Abstract. – Background: Many clinicians acknowledge the importance of genetics in drug response and are favourable about using genetic tests to guide therapy. The 5-Fluorouracil (5-FU) is the backbone of different regimens for the treatment of several solid tumours. Unlikely, some patients develop gastrointestinal and hematologic toxicities when treated by the 5-FU, leading to the suspension of therapy. Current evidences of pharmacogenomics, have reported different polymorphisms associated to genes involved with fluoropyrimidine biotransformation. A multitude of methods has been applied to assess the mutation-status of these genes, without defining a golden standard for the daily diagnostic routine, so far.

Aims: Some adverse drug response due to the administration of 5-FU can be predicted through pharmacogenomics testing tools. This report reviews the recent findings on the polymorphism for genes that are involved in the biotransformation of the drug and its association with fluoropyrimidine biotransformation. A multitude of methods has been applied to assess the mutation-status of these genes, without defining a golden standard for the daily diagnostic routine, so far.

Introduction

For decades, 5-fluorouracil (5-FU) was the sole active agent in the treatment of several solid tumours including gastrointestinal malignances1. This has changed markedly since the year 2000, with the approval of irinotecan, oxaliplatin, and several humanized monoclonal antibodies (MoAbs) that target growth factor receptors as the epidermal growth factor receptor (EGFR).

The best way to combine and sequence these agents is still undergoing validation. Despite its clinical benefit, 5-FU and its pro-drugs (capecitabine and tegafur-Uracile) are associated with frequent gastrointestinal and hematologic toxicities2, which often lead to treatment discontinuation. It is known that, fluoropyrimidine drugs undergo complex metabolic biotransformation. The metabolic fate of Fluoropyrimidine starts by conversion of 5-FU into active metabolite, 5-fluoro-2-deoxyuridine monophosphate (FdUMP) that leads to inhibition of thymidylate synthase (TYMS) and 5-fluoro-uridine monophosphate (FUMP). Both pathways cause inhibition of DNA “de novo” synthetized. 5-fluorouracil is converted to FdUMP and FUMP through two pathways (Figure 1): (1) Oratate phosphoribosyltransferase (OPRT); and (2) Thymidine phosphorylase (TP). The vast majority (about 80%) of administered 5-FU is metabolized by the enzyme dihydropyrimidine dehydrogenase (DPYD) into the inactive form, dihydro-fluorouracil (FUH2), and excreted as a fluoro-β-alanine (FBAL).

Gene products involved in this biotransformation and related to tolerance and response to 5-FU-based chemotherapy have been well documented3,4. They include several enzymes carrying
well-known mutations, as DPYD and TYMS. Additional genetic variation in 5-FU-metabolizing genes includes enzyme which adds the thiol group for elimination glutathione S-transferase (GSTP1) and those drug targets such as methylene-tetrahydrofolate reductase (MTHFR). However, if the detection of these genetic variants on DPYD and TYMS genes is routinely incorporated either into clinical practice or large clinical trials, knowledge concerning the predictive value of pharmacogenomics and pharmacogenetics (PGx) which will eventually enable the individualization of optimized therapy could be gained. However, we still need a precise demonstration that PGx tests offer an added value, in terms of relative cost and benefit.

Furthermore, trials evaluating the pharmacoeconomic impact of genotyping testing in fluoropyrimidine based-therapy will likely provide answers for policy making in the incorporation of PGx testing into clinical practice. The primary aim of a cost-effectiveness analysis is to provide sufficient-ly robust information for decision-makers to allocate resources to healthcare interventions. Overviews of cost-effectiveness studies on PGx technologies are now available. A relevant example is the National Institute for Health and Clinical Excellence (NICE). NICE forms a Diagnostic Advisory Committee, which is willing to stimulate Pharma and Academic communities to produce a robust set of data, including the design and data source in economic models of healthcare.

We review here the clinical use of fluoropyrimidines and the methods for detecting genetic variations related to therapy. In addition, an overview of the cost of genotyping specific germline polymorphisms in drug-metabolizing gene (DPYD), and the primary drug-target (TYMS) associated with 5-FU treatment, were evaluated. Variation in the activity of these enzymes illustrates the proof of principle of PGx in the design of appropriate therapeutic interventions. The goal of this review is to provide information for the oncologist on the advantage and

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**Figure 1.** Schematic metabolic fate of 5-FU. Key candidate genes involved in metabolic activation and drug target of 5-FU are shown in squared box. Drug-detoxification enzymes, are shown by trapezoid box. *Current available pharmacogenomic tests. Abbreviations: Carboxil exterase (CES); Cytidine Deaminase (CDA); Oratate Phosphoribosyltransferase (OPRT); 5-fluoro-uridine monophosphate (FUMP); 5-fluoro-uridine diphosphate (FUDP); 5-fluoro-uridine monophosphate (FUTP); Ribonucleotide reductase (RNR); 5-fluoro-2-deoxuryridine monophosphate (FdUMP); Thymidylate Synthase (TYMS); Thymidine Phosphorylase (TP); Dihydropyrimidinase Dehydrogenase (DPYD); Dihydropyrimidinase (DHP); dihydrofluorouracil (FUH2); fluoro-β-alanine (FBAL); Glutathione S-Transferase (GSTP1); Methylene-tetrahydrofolate Reductase (MTHFR); Deoxuryridine monophosphate (dUMP); Deoxythymidine monophosphate (dTMP); Deoxythymidine Diphosphate (dTDP); Deoxythymidine Triphosphate (dTTP).
limitations, in terms of suitability, of the most common available methods for molecular detection either of the acquired genetic variations or inherited synthase gene polymorphisms (SNPs) of DYPD, TYMS and other additional candidate genes. Moreover, we think that this way could have a key role for the treatment choice in so-called frail patients (i.e. elderly and HIV-positive patients) for whom the efficacy and especially the toxicity profile are important aspects. However, one should keep in consideration that it will not be feasible to conduct randomized trials on each and every diagnostic test, and the economic value of such tests can be modelled using decision analysis techniques.

Dosing and Toxicity of Fluoropyrimidines

The toxicity profile differs between bolus and infusional 5-FU. Bolus 5-FU mono-therapy has limited activity; only the 10% of patients achieve an objective response. Higher response rates can be achieved with infusional regimens, but the survival impact is minimal. While rates of gastro-intestinal (GI) toxicity are similar, grade 3-4 neutropenia is more common with bolus 5-FU (31% bolus vs. 4% infusional), as is hand-foot syndrome (34% vs. 13%, respectively). Compared to bolus 5-FU alone, FU plus Leucovorin (LV) is associated with a twofold higher response rate (RR) (21% vs. 11%)12. LV enhances 5-FU cytotoxicity by prolonging inhibition of the TYMS enzyme forming a stable complex.

Several dose and schedules of 5-FU and LV are currently used in clinical practice (Table I): bolus regimens and infusional regimens (short-term and chrono-modulated). At least three trials have directly compared both approaches13. The monthly schedule causes more neutropenia and stomatitis14, while the weekly schedule causes more diarrhoea (Mayo-clinic regimen). Furthermore, five-day bolus regimen appears to be more toxic in women than in men. Given the lower rates of neutropenia and stomatitis, and the ability to stop or to modify therapy if toxicity occurs, the weekly RPMI regimen is generally preferred.

The equivalence of low-dose (i.e., 20 mg/m² per dose) versus high-dose (i.e., 500 mg/m² per dose) LV for the weekly schedule has not yet been firmly established, although at least one trial suggests similar outcomes and a more favourable toxicity profile and low cost issues for the lower dose regimen15.

Response rates with 5-FU/LV have been further improved by the use of short-term infusional schedules of 5-FU. This was demonstrated in a trial of 448 patients who were randomly assigned to a monthly regimen of LV (20 mg/m²) plus bolus 5-FU (425 mg/m²) on days 1 to 5 every four weeks or a bimonthly regimen, the de Gramont regimen16, of LV (200 mg/m² over two hours) followed by bolus 5-FU (400 mg/m²) and a 22-hour infusion of 5-FU (600 mg/m²)17, both drugs are given daily for two consecutive days, every two weeks18. The infusional regimen was associated with a significantly better response rate (33 vs. 14%) and a trend toward longer median survival (62 vs. 57 weeks, p = 0.067). Infusional therapy also caused less hematologic and GI toxicity. For these reasons, the majority of treatments incorporating 5-FU into irinotecan- and oxaliplatin-based regimens now use short-term infusional schedules. Until the development of combination regimes of LV-modulated 5-FU with either irinotecan or oxaliplatin, 5-FU+LV was considered the standard first-line therapy for metastatic colorectal cancer (mCRC), and it is still used in patients who cannot tolerate these triple drug regimens19,20. If it is to be used alone, because of the more favourable toxicity profile, should be recommended short duration infusional 5-FU+LV (i.e., the de Gramont regimen)18 rather than the Mayo regimen of treatment for five consecutive days, once per month.

Chrono-modulated administration schedules of 5-FU, with or without LV, generally improve response rates and lessen toxicity as compared to non chrono-modulated schedules21. Chrono-modulation is a method where drug administration varies over a 24-hour period. It is based upon the following principles: i) drug absorption, transport, metabolism and/or elimination usually show diurnal changes; ii) most cellular detoxification rhythms appear to be coupled to the rest-activity cycle; iii) these variations in target cell exposure to drugs and the diurnal rhythms that modulate cellular detoxification functions may impact on the pharmacology of administered 5-FU. However, the true value of this field, still remains an experimental approach. Schedules using orally active 5-FU analogs as capecitabine (Xeloda®, Roche pharmaceuticals, Monza (MB), Italy) and tegafur plus uracil (UFT®, Bristol-Myers Squibb, Italy), provide RRs comparable to intravenous 5-FU+LV. Oral fluoropyrimidines are a more convenient (but not
## Table I. Most common regimens including fluoropyrimidines.

<table>
<thead>
<tr>
<th>Regimens (references)</th>
<th>Schedule*</th>
<th>Timely</th>
<th>Application</th>
</tr>
</thead>
</table>
| Mayo Clinic           | LV 20 mg/m²/die iv day 1-5  
5-FU 425 mg/m²/die iv day 1-5 | q 4-5 wks | Metastatic colon cancer carcinomas |
| Poon et al¹⁴           |           |        |             |
| RPMI                  | LV 500 mg/m² over 2 h iv day 1-5  
5-FU 500 mg/m²/die iv by bolus day 1-5 | q 1 wks | Metastatic colon cancer carcinomas |
| Hotta et al¹⁵          |           |        |             |
| Machover              | LV 200 mg/m²/die iv bolus day 1-5  
5-FU 340-400 mg/m²/die iv for 15' day 1-5 | q 3 wks | Metastatic colon cancer carcinomas |
| Machover et al¹⁷       |           |        |             |
| De Gramont            | LV 200 mg/m²/die iv for 2 h day 1, 2  
5-FU 400 mg/m²/die iv bolus day 1, 2  
5-FU 600 mg/m²/die iv bolus for 46 h | q 2 wks | Metastatic colon cancer carcinomas |
| De Gramont et al¹⁶     |           |        |             |
| FOLFOX 4              | OxaIplatin 85 mg/m² iv for 2 h day 1  
LV 200 mg/m² iv bolus day 1, 2  
5-FU 600 mg/m² iv cont. for 46 h | q 2 wks | Metastatic colon cancer carcinomas |
| Maindrault-Goebel et al²² | | | |
| FOLFOX 6              | OxaIplatin 100 mg/m² iv for 2 h day 1  
LV 400 mg/m² iv bolus day 1  
5-FU 2400-3000 mg/m² iv bolus for 46-48 h | q 2 wks | Metastatic colon cancer carcinomas |
| Hebbar et al²³         |           |        |             |
| FOLFIRI               | Irinotecan 180 mg/m² iv for 90’ day 1  
LV 200 mg/m² iv bolus day 1, 2  
5-FU 600 mg/m² iv cont. Infus for 46 h | q 2 wks | Metastatic colon cancer carcinomas |
| Douillard et al¹⁹      |           |        |             |
| FOLFOXIRI             | Irinotecan 165 mg/m² iv for 90’ day 1  
OxaIplatin 85 mg/m² iv for 2 h day 1  
LV 200 mg/m² iv bolus day 1  
Fluorouracile 3200 mg/m² iv-ci in 48 h | q 2 wks | Metastatic colon cancer carcinomas |
| Masi et al²⁰          |           |        |             |
| XELOX                 | OxaIplatin 130 mg/m² iv over 2 h day 1  
Capcitabine 1000 mg/m² die per os day 1-14 | q 3 wks | Metastatic colon cancer carcinomas |
| Cassidy et al²⁹       |           |        |             |
| XELIRI                | Irinotecan 250 mg/m² iv for 90’ day 1  
Capcitabine 1000 mg/m² per os day 1-14 | q 3 wks | Metastatic colon cancer carcinomas |
| Kim et al²¹           |           |        |             |
| XELOX/Bevacizumab     | Capcitabine 850 mg/m² per os day 1-14  
OxaIplatin 130 mg/m² iv for day 1  
Bevacizumab 7.5 mg 7 kg day 1 | q 3 wks | Metastatic colon cancer carcinomas |
| Van Cutsem et al²⁰    |           |        |             |
| XELIRI/Bevacizumab    | Capcitabine 1000 mg/m² per os day 1-14  
Irinotecan 200 mg/m² iv day 1  
Bevacizumab 7.5 mg/kg day 1 for 90’ | q 3 wks | Metastatic colon cancer carcinomas |
| Van Cutsem et al²⁰    |           |        |             |
| FOLFIRI/Cetuximab     | Irinotecan 180 mg/m² iv for 90’ day 1  
LV 200 mg/m² iv for 2 h day 1, 2  
5-FU 400 mg/m² iv bolus day 1, 2  
5-FU 2400 mg/m² iv-ci over 46 h  
Cetuximab 250 mg/m² iv day 1 for 90’ | q 2 wks | Metastatic colon cancer carcinomas |

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*Table continued*
necessarily less toxic) alternative to LV-modulated 5-FU in clinical settings where fluoropyrimidines alone are indicated24.

Capecitabine is a fluoropyrimidine carbamate with the intestinal absorption and then converted to 5-FU by carboxyl-esterase (CES) and cytosine deaminase (CDA) enzymes (Figure 1). The final requisite enzyme, thymidine phosphorylase (TP), is present at consistently higher levels in tumour compared to normal tissue, thereby providing the basis for enhanced selectivity for tumour cells and better tolerability25.

Two identically designed randomized trials (602 and 605 patients, respectively) have shown similar efficacy for mono-therapy capecitabine (1250 mg/m² twice daily for 14 of every 21 days) compared to intravenous 5-FU+LV as first-line treatment of mCRC26. The prevalence of grade 3 or 4 diarrhoea, stomatitis, nausea, and neutropenic sepsis were significantly lower in the capecitabine group; only hyper-bilirubinemia and hand-foot syndrome were more frequent. In contrast to these data, objective response rates with second-line mono-therapy capecitabine are quite low in patients with 5-FU+LV-refractory disease. Capecitabine alone is an inappropriate treatment strategy for patients who have failed 5-FU-based regimens. The approved dose is 1250 mg/m² twice daily for 14 days, every 3 weeks. Some Authors have suggested that lower doses (beginning at 1000 mg/m² twice daily for 14 days, every 3 weeks) improve the therapeutic index27. However, randomized trials have not established the comparative efficacy of these lower capecitabine doses. Some data suggest that the frequency and severity of capecitabine-associated toxicity are substantially higher when it is given after 5-FU plus LV28. The mechanism underlying this sequence-specific exacerbation of toxicity is unclear. The potential for excess toxicity is a consideration for patients who are being considered for crossover from 5-FU+LV to capecitabine. However, these information are based on a relatively small number of patients and need confirmation29. Randomized trials have not yet established the comparative efficacy of the addition of bevacizumab30.

An international phase III trial compared efficacy and tolerability of capecitabine/docetaxel therapy with single-agent docetaxel in anthracycline-pre-treated patients with metastatic breast cancer (mBC). Capecitabine/docetaxel resulted
in significantly superior efficacy in time to disease progression ($p = 0.0001$; median, 6.1 vs. 4.2 months), overall survival ($p = 0.0126$; median, 14.5 vs. 11.5 months), compared with docetaxel. The results achieved with the addition of capecitabine to docetaxel (75 mg/m²), and the manageable toxicity profile, indicate that this combination provides clear benefits over single-agent docetaxel (100 mg/m²)³²; same results were obtained in REAL II/III trial³³.

Another study was designed to compare ixabepilone plus capecitabine versus capecitabine alone in 752 patients, anthracycline-pre-treated or -resistant and taxane-resistant with locally advanced or metastatic breast cancer. The results displayed a prolonged progression-free survival relative to capecitabine (median, 5.8 v 4.2 months), with a 25% reduction in the estimated risk of disease progression ($p = 0.0003$)³⁴.

Tegafur-Uracil is a 4:1 molar combination of fторafur and uracil, which competitively inhibits the degradation of 5-FU, resulting in sustained plasma and intra-tumoural concentrations. Response rates are approximately 25% with UFT mono-therapy, and 40% in combination with oral LV (150 mg daily)³¹. In phase III studies, UFT+LV has similar efficacy and better tolerability compared to bolus 5-FU³¹. The dose limiting toxicity is diarrhoea, while myelosuppression and hand-foot syndrome are infrequent.

Raltitrexed, a folate analog, is a pure TYMS inhibitor. In at least one randomized trial assigning 905 patients with mCRC to either raltitrexed, infusional 5-FU, or bolus plus short-term infusional 5-FU/LV (the de Gramont regimen), Raltitrexed was associated with the greatest toxicity and worst health-related quality of life¹². Raltitrexed, may be a useful substitute for 5-FU in patients with DPYD deficiency (which markedly increases 5-FU toxicity), or possibly as a component of second-line therapy in patients failing irinotecan or oxaliplatin.

An investigational protocol of 43 patients with adenocarcinomas in the pancreatic head, consisting of external-beam irradiation, continuous infusion of low dose 5-FU (200 mg/m² daily), weekly intravenous bolus cisplatin (30 mg/m² daily), and subcutaneous interferon-alpha (3 × 10⁶ units, days 1 to 35) was associated with an improvement in overall survival³⁵.

An Italian study protocol combining Fluoropyrimidine with highly active antiretroviral therapy (HAART) demonstrated that Xelox regimen with concomitant HARRT was feasible and that HIV infection was not a limiting factor for its use. Moreover, the concomitant use of HAART did not seem to increase the toxicity of this regimen, although it is too early to evaluate the Xelox activity in these particular setting of patients¹⁰.

### Genetic Variation in 5-FU Target/Metabolizing Enzyme Genes

Applied researches based on candidate gene approaches screening have demonstrated the associations between Fluoropyrimidine treatment outcomes and polymorphisms in $DPYD$, $TYMS$, and several additional candidate genes (Table II).

#### $DPYD$

The human $DPYD$ gene consists of 23 exons, and includes 3 kb in length of coding sequences³⁶. To date, more than 30 SNPs and deletion mutations have been identified within $DPYD$ gene, although the majority of these variants have no functional consequences on enzymatic activity³⁷.

Expression of DPYD enzyme has been related to tolerance and response to 5-FU-based chemotherapy. Specifically, low expression of DPYD has been associated with accumulation of 5-FU, thereby exposing patients to increased risk of severe or lethal toxicities, while high expression of DPYD has been associated with poor response to 5-FU. The frequency of low DPD enzymatic activity has also been shown to vary significantly among different ethnic subpopulations³⁸. A prospective study conducted by Schwab et al, evaluated all these potential genetic predictors of 5-FU treatment-related toxicity¹. The most known $DPYD$ SNPs associated with grade 3 and 4 toxicities are intronic variant IVS14 + 1 G > A (also named $DPYD*2A$), and mutation A1627G ³⁷. Several clinical assays have been developed assessing $DPYD$ enzyme activity, mRNA expression, and metabolite formation, as well as SNPs within $DPYD$³⁸: as important results previously demonstrated that a homozygote $DPYD*2A$ genotype results in complete deficiency (high-risk patients) while the heterozygous $DPYD*2A$ genotype results in partial deficiency of DPYD enzyme³⁹. Several genotyping methods to screen the known $DPYD$ gene germline mutations have been developed, without defining better platforms for their use in the daily diagnostic routine. Current methodolo-
Pharmacogenomics tests for managing patients who receive 5-FU-based therapy

Table II. Pharmacologic and clinical characteristics of triptans in comparison with 100 mg of sumatriptan, from 34*

<table>
<thead>
<tr>
<th>Genetic variants</th>
<th>Activities</th>
<th>Annotation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPYD</td>
<td>Mucosites</td>
<td>Heterozygosity for the A allele no A/A homozygotes were observed</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Leukopenia</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A1627G</td>
<td>Severe nausea vomiting</td>
<td>The elimination constant (Ke) for 5-FU was significantly lower in patients homozygous for the G allele</td>
<td>69</td>
</tr>
<tr>
<td>TYMS</td>
<td>Neutropenia grade 3-4</td>
<td>Allele with the triple tandem repeat (3R) has increased TYMS expression compared with those with the double repeat (2R). Low TYMS levels are postulated to be markers of more favourable therapeutic response in advanced colorectal cancer</td>
<td>43</td>
</tr>
<tr>
<td>1494deletion</td>
<td></td>
<td>This deletion alters TYMS mRNA stability.</td>
<td>49</td>
</tr>
<tr>
<td>(TAAAG)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Additional candidate genes*

<table>
<thead>
<tr>
<th>Genes</th>
<th>Activities</th>
<th>Annotation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTHFR</td>
<td></td>
<td>Responder were more likely to carry the T allele than non-responders.</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Was significantly linked to specific survival, with homozygous mutated patients having the worst prognosis.</td>
<td></td>
</tr>
<tr>
<td>GSTP1</td>
<td>Neurotoxicity, neutropenia</td>
<td>Elderly rectal cancer patients homozygous for the G (Val) allele of this SNP were less likely than those homozygous for the A (Ile) allele</td>
<td>56</td>
</tr>
<tr>
<td>CDA</td>
<td>Grade 3 or higher neutropenia</td>
<td>Double variants (Lys27Gln and, Ala70Thr) in combination to gemcitabine</td>
<td>60</td>
</tr>
<tr>
<td>GSTP1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ORPT</td>
<td>Grade 3 to 4 neutropenia and diarrhea</td>
<td>The Ala/Ala allele homozygous of this SNP is associated in patients with colorectal neoplasms</td>
<td>61</td>
</tr>
<tr>
<td>NOS 3</td>
<td>Increased risk of recurrence</td>
<td>Study on 1153 women with breast cancer receiving 5-FU in combination with cyclophosphamide and methotrexate or doxorubicin who had −786 CC genotypes,</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TP53</td>
<td></td>
<td>Low value of overall survival</td>
<td>Asian gastric cancer patients carrying Pro/Pro genotype had shorter survival following 5-FU based treatments.</td>
</tr>
<tr>
<td>ERCC2</td>
<td>Increased risk of early relapse</td>
<td>Asian colorectal cancer patients carrying the 2251AC or 2251CC genotypes had significantly increased risk of relapse</td>
<td>64</td>
</tr>
</tbody>
</table>

TYMS

*TYMS* is the primary target of 5-FU. Two major polymorphisms have been reported to be associated with altered *TYMS* expression and clinical response to fluoropyrimidine-based therapy 43,44: (1) polymorphic 28bp tandem repeat polymorphism in the 5’-untranslated region (5’UTR) into *TYMS* sequence enhancer region (TSER) and; (2) *TYMS* 1494del, is a 6-base pair (bp) deletion polymorphism in the 3’-UTR.

Data from both genetic variations suggest increasing *TYMS* mRNA and protein expression 45.

Several studies have identified links between TSER genotype (predominantly TSER*2 and *3) and response to chemotherapy 46. Patients who carry homozygous TSER*2 (carrying double 28bp tandem repeat), are defined at high-risk for toxicity from 5-FU 47.

In addition, polymorphism of *TYMS* 1494del, suggests that this deletion alters *TYMS* mRNA stability 48. A study of *TYMS* mRNA expression...
from tumour cells, in 43 colorectal cancer patients suggested that patients homozygous for the 6 bp deletion express around 3-fold less TYMS mRNA than patients homozygous for the presence of the 6 bp.

In both case, extensive studies on the standardization method for detection of these genetic variations are lacking.

Additional Candidate Genes Related to Fluoropyridines Toxicity and Response

**MTHFR**

MTHFR is a key enzyme forming the reduced folate cofactor essential for TYMS inhibition by 5-FU. Two SNPs, 677C > T and 1298A > C, have been shown to alter enzyme activity and possibly 5-FU sensitivity. There is a large group of work on MTHFR 677C > T variant, in association with a variety of drugs, phenotypes and diseases, and much of it is contradictory. In general, a study of 43 patients with mCRC treated with fluoropyrimidine-based chemotherapy, responders were more likely to carry the T allele than non-responders.

MTHFR 1298A > C has been shown in treated colorectal cancer patients. In addition, results showed 1298A > C polymorphism was significantly linked to specific survival, with homozygous mutated patients having the worst prognosis.

Several methods have been developed to detect common MTHFR mutations, including the older restriction fragments length polymorphism (RFLP) and SSCP. Sequencing method was used as the reference standard method, but it had a limited reliability for assessing allelic discrimination. Fluorescent resonance energy transfer (FRET) probe-based assay is easy to perform and displayed higher resolution than RFLP. Additionally, FRET probe assay is faster and less tedious than other platforms because several commercial kits are now available.

**GSTP1**

Polymorphism GSTP1 Ile105Val (313A > G in exon 5, sometimes labelled GSTP1*B) has been associated with reduced enzyme activity and anticancer drug resistance and toxicity. The allele frequency of the Ile105Val polymorphism varies widely among populations. However, in 166 colorectal cancer patients receiving oxaliplatin and 5-FU, the GSTP1 I105V allele was associated with increased risk of neutropenia and neutrotoxicity. This SNP in position 313 of GSTP1 gene could be detected by allelic discrimination methods as germline mutation. While, mutation detection in tumour cells, needs more sensitivity assay able to enhance mutant allele in a large excess of wild-type alleles.

Cytidine deaminase (CDA): two important polymorphism Lys27Gln and Ala70Thr. Patients receiving 5-FU in combination with gemcitabine with phenotype-associated allele CDA*3 and CDA*2 haplotype alleles (Lys27Gln and, Ala70Thr) were associated with increased frequency of grade 3 or higher neutropenia, (p = 0.0017, n = 177).

Orotate phospho-ribosyl transferase (OPRT): Gly213Ala. The Ala/Ala allele of this SNP is associated with grade 3 to 4 neutropenia and diarrhoea and is an independent predictor of diarrhoea in patients with colorectal neoplasms.

Nitric oxide synthase 3 (NOS3): mutation in non-coding region –786T > C. Study population on 1153 women with breast cancer receiving adjuvant therapy with 5-FU in combination with cyclophosphamide and methotrexate or cyclophosphamide and doxorubicin who had NOS3 –786 CC, had an increased risk of recurrence compared with those with common TT genotypes alleles (p = 0.008).

TP53: Arg72Pro. Asian gastric cancer patients carrying Pro/Pro genotype had shorter survival following 5-FU based treatments (n=110; relapse-free survival = 3.049).

ERCC2 transcription-coupled nucleotide excision repair: 2251A > C. Asian colorectal cancer patients carrying the ERCC2 2251AC or 2251CC (Lys751Gln) genotypes had significantly increased risk of early relapse following treatments including 5-FU and LV (n=201).

**Genotyping of 5-FU Pharmacogenomics Variants**

Generally, genotyping is performed either by custom service laboratories or academic referenced laboratories, as well as by using commercial kits (when available). In the USA, diagnostics products are regulated by the Food and Drug Administration (FDA), whereas diagnostic services are under the rules of the Clinical Laboratory Improvement Act (CLIA). In Europe this...
field is covered by in vitro Diagnostic (IVD) directive, without a distinction between commercial products (used by laboratories) and diagnostics service. For information on assay technical performance, the grey literature was searched, in particular websites of commercial laboratories offering DPYD and/or TYMS genotyping. In both circumstance, a voluntary list of international laboratories (with CLIA certification in the US and CE mark in Europe) that perform genetic tests can be found on a National Institute of Health-funded website named GeneTests™ [http://www.ncbi.nlm.nih.gov/sites/GeneTests?db=GeneTests], although only a small minority of genetic tests listed on this site are PGx tests. At the moment of writing, to our knowledge, there are no available tests approved by the FDA or the CE for genotyping detection of all variants listed in Table I. Clinical laboratories may develop and validate tests in-house (“home-brew”) and perform them as a laboratory service; which may further reduce the cost of analysis).

A panel of diagnostic kits being actively manufactured and marketed for distribution have been developed to detect genomic profile of the patients receiving 5-FU (Table III). Currently, of note there are no assay kits approved by the FDA or marked IVD for genetic testing for DPYD or TYMS genotypes. These commercial tests were searched (via google) using the following two strategies: (1) “Fluorouracil” [MeSH] AND “Thymidylate Synthase” [MeSH] OR “dihydropyrimidine dehydrogenase” [MeSH], limited to, diagnostics test, and the English language; (2) “Fluorouracil” [MeSH] AND “thymidylate synthase” [MeSH] OR “dihydropyrimidine dehydrogenase” [MeSH], limited to, gene AND toxicity, limited to human subjects and the English language. In addition, bibliographies from recent review articles and clinical studies were hand-searched for relevant studies, excluding letters and editorials.

**Discussion**

Genetic variants and predictive markers allow physicians to improve the efficacy of cancer therapy. The clinical utility of described polymor-

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**Table III.** Widely used commercial kits for genotyping of most common known genetic abnormalities in the Fluoropyridines pharmacotherapy.

<table>
<thead>
<tr>
<th>GENE (Polymorphisms nucleotides variant)</th>
<th>Commercially available kit (Vendor)</th>
<th>Method-Based test</th>
<th>Website</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPYD (IVS14 + 1G &gt; A)</td>
<td>SALSA* (MRC-Holland)</td>
<td>Multiplex</td>
<td><a href="http://www.mlpa.com/WebForms/WebFormProductDetails.aspx?Tag=t2fAPLApupKyMjaDFVt9bnu5qklhe/LgqIk8">www.mlpa.com/WebForms/WebFormProductDetails.aspx?Tag=t2fAPLApupKyMjaDFVt9bnu5qklhe/LgqIk8</a></td>
</tr>
<tr>
<td></td>
<td>MLPA (RUO)</td>
<td>Ligation-dependent Probe</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(MRC-Holland)</td>
<td>Amplification</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DPYD*2A (EntroGen)</td>
<td>PCR+gel</td>
<td><a href="http://www.entrogen.com/web/dpyd-genotyping-reagents-fluorouracil-toxicity.php">www.entrogen.com/web/dpyd-genotyping-reagents-fluorouracil-toxicity.php</a></td>
</tr>
<tr>
<td></td>
<td>Genotyping kit Fluorouracil</td>
<td>electrophoresis</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Toxicity RUO CE (EntroGen)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AmpliDPYD<em>2 RUO</em>2 CE (Diachem-srl)</td>
<td>PCR+ sequencing</td>
<td><a href="http://www.diachem-srl.it/home.php">www.diachem-srl.it/home.php</a></td>
</tr>
<tr>
<td>TYMS (28 tandem repeats)</td>
<td>AmpliT S 2R RUO CE (Diachem-srl)</td>
<td>PCR+ sequencing</td>
<td><a href="http://www.diachem-srl.it/home.php">www.diachem-srl.it/home.php</a></td>
</tr>
</tbody>
</table>

1We systematically searched the English literature using Google. Combination of Bio-Medical Subject Headings terms (5-FU genetic variants, 5-FU pharmacogenomics) combined with manufacturing tests, DPYD and TYMS were used. Each vendor’s website was screened by visualizing the home page and list of product for sale; 2No FDA or CE (Conformité Européenne) marked; 3RUO: Research Use Only.
phisms involved in fluoropyrimidines based-therapy is in part limited by: (1) less wide diffusion of genotyping methods in routine clinical diagnostics; (2) the evidence that PGx testing improves clinical outcomes is still an open question; and (3) the cost-effectiveness of the testing being unknown.

The technology platform needed for DPYD and TYMS traits are different; it does depend from the type of mutation: acquired genetic change or the analysis of inherited SNPs. This is due to the heterogeneity of the sample sources. Either the tumour itself or the sample may contain a large excess of wild type (wt) DNA, so highly specific and sensible techniques are required that can detect mutant tumour genomes in a background of normal DNA. Whereas for inherited SNPs there is a copious of suitable methods for genotyping able to detect mutant allele either in heterozygosis or homozygosis. Rational selection of the best method to detect them is dependent from the specifics aims of different laboratories.

Furthermore, a less pronounced genetic contribution of DPYD and TYMS polymorphism has been demonstrated in a larger prospective study conducted in 683 patients, in whom the TYMS 2/2 genotype was found to increase the risk of toxicity 1.56-fold compared with the findings of Lecomte et al in a study of 90 patients, in whom the TYMS 2/2 genotype was found to have a grade 3 or 4 toxicity rate of 43%, whereas only 3% of patients who had the TYMS 3/3 genotype developed those toxicities.

Only few studies have addressed the cost-effectiveness of pharmacogenomics testing implication in clinical practice. For example van den Akker et al, included thiopurine S-methyltransferase (TPMT) genotyping prior to 6-mercaptopurine treatment in paediatric acute lumphoblastic leukemia (ALL); the mean calculated cost from 4 European countries was € 2100,00 per life-year considering low myelosuppression-related hospitalization; the cost for genotyping of TMPT mutation averaged around € 150,00. Early outline of genotyping cost for “home brew” pharmacogenic tests averaged about € 20,00 per SNP.

Furthermore, the major issues to consider for the clinical laboratories (who are responsible for providing PGx services), are: (1) the availability of FDA-cleared tests; (2) the current absence of public reimbursement; (3) the need for genotyping accuracy; and iv) the need to find clinical expertise to interpret laboratory results. In addition to the issues related to assay validation, addition- al equipment requirements, and time consumption, one main issue preventing the clinical application of these assays is their limited sensitivity and specificity. For example, Morel et al have shown that the sensitivity, specificity, and positive and negative predictive values of the detection of the 3 major SNPs (IVS14 + 1 G > A, 2846A > T, and 1679T > G) in DPYD, as factors predictive of 5-FU toxicity, were 0.31, 0.98, and 0.62 and 0.94, respectively. They found that only about 60% of patients who carry genetic variations in DPYD develop severe 5-FU toxicity.

Conclusions and Future Outlook

The usefulness of the described genetic variants for clinical practice will depend on their improving diagnostic prediction or fostering changes in prevention or treatment strategies. Particularly, the molecular testing for mutation in DPYD, TYMS, MTHFR and GSTP1 genes, allows the identification of patients who are most likely to respond to 5-FU. To meet this need, scientists and clinicians must collect information, informed consent, and tissue samples in the expectation of future studies addressing potential future questions.

Over the next few years, the emergence of molecular resistance in the new therapies as results of the genomic alterations in cancer will drive diagnostics companies to develop new tests able to produce results for tailoring patient’s treatment. Hopefully, the future implementation of the methods for genotyping of variants influencing fluoropyrimidine-based therapy will result in personalized treatments and eventually, in shifting the clinical benefit from disease relapse towards disease eradication. Therefore, it is fundamental that pharmaceutical and biotechnology companies join together, in order to develop an extensive study on the standardization method to validated tests suitable for routine diagnostics in pharmacogenomics of 5-FU.

In summary, although there are still questions to be answered, PGx researchers now have improved tools with which to take cancer treatment to the next level. With the increasing number of novel PGx markers being identified and validated, oncologists will have new means based on the individual genetic profile to make treatment decisions, and may eventually be personalized on the patients in order to minimize toxicity.
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References


