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ORIGINAL ARTICLE

Cell-free DNA as biomarker and source for mutation detection in primary colorectal cancer

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Summary

Purpose: *To analyze if cell-free (cf)DNA levels and the presence of KRAS and BRAF mutations in serum could be used as diagnostic biomarkers in patients with primary colorectal cancer (CRC).*

Methods: *This study included 92 individuals who were operated due to primary CRC (N=52;study group) and to hemorrhoids (N=40;control group). Serum cfDNA levels were measured with real-time PCR (RT-PCR) using PicoGreen dsDNA quantitation reagent. Colorectal tissue and related blood and serum samples taken at the time of surgery were subjected to DNA extraction and analysis of KRAS and BRAF mutations based on multiplex SNaPshot assay and DNA sequencing.*

Results: *The average cfDNA concentration was lower in patients of the study group (20±7 ng/μL) in comparison to controls (34±9 ng/μL) and this difference was statistically significant (p<0.001). The SNaPshot analysis detected KRAS c35 mutations in colorectal tumor tissue in 14 cases, but the presence of the mutation was not confirmed in cfDNA extracted from blood samples of these patients.*

Conclusions: *The level of serum cfDNA in CRC is decreased in comparison to patients with hemorrhoids, which questions the usefulness of cfDNA as cancer biomarker. Also, cfDNA does not appear to be suitable as a source for mutation detection in this disease.*

Key words: *BRAF, cell free DNA, colorectal cancer, KRAS, mutation*

Introduction

CRC is the third most common malignant neoplasm worldwide, with higher incidence in men than in women, with its incidence on the increase in developing countries in recent years [1]. In Serbia, the incidence of CRC is approximately 40/100,000 in men and 20/100,000 in women [2]. Surgical removal of the primary tumor is the mainstay treatment for localized disease, while other modalities of adjuvant and palliative therapy include chemotherapy, external irradiation and molecular targeted therapy in selected groups of patients. Most commonly used pharmacotherapy in Serbian patients with CRC is 5-Fluorouracil that is applied according to standard protocols. Recently, epidermal growth factor receptor (EGFR) targeting has become an integral part for progression control in advanced-stage disease in developed countries. Monoclonal antibodies targeting EGFR in patients with metastatic disease have markedly improved disease control and survival, but only a subgroup of patients with unaffected *KRAS* and *BRAF* status responds to this type of therapy [3].

Predictive and prognostic molecular markers are of great importance for patients with CRC and can serve as precious tools in disease diagnosis

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and prognosis, as well as in the choice of treatment and follow up of response to the administered therapy. cfDNA in the plasma or serum of CRC patients has been recently proposed as biomarker for disease monitoring, as well as a source of analyzable cancer-related gene sequences [4-9]. Although *KRAS* and *BRAF* status is extensively analyzed in CRC tissues, their value as biomarkers is not well defined in the literature [10]. It seems that *KRAS* codon 12 mutations are associated with unfavorable prognosis, independent of tumor stage, while the prognostic value of *BRAF* V600E mutation remains unclear. Serum or plasma levels of cfDNA, as well as *KRAS* and *BRAF* mutations could be used to diagnose CRC, monitor patients after surgery and/or during pharmacotherapy and predict disease recurrence [11,12]. Loss of mutations may explain the observed benefit from treatment, whereas appearance of mutations during therapy may be responsible for acquired resistance in primary wild-type disease.

The utility of cfDNA as a CRC biomarker remains unclear and is also limited without mutation analysis. Combined quantitative and qualitative analyses of serum DNA are necessary to confirm the presence of CRC, define disease-free status and indicate the presence of relapse in advanced cancer stages.

The aim of this study was to analyze if cfDNA levels and the presence of *KRAS* and *BRAF* mutations in serum could be used as diagnostic biomarkers in patients with primary CRC.

Methods

Study subjects

This study has included 52 patients with primary CRC and 40 controls. All subjects were enrolled in this study with written informed consent between 2012 and 2014 and were treated at the First Surgical Clinic of the Clinical Centre of Serbia after study approval by the Ethics Committee of the Clinical Centre of Serbia. All patients were treated by the same surgical team with standard oncologically radical procedures for the location and the stage of disease. Patients were interviewed for basic demographic and epidemiological data and all relevant clinical data were collected. The control group consisted of individuals who underwent surgical removal of hemorrhoids. From each subject colorectal tissue, peripheral blood and serum were taken.

Measurement of DNA in serum

Serum samples were stored at -80°C and vortexed before use for DNA quantification. Genomic DNA from K562 cell line of known concentration was used as control and diluted with 1x TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) to make standard DNA samples. The PicoGreen dsDNA quantitation reagent (ThermoFisher Scientific, Foster City, USA) was prepared in a plastic tube protected from light on the day of the experiment by making a 300-fold dilution of the concentrate solution in 1x TE buffer. We used 1μL aliquots of samples mixed with 9μL of TE buffer and 10μL of the working dilution of the PicoGreen reagent and subsequently incubated for 15 min in the dark. After the incubation, all samples were put in 96-well microplates with standard samples loaded on each plate. All samples and standards were prepared in triplicate and analyzed on the 7500 Real Time PCR instrument (ThermoFisher Scientific, Foster City, USA) set to work with the SYBR Green determination program. The apparatus was set to perform three cycles of 94°C for 30s and 65°C for 35s with fluorescence reading. Concentration of DNA was calculated from average fluorescence readings by extrapolating the standard curve.

Analysis of KRAS and BRAF mutations

Genomic DNA was extracted using specific commercial kits for different types of samples. From colorectal tumor tissue, adjacent normal tissue and peripheral blood, DNA was extracted by GeneJET genomic DNA Purification kit (ThermoFisher Scientific, Foster City, USA), while DNA from serum samples was extracted by QIAMP Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany).

Codons 12 and 13 of *KRAS* and V600E mutation of *BRAF* were analyzed using commercial SNaPshot Multiplex System (ThermoFisher Scientific, Foster City, USA) using previously published primers [13]. Fragments of *KRAS* and *BRAF* genes were amplified in separate reactions. The amplifications were performed in the reaction mixtures containing (in total volume of 50μL): approximately 500ng of DNA, 1U of KAPA Taq DNA Polymerase (Kapa Biosystems, Wilmington, USA), 1X KAPA Taq Buffer A (Kapa Biosystems, Wilmington, USA), 2.5mM $MgCl_2$, 0.2mM each dNTP and 10pmol of each primer (Table 1). The amplification was performed under the following conditions: initial denaturation at 94°C for 5min, 35 cycles of 94°C for 1min, 55°C for 1min and 72°C for 1min, and final elongation at 72°C for 10min. The obtained *KRAS* and *BRAF* products were mixed and purified using PureLink PCR Purification Kit (ThermoFisher Scientific, Foster City, USA). SNaPshot analysis was carried out in the reaction mixture containing (in total volume of 10μL): 1μL of SNaPshot Multiplex Ready Reaction Mix (Life Technologies), 1μL of purified PCR products and 0.5μL of each of the 5 primers for the detection of mutations in hotspot nucleotides: c34, c35, c37 and c38 in the *KRAS* gene and c1799 in the *BRAF* gene (Table 1). Products of the SNaPshot reaction were

Table 1. Primers used for PCR amplification and SNaPshot analysis of *KRAS* and *BRAF* genes

Primer name	*Nucleotide sequence*	*Product length (bp)*
KRAS F	5'-TCCCAAGGAAAGTAAAGTTCCCATATTAATG-3'	616
KRAS R	5'- CGCAGAACAGCAGTCTGGCTATTTAG-3'	
BRAF F	5'-GTGGATCACACCTGCCTTAAATTGC-3'	880
BRAF R	5'-GAGAATATCTGGGCCTACATTGCTAAAATC-3'	
c34 KRAS	5'-AACTTGTGGTAGTTGGAGCT-3'	21
c35 KRAS	5'-C(10)ACTTGTGGTAGTTGGAGCTG-3'	31
c37 KRAS	5'-C(20)TTGTGGTAGTTGGAGCTGGT-3'	41
c38 KRAS	5'-C(31)TGTGGTAGTTGGAGCTGGTG-3'	51
c1799 BRAF	5'-C(38)GGTGATTTTGGTCTAGCTACAG-3'	61

Table 2. Demographic and clinical characteristics of the study subjects

Characteristics	*Patients*	*Controls*
Number of subjects	52	40
Male gender (%)	67	60
Average age (years±SD)	61±12	55±13
Age range (years)	37-80	35-80
Tumor localization in rectum (%)	72	
Metastases (%)	30	
Chemotherapy (%)	41	
Radiotherapy (%)	23	
Tumor stage (%)		
T1	5	
T2	21	
T3	62	
T4	12	

analyzed by capillary electrophoresis on 3130 Genetic Analyzer (ThermoFisher Scientific, Foster City, USA) using GeneMapper software v4.0.

The presence of mutations in *KRAS* gene was confirmed by DNA sequencing. Purified PCR products were sequenced using the ABI Prism BigDye Terminator Kit (Kapa Biosystems, Wilmington, USA) and primers used for PCR on 3130 Genetic Analyzer (Kapa Biosystems, Wilmington, USA). Sequences were analyzed using the Sequencing Analysis software v5.2 (Kapa Biosystems, Wilmington, USA).

Statistics

Statistical analysis was performed by Student's t test for numerical variables and Chi-square test for categorical variables. Values of $p \leq 0.05$ were considered statistically significant. Statistical analysis was performed using SPSS statistical software (SPSS for Windows, release 10, SPSS, Chicago, USA).

Results

Demographic and clinical characteristics of the 2 groups are shown in Table 2.

The values of cfDNA concentration measured in serum samples ranged from 10 to 60 ng/μL (median 25.9). The calibration curve was linear (r^2=0.988) from 10 to 70 ng. The average cfDNA concentration was decreased in patients (20±7 ng/μL) in comparison to controls (34±9 ng/μL) and this difference was statistically significant ($p<0.001$).

All colorectal tumor tissue samples were subjected to DNA extraction and analysis of *KRAS* and *BRAF* mutations based on multiplex SNaPshot assay. The SNaPshot analysis detected *KRAS* mutations in colorectal tumor tissue in 14 of 52 cases (27%) (Table 3). All of the results were confirmed by direct DNA sequencing.

In cases with tumors positive for *KRAS* mutations, cf DNA was extracted from serum and screened for a specific somatic mutation present in the primary tumor. The presence of the mutation was not confirmed in any of these cases.

Discussion

The purpose of this study was to investigate the profile of cfDNA in serum of patients with primary CRC and to clarify whether if it can be used as biomarker and source for mutation detection in order to serve as an additional tool for diagnosis, staging and prognosis in this disease. Sensitive and specific methods were used for DNA analysis. Serum levels of cfDNA were measured by a fluorescence-based assay, using PicoGreen reagent and RT-PCR. Extraction of cfDNA was performed with a commercial kit and the presence of *KRAS* and *BRAF* mutations was analyzed by a SNaPshot assay. The main findings of this study were decreased levels of cfDNA in patients in comparison to the controls and inability to detect mutations in cfDNA in cases where mutation was present in colorectal tumor tissue.

The method applied for the measurement of cfDNA in serum samples is very sensitive and al-

Table 3. *KRAS* mutations detected in the tumor tissue of patients with colorectal cancer

Mutation	*Number of patients*	*%*
G12D	8	15.4
G12V	4	7.7
G12A	1	1.9
G13D	1	1.9
No mutations	38	73.1
Total	52	100.0

lows reliable determination of minimal amounts of DNA from biological samples. Taqman chemistry is superior in serum DNA levels measurement, since it can allow precise determination of the number of DNA molecules per unit of volume [9]. Picodroplet digital PCR was proven useful for multiple mutation screening with a sufficient sensitivity to detect mutations in plasma DNA samples [14]. However, the method based on fluorescence using PicoGreen on RT-PCR instrument has much lower cost and follow up of cfDNA levels by this method can be potentially used in both future studies and potentially clinical setting for follow up of disease activity and response to treatment.

Decreased levels of cfDNA in CRC patients in comparison to the control group represent a finding opposite to most of the studies, since cfDNA levels are found to be increased in patients with primary CRC, as well as other solid tumors [15,16]. In patients with CRC cfDNA levels were found to be up to 50 times higher in comparison to healthy individuals at the time of surgery [4-8]. The level of serum DNA was found to gradually increase with the CRC progression, with advanced tumor stages displaying significantly higher serum DNA levels [6,9]. Also, during follow up after surgery, serum DNA levels decrease progressively and increase rapidly when a relapse occurs [4,5]. Although cfDNA quantification was not able to detect premalignant lesions, its use to predict adenocarcinoma at an early stage seems to be promising but needs more sensitive methods to improve cfDNA detection [17]. One possible explanation for the decreased cfDNA levels in CRC patients in comparison to controls may be the activity of the serum deoxyribonuclease (DNase), known to be increased in some types of malignant diseases, but yet uninvestigated in CRC [18]. Assessment of serum DNase activity along with cfDNA levels measurement can therefore be suggested for future studies. Another possible explanation of unexpectedly low cfDNA levels in the patient group may be the fact that most patients in this study were subjected to preoperative radiotherapy and/or chemotherapy, which could abolish malignant cells as the source of cfDNA to a significant extent. However, when measured cfDNA levels from this study were compared to the other studies, it was observed that the average cfDNA concentration in patients (20±7 ng/μL) correlated with other findings [6-8]. On the other hand, the controls from this study had extremely high cfDNA levels (34±9 ng/μL) in comparison to the controls from other studies (below 0.015 ng/μL), both healthy and with non-cancerous diseases [19]. Therefore, it is most likely that the explanation for our finding lies in the fact that the control group in our study consisted of individuals who underwent surgical removal of hemorrhoids instead of healthy individuals used by most studies. Increased release of cfDNA to the bloodstream in our control group may be a consequence of disintegration or deterioration of the tissue that surrounds hemorrhoids, characterized by inflammation. Our finding questions the usefulness of cfDNA as cancer biomarker, indicating that it can be used only as an additional screening tool since it appearantly can not differentiate between malignant disease and inflammation.

Mutation analysis of the DNA extracted from colorectal tumor tissue based on SNaPshot analysis detected *KRAS* mutations in colorectal tumor tissue in 27% of the cases, with the freqeuncy slightly lower than in other studies. Unlike other studies conducted in Serbian population, this study detected no *BRAF* mutations, probably due to the relatively small number of subjects. The spectrum of *KRAS* mutation in different cancers has been studied in Western populations, but comparatively little information is available for developing countries. Analysis of *KRAS* and *BRAF* mutations in the Serbian population performed with DxS TheraScreen KRAS mutation kit, KRAS StripAssay and High Resolution Melting (HRM), has shown that *KRAS* mutations were present in 35%, while among the wild type *KRAS* patients 18% carried the *BRAF* V600E mutation [20,21]. The most frequent mutation type observed in the Serbian population was G12D (between one third and half of detected mutations), while the mutations G12V, G12A and G13D were also detected with relatively high frequency (over 10% of the detected mutations). Mutation distribution in our study was similar, with G12D mutation being by far the most frequent (57% of detected mutations). Higher frequencies of *KRAS* and *BRAF* mutations were found in neighboring populations (Greek and Romanian) [22]. In

Moroccan population, 24% patients with CRC were *KRAS* mutants, while 5% were *BRAF* mutants [23]. In Turkish population, *KRAS* gene mutations were detected in 49% of the samples, and the most frequent mutation was in the G12D codon [24].

The results of the present study confirm the differences in the mutational status between primary tumors and matched serum samples, indicating that cfDNA mutation profile could not be used as a reliable marker in primary CRC. Inability to detect mutations in cfDNA in cases where mutation was present in colorectal tumor tissue could be due to the limitations of the applied method. Although SNaPshot analysis is highly sensitive, it requires PCR amplification using cfDNA as template and the degree to which it is degraded can influence the outcome of the analysis [25]. A previous study has investigated a correlation between *KRAS/BRAF* mutation in primary tumors and serum [26]. *BRAF* mutations were present in 3.5% of the primary tumor tissue samples and 0.9% of the serum samples. In the primary tumors with *BRAF* mutations identical mutations were not observed in the corresponding serum samples, while *KRAS* mutations were observed in 32% of the primary tumors and 11% of the serum samples. For the tumor cases with *KRAS* mutations, a concordance rate was 24%, while discordance was observed in 28% of the patients. The concordance between *KRAS* mutations in the primary tumors and *KRAS* mutations in the matched sera was low (κ=0.231).

Although the levels of tumor DNA in serum were generally found to be significantly higher in patients with CRC, this study has detected significantly decreased cfDNA levels in comparison to the control group. The fact that patients with hemorrhoids display increased cfDNA levels in their blood in comparison to healthy individuals may be a consequence of inflammation and questions the usefulness of cfDNA as cancer biomarker. The level of cfDNA in serum can therefore be used only as a preliminary screening method or an additional tool in clinical practice. Also, cfDNA does not appear to be suitable as a source for mutation detection in CRC.

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Conflict of interests

The authors declare no confict of interests.

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