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ORIGINAL ARTICLE

Pharmacogenetics in cancer therapy – 8 years of experience at the Institute for Oncology and Radiology of Serbia

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Summary

Purpose: *Pharmacogenetics is a study of possible mechanism by which an individual's response to drugs is genetically determined by variations in their DNA sequence. The aim of pharmacogenetics is to identify the optimal drug and dose for each individual based on their genetic constitution, i.e. to individualize drug treatment. This leads to achieving the maximal therapeutic response for each patient, while reducing adverse side effects of therapy and the cost of treatment. A centralized pharmacogenetics service was formed at the Institute for Oncology and Radiology of Serbia (IORS) with the aim to provide a personalized approach to cancer treatment of Serbian patients.*

Methods: *Analyses of KRAS mutations in metastatic colorectal cancer, EGFR mutations in advanced non-small cell lung cancer, CYP2D6 polymorphism in breast cancer, DPD polymorphism in colorectal cancer and MTHFR polymorphism in osteosarcoma have been performed by real time polymerase chain reaction (PCR) and PCR-restriction fragment length polymorphism (PCR-RFLP).*

Results: *Mutation testing analyses were successful for 1694 KRAS samples and 1821 EGFR samples, while polymorphism testing was successful for 9 CYP2D6 samples, 65 DPD samples and 35 MTHFR samples.*

Conclusions: *Pharmacogenetic methods presented in this paper provide cancer patients in Serbia the best possible choice of treatment at the moment.*

Key words: *EGFR, KRAS, personalized therapy, pharmacogenetics*

Introduction

Pharmacogenetics is defined as the study of how an individual's response to drugs is genetically determined by variations in DNA sequence. Germline mutations in genes that encode for drug-metabolizing enzymes (DMEs), drug transporters, receptors and other molecules play an important role in individual variations of the response and tolerance to drug treatment. Beside germline alterations, somatic mutations acquired in cancer tissues also contribute to treatment outcome in oncology. The aim of pharmacogenetics is to identify the optimal drug and dose for each individual based on his/her genetic constitution, i.e. to individualize drug treatment. This leads to achieving the maximal therapeutic response for each patient, while reducing adverse side effects of the therapy and the cost of treatment.

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KRAS

The idea of establishing a pharmacogenetic service at the Institute for Oncology and IORS dates back to 2008, when the first mutation detection analysis was performed in the *KRAS* gene in patients with metastatic colorectal cancer (mCRC). Patients with mCRC have a very poor prognosis despite many improvements in chemotherapeutic and surgical approaches. Since epidermal growth factor receptor (EGFR) was found to be overexpressed in mCRC, targeted blockage of its signaling pathways with monoclonal antibodies Cetuximab (Erbitux®, ImClone/Merck/Bristol-Myers-Squibb) and Panitumumab (Vectibix®, Amgen) proved to be successful in prolonging the survival of patients [1]. However, Cetuximab was effective only in a subset of patients, so it was important to establish molecular diagnostic tests to define the group that will benefit the most from the treatment. In 2006 it was shown that patients with a mutation in the *KRAS* gene giving rise to a constitutively active KRAS (a downhill EGFR signaling molecule), showed no response to the blockage of EGFR and even had a poorer overall survival (OS) compared to the wild-type *KRAS* patients [2]. Approximately 25-50% of mCRC tumors are known to have a mutated *KRAS* gene. The most frequent mutations in *KRAS* occur in codons 12, 13 (exon 2) and 61 (exon 3) (35-45% of mCRC cases) and lock this effector molecule in the GTP-bound activated form resulting in constitutive signaling.

In 2008, Cetuximab was approved for the treatment of mCRC in Serbia, and *KRAS* mutation testing was included into routine clinical setting in the Laboratory for Molecular Genetics at IORS (certified through external quality assurance by German Society of Pathology and Association of German Pathologists - QuIP). In the following years, this and other molecular genetic tests had been introduced as a part of the project named *˝Establishing pharmacogenomic services for oncology centers in Serbia˝* financed by the Serbian Ministry of Health.

EGFR

In non-small cell lung cancer (NSCLC), which accounts for 80-85% of all lung cancers, 5-year survival rates for advanced-stage patients are very low (less than 15%) on standard platinum-based chemotherapy [3]. In NSCLC, EGFR is overexpressed, so in less than 10 years, two types of drugs, small molecular tyrosine kinase inhibitors (TKIs) [4] and monoclonal antibodies [5] found their way into clinical practice. Two TKIs used for targeted treatment (Gefitinib, Iressa®, AstraZeneca and Erlotinib, Tarceva®, Roche) showed a significantly lower hazard of progression and were less toxic compared with chemotherapy [6]. These TKIs compete with ATP for the ATP-binding site within the intracellular tyrosine kinase domain of EGFR thus blocking its signaling cascade. However, TKIs were found to be effective in patients harboring *EGFR* mutations [7,8], as mutated *EGFR* is more susceptible to binding TKIs. *EGFR* mutations are present in about 10-15% of Caucasians, and are more frequent in women, non-smokers and Asians [9]. In 2011 TKIs were approved in Serbia for the treatment of advanced NSCLC so *EGFR* mutation testing became a prerequisite for all patients who were candidates for this treatment. The Laboratory for Molecular Genetics at IORS was also certified for this analysis through external quality assurance by the European Molecular Genetics Quality Network.

Beside these two main diagnostic analyses, other analyses have also been performed in the Laboratory for Molecular Genetics at IORS.

CYP2D6

Most of the 57 cytohrome P-450 proteins (CYP) have an important role in the metabolism of xenobiotic chemicals, sterols, vitamins, fatty acids and eicosanoids [10]. CYP2D6 is highly polymorphic and it's responsible for the metabolism of about 20-25% of all prescribed drugs [11]. Since the early 1980s, tamoxifen has been the standard approach for reducing the risk of recurrence in women with estrogen receptor (ER) positive early-stage breast cancer. Tamoxifen is metabolized via CYP2D6 into endoxifen (4-OH-N-desmethyl-tamoxifen), its primary active metabolite. Certain CYP2D6 alleles result in the poor metabolizer phenotype, characterized by a decreased ability to metabolize the enzyme's substrates [12]. Since CYP2D6 genotyping prior to tamoxifen treatment has been introduced, an increase in drug efficacy has been noted, and the risk of drug-induced toxicity has been minimized.

DPD

Dihydropyrimidine dehydrogenase (DPD) is the rate-limiting enzyme in the catabolism of pyrimidine bases uracil and thymine. It also affects the metabolism of the pyrimidine analogue 5-fluorouracil (5-FU), one of the most frequently used chemotherapeutic drugs for the treatment of a variety of solid cancers [13]. The 5-FU me-

tabolism leads to creation of 5-fluoro-2'-deoxyuridine-5'-monophosphate (FdUMP), which acts as an inhibitor of the thymidylate synthetase (TS) resulting in the arrest of DNA synthesis [14]. 5-FU is metabolized to its inactive form by DPD which has been reported to be responsible for the degradation of more than 80% of 5-FU [15]. Impaired DPD activity can lead to the accumulation of 5-FU and severe toxicities [16]. The genetic variant in *DPD* gene IVS14+1G>A (DPYD*2A), which is the result of a G to A transition of the invariant splice site in exon 14, leads to skipping of exon 14 immediately up-stream of the mutated splice donor site and formation of a truncated protein with no apparent residual activity. Homozygosity for DPYD*2A can lead to complete DPD-deficiency [13], thus this analysis should precede the administration of 5-FU.

MTHFR

Methotrexate (MTX), an antifolate chemotherapeutic agent, is widely used for the treatment of a variety of adult and childhood cancers, however it is associated with various toxicities [17]. Its cytotoxicity is mainly based on the inhibition of the *de novo* synthesis of purines and pyrimidines [18]. Methylenetetrahydrofolate reductase (MTHFR) is a key enzyme in folate and homocysteine metabolism and is essential for DNA synthesis and DNA methylation. The C677T polymorphism in exon 4 of *MTHFR* gene results in an Ala to Val substitution at codon 222, which codes for the binding site for the MTHFR cofactor, flavin adenine dinucleotide (FAD) and facilitates the separation of the enzyme from its co-factor. Individuals with the *MTHFR* T677T genotype have been shown to have ~30% *in vitro* MTHFR enzyme activity compared with the wild-type, whereas those with the heterozygous CT genotype showed ~60% of wild-type MTHFR enzyme activity [19]. It has been shown that this variant in the *MTHFR* gene increases the risk of both hematopoietic and hepatic MTX-induced toxicities [20], so this analysis should be performed before MTX treatment.

Methods

The procedures in this study were performed in accordance with the ethical standards approved by the Ethics Committee of the IORS and with the Helsinki Declaration of 1975, as revised in 2000.

KRAS testing

From 2008 to 2015 (6 years 1 month), 1694 patients diagnosed with mCRC have been tested for *KRAS* mutations at the IORS. The group consisted of 1083 males (63.9%) and 611 females (36.1%), age range 21-84 (median 63), all of Caucasian descent. Formalin-fixed paraffin-embedded tissue samples (FFPE) were sent from various centers in Serbia and DNA was isolated either by QIAamp DNA FFPE Tissue Kit (Qiagen, Manchester, UK), or by Cobas® DNA Sample Preparation Kit (Roche Molecular Diagnostics, Mannheim, Germany). *KRAS* mutation detection was done by several methods: KRAS StripAssay™, TheraScreen® K-RAS Mutation Kit on Applied Biosystems 7500 Real-Time PCR, Therascreen® KRAS RGQ PCR Kit on Qiagen Rotor Gene Q and Cobas® KRAS Mutation Test on Cobas® 4800 [21,22].

EGFR testing

In the period from 2011 to 2015 (3 years 6 months), 1821 patients diagnosed with NSCLC had been tested for *EGFR* mutations. The group comprised 1114 males (61%) and 707 females (39%), age range 20-88 (median 62) all of Caucasian descent. DNA was isolated from FFPE samples either by QIAamp DNA FFPE Tissue Kit (Qiagen), or by Cobas® DNA Sample Preparation Kit (Roche Molecular Diagnostics). *EGFR* mutation testing was performed by: DxS EGFR Mutation Test Kit on Applied Biosystems 7500 Real-Time PCR, Therascreen EGFR Mutation Detection Kit RGQ on Qiagen Rotor Gene Q and Cobas® EGFR Mutation Test on Cobas® 4800.

CYP2D6 testing

The starting material was DNA obtained from peripheral blood of 9 breast cancer patients on ABI PrismTM 6100 Nucleic Acid PrepStation using BloodPrep Kit (Applied Biosystems, Foster City, CA). *CYP2D6* genotyping was performed by TIB MOLBIOL LightMix® Kit. This kit is able to detect *CYP2D6**3 allele (a frame shift mutation generated by a 1bp deletion in exon 5- 2637delA), *CYP2D6**4 allele that has an incorrect splicing due to transition 1934G>A at the junction intron 3 to exon 4, and *CYP2D6**5 allele which is characterized by a deletion of the entire *CYP2D6* gene. Analysis was performed on Roche LightCycler® 480 II instrument by identifying genotypes through the specific melting points (Tm) recorded during the melting curve analysis after the amplification with specific primers.

DPD and MTHFR genotyping

For *DPD* genotyping, EDTA-blood was taken from 65 patients with different types of solid cancers (breast, gastric, colorectal, oesophageal, peritoneal, head and neck, liver), who were treated with 5-FU. The group consisted of 45 females (69.2%) and 20 males (30.8%), age range 34-81 (median 63). Genomic DNA was extracted by a standard salting out procedure. The DPYD*2A variant of *DPD* gene was investigated by PCR-RFLP. PCR was carried out in 20 μL reaction volume containing

12 μL DreamTaq PCR Master Mix 2x (Thermo Scientific, Darmstadt, Germany), 200 ng of genomic DNA, and 5 pmol of each primer. PCR products were visualized on 2% agarose gel and digested with FastDigest *Nde* I restriction enzyme (Life Technologies, Darmstadt, Germany). After digestion, the fragments were separated on 8% polyacrylamide gel and the gels were silver-stained. The digested mutant allele (IVS14+1G>A) yielded fragments of 154 bp, 27 bp and 17 bp, the wild-type allele yielded 181 bp and 17 bp fragments and the heterozygous allele yielded fragments of 198 bp, 154 bp, 27 bp and 17 bp.

For *MTHFR* genotyping, genomic DNA was extracted by a standard salting out procedure from peripheral blood of 35 patients with osteosarcoma, 20 males (57.1%) and 15 females (42.9%), age range 10-58 (median 24). PCR-RFLP was used for *MTHFR* genotyping as previously described [23].

Statistics

All graphs and images have been prepared using GraphPad Prism V5.04 (GraphPad Software, San Diego, CA) and Adobe Photoshop V9.0 (Adobe Systems Incorporated, CA, USA).

Descriptive analysis included gene and allelic frequencies. Deviations of the allele and genotype frequencies from those expected under Hardy-Weinberg equilibrium were assessed using the standard x^2 test. Statistical significance was set at $p<0.05$.

Results

KRAS

The analysis was successful for all 1694 samples. We tested 240 patients with KRAS StripAssay™, 766 patients with DxS TheraScreen KRAS Mutation kit, 111 patients with Therascreen® KRAS RGQ PCR Kit and 577 patients with Cobas® KRAS Mutation Test. Overall, 743 (43.9%) patients tested positive for mutations in the *KRAS* gene, while 951 (56.1%) patients had wild-type *KRAS* (Figure 1a). A total of

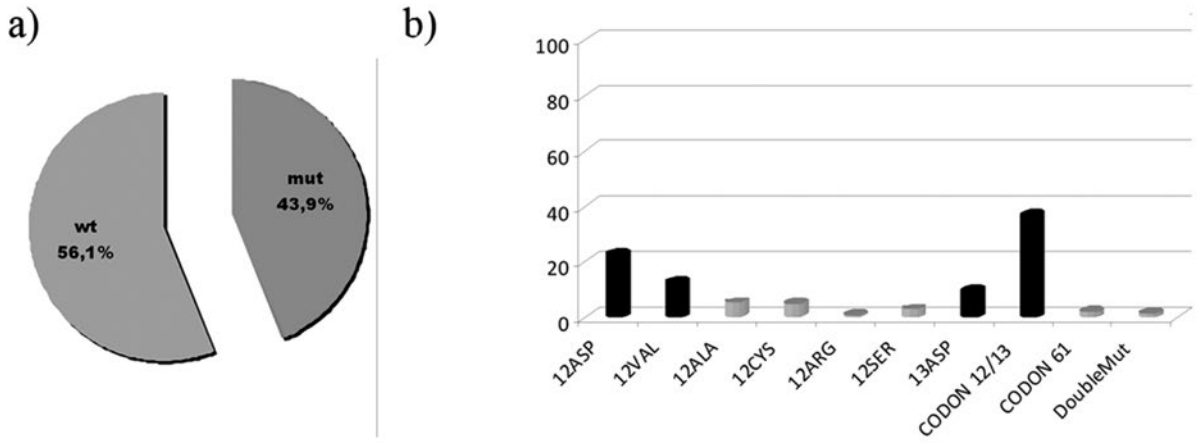

Figure 1. a) Overall *KRAS* mut/wt frequency. **b)** Distribution of *KRAS* mutations (% of total).

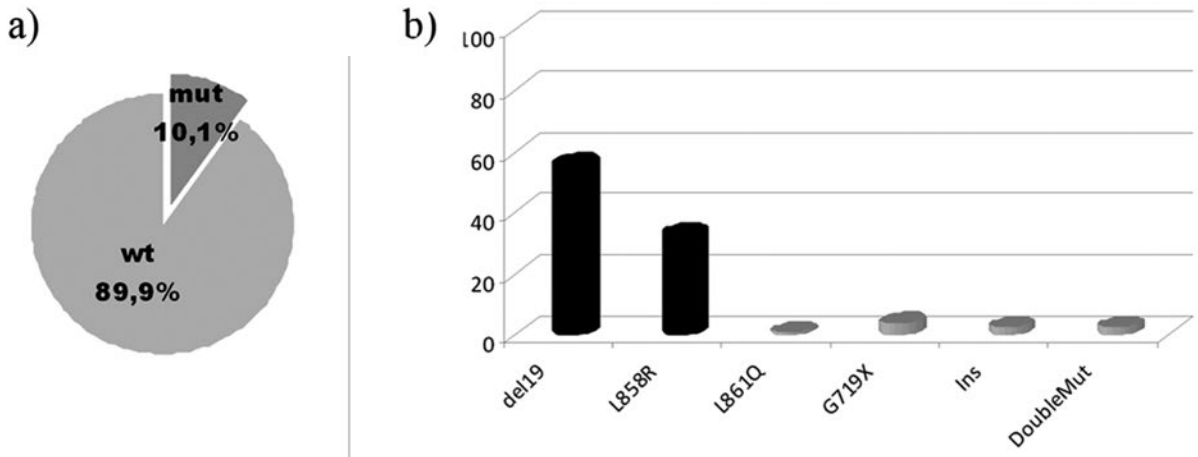

Figure 2. a) Overall *EGFR* mut/wt frequency. **b)** Distribution of *EGFR* mutations (% of total).

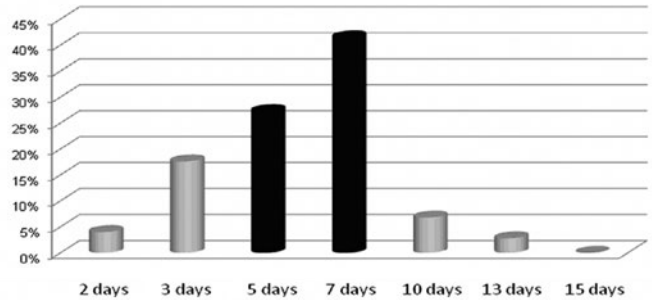

Figure 3. Percentage turnaround time for mutation testing.

728 detected mutations (98%) were in codons 12 or 13 (12ASP, 12VAL, 12ALA, 12CYS, 12ARG, 12SER, 13ASP, 12CYS+12SER, 12CYS+12VAL, 12ASP+12VAL, 12ASP+12VAL+13ASP, 12ASP+13ASP, 12ARG+12ASP, 12SER+12ALA), 14 (1.9%) mutations were in codon 61 and one patient (0.1%) had mutations in both codon 61 and codon 12/13 (Figure 1b).

EGFR

The analysis was successful for all 1821 samples. We tested 779 patients with DxS EGFR Mutation Test Kit, 655 patients with Therascreen EGFR Mutation Detection Kit RGQ and 387 patients with Cobas® EGFR Mutation Test. Overall, 184 (10.1%) patients tested positive for mutations in the *EGFR* gene, while 1637 (89.9%) patients had wild-type *EGFR* (Figure 2a). A total of 164 (89.1%) detected mutations were the two most commonly present mutations, deletion in exon 19 (104, 56%) and point mutation L858R in exon 21 (60, 33%). Other mutations were less present (11% total), of which: L861Q 2 samples, G719X 8 samples, exon 20 insertion 5 samples and double mutants L861Q/G719X 1 sample and G719X/S768I 4 samples (Figure 2b). In the mutated group, 124 (67%) were women ($p<0.001$).

The average turnaround time for *KRAS* and *EGFR* analyses in the diagnostic setting was 7 days from the arrival of FFPE tissue samples to our Laboratory (Figure 3).

CYP2D6

The analysis was successfully performed on 9 patients so far. Only one of the patients had *CYP2D6**4 homozygote genotype which places her into the poor metabolizer phenotype group since this mutation abolishes CYP2D6 enzymatic activity. The rest of the patients had wt genotype for *CYP2D6**3, *CYP2D6**4 and *CYP2D6**5 alleles (Figure 4).

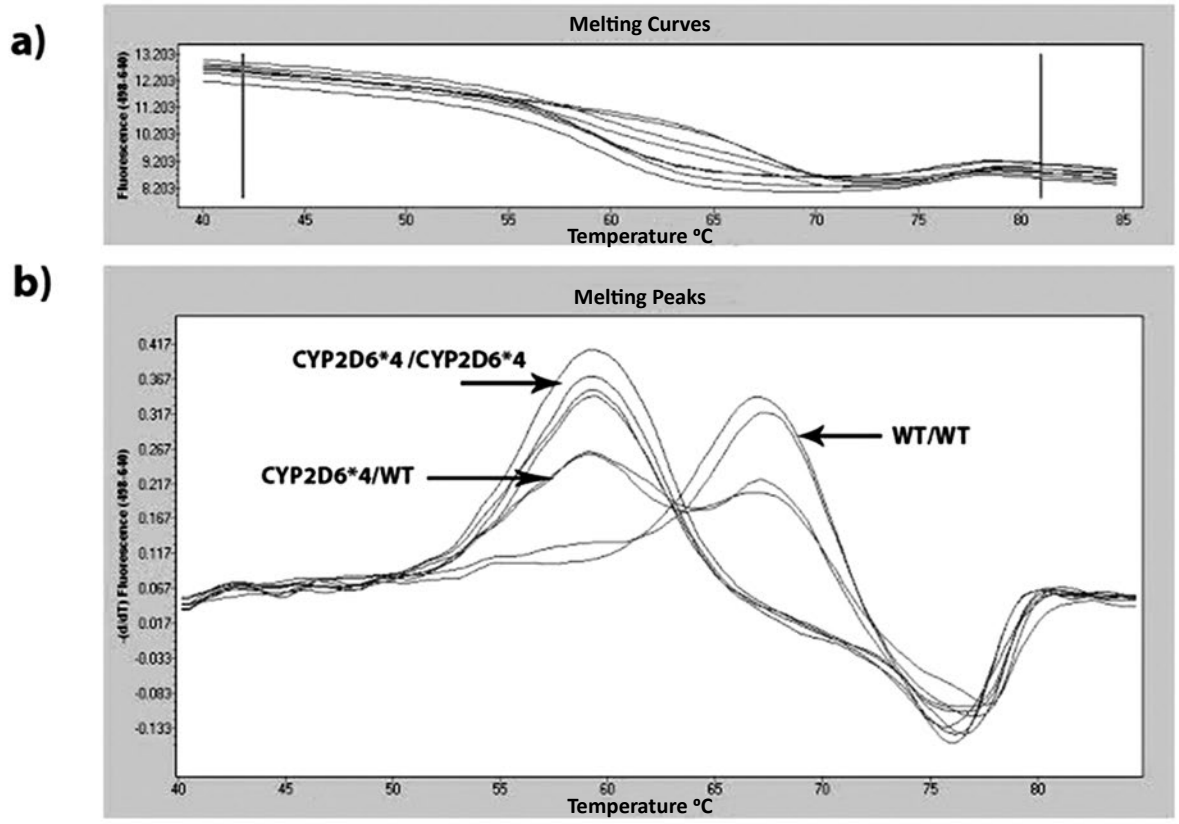

Figure 4. HRM *CYP2D6* analysis. **a)** Normalized and shifted melting curves. **b)** Normalized and temperature-shifted melting peaks.

DPD

PCR-RFLP analysis was successful for all 65 patients. We identified 62 patients without the DPYD*2A variant (GG) and 3 who were heterozygous (GA) for this variant. The wild-type genotype (GG) and allele (G) frequencies of the *DPD* gene were 95.4% and 97.7%, respectively, while the heterozygous genotype (GA) resulted in a frequency of 4.6%. No individuals homozygous for the DPYD*2A variant were detected, resulting in an A allele frequency of 2.3% (Table 1).

Table 1. Genotype and allele frequencies of the DPYD*2A variant among cancer patients

Polymorphism	*Patients (N=65) N (%)*
IVS14+1G>A	
GG	62 (95.4)
GA	3 (4.6)
AA	0 (0)
Alleles	
G	97.7%
A	2.3%

MTHFR

PCR-RFLP analysis was successful for all 35 osteosarcoma patients. *MTHFR* genotyping resulted in 677T and 677C allele frequency of 31.4% and 68.6%, respectively. The frequencies of wild-type CC, heterozygous CT and mutant TT genotypes were 45.7, 45.7 and 8.6%, respectively (Table 2).

Table 2. Genotype and allele frequencies of the MTHFR C677T gene polymorphisms in patients with osteosarcoma

	Patients (N=35) N (%)
Polymorphism	
CC	16 (45.7)
CT	16 (45.7)
TT	3 (8.6)
Alleles	
C	68.6%
T	31.4%

Discussion

KRAS

Traditional *KRAS* mutation testing in mCRC focused only on mutations in codons 12 and 13 of exon 2. Patients who had exon 2 wild-type *KRAS* tumours were treated with anti-EGFR monoclonal antibodies because others were unlikely to benefit. Extended *KRAS* testing revealed additional mutations (exons 3 and 4) in about 15% of what were previously believed to be wild-type *KRAS* patients. Today we should not talk about testing for *KRAS* but rather about *All RAS* testing. Europe has already made the move and the European Medicine Agency has modified its authorization for anti-EGFR agents to exclude patients with any *RAS* mutations. The NCCN guidelines (*http://www.nccn.org/professionals/physician_gls/f_guidelines.asp*) now include all *RAS* testing as mandatory and recommend *BRAF* testing to be performed after detection of *RAS* wild-type status.

The number of mCRC patients sent to IORS for *KRAS* testing in a 5-year period (2008-2013) was 990 (58%), while the number for the next two and a half years (2013 - May 2015) is 704 (42%) which can be explained by the increased incidence of mCRC in Serbia and improved awareness. From 2008 to May of 2015, 743 (41.5%) *KRAS* mutations were detected in Serbian mCRC patients which is in accordance with previously reported mutation rate in Caucasians [24]. Of all *KRAS* mutation carriers, 62% were male, which was also in accordance with literature data.

EGFR

Epidemiologically, lung cancer burden in Serbia (population around 7 million) mirrors the situation in the world, with approximately 5200 of newly diagnosed patients and around 4600 deaths of lung cancer each year [25]. Deletion in exon 19 and point mutation L858R in exon 21 of *EGFR* account for over 85% of all mutations, and these activating mutations bring the highest sensitivity to TKIs. Other, so-called rare mutations, activate EGFR to some extent, but bring limited sensitivity to TKIs, which must be stated on the mutation result [26].

EGFR testing was employed in 2011 in Serbia and so far 1821 patients have been successfully tested. The mutation rate is 10.1%, which is in accordance with literature data for Caucasians [9]. In the mutated group, 67% were women ($p<0.001$), which has also previously been reported as significant for Caucasians, and over 89% of mutations were deletion in exon 19 and point mutation L858R in exon 21. Rare mutations were present in around 11 % of the cases, of which G719X was the most common one (~4.5%).

CYP2D6

Tamoxifen reduces the recurrence rate of breast cancer by blocking ERs [27] and exerts its effects through its metabolites, particularly endoxifen [28]. CYP2D6 is the predominant CYP isoform that catalyses the formation of endoxifen [29] and there is a strong association between *CYP2D6* genotypes and plasma levels of endoxifen. The poor metabolizing (PM) phenotype is represented by two nonfunctional alleles and has been clinically associated with reduced formation of active tamoxifen metabolites, which causes therapy ineffectiveness and increased toxicity. Two-year survival without relapse in patients treated with tamoxifen who are poor metabolizers was 68%, while that of normal metabolizers was as high as 98% [30]. Beside poor metabolizers there are also intermediate metabolizers (IMs) with one wild-type allele and one allele inactivated by mutation which also have reduced ability to metabolize tamoxifen to its active intermediates. Patients with PM or IM phenotypes should either be transferred to alternative therapy (e.g. aromatase inhibitors) or their tamoxifen dose should be adequately adjusted.

CYP2D6 genotyping is the most recent pharmacogenetic test introduced in the Laboratory for Molecular Genetics at IORS. So far, 9 breast cancer patients were sent to our Laboratory for *CYP2D6* genotyping and only one had the PM phenotype. This patient was female, diagnosed with the invasive lobular breast cancer at the age of 47. She was informed about her *CYP2D6* status and about the impairment of CYP2D6 enzyme due to the homozygous mutation in this gene. She decided to switch to aromatase inhibitors treatment instead of tamoxifen due to her PM phenotype.

DPD

Around 25% of all cases of unexpected severe 5-FU toxicity are due to the presence of invariant GT splice donor site flanking exon 14 (IVS14+1G>A) in the *DPD* gene, though its incidence is rare with a population frequency of 0.9–1.8% heterozygotes. Patients identified with mild- to severe deficiency should have their 5-FU dose reduced. Total deficiency should result in other treatment regimens which do not include 5-FU [31].

The prevalence of the DPYD*2A variant is low in the Caucasian population (0.9%) [32]. In the present study only 3 patients (4.6%) carried this variant in their genome (in the heterozygous state). Such a low frequency in patients with severe 5-FU toxicity contrasts the data from two studies [14,32] that reported 24% and 28% DPYD*2A frequency in selected patients with 5-FU toxicity. However, the absence of this mutation in patients with severe 5-FU toxicities was also reported [33,34]. This discrepancy may be due to the different ethnic origins of patients or to the small sample size. Among 65 patients screened for DPYD*2A in our study, 62 developed 5-FU-related toxicities but did not carry this variant. Our data suggest that screening for DPYD*2A alone in the Serbian population may have limited effectiveness in identifying patients at risk of lethal 5-FU toxicity since some patients with normal DPD activity may also develop life-threatening toxicities.

MTHFR

The T allele of the C677T *MTHFR* genetic variant is associated with decreased activity of the MTHFR enzyme, resulting in low folate levels which may have a significant effect on the response of malignant and non-malignant cells to MTX, whose activity depends on cellular composition of folate [35]. Determining C677T *MTHFR* genotype helps identify patients who are at risk of severe treatment toxicity. MTX dosage reductions allow for designing the effective individual chemotherapeutic regimes and avoiding MTX-induced toxicity [36].

MTHFR C677T genotyping was successfully performed in all 35 osteosarcoma patients on MTX therapy sent to our Laboratory. All patients experienced MTX intolerance requiring dose modification and temporary MTX withdrawal. However, only 3 patients were recessive T677T homozygotes which indicated the existence of other mechanisms contributing MTX toxicity. Our previous study [23] showed low frequency of T677T genotype among patients with chronic myeloid leukemia (9.6%) as well as in healthy controls (13.2%) which indicates a generally low frequency of the recessive T allele in the Serbian population. Considering this, and the fact that heterozygotes did not show difference in terms of toxicity compared with wild-type patients, pretreatment screening for this polymorphism in Serbia may not be worthwhile.

Conclusions

The struggle for expanding the knowledge of the underlying genetic mechanisms in cancers will improve the drug development process

and will enable better prediction of the potential toxicity of new drugs. Pharmacogenetic methods presented in this article provide cancer patients in Serbia the best possible choice of treatment at the moment. However, emerging technologies such as digital PCR, the use of circulating nucleic acids for biomarker detection that is not limited to the tumour biopsy and the use of targeted gene panels, are future perspectives that we are relentlessly trying to introduce into everyday laboratory practice in Serbia.

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Conflict of interests

The authors declare no confict of interests.

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