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CfDNA INTEGRITY INDEX AND RCC

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Abstract

Background: Early cancer detection is crucial for successful treatment. Modern screening methods do not allow to detection of many cases of kidney cancer at an early stage. It has been shown that the plasma/serum cfDNA integrity index increases in various malignancies, including kidney cancer. Here, we conducted a retrospective cohort study to evaluate the possibility of using the integrity index for the diagnosis and the postoperative condition of patients with renal cell carcinoma (RCC).

Patients and methods: In the study were analyzed the paired plasma samples from 50 RCC patients were taken before and after (5-6 days) surgery. The Control group included 26 plasma samples from individuals who did not have cancer or any other chronic disease. The cfDNA integrity index was measured by real-time PCR, for which two fragments of the ACTB gene with different lengths (106 and 384 bp) were quantified and their ratios were determined (ACTB-384/106).

Results: Plasma cfDNA integrity index of patients with RCC decreased after tumor removal ($p < 0.0001$), and was significantly higher compared to the control group in both before ($p < 0.0001$) and after surgical resection ($p = 0.0023$) samples. In addition, the integrity index ACTB-384/106 of patients with RCC was significantly higher for patients with Furman's cell atypia G2 ($p < 0.0001$) and G3 ($p < 0.0001$) compared with the control group of healthy individuals.

Conclusion: The obtained results confirm the prospects of using the cfDNA integrity index ACTB-384/106 as a biomarker for the diagnosis of RCC and the possibility of using it as an additional parameter for the monitoring of the patient's postoperative condition.

All human studies were conducted in compliance with the rules of the Helsinki Declaration of the World Medical Association "Ethical principles of medical research with human participation as an object of study". Informed consent was obtained from all participants.

Keywords: *cell-free DNA, RCC, Integrity index, Fuhrman Nuclear Grade*

Introduction

Cancer is the leading cause of death in the world. According to the World Health Organization, in 2020 there were more than 19 million cancer patients and about 10 million deaths from it [1]. Despite advances in medicine that have improved cancer survival since the mid-1970s, cancer is the second leading cause of death among human diseases [2, 3]. Clear cell renal cell carcinoma (RCC) is the most common type of kidney tumors, accounting for 70% of all diagnosed malignancies [4, 5]. Due to progress in surgical techniques and drug development, RCC patients have 85–96% cancer-specific survival 10 years after surgery if disease diagnosed at the early stages [5 – 7]. Thus, it is extremely important to identify biomarkers for the early diagnosis and prognosis of the RCC.

It has been shown that quantitative and qualitative changes in the composition of nucleic acids (DNA, mRNA, non-coding RNA) found in the plasma and serum patients with various pathologies can be used as markers of cancer in the early stages [8 – 10]. The concentration and integrity of cfDNA have been investigated as possible diagnostic or prognostic markers in many diseases [11 – 14], including kidney cancer [15 – 18]. Our previous study showed that the concentration of cfDNA in the plasma of patients with kidney cancer is significantly higher than in healthy individuals, and together with methylation of specific genes it can be used as an addition to serological markers for tumor identification [19].

cfDNA is formed due to apoptosis and/or cell necrosis. DNA released from apoptotic cells is highly fragmented and has a uniform size distribution of 185 to 200 bp as a result of programmed enzymatic cleavage of DNA. DNA released as a result of tumor necrosis circulates in. blood in a form of fragments with different chain lengths, compared to apoptotic cfDNA due to accidental and incomplete digestion of genomic DNA by various deoxyribonuclease [20]. Therefore, a detailed analysis of the size of cfDNA fragments can determine the source of circulating nucleic acid in cancer patients.

cfDNA integrity is calculated as the ratio of the concentration of longer DNA fragments to shorter ones of a particular genetic locus and indicates the degree of cfDNA fragmentation. It has been shown that the integrity index obviously depends on different types of cancer, as well as on the stages of tumor progression [21 – 23].

In this study, we focused on cfDNA integrity as a possible diagnostic marker for patients with RCC. Our results suggest that increased DNA integrity in plasma DNA is associated with kidney cancer, and measuring DNA integrity can provide a simple and inexpensive additional test to diagnose cancer even in the early stages of the disease.

Materials and methods

Patients and samples

The blood plasma used in this study was obtained from 49 patients with kidney tumors who were operated at the Institute of Urology of the National Academy of Medical Sciences of Ukraine from January 2014 till February 2017. Blood sampling was performed one day before surgery and 5-6 days after it. The control group was selected from researchers, doctors, students, and laboratory technicians; they donated blood samples on a voluntary basis. All patients and healthy donors provided signed informed consent prior to enrollment in the study. The samples were collected in accordance with the Declaration of Helsinki and the guidelines issued by the Ethics Committee of the Institute of Urology NAMS of Ukraine. The Ethics Committee of the Institute of Urology approved this study. The mean age of patients was 54.04 ± 1.463 SEM years. The examinations were performed according to the clinical protocols of the Ministry of Health of Ukraine. After pathohistological examination in 49 cases different types of renal cell carcinoma were diagnosed. General information about patients is presented in table 1.

For patients, 28 (57.1%) organ-sparing operations were performed, of which 10 (20.4%) - laparoscopic resection, 18 (37.5%) - open surgery. Nephrectomy was performed in 21 (42.9%) cases, in 11 cases - with open access (22.5%), and in 10 - laparoscopic (20.4%). In 28 (57.1%) cases the left kidney was affected, in 21 (42.9%) - the right.

Blood sampling and cfDNA isolation

9 ml of blood was collected into the K3EDTA-containing tubes. The samples were stored at 4℃ and treated within 3 h after blood collection. To ensure cell-free plasma collection, the supernatant was transferred to 15 ml centrifuge tubes and

centrifuged for 10 minutes at 1000 g and 4°C. The supernatant was then transferred to a new tube and centrifuged for 10 min at 2500 g and 4°C. Thus from 9 ml of blood finally from 2.0 to 4.5 ml of plasma was received from the healthy donors and 3.5-5.5 ml from the patients. Plasma was packed in 1 ml tubes and stored at -80°C until use. The cfDNA was isolated by using the QIAamp Circulating Nucleic Acid Kit (Qiagen, Hildem, Germany) according to the manufacturer's protocol. Isolated cfDNA was eluted in 50 mkL of AVE buffer and stored at –20°C until use.

Quantitative PCR

To determine the integrity index, quantitative PCR was performed using primers to b-actin, with a fragment size of 106 bp (5' – TCGTGCGTGCGTGACATTAAGGAG – 3 ', 5' – GGCAGCTCGTAGCTCTT CTC – 3') and 384 bp. (5' – GCT ATCCCTGTACGCCTCTG – 3 ', R 5' – AGGAAGGAAGGCT GGAAGAG – 3') [24]. PCR was conducted using a 2x SYBR Green PCR Mix (Thermo Scientific, USA) with a CFX96™ Real-Time PCR Detection System (BIO-RAD Laboratories, Inc., Singapore). For each sample, the PCR reaction consisting of a 10 min hot start at 95°C for polymerase activation, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C, was performed in triplicate. The specificity of the PCR products was confirmed by melting curve analysis. Each run included a negative control (without template) and serial dilutions of an external standard. Quantitative standard curves were prepared using serial dilutions (from 20 pg to 100 ng/reaction) of control genomic DNA (Human HCT116 DKO Nonmethylated DNA, Zymo Research Corporation, USA).

Statistical Analysis

Statistical analysis and building of ROC (Receiver-operating characteristics) and evaluation of AUC (Area Under Curve) was based on the GraphPad Prism software (version 7.0a, Inc. San Diego, CA, USA). Parametric statistics (Paired two-tailed t-test) were used for comparison of ACTB384/106 data of patient samples obtained before and after surgery. The nonparametric Mann-Whitney U test was used to compare cfDNA integrity between plasma samples of the RCC patients and healthy donors and explore the relationship with gender, age, tumor stage, and Fuhrman grade. A *p*-value <0.05 was considered statistically significant.

Results

Analysis of cfDNA fragmentation in RCC samples relative to ACTB-384/106 values was performed based on sex, age, tumor size, cartilage cell atypia, and tumor node metastasis (TNM) stage. In the control group, cfDNA (cfDNA-C) was moderately fragmented (median ACTB-384/106 was 0.0486), while patients with RCC showed a significantly greater degree of fragmentation (Table 2). The median ACTB-384/106 for preoperative samples (cfDNA-S) was 0.219, for plasma obtained from patients after surgery (cfDNA-AS) almost 3 times lover with a median of 0.0763. These data confirm the results obtained by other groups and show that the cfDNA integrity index can be used for cancer detection. This also confirms that the levels of macromolecular cfDNA derived from tumor cells decrease after the tumor removal. ROC curve analysis of integrity index showed an AUC = 0.8508 (p <0.0001) and AUC = 0.7115 (p = 0.0026) for healthy individuals and cancer patients before and after 5-6 days of surgery respectively (Fig. 1).

For cfDNA-AS obtained 5-6 days after tumor removal, a significant decrease in the value of the integrity index ACTB-384/106 was observed, compared to cfDNA-S (Paired two-tailed t-test, p <0.0001). ROC curve analysis of index integrity showed an AUC = 0.7172 (p = 0.0002) for samples of cancer patients taken before and after surgery.

Analysis of ACTB-384/106 cfDNA integrity index among different age groups of patients with RCC revealed a difference between the values of cfDNA integrity index of blood plasma taken before and after surgery for patients younger than 54 years (p = 0.0016, n = 18), patients between 55 and 64 years of age. (p = 0.009, n = 27), and in people older than 65 years (p = 0.0173, n = 4) (Fig. 2A).

Differences in the fragmentation levels of ACTB-384/106 of cfDNA-S compared to cfDNA-AS were correlated for patients of different sex groups (Fig. 2B). Significant differences were also observed between the integrity index of cfDNA-S and the control group of healthy individuals of the same sex for women (p = 0.0127) and for men (p = 0.0045) (Fig. 2). However, there was no significant correlation between the group of women and men in the change in ACTB-384/106-S (p = 0. 4194).

The difference between the values of ACTB-384/106 was also found when comparing groups of patients with different Fuhrman nuclear atypia grades. A significant difference in ACTB-384/106 was observed for samples taken before and after surgery for patients with G1 ($p = 0.0027$, $n = 23$), G2 ($p = 0.0082$, $n = 17$) and G3+G4 ($p < 0.0001$, $n = 8$) (Fig. 3). Also, a significant difference was found for ACTB-384/106 values of patients with RCC between groups G1 and G3 ($p = 0.0024$), and between ACTB-384/106 of the control group of healthy individuals and patients with G2 ($p < 0.0001$) and G3 ($p < 0.0001$). The AUC of the ROC curve for distinguishing patients with Fuhrman G2 and Fuhrman G3 from healthy controls was 0.8729 ($p < 0.0001$) and 1.0 ($p < 0.0001$), respectively . There was no significant difference in the cfDNA integrity index between patients with Fuhrman G1 and control (Fig. 4).

Discussion

Recent studies suggest that the "integrity index", as well as the level of cfDNA concentration, can be used as markers for the preliminary diagnosis of cancer, for the monitoring of the presence of metastatic cells in the patient's body not removed during surgery, and for the monitoring of the treatment outcome [12, 13, 25]. To estimate the amount and degree of cfDNA fragmentation, we used the method proposed and used previously [15, 24]. The method is based on the fact that cfDNA, which enters the bloodstream as a result of normal processes occurring in the body (such as apoptosis, secretion), is much smaller than cfDNA derived from the necrosis of cancer cells [26, 27]. Here we used two pairs of primers to the same region of the β-actin gene, which allow amplification of 106 bp and 384 bp size fragments. The primers were selected so that the sites of association of primers ACTB-106 were in the middle of the sequence flanked by primers ACTB-384. As a result, the primers ACTB-106 are specific to both large and small fragments of cfDNA, so the fragment with 106 bp size should be detected at a higher level than the 384 bp size fragment, which is specific only to large cfDNA fragments [24]. Thus, the ratio of the amount of ACTB-384 to ACTB-106, which is called the "integrity index", characterizes the degree of cfDNA fragmentation in blood plasma/serum.

Analysis of the results showed completely different levels of cfDNA fragmentation for cancer patients with RCC and control group (AUC = 0.8508), which confirms the results obtained by other researchers [15, 28]. In the control group of healthy individuals, cfDNA was moderately fragmented (mean 0.04158± 0.0062SEM), while in patients with RCC a significantly greater degree of fragmentation (0.2556±0.0353SEM) was observed. We also examined the opinion of other researchers on changes in the levels of fragmented cfDNA in patients after removal of the tumor as a source of high molecular weight cfDNA due to necrosis of tumor cells. Indeed, the integrity index ACTB-384/106 for cfDNA of blood plasma taken from the same patients on day 5-6 after tumor removal decreased significantly (0.1006 ± 0.1827SEM), although it has not yet reached healthy control values.

It has previously been reported that significantly higher cfDNA concentrations are observed in the older patients as well as in men [21, 29, 30]. Despite this, we didn't observe difference between male and female cfDNA fragmentation. In our study, we found a higher ACTB-384/106 fragmentation in the plasma of patients with RCC compered to healthy donors in both male, and female. Difference between different age groups cannot be analyzed due to small number of patients with the age above 65 years (n=4).

In addition, we found a significant difference in the ACTB-384/106 cfDNA integrity index changes between patients with RCC and the control group of healthy individuals when RCC patients were separated according to Furman's atypia. According to our data, patients with G2 (AUC = 0.8729, $p < 0.0001$) and G3 (AUC = 1, $p = 0.0001$) can be distinguished from the healthy donors by the ACTB-384/106 cfDNA value. Significant difference between this indicator in healthy individuals and the G1 gradation of cell atypia was not found ($p = 0.4108$). However, considering that patients with kidney cancer are often detected at later stages of the disease [6], these results will be useful for creating a non-invasive system for the diagnosis of RCC in the blood.

Among the study group, 9 (18%) patients died due to cancer progression or recurrence of the pathology. However, no correlation between the

ACTB-384/106 cfDNA and disease progression was found. In all but one of these patients (male, 41 years old, pT2N0M0, stage 2 disease, G3), had the integrity index within the mean. Only one of them had a high concentration of ACTB-106 both before surgery (144.6 ng/µl) and 5 days after it (41.7 ng/µl). However, the levels of ACTB-384 were within the average values, and therefore, the ratio of ACTB-384/106 was very low. It should be noted that several other patients had such ultra-high rates, but all of them remain alive and are under the care of doctors. Therefore, we cannot draw conclusions about the use of the integrity index ACTB-384/106 cfDNA as a prognostic marker.

A large number of studies have reported higher DNA integrity in the plasma of patients with differentl cancers compared with healthy controls [12 – 14, 28]. However, other reports have failed to find differences in DNA integrity values in the same tumor types [22, 23]. Our data show that the content of macromolecular cfDNA in the plasma of patients with kidney cancer is higher than in the plasma of healthy donors and that with tumor removal, the content of macromolecular cfDNA begins to decline, which confirms the theory of the necrotic origin of cfDNA [9, 31]. The obtained results indicate the possibility of using the method of determining the integrity index as an additional criterion in the initial diagnosis of kidney cancer, and to monitor the treatment of cancer patients with clear cell carcinoma of the kidney.

Acknowledgments

The authors declsre that there are no conflicts of interest.

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Table 1. Clinicopathological characteristics of patients

Parameter		Number of patients, %
Age, years (n=49):	5-54	18 (36.7)
	55-64	27 (55.1)
	>65	4 (8.2)
Gender (n=49):	Male	33 (67.3)
	Female	16 (32.7)
Side of kidney damage (n=49):	right	21 (42.9)
	left	28 (57.1)
Fuhrman grade (n=49):	G1	23 (46.9)
	G2	17 (34.7)
	G3	8 (16.3)
	G4	1 (2.1)
TNM (n=49):	T1a No Mo-1	18 (36.8)
	T1b Nx-o Mo-1	13 (26.5)
	T2a No-1 Mo	13 (26.5)
	T3a No-1 Mo-1	5 (10.2)
Clinical stage (n=49):	I	29 (59.1)
	II	12 (24.5)
	III	4 (8.2)
	IV	4 (8.2)
Histology (n=49):	Clear cell RCC	45 (91.8)
	Papillary RCC	4 (8.2)

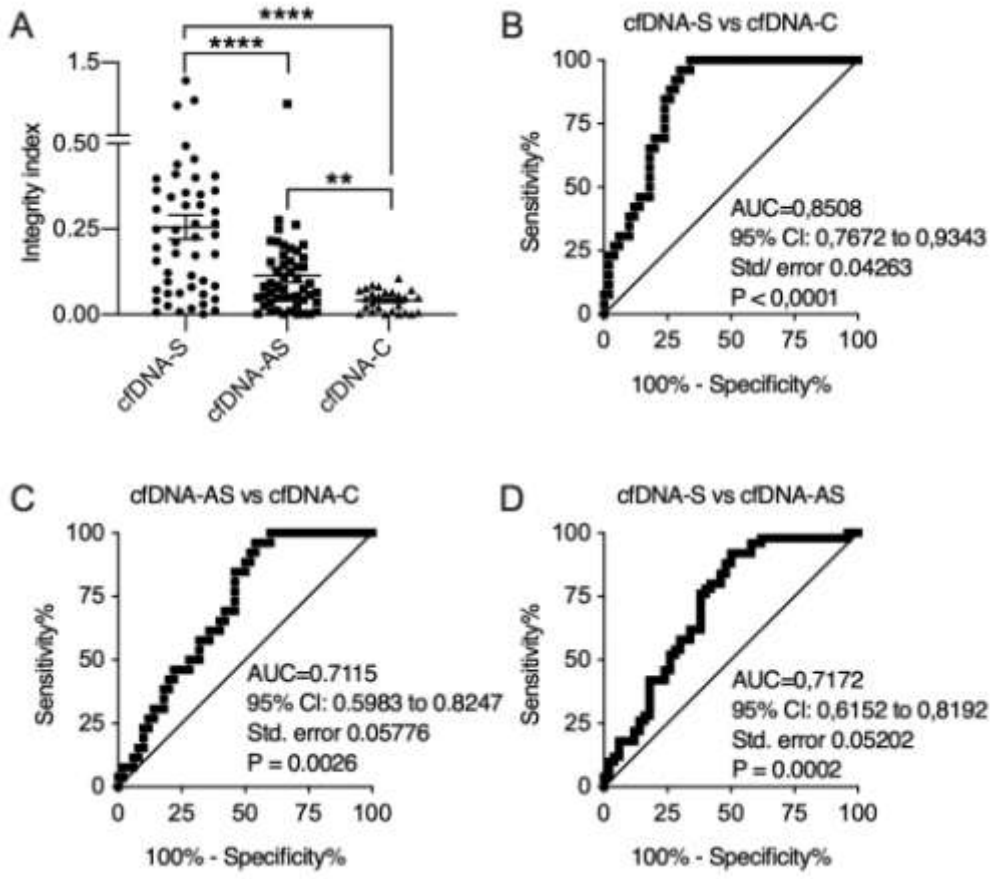

Figure 1. Scatter plots (A) and Receiver-operating characteristics plots of ACTB-384/106 cfDNA integrity index isolated from plasma of patients with RCC (n = 49) before surgery (B) and after surgery (C) in comparison with healthy donors (n = 26) and among themselves (D). ** - p = 0.0023, **** - p <0.0001

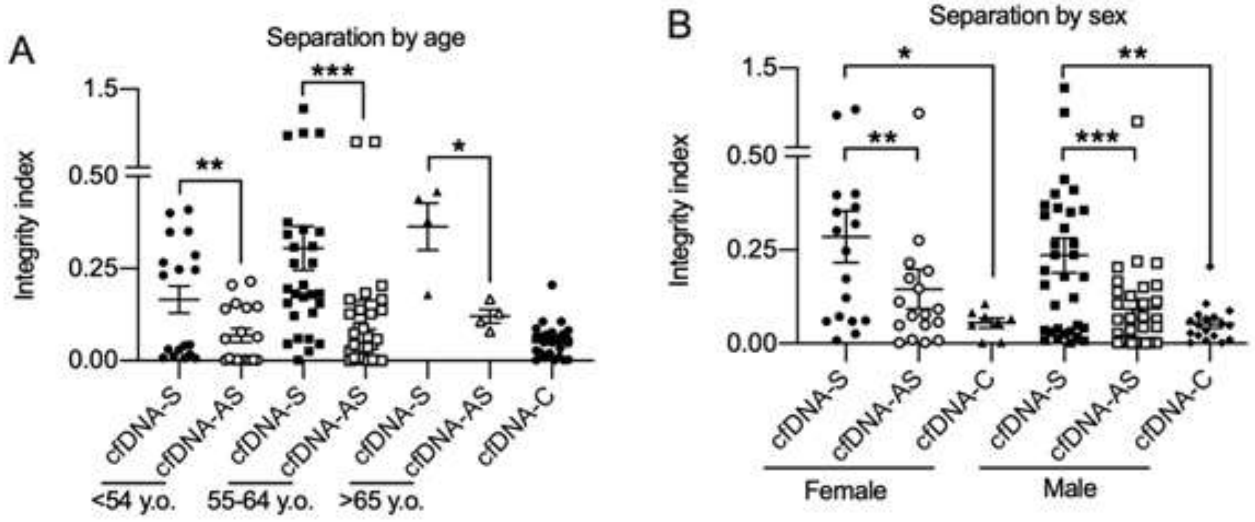

Figure 2. Scatter plots of ACTB-384/106 cfDNA integrity index isolated from plasma of patients with RCC before surgery (S), after surgery (AS), and healthy donors (C) compared by age (A) and sex (B).

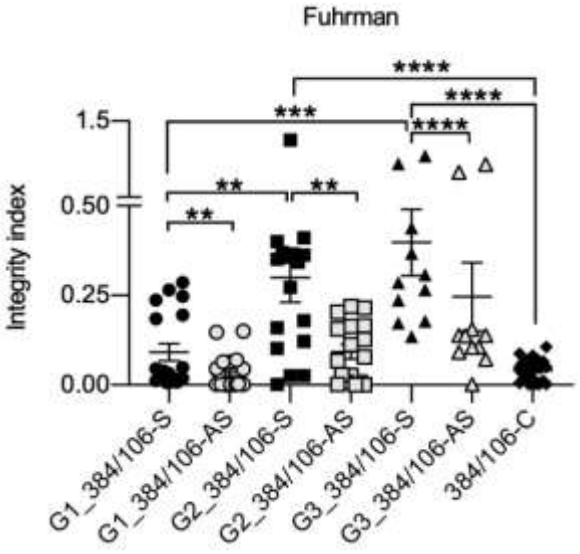

Figure 3. Scatter plots of ACTB-384/106 cfDNA integrity index isolated from plasma of patients with RCC before surgery (B) and after surgery (C) separated by Fuhrman grade and compared with healthy donors

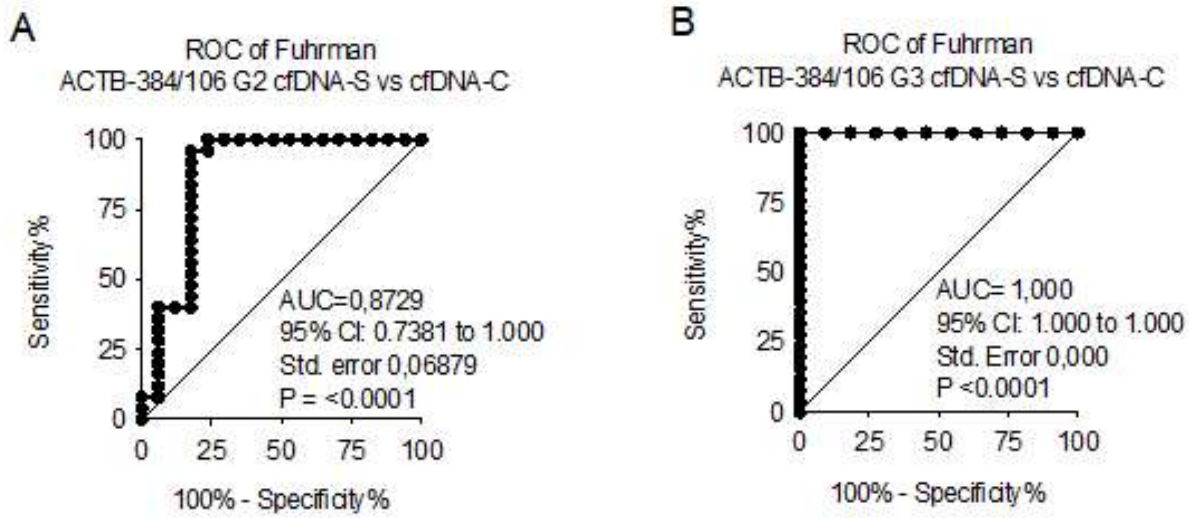

Figure 4. Receiver-operating characteristics plots of ACTB-384/106 cfDNA integrity index, isolated from blood plasma before surgery of patients with RCC characterized by Fuhrman grade G2 (A) and G3 (B) in comparison to healthy donors (C)

Table 2. Statistics for the analysis of integrity index ACTB-384/106 in cfDNA determined for patients with RCC and healthy donors

Index	cfDNA-S, n=49	cfDNA-AS, n=49	cfDNA-C, n=26
Mean	0.2556±0.0353 SEM	0.1135±0.0199 SEM	0.04158±0.0062 SEM
Median	0.2188	0.0763	0.0486
Area under ROC curve vs C	0,8508 95% CI: 0,7672 to 0,9343 SE 0.04263 P value < 0,0001	0.7115 95% CI: 0.5983 to 0.8247 SE 0.05776 P value 0.0026	
Area under ROC curve S vs AS	0,7172 95% CI: 0,6152 to 0,8192 SE 0.05202 P value 0.0002		