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Original scientific article

FREQUENCY OF *NRAS* GENE MUTATIONS AMONG THE PATIENTS WITH WILD TYPE *KRAS* COLORECTAL CANCERS IN SOUTHERN-EASTERN SERBIA

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Colorectal cancer (CRC) is the fourth most frequently diagnosed cancer worldwide, with 1.1 million cases in 2018. *KRAS* and *NRAS* genes are some of the most important molecular biomarkers of CRC. That is why, before starting treatment with anti-epidermal growth factor therapy, patients with CRC are tested for the mutation in those genes. The aim of this study was to evaluate the frequency of *NRAS* gene mutations among patients with wild type (wt) *KRAS* colorectal cancer in Southern-Eastern Serbia. Formalin-fixed paraffin-embedded sample tissues of 55 CRC patients with wt *KRAS* were investigated during the period from 2017 to 2019. Following DNA extraction, the samples were analysed for common mutations of exons 2 (codons 12 and 13), 3 (codon 61), and 4 (codon 117 and 146) of the *NRAS* gene using two diagnostic analyses: real-time PCR and *NRAS* StripAssay. Among these 55 cases of colorectal cancer with wt *KRAS*, there were 3 (5.4%) cases with mutant *NRAS*. One of these patients had mutations in codon 13 and the other two in codon 61. No mutation in codon 12 was found. Moreover, two out of three patients were men with CRC in the T3 stage of tumour infiltration and liver metastases. The third one was a woman with CRC in the T3 stage of tumour infiltration and lung

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metastases. Our results showed that the frequency of *NRAS* mutation in CRC is low, which is similar to other studies covering different geographic areas of the world.

Keywords: NRAS gene, *KRAS* gene, colorectal cancer

INTRODUCTION

Colorectal cancer (CRC) is the fourth most frequently diagnosed cancer worldwide, with an estimated 1.1 million cases and 551 269 deaths in 2018 (RAWLA *et al*., 2019). These numbers are predicted to double by 2030, resulting in over 2.2 million cases, and 1.1 million deaths (YABASIN *et al*., 2018). Although both diagnosis and therapy have significantly advanced in the last ten years, the prevalence of CRC continue to rise, and the 5-year survival rate is still poor (LI *et al*., 2015). Until recently, similarly to the situation with most of the tumours, 90% of the patients with diagnosed CRC was older than 55. (ALBERTS *et al*., 2002). However, new studies observed that CRC prevalence has been rising in the younger population as well (CAMPOS, 2017; CONNELL *et al*., 2017). Because of this, a detailed understanding of the mechanisms underlying the development of CRC, as well as genetic predisposition and lifestyle leading the progression of this disease is of crucial importance.

One of the most important molecular biomarkers of human cancers (including colorectal adenocarcinoma) is the RAS family members (*KRAS*, *NRAS* and *HRAS*), frequently found in their mutated, oncogenic forms in human tumours (LI *et al*., 2015; ALBERTS *et al*., 2002).

RAS genes code four isoproteins: K-RAS4A, K-RAS4B, H-RAS and N-RAS, intracellular guanine nucleotide-binding proteins (G proteins) that belong to the family of small GTPases. These proteins act as a molecular switch, cycling between ON and OFF states during signal transduction (CASTELLANO *et al*., 2011). In normal cells, the downstream signalling is activated after the binding of growth factors to tyrosine kinases receptor, changing their conformation from RAS GDP (inactive form) to RAS GTP (active form). (ROMÅN *et al*., 2018). The transition between these two states is regulated by GTPase-activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs). Namely, GAPs increase the GTPase activity of RAS protein and downregulate its activity, while GEFs increase the release of the GTP. RAS-GTP complex activates several downstream signalling effectors such as the canonical Raf-MEK-ERK and PI3K/AKT/MYC signalling (WHITWAM *et al*., 2007), responsible for the control of multiple cellular functions such as proliferation, motility, survival and apoptosis. The presence of mutations in *RAS* genes favours GTP binding. This results in constitutive activation of Ras, which is associated with hyperproliferative developmental disorders and cancer (QUINLAN and SETTLEMAN, 2009).

Significantly high rates of mutated *Ras* were found in many tumours, such as colon (adenocarcinoma), leukaemia (AML), lymphomas, lung (non-small-cell carcinoma and large-cell carcinoma) and thyroid (anaplastic and follicular carcinoma) (GURUNG and BHATTACHARJEE, 2015). The most frequent of these mutations are the missense ones, found in 80% of mutated *KRAS*, 12% of mutated *NRAS* and 3% of mutated *HRAS* (SIMANSHU *et al*., 2017).

Mutations in *NRAS* causes Ras-GTP to be in a state of continuous activation, which results in malignant proliferation and metastasis (MANDALA *et al*., 2014). Having in mind that *NRAS* induced MAPK signalling leads to the expression of cyclin D1, dysregulation of the cell

cycle and promotion of pro-survival pathways (BOISVERT-ADAMO and APLIN, 2008), it is not of surprise that mutation of *NRAS* mapped on chromosome 1 at the position 13.1 is found not only in a small percent of CRC but also in 94% of melanoma, 94% of acute myeloid leukaemia and 15% of hematopoietic cancers (LANFREDINI *et al*., 2019; FUNCK-BRENTANO *et al*., 2016; DE STEFANO and CARLOMANGO, 2014). Moreover, a low incidence of mutated *NRAS* can be found in bladder cancer, renal cell carcinoma, and in children with myelodysplastic syndromes (LANFREDINI *et al*., 2019, JEKIĆ *et al.*, 2004).

Although the treatment of CRC is still mainly based on conventional methods such as surgical removal of lesions, radiotherapy and chemotherapy, there is rising evidence that molecular approaches such as treatments with monoclonal antibodies, for instance, Cetuximab and Panitumumab might be more efficient. Namely, these monoclonal antibodies prevent activation of epidermal growth factor receptors (EGFR) (WANG *et al*., 2016), that are found to be overexpressed in 80% of CRC cases (POROBESKA *et al*., 2000), and which consequence is excessive activation of *KRAS* (ROMÀN *et al*., 2018).

Additionally, approximately 30-50% of CRCs have *KRAS* mutations and currently, the *KRAS* status is known to be a selective marker of predicting response to anti-EGFR antibodies (DE ROOCK *et al*., 2011).

Clinical studies have shown that patients with *KRAS* mutations do not react to therapy based on EGFR inhibition with monoclonal antibodies, i.e., this therapy is functional only in *KRAS* wild type carriers. Because of that, before receiving Cetuximab (Erbitux, Merck Serono, Geneva, Switzerland) or Panitumumab (Vectibix, Amgen, Thousand Oaks, CA), the tumours of patients with metastatic CRC are now routinely investigated for *KRAS* mutations (DIAZ-FLORES and SHANNON, 2007; VAN CUTSEM *et.al*., 2009; HOYLE *et. al*., 2013).

However, although the absence of mutation is a necessary precondition, it is not the only factor that influences the success of the therapy. Thus, in up to 65% of the patients with wild type *KRAS* gene, CRC still fails to respond to anti-EGFR therapy. This might be explained by the involvement of mutations at other locations of this gene or other genes that act downstream of EGFR in the RAS/RAF/MEK/ERK pathway (SHEN *et al*., 2013; ZHANG *et al*., 2015; MÅRMOL *et al*., 2017). Many studies have shown that the mutations in *NRAS* are associated with a low response rate to the treatment with anti-EGFR antibodies (HU *et al*., 2018; DE ROOCK *et al*., 2010). Moreover, associations of *NRAS* mutation status with specific locations of the colon affected with CRC and with the development of distant metastasis have been observed (DE ROOCK *et al*., 2010; RUSSO *et al*., 2014). The most common mutations in the *NRAS* gene are found in codons 12 and 13 (exon 2), 61 (exon 3), 117 and 146 (exon 4) (ER *et al*., 2014).

The aim of this study was to evaluate the frequency of *NRAS* gene mutation among patients with wild type *KRAS* gene in colorectal cancer in the population of Southern-Eastern Serbia.

MATERIALS AND METHODS

Patients and samples

Mutational status in *NRAS* codons12 and 13 (Exon 2), codons 59, 60 and 61 (Exon 3), and codons 117 and 146 (Exon 4) of 55 patients with wt *KRAS* were analyzed in this study. 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 Clinic of Oncology, University Clinical Center Niš, Serbia during 2017-2019. Ethical approval has been obtained from the Ethics Committee of the Clinical Center, Niš, Serbia No 24722 /6.

The main criteria for including the patients in this study were diagnosis of CRC confirmed by pathohistological analysis and the presence of wt *KRAS* in tumour tissue confirmed using *KRAS* StripAssay and "Easy® KRAS" Kit. These patients were qualified for anti-EGFR therapy with monoclonal antibodies (Cetuximab or Panitumumab monotherapy). The clinical characteristics and localization of CRC adenocarcinoma of patients are summarized in Table 1.

Table 1. Demographic and clinical characteristics of patients under the study

Characteristic	No (55)	%	Characteristic	No (55)	%
Sex			Stage		
male	35	36.3	T1	2	3.6
female	20	63.6	T2	13	23.6
Age			T3	29	52.7
<50	6	10.9	T4	10	18.1
51-60	12	21.8	N0	20	36.3
61-70	25	45.4	N1	22	40
>70	12	21.8	N2	13	23.6
Location					
rectum	24	43.6	Metastasis		
sigmoid colon	17	30.9	liver	31	56.3
ascending colon	6	10.9	lungs	7	12.7
descending colon	4	7.2	both	10	18.1
cecum	4	7.2	other	7	12.7

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.

NRAS testing was performed by using two diagnostic analyses: PCR amplification and hybridization, and Real-time PCR.

PCR amplification and hybridization

NRAS StripAssay (ViennaLab, Austria) which detects 22 mutations in codons 12 and 13 (Exon 2), codons 59, 60 and 61 (Exon 3), and codon 146 (Exon 4) was used for this method (Figure 1). *NRAS* gene sequence was amplified using a mixture made of 15 μL of amplification mix, 5 μL of diluted Taq DNA polymerase (1U) and 5 μL of the DNA template (10 μg/mL). Amplification was performed on iCycler Thermal Cycler (Bio-Rad, USA) starting with an initial denaturation step at 94°C for 2 min, then running 35 cycles of 94°C for 60 s, 70°C for 50 s, 56°C for 50 s and 60°C for 60 s, with a final extension at 60°C for 3 min.

In the end, the amplification products were selectively hybridized to a test strip containing allele-specific oligonucleotide probes immobilized as an array of parallel lines, as shown in Figure 1.

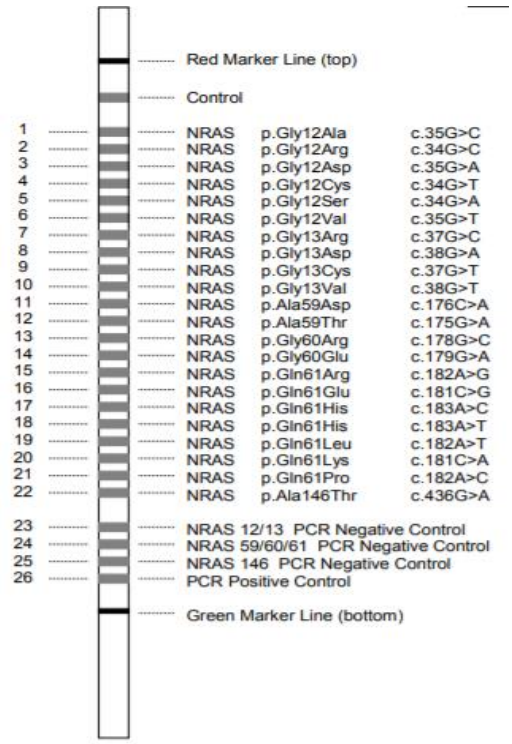

Figure 1. Test strip design (NRAS StripAssayTM Handbook, 2014)

Bound biotinylated sequences were detected using streptavidin-alkaline phosphatase and colour substrates. For each polymorphic position, one of the two possible patterns was obtained: the presence or the absence of *NRAS* mutations hybridization bands.

Real-time PCR

The analysis of the *NRAS* mutation was performed using the "Easy® NRAS" Kit (Diatech Pharmacogenetics, Italy) with a Rotor-Gene 6000 – Corbett RT-PCR device (Diatech Pharmacogenetics, Italy).

All wild type *KRAS* samples were investigated for *NRAS* mutations in codon 12 – Gly12Asp (c.35G>A), Gly12Ala (c.35G>C), Gly12Val (c.35G>T), Gly12Cys (c.34G>T), Gly12Ser (c.34G>A); codon 13 – Gly13Val (c.38G>T), Gly13Arg (c.37G>C), Gly13Asp (c,38G>A) ; codon 59 – Ala59Thr (c.175G>A), Ala59Asp (c.176C>A); codon 61 – Gln61His

(c.183A>C; c.183A>T), Gln61Leu (c.182A>T), Gln61Lys (c.181C>A), Gln61Arg (c.182A>G); codon 117 – Lys117Arg (c.350A>G), Lys117Asn (c.351G>T; c.351G>C); codon 146 – Ala146Thr (c.436G>A);

NRAS gene 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 NRAS pos ctrl). *NRAS* gene 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 *NRAS* mutational analysis were used as categorical variables (presence or absence of the mutation). Comparison between clinical and biological features 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 55 patients' colorectal cancer tissues with wt *KRAS*, collected during 2017-2019. There were 35 male and 20 female patients, aged from 46 to 79 years (Table 1).

Among these 55 cases of colorectal cancer with wt *KRAS*, there were only 3 cases (5.4%) with mutant *NRAS*. The remaining 52 patients showed wild type *NRAS* (Figure 2 (A)).

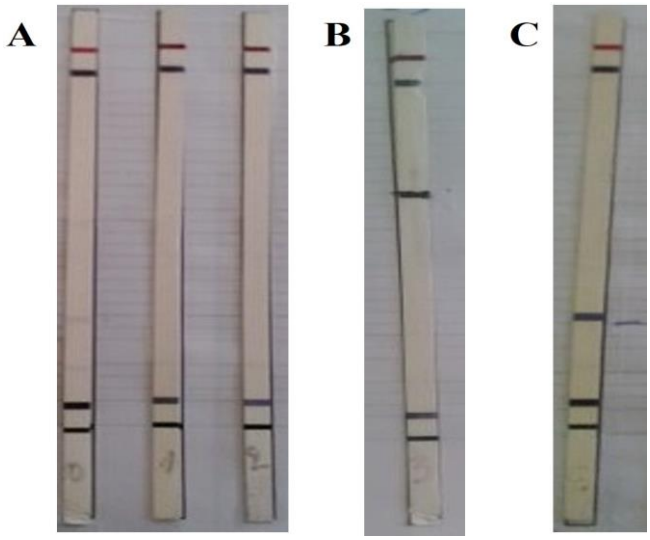

Figure 2. *NRAS* StripAssay. A: wt *NRAS*; B: *NRAS* Gly13Asp (c.38G>A); C:*NRAS* Gln61Lys (c.181C>A

One of the mutations was observed in codon 13 (Figure 2 (B)) and two in codon 61 (Figure 2 (C)). The normal amino acid at codon 13 of the *NRAS* gene is glycine (GGT) and the normal amino acid at codon 61 is glutamine (CAA). In codon 13 came to the substitution of guanine by adenine (38G>A), resulting in the amino acid substitution of glycine to asparagine.

In codon 61 came to the substitution of cytosine by adenine (181C > A), resulting in the amino acid substitution of glutamine to lysine. Two out of the three cases were men with CRC in the T3 stage of tumour infiltration, located in sigmoid colon and liver metastases. The third case was a woman with CRC in the T3 stage of tumour infiltration in rectum and lung metastases.

No mutation in codon 12 was found.

DISCUSSION

Colorectal cancer originates from the epithelial cells of the colon and begins with an adenoma. Accumulation of series of gene changes, i.e., mutations, leads to disorders in the control of epithelial cell growth and proliferation and the transition to cancer (ALBERTS *et al.*, 2002).

According to Global Cancer Observatory (GCO), colon cancer is the fourth and rectal cancer is the eighth most common cancer worldwide (RAWLA *et al.*, 2019). Males are more susceptible than women to the progression of this disease. Interestingly, the prevalence of colorectal cancer is 3 to 4 times higher in developed countries. Namely, a high incidence of CRC was recorded in European countries (Hungary, Slovenia, Slovakia, Netherlands, Norway), Australia/New Zealand, North America and Japan, while the lowest one was observed in African countries (BRAY *et al.*, 2018). The highest incidence of CRC in males was recorded in Hungary, and in females in Norway (70.6 / 100 000 and 29.3 / 100 000, respectively) (BRAY *et al.*, 2018).

In Serbia, colorectal cancer is the second most common localization of carcinomas, as well as the second most common cause of cancer-related death, right after lung cancer in men, and breast cancer in women (BANKOVIĆ-LAZAREVIĆ *et al.*, 2016).

According to the standardized rate of incidence and mortality (27.0 and 16.6 in 100 000, respectively), Serbia belongs to the group of European countries with medium to high incidence rates, and high mortality rates of CRC patients (BANKOVIĆ-LAZAREVIĆ *et al.*, 2016). Moreover, there was an observed increase in the CRC caused mortality in Southern-Eastern Serbia from 1999 to 2015. Namely, the mortality rate increased from 16.7/100 000 and 7.4/100 000 in 1999. to 21.2/100 000 and 8.4 /100 000 in 2015, in males and females, respectively (The Statistical Office of the Republic of Serbia, 2015).

The most often used modern pharmacotherapy in the treatment of CRC is based on the prevention of epidermal growth factor receptor activation by using monoclonal antibodies such as Cetuximab and Panitumumab (WANG *et al.*, 2016).

Clinical trials have shown that carriers of the *KRAS* mutation do not respond to targeted EGFR therapy, meaning that wild-type status is a necessary factor for the positive response. That is the reason that testing for the mutations in *KRAS* is a prerequisite analysis for anti-EGFR therapy in patients with CRC. However, only 40-60% of these patients will benefit, because the absence of mutation in the *KRAS* gene is not the only factor for a successful response to therapy, which suggests that additional molecular markers from the same or other signalling pathways have roles in this process (ZHANG *et al.*, 2015; MÅRMOL *et al.*, 2017). Additionally, many studies

have shown that mutations in the *NRAS* gene can lead to low response on anti-EGFR therapy (IRAHARA *et al*., 2010; WANG *et al*., 2013; DI BARTOLOMEO *et al*., 2014; MODEST *et al*., 2016; AL-SHAMSI *et al*., 2016). That is why it is of crucial importance to additionally test CRC patients with wt *KRAS* for the presence of *NRAS* gene mutation (MOMENZADEH *et al*., 2018).

Although the structures of *KRAS* and *NRAS* genes are identical in the first 85 amino acids (MALUMBRES and BARBACID, 2003), on the contrary to the *KRAS*, *NRAS* is not activated by specific cytokines and growth factors (EHRHARDT *et al*., 2004). A study on the mice model demonstrated significant phenotype differences in colorectal carcinomas caused by mutations in *KRAS* and *NRAS*. Namely, activated *KRAS* promoted the proliferation of cancerous cells and suppressed differentiation, while activated *NRAS* suppressed apoptosis (HAIGIS *et al*., 2008).

The results of our study are in concordance with previous studies, where *NRAS* gene mutations were usually observed in codons 12 and 13 (exon 2), codon 61 (exon 3) and codons 117 and 146 (exon 4).

Also, similarly to previous studies, where the frequency of these mutations ranged between 0 to 7.4 % (IRAHARA *et al*., 2010; AL-SHAMSI *et al*., 2016; MOMENZADEH *et al*., 2018; CHANG *et al*., 2016; BANDO *et al*., 2013; PALOMBA *et al*., 2016; NASERI *et al*., 2016; DOUILLARD *et al*., 2016), we have also noticed that frequency of *NRAS* gene mutation in patients with CRC is relatively low, i.e., of 55 tested patients in this study, only 3 of them (5.4%) had a mutation in *NRAS* gene. The results of the study of Prior and associates, 60% of *NRAS* mutations were found in codon 61 (PRIOR *et al*., 2012), and our study had similar results. Namely, 2 out of 3 found mutations in this research were located in that codon.

Because of the low frequency of this mutation, we did not observe a statistically significant correlation with histopathological characteristics. However, we did observe the trend where *NRAS* gene mutations occur during disease progression mostly on the left side of the colon, which agrees with previous studies (IRAHARA *et al*., 2010; CERCEK *et al*., 2017).

Moreover, the results of this research are in concordance with the meta-analysis of Hu *et al*. (2018) which suggested that the *NRAS* gene could be a prognostic indicator for CRC, especially for patients from western countries. However, more large-sample cohort studies are needed to further confirm this conclusion.

CONCLUSION

This study concluded that the incidence of *NRAS* gene mutation among CRC patients in Southern-Eastern Serbia is low. However, because of its negative influence on anti-EGFR therapy, regardless of the low incidence, it is of crucial importance to test all wt *KRAS* carriers for the possible presence of *NRAS* mutations as well.

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UČESTALOST MUTACIJA U *NRAS* GENU KOD PACIJENATA SA DIVLJIM TIPOM *KRAS* GENA KOLOREKTALNOG KARCINOMA U JUGOISTOČNOJ SRBIJI

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Izvod

Kolorektalni kancer (KRK) je četvrti najčešći kancer na svetu, sa 1.1 miliona dijagnostikovanih slučajeva u 2018. Obzirom da su *KRAS* i *NRAS* najvažniji molekularni biomarkeri KRK-a, pacijenti se, pre početka anti-EGFR terapije, moraju testirati na odsustvo mutacije u ovim genima. Cilj ove studije bila je procena učestalosti mutacija u *NRAS* genu kod pacijenata sa divljim tipom (wt) *KRAS* gena kolorektalnog karcinoma na teritoriji jugoistočne Srbije. Istraživanje je radjeno u Laboratoriji za imunologiju i genetiku, Centra za medicinsku i kliničku biohemiju Kliničkog centra u Nišu, Srbija. Analizirana su 55 parafinski ukalupljena tkiva pacijenata sa KRK wt *KRAS*-om u period od 2017. do 2019. Nakon ekstrakcije DNK, uzorci su testirani na prisustvo uobičajenih mutacija u egzonima 2, 3 i 4 *KRAS* i *NRAS* gena korišćenjem dve dijagnostičke tehnike: 'real-time' PCR i KRAS/NRAS Test Eseja. Od 55 pacijenata kolorektalnog kancera sa wt *KRAS*-om, mutacija u *NRAS* genu nađena je kod tri pacijenta (5,4%). Jedna mutacija uočena je u kodonu 13 a dve u kodonu 61. Nije pronadjena nijedna mutacija u kodonu 12. Dva od tri slučaja bili su muškarci sa KRK u stadijumu T3 infiltracije tumora i metastazama na jetri. Treći slučaj bila je žena sa KRK u stadijumu T3 infiltracije tumora i metastazama na plućima. Naši rezultati pokazuju da je učestalost *NRAS* mutacija u KRK niska, što se slaže sa studijama rađenim na pacijentima drugih geografskih oblasti u svetu.

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