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ORIGINAL ARTICLE

MicroRNA-15a tissue expression is a prognostic marker for survival in patients with clear cell renal cell carcinoma

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Abstract

None of the currently investigated molecular markers demonstrated sufficient accuracy in prognostication of the renal cell carcinoma (RCC) oncologic outcomes; thus, none of them has been recommended for the application in the routine clinical practice. The role of miR-15a as a potential prognostic marker for RCC is still not unveiled. The aim of our study was to assess the expression of miR-15a in tumor tissues of the patients with RCC and to evaluate the possibility of its usage as a prognostic molecular biomarker of this disease. The retrospective included 64 adult patients with clear cell RCC (ccRCC) in whom radical or partial nephrectomy was conducted. After deparaffinization of formalin-fixed paraffin-embedded (FFPE) ccRCC specimens, the tissue expression of miR-15a was measured using the reverse transcription and quantitative polymerase chain reaction in the real time. For the reference, the expression of miR-15a was estimated in 15 FFPE tissue specimens of the normal renal parenchyma. Survival analysis involved all cases of non-metastatic RCCs ($n=57$). Five-year cancer-specific survival (CSS) was estimated by means of the Kaplan–Meier method and was calculated from the date of surgery to the date of death. Patients with the RCC were characterized by significantly upregulated tumor tissue mean levels of miR-15a compared to the healthy controls: 0.10 ± 2.62 relative units (RU) versus $4.84\text{E}-03 \pm 3.11\text{E}-03$ RU ($p<0.001$). Overexpression of miR-15a was strongly associated with poor histologic prognostic features of ccRCC. Poorly differentiated tumors tend to have more pronounced upregulation of miR-15a compared to highly differentiated lesions: Mean expression values were 4.57 ± 3.19 RU for Fuhrman grade 4 versus 0.02 ± 0.01 RU for Fuhrman grade 1 ($p<0.001$). The metastatic involvement of the regional lymphatic nodules (N+) was associated with significantly upregulated miRNA-15a in comparison with N− cases: Mean expression values were 4.92 ± 2.80 RU versus 1.10 ± 2.29 RU, respectively ($p<0.001$). In patients with miR-15a expression in RCC tissues ≤ 0.10 RU, mean 5-year CSS was significantly longer compared to patients with expression levels above this threshold: 92.31% (mean duration of survival—59.88 ± 0.12 months) versus 54.8% (mean duration of survival—49.74 ± 2.16 months), respectively ($p<0.001$). The tissue expression of miR-15a could be used as a potential prognostic molecular biomarker for conventional RCC.

Keywords Cancer · Renal cell carcinoma · Clear cell · miRNA-15a · MicroRNA-15a · Biomarker · Genetic · Prediction · Prognosis · Survival

Introduction

Renal cell carcinoma (RCC) amounts to about 3% among all malignancies in adult population. This type of neoplasia ranks the 3rd in oncologic urology after the prostate and bladder cancers [1]. Despite wide application of modern diagnostic and treatment methods, the morbidity and mortality caused by RCC are constantly growing worldwide [2]. The course and the prognosis of the disease depend on a number of factors. According to the European Association of Urology, they are defined as anatomic, histologic, clinical

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and molecular. The anatomic factors are mainly represented by the TNM classification of American Joint Committee on Cancer (AJCC). The histologic ones are comprised of the degree of nuclear atypia (Fuhrman grades), histologic subtypes of RCC, presence of sarcomatoid and rhabdoid differentiation, microvascular invasion, tumor necrosis and invasion of the collecting system of the kidney. The clinical factors presented by the patients' general performance status, local symptoms, cachexia, anemia, levels of platelets and neutrophils, and neutrophils-to-lymphocytes ratio [3]. Thus, the hazard ratio (HR) of patients with RCC of the stages T2N0M0, T3N0M0 and T4N0M0 (in relation to the stage T1N0M0) is characterized by the significant differences and accounts for 2.71, 5.20 and 18.88, respectively [4]. A 5-year cancer-specific survival (CSS) rates in patients with clear cell RCC (ccRCC), papillary RCC and chromophobe RCC are also significantly different and accounts for 71%, 91% and 88%, respectively [5]. Likewise the HR for II, III and IV RCC Fuhrman grades (correlated with the I grade) amounts to 1.16, 1.97 and 2.82, respectively [6].

For the past decade, numerous potential molecular prognostic markers of RCC have been studied, namely carbonic anhydrase (CaIX) [7], vascular endothelial growth factor (VEGF) [8], hypoxia-induced factor (HIF) [9], cellular marker of proliferation Ki-67 [10], phosphatase and tensin homolog (PTEN) [11], E-cadherin [12], EMT-related transcription factor [13], programmed death ligand-1 (PD-L1) [14] and many others. However, none of the currently investigated molecular markers demonstrated sufficient accuracy in prognostication of the RCC oncologic outcomes, thus, none of them has been recommended for the application in the routine clinical practice [3]. Besides this, none of the existing and validated nomograms for predicting survival for patients with RCC has incorporated genetic molecular markers. For example, one of the most used prognostic systems for renal cell carcinoma—UCLA Integrated Staging System (UISS)—is based on three parameters with the exclusion of molecular: TNM stage, Fuhrman nuclear grade and Eastern Cooperative Oncology Group (ECOG) performance status [15]. Likewise, SSIGN Score for RCC contains similar criteria accompanied by a specification of the presence of tumor necrosis [16]. IMDC (International Metastatic RCC Database Consortium) Risk Model for RCC [17] and Memorial Sloan-Kettering Cancer Center (MSKCC/Motzer) Score for Metastatic RCC [18] both are based exclusively on a clinical factors (e.g., Karnofsky performance status, indices of complete blood count, corrected calcium serum level, lactate dehydrogenase level) and do not include molecular.

Discovery of microRNAs has revolutionized the field of molecular oncology, and scientists have witnessed significant breakthroughs in unraveling essential role of miRNAs in cancer development and progression [19–24]. miRNAs have been shown to modulate repertoire of targets in myriad of cell signaling pathways [25–28]. In recent years, emerging evidence has also highlighted a critical role of miRNAs in RCC. High-quality research has helped us to develop a better knowledge of rapidly upgrading list of miRNAs having diagnostic, prognostic and predicting significance in differentiating and defining the course of RCC [29]. MiRs are noncoding RNAs that regulate the expression of a wide nomenclature of genes by influencing the 3′-untranslated regions of corresponding mRNA. In the context of RCC development, two main classes of the miRs have been accentuated: the oncomirs—miRNAs that promote carcinogenesis—and the anti-oncomirs—molecules that negatively regulate tumorigenesis by suppressing tumor growth. Among other microRNAs, miR-15a was not comprehensively studied and its importance as a promising biological marker in RCC was not comprehensively investigated. The latter miRNA emerging from the cluster of chromosome region 13q14 which is often deleted in cancer [30] is known to act as an anti-oncomir in spectrum of other malignancies such as prostate cancer [31], nasopharyngeal carcinoma [32], esophageal squamous cell carcinoma [33] and malignant melanoma [34] by inhibiting cell proliferation, promoting apoptosis of cancer cells and suppressing tumorigenicity. The existing evidence proves the significance of miR-15a in natural history of RCC [35]. In our previous work, we had provided evidence of high diagnostic performance of miR-15a urine expression in differentiating RCC from benign renal neoplasms such as angiomyolipoma and oncocytoma [36]. Therefore, we assumed that miR-15a expression could be used to predict the survival of RCC patients.

The goal of our study was to assess the expression of miR-15a in tumor tissues of the patients with RCC and to critically evaluate the possibility of its potential as a prognostic molecular biomarker of this disease.

Materials and methods

Ethics statement

This retrospective study was approved by the Ethical Committee of Lviv National Medical University, named after Danylo Halytsky (Lviv, Ukraine; protocol 5, dated 05.25.2015), and was executed in accordance with ethical standards presented in the Declaration of Helsinki (1975). The research was carried out at the Department of Urology of the above-mentioned institution and at the Department of General and Molecular Pathophysiology of Bogomolets Institute of Physiology of National Academy of Sciences of Ukraine during 2015–2019 years.

General data

The research involved the analysis of 210 archived medical histories of the patients having suffered from conventional histologic subtype of the RCC and who had received surgical treatment in the form of partial or radical nephrectomy starting from 2012 until 2014. The exclusion criteria were as follows: non-clear cell histology of RCC; history of neoadjuvant treatment of RCC; absence of postoperative pathologic report; and the reason of patients' death in the distant post-op period that was not associated with the course of RCC (in order to calculate CSS). The final number of cases included in the research was 64 patients with ccRCC who formed the main group. The division by gender was as follows: 37 (57.81%) men and 27 (42.19%) women. The age of the patients ranged from 47 to 68 years (mean—61.2 ± 6.33 years). The size of the tumors ranged from 3.2 to 11.8 cm with average size of 8.52 ± 3.59 cm. There were no cases with distant metastasis. The staging of all patients was performed according to AJCC TNM classification. The grading of RCC was accomplished using four-tiered Fuhrman system. The detailed clinical analysis of the patients is given in Table 1.

Table 1 Detailed characteristics of the patients' groups and subgroups

Characteristics	N	%
Main group	64	100
Division by gender		
Male	37	57.81
Female	27	42.19
Side		
Right	30	46.88
Left	34	53.12
Type of surgical treatment		
Partial nephrectomy	14	21.88
Radical nephrectomy	50	78.12
Classification by TNM		
T1aN0	13	20.31
T1bN0	10	15.63
T2aN0	16	25.0
T2bN0	8	12.5
T3aN0	9	14.06
T3aN1	2	3.13
T3bN0	1	1.56
T3bN1	5	7.81
A total of N−	57	89.06
A total of N+	7	10.94
A total of M−	64	100
Grade of differentiation by Fuhrman		
Grade 1	15	23.44
Grade 2	14	21.88
Grade 3	22	34.38
Grade 4	13	20.31
Presence of tumor necrosis	14	21.88
Control group	15	100
Division by gender		
Male	9	60.0
Female	6	40.0

Paraffin samples

With the aim of investigating the miR-15a expression in the RCC tissues, the formalin-fixed paraffin-embedded (FFPE) tissue specimens with postoperatively preserved RCC samples were obtained from achieve. As the reference, study enrolled 15 FFPE tissue specimens of the normal renal parenchyma from carefully selected persons without renal pathology according to the clinical and postmortem pathologic data (control group). In all 15 cases, death of patients was caused by the acute disorders of cerebral circulation that resulted from the stroke. The additional eligibility criterion was patients' age between 45 and 70 years.

miRNA isolation from paraffin-embedded tissue

All cases involved measurement of the miR-15a expression in the RCC tissues or normal renal parenchyma. For this purpose, the deparaffinization of all tissue samples followed by RNA isolation was executed involving application of the PureLink™ FFPE RNA Isolation Kit (Applied Biosystems, USA) in accordance with the manufacturer's protocol. The determination of RNA concentration was carried out using the spectrophotometer (NanoDrop ND1000, NanoDrop Technologies Inc, USA).

qPCR data

MicroRNA-15a expression was estimated by means of reverse transcription and quantitative polymerase chain reaction (qPCR) in the real-time setting. The reverse transcription was performed using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, USA) with a specific primer for microRNA and 10 ng of total RNA. For the real-time qPCR, TaqMan MicroRNA Assays (Applied Biosystems, USA) were used: U6 snRNA, ID 001973 (as endogenous control) and hsa-miR-15a, ID 000389 (Applied Biosystems, USA). The temperature cycles were as follows: the step of the initial denaturalization 95 °C 10 min; 50 cycles 95 °C—15 s та 60 °C—60 s. The expression level of miRNA was normalized up to U6 snRNA and presented

in the relative units (RU). The amplification was performed using 7500 Fast real-time PCR (Applied Biosystems, USA). Obtained results were analyzed with the help of 7500 Fast real-time PCR software package and presented as the graphs and charts.

Statistical analysis

The evaluation of the difference in the miR-15a expression in the main and control groups as well as in subgroups of patients was performed using analysis of variance (ANOVA) method. The normality of the data distribution was obtained by means of Shapiro–Wilk's test ($W = 0.531$, $p = 0.001$). Since W-statistics was significant ($p < 0.05$), the hypothesis about the normal data classification was rejected and the significance of the differences was defined by the nonparametric Mann–Whitney U test. The result was considered statistically significant in case of $p < 0.05$. The correlation analysis was performed using Pearson method. The software packages SPSS version 22 and Microsoft Excel 2016 were used for the statistical analysis of the received data.

Survival analysis

Survival analysis involved all cases of non-metastatic (N − and M −) conventional RCC ($n = 57$). All patients were stratified to one of the risk groups (low, intermediate or high) using UISS for localized disease, which required assessment of T-stage according to TNM classification, Fuhrman nuclear atypia grade (four-tiered) and Eastern Cooperative Oncology Group (ECOG) performance status. Survival rates (5-year CSS) were estimated by means of the Kaplan–Meier method and were calculated from the date of surgery to the date of death and were compared using log-rank tests.

Results

General analysis of miR-15a expression in tissue samples

The analysis of the obtained data demonstrated the presence of the tissue expression levels of miR-15a in all cases. We observed statistically significant difference between the mean expression values of miR-15a in main and control groups: Patients with the RCC were characterized by significantly upregulated levels of miR-15a compared to the individuals who did not suffer from this renal pathology: Mean expression values were 0.10 ± 2.62 RU versus $4.84\text{E}-03 \pm 3.11\text{E}-03$ RU ($p < 0.001$) (Fig. 1).

There was reverse interconnection between expression levels of miR-15a and RCC Fuhrman nuclear atypia grade: Poorly differentiated tumors tend to have more pronounced upregulation of miR-15a compared to highly differentiated lesions: Mean expression values were 4.57 ± 3.19 RU for Fuhrman grade 4 versus 0.02 ± 0.01 RU for Fuhrman grade 1 ($p < 0.001$) (Fig. 2). Nevertheless, there was no significant difference in mean miR-15a expression between several

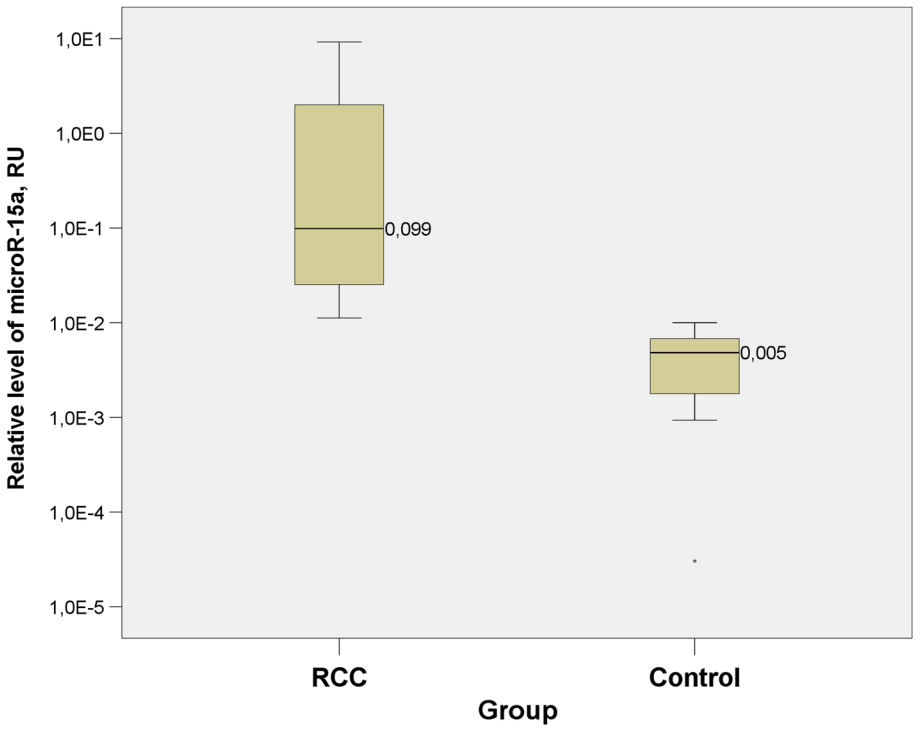

Fig. 1 Box plot of miR-15 tissue expression in main and control groups. *RCC* renal cell carcinoma

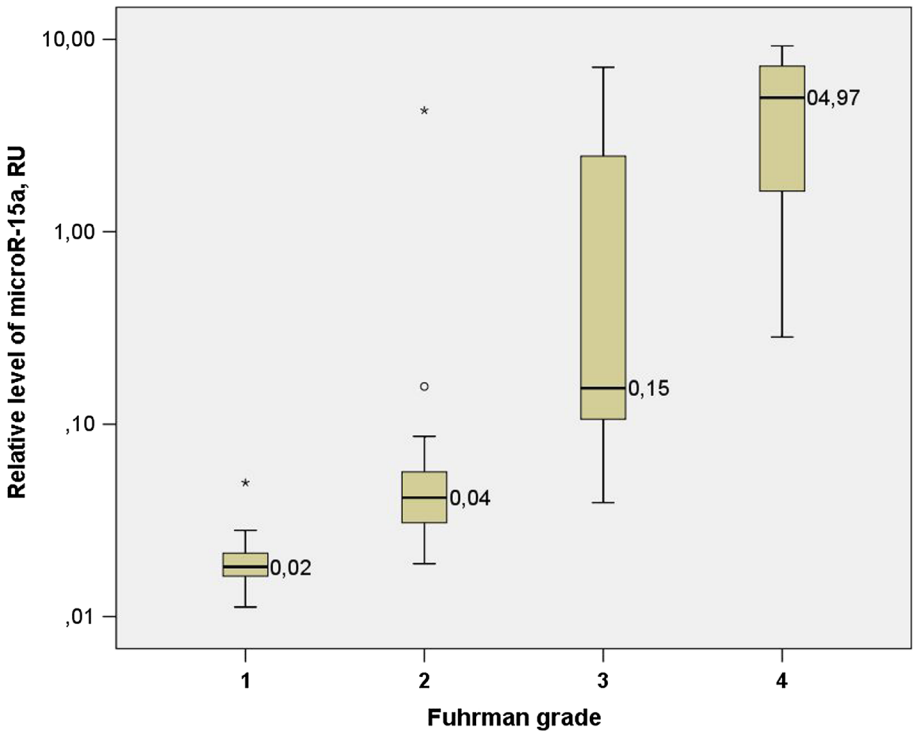

Fig. 2 Box plot of miR-15 tissue expression in patients with ccRCC and different Fuhrman grades

Table 2 Statistical differences in mean miRNA-15a expression values among patients with ccRCC of different Fuhrman grades

Compared subgroups	*P*
RCC grade 1 against grade 2	0.2929 (no difference)
RCC grade 1 against grade 3	0.0095 (there is a difference)
RCC grade 1 against grade 4	0.0002 (there is a difference)
RCC grade 2 against grade 3	0.0671 (no difference)
RCC grade 2 against grade 4	0.0004 (there is a difference)
RCC grade 3 against grade 4	0.0065 (there is a difference)

closest pairs of subgroups of patients: between Fuhrman grades 1 and 2 as well as between Fuhrman grades 2 and 3. The statistical comparison of the following subgroups is given in Table 2.

The study has also revealed interesting phenomena: The mean miRNA-15a expression value in RCC tissues in the presence of the necrotic component was substantially higher compared to cases with the absence of the necrosis—4.01 ± 3.42 RU versus 0.82 ± 1.85 RU, respectively ($p < 0.001$). Likewise, the presence of the metastatic involvement of the regional lymphatic nodules (N+) was associated with significantly upregulated miRNA-15a in comparison with N− cases, and mean expression values were 4.92 ± 2.80 RU versus 1.10 ± 2.29 RU, respectively ($p < 0.001$) (Fig. 3). The detailed statistical characteristics of the miRNA-15a tissue expression in both main and control groups as well as in subgroups are presented in Table 3.

In addition, the correlation analysis showed strong direct positive relation between the tumor size in patients with RCC and the level of miRNA-15a tissue expression: The Pearson correlation coefficient amounted to 0.724 ($p < 0.001$) (Fig. 4).

Survival analysis

As a result of patients' classification according to UISS, all 57 non-metastatic patients with RCC were stratified into intermediate-risk group with predicted 5-year CSS—80.4%. In contrast, according to survival data achieved in this study, 5-year CSS in non-metastatic patients with RCC accounted for 77.19%. The rest 7 patients with RCC and N+ also underwent stratification using UISS for metastatic disease and were all assigned as low-risk group with predicted 5-year CSS—32.0%. However, due to small amount of patients in this subgroup, further survival analysis was postponed.

The comparison of mean miR-15a expression values in RCC tissues of patients who stayed alive during the first 5 years after the nephrectomy ($n = 44$) with the mean expression in case of exitus letalis due to RCC progression during the same time period ($n = 13$) revealed significant difference: 0.92 ± 2.04 RU versus 3.47 ± 3.36 RU, respectively ($p = 0.013$, $t = 2.782$). Moreover, only in one (7.69%) patient among 13 lethal cases, miR-15a expression was below the threshold of 0.10 RU (amounted to 0.05 RU), and in rest 12 (92.31%) patients, it ranged

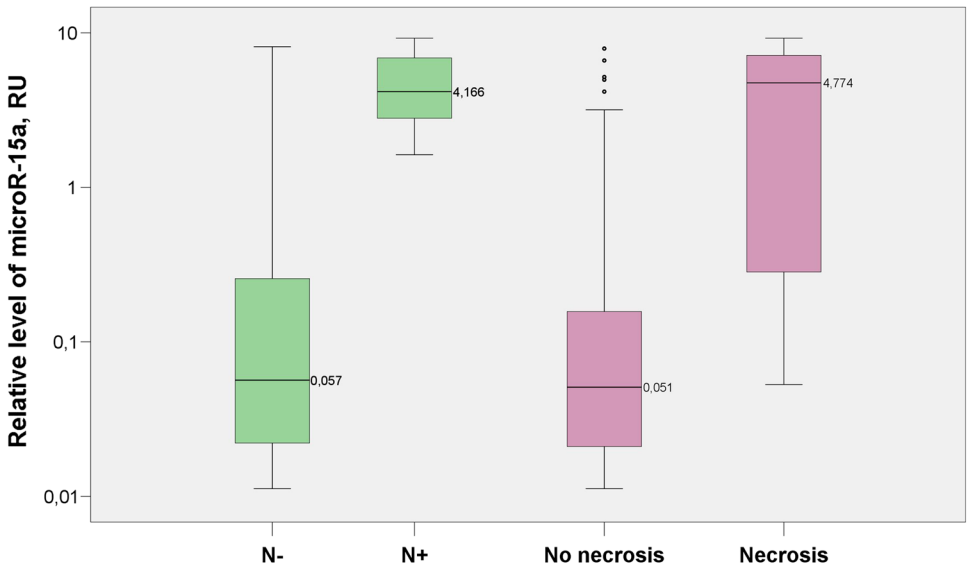

Fig. 3 Box plot of miR-15 expression in ccRCC tissues and N+/N—or presence/absence of necrotic component

Table 3 Statistical characteristics of the miRNA-15a expression in the main and control groups as well as in the subgroups of patients

Patients group/subgroup	*N*	Mean	Median	SD	95% Confidence interval for the mean		Minimum	Maximum
					Lower bound	Upper bound		
RCC	64	1.52	0.10	2.62	0.86	2.17	0.01	9.25
Normal control	15	4.53E−03	4.84E−03	3.11E−03	2.80E−03	6.25E−03	3.04E−05	9.99E−03
RCC with no necrosis	50	0.82	0.05	1.85	0.29	1.34	0.01	7.91
RCC with necrosis	14	4.01	4.77	3.42	2.03	6.0	0.05	9.25
RCC and N−	57	1.10	0.06	2.29	0.49	1.71	0.01	8.13
RCC and N+	7	4.92	4.17	2.80	2.33	7.52	1.63	9.25
RCC FG 1	15	0.02	0.02	0.01	0.02	0.03	0.01	0.05
RCC FG 2	14	0.35	0.04	1.13	0.30	1.0	0.02	4.27
RCC FG 3	22	1.47	0.15	2.39	0.41	2.53	0.04	7.16
RCC FG 4	13	4.57	4.97	3.19	2.64	6.50	0.28	9.25

RCC renal cell carcinoma, *N*− without the spread into lymph nodes, *N*+ with the spread into lymph nodes, *FG* Fuhrman grade

between 0.12 and 9.25 RU. Therefore, we used miR-15a tissue expression value of 0.10 RU as the threshold for further survival analysis. As a result, it was found out that in patients with RCC and miR-15a expression in tumor tissues ≤ 0.10 RU mean 5-year CSS was significantly longer compared to patients with expression levels above this threshold: 92.31% (mean duration of survival—59.88 ± 0.12 months) versus 54.8% (mean duration of survival—49.74 ± 2.16 months), respectively ($p < 0.001$) (Fig. 5).

In addition, the correlation analysis revealed strong reversed interconnection between duration of survival and miR-15a tissue expression levels in patients with RCC: $r = -0.805$ ($p < 0.001$).

Discussion

The recent studies had elucidated an important role of miR-15a in pathogenesis of cancer. This small RNA that origins from chromosomal region 13q14 is involved in multiple cancer-inducing pathways. It is known that nuclear binding of pri-miR-15a is one of the functions of protein kinase Cα (PKCα), which is playing a prominent role in endothelin-1 (ET-1)-mediated signaling system, which is involved in the development of the malignant tumors [37]. Besides this, miR-15a is a part of non-canonical signaling pathway of nuclear factor KappaB (NF-κB), which regulates resistance to apoptosis, angiogenesis and

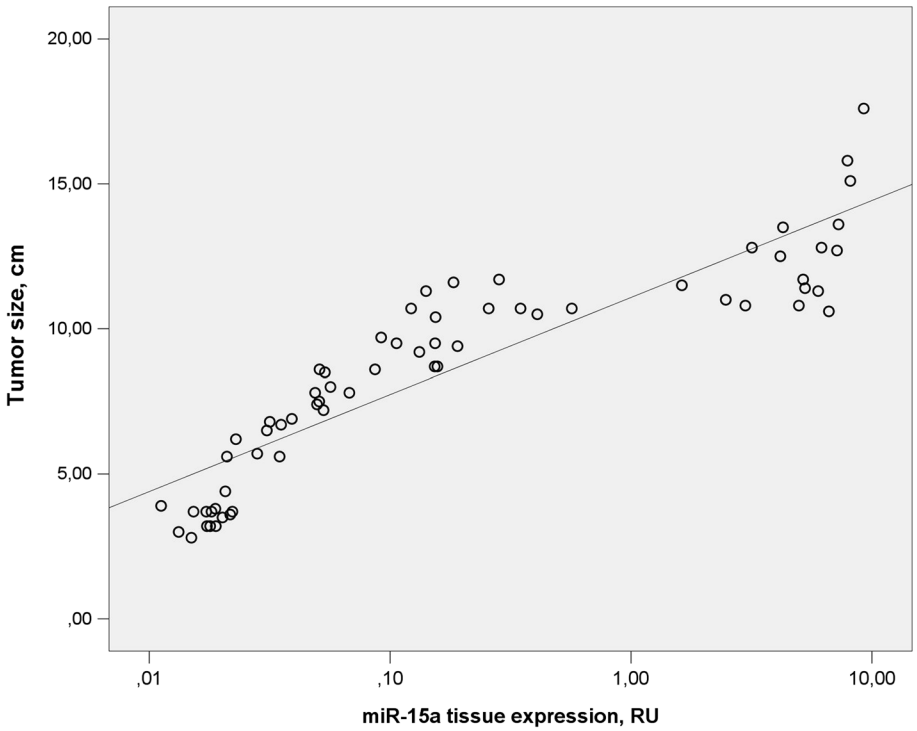

Fig. 4 Scatterplot of correlation between miR-15a expression in tissues and the tumor size in patients with RCC

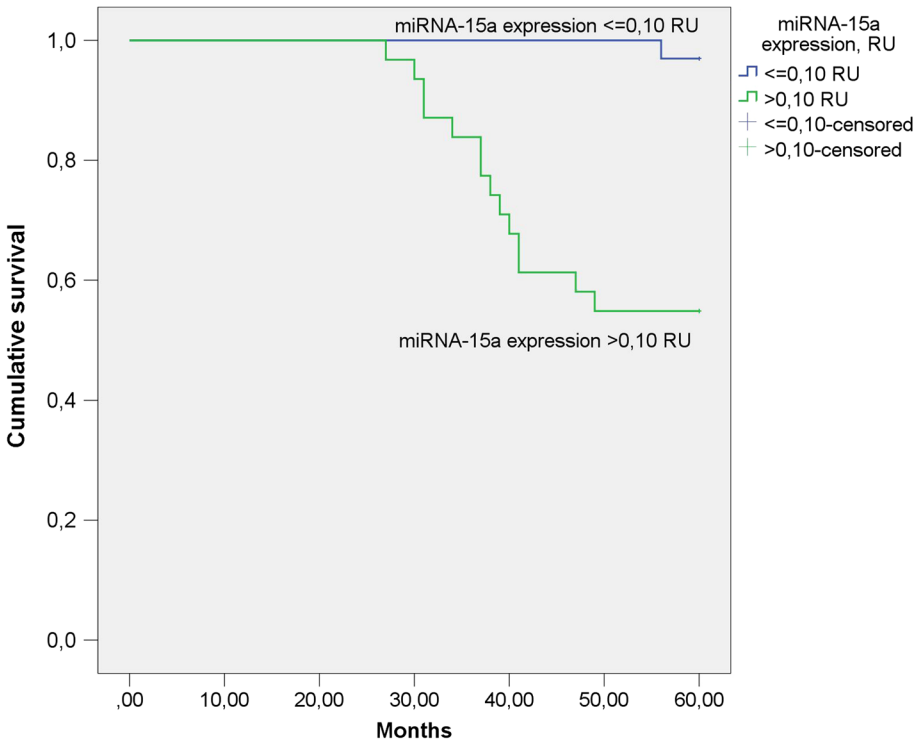

Fig. 5 Kaplan–Meier curves of 5-year CSS of patients with ccRCC and different tissue expressions of miR-15a, difference between both hands $p < 0.001$

multi-resistance to therapeutic agents. Simultaneously, von Hippel–Lindau gene is a negative regulator of NF-κB [38]. A number of researchers evinced a downregulation of miRNA-15a in patients with such malignant tumors as prostate cancer [31], nasopharyngeal cancer [32], malignant melanoma [34], glioma [39], breast carcinoma [40] and others, which was playing a tumor-protective role (anti-oncomir), having targets as genes BCL2, Mcl1, CcnD1 and Wnt3A. However, potential of miR-15a as a prognostic biomarker for RCC is still uncertain.

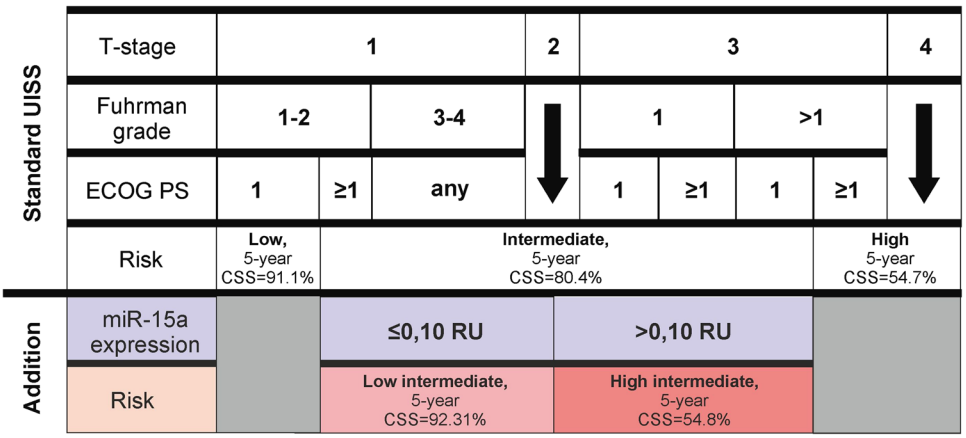

Fig. 6 Modified prognostic system UISS for localized conventional RCC. *CSS* cancer-specific survival, *ECOG PS* Eastern Cooperative Oncology Group performance status

In the presented research, we investigated miR-15a expression levels in normal renal parenchyma, and in conventional RCCs' tissues, the latter miR-15a was significantly overexpressed, conversely acting as oncomir. To some extent, such data correlate with the results of Brandenstein's research which states that miR-15a expression in urine samples was significantly higher in patients with RCC compared to the patients with benign renal tumors [41]. Such divergence may suggest that potential application of miR-15a as a treatment agent may promote RCC tumorigenesis; however, such theory requires profound investigations.

We demonstrated that overexpression of miR-15a was strongly associated with poor histologic prognostic features of ccRCC such as higher Fuhrman grade, presence of necrotic component of the lesion and metastatic lymphatic nodule involvement. The strong direct correlation between RCC Fuhrman grade and miR-15a expression in tumor tissues was observed: $r = 0.727$ ($p < 0.001$). Such tendency completely corresponds with previously received by us data with regard to measurement of miR-15a expression in urine [36]. Noticeably higher expression levels of miR-15a in RCC tissues compared to urine could be hypothetically explained by significantly higher concentration of this molecule in intra- and paracellular compartments.

We invigilated a difference in mean 5-year CSS predicted by UISS and obtained by us as a result of this retrospective study: In non-metastatic patients with ccRCC, it was 80.4% and 77.19%, respectively. Such disparity could be explained by imperfection of UISS prognostic systems' index of concordance (C-index) which is reported to be in the range of 0.76–0.86 for non-metastatic RCC [42]. The results obtained by us entirely fit such boundaries. In our study, 5-year CSS in non-metastatic patients with RCC and miR-15a tissue expression levels above 0.10 RU had much more poor survival rate compared to patients with miR-15a expression below named threshold. This contrasts with other studies where upregulated miR-15a was a predictor of good outcome in non-RCC cancers [31–35]. Such situation we explain by the involvement of miR-15a in broad spectrum of signaling tracts is responsible for the development of different kinds of cancer. Decidedly, further investigations are required for deeper understanding of the miR-15as' role in pathogenesis of RCC and outcome of this disease.

In order to improve prognostic accuracy of UISS for localized disease, we modified it by stratifying patients with intermediate prognosis into one of the two subgroups according to the expression level of miR-15a in RCC tissues: ≤ 0.10 RU—low intermediate risk and > 0.10 RU—high intermediate risk (Fig. 6). Howsoever, this improved prognostic system should be re-assessed with larger cohort of patients and externally validated.

The main limitations of our study were the small amounts of patients with non-metastatic RCC of good or poor prognosis as well as lack of cases with metastatic RCCs. Another important limitation was that only patients with clear cell histologic subtype of RCC were enrolled in the study.

Conclusion

In conclusion, the expression of miR-15a measured in tissues of ccRCC correlates with its poor histologic prognostic features, and upregulation of this molecule is associated with shorter survival rates. The tissue expression of miR-15a could be used as a potential prognostic molecular biomarker for conventional RCC.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human participants and/or animals All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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