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ORIGINAL ARTICLE

MiR429 expression level in renal cell cancer and its correlation with the prognosis of patients

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Summary

Purpose: *To test the hypothesis that miR429 expression in renal cancer patients is increased and plays a role in the pathogenesis of the disease.*

Methods: *Twenty-seven renal cancer patients admitted to our hospital from May 2014 to May 2015 were enrolled as the study group, and 28 non-cancer patients were selected during the same period as the control group. Renal biopsy and serum samples were used to detect miR429 expression levels, and the patient histories were obtained to make relevant associations to clinical outcomes. In addition, the renal cancer cell line SK458 was used for overexpressing or knocking out miR429 in in vitro experiments to observe changes in proliferation and apoptosis rates.*

Results: *The expression levels of miR429 in renal tissues and serum of renal cancer patients were significantly higher compared with control patients ($p<0.05$). In addition, a correlation was found between the levels of miR429 in the serum of renal cell cancer patients and their clinical outcome after conventional treatment, with patients expressing lower miR429 levels showing better clinical outcomes. Finally, experiments with renal cancer cells revealed that the proliferation of cells overexpressing miR429 was increased and their apoptosis rate was significantly reduced, while the opposite was true in miR429-knockout cells.*

Conclusions: *It seems that miR429 can inhibit normal apoptosis rates and lead to high proliferation rates. Accordingly, the higher serum miR429 level in renal cancer patients suggests that it plays a role in the pathogenesis of the disease, while the differential miR429 levels according to the patients' clinical outcomes after treatment suggest that miR429 may be useful as a marker for prognosis.*

Key words: *apoptosis, correlation, miR429, proliferation, renal cancer cell*

Introduction

Renal cell cancer is often difficult to be treated, while the disease is usually diagnosed in late stages [1]. Recent studies have shown that tumor lesions arising in renal tubular epithelial cells are the main culprits of renal cell cancer [2]. Nevertheless, the pathogenesis of renal cell cancer remains unclear and currently there are no efficient drug therapies for treatment. Due to the non-specificity of early symptoms in renal cell cancer, a high level of suspicion is required by physicians in order to reach a diagnosis [3]. According to statistics, the number of renal cell cancer patients in China has reached 2 million and it has been increased by 1.2% yearly in recent years [4]. Complicating matters are the fact that the therapeutic rate of the disease is only about 30%, and 34.5-43.2% of these patients suffer recurrences [5], making the follow-up treatment costs high over the years. Re-

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search on the pathogenesis of renal cell cancer has been essential in the quest for an effective therapy. MicroRNAs play important roles in the regulation of transcription and translation processes. It has been shown, for example, that miR-106b-25 regulates the proliferation and apoptosis of esophageal [6] and colon cancer cells [7], and that the expression level of miR-196b in esophageal cancer cells is 120-fold higher compared with the normal cells [8]. In addition, there are abnormal expressions of miR-429, miR-127-3b and other miRNAs in cervical squamous cancer cells. Recently, miRNAs have also been found to be involved in the regulation of renal cells by altering the expression of some cancer-inducing and tumor suppressor genes [9]. For example, miR-129 can promote the migration of renal cancer cells by changing the expression of the *HOTAIR* gene [10]. Also, in a breast cancer study, miRNAs have been shown to regulate gene transcription at the level of mRNA to affect the expressions of genes such as *RHOA-1* and *THUD-1*, or regulate the translation process at the level of proteins YHD or KISE directly [11].

In this study we looked for a possible correlation between miR429 expression level and the presence and prognosis of disease.

Methods

Subjects

Twenty-seven renal cancer patients admitted to our hospital from May 2014 to May 2015 were selected as the study group. There were 15 males and 12 females, averaging 42.3±15.6 years in age. Twenty-eight non-cancer individuals (cancer-free after biopsy) were enrolled during the same period as the control group, including 14 males and 14 females with an average age of 44.1±17.2 years. The Hospital Ethics Committee approved the study and informed consent forms were obtained from each research subject.

Cells

Cell line SK458 was established in our lab and kept in the hospital's biobank.

RNA extraction from renal biopsy samples

The RNA extraction procedures in this study followed published protocols [11]. Different osteosarcoma cells preserved at -80°C were used to test the RNA extraction quality before the extraction from the experimental biopsy samples.

RNA extraction from patients' serum samples

Blood samples drawn at different times from renal cancer and non-cancer individuals were used to extract RNA for real-time PCR experiments to quantify miR429 levels.

Fluorescence quantitative PCR

To investigate the relative quantitative difference of miR429 expression in renal tissues, we took cDNA translated and transcribed from RNA as template for the fluorescence quantitative PCR experiment, and used the expression levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to normalize the samples. Fluorescence quantitative PCR kits were bought from Sangon Biotech (Shanghai, China) and the manufacturer's instructions were followed. The fluorescence quantitative PCR machine used was purchased from ABI (Germany). The primer sequences used are shown in Table 1.

CRISPR/Cas9 Gene knockout

CRISPR/Case Gene Silencing

In this study, we knocked out the miR429 gene in the SK458 cell line by CRISPR/Cas9. sgRNA sequences for gene knockout were designed in a relevant website (https://chopchop.rc.fas.harvard.edu/index.php) and synthesized by Suzhou Genewiz (China) (Table 2). CRISPR/Cas9 kits were purchased from Nanjing ABM Co. (China); transfection reagents were bought from Suzhou Alpha Biotechnology (China). Experimental procedures were carried out as per instructions and related documents [12].

Construction of cell lines overexpressing miR429

In this study, we took the pAE007-4-carrying plasmid preserved in the lab to overexpress the APC gene, resulting in miR429 overexpression. The primers to amplify miR429 gene were synthesized by Sangon Biotech (Shanghai, China). The sequences are shown in Table 2. Cleavage sites were BamH1 and Kpn1. The transfection experiments were carried out using reagents from Suzhou Alpha Biotechnology (China) as described above.

Detection of cell proliferation by MTT

Cell proliferation rates *in vitro* were measured via MTT assay with kits purchased from AXYGEN (USA), following procedures in references [13,14].

Table 1. Fluorescence quantitative PCR primers

Primer	*Sequence*
miR429-F	ATCGTAGCTAGCTAGCTACGAC
miR429-R	CTACTAGCTGATCGATGCATCGATCG
GAPDH-F	TGCTAGGCTAGGACGCTAGCTAC
GAPDH-R	CTGGGCTAGATCGACGAGAGCTC

Table 2. sgRNA sequences

Primer	*Sequence*
miR429-1	AGTGCTAGCTAGTCGATCGATCG
miR429-2	GTCGTAGCTGCTAGCTAGCTGATC
miR429-3	CGTAGCTAGTCGGCTAGCTAGCAT
miR429-4	CGTAGCTGATATGCTAGATAGCTAG
miR429-5	ATGTCGTAGCTGCGTAGCTAGCTAGC
miR429-6	TCGTAGCTAGCTAGCTACGATGAGTGAC

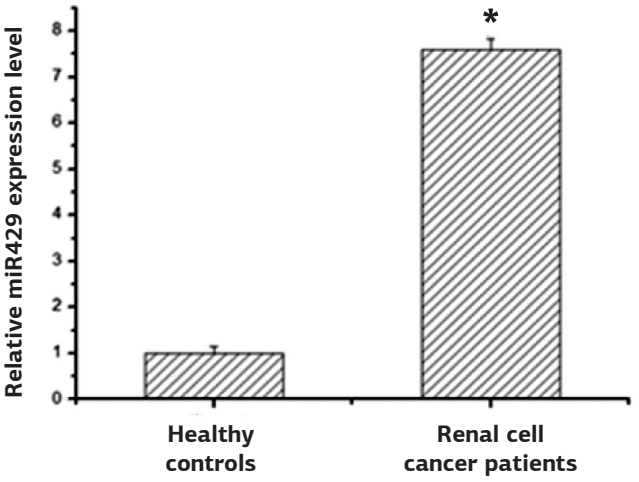

Figure 1. Difference in serum miR429 expression level between renal cell cancer patients and healthy controls. *p<0.05 compared with healthy controls.

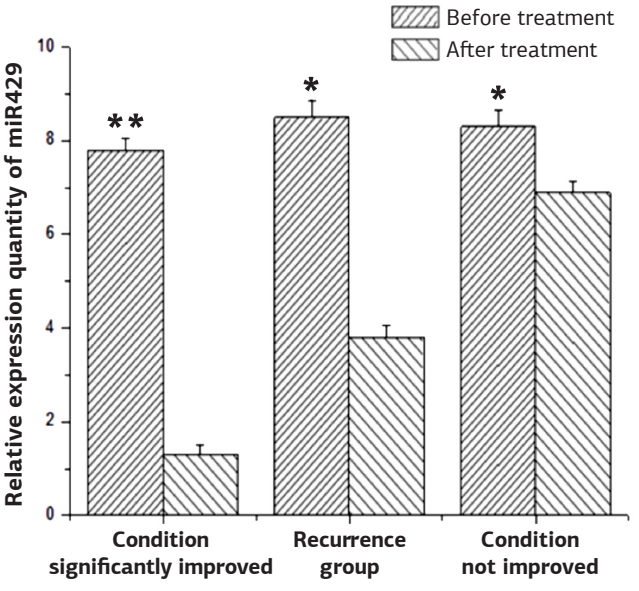

Figure 2. Differences in serum miR429 expression levels before and after different treatments in patients with different prognosis. *p<0.05, **p<0.05.

Detection of cell apoptosis by flow cytometry

Various cell samples (about 1-5×10^6 cells/ml) were collected after centrifugation at 3800 rpm and 4°C. The supernatant was discarded and the cell pellets were washed 2-3 times with sterile PBS (pH 7.2) and then centrifuged again. The rest of the procedures followed those in reference [15] using flow cytometer purchased from THERMO (USA).

Statistics

In this study, experimental data were processed and analyzed using the SPSS 20.0 software. α=0.05 and p<0.05 showed statistical significance. Sometimes, α=0.01 and p<0.01 were used to show extreme significance.

Results

Different miR429 expressions between renal cells of cancer patients and non-cancer individuals

Renal biopsy samples from non-cancer individuals and renal cell cancer patients were used to extract total RNA. Fluorescence quantitative PCR was used to detect the relative mRNA expression levels of miR429 in different samples. Compared with non-cancer subjects, the normalized tissue samples of renal cell cancer patients had 6-7-fold higher miR429 expression levels (p<0.05). Results are shown in Figure 1.

Correlation between the expression of miR429 and prognosis of renal cancer patients

Results are shown in Table 3. The levels of miR429 in 17 patients who were improved were significantly lower than those suffering recurrence after treatment (p<0.05). At the same time, the miR429 levels in patients who were not improved after treatment were significantly higher than in patients who suffered recurrences after treatment, suggesting that the levels in these

Table 3. Correlation between the expression level of serum miR429 and prognosis in renal cell cancer patients

Prognosis	*Expression of miR429 before treatment*	*Expression of miR429 after treatment*	*p value*
Significantly improved	7.8	1.3	<0.05
Recurrence after treatment	8.5	3.8	0.052
No improvement	8.3	6.9	>0.05
p	>0.05	>0.05	

Table 4. Apoptosis detection results in differently treated cell lines

Group	*Cell number*	*Apoptotic cells (%)*	*Apoptotic cells at early stage (%)*	*Apoptotic cells at middle stage (%)*	*Dead cells (%)*
Untreated group	10000	59.8	21.3	18.6	19.9
miR429 knocked out	10000	73.2	32.5	34.5	6.2
miR429 overexpressed	10000	12.1	3.3	5.3	3.5
p		0.1	0.01	0.01	0.01

patients were decreased after treatment but then starts to be increased again with the re-growth of the tumor. Figure 2 shows the graphic results, suggesting that the level of miR429 in renal cell cancer patients can be used as a marker for treatment outcomes.

Construction of cell line with miR429 knockout and overexpression

To construct renal cell lines with overexpressed miR429, the pAV32-6-carrying plasmid was used as the expression vector transfected into SK458 cells. MiR429-3/4 primers were used to amplify miR429 gene, and the digestion of the miR429 amplicon with BamHI and KpnI restriction enzymes was used to verify that the construction of the overexpressed vector was successful (Figure 3A).

MiR429-knockout SK458 cells obtained via CRISPR/Cas9 experiments were verified by PCR with primers for the gene, and the resulting SK458 cells failed to amplify the normal gene (Figure 3B).

Proliferation analysis of cell lines with miR429 knockout and overexpression

MTT assay was used to determine changes in the proliferation of the SK458 cells after miR429 knockout or overexpression. The results are shown in Figure 3. Compared with the normal (untreated) SK458 cell lines of renal cell cancer, the proliferation rate of miR429-knockout cells was significantly reduced ($p<0.05$). In contrast, the proliferation rate of miR429-overexpressing SK458 cells was significantly increased compared to that of the untreated SK458 cell lines ($p<0.05$). These results suggest that the level of miR429 expression is associated with the proliferation rate in the SK458 renal cancer cell line (Figure 4).

Detection of apoptosis of cell lines with miR429 knockout and overexpression

The results displayed in Figure 4 suggest an association between the higher levels of expres-

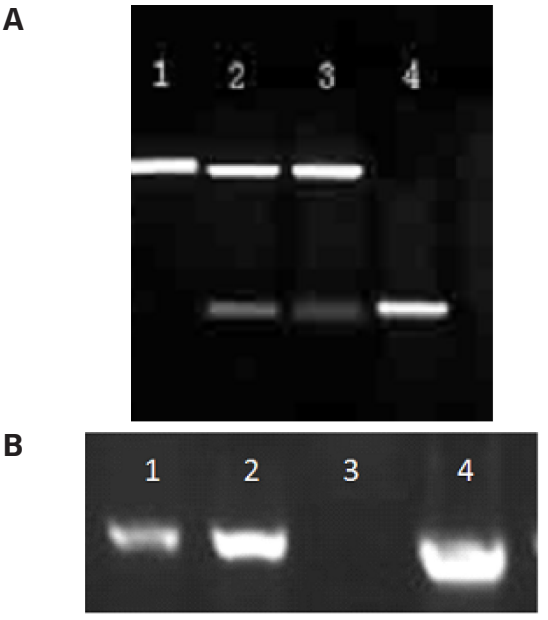

Figure 3. DNA gel analysis to verify cell lines with miR429 overexpression or knockedout. **(A):** miR429-overexpressed cell lines. Lane 1: empty carrier; lane 2: BamHI/ Kpn I digestion of amplicon; lane 3: BamHI/ Kpn I digestion of amplicon; lane 4: PCR product of miR429 cloning. **(B):** cell lines with miR429 knockout. Lanes 1, 2, and 4: original cell lines with miR429 amplified; lane 3: amplification of miR429 in knockout cell line.

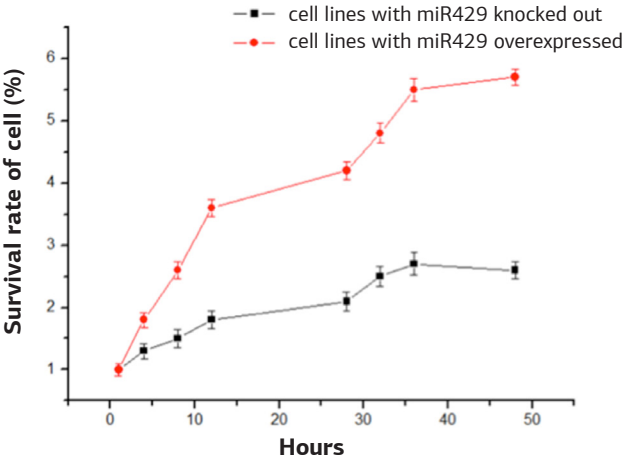

Figure 4. Proliferation of cell lines with miR429 knockout or overexpression. X axis: time, Y axis: survival rate of cells, black line: cell lines with miR429 knockout, red line: cell lines with miR429 overexpression. $p<0.05$.

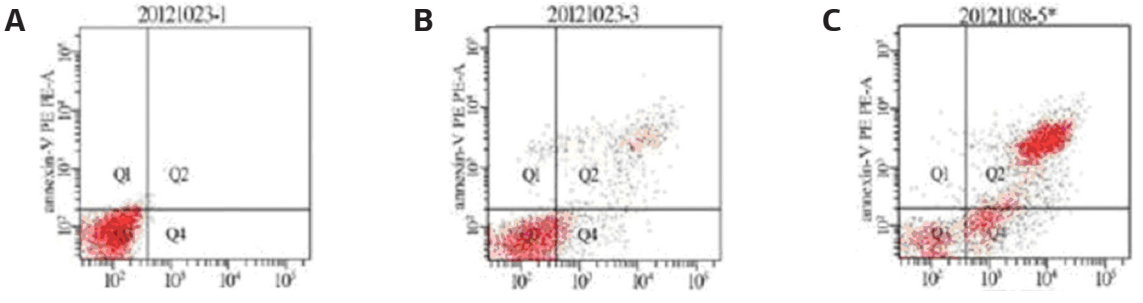

Figure 5. Flow cytometry for apoptosis in cell lines with miR429 knockout or overexpression. **(A)** Untreated cells, **(B)** Cells with miR429 overexpression, **(C)** Cells with miR429 knockout. Cell apoptosis rate was significantly increased ($p<0.05$) in miR429 knockout cells, but significantly decreased ($p<0.05$) in miR429 overexpressing cells.

sion of miR429 and cell proliferation promotion in SK458 cells. Therefore, flow cytometry was used to detect apoptosis rates in the miR429-overexpressing and -knockout cells. The results are shown in Figure 5 and reveal that cell apoptosis rate was significantly increased ($p<0.05$) in miR429-knockout cells, but significantly decreased ($p<0.05$) in miR429-overexpressing cells.

Discussion

The increasing population in China has aggravated the atmospheric pollution and water contamination in recent years, presumably exposing the population to more environmental risk factors for renal cancer. According to statistics, since 1990 the incidence rate of malignant tumors in China has increased year after year, and since the pathogenesis of renal cell cancer remains unclear, there is no efficient drug therapy for this disease as yet. Research findings have revealed multiple genetic factors involved in the pathogenic course of renal cell cancer [16]. For example, the *HOTAIR* gene, an important regulator of embryonic development, has been involved in the pathogenesis of cancers through its expression as a miRNA molecule [17]. MiRNAs are single-chain non-coding RNAs, 18-25bp in length that have been shown to be expressed in the body [18]. The main function of a miRNA is to regulate the transcription and translation of its target genes. A study showed that miR429 is a miRNA molecule known to regulate the transcription of genes related to esophageal cancer, like *CTK1* gene, promoting the migration of esophageal cancer cells [19]. In addition, researchers have found that esophageal cancer cells overespressing miR429 display higher proliferation rates, and detection of apoptosis by flow cytometry showed that cells with miR429 overexpression had a much lower apoptosis rate than that of the normal-miR429-expression counterparts [20].

In this study, we demonstrated that miR429 has close relevance with renal cell cancer for the first time, that is, the overexpression of miR429 in renal cancer cells can promote their proliferation and inhibit cell apoptosis.

In addition, we showed that the renal cell expression level of miR429 was increased in patients with positive biopsy for renal cancer, and we demonstrated that the serum miR429 level in cancer patients was higher than that in non-cancer patients. Furthermore, we showed a correlation between the serum miR429 level and the clinical outcome of renal cell cancer patients after treatment, suggesting that miR429 may be used as a marker for prognosis. Future studies will focus on more specific mechanisms by which miR429 regulates the renal cell cancer and related genes involved in cancer pathogenesis.

Conflict of interests

The authors declare no conflict of interests.

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