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# EXPRESSION OF MICRO-RNA HSA-MIR-30C-5P AND HSA-MIR-138-1 IN RENAL CELL CARCINOMA

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***Aim*: To analyze the expression levels of hsa-miR-30c-5p and hsa-miR-138-1 in tumors of patients with renal cell carcinoma to determine whether they could be used as diagnostic markers. *Materials and Methods*: The relative expression of hsa-miR-30c-5p and hsa-miR-138-1 was compared in the paired samples of kidney tumor tissue and conventionally normal tissue adjacent to the tumor. *Results*: We found a significant decrease in miR-30c-5p and miR-138-1 levels in tumor tissues even in the cases of early stage cancer. In addition, miR-138-1 expression was lower in renal cell carcinoma Fuhrman grade G3 + G4 as compared to Fuhrman grade G2. However, we found no association between miR-30c-5p and miR-138-1 expression in the tumors and the major clinical and pathological characteristics of renal cell carcinoma patients. *Conclusions*: A significant reduction in the expression levels of hsa-miR-30c-5p and hsa-miR-138-1 in renal cell carcinoma indicates the feasibility of further studies on the probable diagnostic utility of these markers.**

***Key Words*: renal cell carcinoma, miRNA expression, miR-30s-5p, miR-138-1.**

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Renal cell carcinoma (RCC) amounts to about 3% among all malignancies in the adult population [1, 2] and ranks the 3rd place among urologic cancers after the prostate and bladder cancers [3].

RCC includes a heterogeneous group of epithelial neoplasms with variability in biological behavior, clinical consequences, and rate of manifestation. Classical symptoms such as low back pain, the presence of volumetric formation and hematuria are characteristic of the late stages of the disease. Moreover, these symptoms appear in only 10%, some in 40% of patients [4].

According to immunohistochemical, morphological, cytological and molecular genetic studies there are several renal cancer subtypes: clear cell RCC (ccRCC, 60–70% of cases), papillary (10–15%), oncocytic (5–7%), chromophobic (3–5%) and about 40 others (collecting tubules, medullary, Xp11 translocation, etc.) [5]. In cases of timely detection of a malignant tumor, 85% of patients live for five years or more. However, due to problems with early detection and predisposition to metastases, the most common ccRCC subtype is usually diagnosed at the stage of metastasis in 40–50% of patients, of whom only 10% survive a five-year period [2]. Despite the wide application of modern diagnostic and treatment methods, the morbidity and mortality caused by RCC are constantly growing worldwide [6].

Factors affecting RCC prognosis can be classified into anatomical, histological, clinical, and molecular [7]. To date, the pathological stage, based on the size and extent of tumor invasion, is the most accurate predictor [8]. Recent data show that molecular signatures can classify RCC subtypes more accurately than morphological characteristics [9]. Identification of the mechanisms that control the pathogenesis of RCC could have a significant impact on the development of new and effective therapeutic approaches for RCC cure.

MicroRNAs are small non-coding RNA sequences (18–24 nucleotides in length) that are capable of negatively modulating gene expression by directly interacting with the 3'-untranslated regions of their target genes and, therefore, induce translational suppression and/or mRNA degradation [10]. miRNAs have been shown to be involved in the regulation of a number of biological processes, including cell proliferation, differentiation, metabolism as well as carcinogenesis [11]. miRNAs can function as tumor suppressors, oncogenes or, in some cases, both. In many cases, this depends on the disease or tissue types [12].

miRNAs are actively involved in the pathogenesis of RCC [13, 14]. Moreover, the potential role of miRNAs as prognostic and diagnostic tools in RCC, as well as its subtypes, has been described in several studies [15, 16]. The previous studies have shown that miRNAs of the miR-30c family are involved in many biological events, including cell apoptosis, growth, and differentiation [17]. The level of miR-30c is deregulated in several types of cancer, such as bladder cancer, invasive micropapillary carcinoma, and peripheral nerve sheath tumors [18, 19]. The expression level of miR-30c may also serve as an independent predictor of the clinical benefit of endocrine therapy in ER-positive breast cancer patients [20]. The decreased miR-30c levels have also been shown in kidney cancer cell lines [21]. The

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*Abbreviations used*: ccRCC – clear cell renal cell carcinoma; RCC – renal cell carcinoma; RU – relative units; qPCR – quantitative polymerase chain reaction.

limited expression of miR-30c-2-3p and miR-30a-3p in ccRCC is thought to be associated with increased HIF2α activity promoting epithelial-mesenchymal transition in these cells [22, 23].

miR-138-1 is also associated with tumor growth inhibition [24]. Its overexpression correlates with a decrease in hTERT telomerase and tumor cell apoptosis [25, 26]. Studies have shown that miR-138 expression decreases in the gallbladder and nasopharyngeal carcinoma [24, 27]. Moreover, its decrease in leukemia and lung cancer is associated with drug resistance [28, 29]. In addition, it has been shown that in ccRCC 786-O cells miR-138 has a negative effect on HIF-1a, overexpression of which promotes carcinogenesis, reducing the proliferation and motility of these cells [30]. Increasing evidence suggests that miRNAs are effective biomarkers and regulators of different types of tumors [9, 13, 15, 16, 31–37], including kidney cancer [38–40], and are therefore of wide relevance in both clinical and therapeutic practice.

The objective of this study was to analyze the levels of hsa-miR-30c-5p and hsa-miR-138-1 in RCC tissue and find out the correlations between these molecular markers and clinicopathological characteristics.

## MATERIALS AND METHODS

***Patients and tissue samples.*** 47 patients treated at the Institute of Urology of the National Academy of Medical Sciences of Ukraine during October 2017 — July 2018 were included to the study. The informed consent of the patients for the use of the samples in research purposes was obtained. The study was performed in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the Institute of Urology of the National Medical Academy of Ukraine. The samples of tumors and conventionally normal tissue adjacent to the tumor tissue (1.5 cm apart from tumor tissue) were taken. Tissue specimens were frozen in liquid nitrogen and stored at –80 °C until use.

The mean age of the patients was 57.09 ± 1.74 years. The clinical examinations were conducted according to the clinical protocols of the Ministry of Health of Ukraine. After pathohistologic conclusion, 41 cases were diagnosed with different types of RCC and 6 with conditionally benign neoplasms. The general information about patients is presented in Table 1.

Most patients underwent organ-saving surgery — 43 (91.5%), of which 23 (48.9%) had a laparoscopic resection, and 20 (42.6%) had open surgery. Nephrectomy was performed in four cases, in one — open access (2.1%) and in three — laparoscopic (6.4%).

***RNA isolation and reverse transcription quantitative polymerase chain reaction (qPCR).*** The preparation of total RNA was carried out using TRI Reagent (Sigma, USA). The synthesis of cDNA was carried out using a High-Specificity miRNA 1st-Strand cDNA Synthesis Kit (Agilent Technologies, USA). RT qPCR was performed using 5XHOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne, Estonia) and the iCycler CFX96 Real-Time PCR system (Bio-Rad Laboratories, Hercules, CA, USA). The relative expression of the gene was measured using the $2^{-\Delta CT}$ method, where $\Delta Ct=Ct_{tmiRNA}-Ct_{R18S}$, Ct: the threshold cycle.

**Table 1.** Clinicopathological characteristics of patients

| Parameter | | Number of patients (%) |
|---|---|---|
| Age, years (n = 47): | 25–54 | 16 (34.0) |
| | 55–64 | 18 (38.3) |
| | >65 | 13 (27.7) |
| Gender (n = 47) | Male | 29 (61.7) |
| | Female | 18 (38.7) |
| Side of kidney damage (n = 47) | Right | 23 (48.9) |
| | Left | 24 (51.1) |
| Fuhrman grade (n = 41) | G1 | 19 (46.3) |
| | G2 | 15 (36.7) |
| | G3 | 3 (7.3) |
| | G4 | 1 (2.4) |
| | NA | 3 (7.3) |
| TNM (n = 41) | T1a N0 M0 | 26 (63.5) |
| | T1b N0 M0 | 8 (19.5) |
| | T2a N0 M0 | 6 (14.6) |
| | T3a N0 M0 | 1 (2.4) |
| Clinical stage (n = 41) | I | 33 (80.5) |
| | II | 6 (14.6) |
| | III | 1 (2.4) |
| | NA | 1 (2.4) |
| Histology (n = 47) | Clear cell RCC | 36 (76.6) |
| | Papillary RCC | 4 (8.5) |
| | Chromophobic RCC | 1 (2.1) |
| | Angiomyolipoma | 2 (4.2) |
| | Angioleiomyofibroma | 1 (2.1) |
| | Leiomyofibroma | 1 (2.1) |
| | Oncocytoma | 2 (4.2) |

The relative expression of miR-30c-5p and miR-138-1 was normalized to R18S level and presented in the relative units (RU). The following primer sequences in RT-qPCR were used: R18S, forward 5'-CGCCGCTAGAGGTGAAATTC-3' and reverse 5'-CATTCTTGGCAAATGCTTTCG-3'; miR-30c-5p, forward 5'- TGTAAACATCCTACACTCTCAGC-3; miR-138-1, forward GCTACTTCACAACACCAGGGCC. A universal reverse primer from a cDNA Synthesis Kit (Agilent Technologies, USA) was used as the reverse of all miRNAs.

PCR was performed under the following conditions: 15 min at 95 °C, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 20 s, and extension at 72 °C for 20 s. Melting curve analysis was performed at a range from 65 °C to 95 °C with stepwise fluorescence acquisition at every 0.5 °C $s^{-1}$. All samples were amplified in triplicate. No-template controls were used as negative controls.

***Statistical analysis.*** To evaluate the statistical significance of differences between the tumors and normal samples of patients we applied the nonparametric Mann —Whitney U test. Wilcoxon test was applied for the comparison between paired tissue samples. All statistical analyses were performed using the GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA). *p*-values < 0.05 were considered to be significant.

## RESULTS AND DISCUSSION

We analyzed the expression of miR-30c-5p and miR-138-1 in tumor tissue of patients with renal cancer. For these miRNAs, a significant decrease in expression

was previously shown in various types of cancer as well as in cell lines of ccRCC [21, 41–47].

The relative expression of miR-30c-5p ranged from 3.14E-04 to 2.33E-02 units in tumor tissues and from 4.62E-03 to 1.24E-01 units in conventionally normal renal parenchyma tissues. Mean expression values were 5.54E-03 ± 7.9E-04 SEM in tumor tissue *vs.* 3.71E-02 ± 3.93E-03 in conventionally normal tissues (Fig. 1, 2, *a*). Statistical analysis of these results confirmed a significant decrease of miR-30c-5p miRNA in the tumor, both when comparing the two study groups (tumor and normal samples, Mann — Whitney U-test, $p < 0.0001$, see Fig. 1), and when comparing paired samples (tumor and conventionally normal adjacent kidney parenchyma tissue) obtained from the same patient (Wilcoxon T-test, $p < 0.0001$, see Fig. 2, *a*).

Our study also confirmed a decrease in the expression of miR-30c-5p and miR-138-1 in RCC tumors (ccRCC, papillary RCC, chromophobic RCC) compared to the normal adjacent tissues.

Similar results were obtained regarding the level of expression of miR-138-1 in RCC tissue and conventionally normal renal parenchyma tissues (see Fig. 1, 2, *b*). Mean expression values were 5.01E-03 ± 8.85E-04 SEM in tumor *vs* 3.77E-02 ± 5.25E-03 in conventionally normal tissues (see Fig. 1). The relative expression of miR-138-1 ranged from 3.75E-04 to 2.97E-02 units in RCC tissues and from 2.65E-03 to 1.72E-01 units in normal renal tissues (Mann — Whitney U-test, $p < 0.0001$, see Fig. 1; Wilcoxon T-test, $p < 0.0001$, see Fig. 2, *b*).

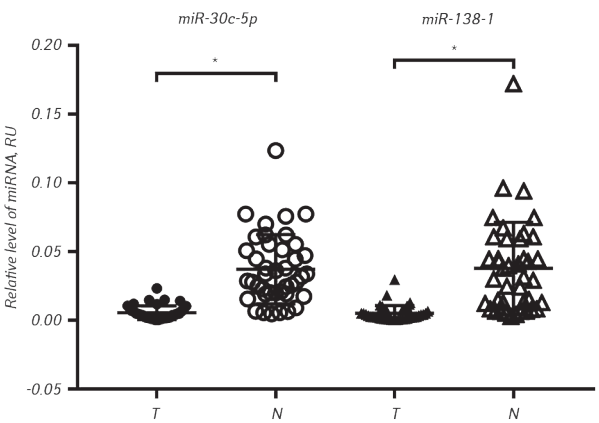


**Fig. 1**. Expression levels of miR-30c-5p and miR-138-1 in tumor (T) and adjacent tissue (N) samples from patients with RCC. * $p < 0.0001$

Nevertheless, no significant correlations were found between the levels of these miRNAs in tumor samples and clinical-pathological parameters (age, stage of the disease, tumor size, differentiation grade, tumor localization, and so on). In particular, no correlation was evident between the levels of miRNAs under study and the extent of the primary tumor according to TNM system (Fig. 3, *a*). The only significant difference was revealed when miR-138-1 level was compared in tumor cells differing by Fuhrman nuclear atypia grade (G2 *vs* G3+G4; $p = 0.0464$) (Fig. 3, *b*).

It is of importance to analyze possible prognostic significance of certain indicators such as the level of miRNA expression. In all subtypes of renal cancer, the prognosis becomes worth with the increase of stage and histological gradation [48]. The 5-year overall survival for all types of renal cancer is 49%, which has improved since 2006, largely due to an increase in early detection of the disease, and an establishment of the TKI inhibitors treatment [49] in addition to organ-sparing, less invasive surgery (nephrectomy, laparoscopic nephrectomy). In our study, we analyzed tumor samples from the patients with the early stages of cancer. Although the follow-up term is less than 3 years, all patients are currently alive, and no recurrence has been detected.

It is of interest that a decrease in miRNA levels was also detected in tumor samples that are associated with benign tumors (n = 6), two of them being diagnosed as angiomyolipoma that under certain conditions might be capable of malignant growth. The relative expression of miR-30c-5p ranged from 3.86E-04 to 7.77E-03 units (mean expression 3.92E-03 ± 1.30E-03 SEM RU) in benign tumor tissues as opposed to normal tissues from 5.19E-03 to 1.04E-01 units (mean expression 3.07E-02 ± 1.53E-02 SEM RU), $p = 0.0411$. For miR-138-1, relative expression was determined from 5.52E-04 to 1.53E-02 (mean expression 4.32E-03 ± 2.44E-03 SEM RU) in benign tumors and from 4.09E-03 to 8.70E-02 in normal kidney tissues (mean expression 3.38E-02 ± 1.37E-02 SEM RU),

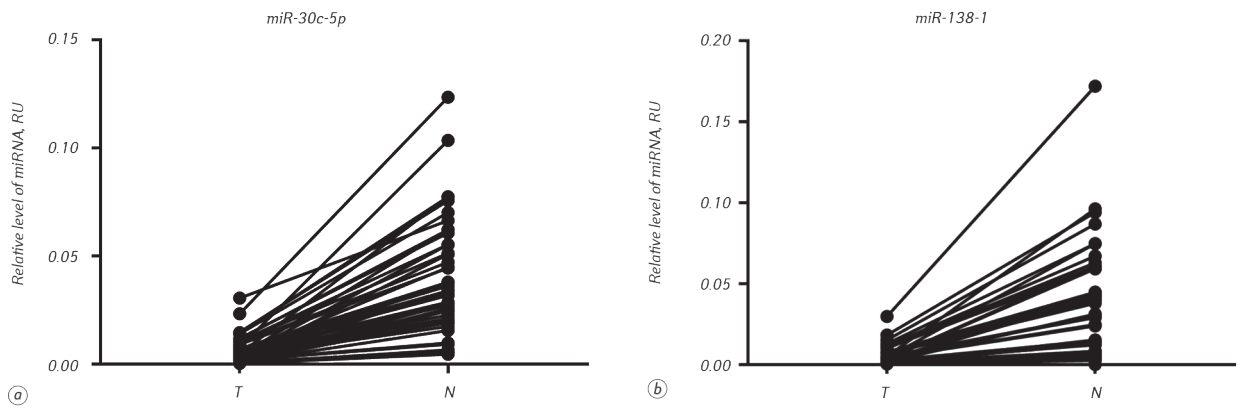


**Fig. 2.** Relative expression level of miR-30c-5p (*a*) and miR-138-1 (*b*) in paired samples of tumors (T) and adjacent tissue (N)

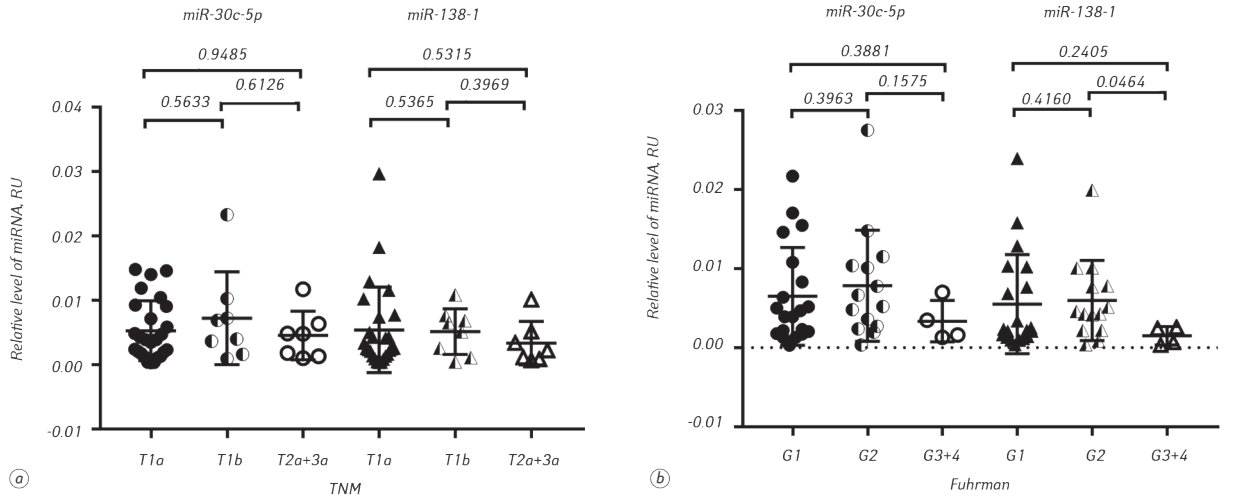


**Fig. 3.** Relative expression levels of miR-138-1 and miR-30c-5p in RCC differing by tumor size (*a*) and Fuhrman grade (*b*)

$p = 0.0411$. No significant difference was found between miR-30c-5p and miR-138-1 levels in malignant tumors of kidney cancer and benign tumors (Fig. 4).

With the exception of angiomyolipoma, most rare kidney tumors cannot be distinguished from RCC based on the results of radiological diagnosis (except for some oncocytoma). Therefore, new and more precise molecular biological markers are needed to improve diagnosis. However, in our study, we were unable to find an association between miRNA levels of miR-30c-5p and miR-138-1 and the specified diagnosis of renal neoplasm, which may be due to a small number of samples used in the study.

Our findings are consistent with those of other researchers who have studied microRNAs of the miR-30 family in tumors of various localization and histogenesis, and RCC cell lines [21, 43–47]. miR-30a and miR-30c have been shown to adversely affect cell proliferation, and their overexpression suppresses tumor formation. Similar results for several types of malignancies and malignant cell lines, including ccRCC cell line, were also obtained for miR-138-1 [48, 49]. Thus, a significant decrease in the expression of miR-30c-5p and miR-138-1 might be associated with an unfavorable course of the disease. These miRNAs may be considered as the candidates for non-invasive diagnosis although their usefulness should be studied in larger cohorts of the patients with different pathological conditions.

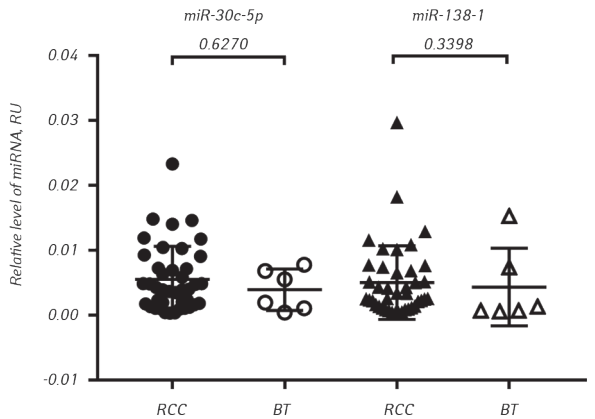


**Fig. 4**. Relative expression levels of miR-138-1 and miR-30c-5p in RCC and benign tumors (BT) samples

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