European Review for Medical and Pharmacological Sciences 2015; 19: 4443-4454

Rational selection of predictive pharmacogenomics test for the Fluoropyrimidine/Oxaliplatin based therapy

R. DI FRANCIA[1], L. DE LUCIA[2], M. DI PAOLO[3], S. DI MARTINO[4], L. DEL PUP[5], A. DE MONACO[1], A. LLESHI[6], M. BERRETTA[6]

[1]Hematology-Oncology and Stem Cell Transplantation Unit, National Cancer Institute, Fondazione "G. Pascale" IRCCS, Naples, Italy
[2]UOC Medical Oncology, AO S. Anna e S. Sebastiano, Caserta, Italy
[3]CETAC Research Center, Caserta, Italy
[4]GORI, Gruppo Oncologico Ricercatori Italiani, Pordenone, Italy
[5]Divison of Gynecological Oncology, CRO National Cancer Institute, Aviano (PN), Italy
[6]Department of Medical Oncology, CRO National Cancer Institute, Aviano (PN), Italy

Abstract. – OBJECTIVE: Both Fluoropyrimidine and Oxaliplatin (FluOx) are the most common anticancer drugs used to treat colorectal, ovarian, and gastrointestinal cancers.

Nevertheless, the efficacy of FluOx-based therapy is often compromised by the severe risk of neurotoxicity, cardiotoxicity, and gastrointestinal toxicity. Stratification of patients for their individual response to drugs is a promising approach for cancer treatment and cost-effectiveness. Here we evaluate the most recent findings on the most appropriate gene variants related to the toxicity in patients receiving FluOx chemotherapy.

MATERIALS AND METHODS: A systematic literature search of the MEDLINE, EMBASE, and Cochrane databases was conducted to identify all clinical studies of any association between DPYD and 5-FU correlated to allelic status of 6 validated polymorphisms in five genes Dihydropyrimidine Dehydrogenase (DPYD), Thymidylate Synthase (TYMS), Glutathione S-Transferase (GSTP1), and DNA-repair genes (ERCC2 and XRCC1).

RESULTS: The stratification of the patients into three genotype profiles group, who are most likely responders to FluOx treatments, provide informations about toxicity and/or resistance before starting therapy. Also, early evaluation cost of panel testing proposed is averaged about €100,00 per sample. The evaluation costs of genotyping before starting treatment could be a good cost-effectiveness strategy.

CONCLUSIONS: Based on the individual genomic profile, the oncologists will have new possibilities, based on the individual genetic profile, to make treatment decisions for their patients and to redefine scheduling and dosage of FluOx-based therapy.

Key Words:
Pharmacogenomics, Toxicity, Fluoropyridine, Oxaliplatin, Genotyping methods, Cost-effectiveness, Genotyping cost.

Introduction

Toxicity profile of Fluoropyrimidine/oxaliplatin (FluOx) is well recognized and often this unfavorable reaction leads to the deferral of therapy and potentially compromises patient benefit[1]. Primarily toxicities include severe gastrointestinal and hematologic events related to the Fluoropyrimidine administrations and peripheral neuropathy associated with acute and cumulative doses of Oxaliplatin[2]. Same adverse drug response associated with the FluOx treatment could be predicted through gene polymorphisms to be known involved with fluoropyrimidine[3] and Oxaliplatin biotransformation[4].

This report reviews the late findings on the validated gene variants that are related to the outcomes of the patients receiving FluOx treatment. In order to early prevent either toxicity or resistance we suggest performing a validated genotyping panel test of the most relevant pharmacogenomics and Pharmacogenetics (PG) markers, including Dihydropyrimidine Dehydrogenase (DPYD), Thymidylate Synthase (TYMS), Glutathione S-Transferase (GSTP1), X-Ray Cross-Complementing group 1 (XRCC1) and Excision Repair Cross-Complementing group 2 (ERCC2 also named XPD).

Corresponding Author: Massimiliano Berretta, MD; e-mail: mberretta@cro.it

Currently, a multitude of methods have been applied to detect the mutational status of these genes, without defining a validated standard for the daily diagnostic routine. We will also take into consideration the usefulness and the costs of the methods used to detect these genetic alterations.

Commonly, there are three main types of economic analysis that differ principally in the assessment of health outcome: cost-effectiveness, cost-utility, and cost-benefit parameters. The first aim of a cost-effectiveness analysis is to provide sufficiently effective informations for decision-makers to allocate resources to healthcare measures. Overviews of cost-effectiveness studies on PG technologies are now available[5]; all the reviews until July 2015 used different inclusion criteria and assessed the quality of analyzed studies using different approaches. Sutton et al[6] report that diverse meta-analysis methods used to assess the accuracy of diagnostic tests can affect and interfere with the economic evaluation. Recently, several methods to assess the quality of cost-effectiveness, cost-utility, and cost-benefit of PG tests have become available. A relevant example is the National Institute for Health and Clinical Excellence (NICE). NICE forms a Diagnostic Advisory committee, which is agreeable to stimulate Pharma and Academic communities to produce a validated set of data, including the design and data source in economic models of healthcare[7]. It is well known that PG tests performed before therapy, reduce overall medical costs and provide higher quality and a longer life expectancy[8].

In this issue, relative costs of a particular panel of PG tests involved in FluOx response were evaluated by "manually curated" criteria due to lack of specific guidelines.

We believe that retrospective and prospective trials evaluating the pharmacoeconomic impact of genotyping testing in FluOx-based-therapy will likely provide answers for policy making on the possibility to incorporate PG testing into daily clinical practice[7].

Materials and methods

Searching Strategy and Genetic Test Inclusion Criteria

A systematic literature search of the MEDLINE, EMBASE, and Cochrane databases was conducted to identify all clinical studies of any association between *DPYD* and 5-FU in Asian, African and Caucasian cancer patient populations from 1 January 2000 to 28 March 2014. The databases were searched using the following medical subject headings or text keywords: i) "Fluorouracil"[MeSH] AND "Thymidylate Synthase"[MeSH] OR "dihydropyrimidine dehydrogenase"[MeSH], limited to, toxicity, diagnostics test, and the English language; ii) "Oxaliplatin"[MeSH] AND "GSTP1"[MeSH] OR "DNA-repair Gene"[MeSH], limited to, polymorphisms, resistance AND toxicity, restricted to the english language.

The requirements for specialized reagents and approximate prices of equipment for each genotyping method are compared by searching the vendor websites. The instrumentation prices given should be used only as an estimate since the actual price will vary due to differences in the manufacturer, model, and local discount. The cost of reagents also varies widely between the genotyping methods; appreciative evaluation costs per SNP detection are scored as very low (< 5,00€), low (< 15,00€), moderate (< 30,00€), high (< 50,00€), very high (> 50,00€), including sample processing and controls. In addition, appreciative time-labour evaluation is referred to perform single sample in a panel assay of six tests within one work session.

We also manually searched the references to identify any relevant Pharmacogenomic studies evaluating the quantitative values of each polymorphism (as odds ratio), excluding letters and editorials. In particular, searching was focused on issues evaluating the pharmacogenomic impact of validated polymorphisms likely providing answers for policy making in the integration of PG markers into clinical practice, including FDA-Cleared Nucleic acid based test (*www.fda.gov/medicaldevices/productsandmedicalprocedures/invitrodiagnostics/ucm330711.htm)*. The selection criteria were described below.

Toxicity and Resistance at Molecular Level

The toxicity and/or resistance can be both acquired because of cellular adaptations as responses to drug insult, and intrinsic caused by polymorphisms in a gene involved in metabolizing enzyme and DNA-repair. Furthermore, it is possible that only one of these mechanisms may lead to FluOx resistance, it is more likely that combination of these mechanisms results

in developing of a resistant cellular tumor clone. Primarily, the toxicity at the molecular level is related to the low activity of metabolic enzymes.

Fluoropyrimidines

About 2-3% of patients who receive fluoropyrimidines, develop marked toxicity due to partial metabolization rate by Dihydropyrimidine Dehydrogenase enzyme (DPD) deficiency. In the rare case of complete DPD deficiency, degradation of 5-Fluorouracil (5-FU) is very low, and the treatment may even result in a lethal outcome. Indeed, partial DPD deficiency could provide a dosage adjustment to minimize toxicity[9].

Several dosages and schedules of 5-FU and other fluoropyrimidines (capecitabine, Raltitrexed, Tegafur-Uracil, etc.) are currently used in therapy as bolus and infusion regimens (short-term and chrono-modulated).

The adverse events differ among bolus and infusional 5-FU therapy. Bolus 5-FU monotherapy has limited activity; only 10% of patients achieve an aim response. Higher response rates can be accomplished with infusional regimens, but the overall survival impact is minimal[10]. While rates of gastro-intestinal toxicity are comparable, grade 3-4 neutropenia is more prevalent 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 dual higher response rate (21% *vs.* 11%)[11].

Oxaliplatin

Toxicities range from mild to severe peripheral neurotoxicity. Despite a modest activity as a single agent, oxaliplatin exerts significant activity in combination with other drugs as like as in combination with fluoropyrimidines[12]. Treatment in conjunction with 5-FU/LV (FOLFOX) has shown enhanced survival in the adjuvant setting among Stage III patients related to 5-FU/LV and 5-FU/irinotecan treatments[13]. Importantly, the occurrence of low neurotoxicity associated with 5-FU is increased with the addition of Oxaliplatin[14]. The *Food and Drug Administration* (FDA) noticed that over 60% of the patients receiving oxaliplatin are affected by some degree of sensory neuropathy[15], including ototoxicity and dysphoric syndrome. Currently, several methods are available to minimize oxaliplatin toxicity[16]. Unusually, neurotoxicity, and not tumor progression, is often the cause of treatment discontinuation.

Resistance to oxaliplatin can develop as a result of increased DNA-repair mechanisms, decreased glutathione conjugation, and increased drug efflux.

Despite these adverse events, FluOx association could have a fundamental liability for the treatment choice in a large set of patients, including in the so-called frail patients (i.e. elderly and HIV-positive patients) for whom the efficacy and, in particular, the toxicity profile are important aspects[17-19].

Selection of Candidate Genes and Polymorphisms

Several criteria were used to select genetic variants associated with toxicity/resistance for FluOx (Table I), in particular: A) searching the most validated genetic variants known to influencing the pharmacokinetics/pharmacodynamics of FluOx, likely providing answers into clinical practice (*www.pharmgkb.org/views/viewGeneticTests.action*); B) reviewing the most topical studies upgrading in clinical research, in particular, trials including pharmacogenomic tests before treatments with FluOx; C) applied study to assess accuracy and costs in routine laboratory applications. In this latter case, scientific literature is very limited.

DPYD

Low expression of DPD enzyme has been linked with the accumulation of 5-FU, thereby, exposing patients to increased risk of severe or lethal toxicities, while high expression of DPD has been associated with resistance events to 5-FU. It has also been shown that the incidence of low DPD enzymatic can vary significantly between different ethnic subpopulations[20]. The main known *DPYD Single Nucleotide Polymorphisms (*SNPs) associated with grade 3 and 4 toxicities are intronic variant IVS14 + 1 G > A rs3918290 (also named *DPYD*2A*) and mutation A1627G rs1801159[21]. Important results have previously demonstrated that a homozygote *DPYD*2A* genotype has resulted in complete deficiency (very high-risk patients) while the heterozygous *DPYD*2A* genotype has led to a partial deficiency of DPYD enzyme[22]. Various genotyping methods to monitor the known *DPYD* gene polymorphism have been validated, without defining better platforms for their use

Table I. Selection of validated pharmacogenomics markers influencing fluoropirimidine/oxaliplatin-based therapy.

Genetic variants (codons)	db SNPrs	Minor Allele Frequency * (MAF) Afr-Eur-Asn	Activities	Annotation
DPYD IVS14+1G>A	rs3918290	(A) 0.00-0.01-0.00	Mucositis severe Leukopenia	Heterozygous for A has been associated with low DPYD enzyme activity, while homozygous A is related to complete DPD deficiency[22].
A1627G	rs1801159	(G) 0.20-0.17-0.26	Severe nausea vomiting	The elimination constant (Ke) for 5-FU was a lot lower in patients homozygous for the G allele[24].
TYMS 28bp tandem repeat		rs34743033	NR	Neutropenia grade 3-4 Allele carrying the triple tandem repeat (3R) has augmented TYMS expression versus those with the double repeat (2R). Low TYMS levels are suggested to be markers of more favorable therapeutic outcome in advanced colorectal cancer[28].
GSTP1 313A>G ATC(Ile)>GTC(Val) (Ile105Val)	rs1695	(G) 0.44-0.32-0.17	Neurotoxicity, neutropenia	Patients homozygous for the G (Val) allele were associated with a lower toxicity and tumor development compared to the homozygous for the A (Ile) allele[35]
ERCC2 2251A>C AAG(Lys)>CAG(Gln) (Lys751Gln)	rs13181	(C) 0.21-0.38-0.09	High activity in nucleotide excision DNA repair.	The CC and the heterozygous AC were associated with reduced response, poor progression-free survival and overall survival in Caucasians. In addition, heterozygous lys751Gln patients had a longer median PFS: 11 months compared with six with unfavorable genotypes, $p < 0.00141$.
XRCC1 1196 A>G CAG(Gln)>CGG(Arg) Exon (Arg399Gln)	rs25487	(A) 0.12-0.35-0.25	High activity in base excision DNA repair	Patients carrying Gln/Arg and Gln/Gln mutant allele were at a 5.2 fold increased risk to fail the 5-FU/oxaliplatin chemotherapy because resistance phenomena[42]

*MAF: Afr (black Africans); Eur (Ancient European-Caucasian); Asn (Indo-Chinese) data from Ensamble (www.ensembl.org/Homo_sapiens/Variation/Population?db=core; r=9:22125003-22126003; v=rs1333049;vdb=variation;vf=1).

in the daily diagnostic routine. Current methodologies include conventional Polymerase Chain Reaction (PCR) followed by sequencing, Single-Strand Conformational Polymorphism (SSCP)[23], Pyrosequencing[24], Fluorescent Resonance Energy Transfer (FRET) probes[22,25].

TYMS

Major polymorphisms have been reported to be associated with altered *TYMS* expression and clinical response to fluoropyrimidine-based therapy. A polymorphic tandem repeat sequence in the 5'-untranslated region (5'UTR) into *TYMS* Se-

quence Enhancer Region (TSER) has been classified in several aplotypes (from TSER*2 up to TSER*9)[26]. Many studies have identified relations between TSER genotype (predominantly TSER*2 and *3) and response to chemotherapy[27].

TYMS is the primary target of 5-FU. Moreover, a less definite genetic contribution of TYMS polymorphism has been demonstrated in a significant prospective study, in which the TYMS 3R/3R genotype was shown to increase the risk for toxicity 1.6 fold (rate of 43% of patients treated with 5-FU), compared with the TYMS 3R/2R genotype. In contrast, only 3% of patients who had the TYMS 3/3 genotype developed 3 or 4 grade of toxicity[28].

Extensive study on based Next Generation Sequencing, GeneChip[29], and Matrix-Assisted Laser Desorption/Ionization Time Of Flight (MALDI TOF) platforms have been developed for research[30], while currently these technologies are rarely used in the clinical laboratory. Methods based on a cheaper PCR could detect these genetic variants in *TYMS* gene, but applied studies to assess accuracy and costs in routine laboratory applications are missing.

GSTP1

Polymorphism rs1695 *GSTP1* Ile105Val (313A>G in exon 5, sometimes labeled GSTP1*B) has been linked to inhibition of enzyme activity and either cancer drug resistance or toxicity[31]. The allele frequency of the Ile105Val polymorphism varies widely among populations[32]. Nevertheless, in 166 colorectal cancer patients receiving Oxaliplatin and 5-FU, the GSTP1 Ile105Val heterozygous allele was associated with increased risk of neutropenia[33] and neurotoxicity[34], while patients homozygous to Val/Val tended to a lower risk of neurotoxicity and tumour progression compared to Ile/Ile phenotypes[35]. This SNP in position 313 of GSTP1 gene could be detected by allelic discrimination methods such as germ-line mutation[36,37].

DNA Repair Genes ERCC2 and XRCC1

Pharmacogenomic studies in cancer cells have consistently shown increased activity of nuclear protein able to remove alien nucleotides from DNA[38]. DNA Repair mechanism is controlled essentially by the Nucleotide Excision repair (NER) and Base Excision Repair (BER) genes family. Furthermore, genetic variants in any of these genes may modulate repair capacity and contribute to individual resistance to chemotherapy. If the cell is clever to repair the DNA being attacked by the platinum agent, then the agent will be unsuccessful in inducing apoptosis. Primary genes involved in DNA adducts repair are Excision Repair Cross-Complementing group (ERCC also named XPD) and the X-Ray Cross-Complementing group (XRCC).

ERCC2 encode for DNA Helicase, which is involved in the unwinding of DNA; variable suppression of this nuclear protein is correlated with resistance to platinating agents. A significant association was detected between the A/A (Lys/Lys) genotype at codon 751 and longer Overall Survivor (OS) in mCRC treated with 5-FU and platinum[39]. In addition, two variants in ERCC2 codon Lys751Gln and Asp312Gln were associated with better treatment outcomes in patients receiving oxaliplatin-based therapy. In a meta-analysis of six studies on a total 625 adults who received Oxaliplatin, an increased risk of relapse was associated with heterozygosis phenotype for Lys751Gln[40,41].

The XRCC1 is an important protein of BER pathway, that interact with Ligase III and Poly (ADP-ribose) Polymerase, which is through to act as a scaffold in the removal alien bases, caused by ionizing radiations and Alkylating agents. Germline variation at codon Arg399Gln of the gene encoding XRCC1 has been associated to decrease in risk of toxicity in cancer: patients carrying Gln mutant allele (heterozygous plus homozygous) were at a 5.2 fold increased risk to fail the 5-FU/oxaliplatin chemotherapy because resistance phenomena in advanced colorectal cancer[42].

However, people possessing of both AA (for ERCC2) and GG (for XRCC1) may have higher DNA repair capacity that could effectively reduce the effect of nucleoside analogues drugs, leading both resistance phenomena and poor prognosis of this patients. To date, we were unable to investigate interaction between DNA-repair gene and fluoropyrimidine because of the limited scientific literature.

Additional Candidate Gene Involved in FluOx therapy

Additional candidate gene variants influencing Fluoropyrimidine and Oxaliplatin-based chemotherapy have been well documented[43,44]. They included TYMS 1494del, "ATP-Binding cassette 1 and 2" (ABCC1 and ABCC2), X-ray repair complementing defective repair in Chinese hamster cells 3 (XCCR3) and "nucleotide exci-

sion DNA repair cross-complementation group 1" (ERCC1). Numerous other polymorphisms, detected by Genomic wide Association study (GWAS), are not mentioned in this issue due to lack of deep validation study. So, they need more evidences in confirmatory studies performed with other methods and platforms.

Other variants of TYMS are known to lead TYMS phenotype: polymorphism of *TYMS* 1494del (TTAAAG) rs34489327, suggest that this deletion alters *TYMS* mRNA stability[45]. A study[46] of *TYMS* mRNA expression from tumour cells in 43 colorectal cancer patients suggests that subjects homozygous for the 6 bp deletion, express about 3-fold less *TYMS* mRNA than patients homozygous for the presence of the 6 bp.

Overexpression of the ABCC1 protein has been linked to resistance to 5-fluorouracil *in vitro*. This could be owing to the ability of ABCC1 to extrude folates and thus depleting their intracellular availability for the activity of 5-fluorouracil. This may explain, in part, the cause of ABCC1 rs35587 on both neutropenia and neurological toxicity. ABCC1-rs35587 might be indicative both of increased function and expression of the ABCC1 transporter.

For ABCC2, three polymorphic traits (rs1885301, rs717620, and rs3740066) have been connected with grade 2-3 neurological toxicity and one of them have been also linked to severe neutropenia. The useful result of these variants is indefinite. In particular, ABCC2 rs717620 has been earlier associated with decreased protein expression *in vitro*[47]. Also, it has been associated with a 13-fold increased risk of grade 2-3 neurological toxicity and to a 5-fold increased risk of severe leukopenia[48]. In addition, ABCC2-rs717620 and rs3740066 have had a combined effect in increasing platinum-related toxicity in colon cancer patients[48].

Further confirmatory studies (both at clinical and molecular level) should be conducted to confirm the clinical associations.

DNA repair protein XRCC3 is part of the double strand break repair machinery. Its reduced activity is associated with significantly higher levels of bulky DNA adducts. Polymorphism XRCC3 rs1799794 is associated with severe non-hematological toxicity. DNA repair is an important mechanism for resistance to platinum-based therapy.

Park et al[39] have described a connection between the *ERCC1* codon Asn118Asn polymorphism rs11615 and clinical output in colorectal cancer subjects treated with platinum-based chemotherapy. This genotype could be a useful predictor of clinical outcome, but many authors have found this association statistically low for colorectal cancer[49], and in NSCLC[50].

However, the fine molecular function of these SNPs remains unclear and controversial. Furthermore, there are many genes whose effects on neurotoxicity to FluOx have to be studied, yet. In addition, emerging new evidence in nutrigenomics field, suggests an accurate evaluation of the diet during therapy[51].

Early Outline Evaluation of Genotyping Costs

Current models in cancer treatments are based primarily on validated multitrack approach that includes, often, the newer expensive patented drugs. In contrast, the global concept of healthcare systems supports that the new healthcare should be given at equal or lower cost with improved patient outcomes. Personalized medicine includes genomic tests of each patient and their illness into their clinical treatments, so as minimize toxicity and maximize benefits due to specific tailored treatments. It is well known that PG tests performed before drug treatment, lower overall medical costs and provided a higher quality of life and a longer life expectancy[5]. NICE, also provide a method for measuring Quality-Adjusted Life-Years (QUALYs); metrics that combine heterogenic information on outcomes, analytical, and cost-effectiveness of each treatment.

The future implementation of the methods for measure the QUALYs will guide to personalized treatment and eventually will shift the balance from disease relapse toward disease eradication.

The cost of a genetic testing includes more than just the cost of the test itself. However, additional costs of genetic counseling, laboratory equipment, time-labour and further diagnostics are potentially of greater magnitude and should be evaluated.

Few studies have addressed the costs of pharmacogenomics testing implication in clinical practice[5]. For example van den Akker et al[52], integrated thiopurine S-methyltransferase (TPMT) genotyping prior to 6-mercaptopurine management in paediatric Acute Lymphoblastic Leukaemia (ALL); the average cost from 4 European countries was € 2100,00 per life-year tak-

ing into consideration low myelosuppression-related hospitalization; the cost for genotyping of TMPT mutation averaged around €150,00. In a recent study[53], an early outline of the genotyping cost for "home brew" tests (based on Fluorescent probes able to perform allele discrimination Assay), was averaged about €20,00 per SNP.

Our specific panel assay includes detection of five SNPs. Based on the wide common platforms available to address allelic discriminations (detection of DNA mutant between the two alleles), the relative cost of the proposed PG panel is averaged about €100,00 per sample, performed in double, including entire sample processing (i.e. DNA isolation) and positive/negative controls. The qualitative assessments of the PG panel are "manually curated" without a necessary gold standard method for the daily diagnostic routine. The requirements for specialized reagents and approximate prices of equipment for each genotyping method are compared in Table II. Rational selection of the best method to detect them is always dependent on the specific aims of different laboratories[54].

Moreover, the major issues to regard for the clinical labs (who are responsible for providing PG services), are: (1) the availability of FDA-cleared tests; (2) the current absence of public reimbursement; (3) the need for genotyping accuracy; and (4) the need to find clinical expertise to interpret laboratory data results[55,56].

Genetic variants and prognostic markers are widley accounted as tools to improve the effectiveness of anti-cancer therapy. In contrast, the clinical utility of the described polymorphisms implicated in FluOx based-therapy is in part limited by: (1) Low, wide diffusion of genotyping methods in routine clinical diagnostics; (2) The evidence that PG testing improves health outcomes is still an open question; and (3) The cost-effectiveness of the testing is unidentified.

The helpfulness of the described genetic variants for clinical practice will depend on their improving diagnostic prediction to plan the better treatment strategies[57]. Particularly, the molecular testing for a mutation in DPYD, TYMS, GSTP1 ERCC2 and XRCC1 genes, could help the oncologist to stratify patients who are most

Table II. Wide diffuses platform allowing allelic discrimination assay. Relative costs for reagents/SNP, and time-labour per sample referred to perform proposed panel for DYPD, TYMS, GSTP1, ERCC1 and XRCC1 polymorphism.

Genotyping methods to detect known SNP	Instrument mean costs[§]	Approximate reagent costs per SNP[$]	Approximate time-labour per sample panel assay[#]
D-HPLC	++++	moderate	fast
SSCP	+	low	laborious
Allele Specific Amplification (ASA)[*]	+	very low	middle
Restriction Fragment Length Polymorphism (RFLP)	+	very low	laborious
FRET probe Allelic Discrimination (Hyb Probe® TaqMan®, Beacons®, Scorpions®)	++	moderate	middle
Locked Nucleic Acid (LNA) probe	++	moderate	middle
Oligo ligation assay (SNPlex®)	+++	high	fast
PCR-Invader® assay	+++	high	fast
High resolution melting (HRM)	++	low	middle
Pyrosequencing[*]	+++	high	fast
Peptide nucleic acid-mediated clamping PCR[*]	+	moderate	middle
Gene Chip technology (LabOnChip)[*].	+++/++++	very high	Laborious
Maldi-TOF Mass Spectroscopy	++++	very high	middle
Next Generation sequencing	++++	very high	fast
Conventional sequencing*	++	low	middle

[§]Approximate instrumentation list price were scored as + (<10,000€); ++ (<50,000€); +++ (<100,000€), ++++ (>100,000€)

[$]Reagent cost/SNP (including controls) were scored as very low (<5 €), low (<15 €), moderate (<30 €), high (<50 €), very high (>50 €).

[#]Time-labour refers to perform a single sample of multiple test. It were scored as very fast (< 1 hour), fast (<4 hours), middle (< 1 day), laborious (>1 day).

[*]Suffers of low sensitivity and specificity in the presence of heterozygous sample.

likely successfull to a better outcome with FluOx. In order to assess an essential profile of good/bad responding patients, a panel assay of 6 SNPs is proposed (Table III). Despite our efforts to make a precise and comprehensive list of polymorphisms, the limit of our proposed panel tests need to be addressed. These issues cause same bias in our estimation, but conclusion criteria could help the clinicians to stratify patients into three genotype profile arbitrarily called FluOx1, 2 and 3. FluOx1 profile, simulate a germline genetic profile, known to be as "favourable enzyme biotransformation" for fluoropyrimidine and lower neurotoxicity for Oxaliplatin (i.e., GSTP1 Val/Val). In contrast, FluOx3 profile stratifies subjects worst responders with high probability of risk of mucositis and neutropenia due to 5-FU adverse reactions, and neurotoxicity caused by oxaliplatin. While FluOx2 profile (all heterozygous) has variable effects, making it unhelpful to stratify a good/bad responder.

Finally, an early outline of the genotyping cost for the proposed panel test (based on "home brew" fluorescent allele discrimination Assay), is averaged about €100,00 per sample[53]. Although,

Table III. the genotyping profile of patients for response to FluOx-based treatment.

	DPYD		TYMS	GSTP1	ERCC1	XRCC1	
PG Profile	rs3918290 G>A IVS14+1	rs1801159 A>G	rs34743033 28 tandem repeat	rs1695 A>G Ile105Val	rs13181 A>C Lys751Gln	rs25487 A>G Arg399Gln	Effects
FluOx1 Favorable	GG	AA	2R/2R	GG (Val/Val)	CC (Gln/Gln)	AA (Gln/Gln)	Favorable biotransformation profile machinery for fluoropyrimidine administration. Lower neurotoxicity and tumor progression for Oxaliplatin, protected by GSTP1 (Val/Val). Resistance phenomena were not observed because ERCC2 phenotype
FluOx2	GA	AG	2R/3R	AG (Ile/Val)	AC (Lys/Gln)	AG (Arg/Gln)	Divergent effects
FluOx3 Risk profile	AA	GG	3R/3R	AA (Ile/Ile)	AA (Lys/Lys)	GG (Arg/Arg)	Very high risk of mucosites and neutropenia becouse to 5-FU administrations. Acute and cumulative Neuropathy. Individual carrying both AA (for ERCC2) and GG (for XRCC1) may have higher DNA repair capacity that could effectively reduce the effect of nucleoside analogs drugs, leading poor prognosis because molecular resistance phenomena

since the number of the samples are limited, the FRET-based methods would seem the most suitable platform for routine clinical use.

This evaluation of genotyping costs before starting treatment could be a good cost-effectiveness strategy, providing a higher quality of life and longer life expectancy (measurable by QUALYs). Clearly, further study is necessary for measuring the QUALYs on a subset of patients genotyped before treatment in order to assess exactly the cost-effectiveness of the our proposed PG panel test.

Optimistically, the future implementation of the methods for genotyping the variants influencing fluoropyrimidine/Oxaliplatin-based therapy will result in personalized treatments[57]. Therefore, it is fundamental that pharmaceutical and biotechnology companies join together, in order to develop a widespread study on the standardization method of validated tests suitable for routine diagnostics in pharmacogenomics of FluOx.

Conclusions

With the growing quantity of new PG markers being identified and validated, oncologists will have new possibilities based on the individual genetic profile, to make treatment decisions, as well as correlation between nutrition and cancer[58,59], that may eventually be personalized on the patient. In the other word, based on the individual genomic profile, the oncologists, in order to minimize toxicity, could redefine scheduling and dosage of FluOx therapy[60].

Based on this rationale, the oncologist, and the lab manager might join together to assess advantages and limits in terms of costs and applicability of the most appropriate methods of setting molecular diagnostics of FluOx pharmacogenomics tests.

Acknowledgements

The authors are grateful to the "Italian Association of Pharmacogenomics and Molecular Diagnostics" for the use of its bibliography resources. We thank Mrs. P. Favetta from "Gruppo Oncologico Ricercatori Italiani" GORI, for her expert assistant in the preparation and correction of the manuscript.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

1) Berretta M, Nasti G, De Diviitis C, Divita M, Fisichella R, Spartà D, Baresic T, Ruffo R, Urbani M, Tirelli U. Safety and efficacy of oxaliplatin-based chemotherapy in the first line treatment of elderly patients affected by metastatic colorectal cancer. WCRJ 2014; 1: e235.
2) Berretta M, Zanet E, Nasti G, Lleshi A, Frustaci S, Fiorica F, Bearz A, Talamini R, Lestuzzi C, Lazzarini R, Fisichella R, Cannizzaro R, Iaffaioli RV, Berretta S, Tirelli U. Oxaliplatin-based chemotherapy in the treatment of elderly patients with metastatic colorectal cancer (CRC). Arch Gerontol Geriatr 2012; 55: 271-275.
3) Di Francia R, Cimino L, Berretta M. Genetic variants influencing fluoropyrimidine based-therapy and available methods to detect them. Eur Rev Med Pharmacol Sci 2012; 16: 285-298.
4) Farina Sarasqueta A, van Lijnschoten G, Lemmens VE, Rutten HJ, van den Brule AJ. Pharmacogenetics of oxaliplatin as adjuvant treatment in colon carcinoma: are single nucleotide polymorphisms in GSTP1, ERCC1, and ERCC2 good predictive markers? Mol Diagn Ther 2011; 15: 277-283.
5) Payne K, Shabaruddin FH. Cost-effectiveness analysis in pharmacogenomics. Pharmacogenomics 2010; 11: 643-646.
6) Sutton A, Ades AE, Cooper N, Abrams K. Use of indirect and mixed treatment comparisons for technology assessment. Pharmacoeconomics 2008; 26: 753-767.
7) Dhalla IA, Garner S, Chalkidou K, Littlejohns P. Perspectives on the National Institute for Health and Clinical Excellence's recommendations to use health technologies only in research. Int J Technol Assess Health Care 2009; 25: 272-280.
8) De Monaco A, Valente D, Di Paolo M, Troisi A, D'Orta A, Del Buono A. Oxaliplatin-based therapy: strategies to prevent or minimize neurotoxicity WCRJ 2014; 1: e232.
9) Boisdron-Celle M. Pharmacokinetic adaptation of 5-fluorouracil: where are we and where are we going? Pharmacogenomics 2012; 13: 1437-1439.
10) Han JJ, Baek SK, Lee JJ, Kim GY, Kim SY, Lee SH Combination of TRAP1 and ERCC1 expression predicts clinical outcomes in metastatic colorectal cancer treated with oxaliplatin/5-fluorouracil. Cancer Res Treat 2014; 46: 55-64.
11) Maughan TS, James RD, Kerr DJ, Ledermann JA, McArdle C, Seymour MT, Seymour MT, Cohen D, Hopwood P, Johnston C, Stephens RJ; British MRC Colorectal Cancer Working Party. Comparison of survival, palliation, and quality of life with three chemotherapy regimens in metastatic colorectal cancer: a multicentre randomised trial. Lancet 2002; 359: 1555-1563.
12) Berretta M, Lleshi A, Di Benedetto F, Bearz A, Spina M, Tirelli U. Oxaliplatin and capecitabine (Xelox) in association with highly active antiretroviral ther-

apy in advanced hepatocarcinoma HIV/HCV-infected patients. Ann Oncol 2006; 17: 1176-1177.

13) Madi A, Fisher D, Wilson RH, Adams RA, Meade AM, Kenny SL, Nichols LL, Seymour MT, Wasan H, Kaplan R, Maughan TS; COIN trial research group. Oxaliplatin/capecitabine vs oxaliplatin/infusional 5-FU in advanced colorectal cancer: the MRC COIN trial. Br J Cancer 2012; 107:1037-1043.

14) Berretta M, Cappellani A, Fiorica F, Nasti G, Frustaci S, Fisichella R, Bearz A, Talamini R, Lleshi A, Tambaro R, Cocciolo A, Ristagno M, Bolognese A, Basile F, Meneguzzo N, Berretta S, Tirelli U. FOLFOX4 in the treatment of metastatic colorectal cancer in elderly patients: a prospective study. Arch Gerontol Geriatr 2011; 52: 89-93.

15) Ibrahim A, Hirschfeld S, Cohen MH, Griebel DJ, Williams GA, Pazdur R. FDA drug approval summaries: oxaliplatin. Oncologist 2004; 9: 8-12.

16) Di Francia R, Siesto RS, Valente D, Del Buono A, Pugliese S, Cecere S, Nasti G, Facchini G, Berretta M. Current strategies to minimize toxicity of oxaliplatin: selection of pharmacogenomic panel tests. Anti-cancer Drugs 2013; 24: 1069-1078.

17) Berretta M, Bearz A, Frustaci S, Talamini R, Lombardi D, Fratino L, Lleshi A, Bonanno S, Spartà D, Palmucci S, Berretta S, Tirelli U. FOLFOX2 in the treatment of advanced colorectal cancer: a comparison between elderly and middle aged patients. J Chemother 2008; 20: 503-508.

18) Berretta M, Lleshi A, Cappellani A, Bearz A, Spina M, Talamini R, Cacopardo B, Nunnari G, Montesarchio V, Izzi I, Lanzafame M, Nasti G, Basile F, Berretta S, Fisichella R, Schiantarelli C C, Garlassi E, Ridolfo A, Guella L, Tirelli U. Oxaliplatin based chemotherapy and concomitant highly active antiretroviral therapy in the treatment of 24 patients with colorectal cancer and HIV infection. Curr HIV Res 2010; 8: 218-222.

19) Berretta M, Di Benedetto F, Bearz A, Simonelli C, Martellotta F, Del Ben C, Berretta S, Spina M, Tirelli U. FOLFOX-4 regimen with concomitant highly active antiretroviral therapy in metastatic colorectal cancer HIV-infected patients: a report of five cases and review of the literature. Cancer Invest 2008; 26: 610-614.

20) Yen JL, McLeod HL. Should DPD analysis be required prior to prescribing fluoropyrimidines? Eur J Cancer 2007; 43: 1011-1016.

21) Catapano O, Barletta O, Di Paolo M, Faioli D, Di Francia R. Impact of DPYD variants in Fluoropyrimidine based-therapy: the state of the art. WCRJ 2014; 1: e279.

22) Toffoli G, Giodini L, Buonadonna A, Berretta M, De Paoli A, Scalone S, Miolo G, Mini E, Nobili S, Lonardi S, Pella N, Lo Re G, Montico M, Roncato R, Dreussi E, Gagno S, Cecchin E. Clinical validity of a DPYD-based pharmacogenetic test to predict severe toxicity to fluoropyrimidines. Int J Cancer 2015. doi: 10.1002/ijc.29654.

23) Okamoto Y, Ueta A, Sumi S, Ito T, Okubo Y, Jose Y, Ninomiya A, Togari H, Nishida M. SSCP screening of the dihydropyrimidine dehydrogenase gene polymorphisms of the Japanese population using a semi-automated electrophoresis unit. Biochem Genet 2007; 45: 713-724.

24) Morel A, Boisdron-Celle M, Fey L, Laine-Cessac P, Gamelin E. Identification of a novel mutation in the dihydropyrimidine dehydrogenase gene in a patient with a lethal outcome following 5-fluorouracil administration and the determination of its frequency in a population of 500 patients with colorectal carcinoma. Clin Biochem 2007; 40: 11-17.

25) Bosch TM, Bakker R, Schellens JH, Cats A, Smits PH, Beijnen JH. Rapid detection of the DPYD IVS14+1G>A mutation for screening patients to prevent fluorouracil-related toxicity. Mol Diagn Ther 2007; 11: 105-108.

26) Yawata A, Kim SR, Miyajima A, Kubo T, Ishida S, Saito Y, Nakajima Y, Katori N, Matsumoto Y, Fukuoka M, Ohno Y, Ozawa S, Sawada J. Polymorphic tandem repeat sequences of the thymidylate synthase gene correlates with cellular-based sensitivity to fluoropyrimidine antitumor agents. Cancer Chemother Pharmacol 2005; 56: 465-472.

27) Liersch T, Langer C, Ghadimi BM, Kulle B, Aust DE, Baretton GB, Schwabe W, Häusler P, Becker H, Jakob C. Lymph node status and TS gene expression are prognostic markers in stage II/III rectal cancer after neoadjuvant fluorouracil-based chemoradiotherapy. J Clin Oncol 2006; 24: 4062-4068.

28) Lecomte T, Ferraz JM, Zinzindohoue F, Loriot MA, Tregouet DA, Landi B, Berger A, Cugnenc PH, Jian R, Beaune P, Laurent-Puig P. Thymidylate synthase gene polymorphism predicts toxicity in colorectal cancer patients receiving 5-fluorouracil-based chemotherapy. Clin Cancer Res 2004; 10: 5880-5888.

29) Xu WH, Long JR, Zheng W, Ruan ZX, Cai Q, Cheng JR, Zhao GM, Xiang YB, Shu XO. Association of thymidylate synthase gene with endometrial cancer risk in a Chinese population. Cancer Epidemiol Biomarkers Prev 2009; 18: 579-584.

30) Meyer K, Fredriksen A, Ueland PM. MALDI-TOF MS genotyping of polymorphisms related to 1-carbon metabolism using common and mass-modified terminators. Clin Chem 2009; 55: 139-149.

31) Lecomte T, Landi B, Beaune P, Laurent-Puig P, Loriot MA. Glutathione S-transferase P1 polymorphism (Ile105Val) predicts cumulative neuropathy in patients receiving oxaliplatin-based chemotherapy. Clin Cancer Res 2006; 12: 3050-3056.

32) Georgiadis P, Demopoulos NA, Topinka J, Stephanou G, Stoikidou M, Bekyrou M, Katsouyianni K, Sram R, Autrup H, Kyrtopoulos SA. Impact of phase I or phase II enzyme polymorphisms on lymphocyte DNA adducts in subjects exposed to urban air pollution and environmental tobacco smoke. Toxicol Lett 2004; 149: 269-280.

33) Ruzzo A, Graziano F, Galli F, Giacomini E, Floriani I, Galli F, Rulli E, Lonardi S, Ronzoni M, Massidda B,

Zagonel V, Pella N, Mucciarini C, Labianca R, Ionta MT, Veltri E, Sozzi P, Barni S, Ricci V, Foltran L, Nicolini M, Biondi E, Bramati A, Turci D, Lazzarelli S, Verusio C, Bergamo F, Sobrero A, Frontini L, Magnani M. Genetic markers for toxicity of adjuvant oxaliplatin and fluoropyrimidines in the phase III TOSCA trial in high-risk colon cancer patients. Sci Rep 2014; 4: 6828.

34) De Monaco A, Faioli D, Di Paolo M, Catapano O, D'Orta A, Del Buono M, Del Buono R, Di Francia R. Pharmacogenomics markers for prediction response and toxicity in cancer therapy WCRJ 2014; 1: e276.

35) Balboa S, Bermudez-Crespo J, Gianzo C, Lopez JL, Romalde JL. Proteomics and multilocus sequence analysis confirm intraspecific variability of Vibrio tapetis. FEMS Microbiol Lett 2011; 324: 80-87.

36) De Monaco A, D'Orta A, Fierro C, Di Paolo M, Cilenti L, Di Francia R. Rational selection of PCR-based platforms for pharmacogenomic testing. WCRJ 2014; 1: e391.

37) Norambuena PA, Copeland JA, Krenkova P, Stambergova A, Macek M, Jr. Diagnostic method validation: High resolution melting (HRM) of small amplicons genotyping for the most common variants in the MTHFR gene. Clin Biochem 2009; 42: 1308-1316.

38) Helleday T, Petermann E, Lundin C, Hodgson B, Sharma RA. DNA repair pathways as targets for cancer therapy. Nat Rev Cancer 2008; 8: 193-204.

39) Park DJ, Zhang W, Stoehlmacher J, Tsao-Wei D, Groshen S, Gil J, Yun J, Sones E, Mallik N, Lenz HJ. ERCC1 gene polymorphism as a predictor for clinical outcome in advanced colorectal cancer patients treated with platinum-based chemotherapy. Clin Adv Hematol Oncol 2003; 1:162-166.

40) Yin M, Yan J, Martinez-Balibrea E, Graziano F, Lenz HJ, Kim HJ, Robert J, Im SA, Wang WS, Etienne-Grimaldi MC, Wei Q. ERCC1 and ERCC2 polymorphisms predict clinical outcomes of oxaliplatin-based chemotherapies in gastric and colorectal cancer: a systemic review and meta-analysis. Clin Cancer Res 2011; 17: 1632-1640.

41) Balboa E, Duran G, Lamas MJ, Gomez-Caamano A, Celeiro-Munoz C, Lopez R, Carracedo A, Barros F. Pharmacogenetic analysis in neoadjuvant chemoradiation for rectal cancer: high incidence of somatic mutations and their relation with response. Pharmacogenomics 2010; 11: 747-761.

42) Stoehlmacher J, Ghaderi V, Iobal S, Groshen S, Tsao-Wei D, Park D, Lenz HJ. A polymorphism of the XRCC1 gene predicts for response to platinum based treatment in advanced colorectal cancer. Anticancer Res 2001; 21: 3075-3079.

43) Lleshi A, Fiorica F, Fisichella R, Spartà A, Di Vita M, Berretta S, Berretta M. Gastric cancer: prognostic aspects, predictive factors to therapy response and real impact on treatment approach. WCRJ 2014; 1: e395.

44) Ceppi P, Volante M, Ferrero A, Righi L, Rapa I, Rosas R, Berruti A, Dogliotti L, Scagliotti GV, Papotti M. Thymidylate synthase expression in gastroenteropancreatic and pulmonary neuroendocrine tumors. Clin Cancer Res 2008; 14: 1059-1064.

45) Acuna M, Eaton L, Cifuentes L, Massardo D. Genetic variants in the enhancer region of the thymidylate synthase gene in the Chilean population. Br J Clin Pharmacol 2006; 61: 778-782.

46) Mandola MV, Stoehlmacher J, Zhang W, Groshen S, Yu MC, Iqbal S, Lenz HJ, Ladner RD. A 6 bp polymorphism in the thymidylate synthase gene causes message instability and is associated with decreased intratumoral TS mRNA levels. Pharmacogenetics 2004; 14: 319-327.

47) Haenisch S, May K, Wegner D, Caliebe A, Cascorbi I, Siegmund W. Influence of genetic polymorphisms on intestinal expression and rifampicin-type induction of ABCC2 and on bioavailability of talinolol. Pharmacogenet Genomics 2008; 18: 357-365.

48) Cecchin E, D'Andrea M, Lonardi S, Zanusso C, Pella N, Errante D, De Mattia E, Polesel J, Innocenti F, Toffoli G. A prospective validation pharmacogenomic study in the adjuvant setting of colorectal cancer patients treated with the 5-fluorouracil/leucovorin/oxaliplatin (FOLFOX4) regimen. Pharmacogenomics J 2013; 13:403-409.

49) Van Huis-Tanja LH, Kweekel DM, Lu X, Franken K, Koopman M, Gelderblom H, Antonini NF, Punt CJ, Guchelaar HJ, van der Straaten T. Excision Repair Cross-Complementation group 1 (ERCC1) C118T SNP does not affect cellular response to oxaliplatin. Mutat Res 2014; 759: 37-44.

50) Yin M, Yan J, Voutsina A, Tibaldi C, Christiani DC, Heist RS, Rosell R, Booton R, Wei Q. No evidence of an association of ERCC1 and ERCC2 polymorphisms with clinical outcomes of platinum-based chemotherapies in non-small cell lung cancer: a meta-analysis. Lung cancer 2011; 72: 370-377.

51) Berretta M, Michieli M, Di Francia R, Cappellani A, Rupolo M, Galvano F, Fisichella R, Berretta S, Tirelli U. Nutrition in oncologic patients during antiblastic treatment. Front Biosci 2013; 18: 120-132.

52) Van den Akker-van Marle ME, Gurwitz D, Detmar SB, Enzing CM, Hopkins MM, Gutierrez de Mesa E, Ibarreta D. Cost-effectiveness of pharmacogenomics in clinical practice: a case study of thiopurine methyltransferase genotyping in acute lymphoblastic leukemia in Europe. Pharmacogenomics 2006; 7: 783-792.

53) Di Francia R, Berretta M, Catapano O, Canzoniero LM, Formisano L. Molecular diagnostics for pharmacogenomic testing of fluoropyrimidine based-therapy: costs, methods and applications. Clin Chem Lab Med 2011, 49: 1105-1111.

54) Di Francia R, Frigeri F, Berretta M, Cecchin E, Orlando C, Pinto A, Pinzani P. Decision criteria for rational selection of homogeneous genotyping platforms for pharmacogenomics testing in clinical diagnostics. Clin Chem Lab Med 2010, 48: 447-459.

55) Di Francia R, Valente D, Catapano O, Rupolo M, Tirelli U, Berretta M. Knowledge and skills needs for health professions about pharmacogenomics testing field. Eur Rev Med Pharmacol Sci 2012; 16: 781-788.

56) Di Francia R, Valente D, Pugliese S, Del Buono A, Berretta M. What health professions in oncology needs to know about pharmacogenomics? WCRJ 2014; 1: e90.

57) Berretta M, Di Francia R, Tirelli U. Editorial – The new oncologic challenges in the 3RD millennium. WCRJ 2014; 1: e133.

58) D'Orta A, Del Buono A, De Monaco A. Nutritional manipulation: epigenetic effect in cancer. WCRJ 2015; 2: e518.

59) Berretta M, Cappellani A, Lleshi A, Di Vita M, Lo Menzo E, Bearz A, Galvano F, Spina M, Malaguarnera M, Tirelli U, Berretta S. The role of diet in gastric cancer: still an open question. Front Biosci 2012; 17: 1640-1647.

60) Berretta M, Tirelli U. The economic face to pharmacogenomic marker in cancer therapy. WCRJ 2014; 1: e354.