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Метилирование 10 генов микроРНК при светлоклеточном раке почки и их диагностическое значение

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Введение. *Светлоклеточный почечно-клеточный рак почки (скПКР) характеризуется высокой частотой (30–40 %) случаев летальных исходов, которая при метастазировании достигает 90 %. Отсутствие эффективной диагностики на ранних стадиях заболевания указывает на необходимость поиска новых маркеров скПКР.*

Цель работы *– определение роли метилирования группы генов супрессорных микроРНК (миРНК) в патогенезе и прогрессировании скПКР и идентификация маркеров для диагностики скПКР и прогноза метастазирования.*

Материалы и методы. *Методом бисульфитной конверсии ДНК с последующей метилспецифичной полимеразной цепной реакцией определено изменение статуса метилирования 10 генов миРНК в образцах ДНК опухоли и парных гистологически неизмененных тканях 70 больных скПКР, а также в образцах ДНК тканей почки 19 умерших от неонкологических заболеваний. Метилирование генов MIR-107, -130b и -148a при скПКР в данной работе исследовано впервые.*

Результаты. *Показано, что 8 генов миРНК (MIR-9-1/3, -34b/c, -124a-1/2/3, -129-2, -130b) метилированы в опухолях скПКР с достоверно более высокой частотой, чем в парной гистологически неизмененной ткани почки. Установлена значимая связь метилирования 4 генов миРНК (MIR-107, -124a-3, -129-2, -130b) с показателями прогрессирования скПКР (стадия, размер опухоли, степень дифференцировки), в том числе для генов MIR-107 и -129-2 – с метастазированием в лимфатические узлы или отдаленные органы. Связь метилирования генов MIR-107 и -130b с прогрессированием заболевания показана впервые. Составлены потенциальные системы маркеров для диагностики скПКР на основе биопсийного материала; по данным ROC-анализа 2 системы маркеров из 4 и 5 генов (MIR-9-1, -34b/c, -124a-3, -129-2; и с добавлением MIR-130b) характеризуются клинической чувствительностью 90 % и специфичностью 94 % (площадь под ROC-кривой 0,93 и 0,94 соответственно).*

Заключение. *Полученные результаты в дальнейшем лягут в основу разработки метода неинвазивной диагностики скПКР. Таким образом, показана связь метилирования ряда генов миРНК с патогенезом и прогрессированием скПКР и их потенциальное диагностическое значение.*

Ключевые слова: *светлоклеточный почечно-клеточный рак, метилирование, ген микроРНК, метастазирование, система маркеров*

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Methylation of 10 miRNA genes in clear cell renal cell carcinoma and their diagnostic value

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Introduction. *Clear cell renal cell carcinoma (ccRCC) is characterized by the high (30–40 % of cases) frequency of lethal outcomes which at metastasis reaches 90 %. Lack of efficient diagnostics at early stages of a disease indicates the need of searching on new ccRCC markers.*

Objective: *for definition of methylation role of some tumor suppressor microRNA (miRNA) genes in ccRCC pathogenesis and progression and marker identification for ccRCC diagnostics and metastasis predictions.*

Materials and methods. *The alterations of methylation status of 10 miRNA genes were determined by methylation specific polymerase chain reaction in tumor DNA samples and matched histologically unchanged tissues from 70 patients with ccRCC, as well as in DNA samples of kidney tissues from 19 post-mortal individuals without cancer history. Methylation of MIR MIR-107, -130b and -148a genes in ccRCC was studied for the first time.*

Results. *It was shown that 8 miRNA genes (MIR-9-1/3, -34b/c, -124a-1/2/3, -129-2, -130b) were methylated in ccRCC tumors with significantly higher frequency than in the matched histologically unchanged kidney tissues. It was established the association of methylation*

of 4 miRNA genes (MIR-107, -124a-3, -129-2, -130b) with ccRCC progression (stage, tumor size, differentiation grade), including metastasis in the lymph nodes or distant organs, revealed for MIR-107 and -129-2. The association of MIR-107 and -130b methylation with progression of ccRCC is shown for the first time. Potential marker systems are made for ccRCC diagnostics using tumor biopsy; according to the ROC analysis, systems from 4 and 5 genes (MIR-9-1, -4b/c, -124a-3, -129-2/with addition of MIR-130b) are characterized by high clinical sensitivity of 90 % and specificity of 94 % (area under ROC curve 0.93 and 0.94).

Conclusion. *The received results will form the basis of noninvasive ccRCC diagnostics further development. To conclude, it is shown the association of methylation of 9 miRNA genes with ccRCC pathogenesis and progression and its potential diagnostic value.*

Key words: *clear cell renal cell carcinoma, methylation, microRNA gene, metastasis, marker system*

Background

Renal cell carcinoma (RCC) accounts for over 90% of renal malignancies and has the highest mortality rate among genitourinary cancers [1]. Approximately 270,000 new cases of RCC are diagnosed annually worldwide, together with 116,000 deaths [2]. The most common histological type of RCC is the clear cell RCC (ccRCC), which represents around 75–80% of all RCC cases. This type of carcinomas is characterized by a more aggressive course (compared to the papillary and chromophobe histological types) and high frequency of metastases (25–30%), reaching 50% in patients after surgery [3]. Metastatic ccRCC is highly resistant to therapy: only 10% of patients respond to chemotherapy, radiotherapy, or immunotherapy [4]. The five-year survival rate in patients with distant metastases does not exceed 9% [4]. In the Russian Federation, the one-year mortality after diagnosis is 16% (as of 2015) [5]. The lack of effective diagnostic tools for early stages of RCC, high mortality rate, and resistance to therapy necessitate the search for new biomarkers for early diagnosis of ccRCC and identification of metastasis.

Hypermethylation of CpG islands located in the promoter of protein-encoding tumor suppressor genes is often detected in malignant tumors and is associated with gene suppression [6]. Hypermethylation of these genes is considered as one of the pivotal genetic alterations in cancer development and as the earliest molecular biomarker for cancer.

MicroRNAs (miRNAs) are a class of small (19–25 nucleotides) single-strand non-coding RNAs involved in the post-transcriptional regulation of protein-encoding genes. The miRNA genes can also be methylated, like any protein-encoding genes [7]. The proportion of miRNA genes regulated through CpG methylation is assumed to be 5–10 higher than that of structural genes [8]. Hypermethylation of genes encoding suppressor miRNA, resulting in their inactivation, was detected in both hematological and solid cancers, including acute myeloid leukemia, melanomas, lung cancer, colon cancer, stomach cancer, breast cancer, etc. [9, 10]. As shown earlier, hypermethylation patterns of miRNA genes can be used as potential biomarkers for diagnosis and prognosis of various cancers [11–14]. However, hypermethylation of miRNA genes in ccRCC was analyzed in very few studies [14–16].

Objective: to analyze hypermethylation patterns of 10 tumor-suppressor miRNA genes in ccRCC using a representative sample and to assess diagnostic and prognostic values of hypermethylated miRNA genes.

Material and methods

All **ccRCC tissue samples** were examined in the N.N. Blokhin Russian Cancer Research Center. We analyzed tumor and matched control (non-malignant) tissue samples collected from 70 patients with ccRCC and 19 kidney tissues specimens from people died for reasons other than cancer (referred to as donors). Specimen collection was performed according to an earlier described procedure [15]. Clinical and histological characteristics of patients (including TNM grading) are shown in the Table 1. We used the phenol-chloroform extraction method to isolate high-molecular-weight genomic DNA from tissue samples.

The study was conducted in accordance with the ethical principles (participation was voluntary and all the information collected was kept confidential) and Russian Public Health Legislation. The study protocol was approved by the Ethics Committee of N.N. Blokhin Russian Cancer Research Center. All patents signed an informed consent before being enrolled.

DNA bisulfite conversion and methyl-specific polymerase chain reaction (MS-PCR) were performed according to an earlier described protocol [18]. The primers for the genes analyzed and MS-PCR conditions were adopted from [15, 19–21]. Three to six CpG dinucleotides were analyzed for each gene. MS-PCR was performed using a T100 Thermal Cycler (Bio-Rad, USA) according to the following cycling protocol: 5 min at 95°C, followed by 35 cycles of 10 sec denaturation at 95°C, 20 sec annealing, and 30 sec extension at 72°C, followed by 3 min at 72°C. False positive results associated with incomplete bisulfite conversion of DNA were avoided by using appropriately designed primers (checked by the absence of MS-PCR products with non-bisulfite converted DNA). The CpG methylated human genomic DNA (#SD1131, Thermo Scientific, USA) was used as a positive control for methylated alleles, whereas the human genomic DNA (#G1471, Promega, USA) served as an unmethylated allele control. The PCR products were analyzed by 2% agarose gel-electrophoresis.

Таблица 1. *Обобщенные данные по клиническим характеристикам больных светлоклеточным почечно-клеточным раком почки (n = 70)*

Table 1. *Pooled data on clinical characteristics of patients with clear cell renal cell carcinoma (n = 70)*

Характеристика Characteristics	*n*	%
Клиническая стадия заболевания: Clinical stage of the disease:		
I	27	39
II	14	20
III	21	30
IV	8	11
Степень дифференцировки опухоли: Tumor grade:		
G_1	18	26
G_2	32	46
G_3	20	28
Размер опухоли по TNM: Tumor size according to the TNM:		
T1	30	43
T2	18	26
T3/T4	22	36
Метастазы: Metastases:		
N0M0	50	71
N1–2/M1	20	29

Примечание. *N – метастазы в регионарные лимфатические узлы; M – отдаленные метастазы.*
Note: *N – regional lymph node metastases; M – distant metastases.*

Statistical analysis included Fisher's exact test performed using the AtteStat software. Differences were considered significant at $P < 0.05$. Optimal biomarkers were identified using receiver operating characteristic (ROC) curve analysis with the Youden index performed at http://www.biosoft.hacettepe.edu.tr/easyROC/.

Results

Hypermethylation pattern of miRNA genes in ccRCC and its association with disease progression. The results of MS-PCR analysis of 10 miRNA genes (miR-9-1, miR-9-3, miR-34b/c, miR 107, miR-124a-1, miR-124a-2, miR 124a-3, miR-129-2, miR-130b, miR-148a) in ccRCC are shown in Table 2. We observed increased methylation of 9 out of 10 miRNA genes (except for miR-148a) in tumor tissue compared to non-malignant tissue from the same patients and healthy tissue from donors. The differences were statistically significant for 8 genes, except for miR-107 and miR-148a ($p < 0.05$ with Benjamini-Hochberg correction for multiple comparisons, Table 2). In donor tissues, the frequency of methylation was 0–10% for the majority of miRNA genes (0–2 samples out of 19).

The results of methylation analysis of 9 selected genes (except for miR-148a) in 70 ccRCC tumor tissue samples were compared with clinical and histological characteristics of the tumor. We found a significantly increased frequency of methylated miR-34b/c, miR-107, miR-124a-2, miR-129–2, and miR-130b in patients with more advanced stages of cancer compared to those with stages I or II ($p < 0.05$) (Figure 1). There was a trend toward a correlation between the clinical stage and the frequency of MIR-124a-3 gene methylation (Figure 1). However, after correction for multiple tests using the Benjamini-Hochberg method, methylation in only 3 miRNA genes (miR-107, miR-129-2, miR-130b) remained significantly associated with stage III–IV RCC.

A significant negative correlation was found between the frequency of miR-107 and miR-124a-3 methylation and the tumor differentiation grade; a trend towards such correlation was observed for MIR-130b gene (Figure 2). The level of miR-124a-2 and miR-130b methylation was significantly associated with tumor size (Figure 3), whereas the methylation status of miR-107 and miR 129-2 genes correlated with regional lymph node metastases and distant metastases (Figure 4). We also revealed a trend towards an increase of miR-130b gene methylation in patients with regional lymph node metastases and distant metastases (Figure 4). However, after the adjustment of the false discovery rate using the Benjamini-Hochberg procedure, several correlations failed to reach statistical significance. However, some of them remained significant, including an association between methylated miR-124a-3 and low differentiation grade, miR-130b and tumor size, and miR-107 and miR-129-2 and metastasis.

Potential diagnostic panels of markers for ccRCC. Analyzing methylation statuses of 8 miRNA genes in 70 ccRCC tissue samples and 19 donor samples, we compiled

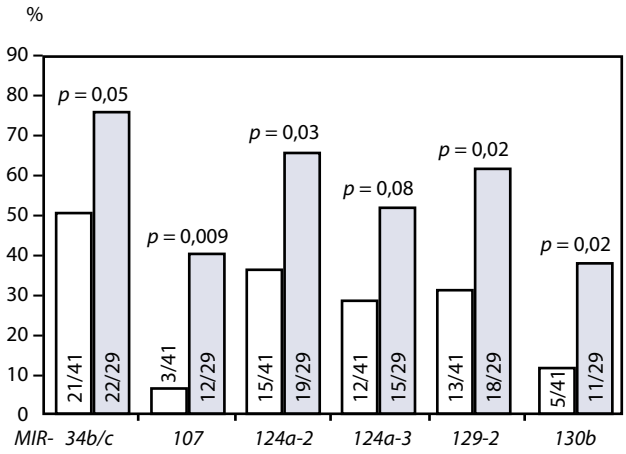

Рис. 1. *Ассоциация метилирования генов микроРНК с клинической стадией светлоклеточного почечно-клеточного рака: стадии I/II – белый прямоугольник, III/IV – серый*

Fig. 1. *Association of microRNA gene methylation with the clinical stage of clear cell renal cell carcinoma: stage I/II is shown by a white rectangle; stage III/IV is by a gray rectangle*

Таблица 2. *Частота метилирования 10 генов микроРНК при светлоклеточном почечно-клеточном раке*

Table 2. *Frequency of methylation of 10 miRNA genes in clear cell renal cell carcinoma*

Ген микроРНК MicroRNA gene	Локализация в геноме Location in genome	*p*	Доля (%) образцов, в которых данный ген микроРНК метилирован, от общего количества образцов Proportion (%) of samples with methylation of this MicroRNA gene of their total number		
			Опухолевая ткань (*n* = 70) Tumor tissue (*n* = 70)	Условно нормальная ткань (*n* = 70) Apparently intact tissue (*n* = 70)	Ткань почки доноров (*n* = 19) Kidney tissue of "donors" (*n* = 19)
MIR-9-1	1q22	**$3{,}2 \times 10^{-5}$**	29 (41)	7 (10)	0 (0)
MIR-9-3	15q26.1	**0,0031**	30 (43)	13 (19)	1 (5)
MIR-34b/c	11q23.1	**$1{,}1 \times 10^{-8}$**	43 (61)	10 (14)	1 (5)
MIR-124a-1	8p23.1	**0,001**	32 (46)	13 (19)	2 (10)
MIR-124a-2	8q12.3	**0,0003**	34 (49)	13 (19)	1 (5)
MIR-124a-3	20q13.33	**$3{,}2 \times 10^{-6}$**	27 (39)	4 (6)	0 (0)
MIR-148a	7p15.2	>0,05	25 (36)	37 (53)	9 (47)
MIR-129-2	11p11.2	**$2{,}6 \times 10^{-10}$**	31 (44)	1 (1)	0 (0)
MIR-130b	22q11.2	0,016	16 (23)	5 (7)	0 (0)
MIR-107	10q23.31	>0,05	15 (21)	6 (9)	1 (5)

Примечание. *Условно нормальной ткани соответствует гистологически неизмененная ткань почки от тех же пациентов. Доноры – умершие от неонкологических заболеваний. Статистически значимые значения p с учетом поправки Бенджамини–Хохберга на множественное сравнение выделены жирным шрифтом.*

Note. *Histologically unchanged kidney tissue from the same patients corresponds to apparently intact tissue. "Donors" are those dying of non-cancer diseases. The statistically significant p-values adjusted using the Benjamini–Hochberg procedure for multiple comparison are bold.*

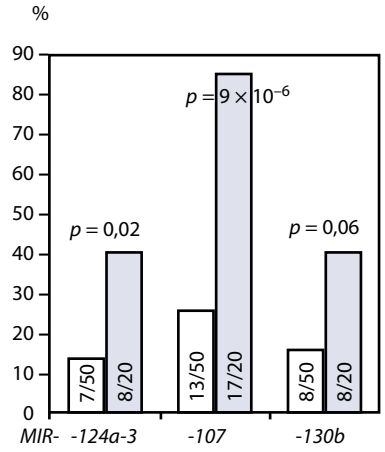

Рис. 2. *Ассоциация метилирования генов микроРНК со степенью дифференцировки опухоли светлоклеточного почечно-клеточного рака: высоко- и умереннодифференцированный, $G_1 + G_2$ – белый прямоугольник; низкодифференцированный, G_3 – серый*

Fig. 2. *Association of microRNA gene methylation with clear cell renal cell carcinoma grade: low- and moderate-grades, $G_1 + G_2$ – a white rectangle; high-grade, G_3 – a gray rectangle*

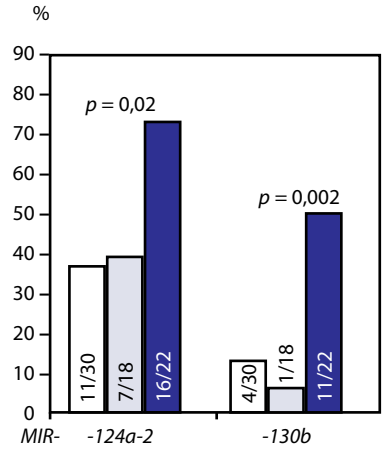

Рис. 3. *Ассоциация метилирования генов микроРНК с размером опухоли светлоклеточного почечно-клеточного рака по TNM-классификации: T1 – белый прямоугольник; T2 – серый; T3/T4 – черный*

Fig. 3. *Association of microRNA gene methylation with the size of clear cell renal cell carcinoma according to the TNM classification: T1 – a white rectangle; T2 – a gray rectangle; T3/T4 – a black rectangle*

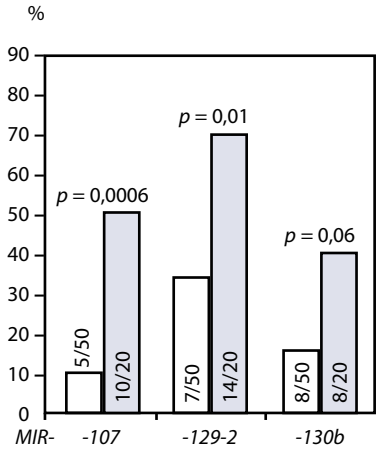

Рис. 4. *Ассоциация метилирования генов микроРНК с метастазированием светлоклеточного почечно-клеточного рака: нет метастазов, N0M0 — белый прямоугольник; N1–2/M1 — серый (N — метастазы в регионарные лимфатические узлы, M — отдаленные метастазы)*

Fig. 4. *Association of microRNA gene methylation with metastases of clear cell renal cell carcinoma according to the TNM classification: N0M0 — a white rectangle; N1–2/M1 — a gray rectangle (N — regional lymph node metastases; M — distant metastases)*

diagnostic panels consisting of 4–5 miRNA genes that can be potentially used for the detection of ccRCC. Diagnostic parameters of four gene panels, assessed using ROC-analysis, are shown in the table 3.

These diagnostic panels allow diagnosing ccRCC by detecting at least one methylated miRNA gene (cutoff criteria 1/4 or 1/5). The sets of 4 or 5 miRNA genes (miR-9-1, miR-34b/c, miR-124a-3, miR-129-2, and miR-130b) have a 90% sensitivity and 94% specificity (the area under the curve (AUC) was 0.93 and 0.94 respectively); therefore, they appear optimal for the diagnosis of ccRCC.

The results of ROC analysis (not provided) suggest that metastasis was strongly associated with miR-107 and miR-129-2 hypermethylation. These miRNAs are highly sensitive, but less specific diagnostic marker for predicting metastasis.

Discussion

We analyzed methylation status of 10 miRNA genes (miR-9-1, miR-9-3, miR-34b/c, miR-107, miR-124a-1, miR-124a-2, miR-124a-3, miR-129-2, miR-130b, and miR-148a) in a representative sample comprised of tumor and matched healthy tissue samples collected from 70 patients with ccRCC and 19 kidney tissues specimens from donors. The methylation status of 7 miRNA genes (miR-9-1, miR-9-3, miR-34b/c, miR-124a-1, miR-124a-2, miR-124a-3, and miR-129-2) was earlier assessed in a smaller sample of patients with ccRCC [15, 16], whereas methylation of miR-107, miR-130b, and miR-148a genes in ccRCC was evaluated for the first time. The role of hypermethylation in these genes was earlier assessed in other types of cancer. For example, miR-148a was found to be hypermethylated in nasopharyngeal carcinoma, hepatocellular carcinoma, and gastric cancer [21–23]. Epigenetic

Таблица 3. *Потенциальные диагностические системы маркеров на основе данных о метилировании 6 генов микроРНК*

Table 3. *Potential diagnostic systems of markers based on methylation of 6 miRNA genes*

Система System	Набор генов микроРНК MicroRNA gene kit	Среднее значение площади под ROC-кривой (95 % доверительный интервал) The mean value of area under the ROC curve (95 % confidence interval)	Оптимальный критерий отсечения Optimal cut-off criterion	Чувствительность Sensitivity	Специфичность Specificity	Положительное предсказательное значение Positive predictive value	Отрицательное предсказательное значение Negative predictive value
№ 1 No. 1	*MIR-9-1; -34b/c; -124a-3; -129-2*	0,93 (0,893–0,981)	1/4	0,900	0,944	0,984	0,708
№ 2 No. 2	*MIR-9-1; -34b/c; -124a-1; -129-2*	0,90 (0,850–0,961)	1/4	0,871	0,833	0,953	0,625
№ 3 No. 3	*MIR-9-1; -124a-3; -129-2; -130b*	0,89 (0,844–0,941)	1/4	0,786	1,000	1,000	0,545
№ 4 No. 4	*MIR-9-1; -34b/c; -124a-3; -129-2; -130b*	0,94 (0,899–0,981)	1/5	0,900	0,944	0,984	0,708

silencing of miR-130b was observed in ovarian cancer [20]; miR-107 was silenced in pancreatic cancer [19]. However, no epigenetic silencing was found in lung cancer and breast cancer [11, 12].

An increased frequency of methylation in the majority of 10 miRNA genes evaluated in this study indicates that these miRNAs function as tumor suppressors in ccRCC. The absence of significant changes in the methylation status of miR-148a gene in ccRCC suggests that its expression is not regulated by methylation. This assumption is supported by evidence showing the suppressor role of miR-148a in ccRCC pathogenesis [24, 25].

Comparing the results of methylation analysis with clinical and histological characteristics of ccRCC samples, we identified four miRNA genes, which methylation was associated with ccRCC progression: miR-107, miR-124a-3, miR-129-2, and miR-130b. Our findings on the relationship between miR-124a-3 and miR-129-2 methylation and cancer progression are consistent with the results obtained by us earlier [15, 16]. In this study, we revealed a significant correlation between miR-107 methylation and advanced clinical stages and metastasis of ccRCC, and an association between miR-130b methylation and advanced clinical stages and increased tumor size.

A significantly increased frequency of methylation in cancer tissue compared to healthy tissue allowed us to identify effective biomarkers for the diagnosis of ccRCC. Two panels of biomarkers that include 4 or 5 miRNA genes (miR-9-1, miR-34b/c, miR-124a-3, miR-129-2, and miR-130b) have a 90% sensitivity and 94% specificity (AUC of 0.93 and 0.94 respectively).

An association between a set of hypermethylated miRNA genes and 11 types of cancer has earlier been reported, but it did not include renal cancer [9]. The possibility of using methylation statuses of 7 miRNA genes (miR-9-1/3, miR-34b/c, miR-124a-1/2/3, miR-129-2) in cancer diagnostics was already discussed in our previous publications [15, 16]. The newly developed panels of biomarkers (assessed in this study) has higher sensitivity and specificity than the system proposed earlier [16]. Some authors described diagnostic systems based on the detection of specific miRNAs in serum to diagnose cancer (including ccRCC). Combination of miR-378 and miR-451 enabled identification of RCC with the sensitivity of 81%, specificity 83%, and AUC = 0.86 [26]. A panel of five serum miRNAs (miR-193a-3p, miR-362, miR-572, miR-28-5p, and miR-378), tested on 79 specimens, demonstrated 80% sensitivity and 71% specificity (AUC ~ 0.8) [27]. Using a panel of two serum miRNAs (miR-141 and miR-1233) ccRCC can be diagnosed with a 100% sensitivity and 73.3% specificity [28]. We identified two panels of miRNAs characterized by quite high sensitivity and specificity (90% and 94% respectively) and AUC over 0.9.

Conclusion

Thus, we recommend further validation of the described panels of miRNAs on larger samples to develop a method for early diagnostics of ccRCC using biopsy material, which is widely used for identification of small renal tumors in foreign countries [29]. Our findings can be used for the development of noninvasive diagnostic methods.

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