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Articles

Relationship Between Salivary and Serum Activity of Beta-Glucuronidase and Multiple Risk Factor for Colorectal Cancer

Ahmed H. Al-Humairi [a, b, *]

[a] Volgograd State Medical University, Russian Federation
[b] University of Babylon, Iraq

Abstract

This study is directed to clarify the role of beta-glucuronidase (GUSB) in the occurrence and development of colorectal cancer (CRC). This issue is closely related to life of intestinal microflora, since we know about the beta-glucuronidase of bacterial origin, to play an important role in carcinogenesis along with its own enzyme.

The study included the results of determining the specific activity of beta-glucuronidase in the blood serum and saliva. We include to research 24 volunteers who did not have or had from one to four risk factors for CRC. Additionally, we performed the fractionation of blood serum and saliva proteins to establish the possibility of native and bacterial enzyme detection.

The study showed the greatest GUSB activity in blood serum and saliva to be a finding in smokers, as well as in the presence of several (three to four) risk factors for CRC. GUSB activity is also correlated with the presence of risk factors such as age and obesity. The peaks of GUSB activity, according to the results of gel chromatography, confirm the fact that bacterial forms contributed to the formation of the overall activity of this enzyme in blood serum and saliva.

The obtained results can be used to create diagnostic algorithms aimed at screening for increased risk of CRC in humans based on salivary GUSB analysis.

Keywords: beta-glucuronidase, blood, saliva, colorectal cancer, oncogenesis.

1. Introduction

Colorectal cancer (colorectal carcinoma, CRC) is one of the most common human cancers with a tendency to increase the number of diseases and deaths in many countries of the world. More than one million people suffer from CRC every year, in developed countries the death rate from this disease is almost 33 % (Cunningham et al., 2010; Siegel et al., 2019). Morbidity rates for men are higher than for women with an approximate 1.4-fold and 1.5-fold difference in morbidity (23.6 vs. 16.3 cases per 100,000 people per year) and mortality (10.8 vs. 7.2 deaths per 100,000 people per year), respectively. These indicators related to the fact that men were affected by environmental factors more than by genetic factors compared to women, so a CRC inheritance is of 45 % for women and 28 % for men (Graff et al., 2017; Ma et al., 2017).

The risk of developing CRC increases after the age of 50, and 90 % of cases are registered in people older than this age. In addition to age, the family history, obesity, diabetes, sedentary

* Corresponding author
E-mail addresses: ahmed.h.mneahil@gmail.com (A.H. Al-Humairi)

lifestyle, smoking, high alcohol consumption, eating high-fat, and low-fiber foods are the main risk factors for CRC (Waszkiewicz et al., 2012, Shuwen et al., 2017).

In terms of ethnic differences, Africans and peoples of African descent are the most susceptible to the disease, while Asian and Pacific islanders are the least susceptible. This is true for both morbidity (43.2 vs. 28.8 cases per 100,000 people per year) and mortality (18.6 vs. 9.9 deaths per 100,000 people per year). Genetic factors may play a role in this difference, for example, several single-nucleotide polymorphisms associated with CRC risk were found in Africans (Ashktorab et al., 2015; Wang et al., 2017).

To date, for more than 50 years of studying the participation of intestinal microflora in the development of gut tumors, many of evidences have been provided for intestinal microorganisms to play a key role in carcinogenesis. Among the microbiota representatives, such species as *Bacteroides, Escherichia, Fusobacterium* and *Porphyromonas* play an important role in CRC occurrence and progression (Tarashi et al., 2019; Thomas et al., 2019). Some specific bacteria, exampling *Bacteroides fragilis, Streptococcus bovis, Escherichia coli, Enterococcus faecalis, and Streptococcus gallolyticus*, are very closely related to CRC (Zeller et al., 2014; Dashnyam et al., 2018; Tarashi et al., 2019). Recent human studies have identified new strains of bacteria involved in CRC carcinogenesis, including bacteria of genera *Parvimonas, Peptostreptococcus, Porphyromonas*, and *Prevotella* (Leystra, Clapper, 2019; Wirbel et al., 2019; Yachida et al., 2019).

Beta-glucuronidase (beta-D-glucuronide-glucuronohydrolase, CF 3.2.1.31, GUSB) is a lysosomal exoglycosidase stimulating the cleavage of beta-D-glucuronides to form D-glucuronic acid, as well as a transglycosylation reaction. This enzyme participates in the degradation of glycosaminoglycans of cell membranes and extracellular matrix of many tissues, including the intestinal mucosa. Tumorous and chronic inflammatory diseases of liver are typical reason to GUSB increase, but high ulcerative colitis, pancreatic, intestinal, breast and cervical carcinomas, and myocardial infarction may accompany by high GUSB activity in serum. Excessive GUSB activity may be a major factor in the etiology of CRC (Muzny et al., 2012; Naz et al., 2013; Sun et al., 2019).

The serum GUSB activity in humans is determined by the intake of its own enzyme from tissues and the activity of intestinal bacteria (Han et al., 2018). Evaluation of the activity of lysosomal hydrolases in serum and urine, according to several studies, can be used in the screening diagnosis of intestinal tumors in the early stages of the disease (Chojnowska et al., 2011; Häuselmann, Borsig, 2014).

Saliva (oral fluid) is a complex filtrate of blood plasma, and it also contains components derived from the mucous membranes, gingival fissures, and dental plaque, including microorganisms and their products (Bhardwaj et al., 2013).

Saliva can change its composition very significantly and properties after exposition of a various stimuli, so the determination of some biomarkers in saliva becomes an important part of laboratory diagnostics to predict not only oral diseases, and also pathology of other tissues and organs (Syndergaard et al., 2014; Ebersole et al., 2015) The diagnostic capabilities of these methods are still far from being exhausted.

The aim of the study was to establish Parallels between the activity of beta-glucuronidase in the blood serum and saliva of healthy volunteers and individuals at high risk of developing intestinal tumors.

2. Materials and methods

Characteristics of the surveyed group

Blood and saliva samples were obtained from 24 individuals (12 men and 12 women) aged 21-60 years who applied for a scheduled medical examination in connection with their current or upcoming professional activities (Table 1).

The exclusion criterion was the presence of a burdened anamnesis for the main kinds of somatic diseases (cardiovascular pathology, respiratory diseases, diabetes, autoimmune disorders, and oncology), or mental disorders. The group also did not includes pregnant or nursing women. Due to the need to study saliva, the subjects must have functionally formed dentitions, fillings and individual crowns are allowed, but the presence of removable orthopedic structures, diseases of the oral mucosa and pronounced periodontal pathology was not allowed.

Table 1. Gender and age characteristics of the surveyed group

Age	Surveyed individuals		
	Number	Including	
		Female	Male
20-29 years	4	2	2
30-39 years	5	3	2
40-49 years	8	4	4
50-60 years	7	3	4
Total number	24	12	12

For each surveyed person we additionally noted the presence or absence of following risk factors for CRC development (Shuwen et al., 2017): age over 50 (7 people), male gender (12), obesity (11), and smoking (10 people). As a result, we found 11 people with at least one risk factor (46 %), and 6 people with three or four risk factors (25 %). The latter were considered separately as individuals with a serious risk of CRC.

Sampling and sample preparation

Blood serum was provided as scrap material for routine biochemical examination of patients (glucose, cholesterol, total protein). To collect saliva, patients were informed about the purpose and conditions of the study, the methods used. This information we included in the text of the voluntary informed consent to medical procedures.

Non-hemolized blood serum was centrifuged at room temperature in the mode of 3200 g for 10 min.

When taking saliva, we additionally took into account a number of requirements, primarily the stability of the results, as well as minimal invasiveness for the patient and simplicity (Syndergaard et al., 2014). Before sampling patients avoided smoking and exercise within two hours, rinsed their mouths twice with drinking water, removed the remaining water with a clean cloth, and then collected saliva through a sterile funnel into a plastic test tubes with a lid (Postnova et al., 2011).

In parallel, the protein concentration was determined using Coomassie dye according to the method of Marion Bradford (Bradford, 1976). The studies were carried out on a SmartSpec Plus spectrophotometer (Bio-Rad, USA) at a wavelength of 595 nm.

Determination of GUSB activity in serum and saliva

To determine the enzymatic activity of SB, we used a Merck KGaA commercial kit (Germany) in accordance with the manufacturer's instructions. The principle of the method is that in the presence of GUSB activity, p-nitrophenyl-β-D-glucuronide is hydrolyzed to p-nitrophenol as a colored compound and glucuronide. The color intensity is proportional to the activity of the enzyme and it is determined spectrophotometrically at a wavelength of 405 nm (Marciniak et al., 2006).

In parallel, we determined the protein concentration using Coomassie dye by Marion Bradford method (Bradford, 1976). The research was performed on a SmartSpec Plus spectrophotometer (Bio-Rad, USA) at a wavelength of 595 nm.

The enzyme activity in the test sample was calculated using the formula:

$$A = K \times E / Pt,$$

where: K is the coefficient calculated from the calibration graph, E is the optical density of the test sample measured against the corresponding control sample, and Pt is the protein concentration (g/l).

The results were expressed in micromol/s×g of protein.

Fractionation of serum and saliva proteins

To carry out gel chromatography we used ENrich SEC 650 column (Bio-Rad, USA) filled with Sephadex G-200. The separated substances were eluted with a borate buffer (pH = 8.80). As an

internal control, for the construction of the calibration curve, a joint injection of a solution containing aliquots of purified human hemoglobin (molecular weight 68 kDa) and colored "blue" dextran (molecular weight 2000 kDa) were used. After identification of peaks on the obtained chromatograms we digitized and characterized its.

Methods of mathematical processing of results

Statistical analysis was performed using the software package Statistica 12.0 (StatSoft Inc., USA). Previously, based on the Kolmogorov-Smirnov and Shapiro-Wilk criteria, the hypothesis of normality of the distribution was rejected, and therefore nonparametric criteria were used for the analysis. In this regard, we used nonparametric criteria. Distribution in the samples was expressed as a median and the interval between the first and third quartile (Me [Q1÷Q3]), the Mann-Whitney criterion (critical significance level $p < 0.01$) was used to analyze the differences between the two research methods, and the Craskell-Wallis criterion for multiple groups ($p < 0.01$) was used to compare the results between several groups. Descriptive statistics were supplemented with Spearman correlation analysis (critical confidence level $p < 0.05$).

3. Results and discussion

GUSB activity in blood serum and saliva

Table 2 shows the results of determining the activity of GUSB due to the absence or presence of risk factors for CRC. As can be seen from the data, we did not find significant differences in GUSB activity in the blood serum and saliva of the examined individuals.

Table 2. Specific activity of GUSB in blood serum and saliva in subjects of the study group and in subgroups of risk expressed as Me [Q1÷Q3]

Group/subgroup	GUSB activity, μM/s × g of protein		P
	Serum	Saliva	
Risk-free group (n = 7)	0.09 [0.05 ÷ 0.12]	0.07 [0.04 ÷ 0.10]	n. r.
Men (n = 12)	0.14 [0.09 ÷ 0.18]	0.11 [0.06 ÷ 0.16]	< 0.01
Over 50 years old (n = 7)	0.18 [0.14 ÷ 0.25] *	0.14 [0.11 ÷ 0.21] *	< 0.01
Obesity (n = 11)	0.18 [0.13 ÷ 0.22] *	0.07 [0.05 ÷ 0.11]	< 0.01
Smoking (n = 10)	0.25 [0.15 ÷ 0.32] *	0.09 [0.06 ÷ 0.14]	< 0.01
High risk (n = 6)	0.22 [0.15 ÷ 0.29] *	0.12 [0.09 ÷ 0.17]	< 0.01

* – significant differences compared to the risk-free group

In the blood serum the GUSB activity in men and in persons over 50 years of age was 2.0 times higher, compared to the value in persons without risk factors. The highest values were found in smokers (2.8 times more than in the risk-free group) and in the presence of several risk factors (2.4 times more). In saliva the GUSB activity was on average 1.8 times lower than in blood serum. The differences between the risk-free subgroup and those over 50 years of age and those with multiple risk factors for CRC, however, were found to be significant.

To assess the involvement of the studied enzyme in the formation of risk for CRC incidence, we performed a Spearman correlation analysis. Table 3 shows the results of this analysis.

As can be seen, eight positive dependencies were found between risk factors and indicators of GUSB activity in serum and saliva, six of them were weak ($r < 0.5$) and two ones were moderate ($0.5 < r < 0.8$). The differences in this case were significant ($p > 0.05$). The relationship between

age and GUSB activity was the most significant, the gender factor did not give such a high dependence.

Table 3. Rank correlations between the values of GUSB activity indicators in blood serum and saliva and the presence of individual risk factors (R)

Indices	Risk factors			
	Over 50 years old (n = 7)	Men (n = 12)	Obesity (n = 11)	Smoking (n = 10)
GUSB activity in serum	0.582 *	0.288	0.064	0.116
GUSB activity in saliva	0.566 *	0.119	0.006	0.009

* – significant differences compared to the risk-free group

Results of activity determination in individual protein fractions

As a result of gel chromatography of 8 samples of blood and saliva, we isolated 2 large fractions in the range of molecular weights: from 225 to 350 kDa (fraction 1) and from 450 to 700 kDa (fraction 2). The 1st fraction approximately includes human GUSB, and the fraction 2 GUSB of bacterial nature.

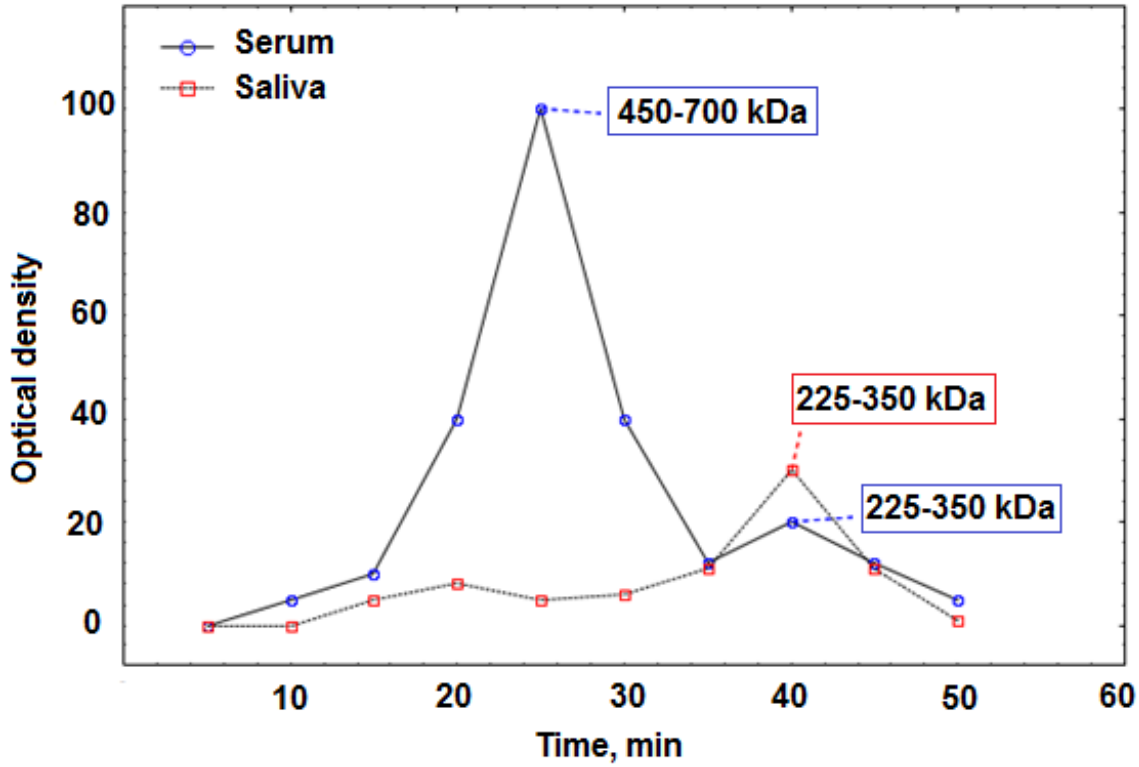

Fig. 1. Chromatogram of GUSB isolation from blood serum and saliva

The Figure 1 represents chromatograms, and we see that there is a peak of activity in the blood serum, which presumably should include GUSB of a bacterial nature. The second peak is found both on the chromatograms of blood serum and saliva samples, and it is presumably associated with the activity of Human GUSB (Liu et al., 2014; Uroy et al., 2019).

At the same time, the second fraction of the blood serum had a high peak in the range of 450-700 kDa, and when the saliva was separated, the 2nd fraction was not detected. The specific GUSB activity was reliably detected in the 2nd fraction of blood serum and amounted to 0.392 μmol/s×g of protein, which is 6 times less than in the 1st fraction. An important point is that in the 2nd fraction after saliva separation, the specific activity of GUSB was practically not detected. This may be due to the fact that there are almost no bacterial enzymes in saliva, since

they have a higher molecular weight, and it is more difficult to penetrate the plasma filtrate, which is the basis of the protein composition of saliva (Zefferman, 2015; Hudson et al., 2017).

4. Conclusion

In this study, we determined the activity and specific activity of GUSB in blood serum and saliva, as well as the differences in these indicators in different groups of people who are not at risk, as well as with a high risk of CRC. In this regard, we can say that the main cause of changes in the activity and specific activity of GUSB is a factor such as Smoking, while changes are observed in both saliva and blood serum. Next in terms of influence on the specific GUSB activity are two factors: age and obesity, but it is important that smoking is the most powerful factor that affects the GUSB activity. The results of correlation analysis confirm the fact about the GUSB activity in blood serum and saliva to depend on the presence of risk factors for CRC, such as age and smoking.

Fractionation of serum and saliva proteins indicates that the main form of GUSB associated with variation in activity due to the presence or absence of risk factors for CRC is Human form of GUSB.

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