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• ORIGINAL PAPER •

Effect of miR-296 on the Apoptosis of Androgen-independent Prostate Cancer Cells

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Objective *To investigate the miR-296's function in prostate carcinoma(PCa) cells.*
Methods *In order to profile the miRNA expression in LNCaP cells, the cultured cells were stimulated with androgen after 48-h starvation, miRNA microarray analysis and Q-RT-PCR assay were performed. To characterize the effects of miR296 on PCa cells, CL-1 and PC-3 cells were transfected with miR-296 and antisense-miR-296, cell growth and apoptosis were then analyzed.*
Results *The miR-296-5p expression was up-regulated by 2.22 folds in the CL-1 cells, which do not express significantly androgen receptor, than in LNCaP cells. Knockdown of miR-296-5p induced apoptosis of CL-1 cells, but not LNCaP cells. However, knockdown of miR-296-5p inhibited the growth rate of LNCaP cells cultured in absence of androgen.*
Conclusion *MiR-296-5p could be important for development of prostate cancer from androgen dependence to androgen independence.*

Key words: prostate cancer cells; miR-296-5p; apoptosis

Most of the primary prostate cancer is androgen-dependent. Therefore, one common clinical therapy of prostate cancer is bilateral orchidectomy, which decreases the levels of body androgen immediately and results in death of huge numbers of PCa cells[1]. This method

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can efficiently reduce the tumor size and alleviate clinical symptoms, and thus it is considered to be one of the most effective palliative surgeries in prostate cancer treatment. The physiological basis of bilateral orchiedectomy is that lower androgen level will induce slower growth rate or even death of prostate cancer cells. However, by the time when tumor relapses, growth of prostate cancer cells are no longer androgen-dependent and finally develop highly graded malignancy, and death of patients [2]. The mechanisms by which prostate cancer cells can become resistant to castration have been heavily investigated. Recently scientists have found that the roles of miRNAs in development of prostate cancer from androgen dependence to androgen-independence[3].

miRNAs are a very prevalent class of cellular short regulatory RNAs that negatively modulate protein expression at the post-transcriptional level and are widely involved in the pathogenesis of several types of cancers. However, very few miRNAs have been functionally characterized[4]. The main objective of our work is to study effects of knockdown of miR-296-5p on growth of prostate carcinoma cells.

Functional characterization of miRNAs functions in mammalian cells is either performed genetically or by delivery of synthetic miRNA or antisense oligonucleotides[5,6]. Because the later is more straight forward and has been proved efficient, it is employed in our study.

Materials & Methods

Cell culture

LNCaP, CL-1 and PC-3 cells are purchased from American Type Culture Collection (Manassas, VA), LNCaP cells are cultured in DMEM media (Invitrogen, Carlsbad, CA) containing 5% normal or Charcoal dextran-stripped fetal bovine serum (FBS; Invitrogen, Carlsbad, CA), 100 u/ml penicillin, and 100 u/ml streptomycin (Shanghai Genebase Gen-Tech Ltd). CL-1 and PC-3 cells are cultured in IMDM media (Invitrogen, Carlsbad, CA) containing 5% Gibco FBS.

Reagents

Glycogen was purchased from Shanghai Beyotime Biotechnology Ltd. Trizol solution, trypsin, Lipofectamine 2000, RNAse and SuperScript II were obtained from Invitrogen, Carlsbad, CA. Glycogen. Methyltrienolone (R1881) was purchased from DuPont, Boston, MA.The oligonucleotides used in these studies were miR-296, AGGGC CCCCCCUCAAUCCUGU, and antisense-miR-296, ACAGGA UUGAGGGGGGCCCU; all the nucleotides in the RNA contain 2'-OMe modifications at every base. MiR-296 and antisense-miR-296 were synthesized by Shanghai GeneChem Co., Ltd.

miRNA transfection

LNCaP and CL-1 cells were seeded at a density of 0.5×10^6 per 60-mm culture dish in DMEM medium supplemented with 5% FBS. After 24 h, cells were transfected with

500 pmol miRNA using Lipofectamine 2000 reagent following manufacturer's instructions. After 48-h transfection, medium was removed and fresh medium supplemented with 5% FBS was added to the cells. All transfections were carried out in triplicate.

Analysis of cell growth

LNCaP and CL-1 cells were seeded in 96-well tissue culture plates at a density of 8×10^3/well, respectively, in their regular medium without added antibiotics. After 24 h, cells were transfected with lipofectamine 2000. After 6 h, medium was changed. At indicated time, cell viability was assessed by direct counting. Values from 4 wells were measured per treatment group for each time point.

Flow cytometry

LNCaP, CL-1 and PC-3 cells were seeded in 6-well tissue culture plates at a density of 2×10^5/well in their regular medium without added antibiotics. The next day, cells were transfected with lipofectamine 2000. After 72 h, cultured cells were washed in protein-free PBS, pelleted, and resuspended in 500 ml cold PBS. Cell fragmentation and cell cycle were studied after the cells were fixed in 1% paraformaldehyde (30 min) and ice-cold 70% ethanol, washed in PBS and finally stained with propidium iodide (PI, Invitrogen, 10 mg/ml, 15 min). All flow cytometry analyses of miR-296 and antisense-miR-296 transfected cells were performed on a Becton Dickinson FACS at Shanghai Institute of Cell Biology.

Light-microscope observations

CL-1 cells were plated in 96-well tissue culture plates at a density of 1.5×10^4/well in their regular medium without antibiotics. The next day, cells were transfected with lipofectamine 2000. After 72 h, cultured cells were washed with protein-free PBS, cells were fixed on plate with ethanol-acetic acid (3：1, 10 min at room temperature) and incubated with Annexin V (0.4 mg/ml, 10 min at room temperature in the dark) and PI (10 mg/ml, 10 min). Cells were observed with a Nikon TE2000 fluorescent microscope (excitation 365 nm, emission 480 nm).

Quantitative real-time polymerase chain reaction (Q-RT-PCR)

To verify the expression level of miR-296-5p in different prostate cancer cell lines, Q-RT-PCR was performed. Equal amounts of total RNA from the cell lines were treated with DNase, and reverse transcribed using random hexamers and SuperScript II to prepare the first strand cDNA samples for Q-RT-PCR analyses. Q-RT-PCR was performed in triplicate using SYBR® Green I chemistry on Rotor-Gene 3000 (Corbett Research) by using the following primers, F: 5'-AGGGCCCCCCCTCAA-3', R: 5'-GTGCGTGTCGTGGAG TCG-3'. The temperature cycle for Q-RT-PCR was set up as following: 50℃ for 2 min; 95℃ for 10 min; followed by 40 cycles at 95℃ for 10 s and 60℃ for 1 min. The CT value for miR-296-5p in the test sample was fitted onto the standard curve to obtain the respective expression level. A smaller CT value indicates a higher expression level, and vice versa. The final miR-296-5p expression data were reported after normalizing to that of U6 RNA.

Statistical analysis

Statistical analysis was performed using Excel and SPSS 11.5 software. Experimental data, where applicable, are represented as $\bar{x} \pm s$. The value of *P*<0.05 was considered statistically significant.

Results

Levels of miR-296-5p is higher in CL-1 cells than in LNCaP cells

CL-1 cell line is androgen-independent subclone of LNCaP cells. Therefore both of the cell lines have been used to compare the difference that is associated with development of prostate cancer to androgen independence. The model of the cell lines was also employed to determine the levels of miR-296-5p. Q-RT-PCR revealed that miR-296-5p expression in CL-1 cells was 2.22 folds higher than that in LNCaP cells (Figure 1).

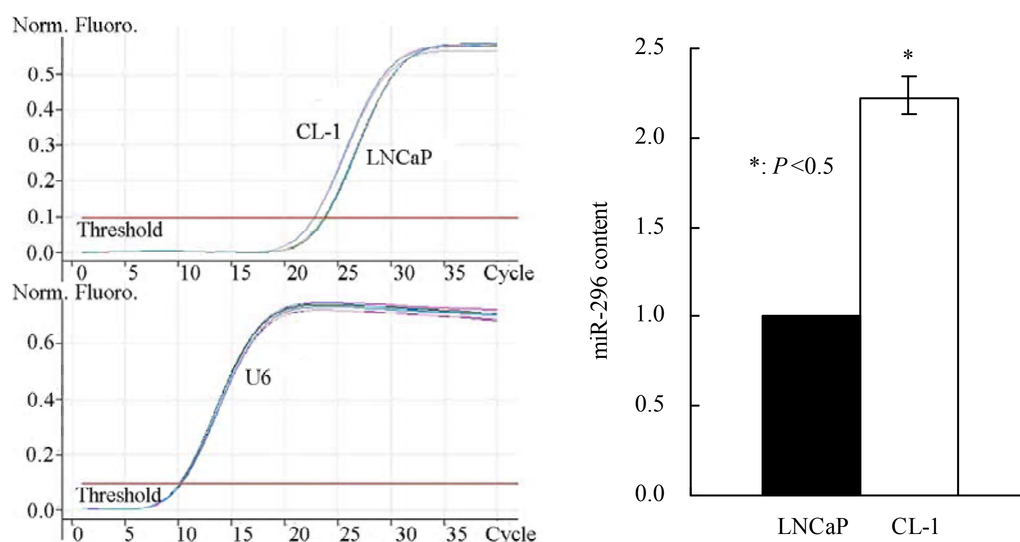


Figure 1 Q-RT-PCR assay of miR-296 expression level in LNCaP and CL-1 cells

miR-296-5p induced apoptosis of CL-1 cells

To investigate the biological function of miR-296-5p in prostate cancer cells, the sense and antisense were transfected oligonucleotides into PCa cells, and the cell numbers were counted at 24 h, 48 h, 72 h after transfection, respectively. The total cell numbers of the mock and miR-296-5p transfected group were increased by 1.70 and 2.20 folds 72 h after transfection, while the numbers of antisence miR296-transfected cells dropped to approximately half of the numbers of cells seeded (Figure 2). The results indicated that CL-1 cells transfected with antisense-miR-296-5p died significantly, while cells transfected with miR-296-5p did not show any significant changes in growth rate.

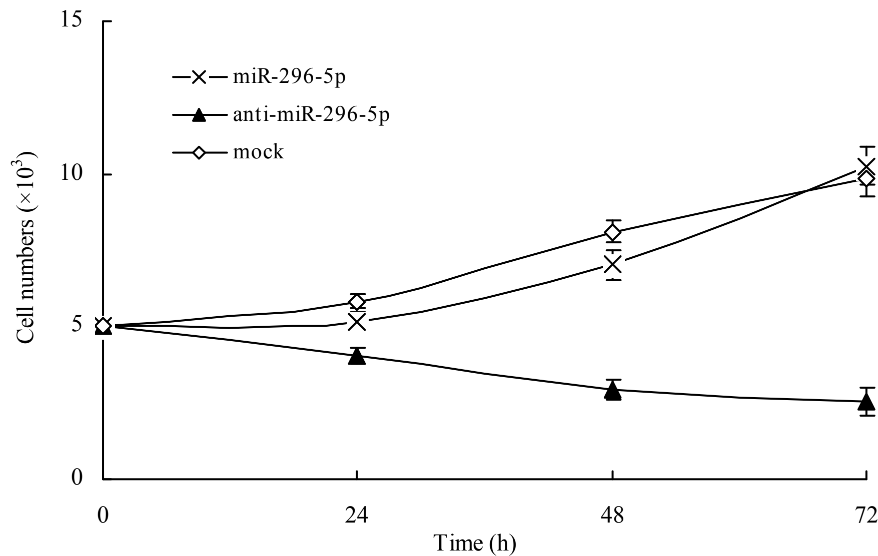
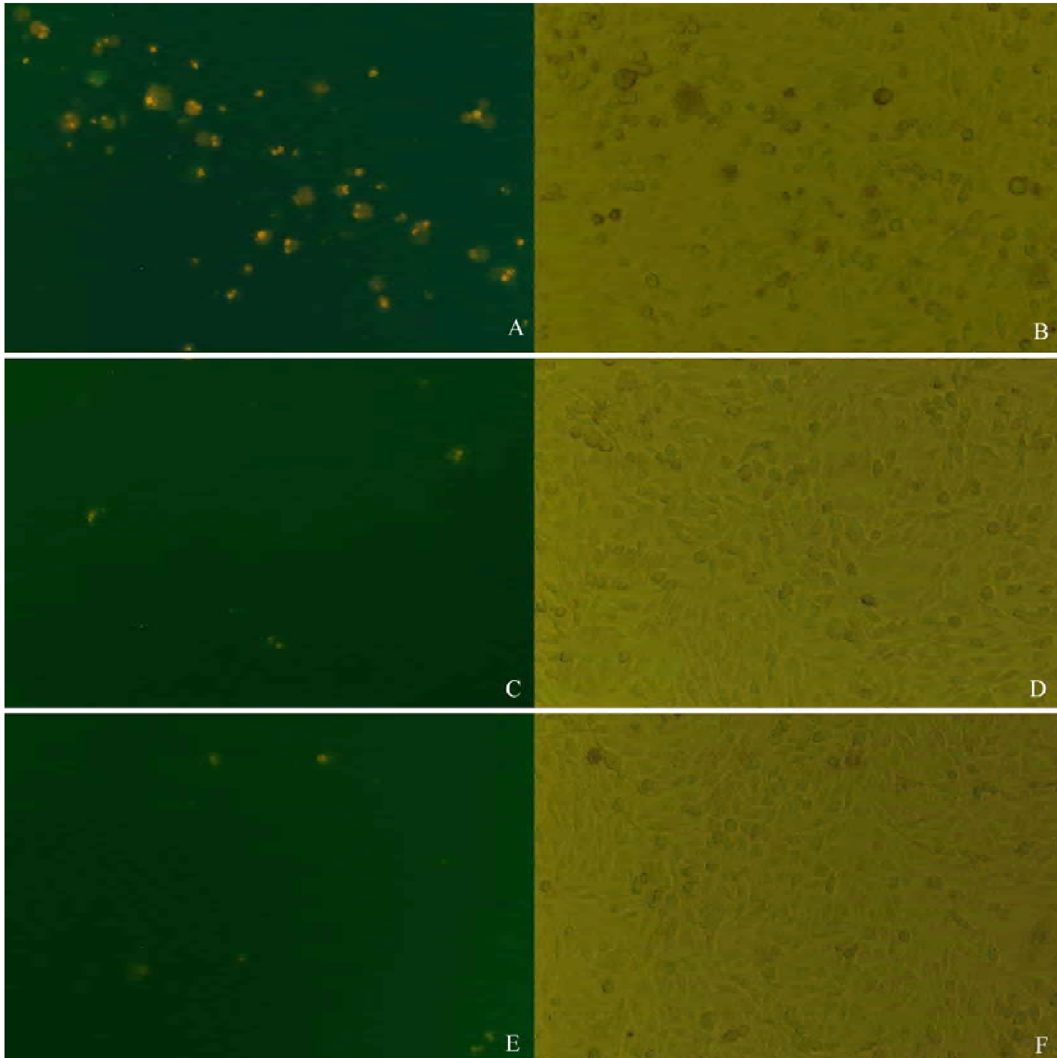


Figure 2 Inhibition of miR-296 induces CL-1 cell's death(P<0.05).CL-1 cells were transfected with miR-296-5p, antisense of miR-296-5p and mock

Reduced number of cells transfected with antisence miR-296-5p suggested that it could be caused by apoptosis of CL-1 cells. Therefore, apoptosis assay was conducted by employing Annexin V/PI staining followed by fluorescent microscopy observation. As shown in Figure 3, Annexin V/PI staining is very obvious in the CL-1 cells transfected with antisense miR-296-5p, and is barely detected in its negative controls. Flow cytometry analyses of antisence-miR-296-5p illustrated that the apoptosis rate of CL-1 cells transfected with antisense miR-296-5p was 2.5−2.7 times higher than those of its negative controls (Figure 4). Therefore, it can be concluded that expression of antisence miR-296-5p induced apoptosis of CL-1 cells. In addition, we tested whether or not antisence miR-296-5p could induce apoptosis in the PC-3 and LNCaP cells. The transfection of antisence miR-296-5p resulted apoptosis of PC-3 cells, but not in LNCaP cells. Therefore, it strongly suggested that miR-296-5p plays a key role in androgen-independent prostate cancer cells.

Since our previously study showed that levels of antisence miR-296-5p is up-regulated by addition of androgen, we further studied whether or not inhibition of miR-296-5p could affect growth rate of LNCaP cells. We first found transfection of antisence miR-296-5p did not significantly change the growth rate of LNCaP cells that were constantly incubated in the regulate FBS, which contains androgen. Additionally, we did the similar transfection assays in the LNCaP cells that had been incubated in charcoal-stripped FBS for 48 h. As expected, the LNCaP cells obviously grew slowly in the absence of androgen, compared with the presence of androgen, and they looked healthy, under light microscope, for as long as 7 d after transfection. The growth rate of antisence-miR-296-5p-transfected cells was



A,C,E pictures observed under blue light; B,D,F pictures observed under natural light

A,B: CL-1 cells transfected with antisense-miR-296; C,D: CL-1 cells transfected with miR-296;

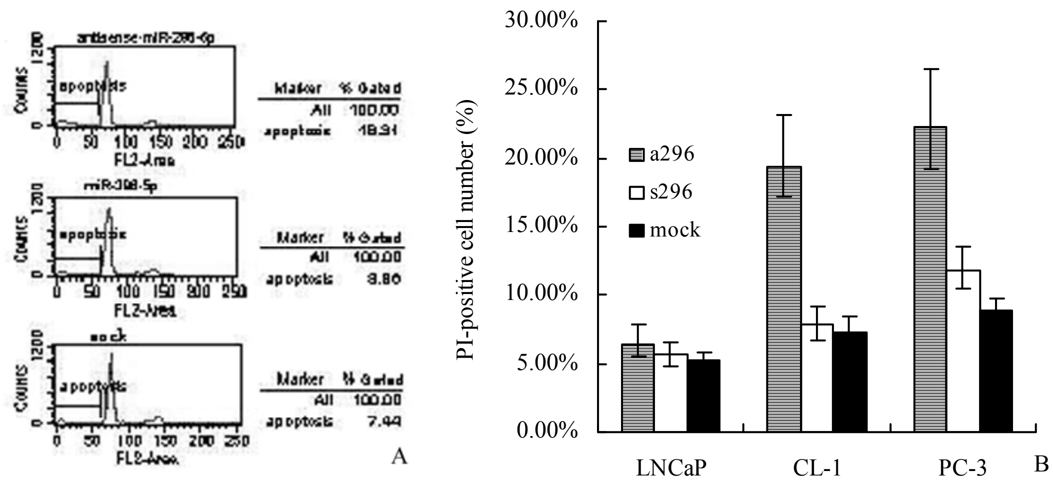
E,F: CL-1 cells tranfected with only the lipofectamine 2000 reagent, mock

Figure 3 Annexin V/PI staining of CL-1 cell transfected with miR-296 and antisense miR-296

approximately 71.4% of that of mock control, and 65.7% of miR-296-5p-transfected cells on d 6 after transfection, indicating that over-expression of antisence-miR-296-5p significantly repressed LNCaP cell growth in absence of androgen (Figure 5).

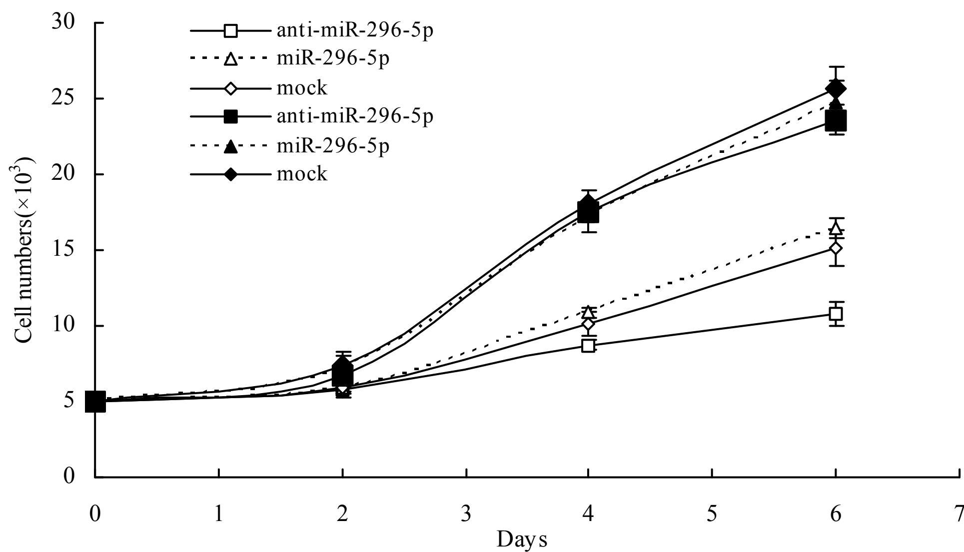
Discussion

As one of androgen-independent subclones of LNCaP cells, CL-1 cell line was previously reported not express significantly androgen receptor. Therefore, CL-1 and LNCaP



A: the original data of apoptosis rate in transfected CL-1 cells; B: the contrast of LNCaP, CL-1 and PC-3 cell apoptosis rate 72 h after transfection (P<0.05)

Figure 4 Apoptosis assay in flow cytometry



The upper three lines represent LNCaP cells cultured in ordinary FBS; while the nether three lines represent LNCaP cells cultured in charcoal-stripped FBS (P<0.05)

Figure 5 Inhibition of miR-296 induces to slowed growth of LNCaP cells cultured in charcoal- stripped FBS

cells have been used as cell model in comparative studies on the difference of androgen -dependent and -independent prostate cancers[7].

We have recently found that expression of several miRNAs were up-regulated in androgen-starved LNCaP cells 48 h after addition of R1881, an analog of androgen

(Run-sheng LI, manuscript prepared). One of them was miR-296-5p, whose expression level was increased by 2.73 folds as compared with that in untreated androgen starved LNCaP cells. This suggested that miR-296-5p expression was androgen responsive.

We have shown that knockdown of miR-296 induces apoptosis of CL-1 cells, and represses grow of LNCaP cells in absence of androgen but not in the presence of androgen. All the results strongly suggest that miR-296 plays a key role in maintaining viability and optimal growth of prostate cancer cells in absence of androgen. This is further supported by our results that knockdown of miR-296 induces apoptosis of PC-3 cells.

One of major features of prostate cancer is its progression of prostate cancer from androgen dependence to androgen independence. The mechanism remains unclear although tremendous studies on it have been reported. Our data show that levels of miR-296 are obviously higher in CL-1 cell than in LNCaP cells. It suggests that up-regulation of miR-296 could gain extra functions in CL-1 cells, compared in LNCaP cells, and by which, could contribute the progression of prostate cancer from androgen dependence to androgen independence. The hypothesis is supported by our results that knockdown of miR-296 induces apoptosis of CL-1 cells and PC-3 cells, but not in LNCaP cells.

Micro-RNAs exert their roles by binding to transcripts of their target genes, and finally reducing levels of expression of target genes. We have shown addition of R1881, an analog of androgen, to androgen-starved LNCaP cells resulted in a raised levels of miR-296 by 270% compared with that in untreated control cells (Unpublished results). Additionally, we reported here that levels of miR-296 are higher in CL-1 cells than in LNCaP cells. All the above data suggest that expression of target genes for miR-296-5p gradually reduces in prostate cancer progression.

Previously, Angie M *et al*. also reported that antisense inhibition of miR-296 decreased cell growth rate and increased apoptosis rate in HeLa cells[8]. Few target genes for miR-296-5p, however, have been reported. We used the software (www.targetscan.org) to search the target genes for miR-296-5p, 110 genes are selected as the potential targets for miR-296-5p. Although the relationship of expression of most of the genes and prostate cancer has not been reported, glutathione peroxidase 3 gene seems to be interesting one to us among the list of genes. The enzyme is a member of glutathione peroxidase and plays a critical role in the detoxification of hydrogen peroxide and other free oxygen radicals. Recent study indicates that glutathione peroxidase 3 acts actually as tumor suppressor, and its gene expression is lost via different mechanisms during progression of prostate cancer[9,10]. Similarly, tumor suppressor gene p53 mutation is also involved in progression of prostate cancer and is associated with advanced (metastatic) stage, loss of differentiation, and the transition from androgen-dependent to androgen-independent growth[11]. Therefore, it will be very interesting to next study whether or not miR-296-5p is a new regulatory mechanism for down-expression of glutathione peroxidase 3 gene, and by which, raised levels of

miR-296 contribute to the development of prostate cancer from androgen dependence to androgen independence.

In conclusion, we have shown that levels of miR-296-5p are lower in LNCaP cells than in their androgen-independent subclone CL-1 cells, and antisence of miR-296-5p induced apoptosis of CL-1 cells. Therefore, miR-296-5p could be a new drug target for clinical treatment of androgen independent prostate cancer, while, there is currently not efficient way to cure these caner patients.

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