INCREASED IL-33 AND IL-17 IN COLORECTAL CARCINOMA PATIENTS WITH SEVERE DISEASE

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POVEĆANE KONCENTRACIJE IL-33 I IL-17 KOD PACIJENATA SA TEŽOM FORMOM KOLOREKTALNOG KARCINOMA

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Received / Primljen: 03. 09. 2018. Accepted / Prihvaćen: 05. 09. 2018.

ABSTRACT

Colorectal cancer (CRC) represents one of the most common cancers. It is frequently diagnosed at advanced stages, indicating on need for new diagnostic markers. The aim of this study was to determine systemic and fecal values of IL-17 and IL-33 in patients with CRC and the relationship with clinicopathological aspects of disease.

The blood samples and feces liquid fraction of 50 patients with CRC were analyzed. Serum and fecal levels of IL-33 and IL-17 were measured using sensitive enzyme-linked immunosorbent assay (ELISA) kits.

Fecal levels of Il-33 and IL-17 were increased in CRC patients with poor tumor tissue differentiation. Serum IL-33 and fecal IL-17 were increased in patients with presence of lung/liver metastasis or peritoneal carcinomatosis, respectively, while enhanced fecal IL-33 was detected only in patients with peritoneal carcinomatosis.

Positive correlation between IL-33 and IL-17 values in sera and feces, respectively was also observed.

We believe that increased local values of IL-33 and IL-17, reflected trough higher fecal concentration, in CRC patients with poor tumor tissue differentiation and with presence of lung/liver metastasis or peritoneal carcinomatosis may be considered as a sign of the tumor's malignant progression and, consequently, of a poor prognosis for patients.

Keywords: *Colorectal carcinoma, IL-17, IL-33.*

SAŽETAK

Kolorektalni karcinom (engl. Colorecral carcinoma- CRC) predstavlja jedan od najčešćih karcinoma. Često se dijagnostikuje u uznapredovalim stadijumima, ukazujući na potrebu za novim dijagnostičkim markerima. Cilj ove studije bio je utvrđivanje sistemskih i fekalnih vrednosti IL-17 i IL-33 kod pacijenata sa CRC i odnosa sa kliničko-patološkim aspektima bolesti.

Analizirani su uzorci krvi i tečne frakcije fecesa 50 pacijenata sa CRC-om. Serumske i fekalne koncentracije IL-33 i IL-17 su merene korišćenjem senzitivnog ELISA (enzyme-linked immunosorbent assay) testa.

Koncentracije IL-33 i IL-17 u fecesu povećane su kod pacijenata sa CRC-om i slabo diferentovanim tumorskim tkivom. Serumski IL-33 i fecesni IL-17 su povećani u pacijenata sa metastazama u plućima/jetri ili peritonealnom karcinomotozom, dok je povećan IL-33 detektovan samo u fecesu pacijenata sa peritonealnom karcinomotozom.

Takođe je detektovana pozitivna korelacija između vrednosti IL-33 i IL-17 u serumu kao i u fecesu.

Verujemo da se povećane lokalne vrednosti IL-33 i IL-17 kod pacijenata sa slabo diferentovanim tumorskim tkivom kolorektalnog karcinoma i prisustvom metastazama u plućima/jetri ili peritonealnom karcinomotozom mogu smatrati znakom progresije maligne bolesti i, posledično loše prognoze za pacijente.

Ključne reči: *kolorektalni karcinom, IL-33, IL-17.*

INTRODUCTION

Colorectal cancer (CRC) is one of the most commonly diagnosed malignancy worldwide. Despite its frequency, it's still one of leading causes of death among women and men. The main cause of death is liver metastasis (1). Colorectal carcinoma is comprised of many types of carcinoma that differ in gene expresion, pathohistological characteristics, primary localisation, and, unfortunately, treatment outcomes (2). Traditonal approaches to therapy of this malignancy - surgery, radiotherapy and chemotherapy up to this day fail to significantly improve survival rate (3). Due to

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UDK: 616.345-006.6:577.175.8
Ser J Exp Clin Res 2020; 21 (3): 239-245
DOI: 10.2478/sjecr-2018-0034

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specific condition in whom CRC arises, many factors such as gut microbiota, chronic inflammation, eating habbits should be further investigated (4). Until this day, it is well known that, no mather what histological type, localisation or genetic characteristics of CRC, inflammation is positively correalted with more invasive types of cancer (5).

Interleukin-33 (IL-33) is a member of the IL-1 family of cytokines, which includes many cytokines, such as IL-1α and β, IL-18, IL-36α, β, γ, IL-37, IL-38 (6, 7). However, in contrast to other IL-1 family cytokines, IL-33 may function as a cytokine, as alarmin, or as a nuclear factor which modulates expression of many genes, especially NF-κB (8, 9). A large body of evidence indicates that IL-33 participate in tissue repair, allergy, autoimmune disease, infectious disease, and cancer. IL-17 is a member of a cytokine family composed of six cytokines and five receptors (10-13). IL-17 is secreted primarily by Th17 cells, but can also be produced by cells other than Th cells, such as invariant NKT cells, $CD8^+$ T cells, and γδ-T cells (14-16). The cytokine has pleiotropic functions with multiple targets. It is shown that IL-17 is involved in several biological processes such as inflammation and neoangiogenesis. The inflammation serves two counteracting functions: promoting tumor growth and antitumor immunity. Interleukins 33 and 17 promotes inflammation and thus may promote both tumor growth and tumor regression.

The aim of this study was to evaluate systemic and fecal values of IL-33 and IL-17 in patients with CRC and the relationship with clinicopathological aspects of disease. In this study we demonstrate enhanced fecal concentration of IL-33 and IL-17 in CRC patients with poor tumor tissue differentiation, with metastatic disease.

METHODS

Ethical Approvals.

The study was conducted at the Clinical center, Kragujevac, Serbia, and Center for Molecular Medicine and Stem Cell Research, Faculty of Medical Sciences, University of Kragujevac, Serbia. All patients gave their informed consent. Ethical approval was obtained from Ethics Committee of the Clinical Center of Kragujevac, Kragujevac, Serbia. All research procedures were made according to the Principle of Good Clinical Practice and the Declaration of Helsinki.

Subjects.

Study included 50 patients with CRC. The diagnosis was based on endoscopic and histopathological criteria, as previously described (17). The study did not include patients with no well-defined pathology, no adequate clinical document available or with previously diagnosed CRC who were treated with radiation and chemotherapy, as previously adopted (18). Clinical data about age, gender, size of cancer and pathologic reports (well/moderate/poor differentiation) and clinical stage (metastasis) were recorded and analyzed in study. Well-differentiated and moderately differentiated tumors (well/moderate) were defined as low-grade lesions, whereas poorly differentiated tumors (poor) were defined as high grade lesions according to the WHO guidelines (19). Grading was based on the evaluation of the worst area, excluding areas of focal dedifferentiation present at the invasive margin of the tumor (20). Poorly differentiated tumors have repeatedly been shown to behave more aggressively than well/moderate- differentiated carcinomas in multivariate analysis (20). Blood and stool samples were taken before the surgery and stored at -80°C until ELISA.

Feces liquid fraction preparation

Stool samples (1-10 g) were collected in the morning in sterile containers and weighed. One gram of fecal samples was diluted, mixed, homogenised in 5 mL of protease inhibitor cocktail (SIGMA, P83401), and then centrifuged, as previously described (21, 22). The supernatant fluid was collected and stored at - 80°C until ELISA.

Determination of IL-17 and IL-33 in sera and feces.

Serum and fecal concentrations of cytokines were measured, as described (23) using sensitive enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, for IL-17 and IL-33; measurement of cytokines according to the manufacturer's instructions). Briefly, the 96-well plates were coated with capture antibody, overnight. The plates were washed with a washing buffer (0.05% Tween-20 in PBS) and incubated with blocking buffer (1% bovine serum albumin in PBS) for 1 hour at room temperature. Serum/faecal samples or standard recombinant IL-17/IL-33 were introduced to the plates for 2 hours before the application of biotinylated detection antibody for 1 hour at room temperature. After introduction of streptavidin peroxidase for 1 hour, the plates were developed with substrate reagent for 20 minutes. The reaction was stopped by adding 4mol/L sulfuric acid, and the absorbance was read at 495 nm by a microplate reader. We measured the exact concentration of mentioned biomarkers by intrapolation of a standard curve made by a series of well-known concentrations as per manufacturer's instruction. Values of measured cytokines are presented as pg/ml of sera and pg/g of feces, respectively.

Statistical analysis

The data were analyzed using commercially available SPSS 20.0 software. The results were reported as mean and standard error of mean (SEM). In determining statistically significant difference between the means of two groups it was used Student's t-test for independent samples if the data had normal distribution or Mann-Whitney U-test for

Table 1. Baseline characteristics of patients

	Number
Gender (male/female)	29/21
Age (mean [range])	65 [50-82] years
Site (P/D/R)	12/29/9
Necrosis (well/moderate/absent)	13/37/0

Note: P: proximal colon; D: distal colon; R: rectum.

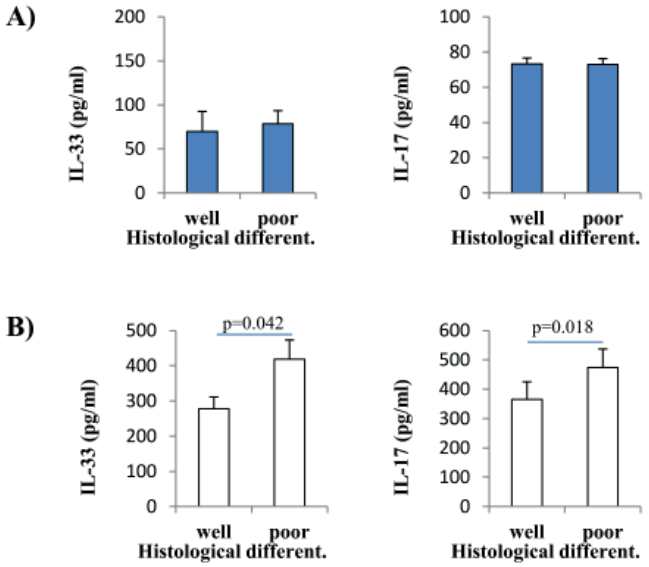

Figure 1. Serum and fecal values of IL-33 and IL-17 in patients with CRC, based on histological differentiation of tumor.
A. *No significant difference in concentration of IL-33 and IL-17 in sera, in patients according to histological differentiation of CRC.* Patients with CRC were divided in two groups, according to histological differentiation rate (well/moderate and poor). Serum and fecal levels of all mentioned biomarkers were determined by ELISA.
B. *Increased concentration of IL-33and IL-17 in feces of patients with poor histological differentiation of CRC.* Statistical significance was tested by Mann–Whitney Rank Sum test or independent samples t-test, where appropriate.

data without normal distribution. Spearman's correlation evaluated the possible relationship between the IL-33 and IL-17. Strength of correlation was defined as negative or positive weak (-0.3 to -0.1 or 0.1 to 0.3), moderate (-0.5 to -0.3 or 0.3 to 0.5) or strong (-1.0 to -0.5 or 1.0 to 0.5). P-value of 0.05 was considered as statistically significant.

RESULTS

Fifty patients with CRC were enrolled in the study. Clinical and pathologic characteristics of these patients are presented in Table 1. There was no significant difference in gender distribution (29 males and 21 females). Patients were similar in age (mean age 65 [50–82]).

Higher fecal IL-33 and IL-17 concentration in patients with poor tumor tissue differentiation

Patients with CRC were categorized into 2 groups according to histological differentiation rate: well/moderate and poor. As shown in Figure 1A, there were no differences in systemic values of IL-33 and IL-17 between defined groups. In patients with poor tumor tissue differentiation, we detected increased fecal IL-33 (poor vs. well/moderate: 418,95 ± 54,33 vs. 278,43 ± 33,50 pg/g; p=0.042) and IL-17 (poor vs. well/moderate: 473,76 ± 62,82 vs. 365,85 ± 148,67; p=0.018; Figure 1B).

Liver, lung and peritoneal metastasis associated with higher IL-33 and IL-17

Further, we divided patients in two categories based on presence of lung/liver metastasis or peritoneal cacrinomatosis, respectively, and analyzed them for values of IL-33 and IL-17. Higher IL-33 was found in sera of patients with detectable liver metastasis (81,01 ± 21,78 vs. 67,16 ± 11,71; p=0.038), lung metastasis (128,37 ± 27,69 vs. 61,51 ± 8,72; p=0.007), or peritoneal cacrinomatosis (105,56 ± 27,04 vs.

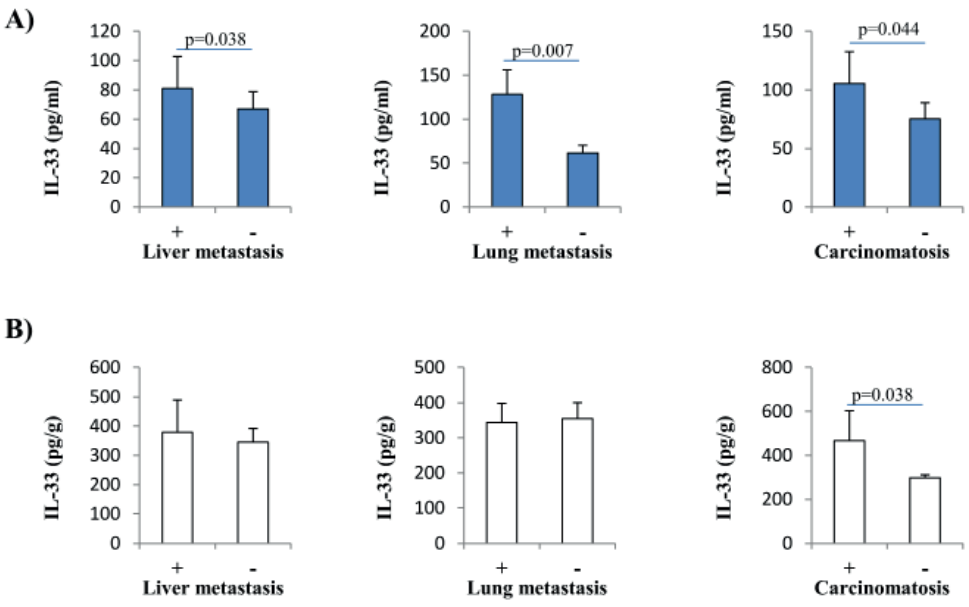

Figure 2. Sistemic and fecal values of IL-33 in patients with CRC, based on tumor progression.

A. *Increased serum IL-33 in patients with detectable liver, lung metastasis and peritoneal carcinomatosis.* Patients with CRC were divided in two groups, based on presence of liver metastasis, lung metastasis and carcinomatosis in peritoneum, respectively (+ and -). Serum and fecal levels of IL-33 were determined by ELISA.
B. *Increased fecal IL-33 in patients with detectable peritoneal carcinomatosis.* Statistical significance was tested by Mann–Whitney Rank Sum test or independent samples t-test, where appropriate.

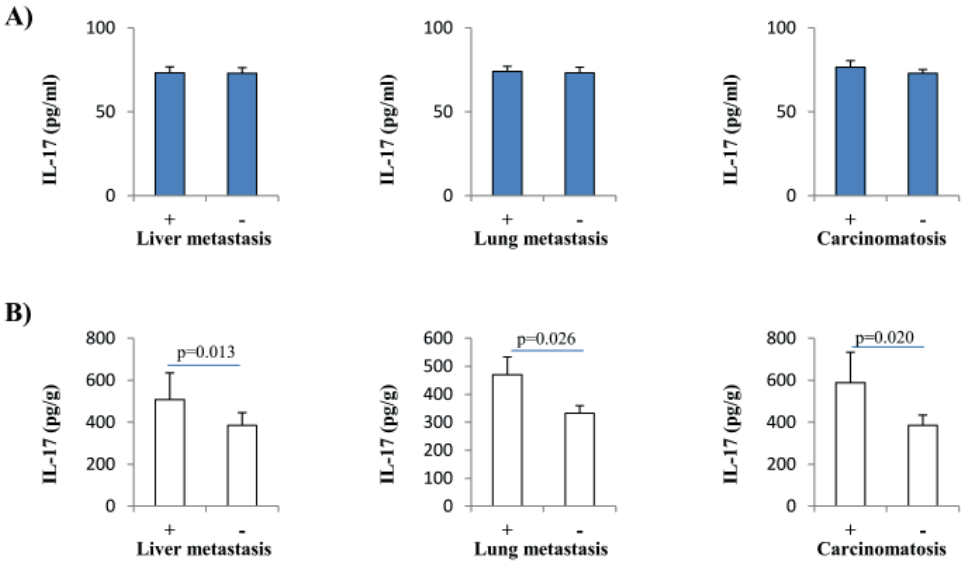

Figure 3. Sistemic and fecal values of IL-17 in patients with CRC, based on tumor progression.
A. *No significant difference in concentration of IL-17 in sera, in patients with detectable liver, lung metastasis and peritoneal carcinomatosis*. Patients with CRC were divided in two groups, based on presence of liver metastasis, lung metastasis and carcinomatosis in peritoneum, respectively (+ and -). Serum and fecal levels of IL-17 were determined by ELISA.
B. *Increased fecal IL-17 in patients with detectable liver, lung metastasis and peritoneal carcinomatosis*. Statistical significance was tested by Mann–Whitney Rank Sum test or independent samples t-test, where appropriate.

75,17 ± 13,91; p=0.044), in comparison to patients without metastasis/carcinomatosis (Figure 2A). Increased IL-33 was detected in feces of patients with detectable peritoneal cacrinomatosis (466,50 ± 136,37 vs. 297,39 ± 14,06; p=0.038; Figure 2B).

In addition, we also found higher IL-17 in feces of patients with detectable liver metastasis (508,78 ± 125,75 vs. 384,41 ± 61,54; p=0.013), lung metastasis (470,34 ± 62,89 vs. 331,76 ± 27,06; p=0.026), or peritoneal cacrinomatosis (588,40 ± 144,95 vs. 385,98 ± 47,53; p=0.020), as illustrated in figure 3.

Serum and faecal IL-33 concentrations significantly correlated with appropriate values of IL-17

Spearman correlation analysis of IL-33 concentration in sera and stool uncovered positive correlation between IL-33 value and IL-17 in sera (r=0.478; p= 0.001) and feces (r=0.675; p= 0.001), respectively.

DISCUSION

Biological role of IL-33 in tumor genesis, progression, immuno-suppression and tumor angiogenesis is well known. IL-33 is known to have protumorigenic role in many malignancies. There are sufficient data that IL-33 is overexpressed in CRC (24, 25). IL-33 is highly expressed in earlier strages of colorectal adenoma-carcinoma, implicating that it might be important for iniciating carcinogenesis. Expression of IL-33 in tumor tissue is higher than in adjacent healthy one. Authors suggest that Il-33 can work in an autocirne manner, especilly when it comes to processes of neoangiogenesis, because it can stimulate secretion of VEGF (24). In the present study, we analyzed systemic and fecal level of IL-33 in different stages of CRC. We didn`t find that sera IL-33 mean values ranged significantly different regard to histological differentiation rate of tumor, while fecal IL-33 showed significant alteration (Figure 1). Recent studies have been exploring usage of feces as a sample for testing different biomarkers (26, 27). For instance, fecal calprotectin (FC), a biomarker of intestinal inflammation that has been in clinical use for years (28), has been also shown to be elevated in CRC and suggested for screening high risk groups for CRC (29). To our knowledge, this is the first study testing fecal IL-33 and IL-17 for detection of severe and progressive forms of CRC. We found increased concentration of IL-33 in stool of CRC patients with poor tumor tissue differentiation (Figure 1). In line with our finding, it has been shown that levels of IL-33 are higher in poor-differentiated human carcinoma cells, and are connected with higher rate of metastasis. Signaling through IL-33/ST2 pathway incerases levels of metaloproteinases (MMP2 and MMP9), IL-6 and CXCR4, molecules that are important for metastasis of human CRC (25). Also, IL-33 is found to be overexpressed on tumor cells and vascular endothelial cells in tumor stroma. Signaling through its receptor ST2, IL-33 can activate JNK kinases, that further activate genes like *NANOG, NOTCH, OCT3/4* which are active in stem cells. Therefore, indirecly, IL-33 promotes poor differentiation of CRC cells and supports its invasivness. It also can recruit macrophaphages in tumor microenviroment that produce prostaglandins, molecules that are also somewhat involved in tumor cell stemness (30). IL-33 via its receptor can also induce higher expression of mRNA and protein levels of COX2 in primary CRC cells. COX2, enzyme crucial in prostaglandin synthesis, indirectly induces proliferation of cancer cells via phosphatidylinositol 3-kinase/Akt pathway (31).

Our study also revealed increased fecal concentration of IL-17 in CRC patients with poor tumor tissue differentiation (Figure 1). IL-17 can stimulate oncogenesis via activating certain genes. In a pancreatic cancer, it has been show that along with IL-17 expression, there are higher levels of stem cell genes that promote poor differentiation. Also, it has been shown that blocking of

IL-17 using a anti-IL-17 antibody significantly downregulates genes that regulate properties of a malignat cell - cellular movement, development, growth and proliferation, even cell-to-cell signaling, cellular assembly and organization (32).

Next, we tested systemic and fecal values of IL-33 and IL-17 as a reliable markers of the disease progression and showed increased IL-33 concentration in sera of patients with CRC with progressive disease: patients with presence of lung/liver metastasis or peritoneal carcinomatosis, respectively, while increased fecal IL-33 was detected only in patients with peritoneal carcinomatosis (Figure 2). Based on these findings, we believe that IL-33 could be a predictor for the advanced stages of colorectal cancer. IL-33 influences differentiation of various immune cells. These changes promote immunosuppressive environment that promotes CRC growth (33). IL-33 expressed on tumor tissue or in tumor stroma enhances infiltration of macrophages, mostly M2 type (TAMs). TAMs work together with IL-33-stimulated Th2 responses thus paving a path to a more malignant disease (34). Also, IL-33 may promote CRC progression through processes of angiogenesis and lymph angiogenesis. Vascular density of CRC is significantly higher in presence of IL-33/ST2 signaling. Also, many markers of neoangiogenesis, such as CD31, LYVE1, and α-SMA are elevated, thus suggesting a role of this cytokine in formation of new blood and lymph vessels and metastasis (35 ,35).

Interestingly, there was no difference in systemic values of IL-17 between patients with and without presence of lung/liver metastasis or peritoneal carcinomatosis, respectively (Figure 3), while increased IL-17 concentration in feces was observed in patients with CRC with progressive disease: patients with presence of lung/liver metastasis or peritoneal carcinomatosis, respectively (Figure 3). The primary role of IL-17 is to recruit and activate neutrophils during an infection. However, when put in a tumor microenviroment, attraction of neutrophiles can lead to unwanted inflammation and consequent exacerbation of the disease (37). Using immnunohistochemistry, Chen et al showed that higer expression of IL-17 in CRC tissue is associated with poorer prognosis (38). It has been shown that IL17 may facilitate progression of colorectal carcinoma by fostering angiogenesis via promoting the vascular endothelial growth factor (VEFG) production from cancer cells (39).

Finally, we found positive correlation between IL-33 and IL-17 in sera and stool, respectively of CRC patients. There is very little data on connection between these two cytokines. Recent study illustrates the ability of IL-33 to directly stimulate mast cells and enhance the Th17 response, in animal model of airway inflammation (40). According to this study, we believe that increased local IL-33 in patients with severe disease may stimulate IL-17 production, and that both cytokines facilitate disease progression.

CONCLUSION

In summary, increased local values of IL-33 and IL-17, reflected trough higher fecal concentration, in CRC patients with poor tumor tissue differentiation and with presence of lung/liver metastasis or peritoneal carcinomatosis may be considered as a sign of the tumor's malignant progression and, consequently, of a poor prognosis for patients. These observations support the idea of potential use of IL-33 and IL-17 as therapeutic targets.

DECLARATION OF INTEREST

The authors declare that they have no competing interests.

ACKNOWLEDGEMENTS

This work was supported by grants from the Serbian Ministry of Science and Technological Development (175071, 175069 and 175103), Serbia and from the Faculty of Medical Sciences Kragujevac (project JP 04/13 and JP 12/12), Serbia. The authors thank Milan Milojevic and Aleksandar Ilic for excellent technical assistance.

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