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# Differential expression of microRNA501-5p affects the aggressiveness of clear cell renal carcinoma

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ABSTRACT

**Renal cell carcinoma is a common neoplasia of the adult kidney that accounts for about 3% of adult malignancies. Clear cell renal carcinoma is the most frequent subtype of kidney cancer and 20–40% of patients develop metastases. The absence of appropriate biomarkers complicates diagnosis and prognosis of this disease. In this regard, small noncoding RNAs (microRNAs), which are mutated in several neoplastic diseases including kidney carcinoma, may be optimal candidates as biomarkers for diagnosis and prognosis of this kind of cancer. Here we show that patients with clear cell kidney carcinoma that express low levels of miR501-5p exhibited a good prognosis compared with patients with unchanged or high levels of this microRNA. Consistently, in kidney carcinoma cells the down-regulation of miR501-5p induced an increased caspase-3 activity, p53 expression as well as decreased mTOR activation, leading to stimulation of the apoptotic pathway. Conversely, miR501-5p upregulation enhanced the activity of mTOR and promoted both cell proliferation and survival. These biological processes occurred through p53 inactivation by proteasome degradation in a mechanism involving MDM2-mediated p53 ubiquitination. Our results support a role for miR501-5p in balancing apoptosis and cell survival in clear cell renal carcinoma. In particular, the downregulation of microRNA501-5p promotes a good prognosis, while its upregulation contributes to a poor prognosis, in particular, if associated with p53 and MDM2 overexpression and mTOR activation. Thus, the expression of miR501-5p is a possible biomarker for the prognosis of clear cell renal carcinoma.**



## 1. Introduction

Renal cell carcinoma (RCC), the most common kidney malignancy with the highest mortality rate of urinary cancers, shows an incidence of 2–4% in the USA population that is steadily rising in the last decades [9,33]. Clear cell renal carcinoma (ccRCC) accounts for about 70% of RCC cases and, approximately one third of these patients commonly develop metastases [10]. Tumor recurrence manifestations are mainly due to the absence of adjuvant therapy in clinical routine, because RCC is both chemotherapy and radiotherapy resistant [31]. Moreover, the lack of specific prognostic biomarkers prevents the development of specific therapy. Thus, the discovery of new biomarkers for the prediction of early metastasis development after nephrectomy could be useful for the clinic and therapeutic follow up of RCC patients [31,35]. Currently, an innovative approach for molecular characterization of tumors is based on the analysis of microRNAs expression [31]. MicroRNAs (miRs) are a class of short non-coding RNAs that regulate gene expression at post-transcriptional level by mRNA

*Abbreviations:* ccRCC, clear cell renal cell carcinoma; MDM2, mouse double minute 2 homolog; mTOR, mammalian target of rapamycin; pRCC, papillary renal cell carcinoma

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degradation or translational inhibition [31]. Recently, miRs have been reported as potential biomarkers for RCC, because they are able to improve the identification of tumor subtypes [29]. For instance, the overexpression of miR-32 is associated with poor outcome of RCC patients [29]. However, in other studies the analysis of circulating serum levels of miR-26a-2*, miR-191, miR-337-3p and miR-378 is unlikely to provide helpful diagnostic/prognostic information in RCC patients [16]. Therefore, the current role of miRs as biomarkers in renal carcinoma is still obscure and deserves further investigation.

In the RCC pathogenesis it is known that mTOR, a protein kinase essential for cell growth, has an important role, because increased mTORC1 activation by the HIF2α pathway is particularly associated to renal cancer progression [14]. Moreover, genetic mutations that enhance mTOR signaling cause increased incidence of metastatic events [19]. Consistently, inhibitors of mTOR have shown to improve survival in poor-prognosis advanced RCC [19,15]. Therefore, also mTOR signaling should be carefully investigated in kidney tumors.

The findings here reported show that low expression level of a specific miR, the miR501-5p, does not affect tumor grading, but is associated with a good prognosis in ccRCC patients. Moreover, the downregulation of miR501-5p induces apoptosis through p53 activation and mTOR inhibition. Consistently, the overexpression of miR501-5p in kidney carcinoma KJ29 cells stimulates cell growth and survival by activation of mTOR kinase and inhibition of p53 via MDM2-associated ubiquitination. The good prognosis in ccRCC patients associated with lower expression of miR501-5p makes this miR a candidate biomarker for clear cell renal carcinoma.

## 2. Material and methods

### 2.1. Reagents

Media and plastic material for cell culture were purchased from EuroClone (Italy). Rabbit polyclonal anti-mTOR, anti-P-mTOR, and anti-Ubiquitin antibodies were obtained from Cell Signaling Technologies (EuroClone, Italy). Anti-p53, anti-MDM2 and anti-β-Actin were acquired from Santa Cruz Technologies (Italy). Enhanced chemiluminescent substrates for western blotting and HRP-conjugated goat anti-rabbit and anti-mouse antibodies were purchased from EuroClone (Italy). Rapamycin was obtained from Sigma–Aldrich (Italy). AntagomiR anti-microRNA501-5p was purchased by Ambion (Life Technologies, Italy), while the recombinant plasmid (PL501) expressing both hsa-miR501-5p and green fluorescence protein (GFP) sequences was produced by OriGene Technologies (Tema Ricerca, Italy).

### 2.2. Collection of sample tissues and kidney cell lines

Renal fresh frozen normal and tumor kidney tissues were collected after surgical resection by the Urology Unit of Sant'Anna Hospital, Ferrara. Paraffin-embedded tissues derived from normal parenchyma as well as from kidney renal carcinoma were obtained from Pathological Anatomy of Ferrara University (Italy). 4/5 human normal as well as 9.7 and 9.12 ADPKD cystic kidney epithelial cells were produced by other laboratories [20], while KJ29 non papillary kidney cancer cells were established and characterized in our laboratory [5]. Cytogenetic analysis of KJ29 cells showed 50 chromosomes including rearrangements of chromosomes 1 and 3. The antigenic phenotype is characterized by co-expression of cytokeratin and vimentin as well as the expression of urothelium differentiation markers, with low levels of class II and the absence of class I MHC antigens. The cell line which is highly tumorigenic in athymic mice displays expression of erbB-2, c-met and Ha-ras oncogenes [5]. Caki-2 cells were kindly provided from Dr. Gagliano (University of Milan, Italy). The study protocol was in line with the 1975 Declaration of Helsinki.

### 2.3. Analysis of microRNA expression by microarray technology

This analysis was performed as described by Negrini et al. [24]. Briefly, after total RNA isolation from cells by TRIZOL reagent (Invitrogen SRL, Italy) as already described [21], RNAs were hybridized on an Agilent Human miRNA microarray (G4470B, Agilent Technologies), which consists of 60-mer DNA probes synthesized in situ and contains 15,000 features specific for 723 human miRNAs. Microarray results were analyzed using the GeneSpring GX software (Agilent Technologies). Differentially expressed miRNA were selected as having a 1.5-fold expression difference between control cells (4/5) and ADPKD cystic cells (9.7 and 9.12). $P$ values <0.05 calculated by Anova test was considered statistically significant. Differentially expressed miRNAs were used for cluster analysis of samples, using the Pearson correlation as a measure of similarity.

### 2.4. RNA extraction, cDNA synthesis and RT-PCR analysis

From fresh frozen tissues and cell pellets, total RNA was extracted by TRIZOL method. RNA extraction from paraffin-embedded tissues was performed by the RecoverAll Total Nucleic Acid Isolation Kit (Ambion, Italy). Four slices from 20 μm in size were treated with 1 mL of xylene 100% and heated for 3 min a 50 °C to melt the paraffin, and the solution was centrifuged at 12000×$g$ for 2 min. After xylene discharge, the pellet was washed twice with 1 mL 100% ethanol and dried in a centrifugal vacuum at 40 °C for 20 min. Next, RNA from samples were obtained following the manufacturer's protocol. Synthesis of cDNA was performed by the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Italy), using RNU6B and hsa-miR501-5p specific primers. Real Time quantitative PCR was carried out by TaqMan method using the ABI Prism 7700 Sequencer Detector system (Applied Biosystems, Italy). The small nuclear U6B was used as endogenous control (reference gene) for the normalization of samples, while the expression level of microRNA501-5p between normal parenchyma and cancer tissue was calculated by delta-delta Ct method as previously described [4].

### 2.5. Cell transfection

The transfection of cells with 30 nM of antagomiR sequences, specific for microRNA501-5p or with 0.75 μg/mL of PL501 was performed by the TurboFect Transfection Reagent (Fermentas, Italy). 200,000, 30,000 or 5000 cells/well were plated in 6-, 24- or 96-well plates respectively, for 24 h in DMEM/F12 medium supplemented with 10% FBS. Next, cells were transiently transfected in DMEM/F12 medium supplemented with 0.4% BSA for at least 6 h following the manufacturer's method. After transfection cells were cultured for 24 h in DMEM/F12 medium in presence of 0.4% BSA for the analysis of apoptosis or for 24, 48 and 72 h in 1% FBS for the evaluation of cell growth.

### 2.6. Analysis of cell cycle, proliferation and survival

For cell cycle analysis, 200,000 cells/well were plated in six well plates, starved for 24 h in medium with 0.4% BSA, transfected with a specific antagomiR and cultured for additional 24 h in medium containing 1% FBS. Then, cells were collected, centrifuged, washed in PBS, stained with a propidium iodide solution and analyzed by flow cytometry using the FACSCalibur Becton Dickinson Immunocytometry System [1]. For cell proliferation analysis, 5000 cells/well were plated in 96 well plates, starved for 24 h in DMEM/F12

0.4% BSA and transfected with PL501 or with an irrelevant plasmid as described above. Cells were cultured for further 24, 48 and 72 h in DMEM/F12 1% FBS in presence or absence of rapamycin (500 nM), and the proliferation was calculated by direct cell counting after trypan blue staining, using a Burker chamber [3]. Cell survival was measured by the CellTiter cell proliferation assay (Promega, Italy), a method based on the quantitation of a colored compound released by cells in culture medium. Color intensity, directly proportional to the living cells, was detected by a plate reader recording the absorbance at 490 nm [8].

### 2.7. Western blotting and p53-ubiquitination analysis

Fresh tissues were lysed in 1% Triton X-100 solution containing a cocktail of protease inhibitors and processed for immunoblots as described previously [2]. Quantitative phosphorylation and protein levels were calculated as the ratio between phosphorylated and un-phosphorylated protein, and among the protein of interest and β-Actin, respectively [3]. For detection of p53 conjugated with endogenous ubiquitin, transfected cells ($1 \times 10^6$) were lysed in TBS buffer (50 mM Tris, pH 8.0, 150 mM NaCl) containing 1% SDS, 1 mM DTT and protease inhibitor cocktail enriched with proteasome and ubiquitin peptidase inhibitors MG132 (10 μm) and *N*-ethylmaleimide (10 mM), respectively. Next, protein lysate was boiled and diluted 10 times in TBS with protease inhibitors, and p53 was immunoprecipitated with a monoclonal anti-p53 antibody (Santa Cruz, Italy). After washing, immunoprecipitates were analyzed by western blotting with both monoclonal anti-p53 and polyclonal anti-Ubiquitin antibodies [26].

### 2.8. Apoptosis detection

Apoptosis was evaluated by Hoechst and caspase-3 assay methods. For apoptotic nuclei detection, 200,000 cells were plated on 24 mm coverslips, starved 24 h in DMEM/F12 0.4% BSA and transfected. After transfection, cells were fixed, permeabilized, and stained with Hoechst 33,258 (10 mg/mL) in the dark [8]. Images were acquired at 40× magnification through a Zeiss Axiovert 200 fluorescence microscope, equipped with a back-illuminated CCD camera (Roper Scientific, Tucson, USA). Caspase-3 activity was evaluated using EnzChek® caspase-3 Assay Kit (Invitrogen, Italy). After transfection, cells were cultured with DMEM/F12 0.4% BSA for 24 h, lysed for 30 min and centrifuged according with the manufacturer's instructions. 50 μL aliquots of supernatant were incubated with 50 μL of 2× substrate working solution containing 5 mM Z-DEVD-R110 and fluorescence was measured every minute for 90 min at 520 nm by the fluorimeter VICTOR3 1420 Multilabel Counter (PerkinElmer, Italy). Values were normalized to the protein content detected by Bradford method [8].

### 2.9. Cell imaging

For GFP detection, cells were seeded on 24 mm coverslips, transfected with PL501 and washed three times with PBS buffer before GFP recording. GFP fluorescence signal was captured by a fluorescence microscope as described above. For immunofluorescence analysis, cells were cultured on 24 mm coverslips in DMEM/F12 0.4% BSA for 24 h and transfected with antagomiR. After transfection, cells were fixed in 0.4% formalin, washed twice in PBS buffer and permeabilized in a PBS solution containing 0.2% Triton X-100. Then, cells were washed twice with PBS buffer and incubated with an anti-p53 monoclonal antibody solution. After washing, cells were treated with a secondary anti-mouse rhodamine-conjugated antibody and washed three times in PBS buffer [4]. Immunofluorescent p53 protein and DAPI-stained nuclei were detected by a fluorescence microscope as previously described.

### 2.10. Statistical analysis

Analysis of data was performed using Student's *t* test (unpaired analysis). Patients survival was measured by Kaplan–Meier estimator and by multivariate analysis with Cox regression. Statistical analysis of microarray data was performed by Anova test. Differences are considered significant at a value of $p < 0.05$. All data are reported as mean ± SD (standard deviation) of at least three independent experiments.

## 3. Results

### 3.1. miR501-5p expression in kidney carcinomas

Microarray data performed in 4/5 normal renal epithelial cells as well as in 9.7 and 9.12 cystic kidney cell lines [20] derived from patients with autosomal dominant polycystic kidney disease (ADPKD), an hyperproliferative pathology, showed statistically significant changes in the expression of six different microRNAs (Fig. 1A). In particular the expression of microRNA501-5p was found 3 times higher in 9.7 and 9.12 cystic cells than in 4/5 control cells (Table in Fig. 1A). By Real Time RT-PCR analysis, an overall higher expression of miR501-5p was also observed in 63 clear cell kidney carcinoma tissues compared with their paired normal renal parenchyma (Fig. 1B), while the other miRNAs did not show significant changes (data not shown). On the contrary, in 19 papillary kidney carcinoma tissues the expression of this miR was markedly lower than in normal tissue (Fig. 1C). As shown in Fig. 1D, ccRCC samples showed a variable distribution of miR501-5p expression that could be associated with age, because this correlation is very close to significance. No association between miR-501-5p expression and tumor grade was observed (Fig. 1E). Based on this variable distribution, a possible link between miR501-5p expression and patient outcome in ccRCC subjects was then investigated. Follow up analysis (at least 5 years) in 45 ccRCC samples (Table 1) showed that patients with low expression of this miR (<1; tumor vs normal tissue) exhibited a good prognosis. Conversely, 66% of patients with higher levels of miR501-5p (⩾1; tumor vs normal tissue) showed a significant higher probability of developing metastases than subjects with low expression of this miR; (miR501-5p expression was 5.34 ± 7.31 in metastatic samples vs 1.73 ± 2.47 in not-metastatic ccRCC patients; $n = 45$, $p < 0.05$). This finding was also confirmed by Kaplan–Meier plot that indicates a significant lower frequency of metastases for patients which express low levels of microRNA501-5p compared with subjects with high expression of this microRNA (Fig. 1F). However, Kaplan–Meier chart did not show significant association with the overall survival (data not shown). In addition, a multivariable Cox regression analysis was performed on ccRCC patients stratified for age in two groups (<65 years ($n = 10$) defined "young" and >65 ($n = 27$) called "old"). This analysis indicates that expression of miR501-5p showed a significant and independent association with time to metastasis (HR 1.103; 95%CI 1.004–1.211, $p < 0.05$).

These data suggest a possible role of miR501-5p in the development of clear cell renal carcinoma.

### 3.2. The upregulation of miR501-5p stimulates cell growth and survival

Since TSC1, an inhibitor component of mTOR complex [37], is a target of miR501-5p (Table 2), the upregulation of this miR should activate the mTOR kinase. To confirm this assumption, we have overexpressed the miR501-5p in two different kidney carcinoma cell lines (KJ29 and Caki-2), by transfection with a plasmid (PL501) containing specific miR501-5p sequences. In basal conditions (Fig. 2A), miR501-5p levels were higher in KJ29 cells than

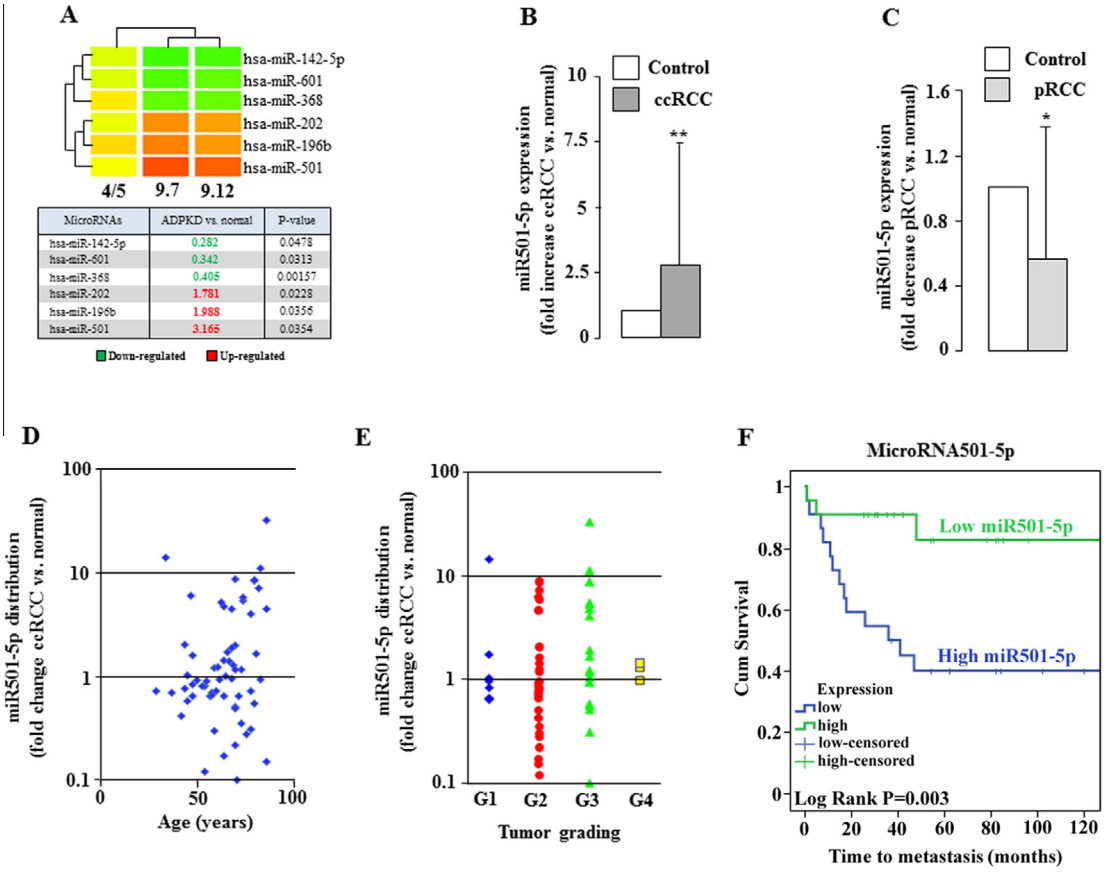


**Fig. 1.** miR501-5p expression in kidney carcinomas. (A) Map of cluster analysis in normal kidney epithelial cells (4/5) and in two different ADPKD cystic cell lines (9.7 and 9.12) performed by microarray technology showed changes in the expression of six different microRNAs. Expression values and statistical significance of different miRNAs (calculated as fold change ratio between ADPKD cystic cells vs normal kidney cells), were indicated in the table. Levels of miR501-5p were analyzed by Real Time RT-PCR in 63 pairs of ccRCC and normal kidney tissues (B) and, in 19 pairs of pRCC and normal kidney parenchyma (C) of patients from Northern Italy. Data from fresh frozen and paraffin embedded tissues were pooled together because miR501-5p values obtained from both samples were comparable. Real Time RT-PCR values, expressed as ratio between cancer tissues and normal kidney parenchyma, were calculated by delta-delta Ct method. In ccRCC tissues the expression of miR501-5p was 2.76 ± 4.86-fold increased compared with control tissues (**$p < 0.01$). In pRCC tissues miR501-5p expression with respect to normal tissue was 0.56 ± 0.87 (*$p < 0.05$). (D) Pearson analysis showed a weakly positive, albeit not significant, correlation between miR501-5p expression and age of patients ($p = 0.0519$). (E) No association between miR501-5p expression and tumor grade, as evaluated by *t*-test, was observed. (F) Kaplan–Meier plot indicated a lower frequency of metastatic events in ccRCC patients with low expression of miR501-5p compared with those expressing greater levels of this microRNA. Kaplan–Meier estimator was performed by using as cut off the median expression of miR501-5p; median = 1.24 (tumors vs normal tissue). Statistical analysis of miR501-5p expression was performed by *t*-test in three experiments in duplicate. Values are shown as mean ± standard deviation. Control = normal kidney parenchyma (1× expression level); ccRCC = clear cell renal cell carcinoma; pRCC = papillary renal cell carcinoma; G = tumor grading (Fuhrman scale).

in Caki-2 cells. The transfection with 0.75 and 1.5 μg/mL of PL501 increased the expression of miR501-5p in both KJ29 and Caki-2 kidney carcinoma cells (Fig. 2B and 2C). Because cell detachment might occur after transfection with 1.5 μg/mL of PL501, we performed further experiments with 0.75 μg/mL or less. As we speculated, the increased expression of miR501-5p increased the phosphorylation of mTOR kinase in PL501-transfected KJ29 cells (Fig. 2D). Consistently, similar data were obtained by using Caki-2 cells (Fig. 2E). Since mTOR signaling stimulates several biological processes including cell proliferation and survival [34], the upregulation of miR501-5p in both KJ29 and Caki-2 PL501-transfected cells was associated with significantly increased cell proliferation and survival (Fig. 2F–H). Transfection with lower doses of PL501 (<0.75 μg/mL) still enhanced cell growth and survival, even if with milder effects (data not shown). Interestingly, mTOR signaling is known to stimulate MDM2 expression [23,13], an inhibitor of the oncosuppressor p53 in metastatic kidney cancer [25]. Therefore, the upregulation of miR501-5p that stimulates mTOR, should also affect the expression of MDM2 and p53 proteins. As expected, the transfection of KJ29 and Caki-2 cells with PL501 not only caused an enhanced expression of MDM2, but also induced a significant reduction of p53 levels (Fig. 3A and B). MDM2 is an E3 ubiquitin-protein ligase that on interacting with p53 causes polyubiquitination and degradation of MDM2/p53 complex by proteasome activation [30]. Consistently, in KJ29 cells overexpressing miR501-5p sequences an increased p53 protein ubiquitination was detected (Fig. 3C). Pharmacological inhibition of mTOR activity by rapamycin treatment of KJ29 cells transfected with PL501 (Fig. 3D) raised the levels of p53 protein by inhibition of MDM2 expression (Fig. 3D). Moreover, the treatment with 500 nM rapamycin in PL501-transfected cells also caused a significant reduction of cell proliferation and survival compared with untreated cells (Fig. 3E–G). These data suggest that miR501-5p, likely through TSC1 mRNA degradation, stimulates cell proliferation and survival by degradation of p53 in a mechanism involving the activation of mTOR kinase and MDM2 expression in kidney carcinoma cells.

### *3.3. MicroRNA501-5p downregulation induces cell death by activation of apoptotic machinery*

Since the upregulation of miR501-5p stimulates cell proliferation and survival, it is presumable that its reduction may be asso-

**Table 1**
Follow-up analysis shows that miR501-5p downregulation is associated with a good prognosis in clear cell renal carcinoma patients.

| Samples | Age | Grading | Metastasis | miR501-5p | Therapy | Outcome |
|---|---|---|---|---|---|---|
| ccRCC1 | 89 | G2 | No | 0.15 | No | Dead |
| ccRCC2 | 85 | G2 | No | 0.17 | No | Alive |
| ccRCC3 | 61 | G2 | No | 0.2 | No | Alive |
| ccRCC4 | 70 | G2 | No | 0.22 | No | Alive |
| ccRCC5 | 76 | G2 | No | 0.28 | No | Alive |
| ccRCC6 | 60 | G2 | No | 0.3 | No | Alive |
| ccRCC7 | 78 | G2 | No | 0.31 | No | Alive |
| ccRCC8 | 73 | G1 | No | 0.32 | No | Alive |
| ccRCC9 | 76 | G2 | No | 0.35 | No | Alive |
| ccRCC10 | 73 | G3 | No | 0.51 | No | Alive |
| ccRCC11 | 46 | G3 | No | 0.58 | No | Alive |
| ccRCC12 | 54 | G2 | No | 0.81 | No | Alive |
| ccRCC13 | 56 | G1 | No | 0.82 | No | Alive |
| ccRCC14 | 82 | G2 | No | 0.82 | No | Alive |
| ccRCC15 | 76 | G4 | Lung | 0.97 | ND | Dead |
| ccRCC16 | 67 | G2 | No | 1.02 | No | Alive |
| ccRCC17 | 53 | G3 | Brain/lung | 1.03 | Anti-VEGFR | Dead |
| ccRCC18 | 86 | G2 | Lung | 1.05 | Anti-VEGFR | Alive |
| ccRCC19 | 68 | G1 | No | 1.08 | No | Alive |
| ccRCC20 | 73 | G3 | No | 1.17 | No | Alive |
| ccRCC21 | 59 | G3 | Kidney | 1.22 | ND | Alive |
| ccRCC22 | 62 | G2 | No | 1.24 | No | Alive |
| ccRCC23 | 69 | G4 | Lung/liver | 1.3 | ND | Dead |
| ccRCC24 | 79 | G3 | Lung/liver | 1.31 | No | Alive |
| ccRCC25 | 70 | G2 | Lung | 1.41 | Anti-VEGFR | Alive |
| ccRCC26 | 64 | G4 | Bones/liver | 1.43 | No | Dead |
| ccRCC27 | 60 | G3 | Liver | 1.48 | Anti-VEGFR | Alive |
| ccRCC28 | 48 | G2 | No | 1.62 | No | Alive |
| ccRCC29 | 71 | G3 | Bones | 1.86 | ND | Dead |
| ccRCC30 | 73 | G2 | No | 2.03 | No | Alive |
| ccRCC31 | 44 | G2 | No | 2.06 | No | Alive |
| ccRCC32 | 78 | G3 | No | 4.06 | No | Alive |
| ccRCC33 | 86 | G2 | Larynx/urothelium | 4.53 | ND | Dead |
| ccRCC34 | 69 | G2 | Bones | 4.58 | ND | Alive |
| ccRCC35 | 67 | G3 | Brain/liver/lung | 4.77 | Anti VEGFR | Dead |
| ccRCC36 | 66 | G3 | Lung/liver | 5.24 | Anti-VEGFR | Alive |
| ccRCC37 | 74 | G3 | Liver | 5.49 | ND | Dead |
| ccRCC38 | 75 | G2 | No | 5.86 | No | Alive |
| ccRCC39 | 50 | G2 | Lung/bones | 6.16 | Anti-VEGFR | Alive |
| ccRCC40 | 83 | G2 | Lymph nodes | 7.17 | No | Dead |
| ccRCC41 | 81 | G2 | No | 8.59 | No | Dead |
| ccRCC42 | 83 | G3 | Lung/bones | 8.7 | Anti-VEGFR | Dead |
| ccRCC43 | 73 | G2 | No | 8.82 | No | Alive |
| ccRCC44 | 35 | G1 | Lung | 14.36 | ND | Alive |
| ccRCC45 | 87 | G3 | Bones | 32.81 | ND | Dead |

For follow-up studies, 45 ccRCC patients with at least 5 years from surgical resection were considered. Biological and clinical parameters matched with miR501-5p expression are indicated.

ciated with the inhibition of cell growth and possible activation of apoptosis. To evaluate this hypothesis the expression of miR501-5p in KJ29 and Caki-2 kidney carcinoma cells was reduced by transfection with a specific antagomiR. As expected, this transfection caused a marked reduction of miR501-5p expression as compared to cells transfected with scramble sequences (Fig. 4A). Moreover, the decreased expression of miR501-5p in these cells induced a significant reduction of cell survival (Fig. 4B and C) as well as a cell accumulation in $G_0/G_1$ phase of cell cycle in KJ29 cells treated with antagomiR, as compared to same cells transfected with scramble sequences (Fig. 4D). Furthermore, an activation of caspase-3, the last enzyme of caspase cascade triggering the apoptotic pathway, was also observed (Fig. 4E). Consistently, in antagomiR-transfected KJ29 cells the formation of apoptotic nuclei, not detected in control cells, was shown (Fig. 5A). Therefore, the decrease of miR501-5p expression stimulates apoptotic activity in kidney carcinoma cells. As previously shown, the upregulation of miR501-5p stimulated the activity of mTOR in KJ29 and Caki-2 cells, therefore, it is reasonable to think that the reduction of this microRNA may affect the mTOR signaling reducing the activity of this protein kinase. Actually, the downregulation of miR501-5p in both KJ29 and Caki-2 cells transfected with antagomiR induced a marked reduction of mTOR protein phosphorylation compared with cells transfected with scramble sequences (Fig. 5B). Furthermore, the decreased activity of mTOR in these cells caused a significant reduction in the expression of MDM2 protein (Fig. 5C) that in turn induced a rising of p53 levels, not detected in control cells (Fig. 6A and B). In particular, antagomiR-transfected KJ29 cells also showed a most p53 nuclear translocation (Fig. 6C) compared with KJ29 control cells, where p53-staining was mainly confined to the cytoplasm (Fig. 6C).

These results suggest that miR501-5p downregulation in KJ29 kidney carcinoma cells activates apoptosis by increased expression and function of p53 and inhibition of both mTOR activity and MDM2 expression.

### *3.4. Role of miR501-5p expression in clear cell kidney carcinoma tissues*

As observed in KJ29 kidney carcinoma cells, also in ccRCC tissues the low expression of miR501-5p (<1 vs normal parenchyma) was associated with higher expression of the tumor suppressor

**Table 2**
Putative gene targets of miR501-5p involved in cell proliferation and apoptosis.

| Gene | Name | Function | Biological processes |
|---|---|---|---|
| TSC1 | Tuberous sclerosis 1 | Tumor suppressor | mTOR signaling |
| Casp1 | Apoptosis-related cysteine peptidase | Caspase cascade | Apoptosis |
| Casp2 | Apoptosis-related cysteine peptidase | Caspase cascade | Apoptosis |
| Casp8 | Apoptosis-related cysteine peptidase | Caspase cascade | Apoptosis |
| Fas | Cell surface death receptor | TNF receptor superfamily | Apoptosis |
| GAS2 | Growth arrest-specific 2 | Caspase-3 substrate | Apoptosis |
| CARD16 | Caspase recruitment domain family, member 1 | Caspase activator | Apoptosis |
| BLID | BH3-like motif containing, cell death inducer | Caspase activator | Pro apoptotic |
| BCL2L11 | BCL2-Like 11 (Apoptosis Facilitator) | Bcl-2 family proteins | Pro apoptotic |
| ING3 | Inhibitor of growth family, member 3 | Tumor suppressor | Apoptosis |
| MCU | Mitochondrial calcium uniporter | Calcium channel | Apoptosis |
| PTEN | Phosphatase and tensin | Tumor suppressor | Apoptosis |
| DCC | Deleted in colorectal carcinoma | Tumor suppressor | Apoptosis |
| MTSS1 | Metastasis suppressor 1 | Tumor suppressor | Cell migration inhibition |
| BRCA1 | Breast cancer 1, early onset | Tumor suppressor | DNA damage repair |
| Rictor | Rapamycin-insensitive companion of mTOR | Subunit of mTORC2 | Cell growth |
| MAPK6 | Mitogen-activated protein kinase 6 | Protein kinase | Cell proliferation, differentiation and development |
| MAP2K1 | Mitogen-activated protein kinase kinase 1 | Protein kinase | Cell proliferation, differentiation and development |
| JUN | Jun proto-oncogene | Oncogene | Gene expression regulation, tumorigenesis |
| KRAS | Kirsten rat sarcoma viral oncogene homolog | Oncogene | Tumorigenesis |
| NRAS | Neuroblastoma RAS viral (v-ras) oncogene homolog | Oncogene | Tumorigenesis |
| MYB | V-myb avian myeloblastosis viral oncogene homolog | Oncogene | Tumorigenesis |
| EGFR | Epidermal growth factor receptor | Protein tyrosine kinase | Gene expression regulation, cell proliferation |

Molecular targets of miR501-5p were identified by using TargetScan Human 6.0, miRBas, miRNAMap, Miranda and DIANA-MICROT programs. Gene names, functions and involved biological processes are indicated.

protein p53 compared with the corresponding normal samples. Moreover, in these tissues a significant reduction of MDM2 expression as well as mTOR protein phosphorylation compared with the matched normal tissues was observed (left part of the panel in Fig. 7A and B). Conversely, non-metastatic ccRCC samples with high levels of microRNA501-5p (>1) with respect to normal renal parenchyma did not show any significant changes in p53 and MDM2 expression or in mTOR activity compared with the paired normal kidney tissue (right part of the panel in Fig. 7A and B). In metastatic ccRCC tissues which usually expressed unchanged or higher levels of this miR, a variable expression of p53 protein was observed (Fig. 7C and D), even if in some of these metastatic kidney cancers a tremendous expression of this protein was shown (asterisks in Fig. 7C). However, metastatic ccRCC which express greater levels of p53 also showed a markedly increased mTOR activity with consequent enhanced expression of MDM2 protein as compared to those with unchanged expression of p53 (Fig.7C and 7E).

Taken together, these findings suggest that miR501-5p might play an important role in clear cell kidney carcinoma favoring a good prognosis in patients expressing low levels of this microRNA.

## 4. Discussion

Recent advances in understanding the molecular biology processes of RCC is not sufficient to approach a successful therapy for this kind of cancer and patients with metastatic kidney disease still have an extremely short life expectancy [7]. Therefore, the research of new biomarkers able to predict possible development of tumor metastases is a high priority not only for the prognosis and the clinical follow up of RCC, but also for defining novel therapeutic strategies. In this regard, miRs, small noncoding RNAs involved in human cancers [11] can serve as biomarkers for cancer risk stratification, outcome prediction and classification of histological subtypes [31,12,17]. Differently from other cancers (lung, colorectal carcinomas and melanoma), currently no predictive molecular biomarkers are available for routine use in kidney carcinoma [22].

Here, we describe miR501-5p, a non-conserved miRNA, as a candidate biomarker for the ccRCC. In fact, the lower expression of miR501-5p in ccRCC cancer tissues implied a better prognosis for the patients (Table 1). Conversely, high expression levels of this miR seems to be less accurate for prognosis prediction, even if most of ccRCC patients with greater expression of this miR developed metastases after surgical resection (Table 1). Consistently, clinical data processed by Kaplan–Meier test showed a significant correlation with the development of metastatic events in ccRCC patients expressing high levels of microRNA501-5p compared with those with low expression (Fig. 1F), but not a significant association with the overall survival (data not shown). The non-positive correlation with patient survival, even if close to significance ($p = 0.072$), could be due to the small number of cases evaluable in this cohort. Also data from "in vitro" experiments in kidney cells showed that the overexpression of miR501-5p by PL501 transfection caused an increased cell survival in both KJ29 and Caki-2 renal cancer cell lines (Fig. 2G and H). Similar features were also reported for different microRNAs belonging to the same cluster of miR501 on locus Xp 11.23, as miR532-5p, miR-500, miR362-5p, and miR502-3p. These miRs were associated with triple-negative breast cancer profile that is often related with high proliferation rate, high tumor grade and aggressive clinical behavior [18]. Moreover, the oncofetal miR500 was found overexpressed in human hepatocellular carcinoma and could be relevant for its diagnosis [39]. Then, the upregulation of miR362 in gastric cancer tissues induced cell proliferation and apoptosis resistance [38]. These features may be associated with the activation of mTOR signaling in different cancer types including renal carcinoma [32]. In this regard, miR501-5p should affect the activity of mTOR kinase, because TSC1 mRNA that codifies for hamartin, a component of mTOR inhibitor [37], is a target of this miR (Table 2). Consistently, we found that the overexpression of miR501-5p in KJ29 and Caki-2 kidney carcinoma cells increased the activation of mTOR (Fig. 2D and E), and consequently their growth and survival. Rapamycin, an inhibitor of mTOR, slowed indeed these biological processes (Fig. 3E–G). Interestingly, mTOR signaling is known to positively modulate the p53 repressor MDM2 [23,13], which is overexpressed in advanced kidney cancer [25]. Reliably, the overexpression of miR501-5p in both KJ29 and Caki-2 renal carcinoma cells, via the mTOR kinase, caused a strong increase in MDM2 content and consequently a marked reduction of the tumor suppressor p53 (Fig. 3A

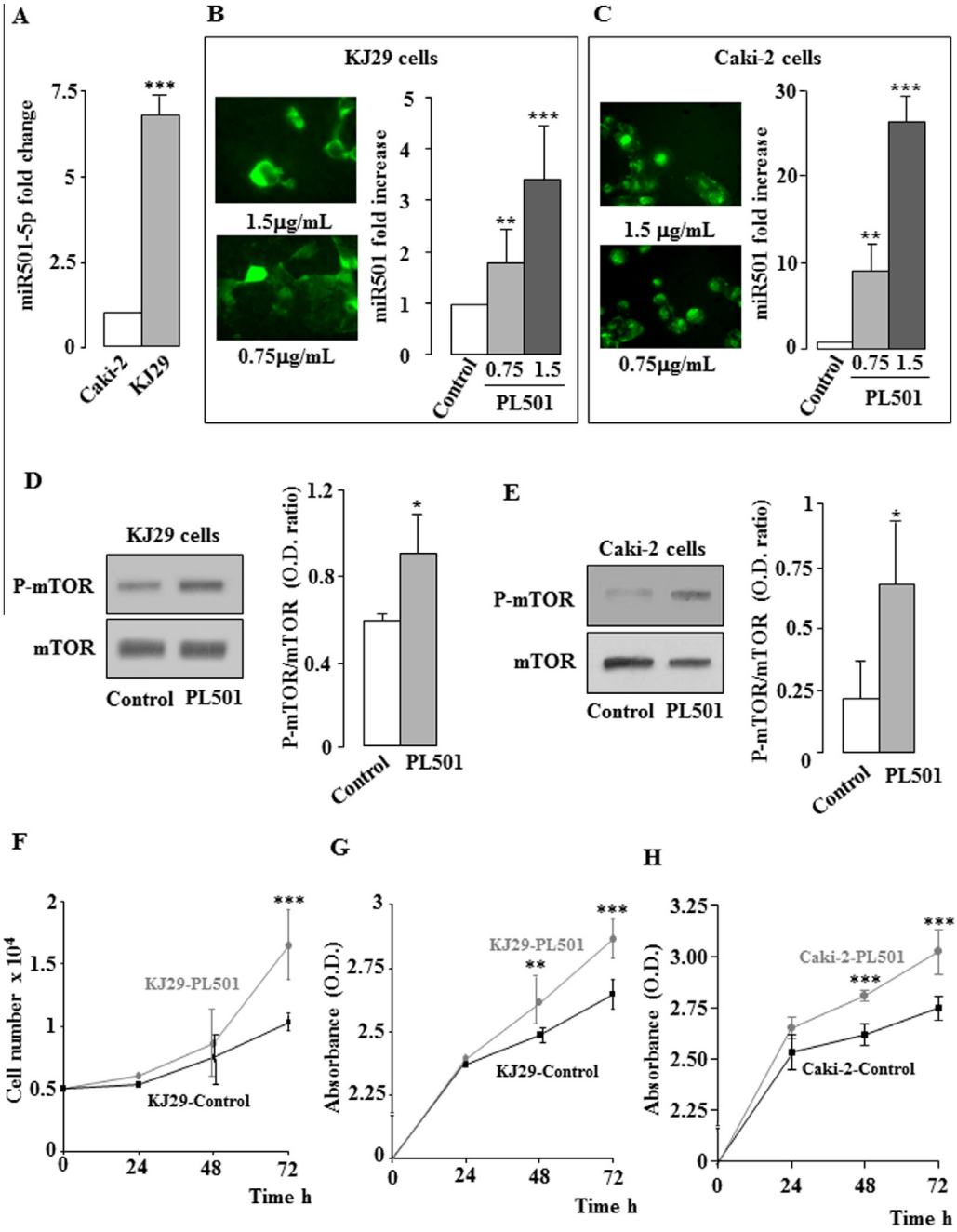


**Fig. 2.** miR501-5p overexpression promoted cell growth and survival by mTOR activation. (A) Real Time RT-PCR analysis of miR501-5p shows that levels of this miR in KJ29 cells were higher than in Caki-2 cells (6.75 ± 0.64-fold increase; $^{***}p < 0.001$). KJ29 (B) and Caki-2 (C) cells were transfected for 24 h with 0.75 and 1.5 μg/mL of a recombinant plasmid expressing both miR501-5p and GFP sequences (PL501) or with an irrelevant plasmid (control). To evaluate cell transfection efficiency, cells were seeded on glass coverslips, transfected for 24 h with PL501 and acquired by a fluorescence microscope at 40× magnification. Green stained cells in the boxes indicate the occurred transfection. The expression of miR501-5p measured by Real Time RT-PCR in PL501-transfected KJ29 and Caki-2 cells was higher than in cells transfected with an irrelevant plasmid (control). Data are expressed as fold increase ratio between PL501- and control plasmid-transfected cells. Values for KJ29 cells were: 1.82 ± 0.6 and 3.4 ± 1.2 for cells transfected with 0.75 and 1.5 μg/mL of PL501, respectively ($^{**}p < 0.01$; $^{***}p < 0.001$). For Caki-2 cells values were: 9.2 ± 3.12 for cells transfected with 0.75 μg/mL and 26.4 ± 3.08 for those transfected with 1.5 μg/mL of PL501 plasmid ($^{**}p < 0.01$; $^{***}p < 0.001$). (D and E) The activity of mTOR kinase, analyzed by western blotting, was higher in both PL501-transfected than in control KJ29 and Caki-2 cells. Values calculated as ratio between the phosphorylated and un-phosphorylated form of mTOR for KJ29 cells were: 0.58 ± 0.019 for control cells and 0.89 ± 0.17 for cells transfected with 0.75 μg/mL of PL501 ($^{*}p < 0.05$). Values for Caki-2 cells were: 0.21 ± 0.14 for control cells and 0.67 ± 0.25 for cells transfected with 0.75 μg/mL of PL501 ($^{*}p < 0.05$). (F) Analysis of cell proliferation, performed by direct cell counting using a Burker chamber, showed an increased cell growth in KJ29 cells transfected with PL501. Cells were transfected with 0.75 μg/mL of PL501 (KJ29–PL501) or with an irrelevant plasmid (KJ29-Control) and cultured in DMEM/F12 supplemented with 1% FBS for 24, 48 and 72 h. After 72 h of culture the number of cells transfected with PL501 or control plasmid was 16,407 ± 2921 and 10,400 ± 350, respectively; $^{***}p < 0.001$). The upregulation of miR501-5p by cell transfection with PL501 enhanced cell survival in both KJ29 (G) and Caki-2 (H) compared with control cells. After transfection with either PL501 or irrelevant plasmid, cell survival was analyzed by CellTiter assay as described in method section. For KJ29 cells cultured for 48 h, the values were 2.62 ± 0.09 in presence of PL501 and 2.49 ± 0.03 in presence of control plasmid ($^{**}p < 0.01$). After 72 h of culture, the absorbance values of KJ29 cells treated with PL501 or control plasmid were 2.87 ± 0.08 and 2.65 ± 0.06, respectively ($^{***}p < 0.001$). For Caki-2 cells cultured for 48 h, the values were 2.81 ± 0.03 in presence of PL501 and 2.62 ± 0.05 in presence of control plasmid ($^{***}p < 0.001$). For cells treated for 72 h with either PL501 or irrelevant plasmid, the values were 3.02 ± 0.11 and 2.75 ± 0.06, respectively ($^{***}p < 0.001$). Reported data are represented as mean ± standard deviation (SD) from three independent experiments for the analysis of mTOR activity and from three independent experiments in duplicate for the other findings in KJ29 cells, while data of Caki-2 cells were obtained from two different experiments in duplicate.

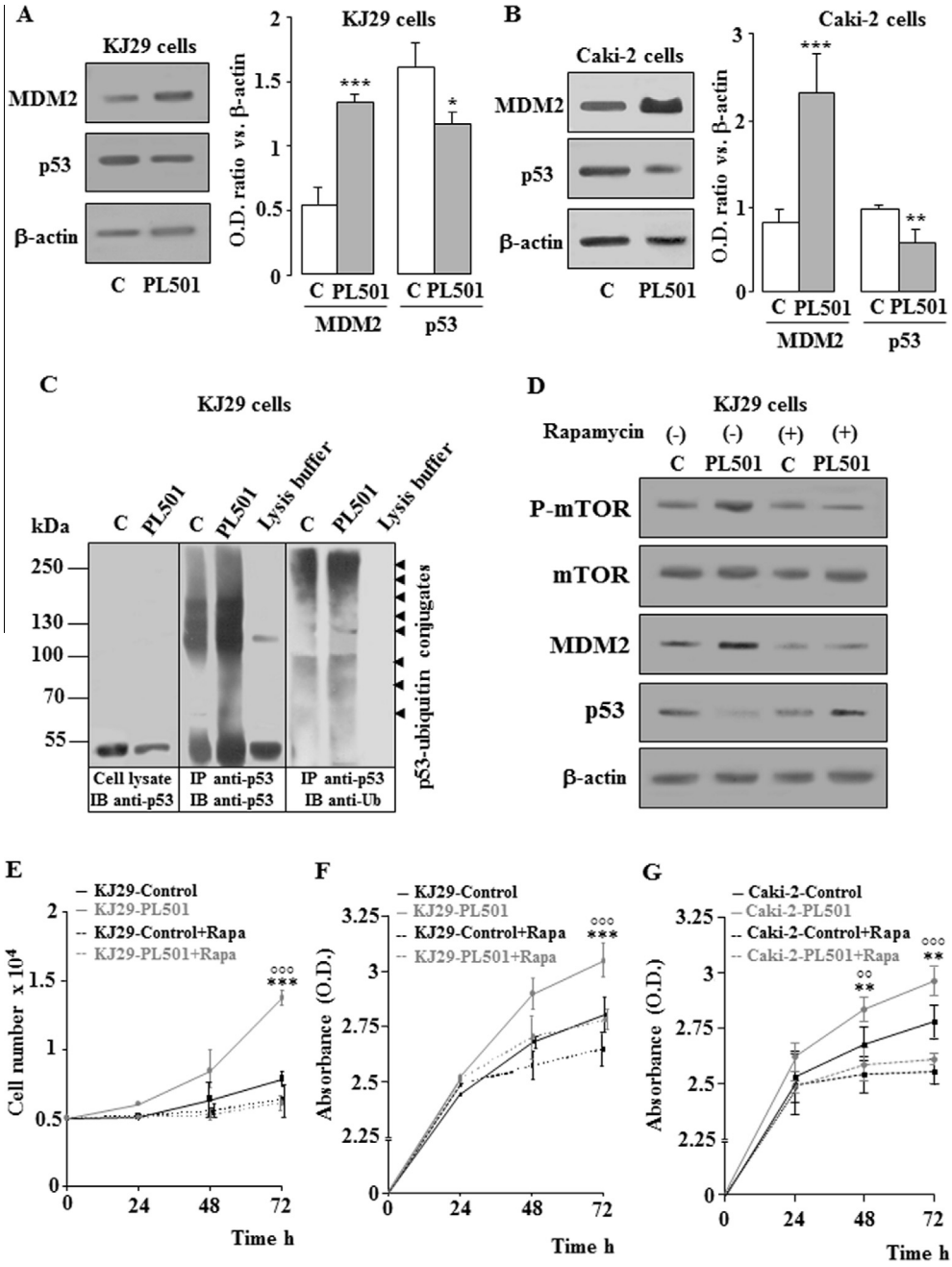


**Fig. 3.** The increased cell proliferation and survival in KJ29 and Caki-2 cells overexpressing miR501-5p is modulated by mTOR signaling in a mechanism involving MDM2 and p53 proteins. (A and B) The overexpression of miR501-5p enhanced the levels of MDM2 protein (0.53 ± 0.12 in KJ29 control vs 1.32 ± 0.068 in KJ29 PL501-transfected cells, $^{***}p < 0.001$; 0.81 ± 0.16 in Caki-2 control vs 2.33 ± 0.47 in Caki-2 PL501-transfected cells, $^{***}p < 0.001$) and reduced the expression of p53 (1.62 ± 0.17 in KJ29 control cells vs 1.17 ± 0.10 in KJ29 PL501-transfected cells, $^{*}p < 0.05$; 0.95 ± 0.07 in Caki-2 control cells vs 0.58 ± 0.11 in Caki-2 PL501-transfected cells; $^{**}p < 0.01$). Protein levels, analyzed by western blot technique, were calculated as ratio between the band corresponding to the protein of interest and actin band, used as housekeeping gene for sample normalization. (C) The overexpression of miR501-5p promoted the ubiquitination of p53 protein in KJ29 cells. After transfection with either PL501 (PL501) or control plasmid (C), cells were treated with the proteasome inhibitor MG-132 (10 μm) for 4 h, lysed and part of protein solution was immunoprecipitated with an anti-p53 antibody. Next, cell lysates and immunoprecipitated proteins were analyzed by western blotting using both anti-p53 and anti-Ubiquitin antibodies to detect p53 polyubiquitinated protein bands. (IB = immunoblot; IP = immunoprecipitation). (D) The treatment with rapamycin enhanced p53 protein levels by reduction of both mTOR activity and MDM2 expression in PL501-transfected KJ29 cells. Western blot analysis was carried out in KJ29 cells transfected with PL501 or control plasmid and treated for 24 h in presence (+) or absence (−) of 500 nM rapamycin. (E) The mTOR inhibitor rapamycin caused a decrease of cell proliferation in PL501-transfected KJ29 cells. Cells were transfected with PL501 (gray line) or control plasmid (black line) and cultured in presence (dotted line) or absence (solid line) of 500 nM rapamycin for 24, 48 and 72 h in DMEM/F12 medium supplemented with 1% FBS. As already observed in Fig. 2F, after 72 h of culture, PL501-transfected KJ29 cells grew more quickly than cells transfected with control plasmid (13,716 ± 542 cells vs 7822 ± 534 cells; $^{***}p < 0.001$). The treatment with rapamycin caused a significant reduction of cell proliferation in KJ29 cells transfected with PL501 compared with untreated cells (13,716 ± 542 for untreated cells vs 6055 ± 550 for rapamycin-treated cells; $^{\circ\circ\circ}p < 0.001$). (F and G) Cell survival, analyzed by CellTiter assay in cells cultured as described above, is increased in both KJ29 and Caki-2 cells treated with PL501 with respect to control cells (3.04 ± 0.078 for KJ29–PL501 vs 2.80 ± 0.076 for KJ29-control cells analyzed after 72 h of transfection, $^{***}p < 0.001$; 2.84 ± 0.05 for Caki-2-PL501 vs 2.68 ± 0.12 for Caki-2-control cells, transfected for 48 h, $^{**}p < 0.01$; 2.97 ± 0.05 for Caki-2-PL501 vs 2.78 ± 0.08 for Caki-2-control cells, transfected for 72 h, $^{**}p < 0.01$). Rapamycin treatment reduced cell survival compared with untreated cells (3.04 ± 0.078 for KJ29–PL501 untreated cells vs 2.77 ± 0.04 for KJ29–PL501 cells treated with 500 nM rapamycin for 72 h, $^{\circ\circ\circ}p < 0.001$; 2.84 ± 0.05 for Caki-2-PL501 untreated cells vs 2.59 ± 0.08 for Caki-2-PL501 cells treated with 500 nM rapamycin for 48 h, $^{\circ\circ}p < 0.01$; 2.97 ± 0.05 for Caki-2-PL501 untreated cells vs 2.61 ± 0.03 for Caki-2-PL501 cells treated with 500 nM rapamycin for 72 h, $^{\circ\circ\circ}p < 0.001$). Data are reported as mean ± standard deviation (SD) from three independent experiments for the analysis of MDM2 and p53 protein levels and from three independent experiments in duplicate for cell growth and survival measurements in KJ29 cells, while data for Caki-2 cells were obtained from two different experiments in duplicate.

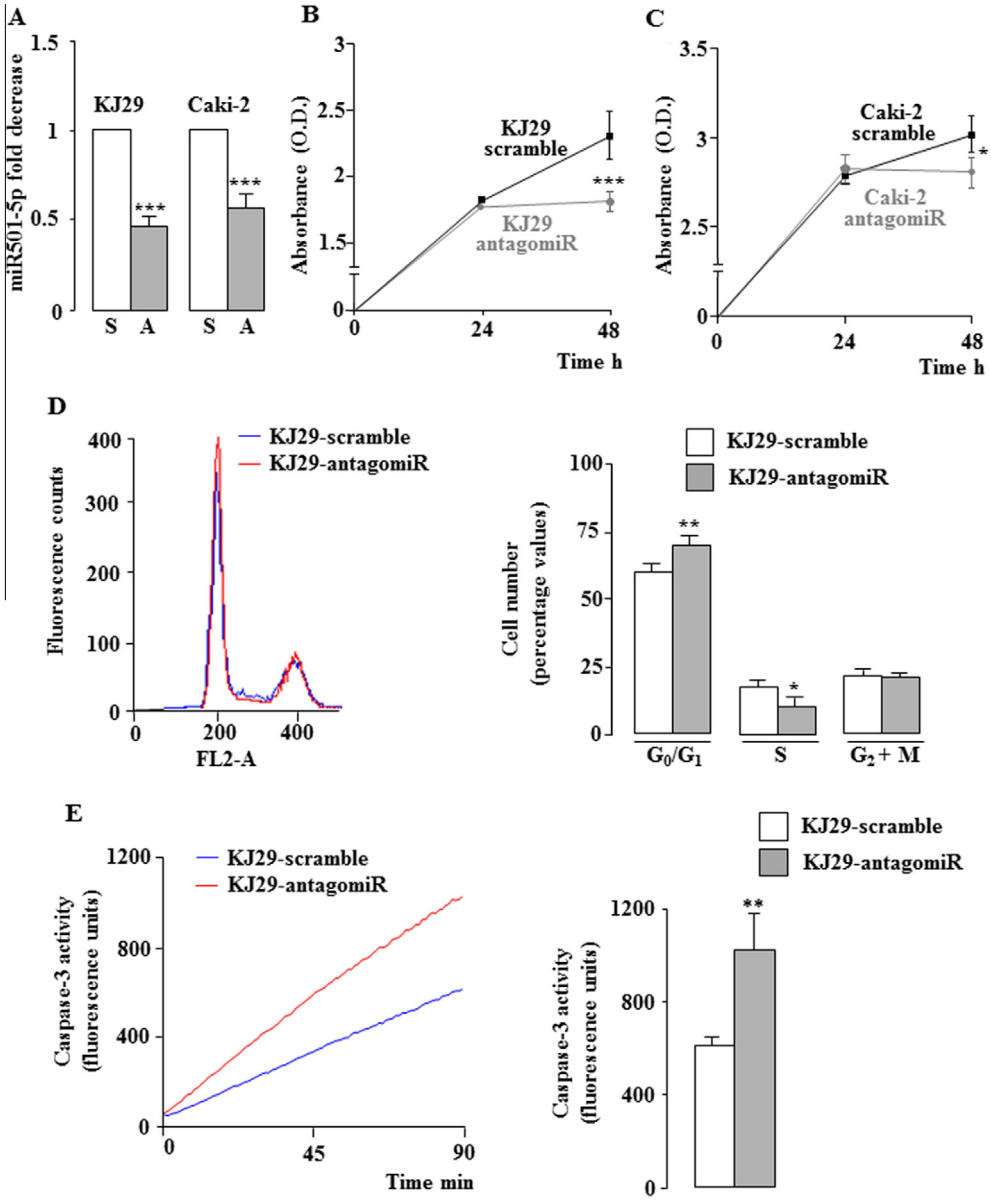


**Fig. 4.** MicroRNA501-5p downregulation reduced cell survival and promoted apoptosis by cell accumulation in $G_0/G_1$ phase of cell cycle and caspase-3 activation. (A) Real Time RT-PCR analysis showed a reduction of about 50% in miR501-5p sequences in both KJ29 and Caki-2 cells transfected for 24 h with a specific antagomiR at 30 nM concentration, that is the optimal molarity suggested by the manufacturer's protocol. Data were expressed as ratio among cells transfected with antagomiR (A) and cells treated with scramble sequences (S) calculated by delta-delta Ct method (0.47 ± 0.03-fold decrease; $^{***}p < 0.001$ for KJ29 cells and 0.57 ± 0.05-fold decrease; $^{***}p < 0.001$ for Caki-2 cells). (B and C) Cell survival was inhibited in KJ29 and Caki-2 cells transfected with an antagomiR specific for miR501-5p (gray line) compared with control cells (black line). Cells, transfected for 24 and 48 h with antagomiR or scramble sequences, were analyzed by CellTiter assay. Values after 48 h of transfection were 1.81 ± 0.077 and 2.31 ± 0.17 for KJ29 cells treated with antagomiR and scramble oligonucleotides, respectively ($^{***}p < 0.001$), while for Caki-2 cells values were 3.02 ± 0.10 in cells treated with scramble sequences and 2.81 ± 0.09 in antagomiR transfected cells ($^{*}p < 0.05$). (D) Cell cycle analysis showed a significant increase of $G_0/G_1$ phase in KJ29 cells treated with antagomiR compared with control cells. After transfection, cells were cultured for 24 h in DMEM/F12 supplemented with 0.4% BSA, resuspended in propidium iodide and analyzed by flow cytometry. Cell percentage in $G_0/G_1$ phase was 67.5 ± 2.5% for KJ29-antagomiR cells and 60.5 ± 1.7% for KJ29-scamble cells ($^{**}p < 0.01$). Cell percentage in S phase was 11 ± 2.2% for KJ29-antagomiR cells and 17.5 ± 3.1% for KJ29-scramble cells ($^{*}p < 0.05$). (E) KJ29 cells transfected with the antagomiR showed higher caspase-3 activation than control cells (1027 ± 155 in cells treated with antagomiR vs 612 ± 41 in cells transfected with scramble sequences; $^{**}p < 0.01$). After transfection with either antagomiR or random sequences for 24 h, cells were collected, lysed and analyzed for caspase-3 activity by the EnzChek® caspase-3 assay using a fluorimeter for data acquisition. Data are expressed as mean ± standard deviation from three different experiments in duplicate for Real time PCR and CellTiter analysis in KJ29 cells, and from two different experiments in duplicate for cell cycle, caspase-3 assays and for findings achieved by Caki-2 cells.

and B). Moreover, MDM2 functioning as an E3 ubiquitin-protein ligase and proteasome activator [30], caused the degradation of p53 by protein polyubiquitination in miR501-5p-overexpressing KJ29 cells (Fig. 3C). Finally, the reduction of mTOR activity by rapamycin treatment in miR501-5p-overexpressing KJ29 cells, through the inhibition of MDM2 expression, restored the levels of p53 protein (Fig. 3D). Therefore, mTOR kinase may negatively modulate p53 protein expression in kidney carcinoma cells. This is also supported by the observation that downregulation of miR501-5p in two different renal carcinoma cell lines (KJ29 and Caki-2) increased the expression of p53 (Fig. 6A and B). Consistently, the reduction of microRNA501-5p expression in KJ29 cells treated with antagomiR, also induced a marked nuclear translocation of p53 (Fig. 6C), which were associated to the reduction of mTOR activity (Fig. 5B) as well as MDM2 protein levels (Fig. 5C). The enhanced activity of p53 tumor suppressor protein triggers

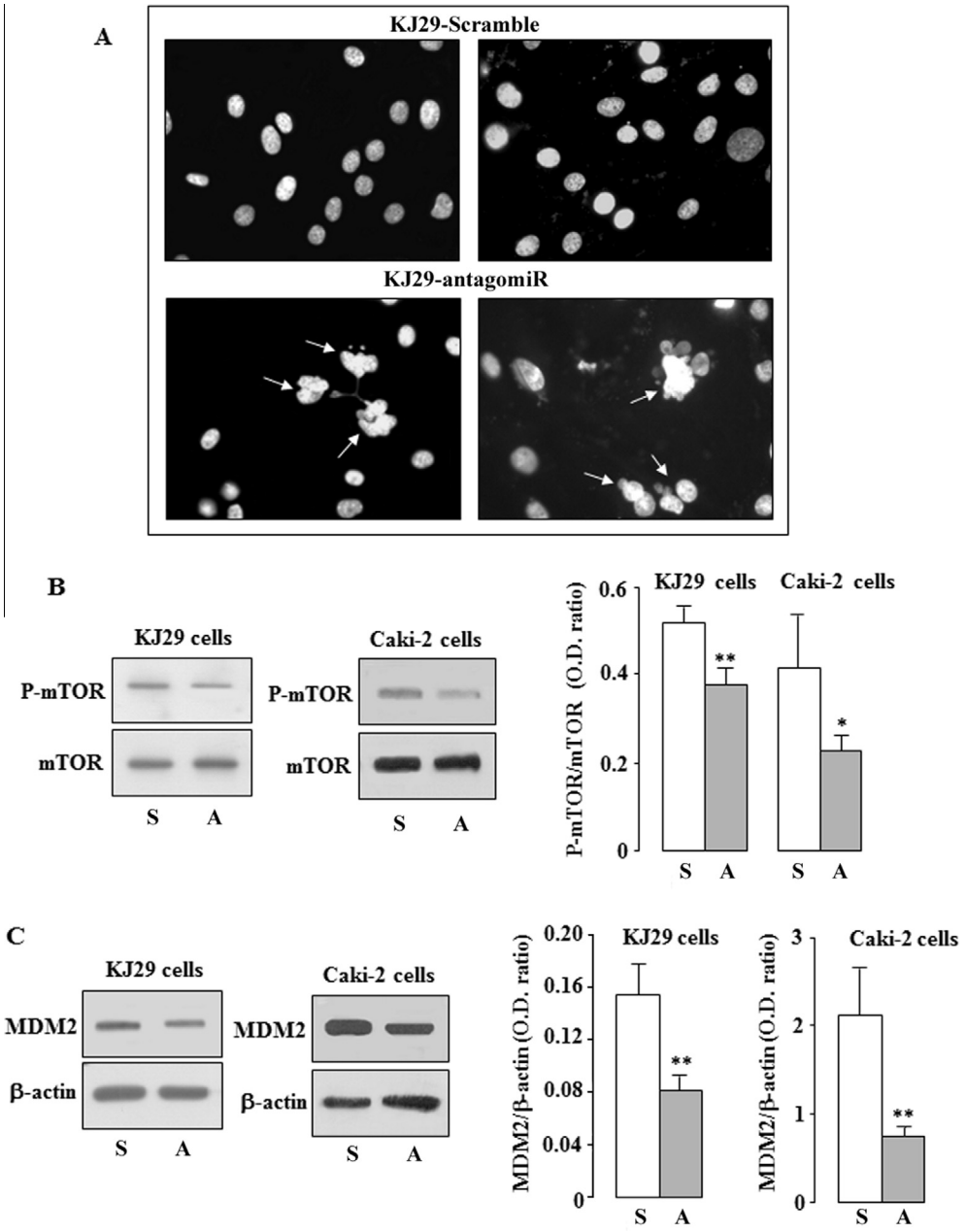


**Fig. 5.** The downregulation of miR501-5p induced apoptosis, reduction of mTOR activity and decreased the expression of MDM2 protein. (A) The analysis of apoptosis by Hoechst method showed an increased number of apoptotic nuclei in KJ29 cells treated with the antagomiR as compared to control cells. KJ29 cells, seeded on coverslips, were transfected with either antagomiR or scramble sequences in DMEM/F12 medium containing 0.4% BSA for 24 h. Next, cells were fixed, permeabilized and stained with Hoechst solution. Images were acquired by a fluorescence microscope equipped with a CCD camera at 40× magnification. Arrows in the squares indicated the apoptotic nuclei. (B) The reduction of miR501-5p expression decreased the activity of mTOR kinase in KJ29 and Caki-2 cells. Values of mTOR activity were 0.52 ± 0.044 in KJ29 cells transfected with scramble sequences and 0.37 ± 0.032 in KJ29 cells treated with antagomiR ($^{**}p < 0.01$); for Caki-2 cells values were 0.41 ± 0.11 in scramble cells and 0.225 ± 0.03 in cells transfected with antagomiR ($^{*}p < 0.05$). (C) MicroRNA501-5p downregulation reduced the expression of MDM2 protein in both KJ29 and Caki-2 kidney carcinoma cells. Values for MDM2 expression were 0.15 ± 0.02 in KJ29 scramble transfected cells and 0.08 ± 0.01 in KJ29 cells treated with antagomiR ($^{**}p < 0.01$); for Caki-2 cells values were 2.12 ± 0.53 in scramble cells and 0.73 ± 0.10 in cells treated with antagomiR ($^{**}p < 0.01$). The activity of mTOR kinase and MDM2 expression, analyzed by western blotting in KJ29 and Caki-2 cells transfected for 24 h with either antagomiR (A) or scramble sequences (S) was calculated as described in Fig. 2Dand E and Fig. 3A and B, respectively. Data are represented as mean ± standard deviation (SD) from three independent experiments for KJ29 cells and from two different experiments in duplicate for Caki-2 cells.

the apoptotic pathway not only by the activation of caspase-3, but also through the formation of apoptotic nuclei (Figs. 4E and 5A). It is interesting to note that the downregulation of miR501-5p may also stimulate the activation of apoptosis by enhancing caspase-1, 2 and 8 activity, being these proteases target of this miR (Table 2). Since caspase-2 is also able to mediate the cleavage of MDM2 [27], a greater activation of this enzyme through the miR501-5p reduction would cause an increased MDM2 degradation with a more powerful activation of p53. Thus, the activation or inhibition of p53 in kidney carcinoma cells seems to be driven by the expression of miR501-5p through the sequential modulation of mTOR activity and MDM2 expression.

The relationship between expression of miR501-5p, mTOR pathway and p53 modulation observed in vitro is supported by studies on ccRCC tissues. In fact, ccRCC tissues with expression levels of miR501-5p lower than the paired normal kidney parenchyma (<1) exhibited increased levels of p53, reduced mTOR activity and decreased expression of MDM2 (Fig. 7A and B). This

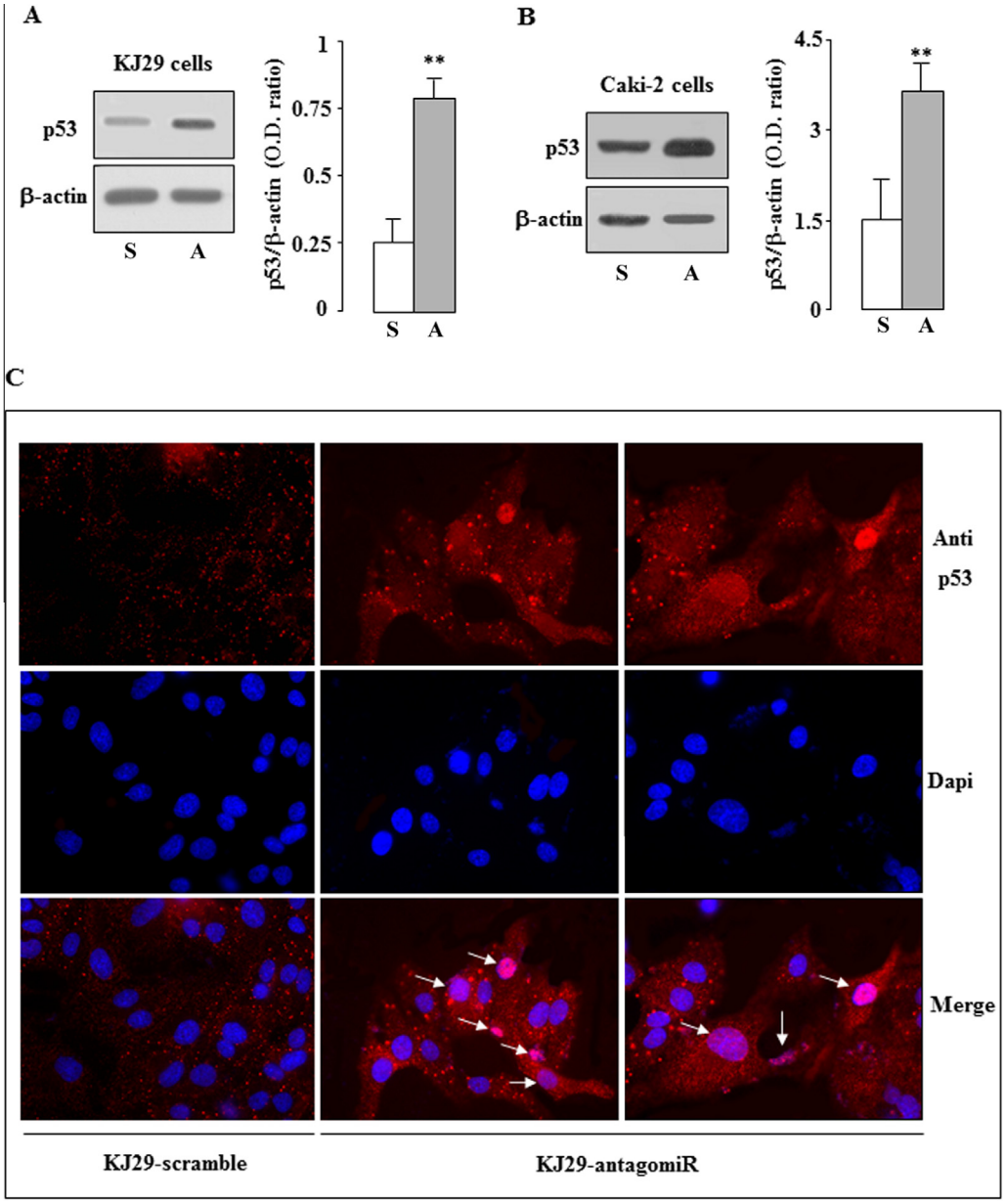


**Fig. 6.** The reduction of miR501-5p enhanced both expression and function of tumor suppressor p53. (A and B) KJ29 and Caki-2 cells transfected with the antagomiR (A) showed higher expression of p53 protein than cells treated with scramble sequences (S). Values were: 0.255 ± 0.08 for KJ29 control cells and 0.789 ± 0.07 for the same cells transfected with antagomiR (**$p < 0.01$). For Caki-2 cells values were 1.5 ± 0.70 for control cells and 3.66 ± 0.47 for cells transfected with the antagomiR (**$p < 0.01$). Protein levels were calculated as described in Fig. 3A and B. (C) The analysis of p53 by immunofluorescence in KJ29-antagomiR cells highlight a greater nuclear translocation of p53 than in KJ29 control cells. Cells, seeded on 10 mm coverslips and transfected with either antagomiR or random sequences, were fixed, permeabilized and treated with an anti-p53 monoclonal antibody. After several washes, cells were treated with a secondary antibody conjugated with rhodamine. Nuclei were marked with Dapi and images were acquired at 40× magnification by a fluorescence microscope equipped with a CCD camera. Arrows show the immunopositive nuclei. Data are represented as mean ± standard deviation (SD) from three independent experiments in KJ29 cells and from two different experiments in duplicate for Caki-2 cells.

molecular mechanism could support the good prognosis observed in ccRCC patients with low levels of miR501-5p. However, as shown in Fig. 7A (right part), 7B and 7C, the upregulation of this miR in ccRCC tissues not always affected mTOR activity neither the expression of MDM2 and p53 proteins. Nevertheless, this finding could be consistent with the lower informativeness on prognosis prediction observed in patients with high levels of this miRNA, the most of which, anyway, may develop metastases (Table 1 and Fig. 1F). Thus, differently from "in vitro" models, in ccRCC tissues with higher expression of microRNA501-5p other mechanisms able to modulate mTOR signaling could be involved. It is possible that in these tissues, which are not protected by apoptosis anymore, the disease may adversely progress in case of somatic mutations that activate oncogenic signals or inhibit tumor suppressor proteins. In these patients, other biomarkers such as p53 expression should be taken into account because the role of this tumor suppressor protein in advanced kidney carcinoma is currently discussed, being often associated with a poor prognosis [40]. In fact, in some advanced ccRCC tissues a tremendous overexpression of p53 oncosuppressor protein was shown (samples identified by asterisk in Fig. 7C). But, differently from other cancers, few somatic p53 mutations in ccRCC were reported [36], suggesting that the inactivation of p53 might be due to other factors. Actually, in metastatic ccRCC tissues the overexpression of p53 is associated with an increased level of MDM2 protein that should induce p53 degradation [36,25]. Also the present findings show that advanced

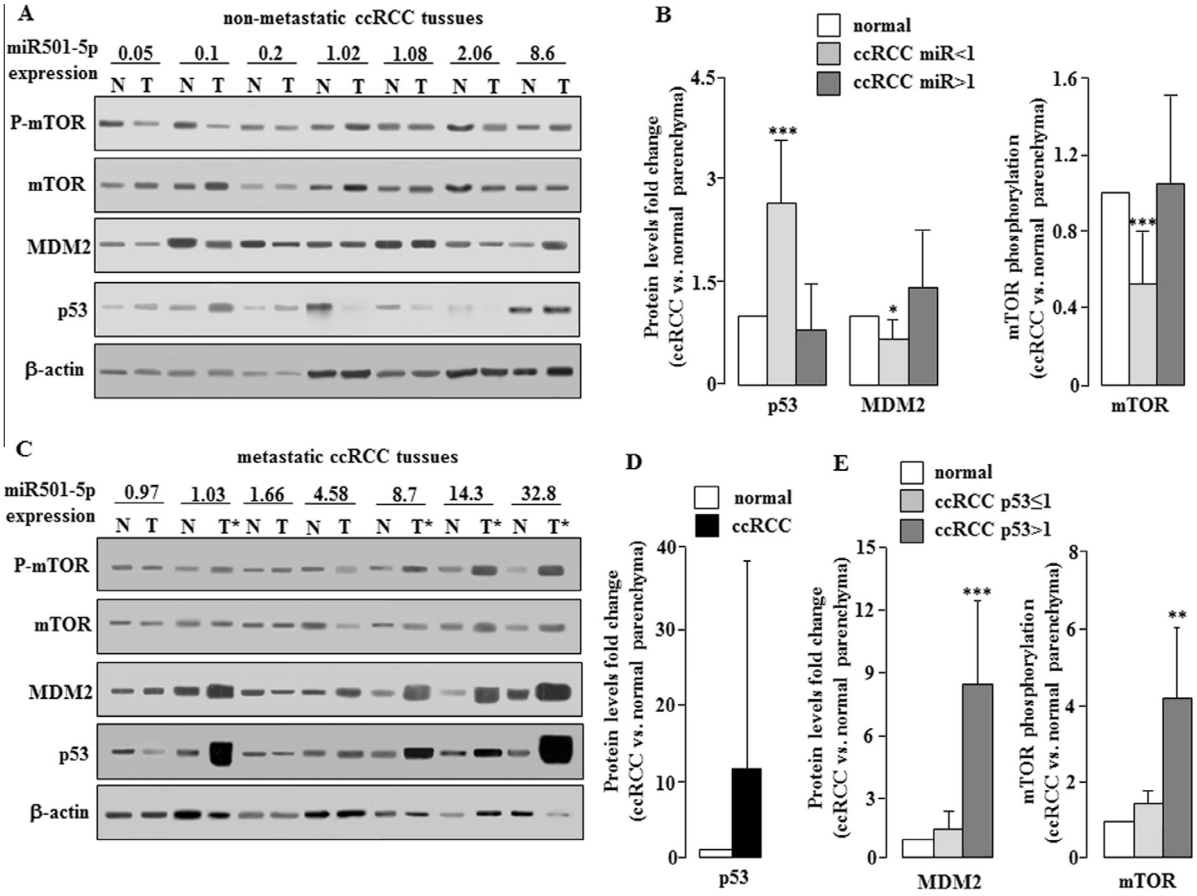


**Fig. 7.** Analysis of mTOR activity, p53 and MDM2 protein expression in clear cell renal cell carcinoma tissues (ccRCC). (A) Western blot analysis of mTOR, MDM2 and p53 proteins in normal kidney parenchyma (N) and non-metastatic ccRCC tissues (T) expressing variable levels of microRNA501-5p. (B) Bars indicating western blot analysis values of different kidney sample tissues (10 normal and 10 paired non-metastatic ccRCC) showed increased p53 expression, reduction of mTOR activity and decreased MDM2 protein levels in ccRCC with low expression of miR501-5p (<1) compared with normal kidney tissues (values calculated as intensity band ratio between ccRCC and normal kidney were: 2.67 ± 0.9-fold change for p53 protein levels, $^{***}p < 0.001$; 0.71 ± 0.34-fold change for MDM2 expression, $^{*}p < 0.05$; 0.53 ± 0.27-fold change for mTOR activity, $^{***}p < 0.001$). No significant changes were observed in 11 ccRCC which express greater levels of miR501-5p (>1) compared with 11 matched normal tissues. (C) Western blot analysis of mTOR, MDM2 and p53 proteins in normal kidney parenchyma (N) and metastatic ccRCC tissues (T) which, usually, expressed unchanged or higher levels of miR501-5p. Tumor samples with the higher expression of p53 protein were marked by the asterisk. (D) No significant changes of p53 expression in 15 metastatic ccRCC tissues compared with 15 paired normal kidney parenchyma samples were shown. (E) The activity of mTOR as well as the expression of MDM2 in metastatic ccRCC tissues expressing high levels of p53 was greater than those with unchanged expression of p53. Values for MDM2 were: 8.36 ± 4.08-fold change in 7 ccRCC with p53 > 1 vs 1.46 ± 0.86 in 8 ccRCC with p53 ⩽ 1 ($^{***}p < 0.001$); for mTOR activity were: 4.16 ± 1.88-fold change in 7 ccRCC with p53 > 1 vs 1.46 ± 0.29 in 8 ccRCC with p53 ⩽ 1 ($^{**}p < 0.01$). The activity of mTOR and the levels of MDM2 and p53 proteins were calculated as described in Fig. 2D and Fig. 3A. Data are expressed as fold change ratio between normal and ccRCC tissue.

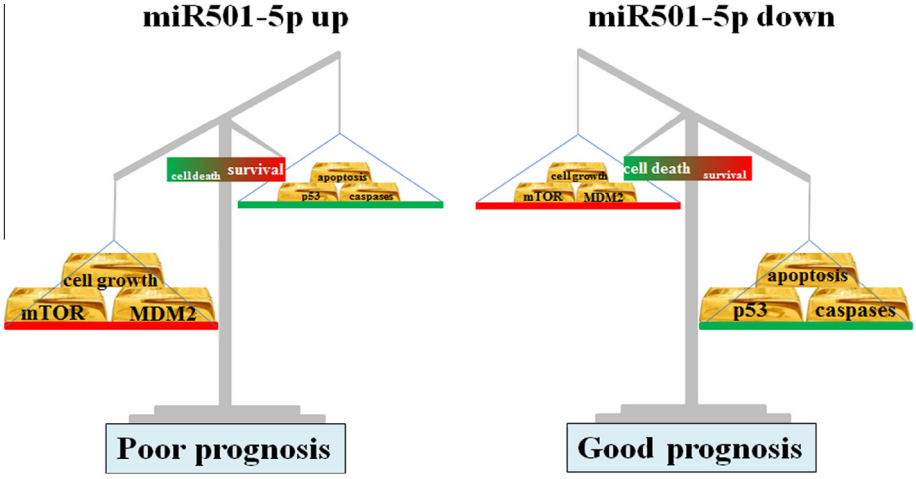


**Fig. 8.** miR501-5p functions as a balance in the modulation of cell survival and apoptosis in clear cell renal carcinoma. The overexpression of miR501-5p promoted cell growth and survival by activation of mTOR signaling. The reduction of miR501-5p expression caused the activation of the apoptotic pathway by p53 activation and mTOR inhibition.

ccRCC tissues overexpressing p53 showed higher MDM2 levels as well as mTOR phosphorylation compared with ccRCC tissues with lower or unchanged p53 expression (samples marked with asterisk in Fig. 7C and E). The enhancement of mTOR signaling may be associated with unfavorable progression of kidney cancer [28] and, the treatment with mTOR inhibitors might improve the survival in patients with metastatic kidney carcinoma [6,15], as also observed in our data (Fig. 3C–E). Therefore, as suggested in other studies, these factors could be considered as candidate biomarkers useful to detect poor prognosis for ccRCC patients [40,28,25].

In conclusion, we can speculate that miR501-5p may have a role of balance among apoptosis and cell survival in kidney cancer cells (Fig. 8). If expressed at low levels, miR501-5p could be considered a new biomarker associated with good prognosis for ccRCC, just because the expression of MDM2 remains low while p53 expression and apoptosis are stimulated. Conversely, the overexpression of this miR caused cell growth and survival that could expose ccRCC patients to development of metastasis and its informativeness on tumor prognosis becomes inaccurate. In this case, other markers, like mTOR-associated elements which are involved in advanced ccRCC, should be taken in account as important tool for poor prognosis detection in ccRCC patients. Finally, the findings here reported suggest that ccRCC patients who express high levels of miR501-5p could be treated with specific miR501-5p antagonists as new therapeutic approach for the treatment of ccRCC.

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