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 promotor 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 (LRRC3B) and Von Hippel-Lindau (VHL) genes possess the highest frequency of deletions and/or methylations in renal carcinoma [14, 15]. Adenomatosis-polyposis-colon (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 α9β1)) 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 diagnostic, 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 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′-CCACACTTGCGCCCATCTACG-3′ and 3′-AGGATCTTCATGAGGTAGTCACTGTCAG-5′; 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 0.3μM of each primer (5′-CCACACTTGCGCCCATCTACG-3′ and 5′-AGGATCTTCATGAGGTAGTCACTGTCAG-3′) and a 0.25μM fluorescent probe (5′-FAM-ATGCCCTCCCCCATGATCTGGT-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 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 (R²), 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” 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 (R² was 0.97).

2.5. Evaluation of Gene Methylation Status. Bisulfitite 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'-GTG- 
TTAACGGCTTTGCGTATC-3' and reverse, 5'-AACCCCG- 
GGCACTAAAAAACGA-3' (60°C, 93 bp) [26]; FHIT, 5'- 
TTGGGGCGCTTTGTTTACTCGC-3' and 5'-CGTAAACGAGCCGGACCCCCTA-3' (62°C, 74 bp) [27]; 
APC, 5'-TATTGGCGAGTTGCGGTTC-3' and 5'-TCGACG- 
AACTCCCGAGCA-3' (60°C, 98 bp) [28]; LRRC3B, 5'- 
GGTGGCGAGGAGGTAGGC-3' (64°C, 149 bp) [29]; VHL, 5'-TGGAGG- 
ATTTTTTGTGCTAGCG-3' and 5'-GAACCGAAGGCGCC- 
GGAA-3' (60°C, 158 bp) [30]; ITGA9, 5'-TGGAGTTTT- 
TTTACGATTATCGCG-3' and 5'-AAAACCGAAA- 
AAGCAGCA-3' (64°C, 116 bp) [31]. Two μl of bisulfite- 
modified DNA was subjected to PCR amplification in a final 
reaction volume of 25μl. 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 
was assayedintriplicate,andeachrunincludedwaterblanks 
and all their possible combinations using SPSS version 22 
for the selected predicting variables 

Table 1: Patient and tumor characteristics.

| Age at diagnosis: | Number of patients |
|------------------|--------------------|
| 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%)           |

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 qPCR 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% CI: 0.6602–0.9497) | 0.7679 (95% CI: 0.6242–0.9116) |
| Median (renal cancer)               | 80.96         | 235.5                                  |
| Median (control)                    | 35.1          | 53.7                                   |
| p value (by Mann-Whitney U test)    | $p < 0.0008$  | $p < 0.0037$                           |

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 α9β1 (ITGA9) genes in plasma cfDNA.

Analysis of simultaneous methylation of CpG islands of the LRRC3B, FHIT, APC, 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, RASSFI, FHIT, and APC CpG islands in cfDNA.*

| Number | Pathology | Age (y) | Sex | pTNM | Clinical grade | Fuhrmann nuclear grade | Methylation |
|--------|-----------|---------|-----|------|----------------|------------------------|-------------|
|        |           |         |     |      |                |                        | LRRC3B      |
| 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   | pT1n0N0MX | III | 1            | –          | –          |
| 5      | ccRCC     | 47      | F   | pT3aN0M1 | II   | 3            | +          | +          |
| 6      | ccRCC     | 64      | M   | pT3aN0M1 | III  | 3            | +          | +          |
| 7      | ccRCC     | 58      | M   | pT2N0MX | III  | 3            | –          | –          |
| 8      | ccRCC     | 61      | M   | pT1n0M0 | III  | 2            | +          | –          |
| 9      | ccRCC     | 75      | M   | pT1n0N0MX | 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   | pT1aN0MX | 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   | pT1aN0MX | 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   | pT1n0N0MX | III | 2            | +          | –          |
| 24     | Sarcoma-like | 60      | F   | NA    | III  | 4            | +          | –          |
| 25     | ccRCC     | 45      | M   | pT1n0N0MX | 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) | $\chi^2$, p value | Sensitivity*, % | Specificity**, % |
|---------|-----------------------------|---------------------------|-------------------|-----------------|-----------------|
| LRRC3B  | 20 (74.1%)                  | 5 (33.3%)                 | 0.01              | 74.1            | 66.7            |
| RASSFI  | 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                 | 0               | 100             |
| ITGA9   | 0 (0%)                      | 0 (0%)                    | 0                 | 0               | 100             |
| RASSFI or FHIT or APC | 25 (92.3%) | 2 (13.3%) | <0.0001 | 92.3 | 86.7 |
| RASSFI or FHIT | 21 (77.8%) | 1 (6.7%) | <0.0001 | 77.8 | 93.3 |
| RASSFI 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 RASSFI genes was found in 1 out of 15 (6.7%) healthy donors. Methylation of APC and RASSFI 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 RASSFI, and 55.6% for FHIT, which exhibited the best specificity in our test (100%).
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.

### 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, ovarian, 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
Data from many studies show that the previously identified tumor suppressor genes in cfDNA (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 RASSFI 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 RASSFIA is methylated in 22.9% of patients; the study of De Martino et al. [19] demonstrated methylation of RASSFIA 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 RASSFI was detected in 62.9% of patients. The differences in methylation levels of the RASSFI 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 RASSFIC, 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 RASSFIA promoter and papillary RCC. However, De Martino et al. [19] analysed 31 samples of papillary RCCs and found no association of RASSFIA methylation with the histological subtypes of RCC. In our study RASSFI 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 α9β1 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 RASSFI, FHIT, and APC genes demonstrated their high specificity (93.3% for RASSFI 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 RASSFI (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 RASSFI, 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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