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Original Article

The frequency and type of mutations in the *EGFR* gene of colorectal carcinoma patients in Southeastern Serbia

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Abstract:

Colorectal carcinoma (CRC) represents a global health problem. The EGFR signaling pathway is very important in the initiation of different human epithelial cancers and plays a key role in colorectal carcinogenesis. Considering the role of *EGFR* as a mitogenic inducer, this study aimed to determine the frequency and type of mutations in the *EGFR* gene and its correlation with clinicopathological characteristics of patients with CRC on the territory of Southeastern Serbia. The genomic DNA of patients was isolated from formalin-fixed and paraffin-embedded tissues. The presence of mutations in exons 18, 19, 20, and 21 was determined by the Real-Time PCR method. The frequency of mutations in the *EGFR* gene in CRC patients of Southeastern Serbia was 7.9%. All detected mutations were G719X. No statistically significant correlation was found between mutational status in the *EGFR* gene and the clinicopathological characteristics of patients.

Key words:
colorectal carcinoma, *EGFR*, mutations

Apstrakt:

Učestalost i tip mutacija u *EGFR* genu kod pacijenata sa kolorektalnim karcinomom u jugoistočnoj Srbiji

Kolorektalni karcinom (KRK) predstavlja globalni zdravstveni problem. EGFR signalni put je veoma važan u inicijaciji različitih humanih epitelnih karcinoma i ima ključnu ulogu u kolorektalnoj kancerogenezi. S obzirom na ulogu *EGFR*-a kao mitogenog inducera, ovo istraživanje imalo je za cilj da utvrdi učestalost i tip mutacija u genu za *EGFR*, kao i hjihovu korelaciju sa kliničkopatološkim karakteristikama pacijenata sa KRK-om na teritoriji Jugoistočne Srbije. Genomska DNK pacijenata izolovana je iz tkiva fiksiranih u formalinu i ukalupljenih u parafin. Prisustvo mutacija u egzonima 18, 19, 20 i 21 određivano je Real-Time PCR metodom. Učestalost mutacija *EGFR* gena kod pacijenata sa KRK-om na teritoriji Jugoistočne Srbije iznosila je 7,9%. Sve otkrivene mutacije bile su tipa G719X. Nije pronađena statistički značajna korelacija između mutacionog statusa u *EGFR* genu i kliničkopatoloških karakteristika pacijenata.

Ključne reči:
kolorektalni karcinom, *EGFR*, mutacije

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Introduction

Colorectal carcinoma (CRC) is a malignant epithelial tumor that represents one of the major health problems in the world. Namely, it is the third most common cancer in men and the second most common cancer in women, accounting for about 11% of all diagnosed cancers (Ferlay et al., 2020). Usually, it is diagnosed in the older population, however, recent research shows an increased incidence of CRC in the younger population as well (Campos, 2017; Connel et al., 2017).

Colorectal carcinogenesis is a gradual process where the clonal accumulation of genetic changes results in the loss of cell cycle control, absence of the cells' natural response to genomic damage, further accumulation of mutations and, finally, in general genomic instability, which represents the basic characteristic of malignant cells (Mármol et al., 2017).

The main role in the evolution of CRC has the dysregulation of several intracellular signaling pathways such as mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3 kinase (PI3K)/protein kinase B (AKT) signaling pathway activated by epidermal growth factor receptor

(EGFR) bounded with the ligand. Dysregulation of these pathways leads to the activation of various transcription factors whose disruption can lead to malignant cell transformation and tumor progression through increased cell proliferation, anti-apoptosis, angiogenesis, and invasion (Wee et al., 2017; Koveitypour et al., 2019).

The *EGFR* gene is located on chromosome 7p12–13. It encodes a 170-kDA transmembrane receptor with an intracellular tyrosine kinase domain. It is a member of the ErbB family of receptors that has four closely related members: ErbB-1bB-1 (HER1/EGFR), ErbB-2 (HER2), ErbB-3 (HER3), and ErbB-4 (HER4). All these receptors contain an extracellular ligand-binding domain, a membrane-spanning region, and a cytoplasmic region which contains a tyrosine kinase (Hirsh, 2018).

After epidermal growth factor (EGF) ligand binding, EGFR forms a functionally active dimer (homodimer or heterodimer) that causes phosphorylation of tyrosine kinases in the intracellular domain of EGFR. This phosphorylation initiates different complex intracellular signals in the cytoplasm followed by the activation of a variety of transcription factors responsible for controlling multiple cellular functions including proliferation, differentiation, apoptosis, and cell migration (Koveitypour et al., 2019). The presence of mutations in some genes involved in these signaling pathways often represents a negative predictive biomarker of response to current therapy (Mármol et al., 2017).

The principal mechanism of *EGFR* dysregulation in CRC is *EGFR* overexpression. Protein upregulation is found in 60-80% of CRC, making it an adequate target for anti-EGFR therapy (Porru et al., 2018). Nowadays, in addition to the standard chemotherapy, there are two other therapeutic approaches in the treatment of cancer, i.e., administration of tyrosine kinase inhibitors (TKI) and administration of monoclonal antibodies (mAbs). Although the mechanism of these two therapies is similar, i.e., blocking of the EGFR autophosphorylation, the outcome of the therapy may differ depending on the mutational status of *EGFR*. To wit, studies showed that the administration of TKI failed to improve the outcome of the treatment in CRC patients with *EGFR* mutations in the TK domain located in exons 8,19, 20 and 21 (Saletti et al., 2015). On the other hand, the study of Cho and associates showed that cell lines that express G719S and G724S mutations in exon 18 are sensitive to the cetuximab monoclonal antibody (Cho et al., 2014). Thus, patients that are carriers of these mutations might benefit from the use of cetuximab, even though it is still mainly used only in the treatment of metastatic CRC patients with wild-type (wt) *KRAS*.

Moreover, although somatic mutations are rare in CRC, some of them can cause acquired resistance to anti-EGFR therapy (Montagut et al., 2012; Cho et al., 2014). The most common somatic mutations that lead to the oncogene activation of *EGFR* are missense mutations, insertions, or deletions, mainly in the tyrosine kinase (TK) domain (Liu et al., 2020). According to the study of Metzger and associates, the frequency of mutations in the *EGFR* TK domain of CRC patients in Europe is 3.8% in exon 21 (codons 847, 836), 2.6% in exon 18 (codons 707, 710, 711, 712, 725), 0.8% in exon 20 (codons 795, 796, 787), and only 0.5% in exon 19 (codon 742) (Metzger et al., 2011).

Namely, *EGFR* mutants bypass the mechanisms that initiate termination of the signal transduction by avoiding receptor endocytosis and degradation (Mosesson et al., 2008). Studies have indicated that *EGFR* mutations not only act as oncogenic drivers of CRC but can also serve as genomic predictors of response to anti-EGFR therapy in CRC patients (Kim et al., 2020).

Having in mind such an important role of *EGFR* as a mitogen inducer, the aim of this study was to determine the frequency and type of mutations in the tyrosine kinase domain of EGFR in starting (T1/T2) and final (T4) stages of tumor infiltration and its correlation with clinical characteristics of patients with CRC on the territory of Southeastern Serbia.

Materials and Methods

This study investigated the mutational status in *EGFR* of 38 CRC patients. The analysis was performed on the 5-10 μm thick formalin-fixed paraffin-embedded (FFPE) sample tissue sections of patients with confirmed CRC who were referred to the Laboratory for Immunology and Genetics, The Center for Medical and Clinical biochemistry, University Clinical Center Niš, Serbia during 2019-2021. Ethical approval has been obtained from the Ethics Committee of the Clinical Center, Niš, Serbia No 24722/6.

The following clinical data were collected for each patient: sex, age, tumor location. stage of tumor infiltration, nodal status, and the presence of distant metastases.

The stage of the tumor was determined in accordance with Tumor-Nodes-Metastasis (TNM) classificational system recommended by the American Joint Committee on Cancer staging system (AJCC), where T1 and T2 denote starting stages (T1, when tumor invades submucosa, and T2, when tumor invades muscularis propria) and T4 denotes the final stage of tumor infiltration, when tumor perforates visceral peritoneum and/or directly

invades other organs or structures (Tong et al., 2018). According to the location, the tumors were divided into the left-sided colon (LCC) and right-sided colon (RCC). LCC included splenic flexure, descending colon, sigmoid, and rectum, while the RCC included caecum, ascending colon, hepatic flexure, and transverse colon.

DNA isolation

Genomic DNA was extracted from the FFPE tumour tissue sections using a QIAamp DNA FFPE Tissue Kit (CE-IVD-marked; Qiagen, Hilden, Germany), according to the manufacturer's protocol. The DNA quality was determined with 260/280 optical density (OD) ratios in all samples, which were stored at -20°C until use.

Real-time PCR

The analysis of the *EGFR* mutation was performed using the „Easy® EGFR" Kit (Diatech Pharmacogenetics, Italy) with a Rotor-Gene 6000-Corbett RT-PCR device (Diatech Pharmacogenetics, Italy), where the list detectable mutations were: **EGFR exon 18**: G719S (2155G>A), G719C (2155G>T), G719A (2156G>C) - not distinguishable between them; **EGFR exon 19:** E746_A750del (2235_2249del15), E746_A750del (2236_2250del15), L747_P753>S (2240_2257del18), L747_A750>P (2239_2248TTAAGAGAAG>C), E746_S752>V (2237_2255>T), L747_T751del (2240_2254del15), L747_S752del (2239_2256del18), E746_T751>A (2237_2251del15), L747_T751del (2239_2253del15), L747_T751>P (2239_2251>C), L747_E749del (2239_2247del9), E746_E749del (2235_2246del12), L747_P753>Q (2239_2258>CA), L747_T751>S (2240_2251del12), E746_S752>A (2237_2254del18), L747_A750>P (2238_2248>GC), E746_S752>D (2238_2255del18), E746_T751>I (2235_2252>AAT), L747_T751>Q (2238_2252>GCA), E746_T751del (2236_2253del18) - not distinguishable between them.

EGFR exon 20: V769_D770insASV (2307_2308insGCCAGCGTG), D770_N771insG (2310_2311insGGT), H773_V774insH (2319_2320insCAC) - not distinguishable between them, T790M (2369C>T), S768I (2303G>T); **EGFR exon 21:** L858R (2573T>G), L861Q (2582T>A).

EGFR sequence was amplified using a mixture of 10 µl Taq Premix, 4 µl double distilled (dd) water, 1 µl mutation primers, and 5 µl DNA template. Each run included at least one amplification of the negative control (dd water) and one amplification of the positive control („Easy® EGFR" Kit positive control).

EGFR sequence was amplified using the following cycling conditions initial denaturation step at 95 °C for 2 min, then running 40 cycles of 95 °C for 10 seconds/58 °C for 60 seconds.

The mutation analysis was carried out in relation to the amplification of positive and negative control tests provided by the manufacturer and according to the included protocol.

Statistical analysis

Statistical analysis was done using Microsoft Excel 2010 (Microsoft Corporation, USA). Results of *EGFR* mutational analysis were used as categorical variables (presence or absence of the mutation). Comparison between clinical characteristics and mutational status was done using Fisher's exact test or χ^2 test where appropriate. Statistical significance was accepted if $p<0.05$.

Results

This study included samples of 38 patients with CRC, collected during 2019-2021. There were 24 male and 14 female patients, aged from 42 to 79 years (**Tab. 1**).

Of all 38 tested patients, 35 of them (92.1%) did not have any detectable mutations in the *EGFR*. *EGFR* mutations (G719X) were observed in exon 18 in 3 (7.9%) patients. The G719X mutation in *EGFR* refers to point mutations that result in substitutions of the glycine at position 719 to other residues, primarily alanine (G719A), cysteine (G719C), and serine (G719S) (**Fig. 1**). No mutations were detected in exons 19, 20, and 21.

Two out of the three patients with detected mutations have the T2 stage of CRC, located in the left colon, and with no metastases in lymph nodes. The third case was a patient with the T4 stage of the tumour, located in the right colon and with metastases in lymph nodes.

Mutation in *EGFR* (G719X) was detected in one patient with distant metastases in the lungs, one patient with distant metastases in both lungs and liver, and in one patient with distant metastases in the pelvis.

There was no statistically significant correlation between the mutational status in the *EGFR* and the clinical characteristics of patients (**Tab. 1**).

Discussion

In Serbia, colorectal carcinoma is the third most common cancer, as well as the second most common cause of cancer-related death, right after lung cancer (Ferlay et al., 2020). Namely, according to the Cancer Register for Republic of Serbia, there were even 4646 new CRC cases registered in Serbia in 2018. More than a thousand of these new CRC cases

Table 1. Correlation between *EGFR* mutation status and clinicopathological parameters of CRC patients

Parameters		N=38		EGFR status N=38		P value
		n	%	mutated	wt	
Sex	male	24	63.2	2	22	0.89
	female	14	36.8	1	13	
Age at diagnosis	≤50	6	15.8	/	6	
	51-60	6	15.8	/	6	
	61-70	18	47.4	2	16	
	≥71	8	21.0	1	7	
Location	Left colon	23	60.5	2	21	0.82
	Right colon	15	39.5	1	14	
Tumor extent	T1/T2	14	36.8	2	12	0.26
	T4	24	63.2	1	23	
Nodal status	without metastasis	14	36.8	2	12	0.26
	with metastasis	24	63.2	1	23	
Distant metastasis	Liver	19	50.0	/	19	
	Lung	6	15.8	1	5	
	Liver and lung	5	13.2	1	4	
	Other	8	21.0	1	7	

wt-wild type

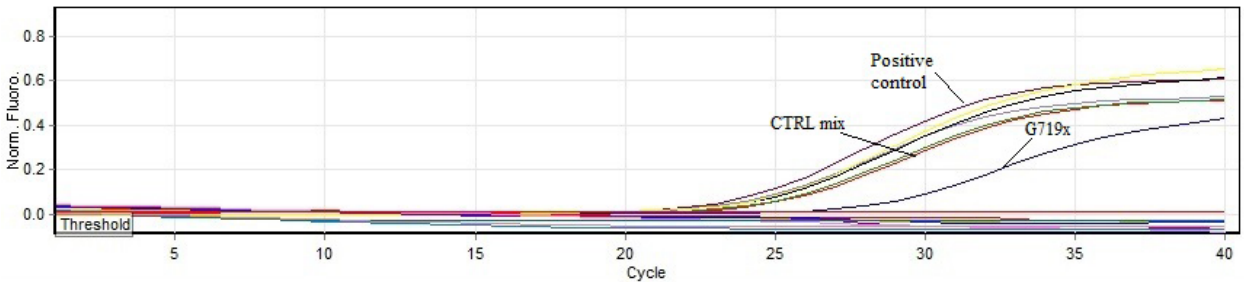

Fig. 1. G719x mutated sample and amplification plot detected by „Easy® EGFR" Kit

were patients from Southern and Eastern Serbia, with 640 males, and 371 females. At the same time, the total number of deaths caused by CRC was 479, with 397 male and 182 female patients (Institute of Public Health „Dr. Milan Jovanovic Batut", 2020).

A key role in colorectal carcinogenesis has the EGFR signaling pathway, which is involved in the initiation of human epithelial cancers. The most common pathological changes of *EGFR* are overexpression of EGFR protein and *EGFR* mutations that activate the kinase.

Based on the hypothesis that a monoclonal antibody against EGFR can inhibit receptor tyrosine kinase activity and cancer cell proliferation, proposed in 1980 by Dr. John Mendelsohn and Gordon Sato, years of studies led to the discovery of anti-EGFR treatments for specific carcinomas such as CRC and non-small-cell lung cancer (NSCLC) (Mendelsohn et al., 2015). It has been observed that EGFR-directed therapy such as cetuximab was more successful in CRC patients with highly expressed *EGFR* than in patients with low *EGFR* expression (Liu et al., 2016).

Many studies evaluated the connection between over-expressed *EGFR* with a variety of carcinomas such as breast cancer (Silva Rocha et al., 2021),

stomach adenocarcinoma (Wang et al., 2017), prostate cancer (Hashmi et al., 2019), NSCLC (Tasdemir et al., 2019), head and neck cancer (Barnes et al., 2020). In all these cancers, the level of expression was different with a tendency to increase, but in all cases, *EGFR* expression was recognized as an unfavorable prognostic biomarker of the disease. Furthermore, many scientists have been researching overexpression and abnormal activation of the *EGFR* in CRC patients. These studies have shown the prognostic value of *EGFR* expression visible as the key factor in the invasion and development of metastasis in CRC patients (Du et al., 2017; del Carmen et al., 2020; Uhlyarik et al., 2020).

In addition, many studies evaluated the frequency of mutations in the *EGFR* gene in a variety of cancers. For example, it has been observed that in NSCLC patients mutations of the gene were highly frequent in exons 19 (over 50%) and less frequent in exons 18 and 20 (8.1% and 3.5%) (Skřičková et al., 2020). Interestingly, NSCLC patients with a mutation in exon 19 had higher overall survival (OS) rate compared to NSCLC patent with mutations in exons 18 and 20 (Syrigos et al., 2018). Also, a high frequency of mutations was found in glioblastoma multiforme (26.8%), lung adenocarcinoma (14.4%), while a low frequency of mutations was found in breast invasive carcinoma, kidney renal papillary cell carcinoma, and prostate adenocarcinoma (Liu et al., 2020).

The results of this study showed that of 38 patients only 3 of them had mutations in the kinase domain of the EGFR. Two out of these three patients had T2 stage of CRC, located on the left side of the colon and the absence of metastasis in lymph nodes. Similarly to our results, the study of Oh and associates showed that the presence of *EGFR* mutations is more frequent in the earlier stages and the absence of lymph node metastasis (Oh et al.,2011). Moreover, our results showed that all these three patients had the same mutation, i.e., in exon 18 (G719X).

Ruhe and colleagues showed that one of the three mutations in exon 18 (G719S) identified in the SW48 colon cancer cell line had oncogenic potential (Ruhe et al., 2007). At the same time, according to Kim and associates, inhibitors of EGFR tyrosine kinases, such as cetuximab and panitumumab, efficiently block the growth of the same cell line with the same mutation (Kim et al. 2020, Cho et al., 2014). Having in mind all these, it might be concluded that CRC carriers of *EGFR* mutation in exon 18 might benefit from cetuximab or panitumumab applied as first-line therapy.

The incidence of mutated *EGFR* in CRC patients included in our study was only 7.9%, while its frequency in the Korean population was even 22.41%. On the contrary to the population of Southeast Serbia, their population had mutations only in exon 20 (Oh et al., 2011). Additionally, the frequency of mutations of in exon 18 (codon 719) in our study was higher than the frequency of mutations in the same exon in the European population (2.6%, codons 707, 710, 711, 712, 725) (Metzger et al., 2011).

Our results are different from the study of Phua and associates who did not detect any mutations of *EGFR* in codons 18, 19, and 21 in a group of 45 patients with CRC (Phua et al. 2015). Similarly, the study that included 31 Caucasians did not detect any mutations in exons 18 and 19, while only one of them had a missense heterozygous mutation in exon 21 (Gly857Arg) (Moroni et al., 2005).

On the other hand, Barber and colleagues found only one carrier of G719S out of 293 examined CRC patients (0,34%) (Barber et al., 2004), while the study of Ogino and associates showed that the incidence of this mutation was 3.33% (1/30) (Ogino et al., 2005).

In Romanian population, the frequency of *EGFR* mutation in exon 19 was 5% (3/60), and it was positively correlated with lymphovascular invasion, perineural invasion, tumor budding of all grades as well as with the invasion of tissues in the proximity of the tumor (liver, visceral, parietal pleura, etc.) (Ionescu et al., 2022). In our study, we did not identify any mutation in exon 19.

However, we noticed a trend of *EGFR* mutation appearance in the left-sided CRC (2/3), while the study of Randon and associates observed a statistically important correlation between the amplification of *EGFR* and wt RAS/BRAF in left-sided CRC (Randon et al., 2021).

Over 60% of CRC patients develop metastases in the liver, while the second most common site of metastasis is the lungs (Uhlyarik et al., 2020). The presence of distant metastases greatly reduces the five-year survival rate, and the development of these metastases is associated with the mutation of many genes (Tanaka et al., 2019, Hao et al., 2022).

According to the results of our study, the presence of *EGFR* mutation was detected in patients with distant metastases in both lungs and liver, metastasis only in lungs, and metastasis in pelvis. However, contrary to the study of Zou and associates that detected a G735C mutation of *EGFR* in one patient with liver metastasis, we found no mutation in patients with metastasis in the liver only (Zou et al., 2018).

To the best of our knowledge, this is the first report on the evaluation of frequency and type of mutations in the gene and its correlation with clinicopathological characteristics of patients with

CRC on the territory of Southeastern Serbia.

Conclusion

The incidence of mutations in the *EGFR* in 38 CRC patients of the Southeastern Serbia was relatively low, and there was no significant correlation between *EGFR* mutational status with clinicopathological characteristics. All detected mutations were G719X. However, we did observe a trend of mutations appearing in the lower stages of tumor infiltration, especially on the left side of the colon without the presence of metastases in the lymph nodes. Since our study was conducted on relatively small number of patients, more cohort studies are needed.

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