

Altered Expression of DDR1 in Clear Cell Renal Cell Carcinoma Correlates With miR-199a/b-5p and Patients' Outcome

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Abstract. *Background/Aim.* Accumulating evidence suggests that discoidin domain receptor tyrosine kinase 1 (DDR1) has an oncogenic role. Therefore, the aim of this study was to evaluate the potential utility of DDR1 and its post-transcriptional repressors, miR-199a-5p and miR-199b-5p, as prognostic factors in clear cell renal cell carcinoma (ccRCC). *Patients and Methods.* The expression of DDR1 in tumor and normal renal tissues of 56 patients with ccRCC was assessed by reverse transcription quantitative polymerase chain reaction, western blotting and immunohistochemistry. Renal cancer cells were transfected with specific RNA sequences to validate DDR1 as a putative miR-199a/b-5p target. *Results.* Decreased DDR1 mRNA and protein, as well as miR-199a/b-5p levels were found in ccRCC. Low DDR1 protein was associated with higher nuclear grade and shorter overall survival. DDR1 immunoreactivity was elevated in the nuclei and unchanged in the membrane/cytoplasmic compartment of tumor cells. DDR1 levels correlated with those of miR-199a/b-5p. In addition, we validated DDR1 as a target gene for miR-199a/b-5p in renal cancer cell lines. *Conclusion.* DDR1 expression is altered in

ccRCC, but our findings do not support its oncogenic role. In-depth investigation will be necessary to elucidate the exact role and potential utility of miR-199a/b-5p in ccRCC.

Renal cell carcinoma (RCC) is considered one of the most lethal urological malignancies representing the sixth and tenth most frequently diagnosed cancers in men and women, respectively (1). The most prevalent histological subtype of RCC, clear cell renal cell carcinoma (ccRCC) comprises 70-80% of renal tumors (2). It is believed that clear cell tumors originate in the proximal convoluted tubule (PCT) (3, 4). The majority of ccRCC sporadic cases are characteristically associated with the loss of function of von Hippel-Lindau (VHL) tumor-suppressor gene by somatic mutations, chromosomal loss or promoter hypermethylation (5). Loss or inactivation of VHL results in a sequence of events comprising accumulation of hypoxia inducible factors (HIFs), overexpression of HIF-driven genes such as vascular endothelial growth factor (VEGF), promotion of angiogenesis, cell proliferation and tumor growth (5). Surgical resection is the mainstay of treatment of localized renal tumors, although a significant number of these patients will eventually develop recurrent or metastatic disease (2). Better understanding of ccRCC molecular background allowed for introduction of new therapeutics that target VEGF and mammalian target of rapamycin (mTOR) pathways and can be applied in the adjuvant pharmacotherapy of advanced or metastatic tumors. However, the benefits of ccRCC adjuvant therapies are still far below expectations and complete and durable responses are rare (4). At present, RCC recurrence risk stratification models are primarily based on the American Joint Committee on Cancer (AJCC) pathological tumor-node-metastasis (TNM) classification and histopathological features (2, 4, 6). To date,

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despite many efforts, there is no consensus on the routine use of molecular biomarkers to improve the surveillance protocols for follow-up of ccRCC patients after nephrectomy and predict of pharmacotherapy responsiveness (2, 4, 6).

The discoidin domain receptor 1 (DDR1) is a unique collagen receptor that belongs to the tyrosine receptor family (7-9). DDR1 exists as homodimers at the plasma membrane which act as a collagen sensors that bind to native triple helix form of collagen types I to IV and VIII (10, 11). *DDR1* expression is predominant in epithelial cells and this receptor is a key molecule that maintains cell-to-extracellular matrix (ECM) crosstalk. DDR1 influences a number of physiological processes and also plays a significant role in a number of pathological processes including tissue fibrosis and neoplastic diseases (11). *DDR1* is often deregulated in many human neoplasms including the cancers of lung, breast, liver, pancreas and ovary as well as leukemias (12, 13). The involvement of DDR1 in the processes of tissue remodeling and its central role in the development of fibrotic lesions strongly suggest that abnormal expression of this receptor may affect, besides intracellular pathways, interactions of cancer cells with the ECM components (11). Therefore, in addition to the control of adhesion, proliferation and survival of cancer cells, DDR1 was proposed as a trigger of epithelial to mesenchymal transition (EMT) and its pro-invasive activity was proved in a variety of human cancer cell lines (12, 13). However, the precise mechanisms by which DDR1 may contribute to oncogenesis have not been fully elucidated. The experimental data are often conflicting and the exact impact of DDR1 on carcinogenesis remains disputable (14, 15).

So far, the prognostic significance of DDR1 in ccRCC was investigated in only one study performed on a Chinese population (16). It was reported that high levels of DDR1 immunoreactivity correlated with the progression of ccRCC and shorter overall survival (OS) of the patients (16). Interestingly, these findings (16) are discordant with the survival analysis available at the Human Protein Atlas website, demonstrating that high expression of *DDR1* correlates with favorable prognosis in ccRCC (17). Therefore, the main purpose of the present study was to assess *DDR1* expression in the paired, tumor and non-cancerous tissue samples of 56 patients with ccRCC by reverse transcription quantitative polymerase chain reaction (RT-qPCR), western blotting (WB) and immunohistochemistry (IHC), and evaluate the prognostic significance of *DDR1*. In addition, the mechanisms that could underlay altered *DDR1* expression in ccRCC, comprising miR-199a-5p and miR-199b-5p, were investigated in ccRCC tumor tissue samples and renal cancer cell lines.

Patients and Methods

Ethical approval. This study was approved by the Bioethics Committee for Scientific Research at the University of Warmia and Mazury in Olsztyn, Poland (agreements no. 4/2010 and 44/2011).

Written informed consent as specified in the Declaration of Helsinki (1964) was obtained from each patient.

Patients and the collection of samples. Specimens were obtained from postoperative material of 56 patients with histologically confirmed ccRCC (27 men and 29 women; mean age \pm SD, 63.9 \pm 11.0; range=27-83 years) who were operated on at the Department of Oncological Surgery, Warmia and Mazury Oncological Center in Olsztyn, Poland, between March 2010 and July 2014. None of patients included in the study had a second neoplastic disease or had previously undergone chemo- or radiotherapy. Clinical and demographic data were obtained at the time of enrollment. Data on the OS were collected and the median follow-up time was 40.6 months. During this time 22/56 (39.3%) patients died. Clinical staging was based on the AJCC criteria (18). The tumor and matched macroscopically unchanged renal tissue samples were obtained from surgically resected kidneys as previously described (19). Specimens for RT-qPCR or western blotting were immediately frozen in a liquid nitrogen and stored at -80°C . Tissue fragments for routine histological evaluation and immunohistochemistry were fixed in 4% buffered formaldehyde, dehydrated in a series of ethanol solutions in ascending concentrations, cleared in xylene and processed into paraffin blocks. The tumor nuclear grading was characterized by pathologist according to the Fuhrman system (20).

***DDR1* mRNA quantification.** Total RNA was extracted and reverse transcribed as previously described (21, 22). The levels of *DDR1* transcripts in homogenates of paired tumor and renal tissue specimens were determined by RT-qPCR and normalized to *peptidyl-prolylisomerase A (PPIA)* and *TATA box binding protein (TBP)* mRNAs content. The reactions were carried out using TaqMan Fast Advanced Master Mix, and the respective TaqMan Gene Expression Assay (*DDR1*, #Hs01058430_m1; *PPIA*, #Hs99999904_m1; *TBP*, #Hs00427620_m1) in an ABI 7500/7500 Fast Real-Time PCR System (all: Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The thermocycling conditions were as follows: polymerase activation for 20 sec at 95°C , followed by 40 cycles of denaturation for 3 sec at 95°C and annealing/extension for 30 sec at 60°C . All samples were amplified in duplicates. The $\Delta\Delta\text{Cq}$ method (23) was used to determine the fold differences in *DDR1* expression between the paired samples of ccRCC and unchanged renal tissue. Based on the median *DDR1* transcript content in the tumor samples, patients were divided into two groups regarded as having 'low' or 'high' levels of *DDR1* mRNA.

***miR-199a-5p* and *miR-199b-5p* quantitation.** Total RNA was extracted and reverse transcribed as previously described (24). The levels of miR-199a-5p and miR-199b-5p in homogenates of paired tumor and normal renal tissue specimens were determined by RT-qPCR and normalized to small nucleolar RNA RNU48 content. The reactions were performed using TaqMan Universal PCR Master Mix and the respective TaqMan MicroRNA Assay (miR-199a-5p, #000498; miR-199b-5p, #000500; RNU48, #001006) in an ABI 7500/7500 Fast Real-Time PCR System (all: Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions were as follows: polymerase activation for 10 min at 95°C , followed by 40 cycles of denaturation for 15 sec at 95°C and annealing/extension for 1 min at 60°C . All samples were amplified in duplicates. The $\Delta\Delta\text{Cq}$ method (23) was used to determine the fold differences in miRs expression between the paired samples of ccRCC and

unchanged renal tissue. Based on the median miR-199a-5p or miR-199b-5p content in the tumor samples, the patients were divided into two groups regarded as having 'low' or 'high' levels of miR-199a-5p or miR-199b-5p.

DDR1 protein quantification. Protein extraction, SDS-PAGE and WB were carried out according to the modified method described previously (21). The levels of protein in homogenates of paired tumor and normal renal tissue specimens were determined using rabbit anti-human antibodies against DDR1 (diluted 1:500; #sc-532; Santa Cruz Biotechnology, Dallas, TX, USA) and actin beta (ACTB; diluted 1:100; #A2066; Sigma-Aldrich) as internal loading control. DDR1/ACTB optical density (OD) ratios were used to determine fold differences in expression between the paired samples of ccRCC and unchanged renal tissue. Based on the median DDR1 protein content in tumor specimens, patients were divided into two groups regarded as having 'low' or 'high' levels of DDR1 protein.

IHC and evaluation of immunoreactivity. DDR1 immunostaining of the tumor and non-cancerous kidney sections was carried out using the Autostainer Link48 (Dako Cytomation, Glostrup, Denmark) according to the previously described method (25). Rabbit antibody directed against human DDR1 was applied (1:300; #sc-532; Santa Cruz Biotechnology) whereas negative controls were obtained by omitting the primary antibody. The DDR1 immunostained sections were evaluated using Olympus BX53 light microscope (Olympus, Tokyo, Japan) by two independent pathologists in a blinded manner regarding the clinical data of the patients. In doubtful cases, re-evaluation was performed until a consensus was achieved. Immunoreactivity of DDR1 was assessed in the cytoplasm of cancer cells and non-transformed, normal epithelial cells of the proximal convoluted tubules (PCTs). The membrane/cytoplasmic immunoreactivity of DDR1 was evaluated according to the immunoreactive score (IRS) of Remmele and Stegner (26). The IRS scale is based on the percentage of cells exhibiting positive reaction (0 points, absence of cells with positive reaction; 1 point, 1-10%; 2 points, 11-50%; 3 points, 51-80%; 4 points, >80% cells with positive reaction) and reaction intensity (0, no reaction; 1, low intensity reaction; 2, moderate intensity reaction; 3, intense reaction). The final score is the result of multiplication of both parameters and ranges from 0 to 12 points. The nuclear DDR1 immunoreactivity was evaluated semi-quantitatively based on the percentage of cells presenting positive expression of DDR1 and encoded as follows: 0 - absence of staining, 1 point when 1-10% cells stained, 2 points when 11-50%, 3 when 51-80%, and 4 points when 81-100%. Based on their DDR1 immunostaining in membrane/cytoplasm of ccRCC cells the patients were divided into two groups regarded as 'negative' (IRS=0) or 'positive' (IRS≥1). Similarly, depending on the absence or presence of DDR1 immunoreactivity on the nuclei of ccRCC cells, patients were divided into two groups regarded as 'negative' (score=0) or 'positive' (score≥1), respectively.

Cell lines and cell culture. Human RCC cell lines Caki-1 (HTB-46), Caki-2 (HTB-47), ACHN (CRL-1611) and a control line, HK-2 (CRL-2190; immortalized cells established from normal epithelium of proximal tubule) were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Caki-1 and Caki-2 cells were maintained in McCoy's 5A medium (Sigma-Aldrich; Merck, KGaA, Darmstadt, Germany), ACHN cells in Eagle's Minimum Essential Medium (ATCC) and HK-2 cells in Dulbecco's Modified

Eagle's Medium/Nutrient Mixture F-12 Ham (Sigma-Aldrich; Merck, KGaA). All culture media were supplemented with 10% fetal bovine serum (Sigma-Aldrich; Merck, KGaA). Cell lines were incubated at 37°C in 5% CO₂.

DDR1 silencing by miR-199a-5p and miR-199b-5p. The transfections were carried out according to the manufacturer's instructions using reverse transfection protocol adjusted for a 6-well plate culture. 2×10⁵ cells were seeded into a well containing pre-activated mixture of 50 pmol mimic (miR-199a-5p, #MC10893; miR-199b-5p, #MC10553; Ambion; Thermo Fisher Scientific, Inc., Waltham, MA, USA) or inhibitory (miR-199a-5p, #MH10893; miR-199b-5p, #MH10553; Ambion; Thermo Fisher Scientific, Inc.) RNA diluted in Opti-MEM Reduced Serum Medium (Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 7 μl of Lipofectamine RNAiMAX and incubated for 16 h prior to replacement with fresh medium. Nonsense mimic or inhibitor RNA sequences were used as negative controls (mirVana miRNA mimic Negative Control #1; mirVana miRNA inhibitor Negative Control #1; Ambion, Thermo Fisher Scientific, Inc.). Three independent transfections were carried out for every cell line. All transfections within a single experiment were performed in duplicates. The cells were harvested 72 h post-transfection and assayed for DDR1 expression levels using qPCR and WB as described above.

Statistical analyses.

The statistical analyses were carried out with Prism 6.04 (Graphpad, LA Jolla, CA, USA) and Statistica 13.1 (Statsoft, Tulsa, OK, USA). The DDR1 and miRs expression levels are expressed as mean±SEM. The differences in DDR1 or miR expression levels between the paired tumor and unchanged renal tissue specimens were examined by the Wilcoxon matched-pairs test. Fisher's exact, Mann-Whitney *U*-test and Spearman's rank correlation were used to assess correlations between the patients' data and DDR1 or miR expression levels. Spearman's correlation coefficient was used to determine the relationship between the DDR1 and miR-199a-5p or miR-199b-5p expression levels. Survival curves were plotted according to the Kaplan–Meier method while the significance of differences in OS between groups of patients was evaluated by the log-rank test. The uni- and multivariate survival associations were analyzed using the Cox proportional hazards regression model. The differences in DDR1 expression levels between the cells transfected with mimics or inhibitor RNA sequences and respective control incubations were examined by Analysis of Variance followed by the Least Significant Difference post hoc test. In all performed analyses results were considered statistically significant for *p*<0.05.

Results

DDR1 expression is down-regulated in ccRCC homogenates. DDR1 transcripts were found in tumor and renal tissue samples of all patients included in the study. The tumor samples showed over 2-fold decrease in the expression levels of DDR1 mRNA as compared to the corresponding unchanged renal tissues (*p*<0.0001; Figure 1A). Western blotting analysis revealed that DDR1 protein was present in 84% (47/56) of ccRCC tumors and 95% (53/56) of normal kidney tissues samples. Consequently, the average DDR1 content in ccRCC specimens was reduced by nearly 1.4-fold

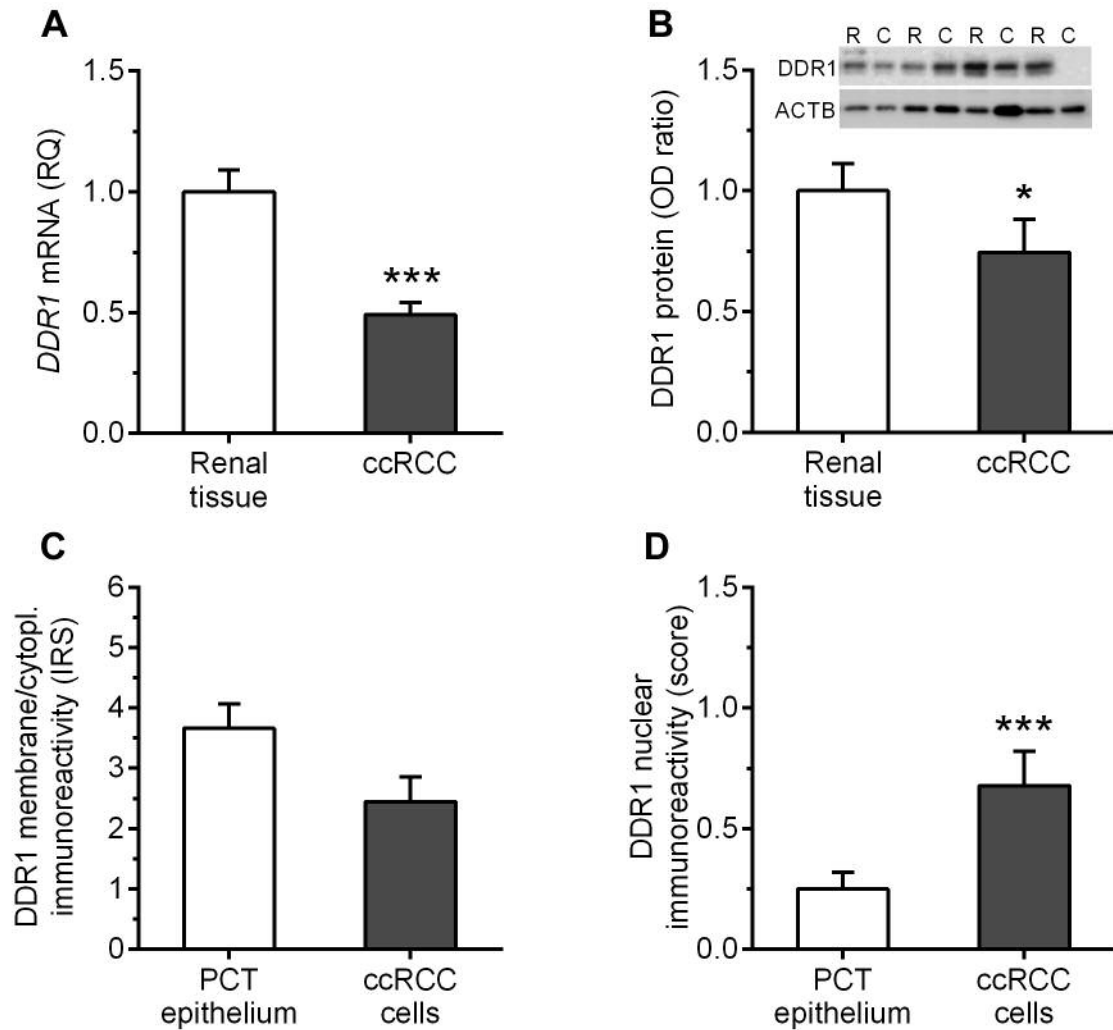


Figure 1. Average levels of discoidin domain receptor tyrosine kinase 1 (DDR1) in the clear cell renal cell carcinoma (ccRCC) specimens and corresponding renal tissue samples were determined by (A) reverse transcription quantitative polymerase chain reaction, (B) western blot and (C, D) immunohistochemistry. * $p < 0.05$; *** $p < 0.001$; RQ: Relative quantification; OD: optical density; ACTB: actin beta; IRS: immunoreactivity score; PCT: proximal convolute tubule.

as compared to the corresponding renal tissues ($p = 0.0113$; Figure 1B).

Decreased DDR1 protein levels in ccRCC are associated with higher nuclear grade. Low content of DDR1 protein in homogenates of ccRCC specimens correlated with higher Fuhrman nuclear grade ($p = 0.0142$; Table I). Furthermore, the average DDR1 protein levels were over 2-fold lower in samples derived from G3/G4 tumors compared to G2 ($p = 0.0095$).

Altered membrane/cytoplasmic and nuclear DDR1 immunohistochemical reactions in ccRCC. DDR1 immunoreactivity was observed in membrane/cytoplasmic and nuclear

compartments of PCT epithelial cells (Figure 2A) and in the cancer cells of the analyzed specimens (Figure 2B). In addition to the PCT epithelial cells, IHC revealed the presence of DDR1 protein in cells of distal convoluted tubule and glomeruli in the sections of unchanged renal tissue. Among 56 tumor sections tested, the cancer cells of 29 (52%) cases exhibited membrane/cytoplasmic DDR1 immunoreactivity. The average levels of DDR1 immunoreactivity in the membrane/cytoplasm of cancer cells and PCT epithelial cells of corresponding renal tissues were similar and did not differ significantly ($p = 0.0712$; Figure 1C). DDR1 immunoreactivity in the nuclei of cancer cells was noted in 20/56 (36%) of ccRCC specimens. IHC revealed over 2.7-fold increase in nuclear immunoreactivity of DDR1 in cancer cells as compared

Table I. Associations between demographic and clinical-pathological features of clear cell renal cell carcinoma (ccRCC) patients and expression of discoidin domain receptor tyrosine kinase 1 (DDR1) gene at the mRNA and protein levels, as determined by quantitative polymerase chain reaction and western blotting, respectively, in homogenates of ccRCC.

Parameter	Number of cases n (%)	DDR1 mRNA level in ccRCC			DDR1 protein OD level in ccRCC		
		Low n (%)	High n (%)	<i>p</i> -Value	Low n (%)	High n (%)	<i>p</i> -Value
Total	56	28 (50)	28 (50)		28 (50)	28 (50)	
Men	27 (48)	16 (59)	11 (41)	0.2847	15 (56)	12 (44)	0.5932
Women	29 (52)	12 (41)	17 (59)		13 (45)	16 (55)	
Age							
<62 years old	26 (46)	11 (42)	15 (58)	0.4218	13 (50)	13 (50)	1.0000
≥62 years old	30 (54)	17 (57)	13 (43)		15 (50)	15 (50)	
Tumor size							
<6 cm	27 (48)	16 (59)	11 (41)	0.2847	10 (37)	17 (63)	0.1078
≥6 cm	29 (52)	12 (41)	17 (59)		18 (62)	11 (38)	
T status							
T1+T2	30 (54)	15 (50)	15 (50)	1.0000	11 (37)	19 (63)	0.0598
T3	26 (46)	13 (50)	13 (50)		17 (65)	9 (35)	
Fuhrman grade							
G2	41 (73)	20 (49)	21 (51)	1.0000	16 (39)	25 (61)	0.0142
G3+G4	15 (27)	8 (53)	7 (47)		12 (80)	3 (20)	
Distant metastases							
Absent	41 (73)	21 (51)	20 (49)	1.0000	44 (18)	23 (56)	0.2270
Present	15 (27)	7 (47)	8 (53)		67 (10)	5 (33)	

Significant *p*-values (<0.05) are shown in bold.

to PCT epithelium of non-cancerous kidney sections ($p=0.0044$; Figure 1D). No correlations between DDR1 immunoreactivity and demographic or clinicopathological parameters of the patients were identified (Table II).

miR-199a-5p and *miR-199b-5p* are down-regulated in ccRCC homogenates. Both analyzed miRs were found in tumor and renal tissue samples of all patients included in the study. The average expression levels of miR-199a-5p and miR-199b-5p were nearly 2-fold reduced in ccRCC homogenates compared to corresponding renal tissues ($p=0.0002$ and $p=0.0134$; Figure 3A and B, respectively).

Expression levels of miR-199a-5p are associated with tumor growth. Higher content of miR-199a-5p correlated with bigger tumor size ($p=0.0315$; Table III). In addition, the average expression levels of miR-199a-5p were over 2-fold higher in tumors equal or bigger than 6 cm compared to those characterized by a smaller diameter ($p=0.0249$).

Low levels of DDR1 protein are associated with an unfavorable prognosis. Kaplan–Meier plots presenting the OS of patients with ccRCC classified in respect to the DDR1 expression levels are presented in Figure 4. Low levels of DDR1 protein expression were associated with shorter OS of the patients with ccRCC (median survival 37.7 months for

low level of DDR1 protein group vs. ≥82.6 months for high levels of DDR1 protein group; Figure 4B). The expression of *DDR1* transcript and DDR1 immunoreactivity did not correlate with OS of the patients (Figure 4A, C and D, respectively). No statistically significant relationships were disclosed between the expression of miR-199a-5p and miR-199b-5p and patients' outcome (Figure 4E and F, respectively). Univariate Cox proportional hazards regression revealed that low levels of DDR1 protein, bigger tumor size, higher T-status of the primary tumor, higher Fuhrman nuclear grade and presence of distant metastases are significantly associated with OS of the patients (Table IV, left panel). However, the subsequent multivariate analysis revealed that none of the parameters achieved a status of an independent prognostic factor in the analyzed group of patients with ccRCC (Table IV, right panel).

miR-199a-5p and *miR-199b-5p* correlate with expression of *DDR1* at the protein level. Significant negative correlation was found between the levels of miR-199b-5p and DDR1 protein in ccRCC specimens ($Rho=-0.3438$, $p=0.0095$; Table V). The content of miR-199a-5p showed a tendency to be negatively associated with DDR protein levels ($Rho=-0.2474$, $p=0.0660$; Table V). In addition, the specimens with higher content of DDR1 protein (>median) were characterized by over 2 and 9-fold decrease in miR-199a-5p and miR-199b-5p expression

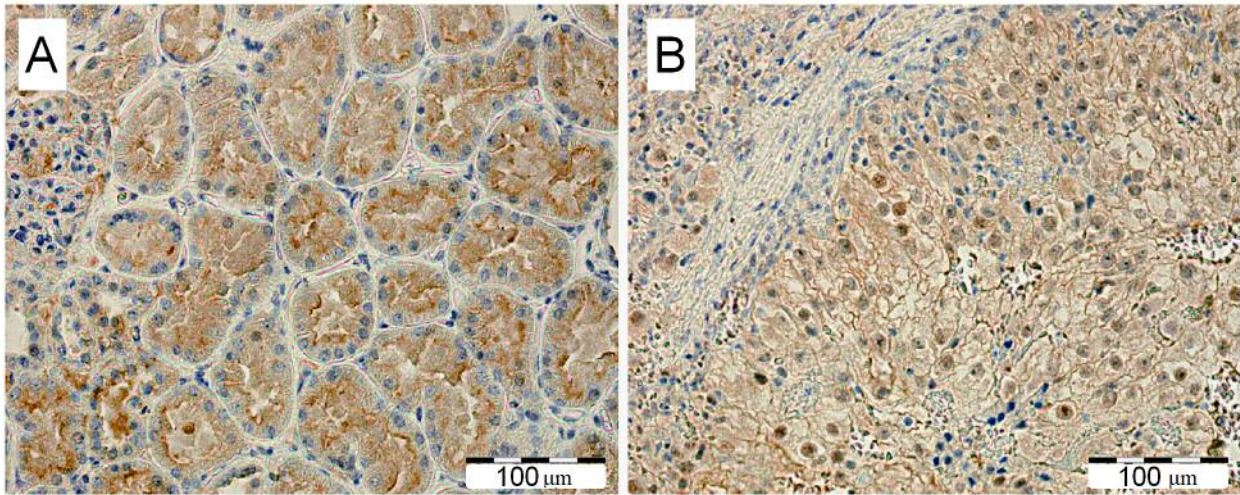


Figure 2. Evaluation of discoidin domain receptor tyrosine kinase 1 (DDR1) immunoexpression in the sections of (A) unchanged renal tissue and (B) tumor of representative patient with clear cell renal cell carcinoma was determined by immunohistochemistry. DDR1 protein was present in the membrane/cytoplasm of proximal convoluted tubule epithelium and cancer cells. Most renal tissue specimens did not reveal nuclear immunoreactivity of DDR1 while approximately one third of tumor sections exhibited weak to moderate DDR1 immunoreactivity in the nuclei of cancer cells.

Table II. Associations between demographic and clinical-pathological features of clear cell renal cell carcinoma (ccRCC) patients and immunoreactivity (IR) of discoidin domain receptor tyrosine kinase 1 (DDR1) in the membrane/cytoplasm and nuclear compartments of ccRCC cells, as determined by immunohistochemistry.

Parameter	Number of cases n (%)	DDR1 membrane/cytoplasmic in ccRCC cells			IR DDR1 nuclear IR in ccRCC cells		
		Negative n (%)	Positive n (%)	<i>p</i> -Value	Negative n (%)	Positive n (%)	<i>p</i> -Value
Total	56	27 (48)	29 (52)		36 (64)	20 (36)	
Men	27 (48)	14 (52)	13 (48)	0.7893	16 (59)	11 (41)	0.5785
Women	29 (52)	13 (45)	16 (55)		20 (69)	9 (31)	
Age							
<62 years	26 (46)	11 (42)	15 (58)	0.4357	18 (69)	8 (31)	0.5796
≥62 years	30 (54)	16 (53)	14 (47)		18 (60)	12 (40)	
Tumor size							
<6 cm	27 (48)	13 (48)	13 (52)	1.0000	16 (59)	11 (41)	0.5785
≥6 cm	29 (52)	14 (48)	15 (52)		20 (69)	9 (31)	
T status							
T1+T2	30 (54)	14 (47)	16 (53)	1.0000	19 (63)	11 (37)	1.0000
T3	26 (46)	13 (50)	13 (50)		17 (65)	9 (35)	
Fuhrman grade							
G2	41 (73)	21 (51)	20 (49)	0.5523	28 (68)	13 (32)	0.3539
G3+G4	15 (27)	6 (40)	9 (60)		8 (53)	7 (47)	
Distant metastases							
Absent	41 (73)	21 (51)	20 (49)	0.5523	26 (63)	15 (37)	1.0000
Present	15 (27)	6 (40)	9 (60)		10 (67)	5 (33)	

levels, respectively, compared to tumors with lower DDR1 protein levels ($p=0.0304$ and $p=0.0163$; Figure 5A and B, respectively). In contrast, significant positive correlations were disclosed between the levels of miR-199a-5p and miR-199b-5p and DDR1 nuclear immunoreactivity score ($Rho=0.3136$,

$p=0.0186$ and $Rho=0.3179$, $p=0.0170$, respectively; Table V). Furthermore, the average levels of miR-199b-5p in the specimens positive for nuclear DDR1 immunoexpression were approximately 7-fold increased compared to the tumors that did not reveal nuclear DDR1 immunoexpression ($p=0.0457$; Figure

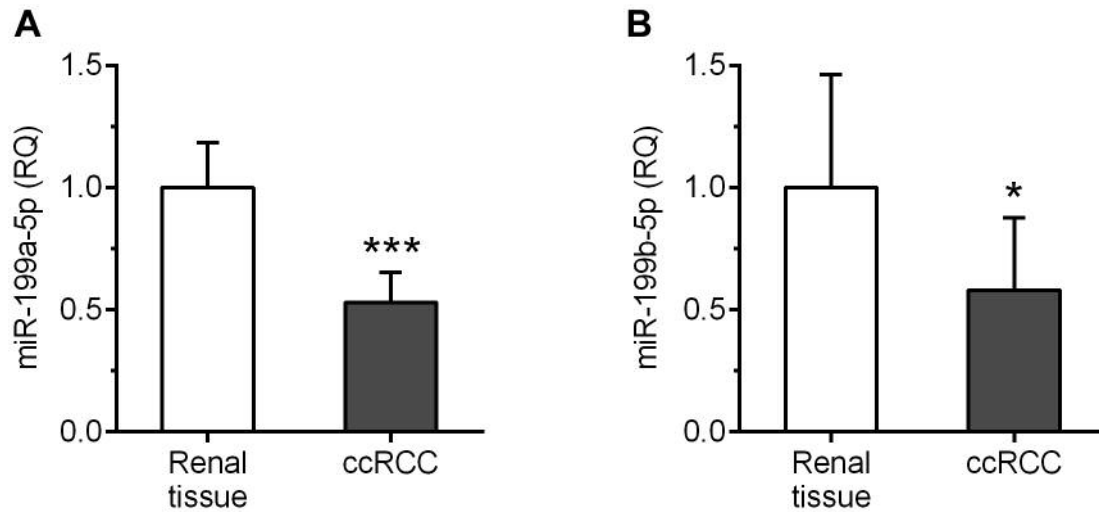


Figure 3. Average expression levels of miR-199a-5p and miR-199b-5p in the clear cell renal cell carcinoma (ccRCC) specimens and the corresponding renal tissue samples as determined by reverse transcription quantitative polymerase chain reaction. *** $p < 0.001$; * $p < 0.05$; RQ: Relative quantification.

Table III. Associations between demographic and clinical-pathological features of clear cell renal cell carcinoma (ccRCC) patients and expression levels of miR-199a-5p and miR-199b-5p in ccRCC, as determined by quantitative polymerase chain reaction in ccRCC homogenates.

Parameter	Number of cases n (%)	miR-199a-5p level in ccRCC			miR-199b-5p level in ccRCC		
		Low n (%)	High n (%)	<i>p</i> -Value	Low n (%)	High n (%)	<i>p</i> -Value
Total	56	28 (50)	28 (50)		28 (50)	28 (50)	
Men	27 (48)	15 (56)	12 (44)	0.5932	15 (56)	12 (44)	0.5932
Women	29 (52)	13 (45)	16 (55)		13 (45)	16 (55)	
Age							
<62 years old	26 (46)	14 (54)	12 (46)	0.7891	13 (50)	13 (50)	1.0000
≥62 years old	30 (54)	14 (47)	16 (53)		15 (50)	15 (50)	
Tumor size							
<6 cm	27 (48)	18 (67)	9 (33)	0.0315	17 (63)	10 (37)	0.1078
≥6 cm	29 (52)	10 (34)	19 (66)		11 (38)	18 (62)	
T status							
T1+T2	30 (54)	17 (57)	13 (43)	0.4218	17 (57)	13 (43)	0.4218
T3	26 (46)	11 (42)	15 (58)		11 (42)	15 (58)	
Fuhrman grade							
G2	41 (73)	24 (59)	17 (41)	0.0683	23 (56)	18 (44)	0.0683
G3+G4	15 (27)	4 (27)	11 (73)		5 (33)	10 (67)	
Distant metastases							
Absent	41 (73)	23 (56)	18 (44)	0.2270	23 (56)	18 (44)	0.2270
Present	15 (27)	5 (33)	10 (67)		5 (33)	10 (67)	

Significant *p*-values (<0.05) are shown in bold.

5D). Similarly, homogenates derived from nuclear-DDR1 positive tumors exhibited over 2-fold elevation in miR-199a-5p expression levels compared to tumors which lacked nuclear DDR1 immunoreactivity, however, this difference did not achieve statistical significance ($p=0.0517$; Figure 5C).

miR-199a-5p and *miR-199b-5p* regulate the expression of *DDR1* in RCC cells. The expression of *DDR1* transcript and protein was present in all analyzed cell cultures under control conditions, *i.e.* untreated or transfected by a nonsense RNA sequences (Figure 6E). Transfection with miR-199a-5p or

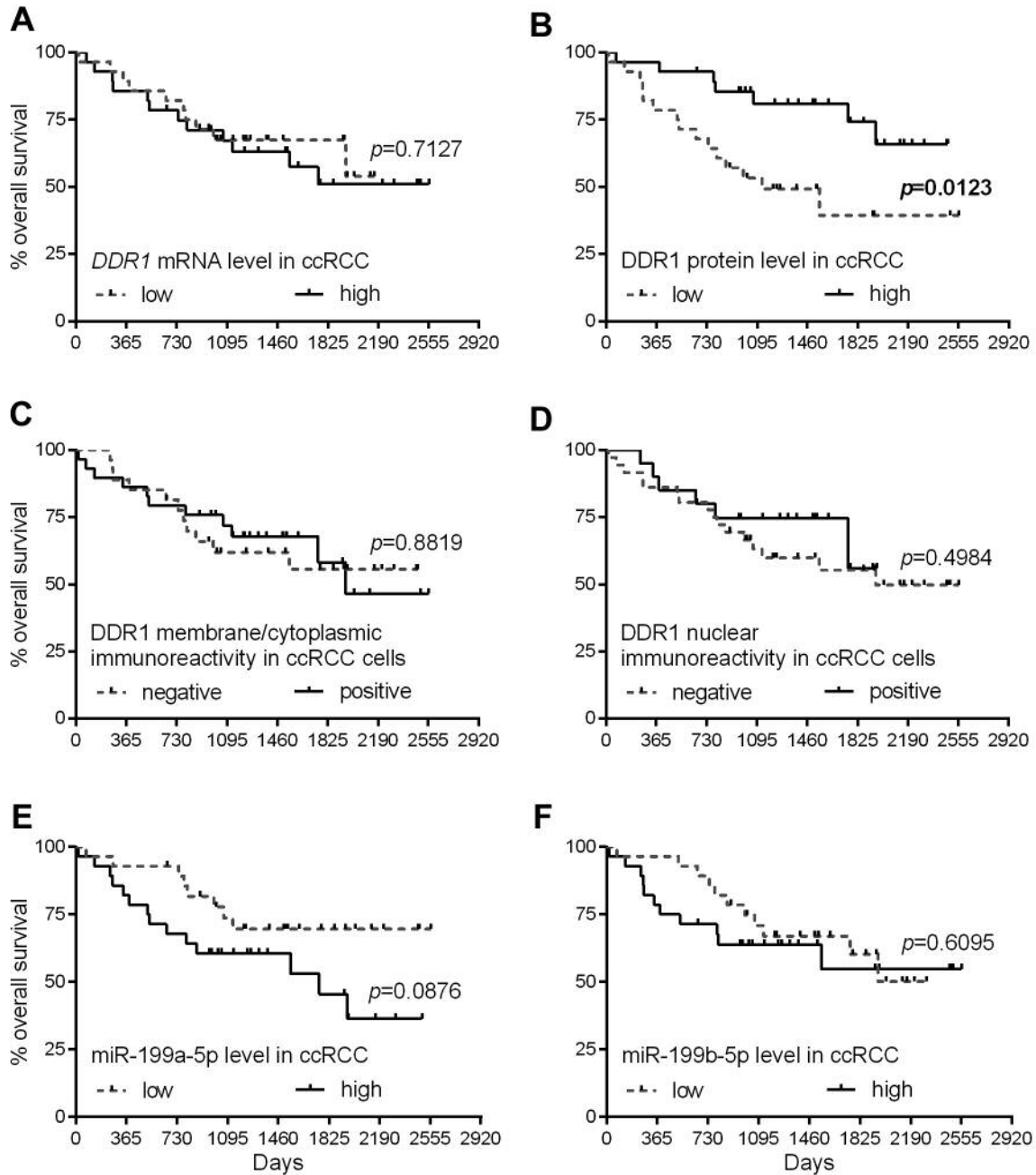


Figure 4. Kaplan–Meier diagrams of overall survival of patients with clear cell renal cell carcinoma (ccRCC) regarding discoidin domain receptor tyrosine kinase 1 (DDR1) expression at the (A) mRNA and (B) protein OD levels, DDR1 immunoreactivity in (C) membrane/cytoplasm and (D) nuclear compartments, as well as (E) miR-199a-5p and (F) miR-199b-5p content in ccRCC tissue homogenates. *p*-Values for the corresponding log-rank test are shown.

miR-199b-5p mimics significantly reduced *DDR1* mRNA in Caki-1 ($p=0.0009$ and $p<0.0001$, respectively), Caki-2 ($p<0.0001$), ACHN ($p=0.0004$ and $p=0.0065$, respectively) and HK-2 ($p<0.0001$) cell lines, ranging from 3.9 to 8.6-fold decrease compared to the cells transfected with nonsense mimic RNA sequences (Figure 6A). Furthermore, all the cell

lines transfected with miR-199a-5p or miR-199b-5p exhibited reduced levels of DDR1 protein expression that ranged from 1.8 to 11.9-fold decrease as compared to negative controls, but this repression achieved a statistical significance in Caki-2 ($p=0.0023$ and $p=0.0087$, respectively) and ACHN ($p=0.0022$ and $p=0.0039$) cells only (Figure 6B and E). In contrast,

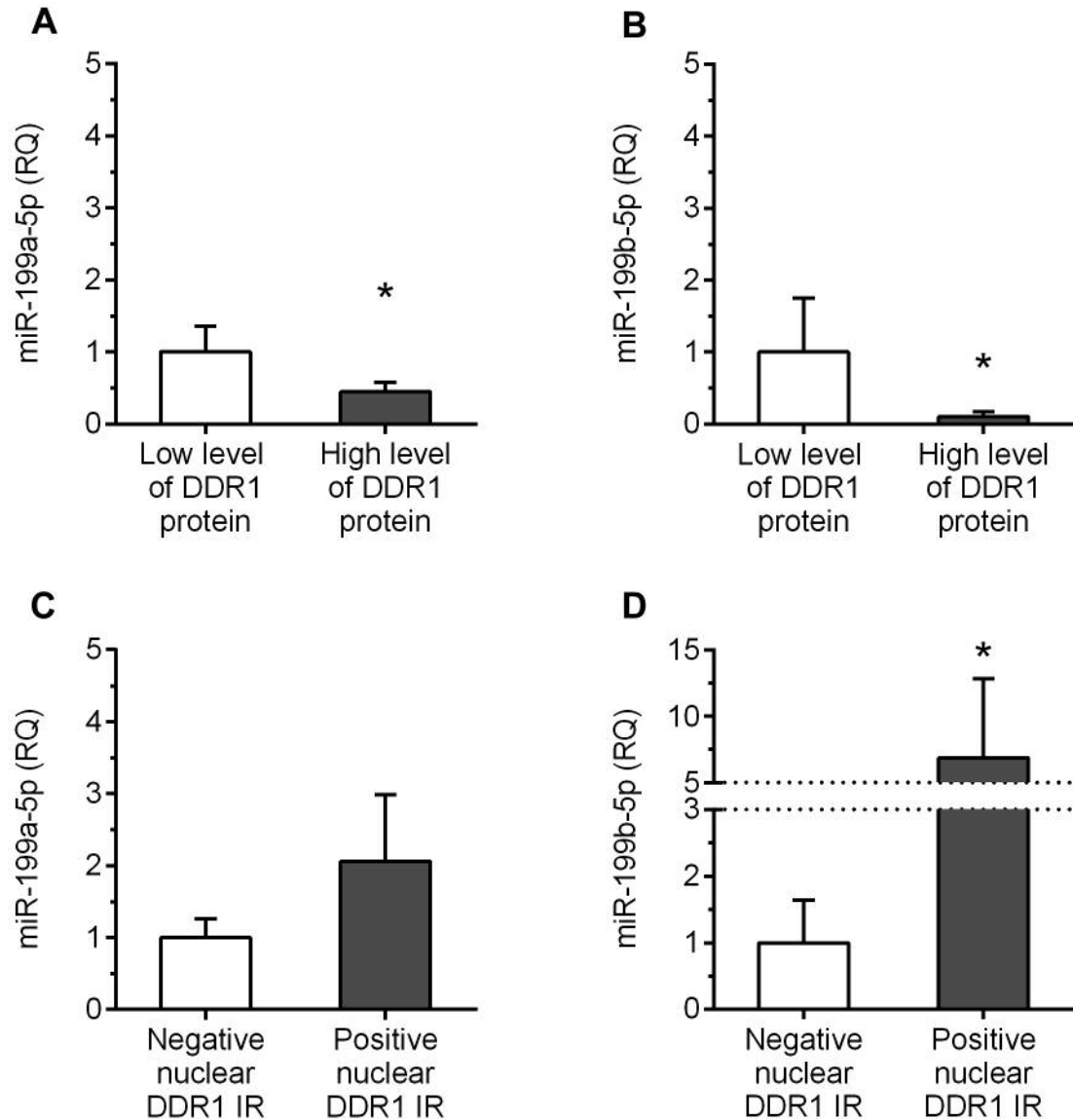


Figure 5. Correlations between the expression levels of discoidin domain receptor tyrosine kinase 1 (DDR1) protein and miR-199a-5p/miR-199b-5p in the clear cell renal cell carcinoma (ccRCC) specimens. Average expression levels of miR-199a-5p and miR-199b-5p in the tumors of patients with ccRCC in respect to (A and B) DDR1 protein OD and (C and D) nuclear DDR1 immunoreactivity. * $p < 0.05$; RQ: Relative quantification; IR: immunoreactivity.

transfection of the cell lines with miR-199a-5p or miR-199b-5p inhibitors had no significant influence on *DDR1* expression levels (Figure 6C, D and E).

Discussion

This study provides a novel insight into the significance of *DDR1* as a putative prognostic factor in ccRCC. Reduced levels of *DDR1* expression in the tumor tissues correlated with

higher nuclear grade and shorter OS of the patients. Tumor cells exhibited altered *DDR1* immunoreactivity reflected by the up-regulated nuclear expression of this protein. In addition, the putative post-transcriptional repressors of *DDR1* expression, miR-199a-5p and miR-199b-5p, were down-regulated in the tumor specimens. To the best of our knowledge the present study is the first to demonstrate that miR-199a-5p and miR-199b-5p expression levels correlate with the content of *DDR1* protein in tumor tissue samples collected from patients with

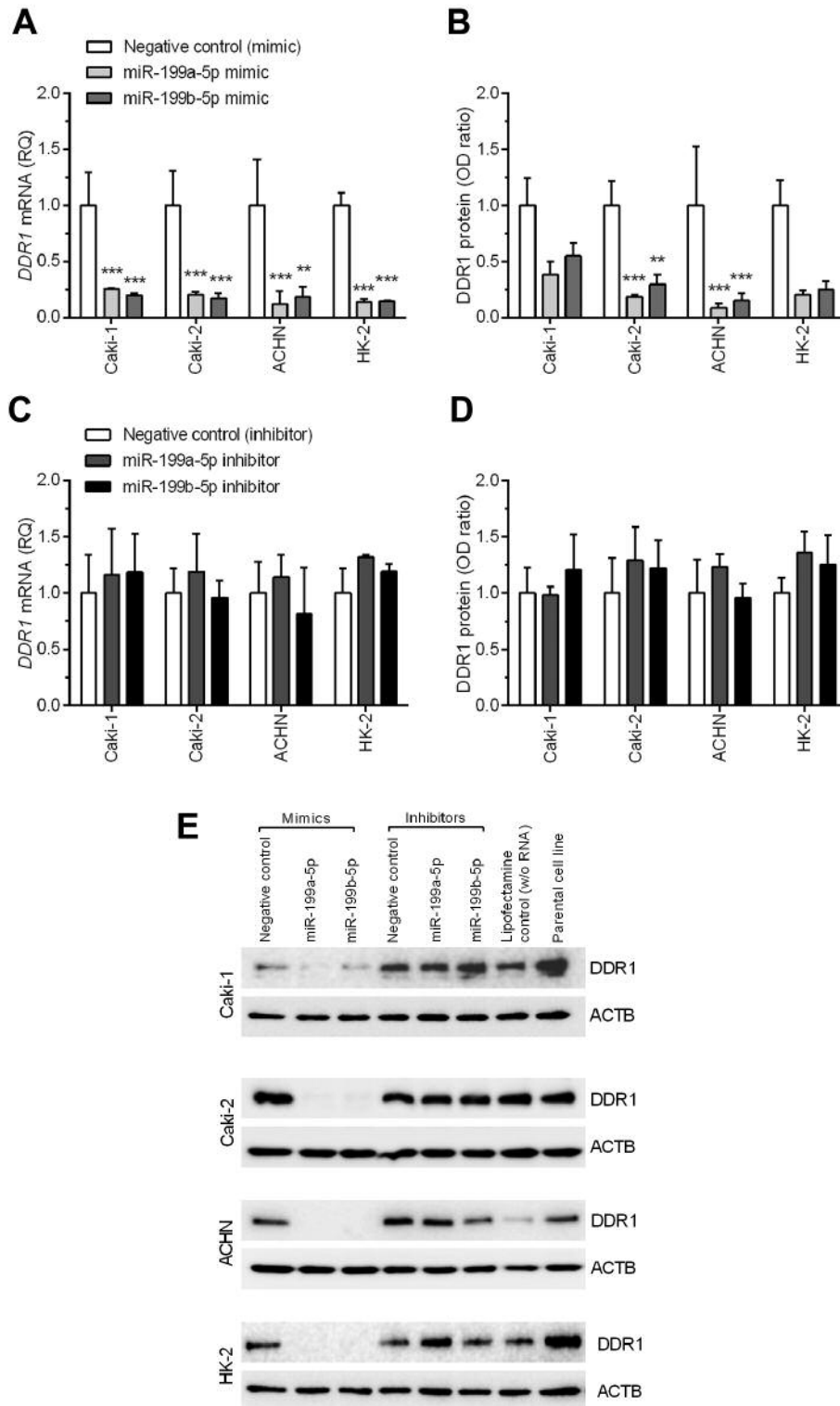


Figure 6. Effect of transfection with miR-199a-5p or miR-199b-5p mimics (A and B) or inhibitors (C and D) on discoidin domain receptor tyrosine kinase 1 (DDR1) expression in Caki-1, Caki-2, ACHN and HK-2 cells. DDR1 mRNA and protein levels were assessed by quantitative polymerase chain reaction and western blotting, respectively. (E) Representative western blots show DDR1 expression in cells transfected with miR-199a-5p or miR-199b-5p mimics, inhibitors and control RNA sequences as well as cells incubated in control conditions, in media with addition of Lipofectamine RNAiMAX only or without any additives (parental cell line). ** $p < 0.01$; *** $p < 0.001$. ACTB: Actin beta.

ccRCC. Furthermore, these findings were validated *in vitro* by the transfection of three renal cancer cell lines and one PCT-derived epithelial cell line with respective mimic RNA sequences that resulted in significant *DDR1* silencing.

In the present study, RT-qPCR, WB and IHC were used to investigate the expression of *DDR1* gene in the tumor and normal renal tissue specimens collected from patients with ccRCC treated by nephrectomy. Tissue homogenates of ccRCC tumors exhibited consistent decrease in *DDR1* expression at the mRNA and protein levels. Interestingly, IHC analysis revealed a differential pattern of *DDR1* expression in cancer cells, disclosing the two distinct localizations of this protein within the membrane/cytoplasmic and nuclear compartments. These findings are in accordance with the data available at The Human Protein Atlas website that indicate two major subcellular localizations for *DDR1* protein expression in the nucleoplasm and cell membrane as it was shown in human MCF7 cells by immunofluorescence (27). The results of our study indicate that the levels of *DDR1* protein could have a prognostic significance in ccRCC. Lower levels of *DDR1* protein were associated with higher Fuhrman grade and worse patients' outcome, although it did not achieve the status of independent prognostic factor in the analyzed cohort of patients. Our results are supported by the analysis of survival data of 528 patients with ccRCC provided by The Cancer Genome Atlas (TCGA) (28), which showed that low expression levels of *DDR1* measured by RNA-seq is an unfavorable prognostic factor in this type of renal cancer ($p < 0.0001$) (17). However, the latter and our observations are not in line with the results of an immunohistochemical study by Song *et al.* (16) who found that the immunoreactivity of *DDR1* positively correlated with the progression of ccRCC and shorter OS of 119 patients with ccRCC. Although the same antibody directed against human *DDR1* was used as in the study by Song and co-workers (16), similar correlations were not observed for membrane/cytoplasmic or nuclear *DDR1* immunoreactivity. However, it has to be noted that Song *et al.* (16) neither distinguished the *DDR1* immunoreactivity in the cellular compartments nor quantified it, and, moreover, did not show representative microphotographs.

Conflicting results suggesting that *DDR1* could either promote or suppress tumor progression, have been disclosed using other types of human cancers (15). An oncogenic role of *DDR1* was proposed on the basis of a number of studies that investigated patients' samples and/or cancer cell lines derived from the tumors that arose in the lung (29, 30), breast (31, 32), ovary (33), pancreas (34), liver (35), prostate (36), large intestine (37), stomach (38), liver (35), pancreas (34), and skin (39). Analogous observations were made in the case of glioma (40) and acute myeloid leukemia (41, 42). In most of these studies *DDR1* overexpressed and its levels correlated with the progression of disease and worse patients' outcome. Nevertheless, several studies demonstrated that *DDR1* can

have also a putative tumor suppressing role. Low levels of *DDR1* immunoreactivity were associated with worse patients' outcome in non-small cell lung cancer (43) and triple-negative breast cancer (44). Furthermore, it has been shown that loss of *DDR1* protein is associated with aggressive and metastatic potential of breast cancer cells (45). Various mechanisms of tumor suppressing activity of *DDR1* were proposed, including the involvement of *DDR1* in the up-regulation of antiapoptotic factors such as Bcl-2-interacting killer (46) and block of EMT by promotion of E-cadherin stability and E-cadherin-mediated adhesion (47) or decrease of actomyosin contractility at cell-to-cell junctions (48). Interestingly, while the epithelial ovarian cancer generally exhibited *DDR1* overexpression, its silencing by CpG methylation was concomitant with the progression of EMT as observed in the tumor samples and cell lines (49). Altogether, the results of our and other studies emphasize the controversial nature of *DDR1* in human malignancies and denote that its exact role may depend on many factors including stage of the disease and overall pathological or physiological context. Thus, the role of *DDR1* in a given cancer type is of particular interest because this receptor is considered as a putative target of novel adjuvant therapies that are in preclinical development (14), however, lack of consensus regarding the role of *DDR1* in many cancers does not support anticancer use of *DDR1* inhibitors. Indeed, the analysis of *DDR1* phosphorylation in two types of RCC showed that it may be phosphorylated in papillary, rather than in clear cell renal tumors (50). Therefore, *DDR1* inhibitors could be less, if any, beneficial in the treatment of ccRCC than it was expected on the basis of recent *in vitro* studies (16).

In the present study, the expression of miR-199a-5p and miR-199b-5p was found, in line with the previous studies, to be reduced in ccRCC (51, 52). Despite the overall down-regulation of miR-199a-5p in ccRCC, in our study, its higher (as compared to median value) expression levels correlated with bigger tumor size and tended to correlate with higher Fuhrman grade. Nonetheless, none of the analyzed miRs revealed to have a prognostic significance in our cohort of patients. In contrast, in the studies of Si *et al.* (51) and Tsukigi *et al.* (52) down-regulation of miR-199a-5p was associated with higher T status and unfavorable prognosis. However, these discrepancies may be the result of heterogeneity of RCC subtypes included in the cited studies and/or usage of RNU6 for the quantification of miR expression (51, 52), since this small nuclear RNA is not recommended as miRs' normalizer in RCCs (53).

Previous studies documented that *DDR1* expression can be regulated at the transcription level by factors such as tumor protein p53 (TP53) and zinc finger E-box binding homeobox 1 (13) or promoter CpG methylation (49). *DDR1* expression may be also controlled post-transcriptionally by the two highly similar miRs, miR-199a-5p and miR-199b-5p as found in hepatocellular carcinoma (35), acute myeloid

Table IV. Univariate and multivariate Cox regression analysis of the overall survival rates associated with different prognostic variables in patients with clear cell renal cell carcinoma.

Parameter	Univariate Cox regression			Multivariate Cox regression		
	HR	95%CI	p-Value	HR	95%CI	p-Value
<i>DDR1</i> mRNA level in ccRCC (low vs. high)	0.85	0.37-1.98	0.7130			
<i>DDR1</i> protein OD level in ccRCC (low vs. high)	3.04	1.22-7.58	0.0169	1.63	0.58-4.59	0.3526
<i>DDR1</i> membrane/cytoplasmic immunoreactivity in ccRCC cells (negative vs. positive)	1.07	0.46-2.46	0.8818			
<i>DDR1</i> nuclear immunoreactivity in ccRCC cells (negative vs. positive)	1.38	0.54-3.56	0.5003			
miR-199a-5p level in ccRCC (low vs. high)	0.48	0.20-1.14	0.0950			
miR-199b-5p level in ccRCC (low vs. high)	0.80	0.35-1.86	0.6102			
Gender (women vs. men)	0.56	0.23-1.33	0.1889			
Age (≥62 vs. <62 years old)	0.84	0.36-1.93	0.6736			
Tumor size (≥6 vs. <6 cm)	3.61	1.33-9.81	0.0117	1.17	0.27-5.00	0.8303
T status (T3 vs. T1 + T2)	4.00	1.55-10.3	0.0041	1.75	0.45-6.86	0.4198
Fuhrman grade (G3 vs. G1 + G2)	6.44	2.53-16.4	0.0001	2.36	0.66-8.43	0.1854
Distant metastases (present vs. absent)	4.62	1.90-11.3	0.0008	1.94	0.63-5.95	0.2488

Median follow-up time, 40.6 months. Significant *p*-values (<0.05) are shown in bold. HR: Hazard ratio; CI: confidence interval.

Table V. Correlations between the expression of discoidin domain receptor tyrosine kinase 1 (*DDR1*) and miR-199a-5p/miR-199b-5p levels in the clear cell renal cell carcinoma specimens.

Spearman's correlation of:	vs. miR-199a-5p		vs. miR-199b-5p	
	Rho	p-Value	Rho	p-Value
<i>DDR1</i> mRNA level	0.1806	0.1829	0.1814	0.1809
<i>DDR1</i> protein OD level	0.2474	0.0660	0.3438	0.0095
<i>DDR1</i> membrane/cytoplasmic immunoreactivity	0.0134	0.9220	0.1352	0.3205
<i>DDR1</i> nuclear immunoreactivity	0.3136	0.0186	0.3179	0.0170

Significant *p*-values (<0.05) are shown in bold.

leukemia (42), colorectal cancer (37), cutaneous squamous cell carcinoma (39) or triple-negative breast cancer (54). The expression levels of miR-199a-5p or miR-199b-5p were negatively correlated with *DDR1* protein content in the ccRCC tissues, suggesting that those two miRs may repress *DDR1* in ccRCC. To validate this hypothesis, RCC cell lines Caki-1, Caki-2, ACHN and PCT epithelium HK-2 cells were transfected with RNA sequences that mimicked or inhibited miR-199a-5p or miR-199b-5p. Transfection with 199a-5p and miR-199b-5p mimics resulted in a noticeable reduction in *DDR1* expression at the transcript and protein levels. However, no significant effect of miRs inhibitors was observed. The latter could be attributed to the low levels of

endogenous miR-199a-5p and miR-199b-5p expression in the renal cancer cell lines as it was reported previously by Tsukigi *et al.* (52).

The results of the present study disclose that *DDR1* immunoreactivity can be found in two distinct subcellular localizations within the ccRCC cells. Nuclear immunoexpression of *DDR1* was up-regulated in cancer cells and was positively correlated with the contents of miR-199a-5p and miR-199b-5p. These observations suggest that those miRs, besides their ability to repress overall *DDR1* expression, could also have an impact on the trafficking of *DDR1* protein and may promote *DDR1* nuclear localization in ccRCC cells. However, the exact, direct or indirect,

mechanisms underlying this phenomenon remain unknown. To date, five DDR1 isoforms resulting from alternative splicing, deletions and frame-shift mutations have been described (55) and it has been proposed that the role of DDR1 in human cancer can be isoform-specific (13). Herein, we showed that miR-199a-5p and miR-199b-5p target *DDR1* expression in renal cancer cells and the isoform-dependent character of this process cannot be excluded. A study carried out on mouse embryonic stem cells demonstrated that alternative transcript variants of oncologically relevant genes can be targeted by distinct miRs (56). In another study the tumor-associated miRs were shown to act by targeting the splicing machinery and as a result they potentiated the expression of cancer-specific isoforms of pyruvate kinase M1/2 glycolytic enzyme during the tumor development (57). Further studies are necessary to identify the mechanisms and relationships within the miR-199a/b-5p/DDR1 network and evaluate their prognostic and predictive relevance in ccRCC.

In summary, the first comprehensive investigation analyzing the expression of receptor tyrosine kinase *DDR1* in a cohort of patients with ccRCC is presented. Evidence is provided indicating that *DDR1* expression is altered in ccRCC and DDR1 protein levels correlate with clinicopathological parameters and OS of the patients. However, DDR1 could not serve as an independent prognostic factor in ccRCC. Our findings do not support an oncogenic role of DDR1 in this cancer type. In addition, we demonstrated that the expression levels of miR-199a-5p and miR-199b-5p are down-regulated in ccRCC. Since the altered *DDR1* expression is correlated with the levels of miR-199a-5p and miR-199b-5p, the exact role of these miRs in ccRCC should be in-depth investigated.

Conflicts of Interest

The Authors declare that they have no competing interests regarding this study.

Authors' Contributions

BEK, JK and ZK initiated and designed the study. JGo and PK collected clinical samples; JGo collected clinicopathological and survival data. JGr and PD performed immunohistochemistry and evaluated immunoreactivity scores. BEK, JK, ASJ and AEK performed cell cultures, qPCR and western blot assays. BEK performed the statistical analysis. The manuscript was drafted by BEK and JK and proof-read by ZK. JK and BEK were managing the project.

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