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Research Article

Concentration and Methylation of Cell-Free DNA from Blood Plasma as Diagnostic Markers of Renal Cancer

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The critical point for successful treatment of cancer is diagnosis at early stages of tumor development. Cancer cell-specific methylated DNA has been found in the blood of cancer patients, indicating that cell-free DNA (cfDNA) circulating in the blood is a convenient tumor-associated DNA marker. Therefore methylated cfDNA can be used as a minimally invasive diagnostic marker. We analysed the concentration of plasma cfDNA and methylation of six tumor suppressor genes in samples of 27 patients with renal cancer and 15 healthy donors as controls. The cfDNA concentrations in samples from cancer patients and healthy donors was measured using two different methods, the SYBR Green I fluorescence test and quantitative real-time PCR. Both methods revealed a statistically significant increase of cfDNA concentrations in cancer patients. Hypermethylation on cfDNA was detected for the *LRRC3B* (74.1%), *APC* (51.9%), *FHIT* (55.6%), and *RASSF1* (62.9%) genes in patients with renal cancer. Promoter methylation of *VHL* and *ITGA9* genes was not found on cfDNA. Our results confirmed that the cfDNA level and methylation of CpG islands of *RASSF1A*, *FHIT*, and *APC* genes in blood plasma can be used as noninvasive diagnostic markers of cancer.

1. Introduction

Renal cell carcinoma (RCC) is a widespread oncologic disease that accounts for about 3% of all malignancies in adults and 85% of all primarily malignant tumors in kidney [1]. Metastases detected at the time of establishing a diagnosis are present in 25–30% of patients, and even after surgery the disease progresses in 20–30% of patients [2, 3]. An asymptomatic period of the disease makes early diagnosis of this type of tumor difficult to perform. Globally, the incidence rates of kidney cancer are predicted to increase. The International Agency for Research on Cancer claims that this number will rise to 22%, from 337,860 cases in 2012 to 412,929 cases in 2020 [4].

Clear cell carcinoma is the most common type of RCC, accounting for 70–80% of all RCCs [5]. Development of this particular type of RCC is associated with many tumor suppressor genes that are localized in the short arm of human chromosome 3. They can be inactivated as a result of mutations, LOH (loss of heterozygosity), or methylation of CpG islands in promoter regions [6–9]. Identification of aberrantly methylated genes for a particular tumor type can be helpful in early diagnosis of the disease.

Cell-free DNA (cfDNA) enters the blood stream from apoptotic and necrotic tumor cells and is useful in detecting tumor-specific signatures, including the methylation of genes [10, 11]. Aberrant cfDNA methylation has been described

in most cancer types and is being actively investigated for minimally invasive clinical diagnostics [11–13].

Large-scale NotI-microarray analyses of genetic and epigenetic alterations in the genes of chromosome 3 in RCC revealed that leucine-rich repeats containing 3B (*LRRC*3B) and Von Hippel-Lindau (*VHL*) genes possess the highest frequency of deletions and/or methylations in renal carcinoma [14, 15]. Adenomatosis-polyposis-coli (*APC*), Ras association domain family 1 (*RASSF1*), and fragile histidine triad (*FHIT*) genes were shown to have high levels of methylation in cfDNA and/or in renal tumors [16–22].

In this study we determined the plasma cfDNA concentration (by quantitative PCR and the fluorescence test) and analysed methylation of 6 genes (*APC*, *FHIT*, *RASSF1*, *LRRC3B*, *VHL*, and *ITGA9* (Integrin $\alpha 9\beta 1$)) in plasma samples from patients with kidney cancer in order to evaluate the diagnostic value of these markers for cancer detection.

2. Materials and Methods

2.1. Sample Collection. The study included 27 patients undergoing surgery for kidney cancer at the Institute of Urology, National Academy of Medical Sciences of Ukraine in Kyiv, between January 2011 and August 2011. Before surgery all patients were fully examined according to the protocols of the Ministry of Health of Ukraine: laboratory clinical analysis, Doppler ultrasound diagnosis, renal scintigraphy, and spiral computed/magnetic resonance tomography of the retroperitoneal space. For the negative controls, peripheral blood was collected from 15 healthy individuals. All patients gave written 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 specifically approved this study.

2.2. Extraction of cfDNA. Blood (5 mL) was collected in K3 EDTA-containing tubes (Cat. number 2102, APTACA, Italy). The samples were stored at 4°C and treated within 3 h after blood collection. The plasma was isolated by low-speed centrifugation: 250 ×g for 7 min, 350 ×g for 8 min, and 500 ×g for 10 min using Jouan MR23i centrifuge (JOUAN, France). It was then aliquoted and cryopreserved at −70°C.

cfDNA was isolated from 2 mL plasma using the Proba NA Kit (DNA-Technology, Russia) according to the manufacturer's recommendations (final elution volume was 150 μL). The extracted DNA was subjected to PCR with the *ACTB* gene (5′-CCACACTGTGCCCATCTACG-3′ and 5′-AGGATCTTCATGAGGTAGTCAGTCAG-3′; 99 bp fragment) as control, and the PCR products were examined by electrophoresis (see Supplementary Figure S1 in Supplementary Material available online at http://dx.doi.org/10.1155/2016/3693096). PCR conditions were as follows: 95°C for 4 min and then 40 cycles of 95°C for 40 s, 56°C for 20 s, and 72°C for 30 s, with a final extension for 5 min at 72°C.

2.3. Quantification of Plasma cfDNA by Real-Time PCR. To measure the plasma cfDNA concentration, the genomic sequence of β-actin was amplified by quantitative real-time PCR (qPCR). The primers and fluorescent probe used for qPCR were as described in Herrera et al. [23]. 5 μL purified cfDNA was amplified using 0.3 μM of each primer (5′-CCACACTGTGCCCATCTACG-3′ and 5′-AGGATCTTCATGAGGTAGTCAGTCAG-3′) and a 0.25 μM fluorescent probe (5′-FAM-ATGCCCTCCCCCATGCCATCCTGCGT-TAMRA-3′). The length of the amplified fragment was 99 bp. PCR was performed under the following conditions: 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Quantitative standard curves were prepared using serial dilutions (from 20 pg to 100 ng/reaction) of control genomic DNA. Human HCT116 DKO Nonmethylated DNA (Cat. number D5014-1, Zymo Research Corporation, USA) was used as calibrator for quantification. The concentration of control DNA was assessed using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). No-template controls (NTCs) were used as negative controls. The fluorescence of the amplified PCR products was detected using the BioRad iQ5 Optical System (Bio-Rad, USA). The results of the qPCR assays represent the mean of three independent experiments, each consisting of duplicate samples. The analysis was repeated if the difference between duplicate samples was greater than one cycle threshold. The linear dynamic range was determined by the standard curve and correlation coefficients (R^2), which was ≥0.98. A more detailed version of the protocol is given in Supplementary Table S1. The statistical significance of differences between samples was established using the Mann-Whitney U test.

2.4. Quantification of Total Plasma DNA by the Fluorescence Test. Evaluation of the cfDNA concentration was also performed measuring the fluorescence of intercalating dye [24]. Specifically, 5 μL of a sample or the same volume of a standard dilution of genomic DNA (Human HCT116 DKO Nonmethylated DNA) with known concentration (0 ng/mL and 9 serial dilutions from 1 to 256 ng/mL) was added to 195 μL of a SYBR Green I solution (Cat. number S7585, Thermo Fisher Scientific, USA) in PBS buffer (1 : 10,000) and to black 96-well plates (PAA, Cat. number PAA30296X, Austria) and incubated for 10 min. Two to three identical mixtures were prepared from each sample or standard for greater accuracy. The fluorescence of the mixtures obtained was measured by the "VICTOR^3 1420-050" Multilabel Plate Readers (Perkin Elmer, USA) using filters for FITC (485/535 nm) and 1 s acquisition time. The DNA concentration was calculated from the standard curve (R^2 was 0.97).

2.5. Evaluation of Gene Methylation Status. Bisulfite treatment of isolated DNA was performed using the EZ DNA Methylation Kit (Cat. number D5001, Zymo Research Corporation, USA) according to the manufacturer's instructions. The methylation status of the different genes was determined qualitatively by the methylation-specific polymerase chain reaction (MS-PCR) [25]. Real-time MS-PCR was performed in a Bio-Rad iQ5 Real-Time PCR detection System (Bio-Rad, USA). Primer sequences used for MS-PCR analysis, with PCR product size and primer annealing temperature,

are listed: *RASSF1* methylated-specific forward, 5′-GTGTTAACGCGTTGCGTATC-3′ and reverse, 5′-AACCCCGCGAACTAAAAACGA-3′ (60°C, 93 bp) [26]; *FHIT*, 5′-TTGGGGCGCGGGTTTGGGTTTTTACGC-3′ and 5′-CGTAAACGACGCCGACCCCACTA-3′ (62°C, 74 bp) [27]; *APC*, 5′-TATTGCGGAGTGCGGGTC-3′ and 5′-TCGACGAACTCCCGACGA-3′ (60°C, 98 bp) [28]; *LRRC3B*, 5′-GGTGCGAGGAAGGTAGGC-3′ and 5′-ACCAATACCTCGCCGACG-3′ (64°C, 149 bp) [29]; *VHL*, 5′-TGGAGGATTTTTTTGCGTACGC-3′ and 5′-GAACCGAACGCCGCGAA-3′ (60°C, 158 bp) [30]; *ITGA9,* 5′-TGGAGTATTTTTACGATAATACGC-3′ and 5′-AAAAACCGAAAAAACGACGA-3′ (64°C, 116 bp) [31]. Two μL of bisulfite-modified DNA was subjected to PCR amplification in a final reaction volume of 25 μL 1x Maxima SYBR Green qPCR Master Mix (Cat. number K0251, Thermo Scientific, USA) and 0.3 μM of each primer. PCR was performed with an initial 10 min incubation at 95°C, followed by 45 cycles of denaturation at 95°C for 15 s, annealing for 20 s, extension at 72°C for 30 s, and a final 7 min hold at 72°C. Each sample was assayed in triplicate, and each run included water blanks and an external control (universal methylated DNA). A fully methylated positive control was created by treating the DNA of lymphocytes from healthy donors with *SssI* CpG Methyltransferase (Cat. number EM0821, Thermo Scientific, USA) according to the manufacturer's recommendations. The specificity of the PCR products was confirmed by melting curve analysis. To verify MS-PCR data, the MSP sequencing assay was performed using Genetic Analyser 3130 (Applied Biosystems, USA) following manufacturer's protocols.

2.6. Statistical Analysis. Samples sizes were calculated using the formula described in [32] assuming α and β values of 0.05 and 0.2, respectively. We used standard deviation obtained in our preliminary experiments and estimated 150% difference in means.

To evaluate the statistical significance of differences between groups we performed the nonparametric Mann-Whitney U test using the OriginPro 9.1 software (OriginLab, USA) or the Chi-square test (χ^2) using Microsoft Excel 2007 in the case of categorical variables.

Differences were considered statistically significant if $p < 0.05$. To evaluate the discriminative power of the parameters studied for kidney cancer diagnostics we built binary logistic regression models for the selected predicting variables and all their possible combinations using SPSS version 22 (IBM, USA). From these models, the probabilities of positive outcome (i.e., cancer occurrence) were calculated. These probabilities were used for Receiver-operating characteristics (ROC) analysis. Building of ROC and evaluation of AUC (Area Under Curve) was performed using the GraphPad Prism 6.07 (GraphPad Software, La Jolla, CA, USA) or the OriginPro 9.1 software (OriginLab, USA).

3. Results

3.1. Concentration of cfDNA in Blood Plasma of Patients with Renal Cancer and of Healthy Donors. In this study, blood samples from 27 patients with renal cancer and from 15 healthy donors were used. The blood samples were collected before surgery in the Institute of Urology NAMS of Ukraine. The results of the histological examination of tumors showed that 23 patients had clear cell carcinoma, 2 patients had sarcoma-like tumors, 1 patient had mixed type RCC (papillary/clear cell), and 1 patient had cancer of the renal pelvis (Table 1).

Table 1: Patient and tumor characteristics.

	Number of patients
Age at diagnosis:	
Age > 55	20 (74.1%)
Age < 55	7 (25.9%)
Gender:	
Male	13 (48.1%)
Female	14 (51.9%)
Histology:	
Clear cell	23 (85.2%)
Sarcoma-like	2 (7.4%)
Papillary (75%)/clear cell (25%)	1 (3.7%)
Cancer of the renal pelvis	1 (3.7%)
Fuhrman grade:	
G1 + G2	19 (70.4%)
G3 + G4	8 (29.6%)
Clinical stage:	
Stage 2	4 (14.8%)
Stage 3	23 (85.2%)
Tumor-Node-Metastasis (TNM):	
T1a+b N0 M0-X	15 (55.6%)
T2 N0 M0-X	6 (22.2%)
T3 N0-1 M1-X	4 (14.8%)
TNM NA	2 (7.4%)

The concentrations of cfDNA in blood plasma were determined by two methods: by measuring the fluorescence level of intercalated SYBR Green I dye and by quantitative real-time PCR (qPCR) of the β-actin gene.

The results of the SYBR Green I fluorescence measurements showed that the concentrations of cfDNA in patients with renal cancer range from 11.3 to 2249.12 ng/mL (median 235.55 ng/mL). The range of cfDNA concentration in healthy donors was much lower, from 3.29 to 426.75 ng/mL (median 53.66 ng/mL) (Figure 1(a)).

qPCR revealed a statistically significant increase of cfDNA concentration in cancer patients (median 80.97 ng/mL, range 23.3–1176.6 ng/mL of plasma). As can be seen from the box plot (Figure 1(c)), these values are significantly higher in RCC patients compared to healthy donors (median 35.1 ng/mL, 3.0–146.78 ng/mL of plasma) (Figure 1(c)).

Receiver-operating characteristics (ROC) analysis showed that the concentration of cfDNA can be used as diagnostic feature for the detection of renal tumors (Figures 1(b)–1(d)). AUC obtained for qRCR analysis was slightly higher (0.8049, $p = 0.0012$) than for the SYBR Green I fluorescence measurements (0.7679, $p = 0.0044$) (Table 2).

Table 2: Comparative analysis of different methods used to measure cfDNA in blood plasma.

	Method	
	qPCR analysis	SYBR Green I fluorescence measurements
AUC	0.8049 (95% Cl: 0.6602–0.9497)	0.7679 (95% Cl: 0.6242–0.9116)
Median (renal cancer)	80.96	235.5
Median (control)	35.1	53.7
p value (by Mann-Whitney U test)	$p < 0.0008$	$p < 0.0037$

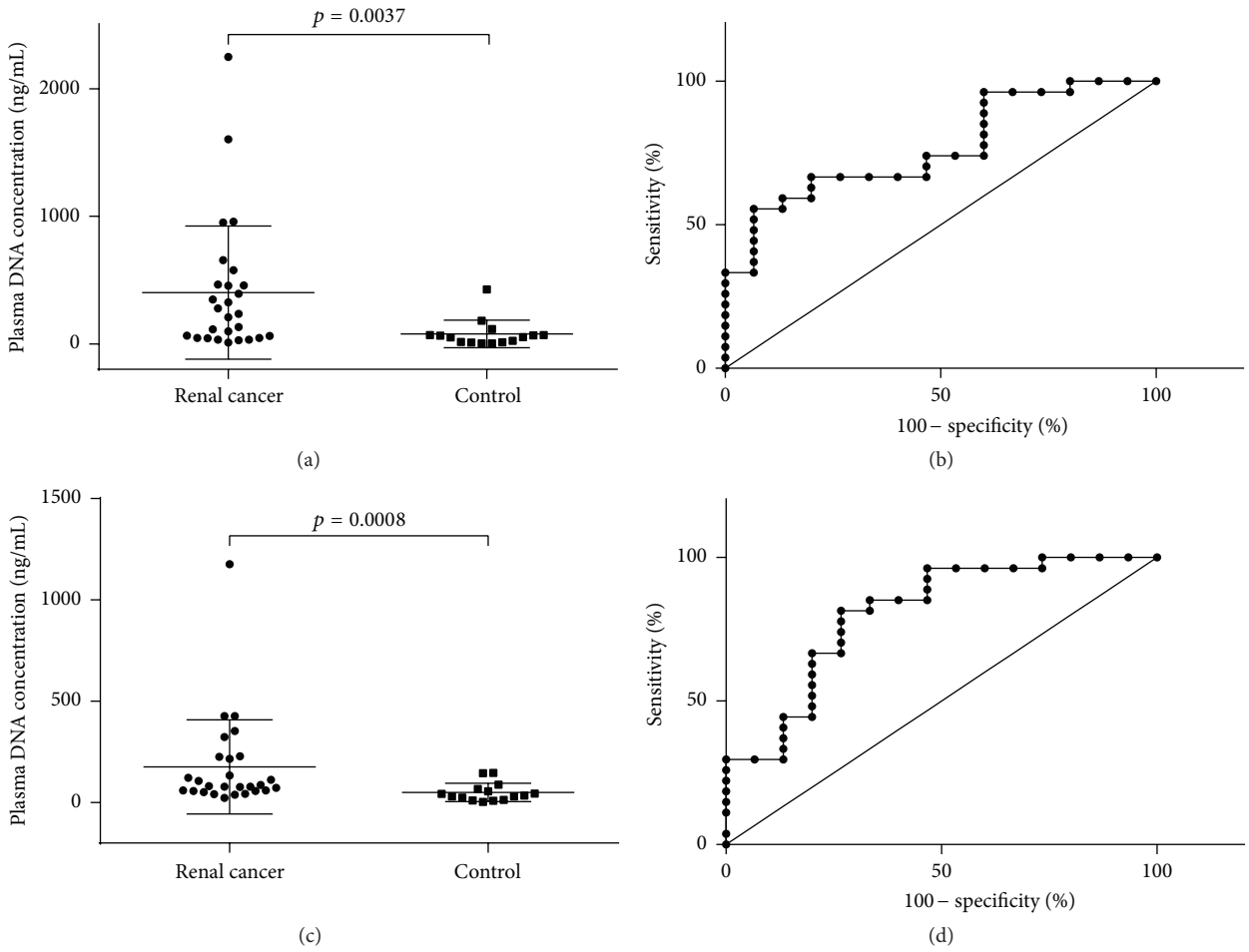

Figure 1: Analysis of cfDNA concentration in plasma of patients with renal carcinoma and controls. cfDNA concentrations were determined by measuring the fluorescence level of intercalated SYBR Green I dye (a) and by qPCR (c). ROC curve analysis of cfDNA concentration in cancer patients compared with the control group ((b) fluorescence test; (d) qPCR).

3.2. Analysis of Methylation of Tumor Suppressor Genes in cfDNA. Since the cfDNA level alone cannot be a specific marker of renal cancer [11], we also analysed the methylation status of CpG islands of 6 tumor suppressor genes in the cfDNA. Using bisulfite treatment followed by MS-PCR we detected methylation of the *LRRC3B, APC, FHIT,* and *RASSF1* genes in the cfDNA of cancer patients. Promoter methylation of the *LRRC3B* gene was detected in 20 out of 27 samples (74%); methylation of the *RASSF1, APC*, and *FHIT* genes was found in 17 (63%), 14 (52%), and 15 (55.6%) patients, respectively (see Table 3 for detailed methylation frequencies). Methylation was not detected in the *VHL* and Integrin $\alpha 9\beta 1$ (*ITGA9*) genes in plasma cfDNA.

Analysis of simultaneous methylation of CpG islands of the *LRRC3B, FHIT,* A*PC,* and *RASSF1* genes showed that all the samples from cancer patients contained at least one methylated promoter; two promoters were methylated in 33.3%, three promoters were methylated in 27%, and four methylated promoters were detected in 11.1% of the samples (Tables 3 and 4).

Table 3: Summary of clinicopathological characteristics of patients with RCC and methylation status of *LRRC3B*, *RASSF1*, *FHIT*, and *APC* CpG islands in cfDNA*.

Number	Pathology	Age (y)	Sex	pTNM	Clinical grade	Fuhrman nuclear grade	Methylation			
							LRRC3B	*RASSF1*	*APC*	*FHIT*
1	ccRCC	54	M	pT2N0M0	II	3	+	+	−	+
2	ccRCC	61	M	T1N0M0	II	2	+	+	−	+
3	Sarcoma-like	66	F	pT2N0MX	II	3	+	−	+	+
4	Papillary/ccRCC	63	M	pT1BN0MX	III	1	−	+	−	+
5	ccRCC	47	F	pT3aN0M1	III	3	+	+	+	+
6	ccRCC	64	M	pT3aN0M1	III	3	+	+	+	+
7	ccRCC	58	M	pT2N0MX	III	3	+	−	+	−
8	ccRCC	61	M	pT1BN0M0	III	2	+	+	−	−
9	ccRCC	75	M	pT1BN0MX	III	2	−	+	−	+
10	ccRCC	65	M	T2N0MX	III	3	−	+	+	+
11	ccRCC	61	F	pT1N0M0	III	2	+	−	+	−
12	ccRCC	63	F	pT3aN1MX	III	2-3	+	−	+	−
13	ccRCC	68	F	pT1 N0 MX	III	1-2	+	−	+	+
14	ccRCC	34	M	pT1aN0 MX	III	1	+	−	+	−
15	Cancer of the renal pelvis	76	M	pT3N0M1	III	4	+	+	+	+
16	ccRCC	56	F	pT1aN0MX	III	1	−	−	+	+
17	ccRCC	62	F	pT1aN0MX	III	1	+	−	+	+
18	ccRCC	46	F	pT1BN0MX	III	1	+	+	−	−
19	ccRCC	55	F	pT2N0MX	III	2	+	+	+	−
20	ccRCC	45	F	T2N0M0	II	2	−	+	−	−
21	ccRCC	61	F	pT1aN0MX	III	2	+	+	−	−
22	ccRCC	60	M	NA	III	2	+	+	−	+
23	ccRCC	63	F	pT1BN0MX	III	2	+	−	−	−
24	Sarcoma-like	60	F	NA	III	4	+	−	−	−
25	ccRCC	45	M	pT1BN0MX	III	2	−	+	+	+
26	ccRCC	63	M	pT1aN0M0	III	1	+	+	−	+
27	ccRCC	73	F	pT1aN0M0	III	1	−	+	−	−

*The results in the Table are presented only for the genes with detected aberrant methylation in cfDNA.

Table 4: Diagnostic data analysis for the discrimination of renal cancer patients and healthy subjects using cfDNA methylation of various genes alone and in combination.

Markers	Renal cell carcinoma (n = 27)	Healthy controls (n = 15)	χ^2, p value	Sensitivity*, %	Specificity**, %
LRRC3B	20 (74.1%)	5 (33.3%)	0.01	74.1	66.7
RASSF1	17 (63.0 %)	1 (6.7%)	0.0058	62.9	93.3
FHIT	15 (55.6%)	0 (0%)	0.0003	55.6	100
APC	14 (51.9%)	1 (6.7%)	0.0034	51.9	93.3
VHL	0 (0%)	0 (0%)		0	100
ITGA9	0 (0%)	0 (0%)		0	100
RASSF1 or *FHIT* or *APC*	25 (92.3%)	2 (13.3%)	<0.0001	92.3	86.7
RASSF1 or *FHIT*	21 (77.8%)	1 (6.7%)	<0.0001	77.8	93.3
RASSF1 or *APC*	21 (77.8%)	1 (6.7%)	<0.0001	77.8	93.3

*Sensitivity was calculated as a percentage of positive results from a number of tested RCC patients; **specificity was calculated as a percentage of negative tests from a given number of healthy donors.

However, *LRRC3B* showed a low specificity as a marker of cancer, since it was methylated in 5 out of 15 (33.3%) healthy donors. Methylation of *FHIT* was not detected in the cfDNA of the control group, while methylation of the *APC* and *RASSF1* genes was found in 1 out of 15 (6.7%) healthy donors. Methylation of *APC* and *RASSF1* was detected in different healthy individuals (Table 4).

The sensitivity of each of these markers exceeded 50% and was 51.9% for *APC*, 63% for *RASSF1*, and 55.6% for *FHIT*, which exhibited the best specificity in our test (100%).

Table 5: Receiver-operating characteristic (ROC) curve analyses of cfDNA marker-based models to discriminate between healthy persons ($n = 15$) and renal cancer patients ($n = 27$)*.

	AUC	Std. error	p value	95% LCL	95% UCL
Conc_qPCR	0.80494	0.06771	0.00119	0.67223	0.93765
Conc_qPCR+*APC*	0.91852	0.04205	$8.61E-06$	0.8361	1.00094
Conc_qPCR+*FHIT*	0.91358	0.04898	$1.10E-05$	0.81759	1.00957
Conc_qPCR+*RASSF1*	0.88148	0.05986	$5.00E-05$	0.76416	0.99881
Conc_qPCR+ *APC*+*RASSF1*	1	0	$1.06E-07$	1	1
Conc_qPCR+*APC*+*FHIT*	0.95802	0.03018	$1.12E-06$	0.89888	1.01717
Conc_qPCR+*RASSF1A*+*FHIT*	0.94568	0.04521	$2.16E-06$	0.85708	1.03428
Conc_qPCR+*APC*+*FHIT*+*RASSF1*	1	0	$1.06E-07$	1	1

*Calculated by binary logistic regression using combination of different markers: concentration of cfDNA determined by qPCR (Conc_qPCR) and methylation marker genes (*APC*, *FHIT*, and *RASSF1*).

The use of the combined analysis of methylation status of three genes (*RASSF1*, *FHIT*, and *APC*) increased the sensitivity (77.8–92.3%), while the specificity remained high (86.7–93.3%) (Table 4). We did not find any correlation between hypermethylation, cfDNA concentration, and clinicopathological parameters (grade, lymph node metastasis, age, and sex) in patients with renal cancer.

To explore the potential of combined cfDNA concentration and gene methylation for RCC diagnostics, we performed binary logistic regression modelling. As a predictor of variables we used cfDNA concentrations measured by quantitative PCR and methylation of *APC*, *FHIT*, and *RASSF1* genes. We built separate models for cfDNA concentration alone and for all possible combinations of cfDNA concentration and methylation of one, two, or three genes. Predictive properties of the models were compared by ROC analysis. As reported above, the AUC value for the cfDNA concentration alone was 0.8. Addition of one of the genes slightly increased the AUC to values 0.88–0.918, although these differences were not statistically significant as can be seen from 95% confidence intervals (Table 5). Addition of two genes led to further increase of the AUC value up to 1 when using *APC* and *RASSF1*. Finally, the AUC value was 1 when we used the cfDNA concentration and methylation of all three genes studied. The results of ROC analysis are summarised in Table 5; some of representative ROC curves are shown in Figure 2.

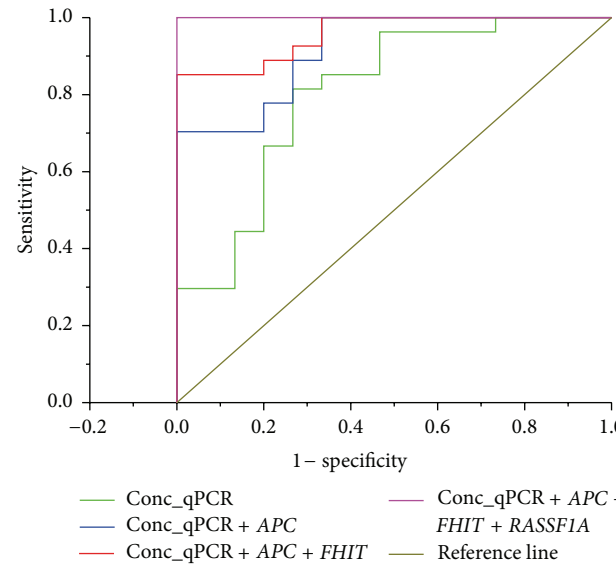

Figure 2: Receiver-operating characteristics (ROC) curves obtained by using different models for the discrimination between healthy controls ($n = 15$) and renal carcinoma patients ($n = 27$). Conc_qPCR: concentration of cfDNA determined by qPCR.

4. Discussion

The level of cfDNA in blood plasma could be a universal marker of malignancy [33]. Many studies have shown that changes in cfDNA concentration can be correlated with development, prognosis, and survival of cancer patients. An increase of cfDNA concentration was observed in patients with breast, gastric, ovary, lung, colon, and prostate cancer [11, 34–39]. It was suggested that an increase of cfDNA concentration in cancer patients is associated with apoptosis and necrosis of cancer cells in the tumor microenvironment [40]. This suggestion was supported by numerous cancer-specific alterations (such as allelic imbalances, methylation, and mutations) that were found in blood cfDNA (for reviews see [11, 41]). It was also demonstrated that monitoring of the cfDNA level in peripheral blood can be used as biomarker of response to therapy in different cancer types [38, 42, 43].

Previous studies demonstrated that the evaluation of concentration of low molecular weight cfDNA (up to 100 bp) is the most representative for detection of malignancies and disease prognosis since the level of fragments of this size increases with disease progression [44–47]. Recently, Lu et al. [47] showed that cfDNA fragments of 67 bp and 180 bp did not differ between the controls and nonmetastatic RCC patients, while the cfDNA integrity index decreased from control to the metastatic group. Significantly higher concentrations of low molecular weight fragments were found in the RCC patients [47]. Here we have shown an increase of cfDNA concentration in RCC patients using genomic cfDNA fragment of β-actin gene of 99 bp. Recent experiments from other laboratories also demonstrated increased cfDNA levels

in the blood of patients with renal cancer compared to healthy individuals [19, 48, 49].

Absolute values of cfDNA concentrations obtained by two distinct methods are different (11–2249 ng/mL of plasma in the fluorescence test compared to 23–1177 ng/mL in qPCR), but in both cases they were significantly higher than in healthy individuals (4–426 ng/mL in the fluorescence test and 3–146 ng/mL in qPCR). The obtained results agree with data from other studies in which the determination of the cfDNA concentration using fluorescent dyes gave higher values than qPCR [24, 41]. The high AUC values obtained for both methods of cfDNA concentration measurement in RCC patients (0.7679 for fluorescent test and 0.80494 for qPCR) demonstrate that these methods can be used for clinical investigations. In addition to quantitative changes, cfDNA also possesses qualitative changes that occur in DNA of tumor cells, such as mutations, microsatellite instability, and methylation [11, 41, 50]. Methylation of gene promoters is a well-known mechanism of regulation of gene expression [51]. Most frequently, aberrant methylation of genes occurs in cancer cells. Aberrant methylation of the promoter detected in cfDNA can be used for noninvasive detection of cancer, differential diagnosis, prognosis of survival, and response to cancer therapy [52–57]. Currently, several diagnostic systems based on the detection of DNA methylation exist that are aimed not only at detecting malignancy (MethylMeter from RiboMed, USA), but also at detecting specific types of cancer (Epi proColon and Epi proLung from Epigenomics AG, Germany; Confirm MDx for Prostate Cancer from MDxHealth, USA; Product series DecisionDx G-CIMP, Melanom, EC, UM, Thymoma from Castle Biosciences). The search for new tools is being pursued because only two of these systems (MethylMeter and Epi proColon) can detect cancer at early stages of development and can monitor treatment since they are based on the detection of cfDNA methylation.

In this study we started investigating methylation of previously identified tumor suppressor genes in cfDNA. Data from many studies show that the *RASSF1* gene plays an important role in cancerogenesis. Hypermethylation of *RASSF1* CpG islands is associated with different types of cancer and with the risk of progression of tumorigenesis [58–64]. It was also shown that rat *RASSF1* is involved in early tumorigenesis of RCC [16, 17]. Studies on methylation of this gene in blood serum led to controversial results. Hauser et al. [18] showed that *RASSF1A* is methylated in 22.9% of patients; the study of De Martino et al. [19] demonstrated methylation of *RASSF1A* in 45.9% of patients; Hoque et al. [20] observed methylation of this gene in 11% of serum samples of patients with RCC. In our study methylation of *RASSF1* was detected in 62.9% of patients. The differences in methylation levels of the *RASSF1* gene can be explained by the use of different CpG islands for analyses. We studied methylation of CpG region located within the first exon of *RASSF1C*, while Hauser et al. [18] and De Martino et al. [19] analysed the region located upstream of the initiation codon. Previously, it was reported that these two CpG islands were differentially methylated in melanoma cell lines and melanoma tumors [65]. Ellinger et al. [66] demonstrated a 100% correlation between DNA hypermethylation of the *RASSF1A* promoter and papillary RCC. However, De Martino et al. [19] analysed 31 samples of papillary RCCs and found no association of *RASSF1A* methylation with the histological subtypes of RCC. In our study *RASSF1* was also methylated in papillary RCC, but it was the only sample of this cancer subtype analysed.

Previously we reported changes in the *LRRC3B* gene promoter during the search for genetic and epigenetic alterations in chromosome 3 in epithelial tumors using NotI-microarrays [14, 67, 68]. *LRRC3B* was identified by Kim et al. [69] as a putative gene suppressor of several tumors that are silenced in gastric cancers by epigenetic mechanisms. Increased methylation of the *LRRC3B* gene promoter was confirmed in samples of clear cell RCC and colorectal, head, and neck cancer [29, 70, 71]. A high level of *LRRC3B* hypermethylation was noted not only in RCC patients (74%), but also in healthy donors (33%) in our study, questioning the use of this gene for the diagnosis of renal cancer on cfDNA.

The promoter of the *APC* gene was methylated in 51.9% of patients, which is in good agreement with the results of Hauser and colleagues [18], who detected methylation of the *APC* gene in 54.3% of patients using cfDNA.

Previously, a significant correlation between *FHIT* expression in clear cell renal carcinomas and patient survival was demonstrated [21]. Kvasha et al. [22] showed a correlation between hypermethylation of the *FHIT* CpG island and a significant decrease of *FHIT* expression in clear cell RCC. The level of aberrant methylation of *FHIT*, obtained in our study on cfDNA (55.6%), was close to the results obtained in the study of Kvasha et al. in samples of RCC tumors (54.6%).

Integrin $\alpha 9\beta 1$ plays an important role in various signal transduction pathways that control proliferation, migration, and differentiation of both normal (reviewed in [72, 73]) and cancer cells (reviewed in [74, 75]). Downregulation of *ITGA9* expression was observed in several cancer types [76–78] that could be caused either by mutations in this gene [79] or by hypermethylation [31, 68, 80]. However, methylation of the *ITGA9* gene was not detected in our experiments. We also have not identified methylation of the *VHL* gene, although NotI-microarray hybridization revealed high levels of changes in this gene (47%) in renal cancer [14]. It is possible that these changes are associated with deletions in the gene rather than with methylation. At the same time, in the study of De Martino et al. where cfDNA was analysed by restriction analysis, methylated *VHL* was detected in 50.3% of patients with RCC [19].

Methylation analysis of the *RASSF1*, *FHIT*, and *APC* genes demonstrated their high specificity (93.3% for *RASSF1* and *APC*, 100% for *FHIT*) for renal tumors. Nevertheless, sensitivity in one gene analysis was just from 51.9% for *APC* to 62.9% for *RASSF1* (Table 4). At the same time the use of a combination of three or two genes (without *LRRC3B* due to the low specificity of this gene) leads to a significant increase in sensitivity (77.9–92.3%) and specificity (86.7–93.3%). All other combinations did not reveal any additional diagnosis information. Simultaneous methylation of the *RASSF1*, *APC,* and *FHIT* genes was identified only in 3 patients with metastases. However, the small sample size does not allow us to draw a conclusion on the correlation

between methylation and disease progression. At the same time binary logistic regression analysis showed the considerable diagnostic potential of combining both approaches used in this study. According to the ROC analysis the use of only cfDNA concentration has moderate diagnostic potential (AUC = 0.8). On the other hand, by using the concentration and methylation of two or three genes, we achieved 100% diagnostic accuracy in our samples. These results, of course, cannot be directly transferred to clinical practice and need verification on a larger number of samples. However, our data demonstrates the potential advantage there is in combining evaluation of cfDNA concentration and gene methylation for RCC diagnostics and provides a basis for further research.

Thus, despite the small sampling, our results confirm the possibility of using the concentration of cfDNA in blood plasma as an additional marker of renal cancer development and show that methylation of three genes, *FHIT*, *APC*, and *RASSF1*, in cfDNA can be used to develop renal cancer diagnostic tools.

5. Conclusion

The results obtained indicate that the concentration of cell-free DNA in plasma and the methylation of specific genes (such as *FHIT*, *APC*, and *RASSF1*) can be a significant addition to serological tumor markers in the identification of patients with renal cancer. However, further studies need to be performed to evaluate their diagnostic value.

Competing Interests

The authors declare that they have no competing interests.

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