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Prospective study on diagnostic and prognostic significance of postoperative FDG PET/CT in recurrent colorectal carcinoma patients: comparison with MRI and tumor markers

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Current guidelines for follow-up after resection of colorectal cancer (CRC) recommend regular measurements of carcinoembryogenic antigen (CEA) and imaging tests. Multidetector computed tomography (MDCT) and magnetic resonance imaging (MRI) are currently primary imaging modalities, while the role of fluorine-18-fluoro-deoxyglucose positron emission tomography/computed tomography (^{18}F-FDG PET/CT), which is recommended in patients with negative MDCT and increased CEA, is still uncertain. Our aim was to compare diagnostic performance and prognostic significance of ^{18}F-FDG PET/CT with MRI and tumor markers CEA and carbohydrate antigen 19-9 (CA 19-9) in detection of recurrent CRC. This prospective study included 35 patients with resected CRC, referred to ^{18}F-FDG PET/CT examination for suspected recurrence. During median follow-up of 24.4±1.5 months ^{18}F-FDG PET/CT and MRI results and tumor marker levels were compared with findings of histopathological examination or with results of clinical and imaging follow-up. Management plan before the ^{18}F-FDG PET/CT scan was considered and compared to the final treatment decision. The sensitivity, specificity, positive and negative predictive value and accuracy of ^{18}F-FDG PET/CT and MRI in detection of recurrent colorectal cancer in patient-based analysis were 92.6%, 75%, 92.6%, 75% and 88.6%, and 65.4%, 66.7%, 85%, 40% and 65.7%, respectively. In lesion-based analysis the sensitivity of ^{18}F-FDG PET/CT and MRI was 83.1% and 68.2%, respectively. The overall accuracy of CEA and CA 19-9 in recurrence detection was 48.6% and 54.3%, respectively. PET/CT induced therapy alterations in 13/35 (37.1%) patients. Progression was observed in 16/35 patients during follow-up, with significantly lower risk of progression in patients with treatment changes based on PET findings (Multivariate Cox regression; p=0.017). In addition, elevated CA 19-9 levels in time of PET scan and male gender carried significantly higher risk of progression (p=0.007 and p=0.016, respectively). Kaplan-Meier Log rank test showed significantly longer progression-free survival time in patients who had treatment plan changed based on PET/CT (p=0.046). We can conclude that ^{18}F-FDG PET/CT showed better sensitivity and accuracy compared to MRI in detection of recurrent colorectal cancer, with much better sensitivity compared to CEA and CA 19-9. Patients with treatment changes based on ^{18}F-FDG PET/CT had significantly better prognosis and longer progression-free survival, while elevated values of CA 19-9 and male gender were associated with worse prognosis.

Key words: FDG PET/CT, colorectal cancer, magnetic resonance imaging, tumor markers, prognosis

Colorectal cancer (CRC) is third among all types of cancer based on incidence and mortality, in males and females both [1]. Although mortality is decreasing by about 3% per annum, changes in risk factors and screening measures account for 88% of the improvement, while advances in therapy are responsible for mere 12% [2]. One of the reasons for such response could be the high frequency of relapses after seemingly curative procedures [3].

Current guidelines for managing patients with CRC after therapy recommend regular measurements of serum carcinoembryogenic antigen (CEA) and imaging tests [4]. CEA, although sensitive in detecting early relapse, gives very often a false-positive result [5]. The role of carbohydrate antigen 19-9 (CA 19-9) in detection of recurrent CRC is still unclear, although there have been studies suggesting its potential prognostic significance [6]. In addition, increased CEA and CA

19-9 values require localization of recurrent disease, which is done with imaging studies [7]. For diagnosis and follow-up of CRC, following modalities are used: multidetector computed tomography (MDCT), magnetic resonance imaging (MRI), and fluorine-18-fluoro-deoxyglucose positron emission tomography/computed tomography (^{18}F-FDG PET/CT). MDCT and MRI are currently primary imaging modalities, by which the progression of colorectal carcinoma is established, while ^{18}F-FDG PET/CT is recommended in patients with negative MDCT and otherwise unexplained CEA increase [4]. In contrast to MDCT and MRI, which use anatomic parameters (lesion size) to evaluate therapy outcome, ^{18}F-FDG PET/CT quantifies functional change, a better predictor to disease development according to some authors [8].

Nevertheless, the status of ^{18}F-FDG PET/CT in evaluation of therapy response in colorectal carcinoma, among other imaging modalities, as well as in different phases of disease, is still uncertain [8,9,10]. Moreover, there is an unsatisfied need for determining the optimal sequence in utilizing different imaging modalities on suspicion of metastasis, in effort to avoid unnecessary interventions, increase the effect of therapy and raise the survival rate [4].

The aim of this prospective study was to compare diagnostic performance of imaging tools (^{18}F-FDG PET/CT and MRI) and biochemical markers (CEA and CA 19-9) in detection recurrent colorectal cancer in clinical practice, with emphasis on impact of ^{18}F-FDG PET/CT on further treatment. The second aim was to assess prognostic significance of imaging and biochemical tests for progression-free survival in recurrent colorectal cancer.

Patients and methods

Study population. This prospective study included consecutive patients with resected colorectal adenocarcinoma referred to ^{18}F-FDG PET/CT examination at National PET Center, Clinical Center of Serbia, Belgrade, from May 2011 to February 2015. The inclusion criteria were: resected colorectal adenocarcinoma and period of at least 6 months after resection, MR imaging of abdomen and pelvis one month prior to PET/CT, follow-up of at least 24 months and glucose level not greater than 11.0 mmol/l. The exclusion criteria included pregnancy and the presence or history of another type of malignancy. The final sample consisted of 35 patients (14 females and 21 males, mean age 60.6±10.6 years).

Procedures. The ^{18}F-FDG PET/CT examination was performed when patients had symptoms and signs suggesting loco-regional recurrence or liver metastases, abnormal or equivocal contrast-enhanced MDCT and/or elevated tumor marker levels. One month prior to ^{18}F-FDG PET/CT all patients underwent MRI imaging and measurements of serum levels of CEA and CA 19-9. During 24 months of follow-up clinical data, results of imaging tests and laboratory data were collected and evaluated. Findings of ^{18}F-FDG PET/CT and MRI were compared with findings of histopathological examination or with results of subsequent clinical and imaging follow-up. Management plan before the^{18}F-FDG PET/CT scan was considered and compared to the final decision for treatment after the PET/CT scan. The primary end-point was progression-free survival, based on imaging findings, clinical examination and/or cancer related death. After PET/CT examination, patients were followed for at least 24 months, with a mean follow-up time of 24.4±1.5 (range 24-32 months). The study was approved by the Ethics Committee of the Faculty of Medicine of the University of Belgrade and written consent was obtained.

Data acquisition and interpretation. The patients underwent ^{18}F-FDG PET/CT examination on a 64-slice hybrid PET/CT scanner (Biograph, TruePoint64, Siemens Medical Solutions, Inc. USA) at National PET Center, Clinical Center of Serbia, Belgrade. After fasting for 6h patients received an intravenous injection of 5.5MBq/kg of ^{18}F-FDG. Following injection of ^{18}F-FDG, patients rested in a quiet and darkened room for 60min, after which images of PET/CT were obtained. Low-dose non-enhanced CT scans (120kV with automatic, real-time dose-modulation amperage, slice thickness of 5mm, pitch of 1,5 and a rotation time of 0.5s) and 3-dimensional PET scans (6-7 fields of view, 3min/field) were acquired from the base of the skull to the mid-thigh. Non-corrected and attenuation-corrected CT, PET and fused PET/CT images were displayed for analysis on a Syngo Multimodality workplace (Siemens AG).

Any lesion with high ^{18}F-FDG uptake on PET/CT was defined as positive for malignancy if any abnormal ^{18}F-FDG uptake was observed after exclusion of benign and physiological lesions, with or without clearly visible corresponding CT malformation. Lesions were analyzed qualitatively and semi-quantitatively. For assessment of glucose metabolism level in metastasis, SUVmax was used, that is singular voxel within volume of interest with maximal standard uptake value, calculated as: activity in tissue (count/pixel/s) multiplied by calibration factor and divided by dose applied (MBq/kg of body weight). Tumor lesions were defined by volume of interest (VOI) placed around every suspected focus of intense FDG uptake, with 50% threshold. The measurements of SUVmax, were done on reconstructed images, after using ordered subsets expectation maximization (OSEM) as statistical reconstruction method, but no absolute cut-off value of SUVmax was used for the diagnosis. Images were interpreted separately by two nuclear medicine physicians, unaware of results of other imaging modalities. In cases of discrepancy, images were presented to multidisciplinary team and experts' opinion was adopted.

PET/CT findings were compared to MR imaging findings. T1 weighted, T2wieghted, diffusion-weighted images (DWI), as well as contrast enhanced images of abdomen, pelvis and basal lungs were acquired in all patients, and were interpreted by experienced radiologists, unaware of PET/CT results. Characterization of lesions was based on standard evaluation criteria by visual characteristics, and all lesions were classified as malignant, benign or equivocal. Equivocal lesions were considered as false-positives or false-negatives after comparison with the gold standard.

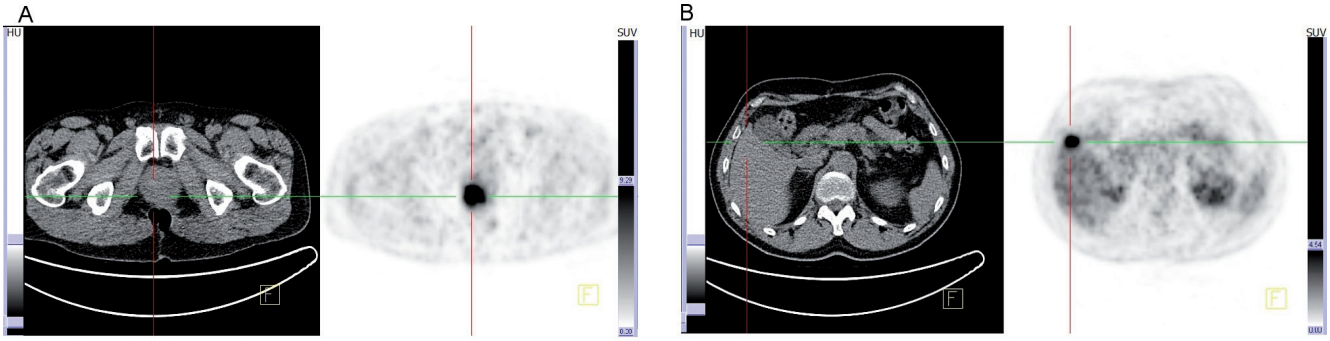

Figure 1. (A) CT and PET image showing local recurrence of rectal cancer in 55 year-old male patient (CEA 6 ng/ml; CA 19-9 97 U/ml); (B) CT and PET image of liver metastasis of CRC in 57 year-old male patient with elevated CEA (50 ng/ml) and normal CA 19-9

Final diagnosis of recurrent disease was made either by histopathological examination, or imaging follow-up during first six months after PET/CT. Histopathological confirmation was obtained in 14 patients, out of who in 10 patients verification came after surgical treatment, while in 4 patients diagnosis was made after biopsy of suspicious lesion. In 18 patients definitive diagnosis was made according to results of imaging and clinical-laboratory results, while in three patients diagnosis was based on clinical and laboratory data (cancer related death or significant tumor marker increase). In patient-based analysis, the imaging study was defined as true-positive (TP) when at least one of suspected lesions was histopathologically confirmed to be malignant or responded to therapy. The ^{18}F-FDG PET/CT study without abnormal ^{18}F-FDG uptake and MRI studies without suspected lesions, if remained so during first six months, were considered as true-negatives (TN). A false-positive (FP) imaging study showed at least one lesion characterized as malignant, but without evidence of disease in first six months. Finally, false-negative (FN) studies had evidence of recurrence on further examination during the first six months despite negative imaging studies at first. In lesion-based analysis all detected lesions by different imaging modalities were compared to reference examination (histopathology or imaging) during first six months after PET/CT and were separately defined as TP, TN, FP or FN.

Progression of the disease was considered in cases of detection of new lesions during follow-up; increase of the existing lesions in size and/or in metabolic activity in any imaging modality; significant increase of tumor marker levels and/or in cases of disease-related death. The date of progression was noted and thus, the progression-free survival time was calculated from the day of PET/CT scan.

Table 1. Demographic and clinical characteristics of patients included in the study (N=35)

Gender	Male	21
	Female	14
Age	Mean 60.6±10.6	
	<61	15
	≥61	20
Localization of primary tumor	Rectum	13
	Colon	22
Chemo-radiotherapy	Yes	31
	No	4
CEA	Normal	20
	Increased	15
CA 19-9	Normal	20
	Increased	15
MRI	Positive	20
	Negative	15
^{18}F-FDG PET/CT	Positive	27
	Negative	8
Progression during follow-up	Yes	16
	No	19

CEA: Carcinoembryogenic antigen; CA 19-9: Carbohydrate antigen 19-9; MRI: Magnetic resonance imaging; ^{18}F-FDG PET/CT: Fluorine-18-fluorodeoxyglucose positron emission tomography/computed tomography

Statistical analysis. The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and diagnostic accuracy of ^{18}F-FDG PET/CT, MRI and tumor markers in detection of recurrent CRC were calculated. The sensitivity of ^{18}F-FDG PET/CT and MRI was determined in lesion-based analysis. Cox proportional hazards regression model was used to determine whether various demographic and clinical factors, such as age (≤61 vs >61), gender (male vs female), CEA and CA 19-9 levels (normal vs increased), MRI results (positive vs negative), ^{18}F-FGD PET/CT results (positive vs negative), and treatment changes based on PET/CT results (yes vs no) were associated with the higher risk of progression of the disease during follow-up. These analyses consisted of determination of hazard ratios (HR) for all factors with 95% confidence interval (CI). Survival analyses were performed using Kaplan-Meier method, and the groups were compared using the Log-rank test. A P value of less than 0.05 was considered significant.

Results

The demographic and clinical characteristics of patients included in the study are given in Table 1.

Patient-based analysis. ^{18}F-FDG PET/CT was positive in 27/35 patients, suggesting recurrent disease, with two patients with false-positive findings. Local recurrence was suggested in 12 patients (Figure 1A), liver metastases in 13 cases (Figure1B), and extra-hepatic disease (lung, peritoneum, bone) in 12 patients (Table 2.). In six out of eight cases with negative PET/CT the presence of disease was not confirmed in follow-up (true-negatives). There were two patients with false-negative findings. Overall, the sensitivity, specificity, positive and negative predictive value and accuracy of ^{18}F-FDG PET/CT were 92.6%, 75%, 92.6%, 75% and 88.6%, respectively.

MRI suggested recurrent disease in 20/35 patients, with 3 cases being false-positive. Local recurrence was observed in 7 patients, liver metastases in 12 cases, and supra-diaphragmal pulmonary metastasis in three patients. In 9/15 patients with normal MRI findings, subsequent histopathological and/or imaging examination detected recurrence, suggesting these patients were false-negative. In patient-based analysis the sensitivity, specificity, positive and negative predictive value and accuracy of MRI in detection of recurrent colorectal cancer were 65.4%, 66.7%, 85%, 40% and 65.7%, respectively.

CA 19-9 was elevated in 15/35 patients, with values ranging from 39 to 660 U/ml, while in 20 patients CA 19-9 values were within reference levels. In regard to imaging tests, among patients with abnormal MRI (20 patients), CA 19-9 was elevated in 9, and normal in 11 patients. CA 19-9 was elevated in 12/27 patients with positive ^{18}F-FDG PET/CT scan. The sensitivity, specificity, PPV, NPV and accuracy of CA 19-9 in detection of recurrent colorectal cancer were 48.1%, 75%, 86.7%, 30% and 54.3%, respectively.

In addition, abnormal values of CEA were also recorded in 15/35 patients, ranging from 5.1 to 711 ng/ml. CEA was elevated in 9/20 patients with abnormal MRI scan, and in 13/27 patients with pathological PET/CT results. The sensitivity, specificity, PPV, NPV and accuracy of CEA in detection of recurrent colorectal cancer were 44.4%, 62.5%, 80%, 33.3% and 48.6%, respectively.

Lesion-based analysis. The total number of lesions detected by ^{18}F-FDG PET/CT was higher compared to MRI. PET/CT identified 84 sites of intensive FDG uptake with suspicion of recurrence, out of which 19 were not confirmed during follow-up (false-positives). However, 14 new sites of recurrent disease were subsequently detected, and these were considered as PET false-negative lesions. Overall, the sensitivity of ^{18}F-FDG PET/CT in lesion-based analysis was 83.1%. MRI detected 53 lesions, overall, out of which 45 lesions were confirmed in follow-up (true-positives). The sensitivity of MRI in lesion-based analysis was 68.2%.

Ten patients were upstaged according to ^{18}F-FDG PET/CT result, out of whom 8 patients were upstaged from no disease to stage I/II (4 patients) or stage IV (4 patients). In two patients PET scan showed distant metastases in addition to local recidive, while in 3 cases extra-hepatic disease was identified on PET, in addition to liver metastatic disease seen on MRI. In 3 patients local recidive was observed on PET, beside known liver metastases. Two patients were down-staged, because although MRI suggested liver disease in one patient and local recurrence in another, ^{18}F-FDG PET/CT scan was negative. Overall, ^{18}F-FDG PET/CT changed the stage of disease in 12/35 patients.

Table 2. ^{18}F-FDG PET/CT results

Result	n
Negative	8
Local recurrence	7
Local recurrence + liver	3
Local recurrence + extra-hepatic disease	2
Liver	5
Liver + extra-hepatic disease	5
Extra-hepatic disease	5

^{18}F-FDG PET/CT and treatment changes. In 7 cases surgical treatment and/or chemo-radiation was applied according to PET findings. In three cases, however, the presence of disseminated disease seen on PET/CT prevented unnecessary and futile surgical treatment. In one patient the planned surgical treatment was altered, with the widening of surgical field since PET detected new lesions in the liver. Finally, in two patients the metastatectomy of liver metastases was accompanied with chemotherapy, because PET/CT detected extra-hepatic disease. In 3 cases treatment was not changed, despite discordance in MRI and PET results. Overall, ^{18}F-FDG PET/CT induced therapy alterations in 13/35 (37.1%) patients.

Progression-free survival. Progression of the disease was observed in 16/35 patients during follow-up. In 12 patients, progression was observed on imaging follow-up, with the clinical data suggesting progression in four of these patients (increase of tumor markers). In three patients progression was considered because of cancer-related death, while in one patient progression was considered due to significant increase of tumor markers. Possible factors associated with disease progression are presented in Tables 3 and 4. These factors

Table 3. Factors influencing possible progression of the disease during follow-up (Univariate Cox proportional hazardous model)

	Univariate Cox regression		
Variable	HR	95% CI	p
Age	0.94	0.35-2.53	0.907
Gender	2.95	0.94-9.18	0.063
CEA	1.34	0.50-3.58	0.558
CA 19-9	2.56	0.95-6.90	0.063
MRI	1.61	0.56-4.65	0.376
^{18}F-FDG PET/CT	4.81	0.64-36.52	0.128
Treatment change	0.30	0.09-1.06	0.062

CEA: Carcinoembryogenic antigen; CA 19-9: Carbohydrate antigen 19-9; MRI: Magnetic resonance imaging; ^{18}F-FDG PET/CT: Fluorine-18-fluorodeoxyglucose positron emission tomography/computed tomography

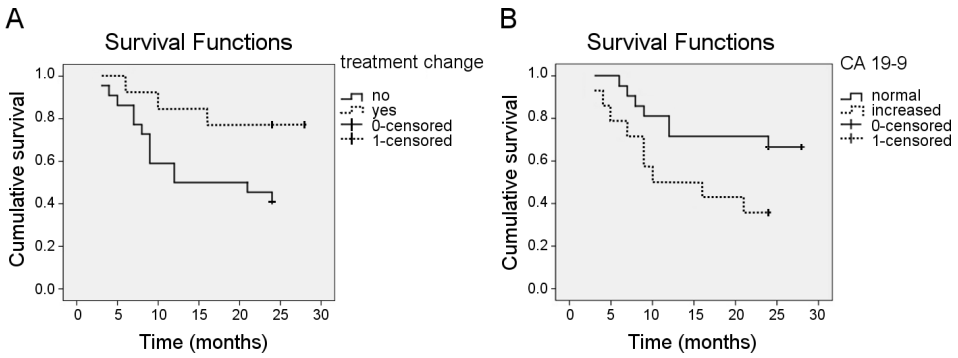

Figure 2. (A) Kaplan-Meier analysis of progression-free survival in patients with the change of planned treatment based on 18F-FDG PET/CT results compared to those without treatment changes (Log-rank test; p=0.046); (B) Kaplan-Meier analysis of progression-free survival between patients with normal and elevated CA 19-9 levels (Log-rank test; p=0.051)

were included in univariate and multivariate Cox regression analysis.

Univariate Cox regression analysis revealed that there was higher risk of progression in patients with increased CA 19-9 levels, as well as in male patients, with lower risk of progression in patients in whom PET/CT influenced on treatment changes, but without statistical significance (p=0.063, p=0.063 and p=0.062, respectively).

However, when all possible influencing factors were included in multivariate analysis, risk of progression was significantly higher in patients with elevated CA 19-9 levels (HR 4.81; CI95% 1.54 – 15.01; p=0.007), as well as in male patients (HR 6.02; CI95% 1.41 – 25.76; p=0.016). Moreover, the risk of diseases progression became significantly lower for those with treatment changes after PET/CT scan (HR 0.15; CI95% 0.03 – 0.71; p=0.017).

Median progression-free survival times in patients with and without treatment alterations after PET/CT scan were 24 and 16.5 months, respectively. Kaplan-Meier analysis, using Log rank test, showed significantly longer progression-free survival time in patients who had treatment plan changed based on PET/CT compared to those in whom treatment was not changed (p=0.046) (Figure 2A).

Progression-free survival was worse in patients with elevated CA 19-9 levels, but this difference did not reach statistical significance (Kaplan-Meier, Log rank test; p=0.051) (Figure 2B).

Table 4. Factors influencing possible progression of the disease during follow-up (Multivariate Cox proportional hazardous model)

	Multivariate Cox regression		
Variable	HR	95% CI	p
Age	1.50	0.43-5.26	0.528
Gender	6.02	1.41-25.76	0.016*
CEA	1.14	0.33-3.90	0.834
CA 19-9	4.81	1.54-15.01	0.007*
MRI	0.91	0.23-3.52	0.889
^{18}F-FDG PET/CT	6.71	0.72-62.63	0.095
Treatment change	0.15	0.03-0.71	0.017*

CEA: Carcinoembryogenic antigen; CA 19-9: Carbohydrate antigen 19-9; MRI: Magnetic resonance imaging; ^{18}F-FDG PET/CT: Fluorine-18-fluoro-deoxyglucose positron emission tomography/computed tomography *p<0.05

Discussion

This study showed higher accuracy and sensitivity of ^{18}F-FDG PET/CT in detection of recurrent colorectal cancer, compared to MRI, with much better diagnostic performance compared to CEA and CA 19-9. The significant impact of ^{18}F-FDG PET/CT based treatment changes and CA 19-9 levels on prognosis of our colorectal cancer patients was noticed.

The diagnostic strength of ^{18}F-FDG PET/CT in detection of recurrent colorectal cancer was proven through number of studies [11, 12]. Contrary to authors suggesting higher specificity of PET in visualization of CRC recurrence [13], our results showed higher sensitivity of this imaging modality (92.6% vs 75%), with only two false-negative studies. In these two patients presence of disease was confirmed despite negative PET/CT scan: in one patient significant increase of tumor markers was noted on first follow-up visit and in another patient metastasis in illiac bone was seen on first follow-up MRI. Also, there were two false positive cases of local recidive and liver metastases, later histopathologically proved to be benign.

The suggested follow-up of patients after resection with curative intent includes serial CT of chest, abdomen and pelvis, as well as regular serum CEA measurements [4]. In addition, intraluminal recurrences can be detected with recto/colonoscopy. However, in large number of cases detection of extra-luminal recurrence and differentiation of postoperative changes from recurrence can be made by imaging tests, with MRI being method of choice by some authors [14], while others stress the role of PET/CT, especially in detection of lymph node metastases [15].

When metastases to the liver are suspected, ^{18}F-FDG PET/CT and MRI are methods most frequently employed in further treatment decision. Whether ^{18}F-FDG PET/CT, which can detect more easily extra-hepatic dissemination, or MRI, which can accurately characterize liver lesions, will be employed depends on various factors (e.g. cost, availability) [4].

In lesion-based analyses, our results also showed higher sensitivity of ^{18}F-FDG PET/CT compared to MRI (83.1% vs 68.2%). This in contrast with other studies [12], although some authors suggest better sensitivity of combined use of PET and contrast-enhanced CT [16]. Most of the lesions observed on PET scan in our study patients were confirmed as recurrence. Nineteen lesions suggested on PET were false-positive (three loco-regional lesions, 5 liver lesions, and 11 suspected lymph nodes), while 14 lesions were missed on PET overall, out of which 8 lesions were in liver, three in lung, one in adrenal gland, one in abdominal lymph node and one in illiac bone. Although, MRI observed lower number of lesions overall compared to ^{18}F-FDG PET (53 vs 84), including false-positive lesions (8 vs 19), there was larger number of missed lesions (21 vs 14). It should be noted that in MRI lesion-based analysis we did not take in account lesions in upper lung and mediastinum, identified on PET in four patients. However, this finding did not influence final result, since there were already present distant metastases, so stage of the disease as well as planned treatment were not changed in these patients. Recent study by Oh et al [17] showed equal sensitivity of PET and gadoxetic acid – enhanced liver MRI in detection of large liver lesions (>2cm), and significantly higher sensitivity of MRI in detection small lesions (<2cm). However, lesion size was not included in calculation of sensitivity in our study, and the number of lesions in the liver missed on PET and MRI was comparable (9 vs 10).

Serial measurements of CEA present inevitable surveillance tool in patients after resection of colorectal cancer. Although the sensitivity was proven in number of studies, it largely depends of threshold values, suggesting better sensitivity with lower cut-off values [18]. In our study, with referent values of 5.0 ng/ml for smokers, and 3.5 ng/ml for non-smokers, CEA showed moderate sensitivity in detection of recurrence. CEA level was increased in around 50% (13/27) of our patients with PET positive findings, which not completely concurred with published results [19]. In addition, CA 19-9 showed low sensitivity (just below 50%), which is in keeping with results of other studies performed on large cohorts [20].

Although the decision on further treatment of our patients was made based on different factors, imaging tests had the most powerful impact. After comparing the treatment plan before and after the ^{18}F-FDG PET/CT scan, we came to conclusion that the management was altered in 13/35 patients. This complies with other publications [12], with the most of the changes being the consequence of detection of disseminated, extra-hepatic disease, which is known to be the advantage of PET scan.

The main objective of hybrid imaging in oncology is more precise and early detection, as well as accurate staging of active disease, in order to optimize the therapy and prolong survival. It has been shown that PET has the most significant impact in the change of preoperative management of rectal cancer [21]. In addition, surveillance of colorectal cancer patients with more than one PET/CT scan leads to the initiation of new treatment in approximately 35% and the change in previous management in 30% [22]. This study also underlined longer survival in patients with all negative PET scans. In comparison with above mentioned, our results showed similar impact of PET/CT scan on treatment alterations (37.1%).

Prognostic significance of preoperative ^{18}F-FDG PET/CT is well known and established through number of studies, and has more often been the objective of investigation compared to postoperative PET scans. It has been shown that pretreatment SUV and ΔSUV after preoperative treatment represent significant predictors of progression-free and overall survival in patients with locally advanced rectal tumors, as well as in patients with liver metastases [23,24]. The value of postoperative ^{18}F-FDG PET/CT in prognosis of survival has not been fully examined and understood. In our study, the detection of recurrence on PET/CT did not influence the prognosis. However, treatment changes induced by PET result significantly improved the prognosis, correlating with lower incidence of progression and longer progression-free survival time in these patients.

Another factor showing significant prognostic ability in our analysis was postoperative level of CA 19-9. Although its role as marker of recurrence was not impressive, the elevated value of CA 19-9 was important predictor of higher risk of progression during postoperative surveillance in our study. The similar results were observed by Abe et al [25], showing that combined elevation of CEA and CA 19-9 was significant predictor of recurrence and shorter recurrence-free survival, while normal CA 19-9 was independent predictor of longer overall survival. Normal CA 19-9 level in time of PET/CT scan was associated with longer survival in our patients, but this correlation did not reach significance. Postoperative levels of CEA and CA 19-9 may serve as indicators of when to perform PET/CT, which was proved in retrospective analysis by Wasserberg et al [26], but still needed prospective confirmation.

To our knowledge this is the first study addressing the prognostic significance of postoperative ^{18}F-FDG PET/CT, MRI and CEA and CA 19-9 levels in patients with recurrent colorectal cancer. However, our study has some limitations. The main drawback of this prospective study was small number of patients, as well as the fact that histopathological verification was not achieved in all suspected lesions, but confirmed through clinical and imaging follow-up. The follow-up period was not long enough to enable evaluation of overall survival in our patients. However, the prognostic significance regarding disease progression was determined. In addition, our study population was not balanced in gender, with higher prevalence of male patients, so results should be

interpreted accordingly. This investigation will continue and include more patients, in order to evaluate the impact and prognostic significance of imaging and biochemical tests in long-term progression-free and overall survival.

In conclusion, the results of our study suggest higher accuracy and sensitivity of ^{18}F-FDG PET/CT, in both patient-based and lesion-based analysis, in detection of recurrent colorectal cancer, compared to MRI, with comparable specificity between these two imaging modalities. Although results of ^{18}F-FDG PET/CT scan did not predict the prognosis of our patients, treatment changes induced by this imaging method significantly improved prognosis and prolonged the progression-free survival, necessitating the need of PET/CT examination in determination of treatment in patients with recurrent colorectal cancer. In addition, despite CEA and CA 19-9 showed lower diagnostic performance in detection of recurrence, compared to imaging tools, elevated values of CA 19-9 in time of PET/CT scan were significantly associated with worse prognosis, indicating its value in postoperative surveillance.

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Distribution of the most common polymorphisms in *TYMS* gene in Slavic population of central Europe

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Thymidylate synthetase (TS) plays a critical role in the *de novo* synthesis of dTMP inside the cell. Therefore, TS is a suitable target for cytotoxic drugs such as fluoropyrimidines. Drug efficacy and toxicity depend on the intracellular level of TS, which is significantly influenced by the polymorphisms in the 5'UTR (TSER – rs45445694, TSER*3G>C – rs2853542) and 3'UTR (1494del TTAAAG – rs151264360) of *TYMS* gene. Polymorphic variants of *TYMS* gene affect TS activity via gene expression and transcript stability. Patients who undergo fluoropyrimidine therapy may benefit from genetic testing prior to the administration of chemotherapy. At the 5' terminus of *TYMS,* there is a polymorphic region represented by a variable number of 28bp long tandem repeats (2-9 tandems) with the G or C nucleotide variant (SNP G>C). The 3'end of *TYMS* gene may decrease the stability of mRNA in the case of 6 base deletion (1494del6, D). In our study, we have focused on testing of *TYMS* gene polymorphisms, determination of *TYMS* variant frequencies in Western Slavic population and comparison of Slovak population with other populations.

We performed identification of 5'UTR (rs45445694 – TSER*2 or TSER*3; rs2853542 – TSER*3G>C; TSER*3+*ins6*) and 3'UTR (rs151264360/1494del6/D) polymorphic regions of *TYMS* gene among 96 volunteers by PCR-RFLP and fragment analysis. Slovak frequencies of selected polymorphisms were established as follows: the frequency of TSER*2, TSER*3, TSER*3G>C, 1494del6/D and I to be 41%, 59%, 34%, 37.5% and 62.5% respectively. The high resolution of the capillary electrophoresis technique allowed among TSER*3 group identification of a subgroup of four individuals with rare 6bp insertion in 3R allele, *id est* 2.1% TSER*3+*ins6* allele frequency. In our study, we have revealed individuals with rare G>C substitution in the first 28bp tandem repeat of TSER*2 promoter enhancer region (rs183205964) as well, the overall frequency of this polymorphic allele in Slovak population was 2.1%.

Our results proved that Slovak population is in Hardy-Weinberg equilibrium and proportion of *TYMS* polymorphisms is in accordance with other published data.

Key words: fluoropyrimidines, thymidylate synthetase, pharmacogenetics, polymorphism, Slovak

Fluoropyrimidine drugs such as 5-fluorouracil (5-FU) and the prodrugs represented by capecitabine and tegafur are extensively used in cancer therapy. The fluoropyrimidine treatment efficiency depends on the intracellular level of the enzyme thymidylate synthetase (TS). If TS is inhibited by fluoropyrimidines, the production of dTMP in the cell is blocked, which leads to the misincorporation of 5-FU metabolites into RNA and DNA, and finally to cell-cycle arrest and apoptosis [1]. Thymidylate synthetase protein is coded by *TYMS* gene consisting of 7 exons located on chromosome 18 (18p11.32). The therapy outcome and also toxicity are influenced by TS cellular content which depends on the interpatient genetic variability [2].

Expression and stability of thymidylate synthetase vary according to the polymorphisms in the variable number of 28-bp tandem repeats and SNP (single nucleotide polymorphism) in the 5'-untranslated region (UTR) and also depend on 6-bp insertion/deletion polymorphism in the 3'UTR of *TYMS* gene (Fig. 1). *TYMS* promoter enhancer region (TSER) contains the variable number of 28bp tandem repeats (CCGCGCCACTT**G**GCCTGCCTCCGTCCCG) which are repeated from 2 up to 9 times (rs45445694 for-

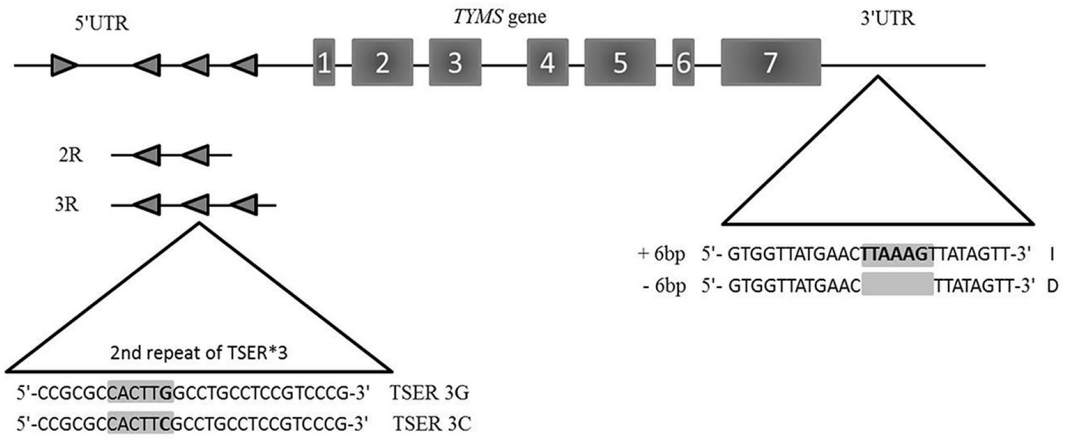

Figure 1. Structure of *TYMS* gene and 5'UTR and 3'UTR polymorphisms. TSER – Thymidylate Synthetase Enhancer Region, R – 28bp tandem repeat, I – I allele and D – D allele.

merly described as rs34743033), more prevalent as duplet (2R, TSER*2) or triplet (3R, TSER*3) [3]. Increased number of repeats raises *TYMS* RNA concentration and protein synthesis [3-6]. Every repeat contains E-box (enhancer box) binding site for upstream stimulatory factors (USF-1/USF-2) functional only when guanine (G) at the last position of CACTTG sequence is present. Both 2R and 3R wild-type alleles contain a cytosine in the last repeat which abolished the USF binding site in the repeat [7]. A clinical association has been found between reduced toxicity and drug efficacy in case of the 3R/3R genotype, contrary patients with the 2R/2R genotype showed an increased risk of toxicity, an increased response to fluoropyrimidine chemotherapy, and an increased survival time compared to individuals with the 2R/3R or 3R/3R genotype. The frequency of TSER*3 (3R) in the world is in the range from 50 to 60% except for Asian populations where the prevalence of TSER*3 is significantly higher (over 80%) [3, 8, 9].

The SNP G>C substitution in the 12th nucleotide in the second repeat of TSER*3 (rs2853542) alters *TYMS* expression by abolishing a transcription factor binding site [7, 10]. Patients with TSER*3 G>C (TSER*3C, 3C) polymorphism have a greater risk of toxicity due to the reduction in *TYMS* expression [10]. The TSER*3 patient might be stratified into high and low *TYMS* expression groups according to the presence of the TSER*3 G>C polymorphism.

Decreased mRNA stability *in vitro* and lower gene expression *in vivo* was revealed by Mandola et al. [11] in association with the 3'UTR 6bp deletion allele located 447bp downstream of the *TYMS* transcription stop codon (rs151264360 formerly described as rs34489327 or rs16430; 1494del6, D) (Fig. 1). Frequency of variant with 6bp deletion in Caucasians is 26-29%, 50% in Africans, and up to 76% in Asians [11, 12].

Other genetic and clinical factors may be also taken into account that may affect patient's risk for toxicity, survival time and therapy response.

The aim of this study was to analyse *TYMS* variants in Slovak population for the first time, to determine the frequency of polymorphisms in *TYMS* gene and to compare our findings with other populations. This study provides valuable information about the genetic variability of *TYMS* gene in Western Slavic population, hitherto missing.

Materials and methods

Samples of healthy unrelated volunteers were analysed by PCR, RFLP and fragment analysis (capillary electrophoresis) to estimate polymorphism frequencies in Slovak population. The analysed group consisted of 96 generally healthy individuals of Caucasian origin, randomly selected from the database of volunteers available at the Institute of Medical Biology, Genetics and Clinical Genetics, participants signed a written informed consent before participation in this study. Acquired data were checked for Hardy-Weinberg equilibrium and genetic linkage between variations.

DNA isolation. Blood samples for DNA extraction were collected in 3 ml tubes containing potassium EDTA. Whole blood DNA was extracted from 200 ml uncoagulated blood using Purification DNA Kit (DNA NucleoSpin Blood Kit; Macherey-Nagel, Düren, Germany).

Allele analysis. The 5'UTR VNTR (variable number of tandem repeats) region and 3'UTR region were amplified by PCR, the primers and PCR conditions used were previously described by Kawakami et al. [5] and by Ulrich et al. [12] were optimized for our laboratory settings. Primers for 5'UTR region (polymorphisms 2R/TSER*2, 3R/TSER*3 – rs45445694; TSER*3+ins6) amplification primers and conditions as follow: 5'-[6FAM] GCGGAAGGGGTCCTGCCA-3' and 5'-TCCGAGCCGGCCACAGGCAT-3', PCR Master Mix (Thermo Fisher Scientific, Massachusetts, USA), PCR conditions: 5 min at 95 °C; 30 cycles: 15 sec at 95 C, 30 sec at 68 °C, 30 sec at 72°C; 5 min at 72 °C.

For 3'UTR region (1494del6/*TYMS*del/D,I – rs151264360) amplification primers and condition as follow: 5'-[HEX] CAAATCTGAGGGAGCTGAGT-3'and 5'-CAGATAAGTGGCAGTACAGA-3, PCR Master Mix (Thermo Fisher Scientific, Massachusetts, USA), PCR conditions: 5 min at 95 °C; 30 cycles: 15 sec at 95 °C, 30 sec at 60°C, 30 sec at 72 °C; 5 min at 72 °C.

PCR products of 5'UTR and 3'UTR region were analysed with the ABI Prism 310 Genetic Analyzer (Applied Biosystems, USA) (Figure 2).

SNP analysis (PCR-RFLP). SNP of G or C in 2R and 3R allele (G>C substitution) [13]: VNTR PCR products were digested 16 hours at 37°C with *HaeIII* (New England Biolabs, Massachusetts, USA). Detection of the SNP was performed after electrophoresis (Origins 2100U, Elchrom Scientific, Switzerland) in Spreadex® EL 300 Wide Mini S-2x13 or Spreadex® EL 300 Wide Mini S-2x25 and staining with GelRed Nucleic Acid Gel Stain (Biotium, California, USA). Briefly, SNP detection: for 3G (3R-GGC) – fragments: 31bp – 28bp – 66bp – 10bp; 3C (3R-GCC): 31bp – 94bp – 10bp; 2GC (wt 2R allele): 31bp – 66bp – 10bp; 2GG: 31bp – 28bp – 38bp – 10bp; 2CG (2R-CG): 59bp – 38bp – 10bp; 2CC (2R-CC): 97bp – 10bp (Figure 3).

PCR-RFLP was used to confirm results of 3´UTR region fragment analysis using PCR product digestion with *DraI* (New England Biolabs, Massachusetts, USA), D allele -142bp fragment, I allele – 60bp and 88bp fragment (Figure 3) [12].

Statistic methods. Chi-square test was used to confirm or rule out whether the Slovak population is in accordance with Hardy-Weinberg equilibrium (Table 1, 2).

Fisher's exact test of contingency tables was applied to detect significant differences in frequency of alleles in Slovak and other population. If the P-value of appropriate test is $P < 0.05$; the corresponding distributions are significantly different at the 5% significance level. In the tables, significantly different P-values are marked bold (Table 3).

Sample power test was used to analyse the strength of Fisher´s exact test for significantly different results of allele distribution (Table 3).

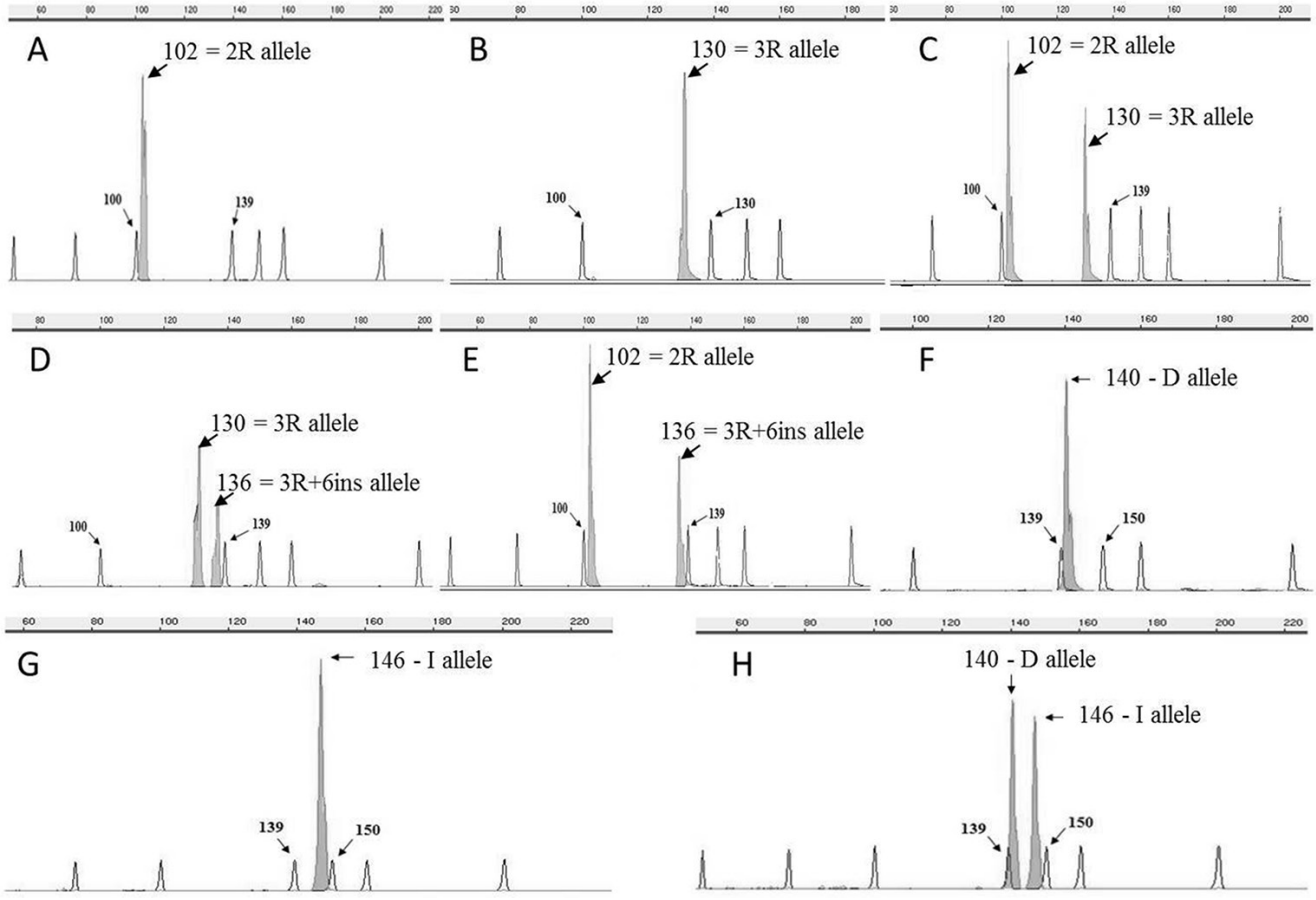

Figure 2. Fragment analysis of 5'UTR TSER region (2R, 3R, 3R+ins6) and 3'UTR region (D and I alleles) of *TYMS* gene. A) 2R/2R homozygote represented by fragment 102bp, B) 3R/3R homozygote – 130bp fragment, C) 2R/3R heterozygote – 102bp/130bp, D) 3R/3R+ins6 heterozygote – 130bp/136bp, E) 2R/3R+ins6 heterozygote – 102bp/136bp. The nearest size standard peaks 100 and 139bp. F) D/D homozygote represented by fragment 140bp, G) I/I homozygote – 146bp fragment, H) D/I heterozygote – 140bp/146bp. The nearest size standard peaks 139 and 150bp.

Results

We have genotyped 96 Slovak DNA samples of healthy volunteers and determined frequencies of *TYMS* polymorphisms in Slovak population for the first time. Using fragment analysis of the 5'UTR VNTR *TYMS* region, we have identified five different genotypes and the following frequencies: TSER*2/TSER*2 homozygotes represented by 20.8%, TSER*3/TSER*3 homozygotes with 35.4% portion, TSER*2/TSER*3 heterozygotes with 39.6% ratio, TSER*3/TSER*3+ins6 with 3.1% and TSER*2/TSER*3+ins6 with 1.1% (Table 1). The overall frequency of TSER*3 (with TSER*3+ins6*)* prevalent allele was 59%, the TSER*2 portion was 41% (Table 3). Within the TSER*3 allele, we have distinguished TSER*3 allele with 6 bp insertion (TSER*3+ins6) with 2.1% portion (Table 1). Based on the Chi-square test the distribution of the allelic variants

Table 1. Genotypes and allele frequencies of 5'UTR TSER region polymorphism (2R, 3R, 3R+ins6) and 3'UTR region polymorphism (D and I) in Slovak population.

Genotype	Number	Frequency (%)	Allele	Frequency (%)
2R/3R	38	39.6	3R	56.8
3R/3R	34	35.4	2R	41.1
2R/2R	20	20.8	3R+ins6	2.1
3R/3R+ins6	3	3.1		
2R/3R+ins6	1	1.1		
D/I	50	52.1	I	62.5
I/I	35	36.5	D	37.5
D/D	11	11.4		

Chi-square test confirmed the Hardy–Weinberg equilibrium in Slovak population (5'UTR TSER P=0.740, 3'UTR polymorphisms P=0.553).

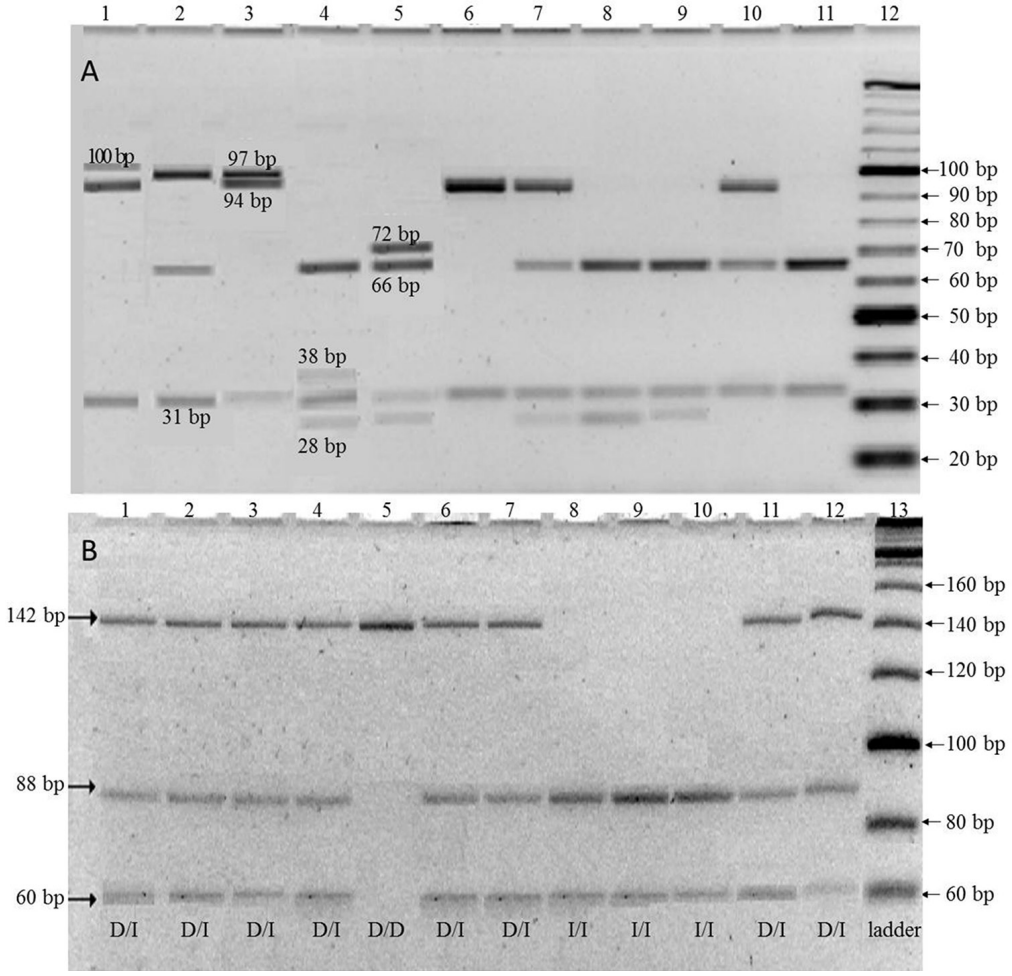

Figure 3. PCR-RFLP A) of 5'UTR region fragment analysis using *HaeIII* digestion of PCR products (sample, genotype, fragments). 1 – 3C/3C+*ins6* – 100bp and 94bp fragments; 2 – 2CC/2GC – 97bp and 66bp, 3 – 2CC/3C – 97bp and 94bp, 4 – 2GG/2GC – 38bp, 66bp and 28bp, 5 – 2GC/3G+*ins6* – 72bp, 66bp and 28bp, 6 – 3C/3C – 94bp; 7 – 3C/3G – 94bp, 66bp and 28bp; 8 – 3G/3G – 66bp and 28bp; 9 – 2GC/3G – 66bp and 28bp; 10 – 2GC/3C – 94bp and 66bp; 11 – 2GC/2GC – 66bp, 12 – DNA ladder. B) of 3'UTR region fragment analysis using *DraI* digestion of PCR product. D allele –142bp fragment, I allele – 60bp and 88bp fragment. PCR-RFLP confirmed results of fragment analysis of all 96 samples. Sample genotypes: 1, 2, 3, 4, 6, 7, 11, 12 – D/I heterozygote; 5 – D/D homozygote; 8, 9, 10 – I/I homozygote; 13 – DNA ladder.

Table 2. Distribution of SNP (G>C substitution) genotypes and alleles of *TYMS* in Slovak population.

Genotype	Number	Frequency (%)	Allele	Frequency (%)
2GC/3C	23	24	2GC	38.5
3C/3G	21	21.9	3C	32.8
2GC/2GC	17	17.7	3G	24.0
2GC/3G	13	13.5	2CC	2.1
3C/3C	7	7.3	3C+ins6	1.6
3G/3G	6	6.3	2GG	0.5
3C/3C+ins6	3	3.1	3G+ins6	0.5
2CC/2GC	2	2.1		
2CC/3C	2	2.1		
2GC/2GG	1	1		
2GC/3G+ins6	1	1		

Chi-square test confirmed the Hardy–Weinberg equilibrium in Slovak population (P=0.999).

of *TYMS* in Slovak population (Table 1) is in Hardy–Weinberg equilibrium. The detection of the TSER*3+ins6 allele was probably not within the detection limit in the studies with which we compared our population. Therefore, when comparing the frequencies of different populations, we have merged the frequency of TSER*3+ins6 allele with TSER*3 allele frequency. The frequencies of investigated polymorphisms correlate with data previously reported for Caucasians. Based on the calculated P-value the significant difference was found in frequency of 2R and 3R polymorphic allele (rs45445694) between Chinese, Kenyan, Ghanaian populations comparing to Slovak population, while the proportion of TSER*2 and TSER*3 was similar in the rest of the analysed populations (Macedonia, African-Americans, American and British Caucasians, Southwest Asian, and Spain) (Table 3). The sample power for comparative analysis of Slovak and: the population of Kenya (0.95), the population of Ghana (0.93) and Chinese population (0.999) is strong.

We have used PCR-RFLP analysis to confirm the distribution of genotypes and allele among Slovaks and to subdivide groups into detailed categories according to the content of G or C within the twelfth nucleotide of the 28bp repeats of TSER*2, TSER*3 or TSER*3+ins6 (Table 2). Frequency of wild-type TSER*2 allele (2GC) was 38.5%, 3C allele (without 3C+ins6) 32.8%, 3G allele (without 3G+ins6) 24%; we have also come across rare alleles, such as the 2CC allele with 2.1% frequency, 3C+ins6 allele with 1.6% portion, 2GG allele 0.5% and 3G+ins6 0.5%. Based on the Chi-square test, the distribution of TSER*2 (2GC, 2CC, 2GG), TSER*3G, TSER*3C allelic variants of *TYMS* in Slovak population is in Hardy–Weinberg equilibrium (Table 2). In order to compare our population with other studies, we merged Slovak 2GC, 2CC and 2GG frequencies to TSER*2 frequency (41%) as well as the 3C+ins6 frequency with 3C frequency (34%) and also the 3G+ins6 frequency with 3G (25%). In addition to this, the detailed investigation of SNP G or C variants in TSER*3 (rs2853542) revealed a significant difference between Slovaks and African-Americans, and between Slovak and Chinese populations as well (Table 3). The sample power for comparative analysis of Slovak and African Americans (0.96), as well as Chinese population (1), is strong.

In our work, the polymorphism rs151264360 in the 3'UTR region of *TYMS* (1494del6/*TYMS*del/D, I) was analysed by fragment analysis and the results were confirmed using PCR-RFLP with 62.5 % presence of I allele and 37.5% of D allele. The proportion of genotypes was as follows: more than half (52.1%) D/I heterozygotes, 36.5% I/I homozygotes, 11.4% D/D homozygotes (Table 1). Distribution of D and I alleles is in Hardy-Weinberg equilibrium in Slovak population. Statistically significant difference in frequency of distribution of D and I alleles was found comparing Slovak and Spanish populations (Table 3). The sample power of Fisher´s exact test comparing the population of Slovakia with the population of Spain is moderately strong (0.726).

In our study, we have analysed the distribution of combined genotypes of 5'UTR TSER region polymorphism (2R, 3R) and 3'UTR region polymorphism (D and I) in 37 volunteers (Table 4). We have found 8 genotype combinations from possible genotypes and following frequencies: most prevalent genotype 3R/3R – I/D with 24.4%; then 2R/3R – I/D 21.6%; 2R/2R – I/I, 2R/2R- I/D and 2R/3R – I/I equally 10.8%; 3R/3R – I/I and 3R/3R – D/D equally 8.1%; and 2R3R – D/D 5.4%. We did not identify individuals with the genotype 2R/2R – D/D.

Discussion

TYMS gene genotyping is a way that can potentially help predict patient response to fluoropyrimidines prior to the use of chemotherapy, thus leading to better-personalized treatment. We have completed the genotype study of *TYMS* gene in Slovak population that shows the distribution of *TYMS* variants among Slovaks. Our study is unique not only because of the analysis of VNTR of *TYMS* gene but also because of G and C determination within the twelfth nucleotide of the 28bp repeat in every tandem. According to our knowledge, such analysis has been not published for any Western Slavic population yet. The *TYMS* allele frequencies in Slovaks are comparable with other published Caucasian populations. The most prevalent allele in our study was TSER*3 (3R) with 59% frequency, less frequent allele was TSER*2 allele with 41% portion, and we have also detected the rare 6-bp insertion in the TSER*3 allele (TSER*3+ins6) in the frequency of 2.1%. The TSER*3+ins6 variant was firstly described by Thomas et al. with the frequency of 0.4% in Caucasians and 1.3% in African-Americans [13]. The functional effect of the 6-bp insertion in 5'UTR of the 3R allele remains to be determined. There were attempts to determine Western Slavic population, but the data are not detailed. In the study of Goricar and colleagues [14], the frequencies of two groups of genotypes for Slovenians are mentioned. The frequency for the first

Table 3. Differences in frequency of 2R and 3R alleles; 2R, 3G and 3C alleles; D and I alleles in Slovak and other populations analysed by Fisher's exact test of contingency tables.

Population	Allele frequency (%)				P-value	Sample power	Reference
	2R	3R	4R	9R			
Slovakia (n =192)	41	59	ND	ND			Current study
Macedonia (n=210)	38	62	ND	ND	0.476	NC	[16]
Kenya (n=196)	44	49	7	ND	**0.000**	0.950	[8]
Ghana (n=496)	40	56	3	1	**0.028**	0.930	[8]
African-Americans (n=184)	46	52	2	ND	0.062	NC	[8]
American Caucasians (n=208)	46	54	ND	ND	0.364	NC	[8]
British Caucasians (n=194)	45	54	1	ND	0.295	NC	[8]
China (n=192)	18	82	ND	ND	**0.000**	0.999	[17]
Southwest Asian (n=190)	38	62	ND	ND	0.532	NC	[17]
Spain (n=250)	47	53	ND	ND	0.248	NC	[34]
	2R	3G	3C	other			
Slovakia (n=192)	41	25	34	ND			Current study
Macedonia (n=210)	38	23	39	ND	0.570	NC	[16]
Spain (n=240)	47	23	30	ND	0.448	NC	[34]
White Ethnicity (n=198)	41	26	33	ND	0.909	NC	[10]
Hispanic (n=196)	42	30	26	2	0.062	NC	[10]
African Americans (n=118)	48	37	15	ND	**0.001**	0.960	[10]
China (n=160)	19	51	30	ND	**0.000**	1	[10]
	D		I				
Slovakia (n=192)	37		63				Current study
Caucasians (n=190)	29		71		0.131	NC	[12]
Northern Ireland (n=888)	32		68		0.150	NC	[35]
Spain (n=256)	26		74		**0.013**	0.726	[34]

Significantly different P-value is marked bold, n = number of alleles, ND = not detected, NC = not calculated.

group of TSER*2/TSER*2 homozygotes is 17.9% and for the other group, consisting of individuals with TSER*2/TSER*3 and TSER*3/TSER*3 genotypes, it is 82.1%. Jakubowska et al. [15] published an analysis of Polish population for the genotype groups: 22% of TSER*2/TSER*2 homozygotes, 30% of TSER*3/TSER*3 homozygotes and 48% of heterozygotes consisting of individuals with TSER*2/TSER*3 or TSER*2/TSER*4 genotypes. Macedonian population, one of the South Slavs, has been studied by Kapedanovska and colleagues [16]. The distribution of Macedonian genotypes is as described: 12% TSER*2/TSER*2 homozygotes, 30% TSER*2/TSER*3C, 20% TSER*2/TSER*3G, 19% TSER*3C/TSER*3C, 10% TSER*3C/TSER*3G.

In our group of volunteers, we have not found individuals with 4 or more 28bp tandem repeats. We have found differences in the distribution of TSER polymorphic alleles between Slovak population comparing the populations of Kenya, Ghana, and China. According to the opinion of Marsh and colleagues [17], polymorphism of TSER in humans may be a by-product of migration rather than allele evolution; environmental and/or other epigenetic factors can shift the allele frequencies in different regional dietary variations. In selective pressure of low intake of thymidine individuals with higher thymidylate synthase, due to the multiplication of TSER tandem repeat have selective advantage. Zhang and colleagues [18] demonstrated that besides humans, *TSER* is length polymorphic in many nonhuman primates while monomorphic in others. They suggested that the most recent common ancestor of hominoids and Old World monkeys probably possessed triple repeats. But now triple and double repeats, via deletion of one repeat, are two dominant types in hominoids and Old Word monkeys.

Table 4. Distribution of combined genotypes of 5'UTR TSER region polymorphism (2R, 3R) and 3'UTR region polymorphism (D and I) in Slovak population.

Genotype (N=37)	Number	Frequency (%)
2R/2R, I/I	4	10.8
2R/2R, I/D	4	10.8
2R/2R, D/D	0	0
2R/3R, I/I	4	10.8
2R/3R, I/D	8	21.6
2R/3R, D/D	2	5.4
3R/3R, I/I	3	8.1
3R/3R, I/D	9	24.4
3R/3R, D/D	3	8.1

N = number of genotypes

There have been numerous attempts to associate TSER polymorphisms with clinical outcomes in cancer patients receiving fluoropyrimidine therapy, conclusions have been inconsistent. Several studies have revealed links between TSER genotype and the response to chemotherapy, and mention that TSER*2/TSER*2 genotype or patients with at least one TSER*2 allele have a better response to fluorouracil as compared to patients with TSER*3/TSER*3 genotype [4, 19-22]. *In vitro* studies have shown up to four times more efficient translation from a construct with three repeats compared with two repeats [3, 23]. In the study of *in vivo* analysis of colorectal cancer tissues, no relation between genotype and transcription has been revealed, but TSER genotype was associated with TS protein expression. Specifically, cancer tissues with the 3R/3R genotype had a significantly higher TS protein expression level than did those with the 2R/3R genotype. Cancer tissue with 2R/2R genotype had the lowest TS protein expression [23].

De Bock and colleagues [7] have analysed thymidylate synthase activity *in vivo* in patients with colorectal cancer according to TSER polymorphism by measurement of 2´-deoxyuridine (dUrd) plasma level, a surrogate marker of TS inhibition. Plasma levels of dUrd were significantly different between genotypes, but in contrast to others, not to the absolute number of functional repeated elements (USF E-box) [7]. This study suggests that not the number of functional sequences, but their position within the promoter determines *TYMS* gene activity [7].

Up to date, there are no prescription and genotyping recommendations of *TYMS* gene prior therapy. Accurate information about the activity of thymidylate synthase depending on TSER genotype is missing as well. There are several studies trying to find out TSER genotype-specific guidelines for fluoropyrimidines dosing. Haller et al. [24] demonstrated regional differences in the tolerability profiles of fluoropyrimidines in the retrospective study. More treatment-related toxicity was reported in the US patients compared with the rest of the world for bolus fluorouracil/leucovorin and capecitabine in first-line metastatic colorectal cancer and adjuvant colon cancer. In the adjuvant setting, a range of fluoropyrimidine tolerability was observed, with East Asian patients having the lowest, and US patients the highest [24]. Soo and colleagues [25] sought to develop TSER specific guideline for capecitabine dosing. In their phase I study, they revealed a possibility to escalate the capecitabine dose of TSER 3R/3R advanced and/or metastatic cancer patients from lower the FDA-approved dose (from 1250mg/m^2 to 1500mg/m^2) [25].

In accordance with previous studies, the significant inverse association between the 5-FU toxicity and number of 28bp tandem repeats in 5'UTR region of *TYMS* gene was reported by some authors [4, 26, 27]. In the Saif's case report, they have described the first case of severe takotsubo cardiomyopathy related to DPD deficiency (heterozygous for the c.85T>C mutation) and homozygous polymorphism of *TYMS* (TSER*2/TSER*2, 2R/2R) in a patient with colon cancer following 5-FU containing regimen [28]. Despite controversy in the literature, overall TSER*2/TSER*2 finding predicts improved survival of patients receiving 5-FU chemotherapy but also increases the risk for 5-FU toxicity. Wang and colleagues have also observed pancytopenia and severe gastrointestinal toxicities in Caucasian TSER*2/TSER*2 homozygous patient with squamous cell rectal cancer after initiated 5-FU therapy in combination with mitomycin-C and radiation therapy followed after surgical excision [29]. The G>C substitution within the 12th nucleotide of the second repeat of TSER*3 (rs2853542) alters the *TYMS* expression due to abolished USF-1 binding site [10]. Morganti et al. demonstrated the reduction in *TYMS* expression in the case of G>C substitution in the second repeat of TSER*3 (TSER*3C) comparing to TSER*3G homozygotes and other genotypes in the colonic mucosa of 48 colorectal cancer patients [30]. The frequency of TSER*3C varies among world population from 15% in African Americans up to 33% in whites [10]. In Slovak population, TSER*3C allele frequency was 34% including TSER*3C+ins6 allele with frequency 1.6%. Marcuello and colleagues showed an improved overall response in metastatic colorectal cancer patients receiving 5-fluorouracil with the low expression genotypes (patients without any TSER*3G alleles) [31]. This SNP may further stratify TSER*3 individuals into high and low *TYMS* expression groups.

Another identified SNP with reduced effect on TS activity was analysed by Meulendijks and colleagues. They studied the G>C substitution in the first 28bp tandem repeat of 2R promoter-enhancer region of *TYMS* (rs183205964 known as the 2RC allele) among 1605 patients of this 28 patients (1.7%) carried the 2RC (our tag 2CC) allele. They observed significantly more frequent early severe toxicity and toxicity-related hospitalization in risk-associated genotype carriers (2RG/2RC, 3RC/2RC and 2RC/2RC). There was only one patient with the rare genotype 2RC/2RC in the study, who had to be hospitalized twice and had severe febrile neutropenia, diarrhoea, and hand-foot syndrome [32]. The G>C substitution in the first 28bp tandem repeat of promoter enhancer region of *TYMS* was firstly described by Lincz and colleagues in 2007 [33]. In our study, we have identified 2 individuals with genotype 2RC/2RG (our tag 2CC/2GC) and 2 with 2RC/ TSER*3C (our tag 2CC/3C), the overall frequency of the rs183205964 polymorphic allele in Slovak population was 2.1%.

On the other hand, Mandola et al. have measured TS mRNA amount in liver metastasis of 43 patients with advanced metastatic colorectal carcinoma and determined genotype – mRNA level correlation according to D and I allele presence. They found that patients with D/D genotype had decreased intratumoral TS mRNA to approximately 24% of TS mRNA amount of intratumoral TS mRNA in individuals with I/I genotype, while TS expression of D/I heterozygotes fell between two extremes with roughly 48% [11].

The knowledge of patient genotype prior fluoropyrimidine may help therapists to adequately set up treatment and prevent undesirable complications and life-threatening conditions in the future, despite the fact that clear predictive strategy has

not been developed for clinical use yet. Other comprehensive haplotype studies involving analysis of all aforementioned polymorphisms in a relationship with therapy effectiveness and the toxicity risks are necessary to achieve the prescription and genotyping recommendations of *TYMS* gene prior to therapy.

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