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EPIGENETIC AND GENETIC ALTERATIONS OF *DPYD* ARE NOT PREDICTIVE OF SEVERE TOXICITY TO 5-FLUOROURACIL-BASED CHEMOTHERAPY IN GASTROINTESTINAL CANCER

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Dissertation to a Master's Degree in Molecular and Oncology Medicine

FACULTY OF MEDICINE OF UNIVERSITY OF PORTO

Porto, January 2010

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Dissertation for applying to a Master's degree in Molecular and Oncology Medicine submitted to the Faculty of Medicine, University of Porto.

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ACKNOWLEDGMENTS

To Professor Carmen Jerónimo, Head of the Epigenetic Group of the Department of Genetics/Research Center – Portuguese Institute of Oncology of Porto, for accepting me as her master student in her group and all the support and encouragement.

To Professor Manuel Teixeira, Director of the Department of Genetics and Research Center – Portuguese Institute of Oncology of Porto, for accepting me in his laboratory.

To Professor Rui Henrique, Director of the Department of Pathology – Portuguese Institute of Oncology of Porto for making tumor tissue samples available, immunoexpression evaluation and all also for the helpful comments to the Thesis.

To Vera Costa, Joao Carvalho and Manuela Pinheiro, my colleagues at the laboratory who helped me in the everyday molecular biology techniques and procedures.

To Natália Salgueiro, for making mutational analysis data available and to Paula Dias, for preparing tumor tissue slides.

This study was funded by a grant from Research Center – Portuguese Institute of Oncology of Porto (CI-IPOP 4-2008).

To André

To my Father

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LIST OF ABBREVIATIONS

5-FU – 5-Fluorouracil

5-FU/LV – 5-FU/Folinic acid

APC – adenomatosis polyposis coli gene

BCRA1 – breast-cancer susceptibility 1 gene

CIMP – CpG island methylator phenotype

CG – control group

CRC – colorectal cancer

CT – control group

DHFU – dihydrofluorouracil

DPD – dihydropyrimidine dehydrogenase

DPYD – dihydropyrimidine dehydrogenase gene

DFS – disease-free survival

DNA – deoxyribonucleic acid

DNMTs – DNA methyltransferases

DYPD – dihydropyrimidine dehydrogenase gene

ECF – epirubicin, cisplatin and 5-FU

ECOG – Eastern Cooperative Oncology Group

ECX – epirrubicin, cisplatin and capecitabine

EOX – epirrubicin, oxaliplatin and capecitabine

EGFR – epidermal growth factor receptor

FAD – flavin-adenine dinucleotide

FMN – flavin mononucleotide

FAP – familial adenomatous polyposis

FdUMP – fluorodeoxyuridine monophosphate

FdUTP – fluorodeoxyuridine triphosphate

FolFIri – folinic acid, 5-FU, irinotecan

FolFOx – folinic acid, 5-FU, oxaliplatin

FUTP – fluorouridine triphosphate

GI – Gastrointestinal

GSTP1 – glutathione S-transferase 1 gene

HGTG – high-grade toxicity group

HNPCC – hereditary non-polyposis colorectal cancer

IGF2 – insulin-like growth factor 2 gene

IHC – immunohistochemistry

IV – Intravenous

KRAS2 – V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog

MLPA – multiplex ligation-dependent probe amplification analysis

MGMT – O6-methylguanine–DNA methyltransferase gene

miRNA – microRNA

MLH1 – homologue of MutL Escherichia coli gene

MTT – microdissected tumor tissue

NADPH – nicotinamide-adenine dinucleotide

OS – overall survival

PAX2 – paired box gene 2

PB – peripheral blood

PFS – progression free survival

QMSP – quantitative methylation-specific PCR

RNA – ribonucleic acid

RFC – reduced folate carrier gene

SAM-CH_3 – S-adenosylmethionine

SCC – squamous cell carcinoma

TNM – tumor, node and metastasis

TYMS – thymidylate synthase

VEGF – vascular endothelial growth factor

WHO – World Health Organization

VHL – Von Hippel–Lindau gene

WRN – Werner syndrome gene

SUMMARY

Introduction: 5-Fluoracil (5-FU) is broadly used in the treatment of gastrointestinal (GI) cancer. Deficiency of the enzyme dihydropyrimidine dehydrogenase (DPD), encoded by the dihydropyrimidine dehydrogenase gene (*DPYD*) has been associated with the development of severe toxicity to 5-FU in GI cancer patients. Promoter hypermethylation has been proposed as an alternative mechanism for DPD deficiency not explained by deleterious mutations. Moreover, thymidylate synthase (TYMS) activity also influences 5-FU cytotoxic effects.

Objectives: The primary objective in this study was to analyze the methylation status of *DYPD* promoter region by quantitative methylation-specific PCR in gastrointestinal cancer patients who developed severe 5-FU toxicity. Secondary objectives were the analysis of large intragenic rearrangements of *DPYD*, the assessment of DPD and TYMS immunoexpression in tumor tissue samples, and, finally, the evaluation of the association between molecular, immunoexpression, and clinical findings.

Material and Methods: This study included 66 patients treated at the Portuguese Oncology Institute – Porto for GI cancer with 5-FU. Forty-five of these patients developed high-grade toxicity and had been previously tested for the IVS14+1G>A mutation in the *DPYD* gene. The control group consisted of the remaining 21 patients, which did not develop severe 5-FU toxicity. *DPYD* hypermethylation was evaluated through quantitative methylation-specific PCR in DNA extracted from peripheral blood (PB) samples and micro-dissected tumor tissue (MTT), using a colon cancer cell line as control. The screening for large *DPYD* intragenic rearrangements was performed with the MLPA method. Specific antibodies were used against DPD and TYMS to assess the immunoexpression of those enzymes in representative tissue sections.

Results: *DPYD* promoter methylation was absent in all the PB and MTT samples from patients experiencing severe 5-FU toxicity, as well as from controls. Large *DPYD* intragenic rearrangements were also not detected in both groups, but the skipping mutation in exon 14 (IVS14+1G>A) previously detected in routine analysis has been confirmed in high-grade toxicity patients. Severe 5-FU toxicity wasn't associated with DPD or TYMS immunoexpression either. Clinically, 5-FU toxicity was more frequent after 5-FU in bolus administration, as well as in gastric/esophageal primary site and patients with higher ECOG score ($P<0.05$).

Conclusions: These results indicate that promoter methylation and large intragenic rearrangements of *DPYD* do not contribute to the development of 5-FU severe toxicity. Additional studies are required to investigate other genetic and/or clinical factors which might be responsible for severe forms of 5-FU toxicity in GI cancer patients.

RESUMO

Introdução: O 5-fluoracilo (5-FU) é um fármaco amplamente usado no tratamento no cancro gastrointestinal (GI). A deficiência da enzima diidropirimidina desidrogenase (DPD) tem sido associada a toxicidade grave ao 5-FU em doentes com cancro GI. A hipermetilação do promotor foi proposta como mecanismo alternativo para a deficiência da DPD não explicada por mutações deletérias. Adicionalmente, a actividade da timidilato sintetase (TYMS) influencia igualmente os efeitos citotóxicos do 5-FU.

Objectivos: O objectivo principal deste estudo consistiu na avaliação dos níveis de metilação da região promotora do *DPYD* através de PCR quantitativo especifico para metilação em doentes com cancro GI que desenvolveram toxicidade grave ao 5-FU. Foram objectivos secundários, a análise de arranjos intragénicos de grandes dimensões do *DPYD*, a determinação da imunoexpressão dos níveis da DPD e TYMS no tecido tumoral, e a avaliação da associação entre os achados moleculares, imunohistoquímicos e clínicos destes doentes.

Material e Métodos: Este estudo incluiu 66 doentes do Instituto Português de Oncologia do Porto com cancro GI tratados com 5-FU. Quarenta e cinco destes doentes desenvolveram toxicidade de alto grau e tinham sido previamente testados para a mutação IVS14+1G>A do gene *DPYD*. O grupo controlo consistiu nos restantes 21 doentes, que não apresentaram toxicidade. A hipermetilação do *DPYD* foi avaliada através de PCR quantitativo específico para metilação em DNA extraído de amostras de sangue periférico (SP) e tecido tumoral microdissecado (TTM), usando como controlo positivo uma linha celular de carcinoma do cólon. A pesquisa de rearranjos genómicos de grandes dimensões foi realizada através do método MLPA. Para a determinação da imunoexpressão das enzimas DPD e TYMS foram usados anticorpos específicos em tecido tumoral fixado com formol e incluído em parafina.

Resultados: A metilação do promotor do *DPYD* não foi detectada em nenhuma das amostras de SP ou TTM dos doentes com toxicidade grave ao 5-FU nem nos controlos. Rearranjos genómicos de grandes dimensões não foram igualmente encontrados em qualquer dos grupos. Contudo, a mutação no exão 14 do *DPYD* (IVS14+1G>A) previamente detectada em análise de rotina foi confirmada nos doentes com toxicidade grave. Os níveis baixos de imunoexpressão da DPD e da TYMS não estavam associados a toxicidade grave ao 5-FU. Clinicamente, constatou-se maior toxicidade após a administração do 5-FU em bólus, na localização gástrica ou esofágica do tumor primário e num ECOG mais avançado ($P<0.05$).

Conclusão: Estes resultados indicam que tanto a metilação do promotor, como os rearranjos genómicos de grandes dimensões do *DPYD* não contribuem para o

desenvolvimento de toxicidade grave ao 5-FU. São, necessários estudos adicionais para investigar outros factores genéticos e/ou clínicos responsáveis pela toxicidade grave ao 5-FU em doentes com cancro GI.

INTRODUCTION

1. GASTROINTESTINAL CANCER

1.1 EPIDEMIOLOGY

Globally, gastrointestinal (GI) cancers are common human neoplasms worldwide. In 2002 colorectal cancer (CRC) accounted for 940,00 million new cases annually, stomach 870,000 and esophageal 410,000[1].

In Portugal, CRC is the most frequent cancer in men (59.7/100,000) and the second most frequent in women (32.3/100,000)[2]. Gastric cancer represents the fifth highest incidence in men (41.1/100,000) and the third in women (19.4/100,000) but has the highest mortality in Europe and it is the third leading cause of death from cancer[3]. On the other hand, there were 8.7/100,000 new cases of esophageal cancer in men and 1.3/100,000 cases in females in 2000. The overall age-standardised mortality rates per 100,000 are similar to those of the European Union average in CRC (11.4), gastric (5.2) and esophageal (3.2) cancers[4].

Risk factors associated with the development of esophageal cancer include ageing, male gender, Caucasian race, body mass index, Barrett's esophagus, and gastroesophageal reflux disease[5]. Risks factors for gastric cancer comprise male gender, cigarette smoking, *Helicobacter pylori* infection, atrophic gastritis, Menetrier's disease and genetic factors such as hereditary non-polyposis colorectal cancer (HNPCC), familial adenomatous polyposis (FAP) and Peutz Jeghers syndrome[6]. Finally, risk factors for colorectal cancer include family history, FAP, HNPCC syndrome, past history of colorectal cancer or adenoma, chronic ulcerative colitis, and Crohn's disease[7].

1.2 DIAGNOSIS AND STAGING

Diagnosis of esophageal, gastric or colorectal cancers requires histopathologic confirmation. The histologic findings should be reported according to the World Health Organizatio (WHO) criteria[8]. The most frequent esophageal cancers are histologically classified as squamous cell carcinoma (SCC) and adenocarcinoma[8]; gastric cancer as adenocarcinoma (intestinal, difuse or mixed types according to Laurèn classification),

adenosquamous, squamous cell or undifferentiated carcinoma[8]; and CRC is classified as adenocarcinoma (which accounts for 95% of cases) and other rare types include squamous cell carcinoma and lymphoma[8].

The histologic grading into well, moderately, poorly, and undifferentiated tumors is applicable to most histologic subtypes. Staging is based on the TNM classification of the American Joint Committee on Cancer[9] .

1.3 TREATMENT

Primary interdisciplinary planning of treatment is mandatory in GI cancers. Surgery is the primary treatment option for all medically fit patients with localized resectable cancers.

1.3.1 Preoperative treatment

The primary treatment in patients with advanced esophageal, gastric and rectal cancers, includes preoperative fluoropyrimidine-based chemotherapy or chemoradiation followed by surgery[10, 11 12].

1.3.1 Postoperative treatment

Postoperative treatment for all these cancers is based on histology, surgical margins and nodal status.

In esophageal cancer, no further treatment is recommended in patients with SCC who have no residual disease at surgical margins, irrespective of their nodal status. Fluoropyrimidine-based chemoradiation is the treatment of choice for patients with adenocarcinoma who have T2N0 tumors with high-risk features and T3N0 tumors. If they had previously undergone preoperative chemotherapy, postoperative chemotherapy may be considered for patients with resectable disease of the lower esophagus. All patients with residual disease at surgical margins may be treated with fluoropyrimidine-based chemoradiation[11].

In gastric cancer, fluoropyrimidine-based chemoradiation is recommended for selected high risk patients with T2, N0 tumors without residual disease at surgical margins,

whereas 5-Fluorouracil (5-FU) based radiosensitization is used for patients with T3, T4 and/or any node positive tumors. Fluoropyrimidine-based postoperative chemoradiation is also recommended for patients with gastro-esophageal junction adenocarcinoma. All patients with residual disease at surgical margins and patients with unresectable disease may be treated with 5-FU-based radiosensitization[13].

In CRC, adjuvant chemotherapy is recommended for advanced stages and may be considered in selected node-negative patients, if high-risk factors for recurrence are present. Standard adjuvant treatment consists of fluoropyrimidine-based chemotherapy as well. The folinic acid, 5-FU, oxaliplatin (FolFOx) regimen improves significantly disease-free survival in stage II and III colon cancer and also increases overall survival in stage III colon cancer. Other options include infusional 5-FU/Folinic acid (5-FU/LV) regimens and capecitabine. Oral capecitabine has been shown to be at least as effective and less toxic as bolus 5-FU/LV[7, 12].

1.3.1 Palliative treatment

In esophageal cancer, concurrent chemoradiation with a fluoropyrimidine-based regimen is indicated for unresectable disease[14].

In stage IV gastric cancer, first-line treatment includes regimens incorporating a platinum, a fluoropyrimidine and an anthracycline. Other options contain docetaxel and irinotecan in combination with 5-FU/LV; epirrubicin, cisplatin and capecitabine (ECX) or epirrubicin, oxaliplatin and capecitabine (EOX). There is no standard second-line chemotherapy regimen and consideration should be given to inclusion in relevant clinical trials[10].

In advanced CRC, first-line palliative chemotherapy consists of a fluoropyrimidine in various combinations as follows: 5-FU/LV, capecitabine, FolFOx, and folinic acid, 5-FU, irinotecan (FolFIri). Second-line chemotherapy in patients with good performance status comprises FolFOx, in patients refractory to irinotecan-based regimens, and FolFOx in patients refractory to FolFIri. Vascular endothelial growth factor (VEGF) and epidermal growth factor receptor (EGFR) antagonists/inhibitors combined with chemotherapy should be considered in selected patients with metastatic colorectal cancer[7, 12].

1.4 OUTCOMES

The survival rates in different gastrointestinal cancer depends on tumor stage at diagnosis, treatment, patient's general health status, and morphological/molecular features of the tumor[8].

Overall, the prognosis of esophageal SCC is poor with 5-year survival rates around about 10%. In esophageal adenocarcinoma the overall 5-year survival rate after surgery is less than 20%. The survival rates are better in superficial adenocarcinoma, ranging from 65% to 80% in different series[8].

Survival in gastric cancer depends basically on TNM staging. Stage 0 has the best prognosis with a 5-year survival of 90-100%. The 5-year survival is 60-70% for stage I, 20-25% for stage II, 5-10% for stage III and less than 1% for stage IV[15].

The overall survival rate in colorectal cancer is about 64%. The 5-year survival rate in early, localized CRC approximates 90% but after spread to adjacent organs or lymph nodes it decreases to nearly 67%. At present, in metastatic colorectal cancer the median overall survival is nearly 30 months and overall 5-year survival has increased to 19%[16].

2. EPIGENETICS

Epigenetics is the study of heritable alterations in gene regulation that do not involve a change in deoxyribonucleic acid (DNA) nucleotide sequence or in its associated proteins. It deals, instead, with an inheritance pattern of information based on gene expression levels[17].

There are at least three epigenetic phenomena known today. DNA methylation, which is often altered in cancer cells, being one of the most studied. Histone modification is another important mechanism that results in dramatic changes in chromatin structure as well as in the accessibility of DNA to transcription factors that mediate gene expression. MicroRNAs (miRNA) are small noncoding ribonucleic acids (RNAs) that comprise a significant transcriptional output from eukaryotic genomes and affect transcriptional and posttranscriptional levels of numerous genes involved in cancer development[18].

2.1. DNA METHYLATION

Genomic DNA methylation is an important epigenetic modification which is essential for life and plays a key role in the regulation of gene expression. Methylation consists in the covalent addition of a methyl group from the methyl donor S-adenosylmethionine to the carbon-5 position of cytosine within the CpG dinucleotide. This enzymatic reaction occurs after DNA synthesis and is performed by DNA methyltransferases (Figure 1)[19].

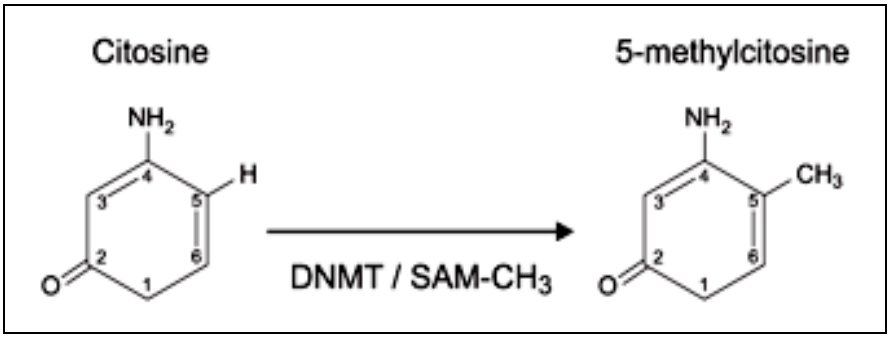

Figure 1 – Mechanism of DNA methylation. Adapted from *Lehbach et al*[19]

In humans, DNA methylation occurs at CpG dinucleotides that are not randomly distributed in the genome. Indeed, there are CpG-rich genomic regions known as CpG islands, which occur in approximately 40% of the promoters of human genes and usually are not methylated in normal cells[20].

These patterns of DNA methylation change substantially when cells become cancerous, as a result of two major phenomena. First, the tumoral genome becomes globally hypomethylated, unlike normal cells, due mainly to the widespread demethylation in the CpGs scattered throughout the body of the genes. Second, the CpGs located at the promoter region of tumor-suppressor genes undergo intense hypermethylation[17].

2.1.1 Hypomethylation

The loss of methylation is mainly due to hypomethylation of repetitive DNA sequences and demethylation of coding regions and introns, which are regions of DNA that allow alternative versions of the messenger RNA that is transcribed from a gene[21].

During the development of a neoplasm, the degree of hypomethylation of genomic DNA increases as the lesion progresses from a benign proliferation of cells to an invasive neoplasm, through the generation of chromosomal instability, reactivation of transposable elements, and loss of imprinting. For instance, loss of imprinting of insulin-like growth factor 2 gene (*IGF2*) is well established in colorectal cancer and in Beckwith–Wiedemann syndrome, which increases the risk for cancer. Disrupted genomic imprinting also contributes to the development of Wilms' tumor[20].

Demethylation of some promotor regions can also lead to expression of usually repressed genes, like the activation of paired box gene 2 (*PAX2*) and *let-7a-3 miRNA* gene, which have been, recently, implicated in endometrial and colon cancers, respectively[22, 23].

2.1.1 Hypermethylation

Hypermethylation of CpG-island promoters can affect genes involved in cell cycle, DNA repair, metabolism of carcinogens, cell-to-cell interaction, apoptosis, and angiogenesis, all involved in the development of cancer[20].

Specifically, hypermethylation of the CpG islands is a well known mechanism of tumor-suppressor gene inactivation, which is a major event in the origin of many cancers

(Figure 2)[17]. This phenomenon is well established in the promoter region of retinoblastoma gene (*RB)*, von Hippel–Lindau gene (associated with von Hippel–Lindau disease) and *p16*^{INK4a} genes. Other examples are the hypermethylation of the promoters of DNA-repair genes homologue of MutL Escherichia coli gene *(hMLH1),* breast-cancer susceptibility gene 1 (*BRCA1),* O6-methylguanine–DNA methyltransferase gene *(MGMT)*, and the gene associated with Werner's syndrome[20]. The silencing of these DNA-repair genes blocks the genetic repair systems of normal cells and opens the way to its neoplastic transformation[20].

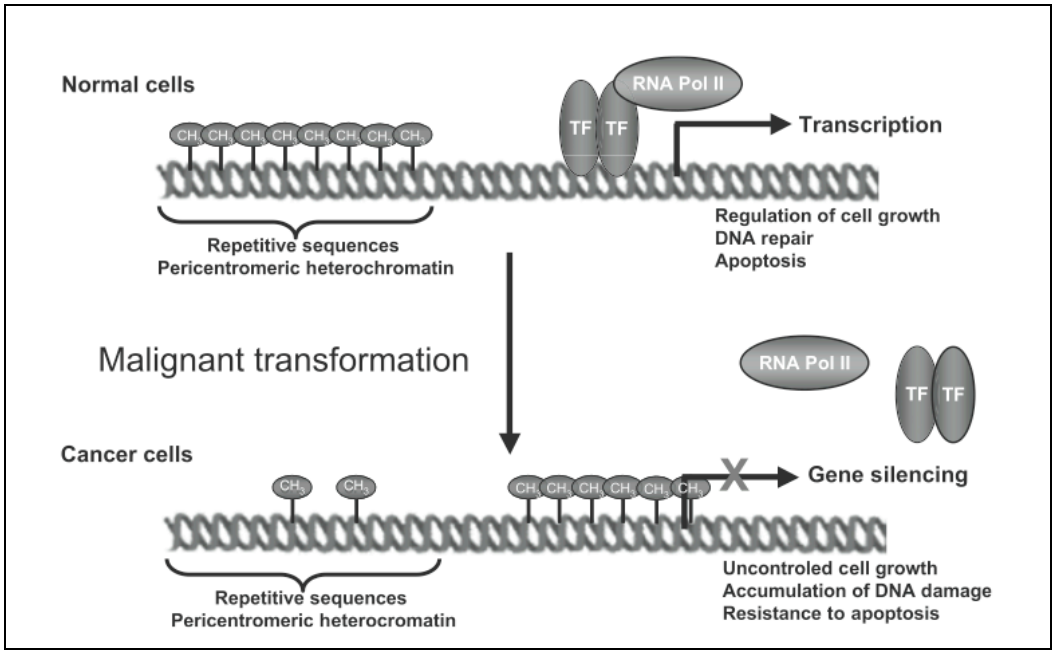

Figure 2 – Hypermethylation of CpG islands. Repetitive DNA sequences and a typical CpG island of a tumor suppressor gene are shown for a normal and a tumor cell. The presence of dense hypermethylation completely changes the molecular environment. Adapted from *Esteller et al*[17].

2.2 HISTONE MODIFICATION

Histone modification by acetylation, deacetylation, and methylation have direct effects on a variety of nuclear processes, including gene transcription, DNA repair, DNA replication, and chromosome organization[20]. Histone acetylation has been associated with an increase in gene activity whereas histone deacetylation usually prevents transcription. Other histone modifications such as histone methylation have a major impact in the initiation of cancer processes and on its progression potentially to malignant cells[18].

2.3 MICRORNA

MiRNA are short noncoding RNA molecules that function as transcriptional regulators of gene expression involved in cell proliferation, differentiation, and apoptosis. Misregulation of miRNA expression might be an important event in cancer development since different miRNA expression profiles were found in normal tissues and derived tumors, as well as between different types of tumor. Some miRNAs may act as tumor-suppressor genes as well[17]. An example is the epigenetic silencing of miR-124a, which induces the activation of cyclin D kinase 6, a bona fide oncogenic factor, and the phosphorylation of the RB tumor-suppressor gene[24].

2.4 EPIGENETICS IN CANCER MANAGMENT

Epigenetic alterations associated with cancer development can lead to the development of new strategies for assessing cancer risk status, early screening, prognosis definition, predicting response to chemotherapy, monitoring follow-up and treatment[17,25]

2.4.1 Early Diagnosis

Hypermethylation of CpG islands can be used as a marker of cancer cells in all types of biologic fluids and biopsy specimens. For example, the detection of glutathione S-transferase 1 gene (*GSTP1)* methylation in urine could help to distinguish between prostate cancer and a benign process[26].

Analysis of hypermethylation of the CpG islands has a potential diagnostic applicability for carriers of high-penetrance mutations in tumor-suppressor genes. For example, identification of DNA hypermethylation in a breast-biopsy specimen from a carrier of a *BRCA1* mutation could be useful when the pathological diagnosis is uncertain, because hypermethylation of the CpG island is an early event in the development of cancer[27].

Several hypermethylated genes can detect twice as many tumor cells in breast ductal fluids as conventional cytologic analysis[28] and hypermethylated genes can be found in exfoliated cells at different stages in the development of cervical cancer[29].

2.4.2 Prognosis

Hypermethylation of a tumor-suppressor gene and DNA hypermethylome profiles can be indicators of prognosis in patients with cancer. Hypermethylation of the death-associated protein kinase (*DAPK*), p16INK4a, and epithelial membrane protein 3 (EMP3) genes have been linked to poor outcome in lung, colorectal, and brain cancer, respectively[20].

2.4.3 Prediction to treatment

The methylation-associated silencing of genes involved in DNA-repair is a potential predictor of the response to treatment. Hypermethylation of *MGMT* is an independent predictor of a favorable response of high grade gliomas to carmustine or temozolomide[30], but in untreated patents with low-grade astrocytoma and other tumor types it is a poor prognosis marker[31]. The potential of the methylation status of *MGMT* and other DNA-repair genes to predict the response to chemotherapy has also been found for cyclophosphamide (with the *MGMT* gene), cisplatin (with the *MLH1* gene), methotrexate [with the reduced folate carrier gene (*RFC* gene)], and irinotecan (with the Werner syndrome gene (*WRN*)[20].

2.4.4 Epigenetic treatment of cancer

Unlike mutations, DNA methylation and histone modifications are reversible. Epigenetic alterations allow the cancer cell to adapt to changes in its microenvironment, but dormant, hypermethylated tumor-suppressor genes can be awakened with drugs. It is possible to re-express DNA-methylated genes in cancer cell lines by using demethylating agents and to rescue their functionality. DNA demethylating drugs in low doses have showed clinical activity against some tumors. Two of such agents, 5-azacytidine and decitabine have been approved recently for the treatment of myelodysplastic syndrome and leukemia, but not still for solid tumors[20].

2.5 EPIGENETICS CHANGES IN GASTROINTESTINAL CANCER

While epigenetic features are not well established in esophageal and gastric tumorigenesis, much is already known for colorectal cancer. Both hypomethylation and hypermethylation are present in CRC development. Hypomethylation occurs at an early stage, which is supported by the loss of the methyl group at CpG dinucleotides in very small adenomas[32]. Hypermethylation is also an important mechanism in transcriptional inactivation of the mismatch repair gene *MLH1*[33], which occurs in approximately 80% of the sporadically occurring microsatellite instability tumors[34]. Moreover, the adenomatosis polyposis coli gene (*APC)* may be inactivated by DNA hypermethylation, although the frequency methylation of *APC* promoter is significantly lower than that of *MLH1* in sporadic tumors[35]. Importantly, the hypermethylation of some of these genes is present both in adenomas and in carcinomas, and may thus represent early changes in tumorigenesis. In a subgroup of colorectal carcinomas that arise from hyperplastic polyp variants there is hypermethylation of CpG islands with subsequent inactivation of *MGMT* combined with mutation of V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*KRAS2)*[36]. The methylation frequency of *MGMT* is similar in colorectal adenomas and carcinomas, further supporting a role for this gene in tumor initiation[37].

2.5.1 The CIMP concept

Presently, cancers might be classified according to their degree of methylation, which seem to define distinct epidemiologic, histologic, and molecular features. The CpG island methylator phenotype, or CIMP, represents those cancers with high degree of methylation at several loci, which are characterized by *epigenetic instability*[38]. In colorectal tumorigenesis, the CpG island methylator phenotype can be observed in 18% to 25% of sporadic colorectal cancers[39] and has been considered a third pathway[40], in addition to chromosomal instability and microsatellite instability[41]. Therefore, CIMP may be an important mechanism for gene inactivation in CRC, as there is a growing evidence that promoter methylation can silence known tumor suppressor genes, including $p16^{INK4a}$, $p14^{ARF}$, *MGMT* and *hMLH1*[42]. Furthermore, early detection of CIMP and subsequent epigenetic intervention could prevent the development of CIMP-positive cancers[38].

3. 5-FLUOROURACIL

The fluoropyrimidine 5-FU is broadly used in the treatment of a wide range of gastrointestinal cancers[43], including colorectal, gastric, and esophageal cancers.

3.1 MECHANISM OF ACTION

5-FU is an analogue of uracil with a fluorine atom at the C-5 position replacing hydrogen, and it enters the cell using the same transport mechanism as the uracil nucleotide[44]. The mechanism of 5-FU cytotoxicity has been ascribed to the misincorporation of fluoronucleotides into RNA and DNA[43].

In brief, 5-FU is converted intracellularly to several active cytotoxic metabolites: fluorodeoxyuridine monophosphate (FdUMP), which inhibits thymidylate synthase; fluorodeoxyuridine triphosphate (FdTMP), which is incorporated in DNA; and fluorouridine triphosphate (FdUTP), which is incorporated in RNA, leading to their disruption (Figure 3)[45].

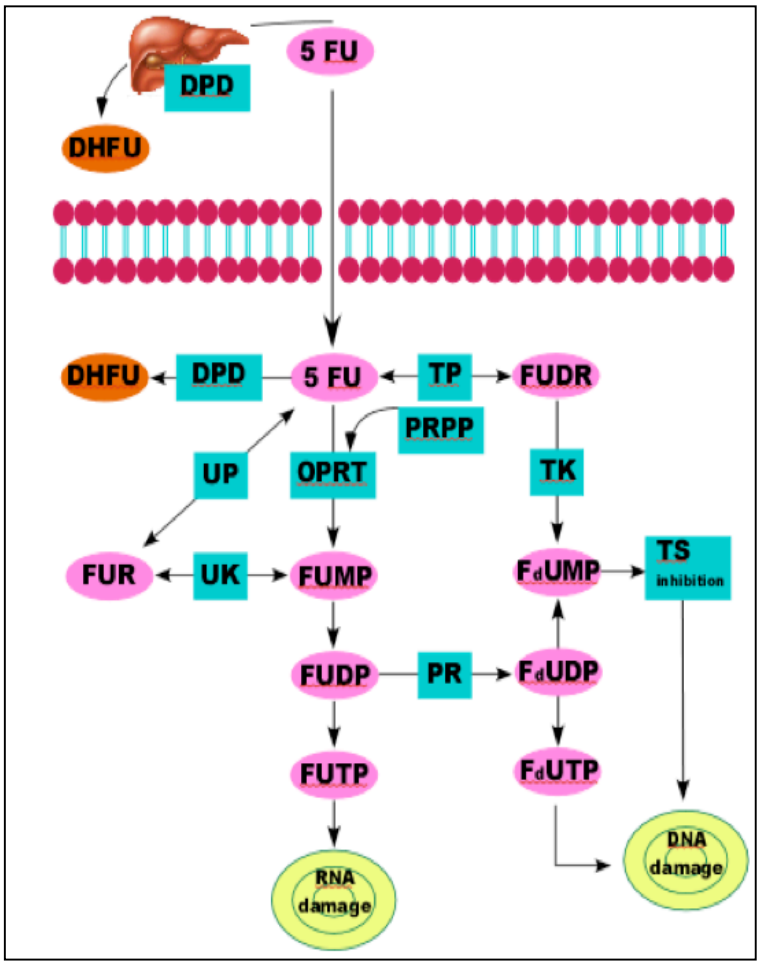

Figure 3 – 5-FU Metabolism. The main mechanism of 5-FU activation is conversion to FUMP, either directly by OPRT with PRPP as the cofactor, or indirectly via FUR through the sequential action of UP and UK. FUMP is then phosphorylated to FUDP, which can be either further phosphorylated to the active metabolite FUTP, or converted to FdUDP by RR. 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 catalysed conversion of 5-FU to FUDR, which is then phosphorylated by TK to FdUMP. DPD-mediated conversion of 5-FU to DHFU is the rate-limiting step of 5-FU catabolism in normal and tumour cells (**FUMP** – Fluorouridine monophosphate, **OPRT** – orotate phosphoribosyltransferase, **PRPP** – phosphoribosyl pyrophosphate, **FUR** – fluorouridine, **UP** – uridine phosphorylase, **UK** – uridine kinase, **FUDP** – fluorouridine diphosphate, **FUTP** – fluorouridine triphosphate, **FdUTP** – fluorodeoxyuridine diphosphate, **RR** – ribonucleotide reductase, **FUDR** – fluorodeoxyuridine, **TK** – thymidine kinase, **DPD** – dihydropyrimidine dehydrogenase, **DHFU** – dihydrofluorouracil). Adapted from *Longley et al* [43].

Thymidylate synthase (TYMS) is the enzyme that converts deoxyuridine monophosphate (dUMP) into deoxythymidine monophosphate. Deoxythymidine monophosphate (dTMP) is then transformed into deoxythymidine triphosphate (dTTP), which is essential for DNA replication and repair. One of the main mechanisms of action of 5-FU is the inhibition of TYMS by fluorodeoxyuridine monophosphate (FdUMP), which forms stable complexes with TYMS and blocks the conversion of dUMP to dTMP and dTTP, leading to DNA damage[46]. On the other hand, TYMS inhibition induces an accumulation of dUMP. FdUMP and dUMP can therefore be converted into their thriphostate forms, fluorodeoxyuridine triphosphate (FdUTP) and deoxyuridine triphosphate dUTP further disrupting the DNA synthesis and repair[47].

The rate-limiting enzyme in 5-FU catabolism is dihydropyrimidine dehydrogenase (DPD), which converts 5-FU to dihydrofluorouracil. More than 80% of administered 5-FU is normally catabolized primarily in the liver, where DPD is abundantly expressed[43].

3.2 PHARMACOKINETICS

The oral absorption of 5-FU is variable and therefore is preferable administrated by an intravenous route. Following intravenous (IV) infusion, 5-FU is widely distributed to the tissues, with its highest concentrations in the bone marrow, GI mucosa, and liver. It can also penetrate into the third space fluid collections and the blood brain barrier. The 5-FU metabolites are excreted by the respiratory tract and kidneys after being catabolized in the liver[48].

3.3 DOSAGE

The usual dosage depends on the different protocols. Intravenous dosing schedules includes weekly bolus, five daily boluses every 28 days, 1 to 5 day continuous infusions, and protracted continuous infusions. Common doses range from 200 to 2600 mg/m^2/d depending upon the dosing scheme[49] (Table 1).

Table 1 – Dosing of 5-FU in adults. The administration is given as IV bolus (a slow push or 5-15 minutes bolus infusion), or as a continuous infusion. Doses >1000 mg/m^2 are usually administered as a 24-hour infusion. Giving the drug as a constant infusion may reduce toxicity. Adapted from *UpToDate*[50]

Mode of administration	Scheme
IV bolus	500-600 mg/m^2 every 3-4 weeks or 425 mg/m^2 on days 1-5 every 4 weeks
Continuous IV infusion	1000 mg/m^2/day for 4-5 days every 3-4 weeks or 2300-2600 mg/m^2 on day 1 every week or 300-400 mg/m^2/day or 225 mg/m^2/day for 5-8 weeks (with radiation therapy)

3.4 SIGNIFICANT ADVERSE REACTIONS

The several types of toxicity associated with IV administration are depicted in Table 2[49, 50].

Table 2 – **Significant adverse reactions to 5-FU. Myelosuppression** is present in a dose limiting effect for the bolus schedules, but is less severe with infusional therapy. Neutropenia and thrombocytopenia are more common than anemia. **Diarrhea and mucositis** may be severe and dose limiting for infusional schedules, but are less severe with bolus schedules. **Hand-foot syndrome**, presenting with painful tingling, erythema, rash, dryness and desquamation of the hands and feet may be severe, and is more commonly seen with infusional therapy. **Cardiac complications** including angina and myocardial ischemia have been associated with 5-FU, especially in patients with preexisting coronary artery disease. Adapted from *UpToDate*[50]

System	Toxicity
Cardiovascular	Angina, myocardial ischemia, nail changes
Central nervous system	Acute cerebellar syndrome, confusion, disorientation, euphoria, headache, nystagmus
Dermatologic	Alopecia, dermatitis, dry skin, fissuring, hand-foot syndrome, pruritic maculopapular rash, photosensitivity, vein pigmentations
Gastrointestinal	Anorexia, bleeding, diarrhea, esophagopharyngitis, nausea, sloughing, stomatitis, ulceration, vomiting
Hematologic	Agranulocytosis, anemia, leukopenia, pancytopenia, thrombocytopenia. Myelosuppression: onset: 7-10 days, nadir: 9-14 days, recovery: 21-28 days
Local	Thrombophlebitis
Ocular	Lacrimation, lacrimal duct stenosis, photophobia, visual changes
Respiratory	Epistaxis
Miscellaneous	Anaphylaxis, generalized allergic reactions, nail loss

3.5. MECHANISMS INVOLVED IN 5–FU TOXICITY

The mechanisms of 5-FU cytotoxicity may depend on genetic and clinical factors. Female gender and mode of administration in bolus are linked to increased toxicity[51]. In 5-FU bolus schedules, the incorporation of fluorouridine triphosphate into RNA appears to be the most important mechanism of action, whereas inhibition of thymidylate synthase is more important as the infusion time is prolonged, therefore becoming less toxic[52].

Thus, the expression levels of thymidylate synthase (TYMS) and dihydropyrimidine dehydrogenase (DPD), which vary among individuals, may be linked to different toxicity profiles. These might also be potentially important as prognostic factors and predictive markers to the response to 5-FU[53, 54]. However, no reliable molecular marker of sensitivity or resistance to 5-FU has been validated until now[55].

3.5.1 Thymidylate synthase

Thymidylate synthase is encoded by *TYMS* gene, which is located at chromosome 18p11.32. The biologically active unit spans about 16 kbp and is composed of seven exons and six introns. TYMS is critical for DNA replication and repair, therefore normal cellular function is disrupted when 5-FU or its metabolites interfere with this function[56].

TYMS expression might depend on germ-line polymorphisms[46]. The 3R/3R genotype was proven to have significantly less toxicity, as it is linked to *TYMS* mRNA over expression levels in both normal and tumor tissue of patients, protecting the cells from damage by 5-FU treatment due to the low efficacy of *TYMS* inhibition. Consequently, the resulting decreased cell death rate leads to resistance in tumor tissue and low toxicity in normal cells. So it seems rational to expect that high expression of *TYMS* would lead to a worse response to 5-FU based chemotherapy regimens and poorer prognosis[57].

On the other hand, it has been shown that patients with the 2R/2R or 2R/3R genotype have lower *TYMS* mRNA levels in the normal tissue, which enhances the cytotoxic effects of 5-FU and leads to more severe side effects in these patients[47]. Hence a lower TYMS expression would point out to a higher sensitivity of tumor cells and better

outcome in patients treated with 5-FU chemotherapy[54, 58].

However additional studies are needed to identify the regulatory factors by which this polymorphism alters *TYMS* expression[46] and their true impact on modulating enzyme activity. As TYMS is considered to be the main intracellular target of fluoropyrimidines it is crucial to make a comprehensive study of its full pathway.

3.5.2 Dihydropyrimidine dehydrogenase

Partial or complete DPD deficiency is a known pharmacogenetic syndrome detected in 3% to 5% of the general population, which has been associated with severe and potentially lethal toxicity following 5-FU administration[59]. Lower DPD levels have also been associated with increased survival and improved prognosis, probably because of a greater responsiveness of cancer cells to 5-FU[54].

DPD, encoded by *DPYD* gene, is present as a single copy gene on chromosome 1p22 and consists of 23 exons. The physical map shows that *DPYD* is at least 950 kb in length with 3 kb of coding sequence and an average intron size of approximately 43 kb[60]. The resulting DPD is a homodimer of 2 x 111 kDa arranged in five distinctive domains (Figure 4)[61].

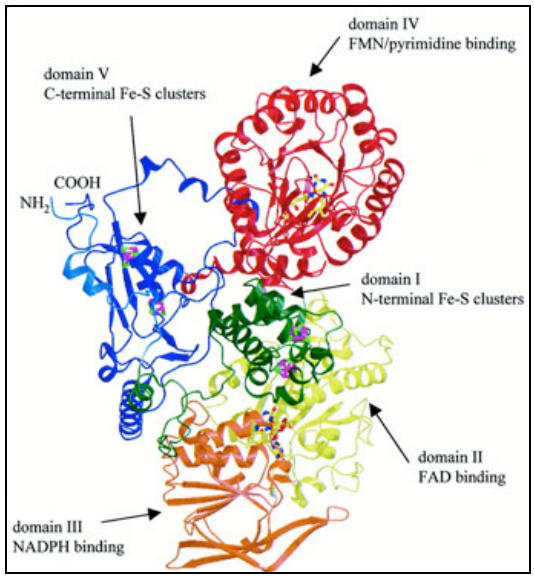

Figure 4 – Structure of liver DPD monomer. Domain I (residues 27– 172) contains two [4Fe-4S] clusters. **Domain II** (residues 173–286, 442–524) and **domain III** (residues 287–441) bind FAD and reduced NADPH, respectively. **Domain IV** (525–847) contains FMN and the uracil/thymine binding site. Finally, the remaining two [4Fe-4S] clusters are bound to the core of the C-terminal domain V (residues 1–26 and 848–1025). Adapted from *Dobritzsch et al*[61].

Over 50 alterations in the *DPYD* gene have been characterized during past decade[62]. The distribution of the mutations along the five domains recognized in DPD shows a slightly higher frequency of mutations in domain I (1.1%) and V (1.1%) compared with domain II (0.8%), domain III (0.9%) and domain IV (0.8%) (Figure 5)[59].

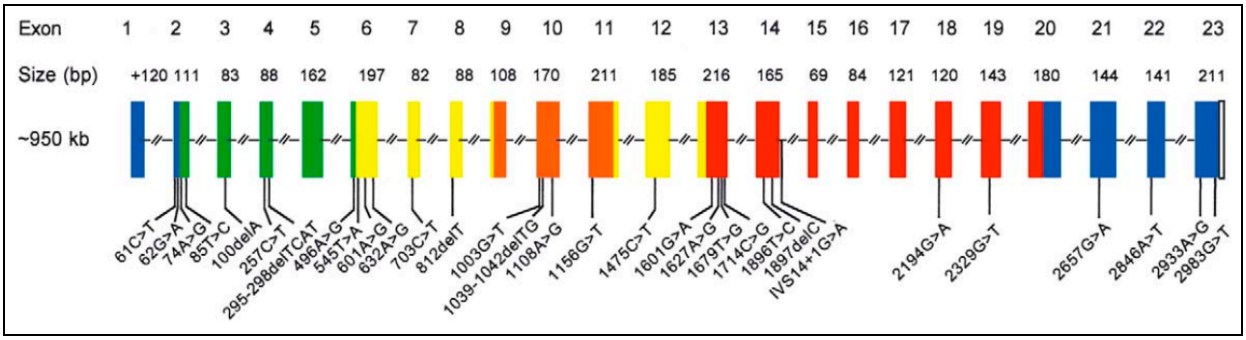

Figure 5 – Organization of the DPD gene (DPYD). DPYD consists of 23 exons with an open reading frame of 3075 bp. The various colors represent the five different domains: green for domain I (N-terminal Fe-S clusters), yellow for domain II (FAD binding), orange for domain III (NADPH binding), red for domain IV (FMN/pyrimidine binding) and blue for domain V (C-terminal Fe-S clusters). The different mutations and polymorphisms identified in patients with a partial or complete deficiency of DPD are indicated, numbers correspond to the cDNA position. Adapted from *Van Kuilemburg et al*[59].

Table 3 – Mutations in patients with DPD deficiency. Adapted from *Omura*[63].

Genotype	Effect	Location
Deletion		
1039–1042delTG	Frameshift	Exon 10
295–298delTCAT	Frameshift	Exon 4
1897delC	Frameshift	Exon 14
Exon skipping		
IVS14+G>A		Exon 14
Missense mutation		
85T>C	Cys29Arg	Exon 2
257C>T	Pro86Leu	Exon 4
496A>G	Met166Val	Exon 6
601A>C	Ser201Arg	Exon 6
632A>G	Tyr211Cys	Exon 6
703C>T	Arg2357Try	Exon 7
1108A>G	Ile370Val	Exon 10
1475C>T	Ser492Leu	Exon 12
1601G>A	Ser534Asn	Exon 13
1627A>G	Ile543Val	Exon 13
1679T>G	Ile560Ser	Exon 13
2194G>A	Val732Ile	Exon 18
2657G>A	Arg866His	Exon 21
2846A>T	Asp949Val	Exon 22
2933A>G	His978Arg	Exon 23
2983G>T	Val995Phe	Exon 23

However, the majority of these genomic alterations represent missense or intronic variants with unknown biological importance and clinical significance[62, 64]. Actually, only a limited number of patients are carriers of mutations (including the most prevalent exon 14 skipping mutation, IVS14+1G>A) which might significantly affect DPD catalytic activity[51, 63, 65, 66] (Table 3).

Recently, large intragenic rearrangements of *DPYD* and a new interstitial deletion [del(1)(p13.3p21.3)] were found in some DPD deficient patients and have been associated with severe 5-FU toxicity[67].

Nevertheless, all the genetic variants reported thus far do not justify most of the cases of DPD deficiency. Thus, epigenetic de-regulation of *DPYD* was hypothesized as an alternative mechanism for reduced DPD activity. In this setting, Noguchi and co-workers found that DPD activity was controlled at the transcriptional level by promoter methylation and thus aberrant methylation might affect the sensitivity to 5-FU in hepatocarcinoma cancer cells[68]. Furthermore, Ezzeldin et al. assessed DPD enzyme activity and *DPYD* promoter methylation status in a small series of clinical samples (n=15). These authors detected *DPYD* methylation in peripheral bloods samples from all (five) DPD-deficient volunteers and in three out of five of the DPD-deficient cancer patients with a previous history of 5-FU toxicity. Interestingly, no evidence of methylation was detected in samples from volunteers with normal DPD activity[69]. Finally, methylation of *DPYD* promoter region of RKO colon-rectal cancer cell line was shown to be associated with decreased gene expression[70].

Taking this data in consideration, it would be relevant to test whether hypermethylation of *DPYD* is, indeed, an alternative mechanism for DPD deficiency and, thus, a major cause of severe 5-FU toxicity.

OBJECTIVES

1. PRIMARY OBJECTIVE

- To analyze the methylation status of *DPYD* promotor region by quantitative methylation-specific PCR in gastrointestinal cancer patients who developed severe 5-FU toxicity in comparison with a control group

2. SECONDARY OBJECTIVES

- To analyze large intragenic rearrangements of *DPYD* in those patients

- To evaluate the immunoexpression of DPD and TYMS in tumor tissue samples from this cohort.

- To determine the association between the molecular, immunoexpression, and clinical findings.

MATERIAL AND METHODS

1. PATIENT SELECTION

The selected patients were those with esophageal, gastric or colorectal cancer who had developed severe toxicity following 5-FU treatment and had been tested for *DPYD* exon 14 skipping mutation (IVS14+1G>A) at the Portuguese Oncology Institute - Porto, Portugal, from January 1994 through December 2008. Randomly selected patients with gastrointestinal cancer who did not develop any severe toxic reaction composed the control group. All patients were treated with chemotherapy regimens based on 5-FU.

2. CLINICAL EVALUATION

Data on patient demographics (gender and age), tumor anatomical site and histopathologic subtype, stage, 5-FU-based chemotherapy scheme, mode of administration, response evaluation, and toxicity profile were assessed by detailed hospital chart review for each case.

Adverse drug effect during the chemotherapy was classified according to the Common Terminology Criteria for Adverse Events (CTCAE) v4.0[71]. Accordingly, toxicity grade III or IV were considered severe.

The outcome clinical variables chosen were disease-free survival DFS) for those receiving adjuvant 5-FU-based chemotherapy, progression-free survival (PFS) in the palliative setting, and overall survival (OS) in both groups. DFS was defined as the interval between a complete disappearance of the cancer (complete response) and the time of relapse, PFS as the time interval from the beginning of treatment to disease progression, and OS as the total amount of time that a patient survived following treatment[72].

3. SAMPLE COLLECTION AND TISSUE PROCESSING

For each patient, a peripheral blood sample was obtained. When available, a representative formalin-fixed, paraffin-embedded tissue block from each tumor (biopsy or surgical specimen) was collected in both severe toxicity and control groups. Informed consent was obtained from all patients.

Routine histopathological evaluation, comprising diagnosis, grading and pathological staging according to WHO[8] was performed in all cases. From each of the representative tissue block, serial sections were cut at 4 μm, and the the first section was stained with hematoxylin and eosin for delineation of the tumor areas by a pathologist. Then, careful microdissection of these areas was performed in ten serial sections and the tissue was collected for molecular analysis. Two additional sections were placed in coated slides for immunohistochemical analysis.

Colon carcinoma cell line RKO [from American Type Tissue Collection (ATTC, USA), kindly provided by Dr. Ragnhild Lothe (Department of Cancer Prevention, Institute for Cancer Research, Oslo, Norway)] was used as control for methylation analysis (this cell line is methylated at the *DPYD* promoter). RKO cells were grown in ATCC-formulated Dulbecco's Modified Eagle Medium (D-MEM) which was supplemented with 10% fetal bovine serum, 100 00 U/L of penicillin and 100 g/L of streptomycin. Cells were incubated at 37ºC in the presence of 5% of CO_2.

4. MOLECULAR ANALYSIS

4.1 DNA Extraction

DNA was extracted from all available samples (RKO cell line, peripheral blood and microdissected tumor tissue) with phenol-chloroform, after overnight digestion in lysing buffer and proteinase K at 55ºC. The aqueous phase was transferred to 1.5 mL tubes and DNA was precipitated at -20ºC overnight by addition of absolute ethanol and 7.5 M ammonium acetate. Then, DNA pellets were washed twice with 70% ethanol and, finally, eluted in distilled water[73].

4.2 DNA Quantification

The extracted and purified DNA from all samples and RKO cell line was quantified using Nanodrop™ ND1000 microspectrophotometer (NanoDrop, USA).

4.3 Bisulfite Modification

Genomic DNA was submitted to a sodium bisulfite reaction, which converts non-methylated cytosine residues to uracil residues, while methylated cytosines residues remain without any modification (Figure 6)[74].

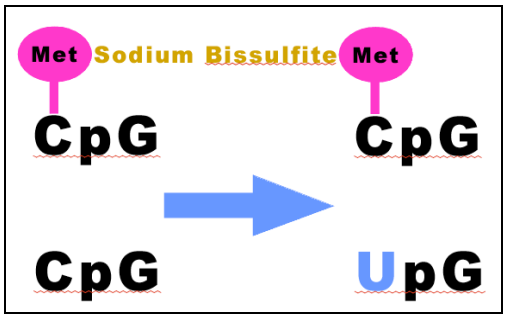

Figure 6 – Sodium bisulfite treatment. Methylated cytosines are protected and remain unchanged (above), while unmethylated cytosines are deaminated to uracil after treatment with sodium bisulfite (below). Adapted from *Esteller et al*[75].

Sodium bisulfite conversion of genomic DNA extracted from peripheral blood, cell line and microdissected tumor was performed using a previously described method[76]. Four µg of DNA were denatured in 0.3 M NaOH for 20 min at 50°C. The denatured DNA was diluted in 450 µL of a freshly prepared solution of 125 mM hydroquinone and 2.5 M sodium bisulfite and then incubated for 3 h at 70°C. After incubation, modified DNA samples were desalted and purified through a column (Wizard DNA Clean-Up System; Promega, USA) treated again with sodium hydroxide for 10 minutes at room temperature, precipitated with 100% ethanol, resuspended in 240 µL of water, and finally stored at -80°C[76].

4.4 Quantitative methylation-specific PCR

The chemically modified DNA from RKO cell line, peripheral blood and microdissected tumor tissue was amplified through quantitative methylation-specific PCR, where all uracils and thymines were amplified as thymines, whereas methylated cytosines appear as cytosines[76].

To accomplish this goal, PCR primer's sequences were chosen for regions containing frequent CpG pairs near the 3' end to discriminate methylated from unmethylated alleles in bisulfite-modified DNA. Primers were designed according to the CpG island of the sense strand of the *DPYD* gene (Genbank accession no. NM_000110) as follows:

forward, 5'-TTTGTTTGTTTTCGATTCGC-3'; and reverse 5'-ATCCGCCGAATCCTTACTAA-3'. A reference gene (*ACTB)* was used to normalize for DNA input in each sample[77].

Fluorescence based real-time PCR assays were carried out in a reaction volume of 20 µL containing 10 mL of SYBR® Green PCR Master Mix (Applied Biosystems, USA), 0.5 mL (10 µM) of each primer, 7 mL of nuclease free water (MP Biomedicals, France) and 2 µL of bisulfite-modified DNA sample. Each sample ran in triplicate and additionally, multiple water blanks were used per plate as a control for contamination (negative control). All amplifications were carried out in 96-well plates on a 7500 Sequence Detection System (Applied Biosystems, USA) under the conditions described in Figure 7.

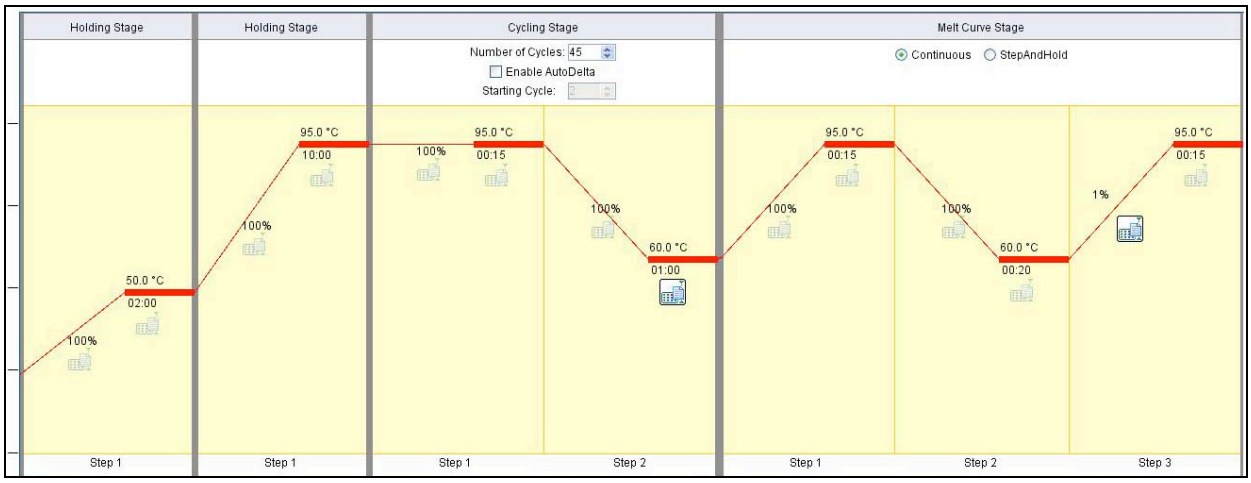

Figure 7 – Quantitative methylation-specific PCR. All amplifications were carried under the following conditions: 50°C for 2 min and 95°C for 10 min (holding phase), followed by 45 cycles of 95°C for 15 s, 60°C for 1 min (cycling phase) and finally 95°C for 15 s, 60°C for 20 s and 95°C for 15 s (melting phase).

A calibration curve was created for each plate by amplifying 10-fold serially diluted universal methylated sperm human DNA samples (Chemicon, USA). Melting curve analysis done after each PCR confirmed that only one product was amplified for all samples.

To determine the relative levels of methylated promoter DNA in each sample, the values obtained by QMSP analysis (mean quantity) for each target gene were divided by the respective values of the internal reference gene (*ACTB*). The ratio thus generated, which constitutes an index of the percentage of input copies of DNA that are fully methylated at the primer site, was then multiplied by 1000 for easier tabulation (methylation level = target gene/reference gene × 1000).

4.5 *DPYD* large genomic rearrangements analysis

Peripheral blood DNA samples were also screened for *DPYD* large genomic rearrangements by MLPA according to the manufacturer's instructions (Salsa P103 kit; MRC, Holland). The MLPA method is based on sequence-specific probe hybridization to genomic DNA, followed by PCR amplification of the hybridized probe (with one FAM-labeled primer), and semiquantitative analysis of the PCR products. Target-specific products were identified according to their differential length on an ABI 310 sequencer using Genemapper software (Applied Biosystems, USA). Peak areas from each patient were then exported to an Excel (Microsoft Office, USA) spreadsheet, which was designed to assess the ratios between each of the test peak area and a normal control peak area. In normal individuals this calculation results in a value of 1.0 representing two copies of the target sequence in the sample. Heterozygote deletions/duplications of the target sequence in the sample should provide a 35-50% reduction/gain in the relative peak area of the amplification product of that probe. The Salsa P103 MLPA kit also contains a probe specific for the exon 14 skipping mutation (IVS14+1G>A) that only generates a signal when the mutation is present[78].

5. IMMUNOHISTOCHEMICAL ANALYSIS

DPD and TYMS expression in tumor tissue samples was assessed by immunohistochemistry, when the corresponding paraffin block was available (30 cases and 16 controls).

Four µm thick sections from paraffin-embedded samples were dewaxed in xylene and hydrated through a graded alcohol series. Antigen retrieval was accomplished by microwaving the specimens at 800 W for 30 minutes with EDTA buffer. After cooling the slides, endogenous peroxidase activity was blocked by incubating the sections in hydrogen peroxide in 3% methanol for 30 minutes. The sections were treated with 5% normal horse serum (VectaStain, USA) in 1% PBS-BSA for 30 minutes to reduce background interference. The primary mouse monoclonal antibodies against DPD (14.3) and TYMS (1.B.926) (Santa Cruz Biotechnology, USA) were applied in 1:50 and in 1:150 dilutions, respectively, with 1% PBS-BSA and left at 4ºC overnight. The

secondary biotinylated horse antibody (VectaStain, UK) at a dilution of 1:50 was added for 30 minutes. In order to enhance the immunohistochemical staining, the sections were incubated in avidin-biotin complexes for 30 minutes. Then, 3,3'-diaminobenzidine (Sigma, USA) was used for visualization and hematoxilin for counterstaining. Finally, the slides were mounted after dehydration and diaphanization.

An experienced pathologist categorized the immunoexpression of both DPD and TYMS according to the stain intensity, using the areas of paired normal epithelium as internal reference: 0 (absent immunoexpression), 1+ (expression lower than normal epithelial tissue), 2+ (expression similar to normal epithelial tissue), and 3+ (expression higher than normal epithelial tissue). Tissue specimens were analysed blinded to clinical and molecular information.

6. STATISTICAL ANALYSIS

Statistical tests were performed to evaluate the relationship between the results of the molecular analysis and clinicopathological data, as follows:

The development of severe 5-FU toxicity was correlated with clinical parameters and immunoexpression of DPD and TYMS using the Pearson chi-square or Fisher exact test. These tests were also applied to examine the association between immunoexpression of DPD and TYMS and clinicopathological variables. Associations with patient's survival were assessed by Kaplan-Meier and Cox regression analysis.

A *p* value less than 0.05 was considered to be the limit of statistical significance. The analyses were performed using Excel (Microsoft Office, USA), GraphPad Prism 5.00 (GraphPad Software, USA), GraphPad Instat 3.05 (GraphPad Software, USA) software and PASW Statistics 18.0 software.

RESULTS

1. CLINICAL DATA AND ITS CORRELATION WITH TOXICITY

This study included 45 patients (24 women and 21 men) with severe (grade III-IV) toxicity and 21 patients (11 women and 10 men) without any severe toxic reaction (control group, CG), following 5-FU treatment. The median age was 56 years (ranging from 34 to 76 years) in the patients with high-grade toxicity group (HGTG) and 46 years (ranging from 25 to 77 years) in the control group. No statistically significant correlation was found between gender, age and severe 5-FU toxicity ($p>0.05$, Table 4).

The primary tumor site was significantly different in both HGTG and CG, as there were esophageal or gastric 23 cases (51%) and 4 controls (19%), and colorectal in 22 cases (49%) and in 17 controls (81%) ($p=0.017$, Table 4). Patients whose primary tumor site was esophageal or gastric experienced more high-grade toxicity events than CRC patients.

A significant difference in tumor staging distribution between HGTG and CG was not found ($p=0.197$, Table 4). Stage II was found in 3 cases (7%) and 4 controls (19%) and stage ≥III in 42 cases (93%) and 17 controls (81%).

The ECOG scored 0 in 20 cases (44%) and 16 controls (76%), and scored ≥1 in 25 cases (56%) and 5 controls (24%). There were significant differences in the distribution of ECOG performance status between HGTG and CG ($p=0.019$, Table 4), with ECOG 0 patients experiencing less toxicity.

In 14 cases (31%) and 12 controls (57%), 5-FU was given in the adjuvant setting and in 31 cases (69%) and 9 controls (43%) it was administered with a palliative purpose. No statistically significant association was found between the chemotherapy setting and toxicity ($p=0.060$, Table 4).

In gastric and esophageal cancer patients, the combination of 5-FU with cisplatinum was given in 23 cases (51%) and 3 controls (14%). In colorectal patients, 5-FU was given in conjunction with levamisole in 2 cases (4%) and in 4 controls (19%), and combined with folinic acid in 12 cases (27%) and 7 controls (33%). FolFOx was administered in 6 cases (13%) and in 4 controls (19%) and FolFIri in 2 cases (4%) and

3 controls (14%). The chemotherapy scheme was statistically associated with toxicity (p=0.017, Table 4), with more toxicity events in the 5-FU/cisplatinum combination.

Thirty-four cases (76%) and 10 controls (48%) were given 5-FU in bolus, 11 (24%) cases and 11 (52%) controls in a mode of administration combining bolus and continuous infusion or continuous infusion alone. The mode of administration was also statistically associated with toxicity (p=0.025, Table 4), with more high-grade toxicity events in the bolus administration.

Table 4 – Association of clinical data with toxicity in patients treated with 5-FU.

Clinical variable	**HGTG (n=45)** n (%)	**CG (n=21)** n (%)	***P value***	**Odds ratio (95% CI)**
Gender			0.942*	1.04 (0.37-2.93)
F	24 (53)	11 (52)		
M	21 (47)	10 (48)		
Age			0.886*	1.09 (0.32-3.73)
<65 years	35 (78)	16 (76)		
≥65 years	10 (22)	5 (24)		
Tumor location			0.017**	4.44 (1.29-15.29)
Esophageal or Gastric	23 (51)	4 (19)		
Colorectal	22 (49)	17 (81)		
TNM stage			0.197**	0.30 (0.06-1.50)
II	3 (7)	4 (19)		
≥III	42 (93)	17 (81)		
ECOG, n (%)			0.016*	0.25 (0.08-080)
0	20 (44)	16 (76)		
≥1	25 (56)	5 (24)		
Treatment Purpose			0.060*	0.34 (0.12-0.99)
Adjuvant	14 (31)	12 (57)		
Palliative	31 (69)	9 (43)		
Chemotherapy scheme			0.017**	4.44 (1.29-15.29)
5-FU/cisplatinum	23 (51)	4 (19)		
Other***	22 (49)	17 (81)		
5-FU Mode of Administration			0.025*	3.40 (1.14-10.15)
Bolus alone	34 (76)	10 (48)		
CI or Bolus+CI	11 (24)	11 (52)		

*Pearson Chi-Square test, ** Fisher's exact test, ***5-FU/LV or 5-FU/FA or FolFOx or FolFIri

2. TOXICITY EVALUATION

The patients in the HGTG developed the following manifestations of toxicities during chemotherapy with 5-FU: mucositis (24 cases, 53%), neutropenia (13 cases, 29%), thrombocytopenia (7 cases, 16%), anemia (6 cases, 13%), nausea/vomiting (6 cases, 13%), diarrhea (5 cases, 11%), and hand-foot syndrome (3 cases, 7%) (Figure 8).

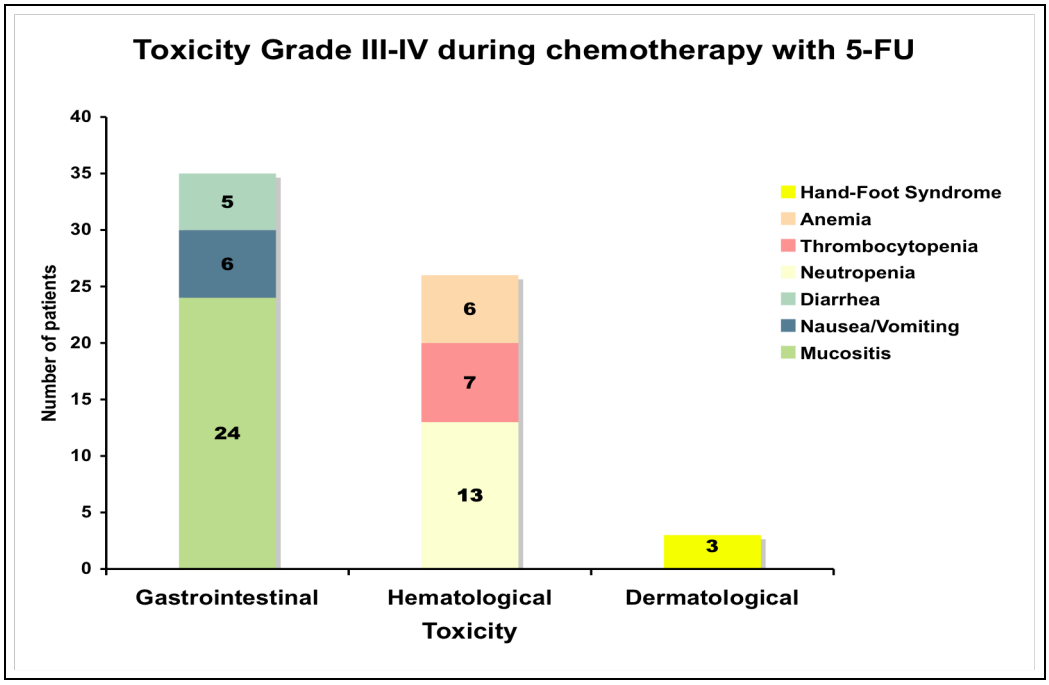

Figure 8 – Toxicity profile of the 45 patients with high-grade toxicity treated with 5-FU according to Common Terminology Criteria for Adverse Events, v4.0[71]

A total of 13 patients in the HGTG were treated for all planned chemotherapy cycles in the adjuvant or palliative setting. Ten patients (22%) had a reduction of 25% and 3 (7%) had a reduction of 50% of the 5-FU dose, mainly at the first (7%), second (9%) or third (4%) cycle. Nineteen patients (29%) stopped the treatment prematurely because of toxicity, mainly at the first (16%), second (7%) or third (11%) cycle. Three palliative (7%) patients stopped the treatment due to disease progression.

3.METHYLATION ANALYSIS

No evidence of *DPYD* promoter methylation was observed in any of the 45 peripheral blood (Figure 9) nor in the 30 microdissected tumor tissue samples (Figure 10) from patients experiencing severe 5-FU toxicity.

The same result was found in the 21 peripheral blood and 16 microdissected tumor tissue samples from control patients. Importantly, *DPYD* promoter methylation was detected in RKO cell line (positive control) (Figure 11).

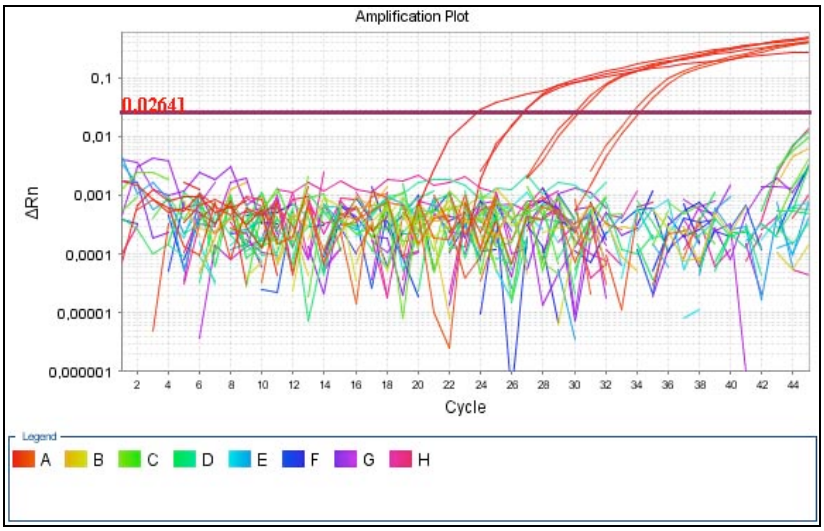

Figure 9 – Quantitative methylation-specific PCR for peripheral blood (PB) from patients experiencing severe 5-FU toxicity. No evidence of *DPYD* promoter methylation was observed (A – red lines correspond to the amplification of the serial 10-fold dilutions of a positive control, as detailed in the text; B, C, D, E, F, G and H lines – correspond to PB samples).

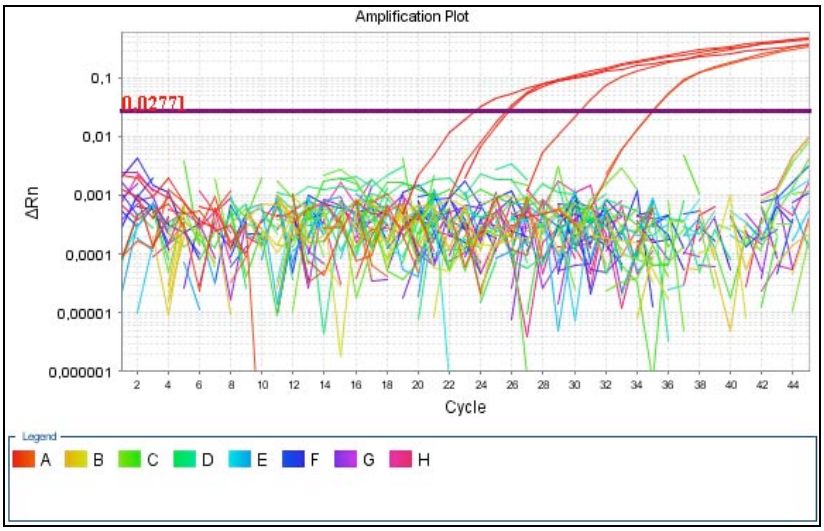

Figure 10 – Quantitative methylation-specific PCR for microdissected tumor tissue (MTT) samples from patients experiencing severe 5-FU toxicity. No evidence of *DPYD* promoter methylation was found (A – red lines correspond to the amplification of the serial 10-fold dilutions of a positive control, as detailed in the text; B, C, D, E, F, G and H lines – correspond to MTT samples).

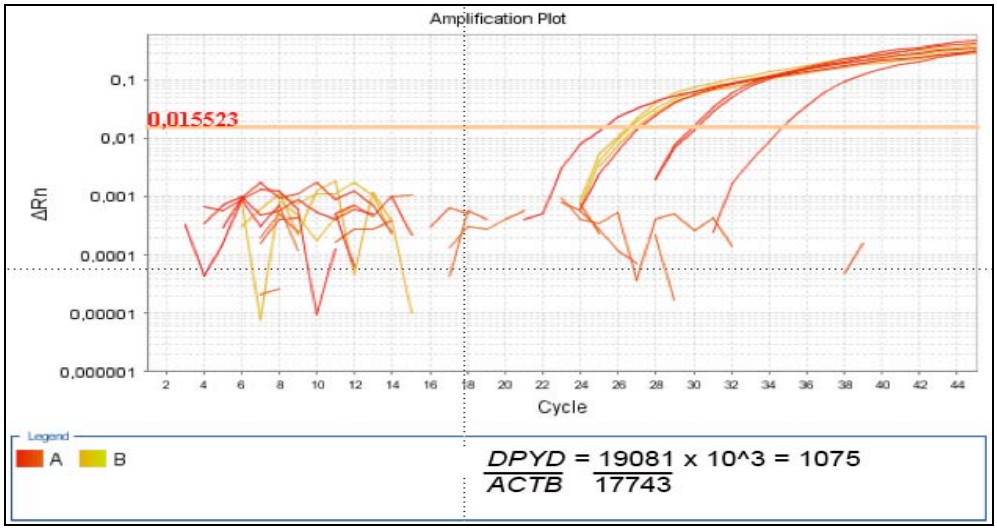

Figure 11 – Quantitative methylation-specific PCR for RKO. The ratio between the mean quantity for the target gene (DPYD) and the internal reference gene (*ACTB*) was 1075. (A – red lines correspond to the amplification of the serial 10-fold dilutions of a positive control, as detailed in the text; B – yellow lines correspond to RKO samples).

4. ANALYSIS OF LARGE INTRAGENIC REARRANGEMENTS OF *DPYD*

Large *DPYD* intragenic rearrangements were absent in the peripheral blood samples of the 45 high-grade toxicity patients and 21 controls tested. Simultaneously, the exon 14 skipping mutations (IVS14+G>A) in 3 patients with high-grade toxicity to 5-FU, which have been previously detected in routine analysis were confirmed (Table 5).

Table 5 – Toxicity profile of the patients carrying the *DYPD* exon 14 skipping mutation (IVS14+G>A) .

Sex, Age (years)	Tumor location, Stage	Neutropenia	Thrombocytopenia	Anemia	Mucositis	Mutation
F, 35	CRC, IV	4	4	4	3	IVS14+G>A, exon 14
M, 64	CRC, III B	4	0	0	4	IVS14+G>A, exon 14
F, 68	Esophageal, IV	0	0	0	4	IVS14+G>A, exon 14

The remaining forty-two patients with severe toxicity to 5-FU treatment and 21 controls showed no clinical relevant mutations of *DPYD*. Thus, the detection of the exon 14 skipping mutation (IVS14+G>A) of DYPD in the GI cancer patients included in this study, identifies those patients that develop severe 5-FU toxicity with 100% specificity and 100% positive predictive value. However, the corresponding sensitivity is 7% and the negative predictive value is only 33%.

5. DPD AND TYMS IMMUNOEXPRESSION

5.1 ASSOCIATION OF DPD AND TYMS IMMUNOEXPRESSION WITH TOXICITY

Table 5 depicts the distribution of DPD and TYMS immunoexpression in the tissue 30 samples available (30 cases and 16 controls). There was no statistically significant association between DPD or TYMS immunoexpression (Table 5).

Table 5 – Association of DPD and TYMS immunoexpression with toxicity in patients treated with 5-FU (**IHC 0** – absent immunoexpression; **IHC 1+** – low immunoexpression; **IHC 2+** – normal immunoexpression).

	HGTG	**CG**	***P value***	**Odds Ratio (95% CI)**
	n (%)	n (%)		
DPD			0.343*	0.682 (0.189-2.464)
IHC 0 or 1+	18 (59)	11 (69)		
IHC 2+	12 (41)	5 (31)		
TYMS			0.202*	0.450 (0.131-1.549)
IHC 0 or 1+	11 (37)	9 (56)		
IHC 2+	19 (63)	7 (44)		

*Pearson Chi-Square test

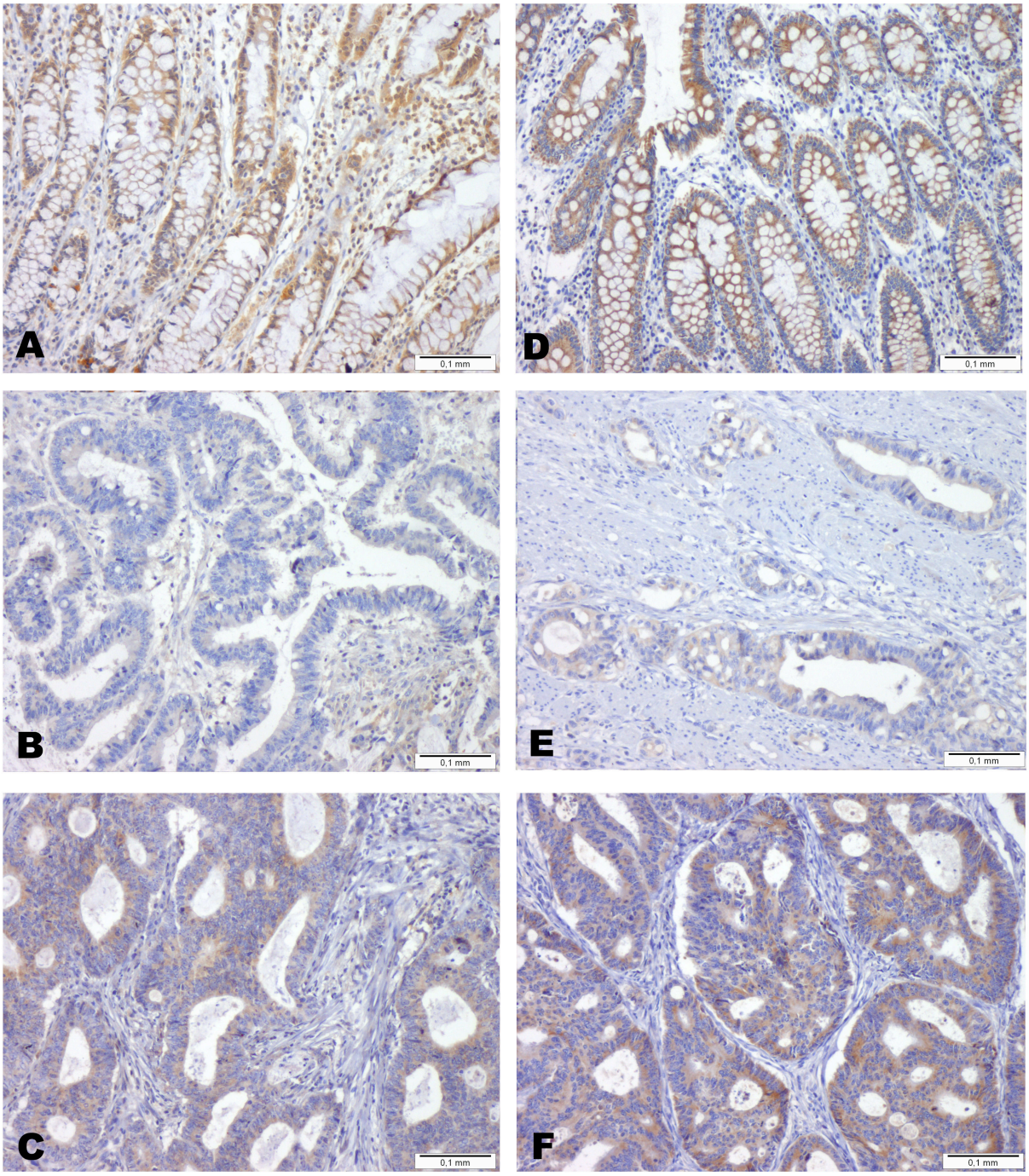

Figure 12 – **Representative images of dihydropyrimidine dehydrogenase (DPD) and thymidylate synthase (TYMS) immunohistochemical stains**. **A** – Colorectal normal tissue with normal DPD expression; **B** – Colorectal cancer tissue with absent DPD immunoexpression (IHC 0); **C** – Colorectal cancer tissue with DPD expression similar to normal colon tissue (IHC 2+); **D** – Colorectal normal tissue with normal TYMS expression; **E** – Colorectal cancer tissue with low TYMS immunoexpression (IHC 1+); **F** – Colorectal cancer tissue with TYMS expression similar to normal colon tissue (IHC 2+).

5.2 ASSOCIATION OF DPD AND TYMS IMMUNOEXPRESSION WITH CLINICOPATHOLOGICAL VARIABLES

In patients with high-grade toxicity, DPD and TYMS immunoexpression were not associated with gender, age, tumor location, tumor stage or ECOG (p>0-05) (Table 6).

Table 6 –Association of DPD and TYMS immunoexpression with clinicopathological variables of patients with high-grade toxicity treated with 5-FU (**IHC < 2** – absent or low immunoexpression; **IHC 2+** – normal immunoexpression; **CI** – continuous infusion).

	DPD Immunoexpression (n=30)			**TYMS Immunoexpression** (n=30)		
	IHC<2	**IHC 2+**	***P value****	**IHC<2**	**IHC 2**	***P value****
	n (%)	n (%)		n (%)	N (%)	
Gender			0.264			0.450
F	7 (39)	8 (67)		4 (36)	11 (58)	
M	11 (61)	4 (33)		7 (64)	8 (42)	
Age			0.660			0.372
<65years	15 (83)	9 (75)		10 (91)	14 (74)	
≥65 years	3 (17)	3 (25)		1 (9)	5 (26)	
Tumor location			0.284			1.000
Esophageal or Gastric	8 (44)	8 (67)		6 (54)	10 (53)	
Colorectal	10 (56)	4 (33)		5 (46)	9 (47)	
TNM stage			1.000			1.000
II	1 (6)	1 (8)		1 (9)	1 (5)	
≥III	17 (94)	11 (92)		10 (91)	18 (95)	
ECOG			1.000			0.702
0	6 (33)	4 (33)		3 (27)	7 (37)	
≥1	12 (67)	8 (67)		8 (73)	12 (63)	

*Fisher's exact test

5.3 ASSOCIATION OF DPD IMMUNOEXPRESSION WITH MUTATION ANALYSIS

From the three carriers of the exon 14 skipping mutation (IVS14+G>A), a tissue block was available in only one patient. DPD immunoexpression was normal in the corresponding tumor and paired morphologically normal epithelium.

6. OUTCOME ANALYSES

6.1 OUTCOME IN THE ADJUVANT SETTING

In this group of patients, the median follow-up was 3.5 years (range 0.5 – 12.4 years, n=14) in the HGTG and 5.9 years (range 2.0 – 12.0 years, n=12) in the CG. Complete remission was achieved only in CRC patients in the HGTG (50%) and in the CG (67%). Recurrent disease was observed in 3 patients (43%) in the former group and in 3 patients (38%) in the latter. Eight patients (57%) in the HGTG and 7 patients (58%) in the CG died from cancer.

6.1.1 Disease free survival

The median disease free survival in the HGTG patients who achieved complete remission initially was 6.5 years (range 1.6 – 11.8 years). No significant difference was noted in DFS with respect to gender, age, 5-FU mode of administration or TYMS immunohistochemical expression (p>0.0.5) (Table 7). DPD immunoexpression was not assessed in this group due to the lack of tissue samples.

Table 7 – Prognostic significance of clinicopathological variables and TYMS immunoexpression in DFS of patients with high-grade toxicity treated with 5-FU in the adjuvant setting. (**IHC < 2** – absent or low immunoexpression; **IHC 2+** – normal immunoexpression; **CI** – continuous infusion).

Clinicopathological variable	No. of cases	*P* value*	Hazard Ratio (95% CI of ratio)
Female *vs* Male	3 *vs* 4	0.075	0.121 (0.01173 – 1.241)
<65 *vs* ≥65 years	6 *vs* 1	0.6485	0.5264 (0.033 – 8.309)
Bolus *vs* IC or IC+Bolus	6 *vs* 2	0.445	3.651 (0.1313 – 101.5)
TYMS IHC <2 *vs* IHC 2+	3 *vs* 2	0.922	1.155 (0.06466 – 20.64)

*Log-rank (Mantel-Cox) test

6.1.2 Overall survival

The median overall survival in the HGTG was 3.5 years (range 0.5 – 12.4 years). Patients with CRC had significantly longer overall survival compared to gastric cancer patients (p=0.014). No significant difference was noted in OS with respect to gender, age, ECOG, mode of administration of 5-FU, DPD or TYMS immunohistochemical expression (p>0.05) (Table 8).

Table 8 – Prognostic significance of clinicopathological variables, DPD and TYMS immunoexpression in OS of patients with high-grade toxicity treated with 5-FU in the adjuvant setting (**IHC < 2** – absent or low immunoexpression; **IHC 2+** – normal immunoexpression; **CI** – continuous infusion).

Clinicopathological variable	No. of cases	*P* value*	Hazard Ratio (95% CI of ratio)
Female *vs* Male	8 *vs* 6	0.954	1.043 (0.252 – 4.321)
<65 *vs* ≥65 years	11 *vs* 3	0.776	1.33 (0.187 – 9.476)
CRC *vs* Gastric	12 *vs* 2	0.014	0.0009 (0.0000037 – 0.247)
ECOG 0 *vs* ≥1	10 *vs* 4	0.119	0.2177 (0.03207 – 1.480)
Bolus *vs* IC or IC+Bolus	9 *vs* 5	0.330	0.3418 (0.0394 – 2.963)
DPD IHC <2 *vs* IHC 2+	7 *vs* 3	0.564	3.794 (0.0042 – 350.6)
TYMS IHC <2 *vs* IHC 2+	5 *vs* 4	0.209	6.089 (0.364 – 101.8)

*Log-rank (Mantel-Cox) test

Although the overall survival at 5 years was higher in patients with normal DPD immunoexpression (Figure 13) and in patients with normal TYMS expression (Figure 14), differences were not statistically significant (p>0.05).

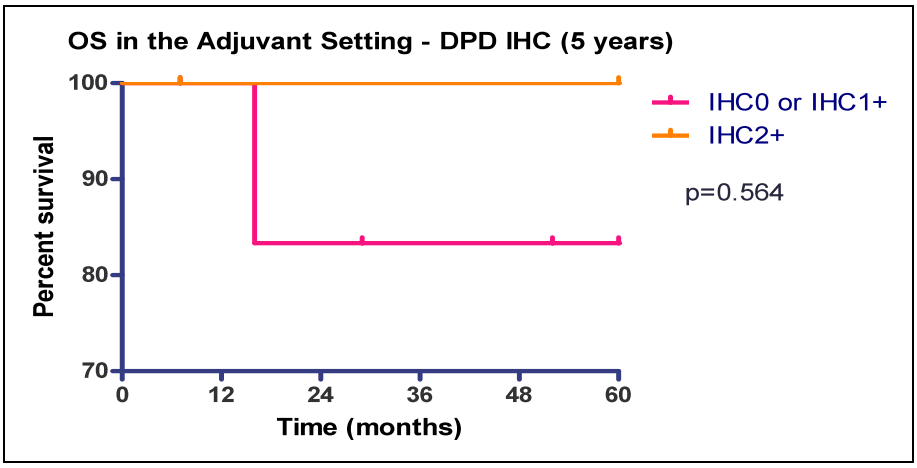

Figure 13 – Overall survival at 5 year in patients with high-grade toxicity treated with 5-FU in the adjuvant setting and with different DPD immunoexpression levels

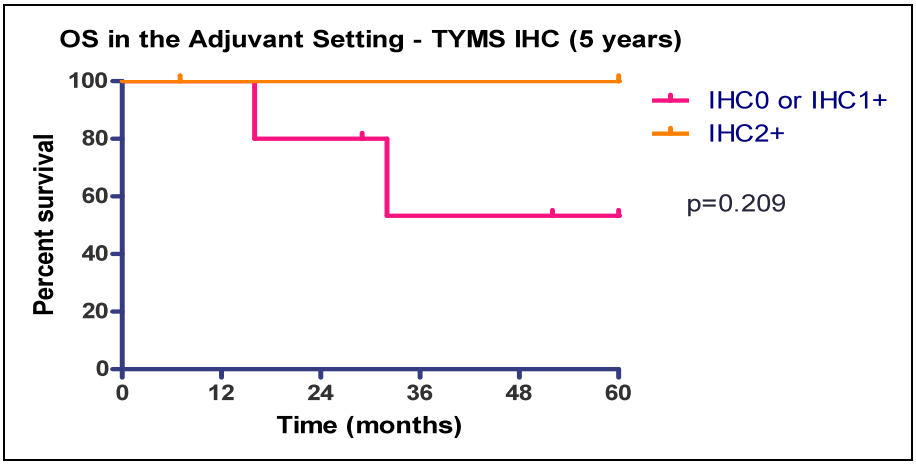

Figure 14 – Overall survival at 5 year in patients with high-grade toxicity treated with 5-FU in the adjuvant setting and with different TYMS immunoexpression levels

6.2 OUTCOMES IN THE PALLIATIVE SETTING

In patients treated with 5-FU with palliative intention (all stage IV), the median follow-up was 0.9 years in the HGTG (ranges 0.1 – 9.3 years and 0.2 – 4.0, respectively). There was disease of progression in 24 patients (77%) (4 esophageal, 8 CRC and 12 gastric) in the HGTG, and in 8 patients (89%) (all CRC) in the CG. Twenty-four patients (77%) in the HGTG and 8 patients (89%) in the CG died from cancer.

6.2.1 Progression free survival

The median progression free survival in HGTG was 9 months (range 1 month – 9.3 years). No significant difference was noted with respect to gender, age, ECOG, 5-FU mode of administration, DPD or TYMS immunohistochemical expression (p>0.05, respectively) (Table 9).

Table 9 – Prognostic significance of clinicopathological variables, DPD and TYMS immunoexpression in PFS of patients with high-grade toxicity treated with 5-FU in the palliative setting (**IHC < 2** – absent or low immunoexpression; **IHC 2+** – normal immunoexpression; **CI** – continuous infusion).

Clinicopathological variable	No. of cases	*P* value*	Hazard Ratio (95% CI of ratio)
Female *vs* Male	16 *vs* 15	0.875	0.930 (0.378 – 2.293)
<65 *vs* ≥65 years	24 *vs* 7	0.448	1.479 (0.538 – 4.067)
ECOG 0 *vs* ≥1	16 *vs* 15	0.415	0.712 (0.314 – 1.610)
Bolus *vs* IC or IC+Bolus	22 *vs* 9	0.739	0.858 (0.347 – 2.116)
DPD IHC <2 *vs* IHC 2+	11 *vs* 10	0.372	0.605 (0.201 – 1.823)
TYMS IHC <2 *vs* IHC 2+	6 *vs* 15	0.450	1.614 (0.465 – 5.596)

*Log-rank (Mantel-Cox) test

6.1.2 Overall survival

The median overall survival in HGTG was 9 months (range 1 month – 9.3 years). No significant difference was noted with respect to gender, age, ECOG, 5-FU mode of administration, DPD or TYMS immunohistochemical expression (p>0.05) (Table 10).

Table 10 – Prognostic significance of clinicopathological variables, DPD and TYMS immunoexpression in OS of patients with high-grade toxicity treated with 5-FU in the palliative setting (**IHC < 2** – absent or low immunoexpression; **IHC 2+** – normal immunoexpression; **CI** – continuous infusion).

Clinicopathological variable	No. of cases	*P* value*	Hazard Ratio (95% CI of ratio)
Female *vs* Male	16 *vs* 15	0.589	0.779 (0.315 – 1.927)
<65 *vs* ≥65 years	24 *vs* 7	0.585	1.326 (0.482 – 3.646)
ECOG 0 *vs* ≥1	16 *vs* 15	0.787	0.881 (0.352 – 2.206)
Bolus *vs* IC or IC+Bolus	22 *vs* 9	0.739	0.858 (0.347 – 2.116)
DPD IHC <2 *vs* IHC 2+	11 *vs* 10	0.121	0.343 (0.089 – 1.328)
TYMS IHC <2 *vs* IHC 2+	6 *vs* 15	0.811	1.194 (0.280 – 5.100)

*Log-rank (Mantel-Cox) test

At 12 months, differences in overall survival categorized for DPD (Figure 15) and TYMS immunoexpression (Figure 16), did not reach statistical significance (p=0.121 and p=0.451, respectively).

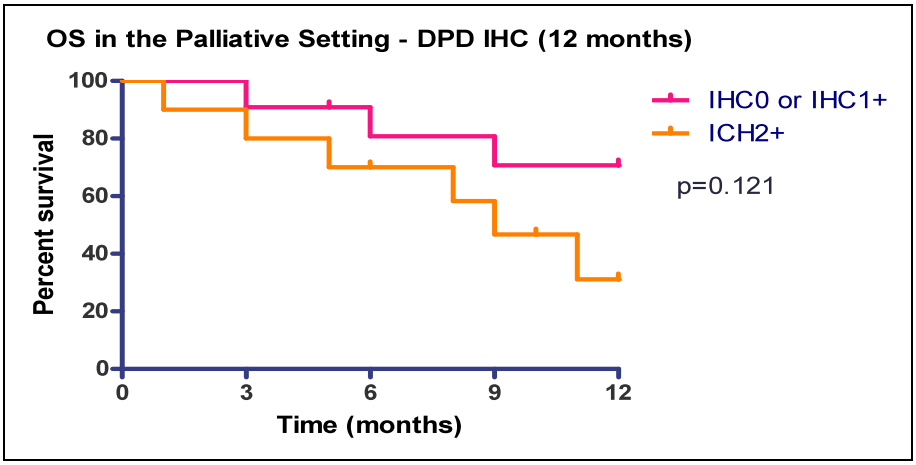

Figure 15 – Overall survival at 12 months in patients with high-grade toxicity treated with 5-FU in the palliative setting and with different DPD immunoexpression levels

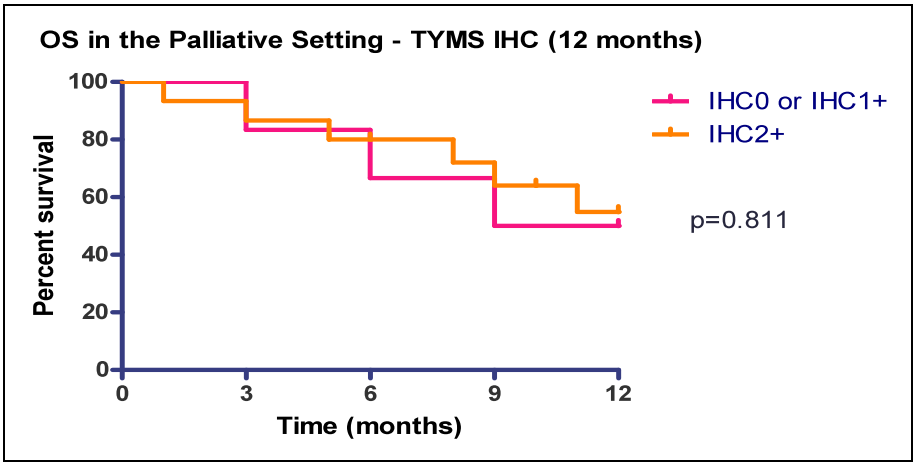

Figure 16 – Overall survival at 12 months in patients with high-grade toxicity treated with 5-FU in the palliative setting and with different TYMS immunoexpression levels

DISCUSSION

5-Fluoracil is broadly used in the treatment of GI cancer. Deficiency of the enzyme dihydropyrimidine dehydrogenase (DPD), encoded by the dihydropyrimidine dehydrogenase gene (*DPYD*) has been associated with the development of severe toxicity to 5-FU in GI cancer patients. Since promoter aberrant methylation has been proposed as an alternative mechanism for DPD deficiency, this study analyzed the methylation status of *DPYD* promoter region by quantitative methylation-specific PCR in GI cancer patients with and without high grade toxicity. Moreover, the analysis of large intragenic rearrangements of *DPYD* was performed and the DPD immunoexpression was assessed in tumor tissue samples. Finally, based on the fact that thymidylate synthase (TYMS) activity also influences 5-FU cytotoxic effects, the TYMS immunoexpression was also evaluated in tumor tissue samples.

Severe toxicity associated with 5-FU has been attributed in a small number of cases to pontual mutations in the *DPYD* gene, which result in lower DPD enzyme activity. However, for most cases of severe 5-FU toxicity no genetic mechanism has been described. Thus, a possible role for epigenetic alteration of *DPYD*, especially promoter methylation, has been hypothesized. The first published study on this issue found that methylation of DPYD promoter in peripheral blood leukocyte DNA from CRC patients was associated with severe 5-FU toxicity[69]. Nevertheless, in our series of 45 patients with GI cancer who developed severe 5-FU toxicity, methylation at the *DPYD* promoter was not found, neither in peripheral blood leucocytes (45 samples) nor in tumor tissue (30 samples). Importantly, a well characterized colon cancer cell line (RKO), known to harbor extensive CpG methylation at the *DPYD* promoter was used as a positive control, thus validating the methodology used in our study. Indeed, while this study was being performed, two independent studies were published stating that no evidence of *DPYD* promoter methylation was found in peripheral blood leucocytes of 28 patients[51] and 17 patients[79] with high-grade toxicity following 5-FU administration. It is noteworthy that the first cited study examined only 15 patients, where the two latter studies and our own comprise a total of 90 patients. Not only our study is the largest single series of patients among these, but it was the first to use a quantitative methylation-specific PCR approach, which is more specific and sensitive than conventional MSP. Thus, our data sustain that *DPYD* promoter methylation is not predictive of sever toxicity in patients treated with 5-FU for GI cancer.

All patients enrolled in this study were screened for the skipping mutation in exon 14 (IVS14+1G>A) of *DPYD* as part of previously published research article[66]. This mutation was found in three patients and all of them developed severe toxicity following 5-FU administration, thus confirming the high specificity and positive predictive value of this genetic analysis, in line with previous observations[51, 63, 65, 66]. However, the low sensitivity (7%) and negative predictive value (33%) of this analysis indicate that the genetic screening of *DPYD* mutations in GI cancer patients before the administration of 5-FU is not effective in preventing serious 5-FU-related toxicity as most of at risk patients will not be identified.

Thus, considering the results obtained for *DPYD* promoter methylation and skipping mutation in exon 14 (IVS14+1G>A) analysis, we decided to determine whether large *DPYD* intragenic rearrangements might explain 5-FU toxicity. However, those large intragenic rearrangements were also not found in any of the 45 cases nor in any of the 21 controls, thus excluding a role for this genetic alteration in the impairment of DPD activity. This result is in agreement with a recently published study in which no large rearrangements were found in series of 68 patients experiencing severe 5-FU toxicity[80].

To test whether tumor cell expression levels of DPD or TYMS might justify the development of severe 5-FU toxicity, representative tumor tissue samples, available from 30 cases and 16 controls, were analyzed for DPD and TYMS immunoexpression. Although immunoexpression might not be a direct representative of enzyme activity, differences in immunoexpression levels might translate into differential tissue ability to metabolize 5-FU. In some studies, lower TYMS immunoexpression levels in tumor tissue were statistically associated with higher toxicity, a finding which might be explained by a higher cellular sensitivity to 5-FU[54, 58]. In the examined series, no differences in DPD or TYMS immunoexpression were disclosed between high-grade toxicity patients and controls, as in a preceding study[81]. Although the referenced study included more than 300 patients, only colorectal tumors were analyzed, whereas 50% of the cases with lower TYMS immunoexpression in our series were gastric and esophageal carcinomas. Thus, modulation of the impact of DPD and TYMS expression in 5-FU toxicity might depend on the primary tumor site. However, because our series is small no definite conclusion can be drawn.

Contrarily to previously published studies[51, 82], no association between severe 5-FU

toxicity and female gender was apparent in our series. The same holds true for age, higher stage disease and treatment intention (adjuvant or palliative). On the other hand, toxicity was more frequent in patients with worse ECOG, primary gastric or esophageal location and 5-FU/cisplatinum regimen and these associations have not been previously reported[51]. The reasons for these discrepancies may lie on (1) different size and characteristics of the studied populations, (2) tumor location as our study included gastric and esophageal tumors, and (3) interaction between the clinical parameters, causing a confounding effect on the statistical analyses. Interestingly, the well-known association between administration of 5-FU in bolus and severe toxicity was also observed in our series. However, 5-FU in bolus was more frequently administered in patients receiving the 5-FU/cisplatinum regimen, which was exclusively prescribed for gastric and esophageal cancer patients. Thus, it is difficult to ascertain whether the risk for severe toxicity depended solely on the mode of administration or if tumor location and/or associated cisplatinum played also a significant role.

Finally, no significant effect of tumor DPD or TYMS immunoexpression was apparent in patient outcome, in our series. Similar results have been reported by Jensen and co-workers although in their series of CRC patients, high DPD immunoexpression was associated, mainly in stage III disease, with increased risk of death both in univariate and multivariate analysis[81]. It is noteworthy that Jensen et al. series comprised a total of 303 patients after complete resection of stage II-III colo-rectal cancer, whereas our series is significantly smaller and heterogenous concerning the primary tumor site and stage. Thus, ours and their results are not directly comparable and additional studies are required to verify the impact of tumor DPD expression in GI cancer patients outcome.

CONCLUSIONS

The main conclusions of this study are:

1. *DPYD* promoter methylation detection in peripheral blood leucocytes is not predictive of severe 5-FU toxicity in GI cancer patients. Because no *DPYD* promoter methylation was found in cancer tissues, this epigenetic mechanism is not likely to regulate DPD expression.
2. Large *DPYD* intragenic rearrangements have not been observed and are also not predictive of severe 5-FU toxicity in GI cancer.
3. DPD and TYMS and immunoexpression intensity in tumor tissue are not associated with the development of severe 5-FU toxicity.

Considering our own and published data, known genetic and epigenetic factors regulating *DPYD* and DPD expression seem to play a very limited role in the development of severe 5-FU toxicity in GI cancer patients.

Although severe 5-FU toxicity is significant clinical concern, additional studies integrating a more comprehensive analysis of 5-FU metabolic pathway are required to uncover the factors underlying the majority of severe 5-FU toxicity cases.

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