the methyl donor. This reaction provides the sole de novo source of thymidylate, which is necessary for DNA replication and repair. TS contains a nucleotide-binding site and a binding site for CH_2THF. The 5-FU metabolite, FdUMP, binds to the nucleotide-binding site of TS, forming a stable ternary complex with the enzyme and CH_2THF which blocks binding of the normal substrate dUMP, thereby inhibiting dTMP synthesis. Inhibition of thymidylate synthesis causes disruption of nucleotide levels that results in DNA damage[402].

which is a critical reaction in maintaining the balance of available deoxynucleotides (dNTPs) in cells, substrates necessary for the synthesis and repair of DNA. The interaction with TS is the main aim of such cytostatic drugs as 5-FU, and the level of *TYMS* gene expression and TS protein is a prognostic marker in the treatment of several types of cancer. Thus, the 5-FU cell sensitivity profile may be affected by genetic variants of the *TYMS* gene, expression level of *TYMS*/TS gene/ -protein, and intracellular concentration of dNTP and CH_2THF[15]. Expression of TS as a sensitivity determinant for fluoropyrimidines has been shown *in vitro*[16] as well as *in vivo*, where intratumour TS expression level was associated with the chemosensitivity of tumour tissue exposed to 5-FU. The most important data collected during the past few years indicate that TS expression varies considerably between different types of cancers and that the degree of tumour response to 5-FU treatment is inversely proportional to the measured level of intratumour mRNA and protein expression[17]. Leichman *et al*[18] first proved that there is an inverse relationship between intratumoural *TYMS* gene expression and the degree of response to 5-FU treatment. CRC patients with low levels of *TYMS* gene expression had a significantly higher rate of response to therapy and longer median survival compared to patients with higher *TYMS* expression in tumour tissue (13.6 mo *vs* 8.2 mo, $P = 0.02$)[19]. A meta-analysis of 13 clinical trials of patients with advanced CRC (total number of patients: 887 cases) carried out by Popat *et al*[20] showed that patients with low TS expression had longer overall survival (OS) than patients with higher TS expression in tumour tissue. Recently, a meta-analysis including 24 clinical trials with more than 1100 CRC patients was also published[21]. The pooled relative risk of overall response

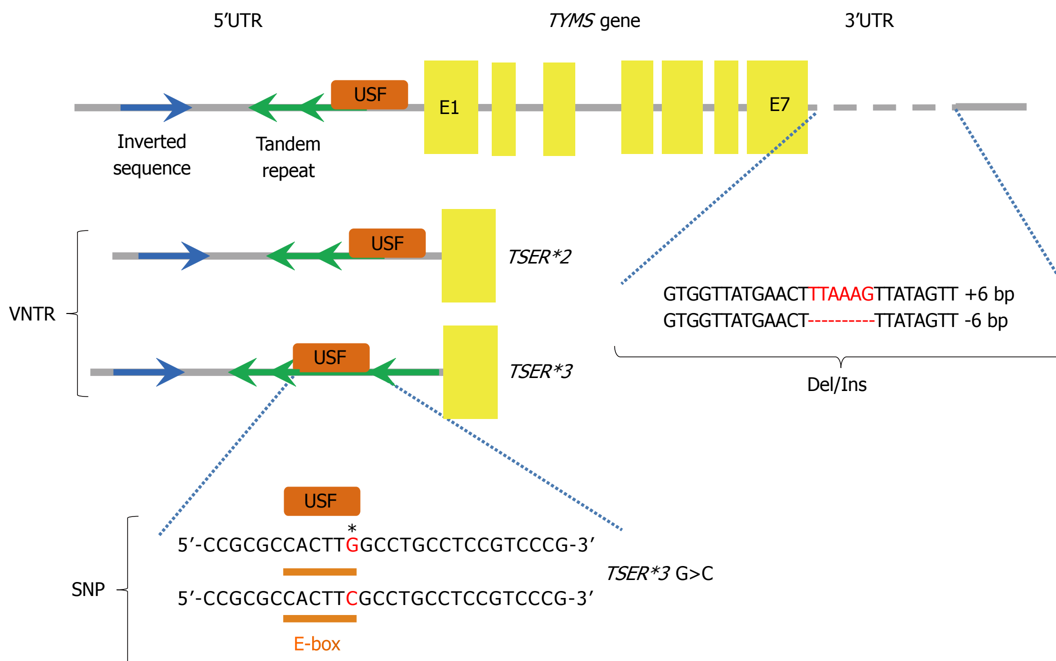

Figure 3 Some of the described polymorphisms affect inter-individual differences in patient sensitivity to 5-fluorouracil treatment. Polymorphisms in the thymidylate synthase gene (*TYMS* gene), 5' and 3' untranslated regions (5'UTR and 3'UTR), exons (E1-E7), binding site for upstream stimulating factor (USF), variable number tandem repeats (VNTR), single nucleotide polymorphism (SNP), deletion/insertion polymorphism (Del/Ins), two-tandem repeats (*TSER*2*), three-tandem repeats (*TSER*3*), *TSER*3* G>C (single nucleotide polymorphism of *TSER*3*). Regulation of *TYMS* gene expression. TSER polymorphism (TS 2R/3R repeat) is a tandem repeat upstream of the TYMS translational start site containing either double (2R) or triple (3R) repeats of 28-bp sequences. These tandem repeats regulate transcription and translation of TYMS. Additional functional variants of the *TYMS* gene have been identified and TSER 2R/3R repeat is now studied together with a G to C SNP within the second repeat of the 3R allele. TSER 3RC/3RC genotype causes lower transcriptional activity of TYMS, comparable with the TS 2R/2R genotype. TS 1494del6bp is another functional variant of the *TYMS* gene and has been shown to decrease RNA stability and therefore influence TS mRNA and TS protein expression *in vitro*[52].

rate (ORR) indicated that the group with lower TS expression had greater sensitivity to fluoropyrimidine-based chemotherapy than patients with high TS expression level[21]. Numerous studies were also carried out to investigate different TS expression levels in tissue derived from primary tumours and metastases[22,23]. Analysis of the two subgroups it was demonstrated that predictive TS expression levels determined in tissue derived from metastases were more pronounced than those determined in primary tumours[21]. Furthermore, during the assessment of the predictive values of TS expression level, the results obtained using RT-PCR techniques were statistically more significant than those in which the expression was determined using immunohistochemistry (IHC) techniques[21].

These results indicated that low TS expression in CRC patients with advanced tumours was associated with increased individual sensitivity to 5-FU therapy[7,17,19,24-39]. Furthermore, *in vitro* studies using cell lines and tumour tissues demonstrated that 5-FU therapy contributes to the induction of TS expression[40,41]. This increase in TS expression upon 5-FU exposure seems to be a result of a negative feedback loop in which ligand-free TS binds to its own mRNA and inhibits its own translation[42]. When stably bound by FdUMP, TS can no longer bind its own mRNA and suppress translation, resulting in increased protein expression. This constitutes a potentially important resistance mechanism, as acute increases in TS would facilitate recovery of enzyme activity[41].

Although, the reason for ontogenetic variation in TS expression is still not clear, one of the main examined hypotheses is the possible influence of *TYMS* gene polymorphisms on TS expression. As it is now known, some of the described polymorphisms affect inter-individual differences in patient sensitivity to 5-FU treatment (Figure 3 and Table 1)[43-52]. Polymorphism of the variable number of tandem repeats (VNTR) located in the *TYMS* gene sequence is one of the studied genetic variants that may have clinical relevance as a predictive marker for the effectiveness of 5-FU treatment. Horie *et al*[53] reported a 28-nucleotide sequence in the 5'-region of the *TYMS* gene, which occurs in the population with a variable number of iterations: two (2R) or three (3R). According to the classification proposed by Kawakami and Watanabe, it is assumed that VNTR in this region is responsible for the occurrence of two alleles, 2R and 3R, and three different genotypes (2R/2R, 2R/3R and 3R/3R)[54]. The results of various studies suggest that the 3R allele is responsible for four times higher mRNA level of the *TYMS* gene observed in tissue tumours obtained from patients with metastatic CRC compared to patients who were carriers of the 2R variant ($P < 0.004$)[55]. Homozygous patients having both alleles with a double repeat (2R/2R) showed a significantly higher percentage of favourable response to 5-FU treatment as compared to those who had the 3R/3R genotype (50% *vs* 9%, $P = 0.04$)[55]. In addition to the predictive values for 5-FU chemotherapy, retrospec-

Table 1 Some common polymorphisms of genes *TYMS*, *MTHFR*, *DPYD* and *UMPS* and their potential impact on the functioning of proteins associated with the pharmacology of 5-fluorouracil

dbSNP rs cluster ID	Type of polymorphism	Function	Ref.
Thymidylate synthase (*TYMS*) (OMIM # 188350)			
rs45445694	VNTR		[43-51,68,409-413]
	TSER*2/ TSER*3	TSER polymorphism (TS 2R/3R repeat) is a tandem repeat upstream of the TYMS translational start site containing either double (2R) or triple (3R) repeats of 28-bp sequences	
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 45445694			
rs34743033	SNP		[44-46,49,50,414]
	TSER*3G>C	TSER*2/*3 repeat is studied together with a G to C SNP within the second repeat of the TSER*3 allele	
		TSER*3C allele = decrease transcriptional activity of TYMS	
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 34743033			
rs151264360	Del/Ins		[44,46,49,51,72,415]
	TS 1494del6bp	-6-bp deletion, decreased stability of TS mRNA	
		+6-bp insertion, increased stability of TS mRNA	
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 151264360			
Methylenetetrahydrofolate reductase (*MTHFR*) (OMIM # 607093)			
rs1801133	SNP		[66-69,72,313,316,362]
	677C>T	At codon 222 in exon 4 (Ala → Val)	
		Reduces enzymatic activity and increased thermolability	
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 1801133			
rs1801131	SNP		[67-69,72,313,316]
	1298A>C	At codon 429 in exon 7 (Glu → Ala)	
		Reduces MTHFR activity	
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 1801131			
rs4846051[a]	SNPs		[71,416]
	1305T>C	At codon 435 (synonymous), effect unknown	
rs201095365[b]	1798G>A	At codon 600 (Glu → Lys), effect unknown	
[a]http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 4846051			
[b]http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 201095365			
Dihydropyrimidine dehydrogenase (*DPYD*) (OMIM # 612779)			
rs3918290	SNP		[88,412,417,418]
	IVS14+1G>A	Exon 14 is skipped as a result of the G → A translocation at intron 14, inactive enzyme is formed	
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 3918290			
rs75017182	SNP		[92,419]
	c.1129– 5923C>G	Cryptic splice donor site leads to a 44 bp fragment of intron 10 insert in mrna, frameshift and premature stop codon in exon 11	
		Associated with toxicity	
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 75017182			
-	SNPs		[92,417]
	IVS5+18G>A	G → A translocation at intron 5, effect unknown	
	IVS6+139G>A	G → A translocation at intron 6, effect unknown	
	IVS9–51T>G	T → G translocation at intron 9, effect unknown	
rs1801265	SNP		[85,420-424]
	85T>C	At codon 29 in exon 2 (Cys → Arg)	
		Decreased expression	
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 1801265			
rs2297595	SNP		[420,421,424-427]
	496A>G	At codon 166 in exon 6 (Met → Val)	
		Significantly conserved site close to the Fe-S motif, may disrupt electron transport	
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 2297595			
rs1801159	SNP		[421,424,427-430]
	1627A>G	At codon 543 in exon 13 (Ile → Val)	
		Decreased expression	
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 1801159			
rs55886062	SNP		[92,422,431-434]
	1679T>G	At codon 560 in exon 13 (Ile → Ser)	
		Might destabilize FMN (flavine mononucleotide) binding domain	
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 55886062			
rs1801160	SNP		[424,428]
	2194G>A	At codon 732 in exon 18 (Val → Ile)	
		Decreased expression	
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 1801160			

rs67376798	SNP		[92,412,417,422,425,426, 432,435-437]
	2846A>T	At codon 949 in exon 22 (Asp → Val) Significantly conserved site near the Fe-S motif, may disrupt cluster formation and electron transport and lead to lower DPD activity	
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 67376798			
Uridine monophosphate synthetase (*UMPS*) [OMIM #613891]			
rs121917890[a]	SNPs		[122-126]
	213A>G	At codon 96 (Arg → Gly), effect unknown	
rs121917892[b]	326T>G	At codon 109 (Val → Gly), effect unknown	
rs1801019[c]	638G>C	At codon 213 (Gly → Ala), increase activity	
rs2291078[d]	1050T>A	At codon 350 (synonymous), effect unknown	
rs121917891[e]	1285G>C	At codon 429 (Gly → Arg), effect unknown	
rs3772809[f]	1336A>G	At codon 446 (Ile → Val), effect unknown	

[a]http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 121917890
[b]http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 121917892
[c]http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 1801019
[d]http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 2291078
[e]http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 121917891
[f]http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 3772809

SNP: Single nucleotide polymorphism.

tive studies have demonstrated that this polymorphism also has the properties of a toxicity marker for fluoropyrimidine-based chemotherapy. Patients who are carriers of the 3R/3R genotype exhibited reduced toxicity as compared to patients with the 2R variant. A high TS expression level related to the presence of 3R/3R genotype accounts for less effective inhibition of TS, which contributes to both an increased likelihood of survival of cancer cells (drug resistance), and a reduced loss of healthy cells and less toxic therapy[55]. Moreover, a single nucleotide polymorphism (SNP) of guanine instead of cytosine (G/C) in 3R determines two different alleles (3C or 3G)[55]. Based on the presence of this polymorphism two different groups of patients can be distinguished with two levels of TS expression: a high expression group with (2R/3G, 3C/3G and 3G/3G genotype carriers) and a low expression group (2R/2R, 2R/3C and 3C/3C genotypes). Taking into account the study results published by Mandola *et al*[56], it is believed that the presence of the 28-bp G>C SNP within the second repeat of the 3R allele *TYMS* promoter enhancer region (TSER) is associated with a weaker bond in the promoter region of USF-1 transcription factor leading to a decreased transcriptional activity of *TYMS* gene. A lower transcription rate of the TSER 3RC allele *in vitro* is also observed when compared with TSER 3RG, comparable with the TSER 2R/2R genotype[56,57]. These results may, at least partly, explain why some patients with 3R/3R genotype have low TS expression and a good response to 5-FU chemotherapy.

The third described polymorphism is an insertion/deletion of hexanucleotide TTAAAG sequence at 1494 position on the 3'-UTR (1494del6)[58]. This polymorphism may contribute to stability changes of secondary mRNA structure as has been demonstrated for alterations of the 3'-region in other genes[59]. Ulrich *et al*[58] analysed the mRNA expression level of *TYMS* gene in 43 patients and showed that homozygous patients with 6-bp deletion had a steady-state TS mRNA level three times lower than patients who were homozygous for the 6-bp insertion alleles (P = 0.017). Furthermore, it was shown that homozygous patients with deletion (del/del) had significantly lower mRNA levels of the *TYMS* gene which was also associated with greater sensitivity to 5-FU-based therapy as compared to homozygous patients with (ins/ins) insertion (P = 0.017)[57,60]. There is a need for further analyses to allow identification of *TYMS* transcription regulatory mechanisms including the role played by combinations of different genetic variants, such as polymorphisms, SNPs and VTNR in *TYMS*/TS expression variability in populations.

A major limitation of correlational research on the pharmacogenetic importance of polymorphisms and *TYMS*/TS expression is an increasing proportion of patients who are treated with combination therapy, for which 5-FU is not the only component in the chemotherapy. Therefore, it is often difficult to determine whether the observed greater sensitivity in a small number of patients to a treatment is associated with the presence of genetic determinants (*e.g.*, 2R/2R homozygous status, 6 bp$^-$/6 bp$^-$ 3'-UTR, allele G of the G>C SNP) or is a result of drugs other than 5-FU used in the combination therapy[50].

Methylenetetrahydrofolate reductase

The use of folic acid in combination with 5-FU has been standard in the treatment of advanced CRC for more than 30 years[61]. The intracellular metabolic balance of folic acid is regulated by methylenetetrahydrofolate reductase (MTHFR), a critical enzyme in the folic acid pathway catalysing irreversible conversion of CH_2THF to 5-methyltetrahydrofolate (CH_3THF) (Figure 2). 677C>T is one of numerous polymorphisms of the *MTHFR* gene described in the literature, which may contribute to activity changes in this enzyme. 677TT genotype is responsible for a 30% reduction in enzymatic activity compared to 677CC genotype associated with reduced thermolability observed *in vitro*[62], which results in a decreased eryth-

rocyte concentration of CH_3THF and accumulation of CH_2THF[63]. The frequency of specific genetic variants of *MTHFR* for SNP 677C>T is ethnically diverse. Analyses of Caucasian and Asian populations suggest that the prevalence of 677TT genotype oscillates between 12% and 15% with a frequency of 677CT homozygotes at the 50% level. Whereas, in a population of African-Americans there was a very low frequency of 677TT genotype[64]. An important consequence of the presence of *MTHFR* 677T variant is the possibility of accumulation of CH_2THF in the cells, which may have a significant effect on the pharmacological efficacy of 5-FU. This is due to the fact that the effect of 5-FU is largely dependent on the concentration of foliants. The 5-FU-5-FdUMP metabolite irreversibly forms a stable complex with TS and CH_2THF. Creation of this complex inhibits the activity of TS, which leads to an intracellular drop in dTMP concentration and finally inhibition of DNA synthesis. Increased concentration of CH_2THF as a consequence of the presence of the *MTHFR* 677C>T polymorphism may therefore contribute to changes in the chemosensitivity of cancer cells exposed to 5-FU by increasing the amount and stability of CH_2THF-TS-FdUMP ternary complex, and thus a stronger inhibition of DNA synthesis. Sohn *et al*[65] in both *in vitro* and *in vivo* studies observed that the presence of 677T allele of the *MTHFR* gene is responsible for greater chemosensitivity in colon cancer cells, suggesting that the genetic variant 677C>T may be a pharmacogenetic factor used to assess the effectiveness of 5-FU-based chemotherapy. However, clinical studies published in recent years have led to contradictory and inconsistent conclusions[64]. In advanced CRC patients undergoing 5-FU-based therapy, in three published studies the presence of the 677T variant of the *MTHFR* gene was associated with a higher percentage of positive responses[66-68], while the results of another study did not confirm the existence of such a relationship (Table 1)[69].

Another frequent polymorphism of the *MTHFR* gene is SNP 1298A>C, which results in substitution of glutamine amino acid by alanine an in enzyme protein sequence[70,71]. Similar to SNP 677C>T, 1298A>C polymorphism contributes to the reduction in enzymatic activity of MTHFR, but has no connection with the thermolabile proteins. The observed frequency of the mutated 1298C allele is approximately 33%[70,71]. Some of the published studies on SNP 1298A>C suggest that the presence of the 1298C variant of the *MTHFR* gene has no impact on the percentage of positive responses to 5-FU treatment[68,69,72], while two studies suggest that it is associated with significantly decreased patient survival time[67,73]. Thus, contrary conclusions concerning both polymorphic variants of 677C>T and 1298A>C of the *MTHFR* gene call into question their practical application as response predictors in 5-FU-based therapy[74]. However, recent reports suggest that the simultaneous assessment of several markers, such as *MTHFR* 1298A>C and *TYMS* 3'UTR ins/del polymorphisms makes it possible to obtain accurate assessments to predict the toxic effects of 5-FU treatment in CRC patients[75]. Large-scale and well-planned clinical trials are necessary to determine if the practical application of *MTHFR* 677C>T and 1298>C gene polymorphisms would be possible to predict treatment efficacy. It is also necessary to assess whether these SNPs may be used as prognostic markers in patients undergoing CRC treatment based on 5-FU.

Dihydropyrimidine dehydrogenase

5-FU as a prodrug, in order to achieve its intracellular cytotoxic activity, requires metabolic activation (with over 80% of the administered dose of 5-FU degrading rapidly)[76]. Considering 5-FU metabolic pathways in cells, it seems important to conduct pharmacogenetic analysis of the molecular factors that are associated with biotransformation of the drug. Inter-individual variability in the response of patients to 5-FU treatment may in fact be associated with a decrease in the activity of enzymes responsible for catabolism of the drug, which will result in an increase in drug concentration and longer half-life, and thus an increased risk of serious toxic effects[77]. Dihydropyrimidine dehydrogenase (DPD) acts as a regulatory enzyme in the 5-FU catabolic pathway responsible for conversion of 5-FU to 5-fluorodihydrouracil (5-FUH_2). After this conversion, 5-FUH_2 is further metabolized to its final metabolite, 5-fluoro-β-alanine, which is excreted in the urine (Figure 1)[78].

Partial DPD activity deficiency in the general population is about 5%, and its total loss is very rare, about 0.2%[79]. Partial or total loss of DPD activity may be associated with the presence of genetic determinants influencing the function of the *DPYD* gene including SNPs[80], deletion mutations[81,82] and methylation[83]. DPD deficit was first described in an autosomal recessive disease in patients with various neurological symptoms and an accumulation of uracil and thymine in the urine[84]. In recent years, several research groups have investigated the genetic variations present in the *DPYD* gene, and DPD expression levels in tumour cells with respect to their use as predictive markers for predicting both the effectiveness and toxicity of 5-FU treatment[85]. So far, more than 15000 genetic polymorphisms have been recorded in NCBI dbSNP in the coding, intronic and untranslated 3' and 5' regions of *DPYD*. Conditions resulting in a mutant *DPYD* allele include base substitutions, splicing deficits and frameshift mutations[85-87]. Taking into account the effect of catabolic processes on the pharmacokinetics of 5-FU and toxicity resulting from dosage, patients with low DPD activity are at an increased risk of serious or even fatal side effects when using the standard 5-FU dose. Also, case reports of severe and fatal toxicity in patients with markedly low DPD activity and treated with capecitabine suggest that DPD deficiency increases the risk of toxicity after oral administration of 5-FU[88].

Meinsma *et al*[89] described the molecular basis of observed DPD activity deficiency by testing the phenotype and genotype of patients with no DPD activity. Among the analysed cases, there was no 165 nucleotide fragment

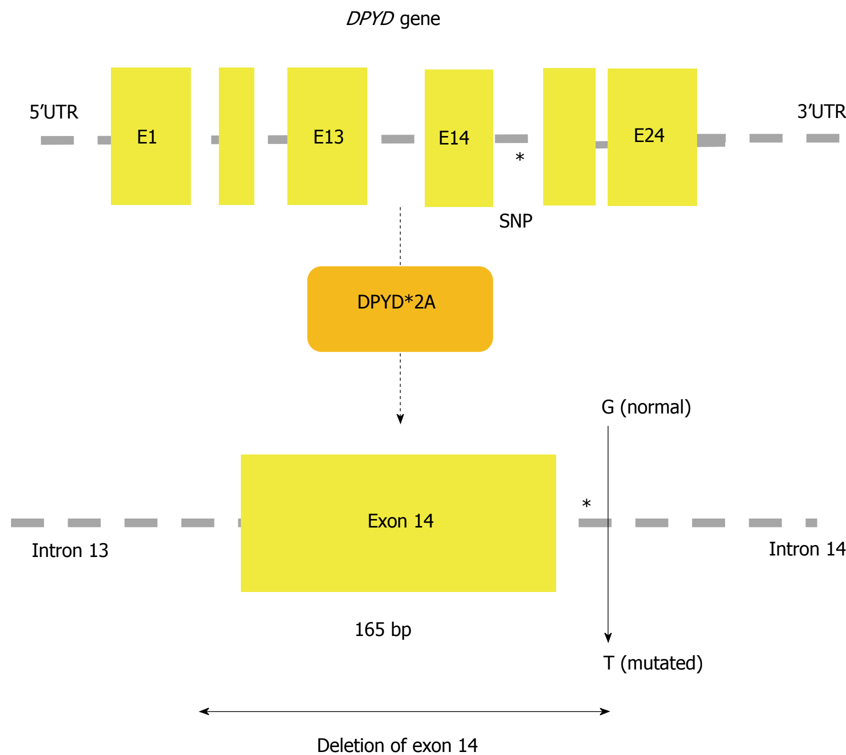

Figure 4 A schematic map of the human *DPYD* gene is shown with the location of SNP *DPYD*2A* (IVS14+1G>A); exon 14 is skipped as a result of the G>A translocation at intron 14.

of mRNA sequence as a result of ejection of one of the exons, moreover, no enzyme DPD protein was detected in these patients[89]. Wei *et al*[90] identified a heterozygous deletion of 165 nucleotides in a British cancer patient, in whom there was no partial DPD activity and who had serious toxicity following administration of 5-FU. They found that a G to A transition within the 5' splice site of intron 14 resulted in exon skipping and an inactive *DPYD* allele (IVS14+1G>A, *DPYD*2A*) (Figure 4)[90]. Other rare (frequency < 0.1%) polymorphisms and mutations have also been identified (85T>C, 496A>G, 1627A>G, 2194G>A, and 2846G>T) as factors possibly affecting the appearance of toxic symptoms after standard 5-FU treatment (Table 1). DPD activity deficiency is observed in approximately 60% of cases occurring in patients with severe toxicity, and *DPYD*2A* polymorphism is found in 50% of patients with the 4th stage of neutropenia as a result of 5-FU treatment[91]. In total, more than 40 *DPYD* polymorphisms were described to have potential use in 5-FU treatment prediction. In addition to single polymorphism changes it has also been demonstrated that the presence of a haplotype consisting of three new intronic SNPs (IVS5+18G>A, IVS6+139G>A, and IVS9-51T>G), and synonymous mutation (1236G>A) may be associated with a decrease in DPD activity[92]. Moreover, hypermethylation of the promoter region of the *DPYD* gene is described as a possible mechanism of variable DPD activity[83,93]. It is believed that only a few of the reasons listed above are responsible for drug resistance and/or toxicity of fluoropyrimidines[94].

Low DPD expression level should lead to reduced catabolism of 5-FU and therefore contribute to a more effective accumulation of the drug inside cells. On the other hand, high DPD activity in tumour tissue should be responsible for the development of drug resistance by reducing the cytotoxic effects of 5-FU. Also, genetic changes in the functioning of other genes encoding enzymatic proteins of the 5-FU metabolic pathway, such as *DPYS* (dihydropyrimidinase)[95] or *UPB1* (β-ureidopropionase)[96] may contribute to a decrease in therapy effectiveness. Furthermore, it was proved that the patients with low expression of three genes, *TYMS*, *DPYD* and thymidine phosphorylase (*TYMP*) have a significantly longer survival time compared to patients with high expression of any of these genes[17]. A similar correlation between low expression of the *DPYD* gene determined using RT-PCR and better response to 5-FU based therapy was found in patients with advanced CRC treated with first-line capecitabine[97]. On the other hand, the results of recent studies in patients with metastatic CRC treated with fluoropyrimidine suggest that this correlation is weak or there is no evidence of an association between the expression of *DPYD* and effectiveness of chemotherapy[37,98,99]. The acquired uncertain evidence is derived mostly from retrospective clinical studies and suggests that low expression of the *DPYD* gene may be a sensitivity marker in tumour cells for fluoropyrimidines and thus allow us to predict the degree of response to treatment. However, currently little good quality clinical data have confirmed the predictive value of *DPYD* expression determination in order to

predict the efficacy of 5-FU therapy in CRC patients[94].

TYMP

TYMP is the gene encoding thymidine phosphorylase (TP), an enzyme that catalyses phosphorylation of thymidine or deoxyuridine to thymine or uracil, and thus is essential for the nucleotide salvage pathway, that recovers pyrimidine nucleosides formed during RNA or DNA degradation[100]. Several studies suggest that TP is a promoter of tumour growth and metastasis by inhibiting apoptosis and induction of angiogenesis[100]. There is evidence that the level of TP expression is connected with angiogenesis, growth and progression of certain types of cancer[101]. An observed increase in TP expression in tumour tissues as compared to that occurring in normal tissues is visible inter alia in CRC[102]. In most of the analysed cases, high TP expression is related to aggressiveness of cancer and poor prognosis, although there are conflicting reports in this regard (Table 2)[100].

TP is involved in the metabolism of 5-FU, where catalysed by TP, 5-FU is converted to 5-fluoro-2'-deoxyuridine (5-FUDR) (Figure 1). This is the first stage of 5-FU activation in tumour cells consequently leading to inhibition of DNA synthesis by reducing the pool of available dTTP to the substrate of this reaction. Capecitabine, an oral form of 5-FU prodrug, is designed to reduce the gastrointestinal toxicity of 5'-deoxy-5-fluorouridine (5'DFUR) and to generate 5-FU preferentially at the tumour site[103]. 5'DFUR may be transformed in cancer cells in a reaction catalysed by TP or uridine phosphorylase[103,104]. Since TP expression is significantly higher in tumour cells, it allows targeted activation which minimizes the toxicity of such therapy[105]. In phase Ⅲ clinical trials, metastatic CRC patients who were treated with capecitabine monotherapy had a significantly lower incidence of toxic effects in comparison to patients treated with 5FU/LV[106]. Moreover, since the enzymatic activity of TP is essential to obtain an adequate level of concentration of an active form of capecitabine, it may be a useful marker for predicting the effectiveness of chemotherapy using this drug[98].

Soong *et al*[107] published a study on the relationship between the expression level of TP (determined by microarrays and immunohistochemistry) and survival time of 945 CRC patients treated with 5-FU. The results of this study suggest that the low level of TP expression may be associated with the improved treatment outcomes observed, and may be a good predictive marker for response to 5-FU chemotherapy[107]. Also, the results presented by Salonga *et al*[17] confirm the link between low TP expression and a positive response to 5-FU. However, results different from the above were obtained by Meropol *et al*[98]. Patients with metastatic CRC treated with combination therapy using CPT-11 plus capecitabine (CAPIRI) were subjected to an assessment of TP protein expression in primary tumour tissues and metastases. Positive results for TP expression confirmed by IHC techniques were associated with a statistically significant longer time to progression (TTP) in comparison with those cases in which a low level of TP expression was found (8.7 mo *vs* 6.0 mo). Conversely, neither TS nor DPD, both enzymes that have been previously shown to correlate with resistance to 5-FU, were able to predict response to CAPIRI[98,108]. Presumably, the cells with higher expression of TP may exhibit an increased sensitivity to 5-FU, due to the increase in FdUMP concentration, which is the result of increased 5-FU activation. On the other hand, low TS expression may lead to serious DNA damage. Since cancer cells are characterized by a higher degree of proliferation compared to normal cells, low TS expression in tumour tissue may lead to a decrease in the dUMP substrate necessary for DNA synthesis, which would inhibit its replication and proliferation. Therefore, the low level of TS expression in tumour cells is associated with a less aggressive course of the disease and a more favourable prognosis in patients. In conclusion, a low level of TS expression may be prognostic rather than a predictor of fluoropyrimidines effectiveness[108,109]. However, the prognostic value of TS expression was not observed in one of the largest retrospective studies[110], which may give rise to questions as to whether further retrospective analysis can provide useful data to confirm the clinical significance of this marker. As highlighted in the meta-analysis by Popat *et al*[20], large methodological differences in individual primary studies make it difficult to come to decisive conclusions. The results of this analysis showed that patients whose tumour tissue had a high level of TS expression were observed to have worse OS compared to the group of patients with a low level of expression. However, as emphasized by the authors of the meta-analysis, the heterogeneity of the studies and possible publication bias do not allow a straightforward conclusion[20].

Uridine monophosphate synthetase

In mammalian cells, the last step of pyrimidine nucleotide synthesis involves the conversion of orotate to uridine monophosphate (UMP) and is catalysed by UMP synthase (UMPS). This bifunctional enzyme has 2 sequential activities, orotate phosphoribosyltransferase (OPRT) and orotidine-5-monophosphate decarboxylase (ODC)[111]. The protein product of the *UMPS* gene is the OPRT enzyme, which catalyses the conversion of 5-FU into FUMP, a common substrate for the production of 5-fluorouridine triphosphate and dUTP, two cytotoxic metabolites that target RNA and DNA, respectively. Muhale *et al*[112] showed that in the anabolic pathway of 5-FU, *UMPS* is the only gene that rounds out a manifestation of the phenotype of resistance to 5-FU. Furthermore, the high OPRT enzyme activity or increased expression of mRNA for *UMPS* gene is associated with longer survival times, suggesting that the *UMPS* may be a clinically useful marker for predicting the effectiveness of treatment with 5-FU[113-121]. In clinical *in vitro* studies carried out by Isshi *et al*[122], OPRT and DPD enzymatic activity was determined by radioassay in tumour tissues taken from

Table 2 Gene/protein expression or metabolic enzyme activity in colorectal cancer cells and correlation with outcome of patients receiving fluoropyrimidine-based chemotherapy

Treatment setting	Method	Patients (*n*)	Better response to chemotherapy	Form of the disease	Ref.
Thymidylate synthase (*TYMS*) [OMIM # 188350]					
5-FU	RT-PCR	29	Low expression	mCRC	Iyevleva *et al*[24]
5-FU	RT-PCR	39	Low expression	CRC	Ishida *et al*[25]
5-FU	IHC	57	Low expression	mCRC	Hosokawa *et al*[26]
5-FU	IHC	62	Low expression	aCRC	Ciaparrone *et al*[27]
5-FU	RT-PCR	92	Low expression	CRC	Nakajima *et al*[28]
5-FU	RT-PCR	309	Low expression	CRC	Kornmann *et al*[29]
5-FU	IHC	391	Not significant	aCRC	Westra *et al*[438]
5-FU	IHC	945	Not significant	CRC	Soong *et al*[107]
FUdR	IHC	36	Low expression	mCRC	Davies *et al*[31]
5-FU/LV or 5-FU	RT-PCR	29	Low expression	mCRC	Kornmann *et al*[32]
5-FU/LV	RT-PCR	33	Low expression	aCRC	Salonga *et al*[17]
5-FU/LV	RT-PCR	36	Low expression	mCRC	Lenz *et al*[7]
5-FU/LV	RT-PCR	42	Low expression	CRC	Leichman *et al*[19]
5-FU/LV	RIA	102	Low expression	mCRC	Etienne *et al*[33]
5-FU/OX	RT-PCR	45	Low expression	aCRC	Shirota *et al*[34]
5-FU/MTX	IHC	108	Low expression	aCRC	Paradiso *et al*[35]
5-FU or 5-FU/MTX or 5-FU/LV	IHC	24	Not significant	aCRC	Belvedere *et al*[439]
5-FU or 5-FU/MTX or 5-FU/LV	IHC	27	Not significant	mCRC	Aschele *et al*[23]
5-FU or 5-FU/MTX or 5-FU/LV	IHC	48	Low expression	mCRC	Aschele *et al*[36]
5-FU/LV/CPT-11	RT-PCR	13	Low expression	aCRC	Yanagisawa *et al*[37]
5-FU/LV/CPT-11	IHC	54	Low expression	aCRC	Bendardaf *et al*[38]
5-FU/LV/CPT-11	IHC	57	Not significant	aCRC	Paradiso *et al*[440]
UFT/LV	RT-PCR	37	Low expression	mCRC	Ichikawa *et al*[39]
Capecitabine	RT-PCR	37	Not significant	aCRC	Vallböhmer *et al*[97]
Capecitabine	IHC	39	Not significant	CRC	Lindebjerg *et al*[441]
Capecitabine/CPT-11	IHC	556	Not significant	aCRC	Koopman *et al*[110]
5-FU-based therapy	IHC	681	Not significant	CRC	Karlberg *et al*[442]
Dihydropyrimidine dehydrogenase (*DPYD*) (OMIM # 612779)					
5-FU	RT-PCR	29	Not significant	mCRC	Iyevleva *et al*[24]
5-FU	RT-PCR	39	Not significant	CRC	Ishida *et al*[25]
5-FU	IHC	62	Low expression	aCRC	Ciaparrone *et al*[27]
5-FU	IHC	303	Low expression	CRC	Jensen *et al*[443]
5-FU	RT-PCR	309	Low expression	CRC	Kornmann *et al*[29]
5-FU	IHC	391	Not significant	aCRC	Westra *et al*[438]
5-FU	IHC	945	Not significant	CRC	Soong *et al*[107]
5-FU/LV	RT-PCR	33	Low expression	aCRC	Salonga *et al*[17]
UFT/LV	RT-PCR	37	Low expression	mCRC	Ichikawa *et al*[39]
5-FU/LV/CPT-11	RT-PCR	13	Not significant	aCRC	Yanagisawa *et al*[37]
Capecitabine	RT-PCR	37	Low expression	aCRC	Vallböhmer *et al*[97]
Capecitabine/CPT-11	RT-PCR	67	Not significant	aCRC	Meropol *et al*[98]
Capecitabine/CPT-11	IHC	556	Low expression	aCRC	Koopman *et al*[110]
5-FU-based therapy	ELISA	64	Low expression	aCRC	Oi *et al*[444]
5-FU-based therapy	RT-PCR	102	Low expression	CRC	Lassman *et al*[445]
5-FU-based therapy	RT-PCR	144	Low expression	aCRC	Gustavsson *et al*[446]
5-FU-based therapy	IHC	150	Low expression	aCRC	Tokunaga *et al*[447]
Thymidine phosphorylase (*TYMP*) (OMIM # 131222)					
5-FU	IHC	62	Not significant	aCRC	Ciaparrone *et al*[27]
5-FU	IHC	945	Not significant	CRC	Soong *et al*[107]
5-FU/LV	RT-PCR	33	Low expression	aCRC	Salonga *et al*[17]
5-FU/LV/CPT-11	RT-PCR	13	Not significant	aCRC	Yanagisawa *et al*[37]
Capecitabine	RT-PCR	37	Not significant	aCRC	Vallböhmer *et al*[97]
Capecitabine/OX	IHC	41	High expression	mCRC	Petrioli *et al*[448]
Capecitabine/CPT-11	RT-PCR	67	High expression	aCRC	Meropol *et al*[98]
Capecitabine/CPT-11	IHC	556	Not significant	aCRC	Koopman *et al*[110]
5-FU-based therapy	RT-PCR	144	Low expression	aCRC	Gustavsson *et al*[446]
5-FU-based therapy	IHC	150	Low expression	aCRC	Tokunaga *et al*[447]
Uridine monophosphate synthetase (*UMPS*) (OMIM #613891)					
5-FU	RT-PCR	38	Not significant	mCRC	Sameshima *et al*[449]
5-FU	RT-PCR	39	Not significant	CRC	Ishida *et al*[25]
5-FU/LV/OX	RT-PCR	58	Not significant	CRC	Dong *et al*[450]
5-FU/LV/cisplatin	RT-PCR	22	High expression	mCRC	Matsuyama *et al*[113]
UFT	RIA	40	High expression	CRC	Ichikawa *et al*[114]
UFT	RIA	124	High expression	CRC	Ochiai *et al*[115]
UFT	IHC	150	High expression	CRC	Tokunaga *et al*[116]

UFT	IHC	160	High expression	CRC	Tokunaga *et al*[117]
UFT/LV	RT-PCR	37	High expression	mCRC	Ichikawa *et al*[118]
UFT/LV	RT-PCR	103	High expression	CRC	Yamada *et al*[119]
5-FU-based therapy	RT-PCR	10	Not significant	CRC	Ishibashi *et al*[451]
5-FU-based therapy	RIA	11	Not significant	CRC	Yamada *et al*[452]
5-FU-based therapy	RT-PCR	52	Not significant	CRC	Kinoshita *et al*[453]
5-FU-based therapy	RIA	54	High expression	CRC	Fujii *et al*[120]
5-FU-based therapy	RIA	90	High expression	CRC	Ochiai *et al*[121]

5-FU: 5-fluorouracil; LV: Leucovorin; FUdR: 5-fluorodeoxyuridine; MTX: Methotrexate; OX: Oxaliplatin; UFT: Compound tegafur tablets; CPT-11: Irinotecan; CTX: Cetuximab; RT-PCR: Reverse trascriptase polymerase chain reaction; IHC: Immunohistochemistry; ELISA: Enzyme-linked immunosorbent ssay; RIA: Radioimmunoassay; CRC: Colorectal cancer; aCRC: Advanced colorectal cancer; mCRC: Metastatic colorectal cancer.

patients diagnosed with CRC (n = 62) and fluorescein diacetate assay (FDA) or histoculture drug response assay (HDRA) were used to determine the chemosensitivity in relation to 5-FU. The chemosensitivity test proved positive in 60% of the specimens with ORPT activity of 0.413 (nmol/min per mg protein) or above and 50% of those with DPD activity of 30 (pmol/min per mg protein) or below. Of the patient specimens showing OPRT activity of 0.413 or above and DPD activity of 30 or below, 88.9% were positive for 5-FU sensitivity, suggesting the possibility that the combination of these two levels may be predictive of positive 5-FU sensitivity[122]. Tokunaga *et al*[116] indicated that high OPRT (IHC) expression in patients with CRC stage Ⅱ-Ⅳ is associated with a longer OS, which was not confirmed in a study using RT-PCR in a smaller study group[37]. The prognostic value of *UMPS*/OPRT expression in both tumour and stromal cells, but each with an opposite effect on outcome, was an unexpected finding in a retrospective analysis of a large trial[110].

There are several described SNPs located in *UMPS*[123-126], including 286A>G (Arg96Gly), 1285G>C (Gly429Arg), 326T>G (Val109Gly), and 638G>C (Gly213Ala). Kitajima *et al*[123] analysed the effects of several SNPs gene *UMPS* (638G>C, 1050T>A, and 1336A>G) on the sensitivity to 5-FU in a group of 31 patients with CRC. They found no relationship between the effectiveness of treatment with 5-FU and frequency of any of the genetic variants among respondents[123]. In clinical *in vitro* trials it was shown that the functional polymorphism, Gly213Ala (638G>C) substitution, contributes to an increase in enzymatic OPRT activity[127]. With reference to the above results, *in vivo* studies showed that patients with substitution of 213Ala in the OPRT protein sequence, after exposure to 5-FU, experience much more severe symptoms of toxicity[124] such as grade 3 diarrhoea (P = 0.031) and grade 2-3 anorexia (P = 0.035)[125]. The probable mechanism of gastrointestinal toxicity is related to the incorporation of 5-FU into RNA (F-RNA), but not with inhibition of the biosynthesis of dTMP by conversion of 5-FU to FdUMP[128]. Therefore, 5-FU/LV administration at a higher OPRT enzymatic activity (especially with the homozygous genotype 638CC) significantly increases the level of F-RNA in enterocytes, which may increase the likelihood of severe diarrhoea[125].

There are still many unknown factors that may participate along with SNPs gene *UMPS* in chemosensitivity or mechanisms of resistance to 5-FU, which makes it necessary to analyse other regions of the gene including the promoter and regulatory region. A lack of confirmed reliable test data from *in vivo* studies on the correlation between the expression of *UMPS*/OPRT and the effectiveness of treatment with 5-FU, makes it now impossible to determine the potential clinical value of this marker.

Other potential factors

A total of 20 polymorphic variants and 20 haplotype systems of the *CYP2A6* gene have been described, which encode P-450 cytochrome isoenzyme involved in the metabolic activation of tegafur (UFT). Based on the results obtained from genotype/haplotype-phenotype association tests, Wang *et al*[129] showed that the variant *CYP2A6*4* is the main determinant contributing to the reduction of formed 5-FU with UFT, and the presence of the allele affects the level of decrease in *CYP2A6* gene expression. A different correlation was observed in the case of 14 haplotype (a novel *CYP2A6*1B* alleles), which was associated with an increase in UTF microsomal activation to 5-FU, and the presence of the haplotype contributed to increased expression of *CYP2A6*. The authors speculate that the phenotype of increased metabolic activity of CYP2A6 may be the result of the sum of three different variants (22C>T, 1620T>C and a gene conversion in the 3'-UTR) included in this haplotype. Wang *et al*[129] conclude that variants *CYP2A6*4* and *CYP2A6*1B* are major genetic factors responsible for inter-individual variation of UTF activation degree to 5-FU.

Microsatellite instability (MSI) is common in many types of tumours and is observed in 10%-14% of sporadic CRC. The MSI phenomenon is caused by mutations located in mismatch repair (MMR) genes, this group of genes are *hMSH2*, *hMLH1* and *hMSH6*. Protein products of these genes are responsible for the repair of DNA damage caused during the replication process. It is believed that the MMR deficiency operation is one of the possible causes of resistance to fluoropyrimidines[130]. Meyers *et al*[131] showed that the restoration of a functional protein MLH1 in an MMR-deficient human colon cancer cell line contributes to increased sensitivity to 5-FU, which suggests that MMR deficiency in cells may be associated with resistance to 5-FU. It is likely that MMR deficiency in cancer cells contributes to increased tolerance to the presence of DNA damage occurring as a re-

sult of replication errors, instead of undergoing cell cycle arrest or death[132]. The results of several studies suggest that the presence of MMR deficit in tumour cells is associated with chemosensitivity to 5-FU based therapy[133]. Most of these studies found low sensitivity to 5-FU in the case of MMR deficiency, which was confirmed by a recent pooled reanalysis of randomized trials[134]. On the other hand, among patients with Ⅱ and Ⅲ stage CRC, prolonged survival time in cases with high MSI was detected[133,135,136]. In addition, when comparing the group of MSI patients with patients who were microsatellite stable it was found that MSI prolongs disease-free time, but is not beneficial in 5-FU adjuvant chemotherapy[137]. Furthermore, it was found that in most of these cases, where the tumours showed positive results for MSI, the expression was observed in wild-type p53[138] which is an important determinant of 5-FU sensitivity.

The tumour suppressor protein p53 plays a key role in the control of cell cycle progression and cell death[139]. It is estimated that in about 50% of cases with various types of tumours a number of mutations in *P53* gene which encodes the p53 can be seen[140]. p53 is responsible for cell cycle arrest and directing cells to the apoptotic pathway in a situation where there is a risk of sustaining damage to the integrity of the genome preventing the transfer of damaged DNA into daughter cells. Longley *et al*[41] demonstrated that p53 and p53-target genes are activated in response to RNA-directed 5-FU cytotoxicity. Moreover, *in vitro* test results indicate that the loss of p53 functionality contributes to reducing chemosensitivity of cells to 5-FU[41,141]. Studies on expression have also shown that overexpression of p53 is correlated with resistance to 5-FU-based chemotherapy[136,142,143] although there is no conformity with the results obtained by other researchers[35]. The impact of the presence of specific mutations of *P53* gene was also described, which may contribute to transformation and drug resistance[144]. Indeed, Pugacheva *et al*[145] suggested that certain p53 mutants may increase dUTPase expression, resulting in 5-FU resistance. Thus, 5-FU chemosensitivity may be dependent on the particular *TP53* genotype.

IRINOTECAN

7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin (CPT-11) is a synthetic analogue of a naturally occurring alkaloid, camptothecin. CPT-11 was first approved for clinical use in Japan in 1994 for the treatment of small-cell lung cancer and hematologic malignancies, and then in 1995 in France for the treatment of advanced CRC. Finally, in 1996, CPT-11 was approved by the US Food and Drug Administration (FDA) and approved for use in the treatment of CRC in 1998. Currently, CPT-11 is mainly used in CRC diagnosed patients with metastases, with recorded relapse or progression after application of standard 5-FU-based therapy[146].

In preclinical screening tests using the HST-1 human squamous carcinoma cell line, SN-38, which is an active CPT-11 metabolite, exhibited the ability to increase the antitumour effect of such cytostatics as cisplatin, mitomycin C, 5-FU, and etoposide[147]. In *in vitro* tests using colon and hepatocellular carcinoma cell lines it was also observed that SN-38 had greater cytotoxic activity compared to cisplatin, mitomycin C, doxorubicin and 5-FU[148]. The *in vivo* tests showed that the positive response rate to CPT-11 monotherapy ranged from 17% to 27% of cases[149]. The effectiveness of CPT-11 based treatment was observed in both the group of patients for which this was the first application of treatment as well as in patients for whom 5-FU therapy was found to be ineffective[150]. The clinical application of the combination of CPT-11 with 5-FU/LV (FOLFIRI) resulted in a significant percentage increase in positive responses, prolonged time to tumour progression and survival. Efficacy was demonstrated both in chemotherapy-naive patients and those who progressed after 5-FU-based chemotherapy when compared with 5-FU/LV alone[151].

Tumour-specific somatic mutations and abnormal gene expression as well as germline genetic variations have been reported to be associated with CPT-11 therapeutic efficacy and toxicity. However, the available studies do not provide unequivocal confirmation that somatic mutations have a significant impact on the outcome of CPT-11 treatment, which prevents their usage as predictive markers. Generally, genetic variations may influence both the pharmacokinetics and pharmacodynamics of CPT-11[152-154]. Taking into account the results of previous preclinical and clinical tests, CPT-11 resistance phenotype may be associated with three different mechanisms: (1) insufficient intra-tumour accumulation of SN-38 (determined by pharmacokinetic factors); (2) a change in TOPI activity that decreases levels of the SN-38-Topo Ⅰ-DNA complex (pharmacodynamic factors); and (3) alterations in the events downstream from the ternary complex, for example, apoptosis, cell cycle regulation, checkpoints, and DNA repair (pharmacodynamic factors)[155,156].

Carboxylesterase

Hydrolysis of the bulky dipiperidino moiety of CPT-11 produces the active metabolite SN-38. The enzymes responsible for these reactions have been identified as human carboxylesterases CES1, CES2 (Figure 5) and the recently described isoenzyme CES3. However, CES3 catalytic activity is low and therefore not likely to play a significant role in the metabolism of CPT-11. Several studies indicated that the CES2 isoenzyme plays a major role in CPT-11 and SN-38 hydrolysis[157].

Resequencing of *CES1* and *CES2* allowed the identification of SNPs and haplotype structure of these genes[158-163]. Numerous SNPs and haplotypes have been described in several populations: Europeans, Africans, and Asian-Americans[163]. Charasson *et al*[158] studied 115 cases (Caucasian population) for sequence analysis of all 12 exons of the *CES2* gene and 5' and 3' untranslated regions, and identified 11 SNPs. One of these SNPs located at position 830 of gene (830C>G) was associated

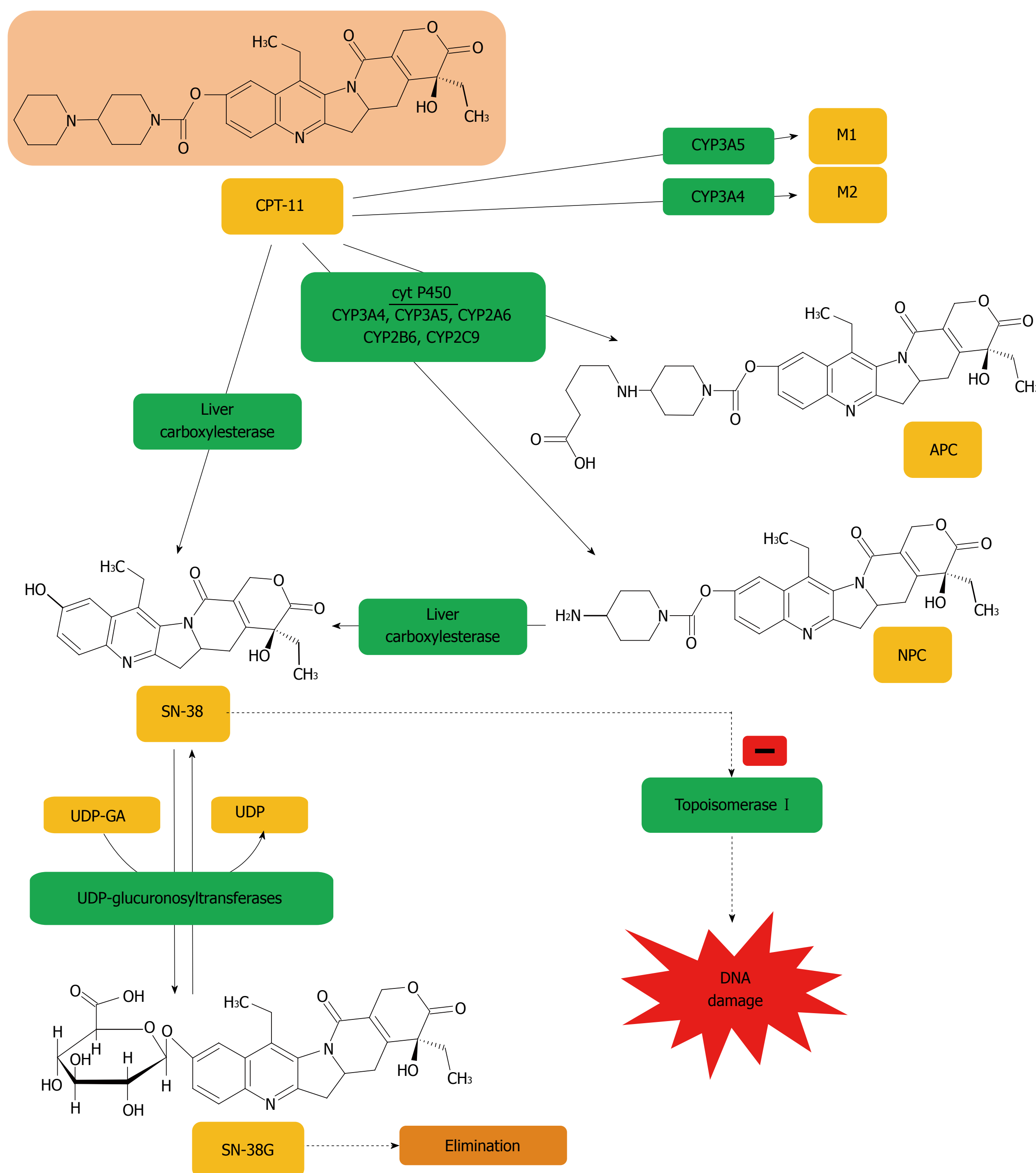

Figure 5 Irinotecan is metabolized to APC or NPC and potential other intermediate metabolites (M1, M2) *via* a cytochrome P450 mediated process. Neither 7-ethyl-10-[4-*N*-(5-aminopentanoic acid)-1-piperidino]carbonyloxycamptothecin (APC) nor 7-ethyl-10-(4-amino-1-piperidino)carbonyloxycamptothecin (NPC) contribute directly to irinotecan activity *in vivo*. NPC is further converted to 7-ethyl-10-hydroxy-camptothecin (SN-38) by carboxylesterase. All irinotecan metabolites are pH sensitive, thus are at risk of transforming from inactive to active products, and *vice versa*. SN-38 is subsequently conjugated predominantly by the enzyme UDP-glucuronosyltransferase 1A1 (UGT1A1) to form a glucuronide metabolite (SN-38G)[403].

with a decrease in *CES2* expression, which has been reported in 60 cases in the North American population[158]. The CPT-11 intra-tumour activation process is partially explained as some authors have provided experimental data indicating that the level of CES2 activity may be a predictor of CPT-11 toxicity[164], while others failed to detect CES2 activity in cultured cells[165].

Kubo *et al*[166] found 12 new SNPs located in the *CES2* gene sequence including the nonsynonymous SNP 100C>T (Arg34Trp) and the SNP at the splice acceptor site of intron 8 (IVS8-2A>G). *In vitro* test results regarding functional characterization of these SNPs, as well as the additional nonsynonymous SNP 424G>A (Val142Met), suggest that the presence of 34_{Trp} and 142_{Met} variants is responsible for the loss of enzyme activity, and IVS8-2_G allele is associated with a significant reduction in metabolic

activity of CES2[166]. Kim *et al*[161], studying a Japanese population, based on linkage analysis of 21 polymorphisms of the *CES2* gene, identified a panel comprising a number of haplotypes and found that some haplotypes were rare in the population, including nonsynonymous SNPs may contribute to the reduction of enzyme activity. Furthermore, Kim *et al*[161] found that patients who are carriers of nonsynonymous SNPs, 100C>T (Arg34Trp) or 1A>T (Met1Leu) have a significantly reduced ratio of (SN-38 + SN-38G)/CPT-11 area under the plasma concentration curve (AUC). *In vitro* test results regarding functional analysis of these SNPs allowed determination of their impact on the efficiency of translation and transcription of the *CES2* gene. It has been shown that the presence of the 1A>T genetic variant does not affect the transcriptional activity of the gene, but it is important for the efficiency of the translation course[161]. These observations are the starting point for further research into *CES2*/CES2 pharmacogenetics, the results of which can be used in future to individualize dosing of CPT-11 and other prodrugs activated by carboxylesterases.

Carboxylesterase hydrolyzes CPT-11 to SN-38 primarily in the liver, but also in plasma and the gastrointestinal tract. It was found that the *CES1* gene is highly expressed in the liver, which is the main organ responsible for the metabolic activation of CPT-11. It is likely that the genetic variants of *CES1* can affect the concentration of CPT-11 metabolites in plasma. However, the clinical relevance of genetic determinants of *CES1* on the pharmacokinetics/pharmacodynamics of CPT-11 is not fully understood. Functional human *CES1* genes include *CES1A1* and *CES1A2* which are inversely located on chromosome 16q. In addition to structural variations of the *CES1* gene family, several SNPs and small deletion/insertion variants were found. The influence of the -816C variant located in the *CES1A2* promoter region on increased transcriptional activity of the *CES1A2* gene was described. Furthermore, Tanimoto *et al*[167] showed that the mRNA expression level of the *CES1A2* gene is related to the sensitivity of tumour cells to CPT-11. Besides, it was found that the polymorphism -816A>C is coupled to several other SNPs (-62T>C, -47G>C, -46G>T, -41C>G, -40A>G, -37G>C, -34del/G and -32G>T) located in the proximal promoter region, which is associated with increased transcription of *CES1A2,* as bound transcription factors such as Sp1 are found in this area[168]. The studies by Yoshimura *et al*[168] suggest that the genetic variant *CES1A* may affect the dose-dependent antitumour activity of CPT-11.

In conclusion, there are certain conditions relating to the impact of polymorphisms located in the *CES1*/*CES2* genes on the metabolism of CPT-11, which, if they are confirmed in large clinical trials, in the future may allow the setting of individual regimens of CPT-11 in patients with cancer (Table 3).

UDP-glycosyltransferase 1 family

SN-38 is glucuronidated, mainly in the liver, to SN-38 glucuronide (SN-38G) by the uridine diphosphate glucuronosyltransferase enzymes (UGTs), primarily the *UDP*-glycosyltransferase 1 family (UGT1As) isoenzyme. SN-38G metabolite is excreted into the bile and urine, where it can be removed from the body. However, rehydrolysis of SN-38G to SN-38, which can take place in the digestive tract under the influence of bacterial β-glucuronidase, can cause acute diarrhoea observed during treatment with CPT-11[169].

UGTs are one of the most important classes of enzyme proteins participating in the coupling reaction phase Ⅱ of xenobiotic metabolism. Currently there are 17 human UGT isoenzymes described that have been assigned to one of two families identified as UGT1 and UGT2, which are further subdivided on the basis of amino acid sequence similarity into UGT1A, UGT2A and UGT2B subfamilies. Members of the UGT1 family are encoded by the *UGT1A* locus on chromosome 2q37, which contains 13 first exons, each having its own promoter and enhancer regions, which are spliced to identical exons 2-5 (Figure 6). UGT1A1 isoenzyme is responsible in humans for bilirubin conjugation with glucuronic acid, and some genetic variants located in the *UGT1A1* gene are associated with the development of hyperbilirubinemic syndromes. These diseases, including Gilbert's syndrome and Crigler-Najjar syndrome type Ⅰ and Ⅱ, are most often described in cases with no or low activity of UGT1A1 as a result of polymorphisms in the sequence of the promoter or coding region[170-172]. Two other isoenzymes, namely the liver UGT1A9 and extrahepatic UGT1A7 are considered important in the SN-38 enzymatic inactivation process. Several research groups have tested *in vitro* the impact of genetic variation in *UGT1A1*, *UGT1A7* and *UGT1A9* on the level of SN-38 glucuronidation[173,174]. Among the frequently occurring genetic variants in the *UGT1A* gene locus 100 SNPs were described, which are located both in the promoter region as well as the coding sequence of the *UGT1A* gene, many of these polymorphisms remain in linkage disequilibrium to the other alleles[175]. Determination of the possible clinical consequences of these functional changes is being studied, and has been fairly well documented for some of the identified alleles. A number of *in vivo* studies were aimed to determine the effect of different *UGT1A* genotypes on the pharmacokinetics and toxicity of CPT-11[176-183].

One of the best known *UGT1A1* polymorphisms is VNTR concerning the number of repetitions of the dinucleotide part of TA [A$(TA)_n$TAA, *n* = 5-8], which is located in the TATA sequence of the promoter region. The wild-type allele contains six repeats $(TA)_6$ (*UGT1A1*1*), which are located between position -53 and -42 of the translational start codon. While $(TA)_7$ (*UGT1A1*28*), an often quoted variant in Gilbert's syndrome[172], in the *in vitro* study was responsible for a 63% reduction in translational activity compared to wild-type alleles[184]. Other variations such as $(TA)_5$ (*UGT1A1*36*), and $(TA)_8$ (*UGT1A1*37*), respectively, contribute to the growth and reduction of transcriptional activity, as observed in *in vitro*

Table 3 Selected common polymorphisms of *UGT1A1, UGT1A7, UGT1A9, CES2, CYP3A4, CYP3A5, MDR1, MRP1, MRP2, BCRP, OATP1B1* genes and their potential impact on functioning of proteins related to CPT-11 pharmacology

dbSNP rs cluster ID	Type of polymorphism	Function	Ref.
UDP-glycosyltransferase 1A1 (*UGT1A1*) (OMIM # 191740)			
rs8175347	VNTR		[177,178,180,182,191,192,197,219,317,356,454-460]
	-53(TA)6>7	UGT1A1*28, reduced activity	
	-53(TA)6>5	UGT1A1*36, increased activity	
	-53(TA)6>8	UGT1A1*37, reduced activity	
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 8175347			
rs3755319	SNP		[187]
	-3279T>G	UGT1A1*60, reduced activity	
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 3755319			
rs10929302[a]	SNP		[192,404]
	-3156G>A	UGT1A1*93, reduced activity	
rs887829[b]	-3140G>A	effect unknown	
[a]http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 10929302			
[b]http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 887829			
rs4148323	SNP		[186,191,461]
	211G>A	Gly71Arg, UGT1A1*6, reduced activity	
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 4148323			
rs35350960	SNP		[172,174,189]
	686C>A	Pro229Gln, UGT1A1*27, reduced activity	
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 35350960			
rs34993780	SNP		[170,174,189]
	1456T>G	Tyr486Asp, UGT1A1*7, reduced activity	
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 34993780			
UDP-glycosyltransferase 1A7 (*UGT1A7*) (OMIM #606432)			
rs17868323[a]	SNP		[188,189,197,237]
	387T>G	Asn129Lys, UGT1A7*2 and *3, increased activity	
rs17863778[b]	391C>A	Arg131Lys, UGT1A7*2 and *3, increased activity	
rs11692021[c]	622C>T	Trp208Arg, UGT1A7*3 and *4, reduced activity	
[a]http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 17868323			
[b]http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 17863778			
[c]http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 11692021			
UDP-glycosyltransferase 1A9 (*UGT1A9*) (OMIM #606434)			
rs45625337	VNTR		[190,197,462]
	–118(T)9>10	UGT1A9*22, increased activity	
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 45625337			
rs2741049	SNP		[197,463]
	IVS1+399C>T	Effect unknown	
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 2741049			
Carboxylesterase 2 (*CES2*) (OMIM #605278)			
-	SNP		[159,161,166]
	1A>T	Met1Leu, CES*5	
rs72547531[a]	100C>T	Arg98Trp, CES*2	
rs72547532[b]	424G>A	Val206Met, CES*3	
rs8192924[c]	617G>A	Arg270His, CES*6	
rs11075646[d]	830C>G	Synonymous	
rs72547533[e]	IVS8-2A>G	Splicing defect, CES*4	
[a] http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 72547531			
[b] http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 72547532			
[c] http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 8192924			
[d] http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 11075646			
[e] http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 72547533			
Cytochrome P450, subfamily IIIA, polypeptide 4 (*CYP3A4*) (OMIM #124010)			
rs2740574[a]	SNP		[211,464,465]
	-392A>G	CYP3A4*1b, altered pharmacokinetics and toxicity	
rs4986907[b]	485G>A	CYP3A4*15, Arg162Gln	
rs4986908[c]	520G>C	CYP3A4*10, Asp174His	
rs12721627[d]	554C>G	CYP3A4*16, Thr185Ser	
rs4987161[e]	566T>C	CYP3A4*17, Phe189Ser, altered pharmacokinetics	
rs55785340[f]	664T>C	CYP3A4*2, Ser222Pro, altered pharmacokinetics and toxicity	
rs28371759[g]	878T>C	CYP3A4*18, Leu293Pro, altered pharmacokinetics and toxicity	
[a]http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 2740574			
[b]http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 4986907			
[c]http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 4986908			
[d]http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 12721627			
[e]http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 4987161			
[f]http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 55785340			
[g]http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 28371759			

rs4986910	SNP		[210,465]
	1334T>C	CYP3A4*3, Met444Thr	
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 4986910			
Cytochrome P450, subfamily IIIA, polypeptide 5 (*CYP3A5*) (OMIM #605325)			
rs776746	SNP		[179,464-467]
	6986A>G	Synonymous	
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 776746			
Multidrug resistance 1 (*MDR1*, *ABCB1*) (OMIM #171050)			
rs1128503	SNP		[210,211,217,460,467-469]
	1236C>T	Synonymous, CTP-11 or SN-38 AUC ↑	
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 1128503			
rs2032582	SNP		[217,468-470]
	2677G>T/A	Ser893Ala or Ser893Thr	
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 2032582			
rs1045642	SNP		[179,217,468-475]
	3435C>T	Synonymous, CTP-11 AUC ↑	
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 1045642			
rs10276036	SNP		[207]
	IVS9-44A>G	Effect unknown	
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 10276036			
Multidrug resistance-associated protein 1 (*MRP1*, *ABCC1*) (OMIM #158343)			
rs35605	SNP		[210,476]
	1684T>C	Synonymous	
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 35605			
rs17287570	SNP		[237]
	c.1677+4951A>C	Effect unknown	
rs3765129	SNP		[207,210,476]
	IVS11-48C>T	Effect unknown	
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 3765129			
rs2074087	SNP		[476,477]
	IVC18-30C>G	Effect unknown	
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 2074087			
Multidrug resistance-associated protein 2 (*MRP2*, *ABCC2*) (OMIM #601107)			
rs1885301	SNP		[477]
	-1549A>G	Effect unknown	
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 1885301			
rs2804402	SNP		[207]
	-1019A>G	Effect unknown	
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 2804402			
rs717620	SNP		[477-479]
	-24C>T	Effect unknown	
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 717620			
rs2273697	SNP		[467,479,480]
	1249G>A	Val417Ile, effect unknown	
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 2273697			
rs3740066	SNP		[477,479,481]
	3972C>T	Synonymous, CTP-11 or APC or SN-38G AUC ↑	
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 3740066			
Breast cancer resistance protein (*BCRP*, *ABCG2*) (OMIM #603756)			
rs2622604[a]	SNP		[237]
	c.-19-17758A>G	Synonymous	
rs3109823[b]	c.-19-3436G>A	Synonymous	
[a]http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 2622604			
[b]http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 3109823			
rs2231142	SNP		[239-244,482]
	421C>A	Gln141Lys, no significant changes in CPT-11 pharmacokinetics	
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 2231142			
rs2231137	SNP		[242,467,482]
	34G>A	Val12Met, higher drug resistance *in vitro* (SN-38)	
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 2231137			
rs1481012	SNP		[483]
	c.841+179T>C	Synonymous	
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 1481012			
Organic anion-transporting polypeptide 1B1 (*OATP1B1*, *SLCO1B1*) (OMIM #604843)			
rs2306283	SNP		[247-249,460,467,484]
	388A>G	Asn130Asp, effect unknown	
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 2306283			
rs4149056	SNP		[247-249,460]
	521T>C	Val174Ala, effect unknown	
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 4149056			

SNP: Single nucleotide polymorphism.

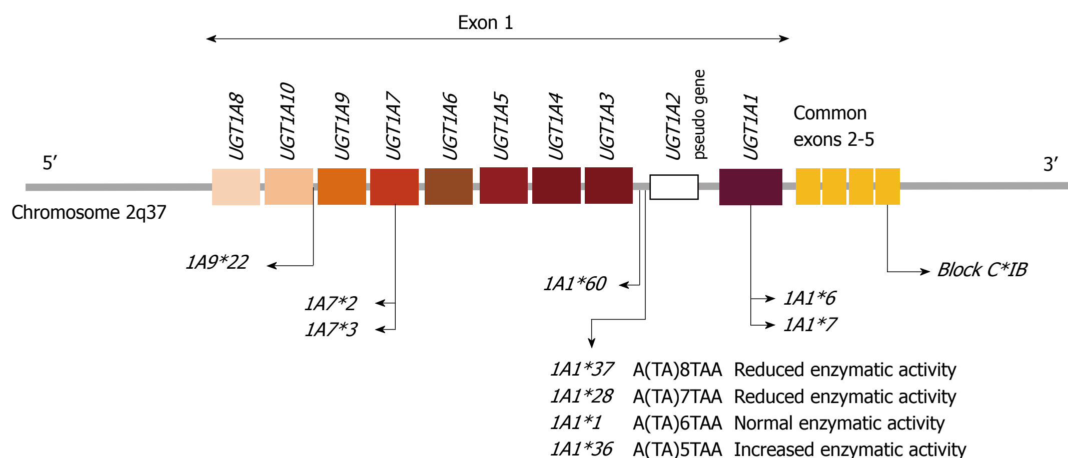

Figure 6 Graphic representation of the human *UGT1A* gene locus encoding the *UGT1A* enzymes and the major *UGT1A1*, *1A7* and *1A9* polymorphisms that are responsible for glucuronidation of SN-38. Individual first exons are positioned at the 5' end of the chromosome and common exons 2-5 at the 3' end. Individual exon 1 sequences are combined with exons 2-5 sequence, which is present in every UDP-glycosyltransferase 1A1 (*UGT1A1*) transcript, the intervening sequence of the primary transcript is eliminated by splicing[404]. The promoter variant, *UGT1A1*28*, **36* and **37* results from a TA insertion/deletion in the $(TA)_6$TAA element of the UGT1A1 promoter region. This alteration leads to decreased/increased gene expression[184].

studies (Figure 6). Iyer *et al*[185] found that human hepatic tissue homozygous for the $(TA)_7/(TA)_7$ polymorphism and tissue heterozygous for the $(TA)_6/(TA)_7$ genotype had a significantly decreased rate of glucuronidation of SN-38 and bilirubin compared with tissue containing the reference sequence allele [$(TA)_6/(TA)_6$]. SN-38 glucuronidation decreased in the following manner: 6/6 > 6/7 > 7/7[185].

In addition, Han *et al*[186] investigated the genetic variation of the *UGT1A* gene. They showed that two SNPs *UGT1A1*6* (211G>A, Gly71Arg) and *UGT1A9*22* were important factors influencing the metabolism of CPT-11 and the toxicity of therapy[186]. Both studied polymorphisms affect the coupling efficiency of SN-38 with glucuronic acid which results in serious toxic effects[186]. The *UGT1A1*60* allele is related to the presence of SNP -3279T>G, and is located in the distal enhancer region [phenobarbital-responsive enhancer module (PBREM)], and is another of the genetic variants of *UGT1A1* which contributes to the reduction in gene transcription activity and an increase in bilirubin concentration in serum[187]. *UGT1A1*27* (686C>A, Pro229Gln) is a rare nonsynonymous polymorphism in the population, *in vitro* studies have shown its relation with a reduced level of glucuronidation of SN-38, and it has been observed in patients with symptoms of Gilbert's syndrome[174]. Another nonsynonymous variant is *UGT1A1*7* (1456T>G, Tyr486Asp) recorded in an Asian population and is associated with Crigler-Najjar syndrome type Ⅱ[170] for which a decrease in activity of the enzyme deactivation pathway of SN-38 was observed[174].

The frequently occurring functional SNPs of the *UGT1A7* gene include: *UGT1A7*2* [387T>G (Asn129Lys), 391C>A, (Arg131Lys)], *UGT1A7*3* [387T>G (Asn129Lys), 391C>A, (Arg131Lys), 622C>T (Trp208Arg)], and *UGT1A7*4* [622C>T (W208R)][188]. For these SNPs in clinical *in vitro* studies conditioned by *UGT1A7*3* and *UGT1A7*4*, the phenotype shows a reduced rate of glucuronic acid conjugation with SN-38[189]. In contrast to these genetic variants, a common VNTR polymorphism -118$(T)_{9>10}$ (*UGT1A9*22*), which is located in the promoter region of the *UGT1A9* gene is associated with increased transcriptional activity, which has been confirmed *in vitro*[190].

First evidence from clinical trials on the role of *UGT1A1*28* in the development of toxicity resulting from administration of CPT-11 was published by Ando *et al*[191]. They studied the relationship of the genetic variants of *UGT1A1* with serious toxic effects (grade 4 leucopoenia and/or grade 3 or 4 diarrhoea) in a group of 118 Japanese patients undergoing CPT-11 therapy in a variety of regimens[191]. Also Innocenti *et al*[192] studying a group of 66 patients (including 50 Caucasians) treated with CPT-11 alone, demonstrated that the *UGT1A1*28* allele is an important factor in the development of grade 4 neutropenia. In this study, it was observed that the incidence of severe neutropenia was much more common in patients with genotype $(TA)_7/(TA)_7$ (50%) compared to heterozygous $(TA)_6/(TA)_7$ (12%) and homozygous $(TA)_6/(TA)_6$ (0%). Moreover, another genetic variant, -3156G>A, is in strong linkage with *UGT1A1*28* and was a better predictor of toxicity than the *UGT1A1*28* polymorphism[192]. Also Marcuello *et al*[182] studied the effect of the *UGT1A1*28* variant on the occurrence of severe toxic effects in a group of 95 cases with CRC (Caucasians) who were treated with CPT-11 containing regimens (5-FU or raltitrexed). In this study, the incidence of acute diarrhoea (grade 3 or 4) was significantly higher in patients who were carriers of *UGT1A1*28* mutations [homozygous (50%) and heterozygous (33%)] in comparison to homozygotes of wild-type (17%). Also, symptoms of neutropenia were more frequently noted in the homozygotes group with the *UGT1A1*28* allele, however, this relationship was not statistically significant[182].

The first systematic analysis of clinical studies on the impact of *UGT1A1*28* on the effectiveness of CPT-11 therapy was published by Dias *et al*[193]. These results were generally supportive of the clinical utility of genotyping *UGT1A1*28* prior to commencement of CPT-11 therapy in order to decrease the risk of severe neutropenia and diarrhoea through the pre-emptive dose reduction of CPT-11 for *UGT1A1*28* homozygotes. The meta-analyses indicate that there is unlikely to be an important association between *UGT1A1* genotype and ORR with CPT-11, however, this does not provide direct evidence that a dose reduction for *UGT1A1*28* homozygotes will not lead to an important reduction in ORR[193]. Hu *et al*[194] published a meta-analysis of the relationship between the presence of *UGT1A1*28* and the incidence of neutropenia induced by CPT-11. It has been shown that the presence of *UGT1A1*28* is associated with an increased risk of developing neutropenia, not only in cases of medium or high CPT-11 dose applied, but also in patients treated with low doses of the drug. The dose-dependent manner of SN-38 glucuronidation explained why the association between *UGT1A1*28* and neutropenia was dose dependent[194]. Also, Hu *et al*[195] published a meta-analysis of clinical studies on the relationship between the presence of the variant *UGT1A1*28* and the risk of severe diarrhoea. Also in this case, in patients who are carriers of one or two mutant alleles [genotypes $(TA)_7/(TA)_7$ or $(TA)_6/(TA)_7$] there was an increased risk of severe diarrhoea induced by CPT-11. However, this increased risk was present only in the group of patients with high and medium drug dose[195]. This evidence supports the assessment of *UGT1A1*28* in routine clinical practice. The FDA-approved diagnostic blood test (Invader®) is available specifically for testing the *UGT1A1*1* (wild-type) and the *UGT1A1*28* genotype. However, the proposed benefit of testing CRC patients for *UGT1A1* genotype is that the risk for adverse drug-related side effects (*e.g.*, severe neutropenia) among patients found to be homozygous for the **28* genotype can be reduced by lowering their initial and/or subsequent doses of CPT-11. The concomitant harm is that a reduction in CPT-11 dosage may also reduce the effectiveness of chemotherapy in tumour suppression and long-term survival[133,196].

In recent years, several studies were published on the effects of *UGT1A* polymorphisms on CPT-11 effectiveness in CRC cancer therapy. Marcuello *et al*[182] observed a trend in reduced OS in patients with genotype $(TA)_7/(TA)_7$ or $(TA)_6/(TA)_7$ in a study of 95 (Caucasians) cases with metastatic CRC who underwent therapy based on CPT-11. The probable reason for poor response to treatment, as concluded by the authors, was the need to reduce the dose of CPT-11 in patients with symptoms of severe diarrhoea, and who were carriers of the mutant allele *UGT1A1*28*. Toffoli *et al*[177] studying a group of 71 patients (Caucasian) with CRC and metastasis observed that in the homozygous group $(TA)_7/(TA)_7$ there was a higher percentage of positive responses to the treatment based on CPT-11 and longer survival time as compared to the homozygous group $(TA)_6/(TA)_6$. The authors suggested that toxicities in $(TA)_7/(TA)_7$ patients could be well-managed during the entire course of treatment without a reduction of CPT-11 dosage[177]. The impact of genetic variants of *UGT1A7* on the effectiveness of therapy with capecitabine/CPT-11 was examined[197]. The analysis of 66 cases of CRC (including 55 Caucasians) demonstrated that the homozygous groups *UGT1A7*2/*2* and *UGT1A7*3/*3* showed low enzymatic activity and a lower incidence of severe diarrhoea (P = 0.003), but a higher percentage of positive responses to treatment (P = 0.013) compared with the other genotypes[197]. Also, considering the impact of another polymorphism located in the sequence *UGT1A9* [-118 $(T)_{9>10}$, *UGT1A9*22*], it was observed that the presence of genotype $(T)_9/(T)_9$ significantly reduced the toxicity (P = 0.002) and increased the degree of response to treatment (P = 0.047)[197]. These results suggest that the low activity phenotype of isoenzymes UGT1A7/1A9 conditioned by the presence of genetic variants is associated with a protective effect against toxicity such as severe diarrhoea. The authors explained that this observation may be due to reduced excretion of SN-38G to the intestine, where it is under the influence of bacterial β-glucuronidase hydrolysed to SN-38, responsible for toxic effects such as severe diarrhoea[197,198]. This finding also raised caution that higher intestinal levels of SN-38G can promote diarrhoea, while hepatic glucuronidation offers protection against neutropenia[197].

Cecchin *et al*[176] performed genotyping of (*UGT1A1*28, UGT1A1*60, UGT1A1*93, UGT1A7*3* and *UGT1A9*22*) in a large group of 250 CRC patients with metastases treated with the FOLFIRI regimen. In addition, the study determined the relationship of these genetic variants with the incidence of severe hematologic and nonhematologic toxicities, the degree of response to therapy, and TTP and OS[176]. The results demonstrated that only the variant *UGT1A7*3* may be a marker of severe hematologic toxicity after the application of the first cycle of therapy (P = 0.04). In addition, *UGT1A1*28* allele and II haplotype (all the variant alleles, but not *UGT1A9*22*) were associated with a response indicator of the therapy (P = 0.01), and the *UGT1A1*28* allele was also the only marker associated with TTP. The authors concluded that genetic variants near *UGT1A1*28* may be predictors in CRC patients treated with FOLFIRI[176]. Liu *et al*[199] examined the impact of a polymorphic variant *UGT1A1*28* on toxicity and the results of treatment in a group of 128 Chinese CRC patients with metastases undergoing therapy with FOLFIRI. It was found that, although the need to reduce the dose of CPT-11 was significantly higher in patients with genotype $(TA)_6/(TA)_6$ (P < 0.01), it had no significant effect on the rate of response to CPT-11 therapy, PFS and OS[199].

The above reports make it difficult to draw clear conclusions whether reduced UGT1A activity conditioned by the presence of genetic variants in the gene sequence only intensifies the anti-cancer activity of CPT-11, or

results in a better response to treatment with the simultaneous increased frequency of severe toxic complications. It seems that the overall balance of the effectiveness/toxicity of the therapy depends primarily on the treatment regimen used. Moreover, the appearance of severe toxicities depends on the exposure levels of SN-38 in the tissues, however, the antitumour responses can be influenced by additional factors related to properties of target tumours, such as the tumour stage, acquisition of resistant factors, and sensitivity to other chemotherapeutic agents when combined.

CYP3A4 and CYP3A5

CYP3A4, which is highly expressed in the liver, is considered one of the major P-450 cytochrome isoenzymes involved in the metabolism of a large group of drugs. CYP3A4 and CYP3A5 are responsible for CPT-11 oxidation to the APC metabolite (7-Ethyl-10-(4-*N*-aminopentanoic acid)-1-piperidino)carbonyloxycamptothecin and inactive NPC (7-Ethyl-10-(4-amino-1-piperidino)carbonyloxycamptothecin), which can be hydrolysed to an active form of SN-38 (Figure 5). Inter-individual variation in CYP3A4 activity may contribute to changes in the pharmacokinetic parameters of CPT-11[200-202].

Several polymorphisms located in genes *CYP3A4* and *CYP3A5* have been described[203-206]. There are different SNPs for *CYP3A4* and the frequencies of genotypes and alleles occurrence in different populations have been published. Relatively frequent SNPs are *CYP3A4*2* (664T>C, Ser222Pro), *CYP3A4*10* (520G>C, Asp174His), and *CYP3A4*17* (566T>C, Phe189Ser) in Caucasians and Mexicans (2%-5%), *CYP3A4*15* (485G>A, Arg162Gln) in African-Americans (2%-4%) and *CYP3A4*16* (554C>G, Thr185Ser) and *CYP3A4*18* (878T>C, Leu293Pro) in East Asians (1%-10%)[207]. Perhaps some of these genetic variants of *CYP3A4* may have impact on the pharmacokinetics of CPT-11. An analysis of gene haplotypes of *CYP3A4* conducted in a group of 416 cases from the Japanese population has allowed the identification of 25 haplotypes[208]. However, the influence of individual haplotypes on the pharmacokinetic parameters of CPT-11 was tested among 177 Japanese patients undergoing chemotherapy[209]. Haplotype **16B* which consists of polymorphisms 554C>G (Thr185Ser) and IVS10+12G>A was present only in male patients, and in this group a significantly lower concentration ratio of APC/CPT-11 (*in vivo* tests of CYP3A4 activity) was observed compared with other patients. However, no relationship was observed between the genotypes and total clearance of CPT-11, and the frequency of toxicity symptoms in the study group[209]. Despite significant individual variability[206] and occurrence of more polymorphisms within genes *CYP3A4* and *CYP3A5*, in the currently published studies there is no significant correlation between genotype CYP3A4/5 and the pharmacokinetics of CPT-11 or toxicity[210,211]. No significant correlation between genotypes *CYP3A4/5* and the pharmacokinetic parameters of CPT-11 may be associated with the low frequency of alleles in most described genetic variants of *CYP3A* in the Caucasian population (*e.g.*, *CYP3A4*17, CYP3A4*18,* and *CYP3A5*1*), or the presence of these variants does not result in measurable changes in enzyme activity *in vivo* (*e.g.*, *CYP3A4*1B*)[157]. In conclusion, the current research findings do not support the clinical use of *CYP3A4/5* genotyping in order to differentiate individual doses of CPT-11.

ABC and SLC transporters

In addition to the importance of the metabolism of CPT-11, the influence of the above-mentioned enzymes on the pharmacokinetics of the drug, and its own influence can also be demonstrated on different transporters, especially from the ABC (ATP-binding cassette transporter superfamily) group of transporters. ABC transporters play an important role in the pharmacology of CPT-11[157], and are one of the major causes of cancer cell resistance observed *in vitro* and *in vivo*[212]. A number of polymorphic variants of genes encoding proteins of ABC transporters and their potential impact on the transcription/expression and changes in transport activity have been described[213]. CPT-11, SN-38 and SN-38G are transported from cells to the extracellular environment *via* ABCB1 multidrug resistance (MDR1), ABCC1 multidrug resistance protein 1 (MRP1), ABCC2 multidrug resistance protein 2 (MRP2), ABCG2 breast cancer resistance protein (BCRP) and SLCO1B1 organic anion-transporting polypeptide 1B1 (OATP1B1) (Figure 7)[214]. Transport proteins which export CPT-11 and its metabolites to bile and urine were examined due to their potential impact on the effectiveness of anticancer therapy, and the occurrence of adverse reactions[215,216].

Studies regarding the influence of transport protein P-glycoprotein encoded by the gene *ABCB1/MDR1* on CPT-11 pharmacology, have given ambiguous results. More than a dozen different polymorphisms have been identified in the sequence of the gene *ABCB1*. Research evaluating the impact of SNPs on the pharmacokinetics of CPT-11 typically focus on three well-known polymorphisms 1236C>T, 2677G>T/A and 3435C>T, which are in strong linkage disequilibrium[157]. Some studies have shown that both single genetic variants and haplotypes of *ABCB1* can increase the bioavailability of CPT-11 and SN-38[210,217], while other studies have come to the opposite conclusion[216,218]. Furthermore, Korean studies found an association between the presence of wild-type *ABCB1* and the occurrence of neutropenia[218], which was not confirmed by the results from American research[216]. Similarly, a lack of correlation between the occurrence of SNPs *ABCB1* and toxicity of CTP-11 therapy was found in French studies[179]. On the other hand, studies by Glimelius *et al*[219] demonstrated that patients who are carriers of the mutated allele *ABCB1* are less responsive to treatment with CPT-11. Carriers of at least one TT genotype of *ABCB1* 1236C>T, 2677G>T/A or 3435C>T were less likely to respond to treatment (OR = 0.32). A *post hoc* analysis showed that fewer patients with at least

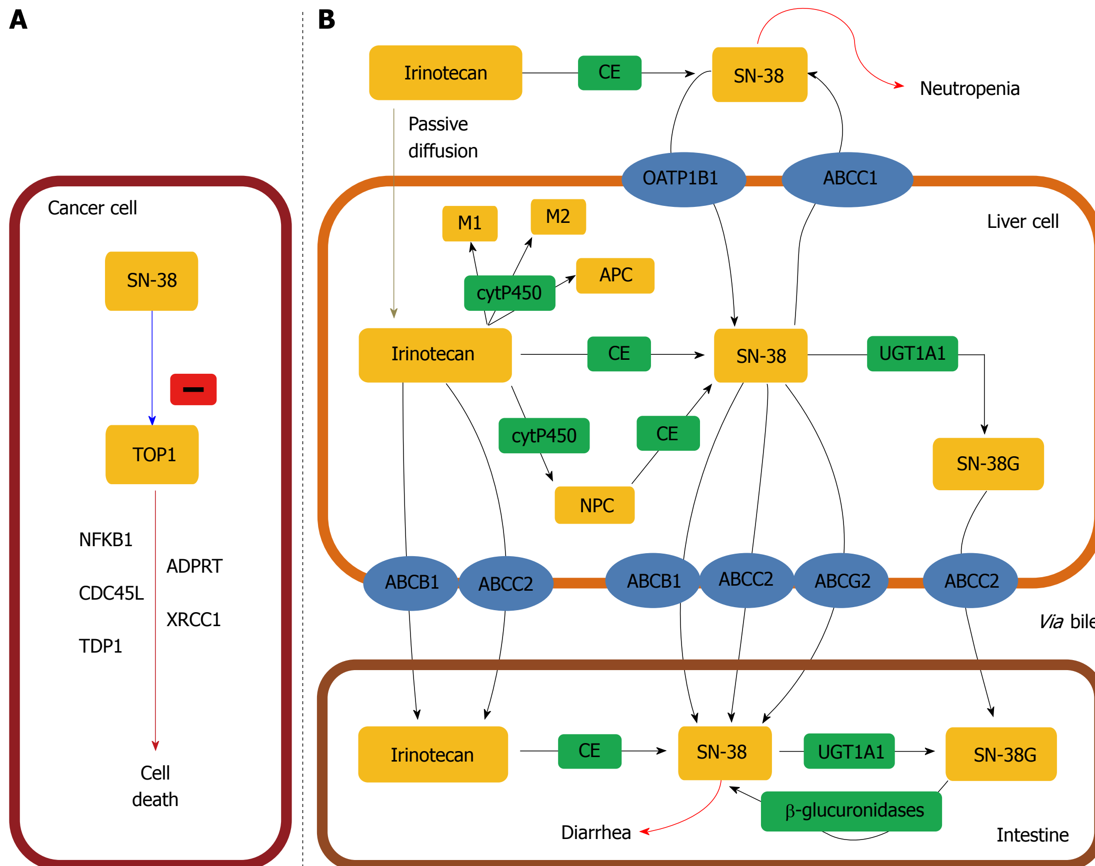

Figure 7 UDP-glycosyltransferase 1 family. A: The active metabolite of irinotecan, SN-38, is a DNA topoisomerase I (TOP1) inhibitor which leads to cancer cell death. TOP1 is a nuclear enzyme required in replication, responsible for unwinding DNA and preventing lethal strand breaks. SN-38 is cytotoxic and destabilizes the TOP1-DNA covalent complex formed in colorectal cancer cells. SN-38 causes irreversible double strand breaks which lead to S phase arrest followed by cell death. To do so, SN-38 attaches to the complexes and blocks future replication forks preventing repair of double strand breaks[405]; B: Irinotecan uptake and transport into the liver is facilitated by: OATP1B1 (SLCO1B1), ABCB1, MRP1 (ABCC1), MRP2 (ABCC2), and MXR (ABCG2). Specifically, ABCB1 is present on the bile membrane and is responsible for the secretion of irinotecan and its metabolites into the liver[406]. Irinotecan is metabolized in the liver and converted to SN-38, the active metabolite and TOP1 inhibitor, by carboxylesterases (CE) mediated hydrolysis. SN-38 is then glucoronized to SN-38 glucuronic acid (SN-38G) and detoxified in the liver *via* conjugation by the UGT1A family, which releases SN-38G into the intestines for elimination[407]. Approximately 70% of SN-38 becomes SN-38G, which has 1/100 of the antitumour activity and is virtually inactive. In the intestinal lumen, bacterial β-glucuronidases can reverse the reaction and transform inactive SN-38G back into the active form SN-38. This factor contributes to varied toxicity, specifically dose limiting diarrhoea[198].

one *ABCB1* 1236T-2677T-3435T haplotype responded to treatment compared with others (43% *vs* 67%, P = 0.027)[219]. Given the conflicting results obtained in earlier research on the impact of genetic variants of *ABCB1* on the effectiveness of CPT-11 therapy[179,210,216-218], the conclusions presented by Glimelius *et al*[219] need to be confirmed in *in vivo* studies in a larger population.

Several *in vitro* studies have shown that ABCC1/MRP1 is involved in the transport of CPT-11 and SN-38. The ABCC1 transporter is responsible for the efflux of SN-38 from the hepatocyte into the interstitial space[220]. Polymorphisms 462C>T, 1684T>C, 4002G>A, 14008G>A, 34215C>G, IVS9+8A>G, IVS30+18A>G, IVS11-48C>T and IVS18-30C>G in the *ABCC1* gene have been identified[210,216]. Two SNPs of *ABCC1*, 1684T>C and IVS18-30C>G, are responsible for differentiated pharmacokinetic phenotypes of CPT-11 as measured by the AUC values for its metabolites: APC and SN-38G/SN-38. Polymorphism 1684T>C contributes to an increase in AUC value for SN-38, and SNP IVS11-48C>T causes a decrease in AUC for APC. The positive association between *ABCC1* 1684T>C and SN-38 AUC is consistent with increased transport of SN-38 from the hepatocyte into the plasma[216]. In comparison to the available data on the role of *ABCB1* in drug resistance and bioavailability of CPT-11, the clinical significance of the genetic variation of *ABCC1* is not sufficiently documented, and therefore further functional studies should be carried out to confirm these preliminary observations[216]. There are several rare variants of ABCC1, which may potentially affect the transport function, but the low frequency of occurrence of these alleles hinders unequivocal conclusions regarding their clinical significance in pharmacotherapy of CPT-11[221-224]. Similarly, there is insufficient evidence regarding the effect of the polymorphisms in the gene expression of *ABCC1* measured by mRNA levels in lymphocytes or duodenal enterocytes[225].

In vivo tests on animals showed that the biliary excretion of CPT-11 carboxylate and SN-38 carboxylate, and both the lactone and carboxylate forms of SN-38G was

lower in ABCC2-deficient rats[226]. Moreover, the impact of gene polymorphisms *ABCC2/MRP2* on the bioavailability of CPT-11 has been described. Innocenti *et al*[192,227] examining a group of 64 cancer patients showed that the silent polymorphic variant 3972T>C was associated with the AUC value of CPT-11 (P = 0.02), for APC (P < 0.0001) and for the APC/CPT-11 ratio (P < 0.0001). Kitagawa *et al*[228] also studied the effects of gene SNPs of *ABCC2* on the toxicity of CPT-11 therapy. However, in the 120 Japanese patients studied, there was no association between genetic variants 1249G>A, or -24C>T gene *ABCC2* and the incidence of severe complications after treatment with CPT-11[228].

There are many studies confirming the important role of protein ABCG2/BCRP in the transport of CPT-11 and its metabolites. Scientific evidence supports the proposition that overexpression of *ABCG2*/ABCG2 leads to the development of drug resistance in tumour cells against drugs that are derivatives of camptothecin such as topotecan[229], CPT-11 and SN-38[230-233]. Several possible mechanisms were described which may contribute to drug resistance conditioned by the activity of gene *ABCG2*, such as: demethylation of CpG islets in the *ABCG2* promoter resulting in increased gene transcription[234], gene amplification[235], and truncation at the 3'UTR of the *ABCG2* mRNA, which is associated with a loss of the miRNA-159c binding site conferring higher mRNA stability[236]. Furthermore, it has recently been demonstrated that the *ABCG2* mRNA content of liver metastatic tumour cells from CRC patients treated with CPT-11 is higher than that from CPT-11-naive patients[207]. Cha *et al*[237] suggested that the presence of introning SNP in gene sequence *ABCG2* (rs2622604) may contribute to changes in transport protein activity which can effect the increase in CPT-11 concentration in cells. This may lead to an increased risk of severe myelosuppression (grades 3 and 4) in patients with this genetic variant[237]. The same research team also identified another SNP (rs3109823), which like the previous one had a strong association with severe myelosuppression[237]. Following this study, Poonkuzhali *et al*[238] showed that a polymorphic variant of rs2622604 was associated with decreased expression of *ABCG2* measured by the level of mRNA. These results support the hypothesis that patients who are carriers of the rs2622604 negative variant, have in their livers, a low level of SN-38 excretion to the bile which leads to the growth of intracellular concentrations of SN-38 in hepatocytes. This, in turn, contributes to accumulation of CPT-11/SN-38 in the blood and an increased risk of severe myelosuppression. On the other hand, although described by Cha *et al*[237], another SNP rs3109823 showed a stronger association with myelosuppression than the variant rs2622604, and Poonkuzhali *et al*[238] did not prove it had an effect on the gene expression level of *ABCG2*.

Functional *in vitro* studies on the importance of amino acid substitution in the sequence of protein ABCG2 (Gln141Lys, 421C>A) have shown that it contributes to the reduction of transport activity substrates such as mitoxantrone, topotecan, SN-38[239,240], and therefore can contribute to an increase in cell chemosensitivity[241,242]. There were also several *in vivo* studies published on the effect of this polymorphism on the pharmacokinetics of CPT-11. de Jong *et al*[243] studied a group of 85 patients diagnosed with solid tumours who received chemotherapy based on CPT-11. They reported greater accumulation of SN-38 and SN-38 glucuronide in one of two homozygous carriers of the 421 variant alleles. However, the AUC of CPT-11 (P = 0.72) and its active metabolite SN-38 (P = 0.67) did not differ significantly between patients carrying the wild-type sequence and patients carrying at least one variant allele[243]. Also, the results of research published by Jada *et al*[244] confirmed the findings that there is no relationship between the presence of genetic variants 421C>A gene *ABCG2*, and the change in the pharmacokinetics of SN-38. Available results from this study suggest that the probable coexistence of SNPs other than 421C>A genetic variants [*e.g.*, 34G>A (Val12Met) and 1322G>T (Ser441Asn)] of the gene *ABCG2* may have some clinical implications for the pharmacology of CPT-11. Furthermore, additional *in vitro* and *in vivo* studies are needed to better clarify the role of the 34G>A polymorphism as this SNP is prevalent in many populations and there are many conflicting reports regarding the functional effects of this polymorphism[245]. Systematic prospective studies with well-chosen and less heterogeneous groups of patients should be conducted to provide more reliable evidence on the role of gene polymorphisms of *ABCG2* on the pharmacokinetics of CPT-11.

Organic anion-transporting polypeptide 1B1 (OATP1B1, SLCO1B1), expressed on the basolateral membrane in hepatocytes, has been reported to contribute to the hepatic uptake of SN-38[246]. SLCO1B1 transports among others, CPT-11, SN-38 and SN-38G from blood to liver cells. Several polymorphic variants of the gene *SLCO1B1*, among them *SLCO1B1*1b* (388A>G) and *SLCO1B1*5* (521T>C), have been described. *In vitro* research on the haplotype *SLCO1B1*15*, which is a combination of the SNPs, showed that it is responsible for a 50% reduction in the intracellular concentration of CPT-11, which may cause intra-individual variability in the toxicity of this drug[246,247]. Another pharmacokinetic study revealed that CPT-11 clearance was 3-fold reduced and systemic exposure to CPT-11 was enhanced in patients with the *SLCO1B1*15* haplotype[248]. The literature also describes the case of a patient with severe toxic complications after CPT-11 treatment and the presence of the haplotype **15*[249]. The effect of these SNPs and haplotype **15* on induction of CPT-11 toxicity should be confirmed by further *in vivo* studies. Other studies on the toxicity of CPT-11 and its effects on different genetic factors were carried by Takane *et al*[250]. By analysing three genetic variants of *UGT1A1*6*, *UGT1A1*28* and *SLCO1B1*15* a strong correlation was found between the presence of these alleles and excessive accumulation of

SN-38, which resulted in severe toxic complications observed with the use of CPT-11.

In summary, it can be stated that frequent polymorphisms in genes encoding ABC and SLC transporters can have a significant impact on changes in the pharmacokinetics and pharmacodynamics of CPT-11. However, the practical application of previously published results will require additional study *in vivo* including CRC patients.

Topoisomerase Ⅰ*, DNA repair genes and cell cycle regulation*

There is substantially less knowledge on CPT-11 pharmacodynamics, including DNA damage repair or cell death pathways, following the formation of camptothecin-TOP Ⅰ-DNA complexes[251]. SN-38 is an inhibitor of topoisomerase Ⅰ (TOP Ⅰ) an enzyme that prevents the unfolding of DNA during transcription and replication. Scientists studying cancer cells which exhibited resistance to CPT-11, found that a possible cause of low sensitivity to the drug may be associated with the presence of mutations or low *TOP1* gene expression[252,253]. The impact of the presence of different genetic variants of *TOP1* gene expression was described, which may be a cause of primary drug resistance[254]. Genetic variation in the drug target of SN-38, as well as in cellular effectors responsible for DNA repair and apoptosis, are a potential source of clinically observed inter-individual variability in the efficacy and toxicity of treatment based on CPT-11[255]. Knowledge of the causes of drug resistance leading to CPT-11 treatment failure, provides the opportunity to better plan treatment and to predict the effects of therapy for an individual patient. The activity of numerous genes and proteins[155,255] and a mutual network of connections between various intracellular pathways are responsible for the phenotype of sensitivity to CPT-11. The molecular factors involved in CPT-11 pharmacodynamics may include: drug target-TOP Ⅰ, cell cycle division 45-like protein (CDC45L), nuclear factor-κB (p50 subunit; NFκB1), poly (ADP-ribose) polymerase Ⅰ (PARP1), tyrosyl DNA phosphodiesterase (TDP1), and X-ray cross complementation factor (XRCC1)[256-260].

XRCC1 plays a key role in base excision repair by forming a complex with DNA repair proteins including PARP1 and DNA polymerase β[261]. Hoskins *et al*[251] studied a group of 107 (European) patients with advanced CRC, treated with CPT-11. They conducted an analysis of the impact of genetic variant 1196G>A (Arg399Gln) of the gene *XRCC1* on the efficacy of CPT-11 therapy. They found that patients who demonstrated a favourable response to treatment more commonly had the genotype 1196_{GG} variant allele than 1196_{T} (genotypes GA or AA) (46% *vs* 26%, $P = 0.10$). Patients homozygous for an *XRCC1* haplotype (GGCC-G) were more likely to show an objective response to therapy than other patients (83% *vs* 30%, $P = 0.02$). This effect was also confirmed in a multivariate analysis (OR = 11.9, $P = 0.04$)[251]. A possible explanation for these findings is that the presence of the allele in the 1196_{G} gene sequence *XRCC1* conditioning the presence of arginine in the protein sequence XRCC1 (399_{ARG}) leads to weaker DNA repair capacity, as compared with 1196_{A} (399_{Gln}). However, these findings, derived from *in vivo* studies, have not been confirmed in numerous *in vitro* studies, which unanimously showed that the presence of glutamine in codon 399 was associated with a reduced ability to repair DNA as assessed by the persistence of DNA adducts, elevated levels of sister chromatid exchanges, increased RBC glycophorin A, *TP53* mutations, and prolonged cell cycle delay[262]. Hoskins *et al*[251] also investigated the effect of the gene variant IVS4+61 *TOP1* on the frequency of severe neutropenia (grade 3/4). The cause of the differences observed *in vivo* in the toxicity of CPT-11 therapy and the frequency of different variants of the *TOP1* gene, can be related to the stability of complexes SN-38-TOP Ⅰ-DNA in bone marrow cells, which may lead to greater sensitivity and increased bone marrow toxicity. Furthermore, Hoskins *et al*[251] found that patients who are carriers of the homozygous CC gene haplotype *PARP1* (with SNPs combination 852T>C-IVS19-297C>T) often suffer toxic effects due to CPT-11 treatment in comparison to patients with different arrangement of alleles in this haplotype. This observation suggests that the presence of the haplotype 852_{C}-IVS19-297_{C} is related to decreased DNA repair capacity by PARP1 protein, leading to increased loss of bone marrow cells and symptoms of neutropenia as a result of the cytotoxic effect of CPT-11[251].

In vitro research using colon/colorectal carcinoma cell lines, showed that there is a link between the presence of functional aberration in p53 and phenotype hypersensitivity to camptothecins[263-266], whereby some of the experimental test models showed only moderate cellular sensitivity[267]. Moreover, HT-29 colon carcinoma cells characterized by mutations in p53 had a much higher sensitivity to CPT-11 than control cells expressing wild-type p53[268]. Also, experiments with cell clones derived from tumour tissues with evidence of impaired activity of p53 showed that the apoptosis induction path is an important determinant of sensitivity to camptothecins. On the other hand, p53 is required for targeting apoptotic proteins in the sensitization of colon carcinoma to TNF-related apoptosis-inducing ligand (TRAIL) pathway therapy using CPT-11[269]. Most experimental data show that the initiation of apoptosis resulting from exposure to camptothecins is much weaker for cells with wild-type p53 compared with mutated p53. Tomicic *et al*[270] proposed that the phenotype conditioned by wild-type p53, formed in the presence of CPT-11 complexed with DNA and TOP Ⅰ is degraded more easily, leading to the reduced DNA transcription/replication effect of camptothecins and contributes to the development of drug resistance. In cells lacking functional p53, TOP1-cc (TOP1-cleaved DNA 3'-phosphotyrosyl intermediates referred to as cleavable complexes) is not efficiently degraded upon transcription stalling, thus TOP1-linked single-strand breaks accumulate, which may interfere with DNA

replication. p53 defective cells are, due to lack of p21 expression, only transiently arrested in G2, having no time to repair excessive camptothecin-induced replication-dependent double-strand breaks (DSB), thus undergoing mitotic cell death accompanied by apoptosis[270].

Malfunction of DSB repair mechanisms is essential for the survival of cancer cells and is one of the major reasons why these cells avoid the cytotoxic effects of camptothecin derivatives. Therefore, it seems reasonable to state that cells with a compromised DSB repair mechanism may have greater susceptibility to therapy based on camptothecins. The main paths of the DSB repair mechanisms include homologous recombination (HR) and non-homologous end-joining (NHEJ). Mutations in genes *RAD51*, *XRCC2*, *BRCA2*, *RAD54* and *MUS81* involved in HR contribute to the hypersensitivity of cells exposed to camptothecins because the protein products of these genes are essential for proper functioning of the HR pathway in S and G2 phases of the cell cycle[270]. The results indicate that DSB induced in cells by derivatives of camptothecin are repaired either by NHEJ or HR[270-272]. As HR requires replication it might even be the predominant route of defence against the killing effects of camptothecins that require replication to elicit cytotoxicity[270]. In conclusion, the decisive role in the creation of phenotype drug resistance to CPT-11 is the status of p53, the degree of degradation of the TOPI complex from DNA, DSB repair by HR on stalled replication forks, and downstream pro- and anti-apoptotic pathways, while the NHEJ pathway seems to be much less important[270].

OX

Within the last 40 years, a few thousand platinum derivatives have been synthesised and tested with regards to their anti-cancer activities. Among these compounds, the most interesting ones seem to be those discovered in the early 70s, such as derivatives of the 1, 2-diaminocyclohexane (DACH) carrier ligand that are non-cross-resistant with cisplatin. In the last two decades, many scientists searching for new and effective cytostatic medicines directed their research efforts towards this platinum derivative group. Interest in the DACH group compounds is associated with their beneficial properties in comparison with other platinum derivatives such as cisplatin or carboplatin. Not only do DACH compounds demonstrate less nephrotoxicity (as opposed to cisplatin) and myelosuppression (as opposed to carboplatin), but they also have higher efficacy in cancer which proved to be resistant to treatment with cisplatin. Research results in both cell lines and *in vivo* observations prove that the efficacy of DACH compounds, in comparison to cisplatin and carboplatin, may be related to breaking inner resistance to these cytostatics. The significant cytostatic activity of OX was proved during tests on several human cancer cell lines and is believed to be the most important platinum derivative from the DACH group[273,274].

Combination therapy with 5-FU/LV plus OX (FOLFOX) is currently a standard in treating gastric cancer and CRC with a 40% positive response ratio during first relapse therapy[275]. Despite the efficiency of combined therapy, a high percentage of patients show drug resistance to a higher or lower degree, which suggest that the therapeutic efficiency of FOLFOX is characterised by high variability. Since approval of the clinical application of OX in the treatment of patients with advanced CRC in 1999 in Europe and then in 2004 in the United States, access to data concerning OX pharmacology has grown significantly. In preclinical studies, OX showed activity towards colon cancer cell lines characterised by primary and acquired resistance to cisplatin[132]. Also, in many other experimental models with a phenotype of resistance to cisplatin it was shown that the sensitivity/drug resistance profiles of both platinum derivatives were different[276].

Resistance to platinum compounds, as is the case with other cytotoxic compounds, is multi-factorial and individual platinum derivatives have different degrees of cross-resistance. Generally, in the majority of studies of experimental cancers, carboplatin has cross-resistance with cisplatin, but not with OX. On the basis of numerous studies, six major cell drug resistance mechanisms towards platinum derivatives, have been identified[277,278]. Processes connected with transporting to and from cells could be included here, as they contribute to lower intracellular drug concentration. Also, an increase in drug detoxication may be of importance (*e.g.*, increased concentration of sulphydril-containing molecules or activity of metabolic enzymes) or an increase in the quenching of DNA monoadducts. Lastly, in the cells with resistance to platinum compounds, a system of recognition and/or DNA damage repair may malfunction[279].

Intracellular drug accumulation

Membrane transporters and channels, collectively known as the transporters, are some of the best known factors determining chemosensitivity and drug resistance and the history of research into their significance in anticancer therapy dates back to the beginning of scientists' interest in the causes of chemotherapy failure[280]. Only a small group of the known transporters have been recognised as relevant for intracellular accumulation of platinum derivatives. There is a broad review concerning membrane transporters and channels that can be found in the publications of Choi and Kim[281], Hall *et al*[282] and Liu *et al*[283].

Potential platinum uptake or influx transporters include copper transporter (CTR) proteins[284], organic cation transporters (OCTs) belonging to the SLC22 family[285] and an undefined cis-configuration specific platinum influx transporter[286]. In addition, some outward-directed drug transporters facilitating the active efflux of platinum compounds have been linked to decreased accumulation of platinum compounds and include adenosine triphosphate (ATP) binding cassette (ABC) multidrug transporters[287], and copper-transporting P-type

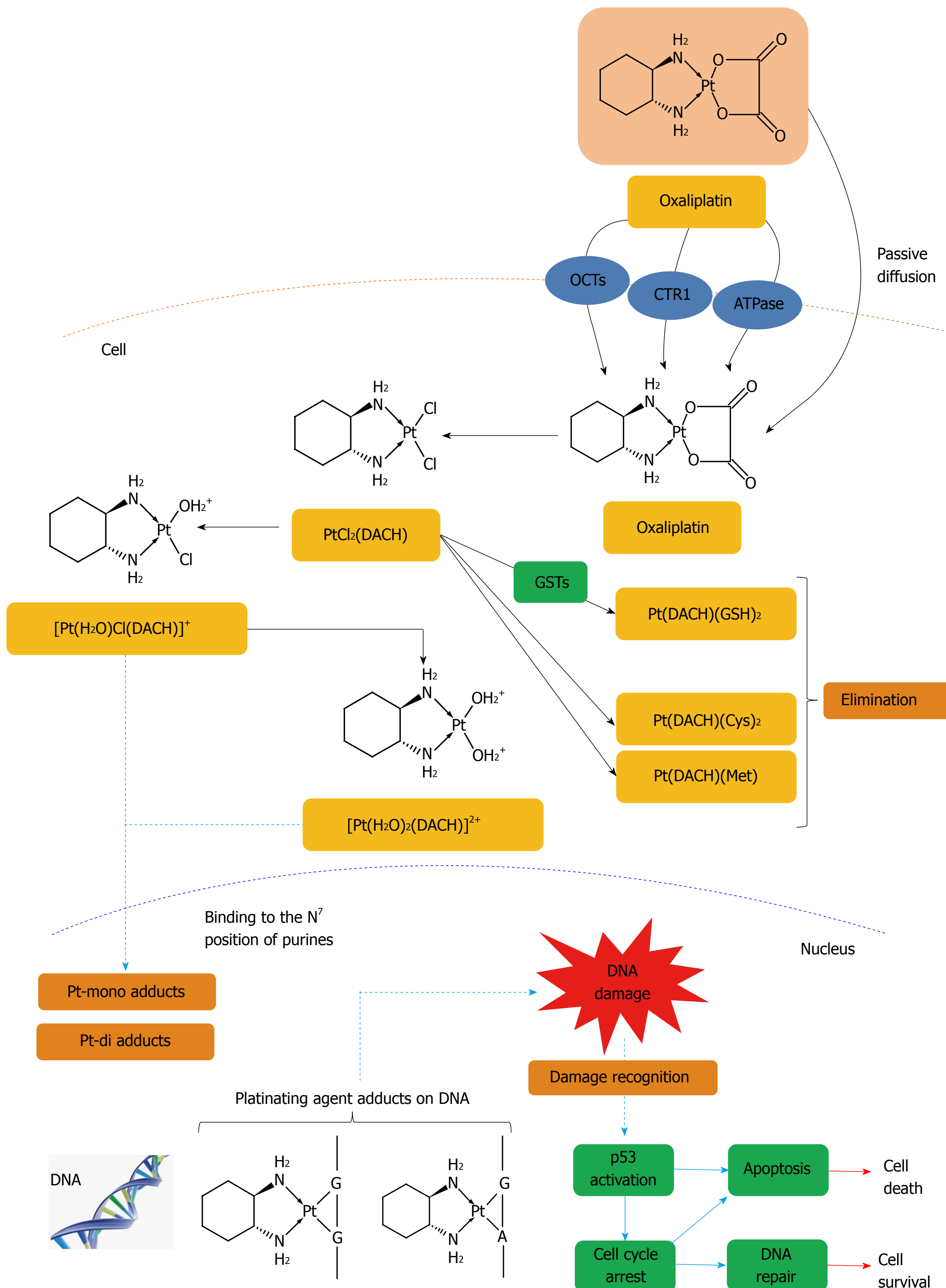

Figure 8 Intracellular drug accumulation. The free fraction of oxaliplatin is biotransformed non-enzymatically and subsequently forms complexes with chloride, glutathione (GSH), methionine (Met) and cysteine (Cys). Oxaliplatin undergoes non-enzymatic conversion in physiologic solutions to active derivatives *via* displacement of the labile oxalate ligand. Several transient reactive species are formed, including monoaquo DACH (1,2-diaminocyclohexane) platinum $[Pt(H_2O)Cl(DACH)]^+$ and diaquo DACH platinum $[Pt(H_2O)_2(DACH)]^{2+}$, which covalently bind with macromolecules. There is no evidence of cytochrome P450-mediated metabolism in vitro. The major route of platinum elimination is renal excretion. The main mechanism of action is mediated through the formation of DNA adducts which is thought to be related to the anti-tumour effects of oxaliplatin. An important factor is the induction of apoptosis by the primary DNA-Pt lesions, which is possibly enhanced by the contribution of targets other than DNA. Several influx and efflux transporters such as organic cation transporters (OCTs) 1, 2 and 3 (SLC22A1, SLC22A2 and SLC22A3), copper efflux transporters (CTRs), P-type ATPases, ATP7A and ATP7B have been identified, which may play an important role in determining tumour sensitivity and/or resistance to oxaliplatin[408].

Table 4 Selected common polymorphisms of *MDR1*, *GSTP1*, *ERCC1*, *ERCC2*, *XRCC1* genes and their potential impact on functioning of proteins related to OX pharmacology

dbSNP rs cluster ID	Type of polymorphism	Function	Ref.
Multidrug resistance 1 (*MDR1*, *ABCB1*) (OMIM #171050)			
rs1128503	SNP		[152,296,318,485]
	1236C>T	Synonymous, effect unknown	
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 1128503			
rs2032582	SNP		[152,296]
	2677G>T/A	Ser893Ala or Ser893Thr, the GG genotype carriers have the highest while the AT genotype carriers have the lowest levels of ABCB1 expression	
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 2032582			
rs1045642	SNP		[152,296,350,485]
	3435C>T	Synonymous, TT genotype carriers have lower intestinal ABCB1 expression	
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 1045642			
Glutathione *S*-transferase π (*GSTP1*) (OMIM #134660)			
rs1138272	SNP		[311,477]
	341C>T	Ala114Val, altered enzyme kinetics, altered toxicity	
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 1138272			
rs1695	SNP		[51,180,311-329,467,477]
	313A>G	Ile105Val, decreased enzymatic activity, altered toxicity	
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 1695			
Excision repair cross-complementation group 1 (*ERCC1*) (OMIM #126380)			
rs11615	SNP		[51,313,344,345,357,486]
	354T>C	Synonymous, decreased transcriptional activity of ERCC1	
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 11615			
rs3212948	SNP		[487]
	321+74C>G	Intronic SNP (intron 2), protective effect of the C allele to cancer risk	
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 3212948			
Excision repair cross-complementation group 2 (*ERCC2*, *XPD*) (OMIM #126340)			
rs13181	SNP		[51,313,336,337,350,351,353, 356,357,486]
	2251A>C	Lys751Gln, the Gln allele is associated with a higher DNA adduct level or lower DNA repair capacity	
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 13181			
rs1799793	SNP		[313,336,337,353]
	862G>A	Asp312Asn, lower DNA repair capacity for the Asn allele than the Asp allele	
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 1799793			
X-ray cross complementation factor (*XRCC1*) (OMIM #194360)			
rs25487	SNP		[51,313,349,350,361-364,486]
	1196A>G	Arg399Gln, reduced base excision repair function for Gln allele than the Arg allele	
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs = 25487			

SNP: Single nucleotide polymorphism.

adenosine triphosphatases (ATPases) (Figure 8). Insufficient intra-tumour concentration of platinum compounds is a critical factor determining both primary and secondary resistance. Lowered inflow and/or increased activity of outward-directed cellular transport is a frequent phenomenon in clones of chemoresistant cancer cells[280] exposed to cisplatin, OX[288] and carboplatin. However, currently, it is not quite clear whether and to what degree transporters help maintain therapeutic platinum concentrations in cancer cells, thus playing a crucial (clinically relevant) role in sensitivity and cell resistance to platinum derivatives[283]. During the last 15 years, a series of clinical studies have been designed to establish the connection between efficiency of chemotherapy based on OX and the level of expression of membrane transporters in both cancer cells and in healthy tissue. These studies of transporters including ATP7A, ATP7B, ABCC2, ABCG2, ABCB1, OCT2 and CTR1 are detailed below and summarized in Table 4.

The first clinical studies concerning the dependency between the results of treatment with platinum compounds in cancer chemotherapy and the expression of transporter concerned the P-type copper transporting ATPases ATP7A and ATP7B. In a study of 50 patients with an advanced stage of CRC and treated with 5-FU/LV/OX (FOLFOX) a correlation was observed between resistance and the level of expression of these transporters[289]. ATP7A and ATP7B involved in the sequestration and extrusion of copper from a compartment localized within the trans-Golgi network to the plasma membrane, have also been implicated in the efflux of platinum compounds[290]. While examining their CRC patients, Martinez-Balibrea *et al*[289] showed that low expression of the *ATP7B* gene measured by its level of mRNA was linked

with significantly longer TTP (P = 0.0009) as opposed to the group of patients with a higher level of mRNA (12.14 mo *vs* 6.43 mo) who also had a greater risk of disease progression (HR = 3.56, P = 0.002). Furthermore, patients with both a low level of mRNA and ATP7B protein noted, had the longest TTP and benefitted from FOLFOX therapy most, as opposed to patients with a high level of mRNA and protein (14.64 mo *vs* 4.63 mo, respectively, P = 0.01)[289].

Various multidrug resistance-associated proteins (MRPs) belonging to the ABCC subfamily of ABC efflux transporters have been implicated in mediating resistance to platinum compounds[291]. Cancer cells resistant to platinum compounds are able to remove OX metabolites that are coupled with glutathione (GSH) into the intracellular environment *via* ATP transport dependent on hydrolysis through biological membranes[292]. On the basis of the above mechanism, it may be assumed that GHS accessibility and the effectiveness of conjunction with GHS are the key factors for the development of such resistance towards OX. Beretta *et al*[293] stated that some of the superfamily ABC transporters (ABCC1/MRP1 and ABCC4/MRP4) had significant expression in ovarian cancer cells with secondary OX resistance. Overexpression of ABCC1 or ABCC4 in cancer cell lines derived from ovarian cancer cells was connected with resistance to cisplatin and OX. The above results prove that the development of OX resistance is induced by the activity of MRP proteins, and it may be conducive to use cytostatics other than platinum derivatives that are not substrates of ABCC1 or ABCC4[293] in patients with relapsing cancer previously treated with OX. Furthermore, in other research it was observed that administering 5-FU inhibits the expression of *ATP7B* and human organic cation transporter 2 (*OCT2*) with a simultaneous 5.8-fold increase in the level of mRNA for the *ABCC2* gene (*MRP2*) coding another transporter from ABCC[294]. Theile *et al*[294] proposed as one mechanism for FOLFOX synergism, the 5-FU mediated suppression of *ATP7B*, the overexpression of glutathione exporters such as *MRP2* and the decrease in glutathione levels by the OX metabolite oxalate.

In studies of another transporter from the superfamily of ABC - ABCG2/BCRP it was found that overexpression may be a negative marker of OX therapy effectiveness[294]. Lin *et al*[295] tested the level of expression of protein ABCG2, measured by the IHC method, in a group of patients with CRC both in the primary and metastatic cancer tissue. They observed that lower expression of ABCG2 was noted more frequently in patients with better response to FOLFOX therapy than in patients with higher protein expression (63.6% *vs* 9.5%, respectively). Moreover, it was found that in the majority of cases the level of ABCG2 expression was higher in tissue derived from metastatic tissue than from primary tumours[295]. Therefore, Lin *et al*[295] concluded that ABCG2 expression is related to response to therapy based on FOLFOX among patients with metastatic CRC and that ABCG2 may be a selective marker in predicting the effectiveness of FOLFOX.

Wu *et al*[296] evaluated the influence of SNPs of *ABCB1/MDR1* gene (1236C>T, 2677G>T/A and 3435C>T) on the outcome of treatment in CRC patients treated with OX-based therapy. Carriers of the 1236C>T variation of the *ABCB1* gene had longer OS following post-operative OX therapy. Additionally, carriers of the 1236TT-2677TT-3435TT genotype combination had worse PFS (P = 0.043) and recurrence-free survival (P = 0.006)[296]. On the other hand, Yue *et al*[297] showed that SNPs of the *ABCB1* gene were not pharmacogenetic factors which determined prognostics for chemosensitivity to OX-based therapy in CRC patients.

The SLC22 family of transporters includes several subgroups of proteins classified on the basis of position and transporting mechanisms. The subgroup of organic cation transporters (OCTs) consists of only three members: SLC22A1 (OCT1), SLC22A2 (OCT2) and SLC22A3 (OCT3)[285]. Currently, we have a limited range of accessible data concerning the connection between genetic variations and the level of *OCT1* or *OCT2* expression in tumour tissue and the results of treatment after administering therapy based on platinum derivatives. It is, however, postulated that these transporters may be of potential clinical importance as predictive markers. In an experimental model using transfected cells it was noted that the expression of the *OCT1* gene significantly increased intracellular OX accumulation[298]. On the other hand, research results showed that OX is an excellent substrate for *OCT2*[298,299]. Zhang *et al*[298] showed that in transfected HEK293-hOCT2 cells, the amount of accumulated OX was 23.9-fold greater than that in control cells. Whereas, in the presence of cimetidine, which is an OCT2 inhibitor, the amount of accumulated OX was significantly lower. They also stated that in the transfected cells, the cytotoxic effect significantly increased following treatment with OX compared with control cells[298]. It is thought that OCT2 expression may modulate the sensitivity of CRC cells to OX. It is also postulated that the level of OCT2 expression may condition drug resistance in CRC patients treated with therapy based on a scheme including platinum[298]. However, the results of the above studies are not fully credible as while testing OCT2 expression in tissue, it was noted that a positive result was obtained in 11 of 20 tissue samples from patients with colon cancer, while a negative effect was obtained in 4 healthy tissue samples[300]. In contrast, all colon cancer cell lines investigated for transporter gene expression were found to lack *OCT2* mRNA expression[298,300]. Therefore, it is worth stressing that if a significant role of OCT2 was proved to mediate transport of platinum derivatives in pre-clinical studies[298], the results of clinical studies do not confirm this observation.

The role and significance of copper influx and transporters efflux (CTRs) in cell accumulation of platinum compounds has been widely discussed in the literature[284,301,302]. CTR1 is an important transporting protein

that is responsible for regulating copper concentrations, ensuring the biological balance of copper ion concentration. When the copper concentration is too low this leads to deactivation of enzymatic systems dependent on copper ions, whereas when the concentration is too high it causes cell toxicity[303]. Holzer *et al*[304] put forward a thesis that CTR1 plays an important role in OX accumulation only when exposed to a relatively low concentration (2 μmol/L) and does not have any relevance at higher OX concentrations. Furthermore, it is postulated that intracellular OX concentration is less dependent on the transporting activity of CTR1 than that of other platinum derivatives, *e.g.*, cisplatin and carboplatin. Additionally, it was shown that similar to CTR1, CTR2 may also have analogical properties as a cisplatin and carboplatin concentration regulator and possibly OX as well[305]. Further *in vivo* research confirming the above hypotheses is necessary.

Clinical studies concerning transporters for platinum derivatives have concentrated on evaluation of the connection between intratumour expression of certain transporters and the results of treatment after chemotherapy based on platinum derivatives. The results of these studies are not completely certain due to many limitations. One of these limitations is the lack of functional research into transporting activity as accessible data focus on gene or protein expression using methods such as RT-PCR or IHC, respectively. Generally, correlations observed in the research were not supported by the analysis of pharmacokinetic variables in relation to accumulation of platinum derivatives in the tumour tissue, and the size of individual groups was small. Furthermore, it is necessary to conduct *in vivo* research into the meaning of genetic variability of membrane transporters and channels for gene expression and their influence on the pharmacokinetics and effectiveness of OX-based therapy.

Glutathione S-transferases

The phenotype of resistance to platinum derivatives may be dependent on the variable activity of detoxification channels. In the cytoplasm, platinating agents become acquated, which then enables them to react with thiol-containing molecules, including GSH and metallothioneins (Figure 8). In the cell, GSH plays the role of antioxidant which helps maintain a reductive intracellular environment by coupling oxidated particles with sulphydryl groups. It is assumed that high GSH concentration and/or metallothionein may cause deactivation of platinum compounds before they have a chance to interact with DNA in the nucleus (it is estimated that only 1% of the dosage that enters the cell stands a chance of bonding with nuclear DNA[306]) to quench Pt-DNA monoadducts before conversion to more lethal diadducts, or the efflux of Pt-glutathione conjugates[307,308]. There is ample evidence to show that glutathione *S*-transferers (GSTs) belonging to the superfamily of dimeric enzymes of the second metabolism phase are responsible for a differential sensitivity profile towards anticancer drugs, including platinum derivatives[309]. GSTs are coded by genes belonging to at least five main groups: α (*GSTA1*), μ (*GSTM1*), π (*GSTP1*), σ (*GSTS1*) and θ (*GSTT1*). Many of these genes have genetic polymorphisms that influence their transcription and/or enzymatic activity of the proteins coded by them[310]. One of the isoenzymes from the GSTs family - GSTP1, has high expression in CRC tissues and partakes in detoxication processes of platinum derivatives, therefore, it may be a source of drug resistance in some patients treated with therapy based on cytostatics that are platinum analogues. The published research suggests a connection between some of the polymorphic variables of *GSTP1* gene and the increase in effectiveness of anticancer therapy[51].

Two major polymorphisms in GSTP1 - 313A>G (Ile105Val) and 341C>T (Ala114Val) - induce amino acid changes in the electrophile-binding active site of the enzyme[311]. SNP 313A>G, responsible for substitution of isoleucine through valine in codon 105 (Ile105Val) causes lowered enzymatic activity of GSTP1[312]. There are a few clinical studies available which refer to the influence of this polymorphism on the frequency of occurrence of toxic effects due to FOLFOX or IROX therapy (CPT-11/OX) in patients with metastatic CRC[180,313,314]. McLeod *et al*[180] state that in a group of patients treated with FOLFOX, who were homozygous for the 105_{Val} variation, treatment discontinuation was more frequent due to symptoms of neurotoxicity (P = 0.01). However, the necessity to discontinue therapy was not dependent on the frequency of occurrence of individual genotypes in groups treated with other combinations (IROX or capecitabine/OX). Most probably, the presence of the 313_{GG} genotype is connected with significant lowering of the catabolic activity of GSTP1 than it is the case of allele 313_{A} carriers (genotypes 313_{AG} or 313_{AA}), which leads to increased OX accumulation and thus a greater risk of 3rd degree neurotoxicity[313,314]. On the other hand, Inada *et al*[315], while examining CRC patients, demonstrated that genotype 313_{AA} carriers were more likely to develop early OX-induced grade 1 peripheral neurotoxicity than patients with 313_{G} alleles (313_{AG} or 313_{GG}), but they did not observe a connection between the frequency of these genetic variations and the risk of grade ≥ 2 neurotoxicity. In addition, the results of other research did not confirm the existence of SNP 313A>G dependence and neurotoxicity of OX therapy[316-321].

As replacing isoleucine with valine (Ile105Val) leads to a lowering of the cell's ability to protect itself against cytotoxic factors, this polymorphism may contribute to an increase in chemosensitivity to OX[312]. A few clinical studies showed that patients with the 313_{GG} genotype benefitted more from combined therapy including OX than patients with the 313_{A} allele[51,322-324]. However, three recently published studies on the efficiency of FOLFOX in patients with advanced CRC, on the basis of genotyping *GSTP1* gene for SNP 313A>G, showed no connection between the presence of the allele and PFS[313,321,325]. Ye *et al*[326] performed a systematic analysis of five clinical

studies[314,325,327-329] involving 415 CRC patients treated with OX. In this analysis, no dependence between the 313A>G polymorphism and the level of response to OX-based therapy (*P* = 0.13) was confirmed[326]. In order to put forward any definite conclusions concerning the predictive significance of SNP 313A>G, it is necessary to carry out clinical research on a large group of patients.

Among the available clinical data, studies on copy number variations (CNV) of *GSTT1* and its potential influence on the toxicity of OX-based therapy have been observed. While investigating CNV of *GSTT1,* Goekkurt *et al*[330] found no statistically relevant dependence between genetic variables of this gene and the frequency of toxic effects due to therapy in patients with gastric cancer, although there was a trend showing that patients with the null variant were less likely to develop hematologic toxicity. Two other clinical studies of patients with metastatic CRC treated with OX did not confirm the hypothesis of the potential influence of CNV of *GSTT1* on therapy toxicity[316,317]. It is necessary to conduct further research which would clearly resolve the role of genetic GSTs variability in the development of toxicity in CRC patients undergoing treatment which includes OX.

Nucleotide excision repair pathway (ERCC1, ERCC2, XRCC1)

Blocking the process of DNA replication using platinum derivatives by creating adducts with nuclear nucleic acid leads to the induction of apoptosis and the death of cancer cells[331,332]. The observed inter-individual variability in the ability to recognise and repair such DNA damage through the nucleotide excision repair (NER) pathway is one of the factors that may influence the success of OX-based therapy. DNA strands are separated and a DNA residue containing the adducts is removed (Figure 9). The mechanism of recognition and repair of the damaged DNA fragments itself is dependent on several factors. Lowered efficiency of the DNA repair system may, in consequence, lead to the increased sensitivity of cancer cells to therapy which includes platinum compounds[333]. excision repair cross-complementation group 1 (ERCC1) and ERCC2 protein [otherwise known as xeroderma pigmentosum group D (XPD)] are the two main compounds of the NER group that play a crucial role in regulation of the activity of other elements that are part of the NER pathway. Together with xeroderma pigmentosum group F (XPF) protein, ERCC1 is responsible for recognising these places in the DNA strand where adducts are located, whereas ERCC2 is a subunit of human transcriptional initiation factor TFⅡH with ATP-dependent helicase activity[334]. Considering the above, it may be assumed that functional SNPs in *ERCC1* and *ERCC2* genes may directly contribute to the phenotype sensitivity to platinum compounds, such as OX, through conditioning congenital suboptimal activity of the NER pathway. For genes *ERCC1* and *ERCC2*, there are several frequent and probably functional SNPs described, among them are 354C>T and 8092C>A in the *ERCC1* gene, which contribute to the changes in activity measured by the level of mRNA[335] and *ERCC2* SNPs 312G>A gene (Asp312Asn), and 2251T>G (Lys751Gln) are recognised as determinants of suboptimal activity of the DNA repair system[336,337]. Study results suggest that ERCC1 is a potential predictive marker of response to therapy based on platinum compounds due to the fact that low ERCC1 expression is connected to cancer cells' sensitivity to chemotherapy with those drugs[34,338-340].

Shirota *et al*[34] were the first research group to study the influence of *ERCC1* gene expression on the results of treatment in 50 patients with advanced stage CRC and the phenotype of resistance in those treated with 5-FU/OX. They stated that patients with high intra-tumour *ERCC1* expression measured by mRNA level had shorter survival time than patients with a lower level of expression (*P* = 0.008)[34]. Uchida *et al*[341], while examining 91 patients treated with a combination of capecitabine/OX stated that a high mRNA level for the *ERCC1* gene was associated with shorter time to treatment failure compared to patients with lower expression (*P* = 0.046). In another study, low expression of the *ERCC1* gene was also associated with better response to both primary (*P* = 0.047) and secondary chemotherapy, although in the latter case this association was on the verge of statistical relevance (*P* = 0.054). Furthermore, high expression of the *ERCC1* gene was related to shorter OS in primary therapy (*P* = 0.014)[342]. The above results from clinical studies support the hypothesis put forward at the beginning regarding the influence of *ERCC1* gene expression on the results of treatment with platinum derivatives, whereas a high level of mRNA may be the cause of clinical resistance to OX.

The literature also describes polymorphisms located in the *ERCC1* gene sequence, one of them being a silent SNP 354C>T (Arg118Arg). Although the mechanism through which this SNP influences the change in ERCC1 activity is not fully known, it is postulated that AAC codon exchange on a rarely occurring AAT influences the effectiveness of the translation process, however, for 354T allele, there is a decrease in protein expression of about 50%[343]. In two clinical studies of patients with advanced CRC, it was observed that carriers of the 354TT genotype had higher response rates to OX treatment[344] and longer PFS[345]. However, in five other studies, the survival time of patients with CRC was longer in genotype 354CC carriers[51,313,314,339,346]. While examining 168 patients, Chang *et al*[346] showed that in a group with genotypes which included allele 354T (354CT or 354TT), poorer treatment results were noted in comparison with those of patients with genotype 354CC [in terms of response (*P* = 0.01), PFS (*P* = 0.01) and OS (*P* = 0.01)]. Additionally, while evaluating the association between genetic variants 354C>T and protein expression determined by IHC, it was shown that a higher level of expression was related to the presence of allele 354T[346]. In addition, Chen *et al*[314], while examining 166 patients, pointed out that carriers of genotypes with at least one 354T allele were characterised by poor re-

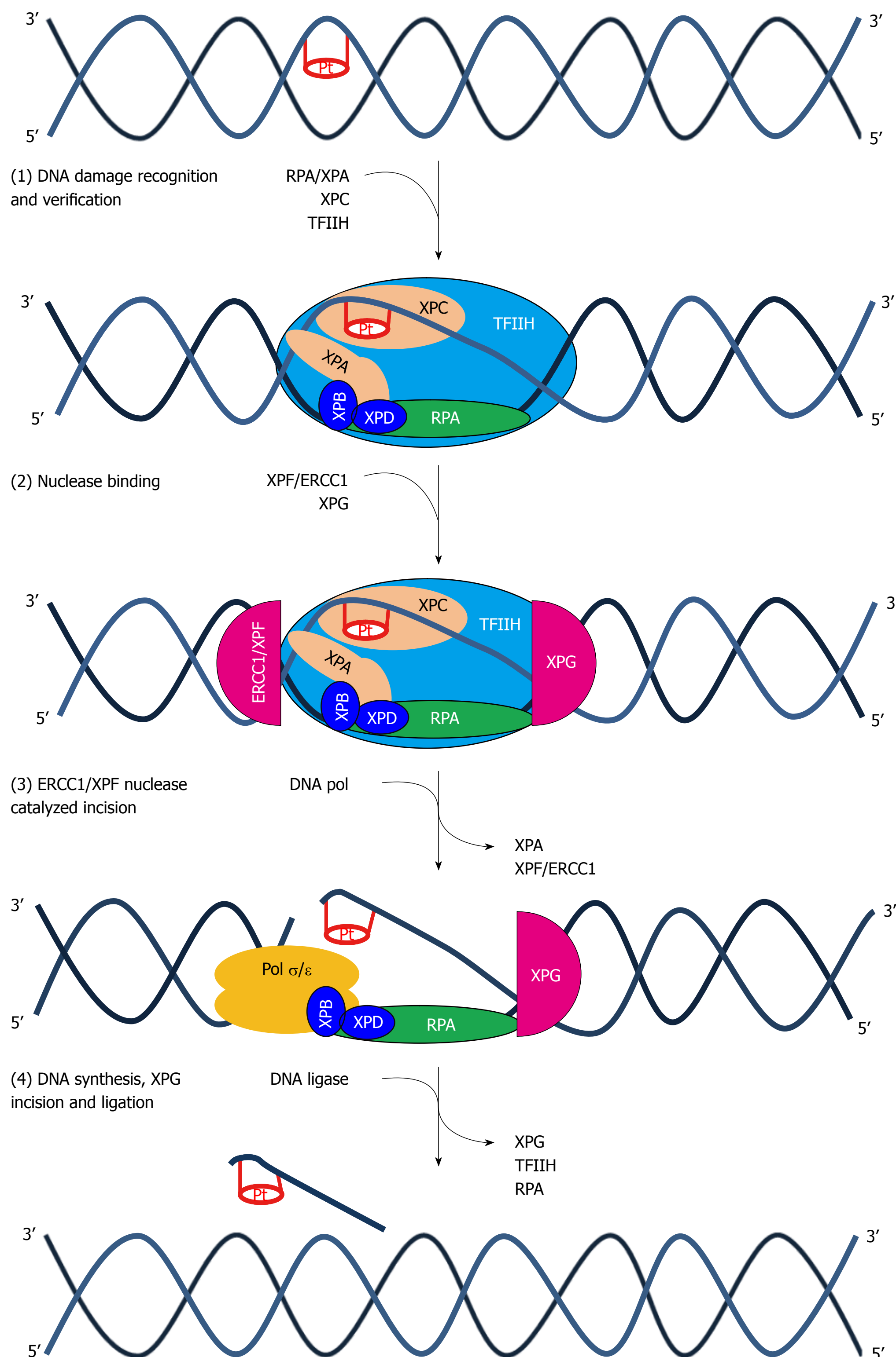

Figure 9 Nucleotide excision repair pathway. (1) DNA damage formed by platinum agents leads to DNA double helix distortion. Several distinct complexes are involved in sequential steps than can be summarized as DNA damage recognition (XPCHR23B), damage demarcation, and verification (TFⅡH), assembly of a pre-incision complex (RPA and XPA) and helix unwinding (XPB and XPD); (2) Endonuclease recruitment with dual incision of the damaged strand on the 5′ side (ERCC1-XPF heterodimers) and 3′ side (XPG) followed by the removal of the excised oligomer; (3) DNA repair synthesis to fill in the resulting gap; and (4) ligation. ERCC1: Excision repair cross-complementation group 1; Pol σ/ε : Polymerase σ/ε; RFC: Replication factor C; TFⅡH: Transcription factor Ⅱ H; XP (A,B,C,D,F,G): Xeroderma pigmentosum complementation group (A,B,C,D,F,G)[340].

sponse (P = 0.01) and shorter OS (P = 0.01). Park *et al*[339] also found a significant correlation between polymorphic variants in codon 118 and treatment outcome in 106 patients with advanced refractory CRC receiving 5-FU/OX. For patients with genotype 354CC, median survival time was 15.3 mo, while in a group of allele 354T (354CT and 354TT genotypes) carriers it was only 11.1 mo.

Partially different from fluoropyrimidine genes previously described, the frequency of these polymorphisms varied with race and may account for reduced response rates in Black patients compared with Caucasian patients, as expressed by Goldberg *et al*[347] and confirmed in more recent studies, as in the subgroup of patients in the CAIRO study[110]. It is postulated that the differences in the observed associations and the strength of the correlations may be connected with inter-population differences in the frequency of occurrence of alleles and genotypes. For instance, the frequency of occurrence of SNP 354C>T (Arg118Arg) in an East Asian population was much lower than that in other ethnic groups[340].

The presence of allele 354T in the *ERCC1* gene is connected with the change in the expression of gene/protein[339], while allele 2251G which is a variation of the *ERCC2* gene was described as having influence on a low number of X-ray induced chromatic aberrations[336]. Carriers of genotype 2251TT had a 7-fold greater risk of suboptimal repair of DNA damage compared to carriers of allele 2251G (genotypes 2251GG or 2251GT)[336]. It is postulated that patients who have both allele 354T (*ERCC1*) and 2251G (*ERCC2*) that are connected with a highly efficient detection system and DNA damage repair, may have resistance to OX, thus contributing to a worse prognosis. However, the results of clinical studies do not confirm the above hypothesis. The 2251T>G (Lys751Gln) polymorphism did not show any relation with survival time compared with the frequency of genotype dispersion in patients with gastro-oesophageal cancer[348,349] and CRC[350,351] who underwent treatment based on various platinum derivatives. Whereas, studies of the synonymous SNP Arg156Arg (C>A) *ERCC2* gene carried out in patients with gastric cancer treated with OX showed that carriers of A allele (genotypes CA or AA) were characterised by a higher response rate and longer TTP compared to patients with genotype CC[352]. A similar trend was observed in the studies by Park *et al*[353], who examined patients with metastatic CRC, and noted that the presence of A allele contributed to better treatment response and longer median survival compared to patients with different variants of the *ERCC2* gene. Functional studies confirmed the SNPs influence of the *ERCC1* (354C>T) and *ERCC2* (2251T>G) genes on the phenotype of NER pathway efficiency[335,354,355]. In a study of 73 patients treated with 5-FU/OX it was observed that in patients with the genotype 2251TT (751Lys/Lys) median survival time was 17.4 mo, while for carriers of genotypes with the 2251G allele it was 12.8 mo (751Lys/Gln) and 3.3 mo (751Gln/Gln) (P = 0.02)[353]. The influence on genetic variants of the genes *ERCC1* and *ERCC2* was also studied in a group of 166 metastatic CRC patients who were treated with a combination of 5-FU/LV/OX (FOLFOX4)[356]. In the analysis of associations between SNPs and the results of treatment it was shown that the occurrence of each of the genotypes *ERCC1*-354TT, *ERCC2*-2251AC and *ERCC2*-2251CC, independently of each other, was related to shorter PFS. The median PFS was 11.2 mo for patients without any of the three genotypes, 9.8 mo for those with one of the high-risk genotypes, and 8 mo for those with both the *ERCC1*-354TT and either *ERCC2*-2251AC or -2251CC genotypes (P = 0.002)[356]. In the meta-analysis published by Yin *et al*[357] it was shown that SNPs 354C>T (*ERCC1*) and 2251T>G (*ERCC2*) may be clinically useful in the evaluation of treatment results in patients with gastric cancer and CRC who underwent treatment which included OX (FOLFOX or XELOX). However, as the authors of this analysis emphasise, it is necessary to carry out wide and well-planned prospective clinical studies to clearly show the utility of these markers in clinical practise[357].

Apart from studies which focused on the analysis of individual determinants of therapy efficiency such as SNPs, a joined analysis of a few potential predictive factors in forecasting the effects of chemotherapy in CRC patients was also carried out. Kim *et al*[358] assessed the expression of proteins ERCC1, TS and GSTP1 using IHC for potential application in predicting the effects of therapy in 70 patients with advanced stage CRC who underwent treatment with 5-FU/OX. They observed that positive expression occurred in 55.7% (ERCC1), 68.6% (TS) and 71.4% (GSTP1) of the analysed cases. It was confirmed that a low level of TS expression was related to better chemotherapy outcome (P = 0.009), however, in the case of ERCC1 and GSTP1 proteins there was no statistically relevant association between the level of expression and efficiency of treatment (P = 0.768, P = 0.589, respectively). The median OS was significantly longer in patients with negative ERCC1 protein expression (P = 0.0474). Additionally, patients with positive expression of both ERCC1 and TS had poorer OS (P = 0.0017). Also, multi-variant analysis confirmed that positive expression of ERCC1 and TS significantly influenced OS (HR = 1.72, P = 0.023), which justifies simultaneous clinical application of the two markers for predicting the efficiency of 5-FU/OX therapy[358].

Apart from the NER pathway, the base pair excision repair pathway (BER) may also influence the efficiency of therapy based on platinum derivatives. XRCC1 plays a key role in the BER pathway and it has been demonstrated that the Arg399Gln (1196A>G) substitution in the *XRCC1* gene is associated with increased levels of DNA damage markers[359]. This relatively frequently occurring polymorphism probably contributes to the change in XRCC1 protein conformation in the domain binding other elements of the BER complex, which may lead to a decrease in the efficiency of the DNA repair system. A deficiency in DNA repair pathways has been shown to confer resistance to several drugs, including platinum

compounds[360]. It was shown that the presence of allele 399_{Arg} (1196_{A}) is associated with better survival time in patients with gastric[349] and lung cancer[361] undergoing chemotherapy with platinum derivatives. Also, Suh *et al*[362] observed that better treatment outcomes in patients with metastatic CRC treated with FOLFOX occurred in those where the presence of allele 399_{Arg} (1196_{A}) was noted. However, the results of other clinical studies published in patients with advanced CRC and gastric cancer treated with OX, did not confirm the above observations[51,313,350]. Liang *et al*[363] attempted to analyse the influence of both polymorphisms on genes engaged in DNA repair processes: *ERCC1* (354C>T) and *XRCC1* (1196A>G). They studied a group of 113 patients diagnosed with metastatic CRC who underwent chemotherapy that included OX. The analysis of individual SNPs showed no significant influence of these polymorphisms on prediction of disease control rates (DCR) or OS (P = 0.662 and 0.631, respectively). However, while evaluating the influence of the combination of both SNPs, a significant correlation between genetic variations of *ERCC1* (354C>T) and *XRCC1* (1196A>G), DCR (P = 0.01) and OS (P = 0.001), were independently observed. This was the first study to prove the importance of the clinical application of genetic determinants located in *ERCC1* and *XRCC1* genes in the selection of patients with metastatic CRC who were expected to benefit most from OX-based therapy[363]. Subsequent results obtained by Stoehlmacher *et al*[364], who studied the influence of Arg399Gln (1196A>G) polymorphism on the efficiency of treatment with 5-FU/OX in 61 patients with metastatic CRC, confirmed the significance of this SNP as a predictive marker. Seventy-three percent of patients with the favourable $399_{Arg/Arg}$ (1196_{AA}) genotype responded to treatment, and patients who possessed at least one 399_{Gln} (1196_{A}) allelic polymorphism in XRCC1 were 5.2-fold more likely to fail 5-FU/OX chemotherapy[364].

Among the available data, one clinical study conducted a multivariate analysis of a few of the predictive factors described above in patients with refractory CRC who underwent treatment with the 5-FU/OX combination. Analysis of multiple gene polymorphisms proved that the efficiency of such therapy may be dependent on the presence of two or more unfavourable variants for genes *ERCC1*, *ERCC2*, *TYMS* and *GSTP1* as the carriers of these SNPs were characterised by a significantly shorter OS[51]. In summary, for the successful prediction of the effectiveness of a particular therapy, a few predictive markers need to be applied where several cytostatic drugs are used in a combination therapy.

MMR and apoptosis regulation

The cytotoxic effects caused by OX are stronger than those caused by cisplatin due to the result of a stronger reduction in DNA damage[365]. Resistance to cytostatic platinum derivatives is probably the result of variable functionality of the proteins responsible for recognising damage resulting from Pt-DNA adducts[366]. MMR is a highly conserved, strand-specific repair pathway which is a multi-stage process initiated when DNA damage is recognised by specific proteins[367]. In many types of cancer, various defects in activity of these proteins are noted, particularly three proteins: MSH2, MSH6 and MLH1[368]. In a situation when MMR shows a deficit in activity, this results in the accumulation of numerous types of DNA damage in the genome, which leads to MSI[369]. Experimental data have shown that MMR deficits are associated with resistance to the cytotoxic activity of alkylating agents[370]. Studies of DNA repair mechanisms after exposure to cisplatin showed that Pt-DNA adducts are recognised by the complex of MMR proteins[371]. The MMR pathway is one of the factors influencing cisplatin activity, which was proved by pre-clinical studies where cells with deficient activity of proteins MLH1, MSH2 and MSH6 had the phenotype of moderate resistance to cisplatin, but remained sensitive to the cytotoxic activity of OX[276,372]. Interestingly, Pt-DNA adducts are recognised by MSH1 protein only when damage occurs after cells are exposed to cisplatin, but not when Pt-DNA adducts are created due to the influence of OX[371,372]. Therefore, even though the MMR pathway is a key element in the mechanism of DNA repair, this system seems not to recognise Pt-DNA adducts created following exposure to OX. Generally, it is assumed that if attempts to repair Pt-induced DNA damage fail, this eventually leads to initiation of apoptosis[373,374]. Adducts induced by OX do not activate JNK (JNK-c-Jun NH_2-terminal kinase, also known as stress activated protein kinase) and c-Abl (a nuclear protein)[375], which allow OX to maintain its cytotoxic activity in both MMR-proficient and -deficient cells[372,375]. Cisplatin depends on an intact MMR system for maximal cytotoxicity and for signalling apoptosis *via* the JNK-mediated pathway[371,375,376]. The binding of the MMR complex to Pt-DNA adducts appears to increase the cytotoxicity of the adducts[377], either by activating downstream signalling pathways that lead to apoptosis[375] or by causing "futile cycling" during translation synthesis past Pt-DNA adducts[372]. Therefore, cisplatin and OX have a different ability for activating signal paths to induce apoptosis in response to Pt-DNA adducts, which may be the basis of the observed differences in the profile of drug resistance in these platinum derivatives[378].

Protein p53 mediates the transduction of a signal induced by DNA damage following exposure to cisplatin[379]. p53 interacts with several significant elements that are part of the NER pathway, such as xeroderma pigmentosum, complementation group C (XPC), TFⅡH and replication protein A (RPA), which points to its role in supervising the DNA repair process[380]. While testing 60 different cell lines, Vekris *et al*[381] showed that the expression of p53 was positively correlated with cell sensitivity to four different platinum derivatives: cisplatin, carboplatin, OX and tetraplatin. As p53 takes part in apoptosis induction and participates in the process of removing Pt-DNA adducts created by platinum derivatives, this protein may contribute to both chemosensitiv-

ity and drug resistance[382]. A systematic analysis of cellular sensitivity to OX in relation to p53 status in pairs of cisplatin-sensitive and -resistant cells showed that OX is less potent than cisplatin in cisplatin-sensitive cell lines, whereas it was capable of overcoming cisplatin resistance in the majority of sublines. Cell sensitivity to OX seems also dependent on the occurrence of genetic variants in gene *TP53*. While studying the cell line A431 which is characterised by a mutation in codon 273 of p53, it was observed that it has high resistance to OX[276].

Clinical application of the above *in vitro* studies to test a various panel of factors influencing the phenotype of chemosensitivity or drug resistance will require a series of *in vivo* studies with the participation of well selected groups of patients. Currently available data from pre-clinical studies show the potential significance of some molecular factors connected with the DNA repair processes and those participating in control of the cell cycle and apoptosis, which could serve as predictive markers in forecasting the efficiency of OX therapy in CRC patients.

FUTURE PERSPECTIVES IN PERSONALIZED MEDICINE FOR THE TREATMENT OF COLORECTAL CANCER

The last few decades have resulted in huge progress in understanding the complex processes regulating the growth and development of tumours. However, the major challenge in basic and clinical research is to solve the problem of primary and secondary drug resistance, which in many cases significantly reduces the antitumour efficacy of therapy. Early research on the development of new chemotherapeutic agents with significant antitumour potency, led to the introduction in oncology practice of few effective drugs, including those currently used in the treatment of CRC. Although they strongly induce apoptosis in intensively dividing cells, their strongest drawback is that they have the same effect on both cancer cells and healthy tissue. Therefore, to maintain the effectiveness of cancer treatment, it is necessary to use a maximum dose that provides a strong cytotoxic effect against tumour tissue, while minimizing toxicity to a patient. On the other hand, the intensive development of molecular tests in the last two decades initiated the development of "targeted" drugs and new treatment strategies such as targeted therapy. These new techniques have increased the hope of achieving substantial benefits in patients for whom the use of cytostatics proved not to be very effective. The main advantage of targeted therapy is the ability to avoid toxic effects of the drug with little impact on healthy cells. However, soon after the first research reports on targeted therapy and its high potential in clinical applications, drug resistance still remains a problem even with these "smart drugs". Similar to conventional cytostatics, resistance to a new class of drugs is an important issue in oncology[383]. It should be noted that drug resistance remains the most critical factor in the success of therapy. Currently, the main problem for researchers working on the effectiveness of cancer treatment is how to produce a rational treatment plan based on the classic cytostatic drugs and targeted drugs. Overcoming resistance in many cases is only possible by selecting an appropriate drug combination and optimal dosing during the treatment cycle. Due to the fact that many of the drug-resistance mechanisms are determined by individual patient characteristics, the key to successful therapy may be personalized cancer medicine. However, in recent years most scientists conducting research in the field of molecular mechanisms of drug resistance have focused on individual processes associated with metabolism, biodistribution, and anticancer drug mechanisms. Such research does not include the wider context and different body processes that constitute the effectiveness of a therapeutic strategy[384].

In the current paradigm accepted by scientists, it is considered that individual differences in response are the results of individual patient features that can be identified at a molecular level. These features are subject to genetic variation and the environmental pattern which are specific for each patient. It can be assumed that understanding the molecular mechanisms of inter-individual differences in the effectiveness of cancer treatment will allow the optimization of cancer therapy. Therefore, in the past two decades there has been a significant research effort to acquire information on the mechanisms responsible for the effectiveness of therapies. The approach that underlies individualized medicine is based on the assumption that by using molecular profiling and a set of biomarkers we can improve treatment efficacy in a patient, prolonging survival time and/or reduce the risk of serious complications[385].

Is it possible to apply these concepts in the individualization of treatment of CRC patients in the near future? In the previous chapters a variety of prognostic and predictive markers were described, which in recent years have been subject to various test procedures in order to determine their potential clinical value in the treatment of CRC. A technological breakthrough in molecular studies, as observed in recent years [single-nucleotide polymorphism arrays, complementary DNA microarrays, DNA methylation and microRNA (miRNA) profiling as well as next-generation sequencing] also made it possible to create individual molecular profiling for patients which is more profitable in economic terms. The data obtained using these high-throughput methods give hope for the practical application of various biomarkers to predict the effectiveness of treatment in individual patients with CRC.

Of the main variables affecting the therapeutic efficacy of cytostatics, the level of DNA synthesis and/or the intensity of cell division are important, and in the case of targeted drugs, the expression level of molecules in a signalling pathway in which the drug is targeted. As in the case of cytostatic drugs, the predominant mechanism

of drug resistance is a wide panel of pharmacokinetic factors, and for targeted therapy it is mainly processes related to pharmacodynamics (genetic alteration/mutation of the target itself, persistent activation of downstream signalling pathways, and bypass mechanisms). Such a clear distinction does not describe the complexity of drug resistance mechanisms. Given the holistic nature of personalized medicine, it is necessary to develop and validate a wider panel of biomarkers which would reflect the multifactor mechanisms of resistance. In addition, when using predictors in clinical practice, we must take into account different therapeutic objectives which are set for specific subgroups of patients. From the point of view of drug resistance in cancer therapy, at least two main objectives require to be met in personalized medicine: (1) risk minimization of inducing resistance; and (2) breaking existing primary or secondary resistance. Finding the optimum combination of drugs and dosage regimen can, in many cases, lead to better efficiency in first-line treatment, and prevent cancer relapse. Furthermore, an equally important problem is the selection of resistant clones during the first treatment cycle, which in the case of relapse can significantly reduce the therapeutic efficacy of new combinations of drugs. Use of dynamic-response markers in clinical practice that would allow monitoring of the course of treatment is a promising line of research in personalized medicine. Changing the level of expression of marker genes or activity of posttranslational protein modification during the course of therapy has been assesssed in several studies. Analysis of molecular changes taking place during treatment may provide information regarding the development of resistance resulting from drug exposure, which is particularly important in the context of the existence of secondary drug resistance mechanisms. In such cases, a change in treatment regimen may be important for the future of a patient.

There are several main obstacles which currently prevent the full application of personalized medicine in clinical practice, despite significant progress in the study of causes of drug resistance in the treatment of CRC. Interindividual differences in the response to treatment in patients with CRC may be subject to genetic and epigenetic features which can be classified as genomic aberrations [*e.g.*, MSI[386,387], chromosomal instability (CIN)[388,389] and CpG island methylator phenotype (CIMP)[390-393]] as well as polymorphic variation (*e.g.*, SNP or VNTR). This multifactor substrate conditioning efficacy in CRC makes it difficult to plan reliable research on predicting markers. In addition, the available clinical data indicate that CRCs are a molecularly heterogeneous group of neoplasms, which is why it is important to plan future studies taking into account this heterogeneity. Only this type of approach will provide a link between specific molecular features and effectiveness of the treatment. Another of the existing barriers for development of personalized medicine is the need for invasive biological sampling. A large part of the results of clinical trials on CRC drug resistance is based on the analysis of biological material derived from tumour biopsy. The possibility of using blood serum may be a way of solving this problem[394]. Another barrier that prevents truly individualized treatment of CRC patients is the small amount of research data that could connect mutation analysis and gene expression during the course of translation and activity of specific marker proteins. The main research stream based on transcriptome analysis does not provide information on protein expression, and mRNA level does not allow the determination of protein activity. It was not until recently when proteome analysis (proteomics) was developed, including important protein-protein interactions, that a number of new drugs for targeted therapy, such as inhibitors of kinases and their substrates were developed. Analysis of the activity of individual proteins involved in intracellular signal transduction is a very important aspect of research on tumour biology, and as shown by Pierobon *et al*[395], the level of protein expression and the level of protein activation (*e.g.*, phosphorylation) do not always correlate, suggesting that the latter could be a better predictive biomarker for patient stratification. In conclusion, due to the heterogeneity of CRC and the complexity of drug resistance, prediction of the effectiveness of treatment in individual patients should be based on prediction biomarkers derived from the genome and proteome. Analysis of multiple markers is also justified as most modern standards of CRC treatment use a combination of several anticancer drugs. Combination therapy is based on the inhibition of tumour cells on several molecular levels. In order to rationally combine different therapies that would presumably be more effective than monotherapy, it is therefore necessary to use an integrated approach for the analysis of multiple pathways simultaneously. In this way, it will be possible to highlight pathway alterations that can be targeted by different agents.

The most recent data in the field of biomarker research show that only the interdisciplinary research approach, using combined analysis of genome and proteome, makes it possible to recognise prognostic and predictive factors which will help select patients in terms of relevant clinical features for individualized therapy[396]. Among a number of potential predictive markers described in the preceding sections of this review, only a small number were found to be clinically useful. In many cases, the analysis of the same marker provided contradictory data sometimes leading to opposing conclusions. There may be several reasons for these discrepancies, including the following: (1) methodological differences (prevalence of retrospective studies); (2) use of different and non-standardized research techniques; (3) use of inappropriate statistical analysis for a given type of data; and (4) diverse and/or insufficiently large groups of patients[385]. Therefore, to increase the credibility of preclinical and clinical prediction, it is necessary when planning research to take into account all variables which can affect the outcome of the analysis. Adoption of uniform research standards in the form of guidelines, such as reporting recommendations for tumour MARKer

prognostic studies[397], provide an opportunity to obtain reliable data. Moreover, the current retrospective analysis, the results of which suggest a correlation should only be used as a source of hypotheses to be verified during the course of later well-designed studies.

In summary, from a clinical point of view, there is a need for innovative patient stratification methods which, based on validated biomarkers, will help clinicians to make correct therapeutic decisions. The effectiveness of anticancer drugs such as classical cytostatics and targeted drugs should be carefully reviewed in properly selected groups of patients whose common molecular profile will determine susceptibility or resistance to treatment[398]. The implementation of new technologies has led to the accumulation of huge amounts of genomic and proteomic data and the identification and validation of predictive biomarkers for existing and new targeted therapies, and will likely improve patient outcomes in the future[399]. Although the initial costs of cancer management and personalized medicine may be high[400], in the future they should result in significant benefits from both a clinical and economical perspective.

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P- Reviewer: Fang BL, Lakatos PL, Nishida T
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ISSN 1007-9327

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