

The Role of microRNA-221 and microRNA-222 in Androgen-Independent Prostate Cancer Cell Lines

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Abstract

Androgen-dependent prostate cancer typically progresses to castration-resistant prostate cancer (CRPC) after the androgen deprivation therapy. MicroRNAs (miR) are noncoding small RNAs (19-25nt) that play an important role in the regulation of gene expression. Recent studies have shown that miR expression patterns are significantly different in normal and neoplastic prostate epithelial cells. However, the importance of miRs in the development of CRPC has not yet been explored. By performing genome-wide expression profiling of miRs, we found that expression levels of several miRs, in particular miR-221 and miR-222, were significantly increased in CRPC cells (the LNCaP-derived cell line LNCaP-Abl), compared with those in the androgen-dependent prostate cancer cell line (LNCaP). Overexpression of miR-221 or miR-222 in LNCaP or another androgen-dependent cell line, LAPC-4, significantly reduced the level of the dihydrotestosterone (DHT) induced up-regulation of prostate-specific antigen (PSA) expression and increased androgen-independent growth of LNCaP cells. Knocking down the expression level of miR-221 and miR-222 with antagonist miRs in the LNCaP-Abl cell line restored the response to the DHT induction of PSA transcription and also increased the growth response of the LNCaP-Abl cells to the androgen treatment. Changing the expression level of p27/kip1, a known target of miR-221 and miR-222, alone in LNCaP cells affected the DHT-independent cell growth but did not significantly influence the response of PSA transcription to the DHT treatment. In conclusion, our data suggest the involvement of miR-221 and miR-222 in the development or maintenance of the CRPC phenotype. [Cancer Res 2009;69(8):3356-63]

Introduction

Androgen and androgen receptor (AR) play crucial roles in prostate cancer (CaP) development and progression. Similar to other secondary sexual organs, the development and homeostasis of prostate epithelial cells requires androgen, even in the transformed state. Initially, almost all metastatic CaP require testosterone for growth and androgen deprivation therapy is an effective therapeutic intervention for metastatic CaP (1). Most patients are initially responsive to androgen deprivation therapy, but eventually, their cancers progress to castration-resistant CaP (CRPC), leading to death (2). Many attempts have been made to characterize the molecular signature of CRPC. It is known that

during CRPC progression, CaP cells use a variety of AR-dependent and AR-independent pathways to survive and flourish in an androgen-depleted environment (2). However, a comprehensive understanding of the involved mechanisms is still lacking, and thus, the further study of CRPC is an important step toward developing therapies for this lethal phenotype.

MicroRNAs (miRs), which are groups of naturally occurring small noncoding RNAs, negatively control gene expression either by regulating mRNA translation or stability posttranscriptionally (3) or via the transcriptional silencing, in a sequence-specific manner (4, 5). It was estimated that the human genome encodes close to 1,000 different miRs, which are predicted to control the activity of ~30% of all protein-coding genes (6). Involvement of miRs has been shown in many cellular functions such as cell proliferation, cell differentiation, stress response, apoptosis, immunity, and transcriptional regulation (7). Cancer, which results from a dysregulation of these pathways, might in part result from aberrant miR expression. Many studies have indicated that miRs could function as oncogenes or tumor suppressors, playing crucial roles in transformation and carcinogenesis (8, 9). MiR expression signatures have been used to classify cancers and to define miR markers that might predict a favorable prognosis (10, 11). Recent miR profiling data implicated the association of specific miR expression patterns with tumorigenesis and degree of tumor differentiation in human CaP (12, 13). However, the precise role of specific miRs in modulating malignant progression is largely unknown.

We are interested in molecular mechanisms or modulators involved in the progression of CRPC. To identify and to understand the role of miRs in the development and/or maintenance of CRPC, we developed comprehensive miR expression profiles in the androgen-dependent LNCaP cell line and the androgen-independent LNCaP-Abl cell line. The initial analysis generated a subset of miRs that was differentially expressed in LNCaP and LNCaP-Abl. Among these differentially expressed miRs, the expression level of miR-221 and miR-222 exhibited the most dramatic difference between androgen-dependent and androgen-independent CaP cell lines, suggesting their importance in the development of CRPC. In this report, we determined miR signatures in CRPC and showed direct evidence for the involvement of miR-221/-222 in CRPC development and/or maintenance.

Materials and Methods

Reagents. Synthetic, chemically modified short single- or double-stranded RNA oligonucleotides Pre-miR-221 molecule, Pre-miR-222 molecule, Pre-miR-negative control, anti-miR-221 molecule, anti-miR-222 molecule, and anti-miR-negative control were purchased from Ambion. Dihydrotestosterone (DHT) and flutamide were purchased from Sigma-Aldrich. Predesigned siRNA targeting p27/kip1 (AM 16704) and siRNA negative control (AM 4611) were purchased from Ambion. P27/kip1

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expression vector, pCMV-SPORT6, inserted with full-length p27/kip1 cDNA was purchased from American Type Culture Collection (ATCC; MGC-5304).

Cell lines. The CaP cell line LNCaP was obtained from the ATCC. LNCaP-Abl cell line was provided by Zoran Culig (Innsbruck Medical University; ref. 14). LNCaP C4-2 cell line was obtained from ViroMed Laboratories (Minneapolis, MN). LNCaP-104R2 cell line was provided by Shutsung Liao (University of Chicago, Chicago, IL; ref. 15), and LAPC-4 cell line was provided by Charles Sawyers (University of California, Los Angeles, CA; ref. 16). LNCaP is a widely used CaP research model cell line, which was initially isolated from a biopsy of a lymph node metastasis from a 50-y-old man (17). The LNCaP-Abl and LNCaP-104R2 were androgen-independent cell lines directly derived from LNCaP by maintaining in an androgen-depleted medium (14, 15). LNCaP-C4-2 cell line was established from castrated mouse xenografts. LAPC-4 cell line contains a wild-type AR and is an androgen-dependent cell line. LNCaP, LNCaP-Abl, LNCaP C4-2, LNCaP-104R2, and LAPC-4 cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS; for LNCaP cells, LNCaP C4-2 and LAPC-4 cells) or 10% charcoal-stripped FBS (for LNCaP-Abl and LNCaP-104R2 cells).

MiRNA microarray experiments. LNCaP and LNCaP-Abl cell were seeded in 10-cm dishes at ~15% confluence and then grew to ~60% confluence for RNA isolation. Biological triplicate total RNAs (15 µg) from each cell line were isolated using *mirVana* miRNA Isolation kit (Ambion). MiR profiling was performed by LC science using µParaflo microfluidics chip (human-MRA-1001, version 10.1), which contains 711 verified human miR probes. Raw data were normalized and analyzed using Gene Cluster 2.0. Average linkage clustering was performed by using uncentered correlation metric. Statistical comparisons were done by using ANOVA tool in SigmaStat 3.1 (SPSS, Inc.).

Transfection. LNCaP, LAPC-4, and LNCaP-Abl cells were cultured to 60% confluence and then transfected with 30 nmol/L of Pre-miR-221 and Pre-miR-222 oligonucleotides or anti-miR-221 and anti-miR-222 inhibitors, or miR-Precursor negative control or anti-miR inhibitors negative control for 24 h using Lipofectamine²⁰⁰⁰ according to manufacturer's instructions. Subsequently, 10 nmol/L DHT with or without 1 µmol/L casodex or 1 µmol/L flutamide was added into media and incubated for another 24 h. To alter the expression level of p27/kip1 in LNCaP, predesigned p27/kip1 siRNA, negative control siRNA, or pCMV-SPORT6-p27/kip1 plasmid were transiently transfected into LNCaP cells using Lipofectamine²⁰⁰⁰ (Invitrogen). Twenty-four hours after transfection, cells were treated either ethanol vehicle or 10 nmol/L DHT for another 24 h. Total RNAs or proteins were then harvested for the analysis of p27/kip1 expression.

RNA extraction and real-time reverse transcription-PCR. Total RNAs were isolated using *mirVana* miRNA Isolation kit (Ambion). MiR expression levels in different cells were quantitated by quantitative real-time PCR (qRT-PCR), using *mirVana* qRT-PCR miRNA Detection kit and Primer Sets

(Ambion), following the manufacture instruction. Prostate-specific antigen (PSA), AR, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) real-time PCR primers and probe sets were inventoried products of Applied Biosystem (Assay ID: Hs01105076_m1, Hs00907244_m1 and Hs02758991_g1, respectively). Analyses were performed using a standard TaqMan PCR kit protocol. Experiments were carried out in triplicate for each data point.

Western blotting. Total proteins from various CaP cell lines were extracted with radioimmunoprecipitation assay buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 5 mmol/L EDTA]. Fifty micrograms of each protein extract were resolved on 10% SDS-PAGE and transferred to Hybond-C nitrocellulose. The level of p27/kip1 expression was evaluated by mouse monoclonal anti-p27 antibody (610241; BD Biosciences) and with an Enhanced ChemiLuminescence detection system (Amersham Life Science). As a loading control, β-actin expression levels were measured using a monoclonal anti-Actin (A 3853; Sigma).

Cell proliferation assay. For all cell growth studies, cells were plated in 96-well plates in androgen-depleted medium. After being cultured for 24 h, cells were transfected with Pre-miR221 or Anti-miR221 for 24 h before adding 10 nmol/L DHT. Tetrazolium salt (WST-1; Roche Applied Science) cell proliferation assay was then carried out at various days after transfection, following the manufacture instruction.

Results

MiR expression screening in the CRPC LNCaP-Abl cell line versus the androgen-dependent LNCaP cell line. To investigate the involvement of miRs in the CRPC development, we chose to perform miR expression profiling in the androgen-dependent LNCaP CaP cell line and its derived castration-resistant counterpart, LNCaP-Abl. The relationship of LNCaP and LNCaP-Abl resembles the progression of CaP from androgen-dependent to CRPC. Additionally, in these two cell lines, the whole chromosome AR binding sites have been mapped and the dynamics of AR transcription complex loading on PSA regulatory regions are well-characterized (18, 19). Thus, we decided to use the LNCaP and LNCaP-Abl cell lines for miR expression profiling and use the expression of PSA, an androgen-regulated gene, to evaluate the AR-mediated transcription in response to the DHT treatment in this study.

Triplicate sets of miR expression profiling were performed. Pairwise significance analysis of the microarray data indicated that the expression of four miRs (miR-221, miR-222, miR-15a, and miR-16-1) was significantly up-regulated in LNCaP-Abl, ranging from

Table 1. The relative expression level of differentially expressed miRs in LNCaP Abl versus in LNCaP

MiRNAs	Chromosomal location	Fold change	P (t test)
hsa-miR-221	Xp11.3	+10.756	<0.001
hsa-miR-222	Xp11.3	+6.512	<0.001
hsa-miR-15a	13q 14.3	+5.171	<0.001
hsa-miR-16-1	13q 14.3	+1.850	0.018
hsa-miR-203	14q 32.3	-4.255	0.004
hsa-miR-23b	9q22.3	-1.626	0.014
hsa-miR-27b	9q22.3	-1.942	0.048

NOTE: Two-sided *t* tests and Q value (false-positive rate) were calculated on three sets of independent experimental data. All differentially expressed miRs have Q value of <0.01 and P value of <0.05 for *t* test. These miRs were identified by predictive analysis of microarrays as predictor with lowest misclassification error. Four miRs are up-regulated (marked with "+") and three are down-regulated (marked with "-") in the CRPC cell line LNCaP-Abl compared with the androgen-dependent LNCaP cells.

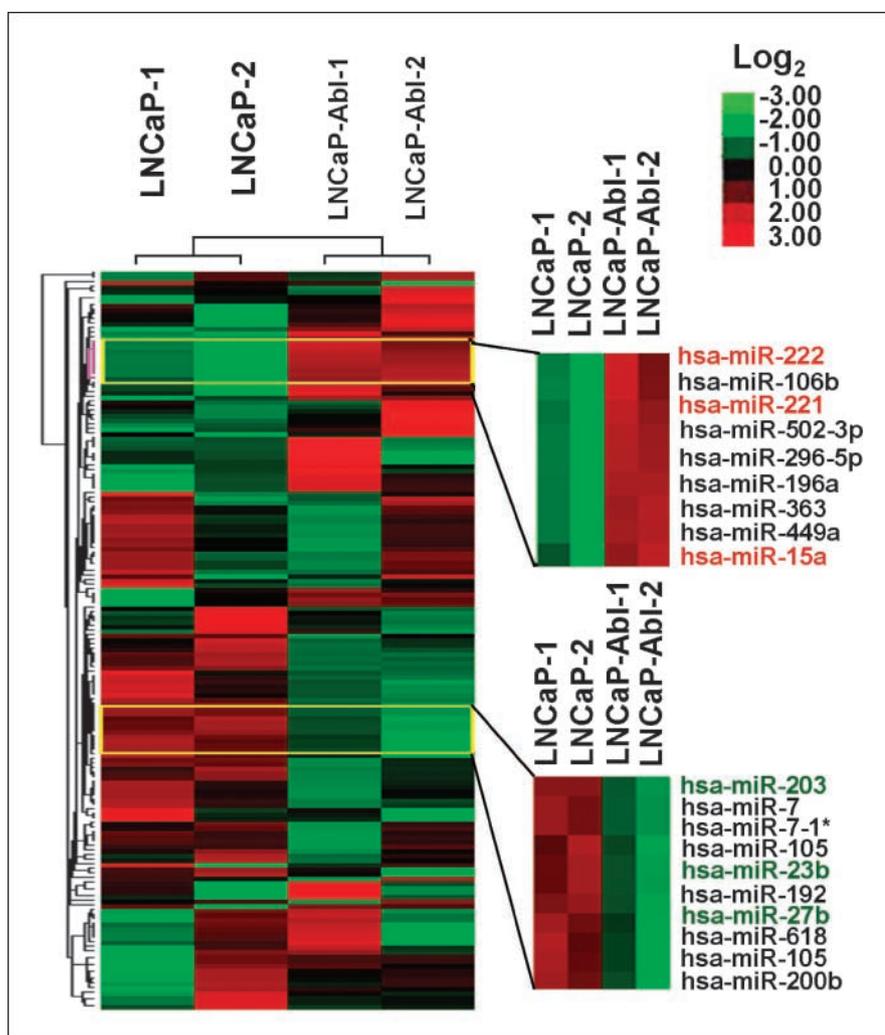


Figure 1. Comparison of miR expression patterns in LNCaP and LNCaP-Abl. Fold changes (LNCaP-Abl versus LNCaP) of the miRs are presented. LNCaP-1/LNCaP-Abl-1 and LNCaP-2/LNCaP-Abl-2 represent two independently performed experiments. The tree graphs display the log₂ transformation of the average fold changes. Arrays were mean centered and normalized by Gene Cluster 2.0. Average linkage clustering was performed by using uncensored correlation metric. *Scale bar*, the color range and the corresponding log₂ transformation of average fold changes (*right top corner*).

1.5- to 10- fold change, compared with those in LNCaP (Table 1; Fig. 1, *right top*; red, ones statistical significantly up-regulated in LNCaP-Abl cells). Remarkably, miR-221 and miR-222 showed a 6- to 10-fold overexpression in LNCaP-Abl compared with LNCaP. Additionally, the expression of miR-203, miR-23b, and miR-27b was reproducibly down-regulated in LNCaP-Abl by 4.3-, 1.6-, and 1.9-fold, respectively, compared with those in LNCaP (Table 1; Fig. 1, *right bottom*, green).

To validate the differential expression patterns of these seven miRNAs, revealed by microarray analysis, we performed qRT-PCR for each individual miR in LNCaP, LNCaP-Abl, and two additional LNCaP-derived androgen-independent cell lines, LNCaP-104R2 and LNCaP-C4-2 (Fig. 2). The analysis confirmed that expression of miR-221, miR-222, miR-15a, and miR-16-1 was up-regulated in CRPC cells and expression of miR-203, miR-23b, and -27b is down-regulated in all three independently derived CRPC cell lines, compared with those in LNCaP. Interestingly, the dramatic up-regulation of miR-221 and miR-222 (5.9- to 7.1-fold increase for miR-221 and 3.9- to 4.7-fold increase for miR-222, respectively) was reproducibly observed in LNCaP-Abl, LNCaP-104R2, and LNCaP-C4-2, suggesting their functional importance in CRPC development. Because of the magnitude of the change in expression, we decided to focus on the significance of miR-221 and miR-222 in the development and maintenance of CRPC.

The effect of miR-221 and miR-222 expression levels on the AR-mediated transcription in response to the DHT treatment.

MiR-221 and miR-222 are encoded in tandem from a gene cluster on the short arm of chromosome X, and their expression is presumably controlled by shared regulatory mechanisms (20). To evaluate the significance of miR-221 and miR-222 in controlling CRPC development, we determined the level of AR-mediated PSA transcription in response to the DHT treatment, when the expression level of miR-221 and/or miR-222 was altered in LNCaP or LNCaP-Abl. To overexpress miR-221 or miR-222, we transiently transfected cell lines with commercially synthesized miR precursors, Pre-miR-221 or Pre-miR-222 (or with the synthetic mature miR-221 duplex). To knock down the expression of miR-221 or miR-222, cell lines were transiently transfected with commercially synthesized anti-miR miRNA inhibitors (Anti-miR-221 or Anti-miR-222). The overexpression or down-regulation of each miR after transfection was confirmed by reverse transcription-PCR (RT-PCR) as shown in the left panel of Fig. 3A and B.

LNCaP expressed a relatively low level of miR-221 and miR-222. Overexpression of miR-221 and/or miR-222 in LNCaP significantly reduced the sensitivity to DHT treatment, as assessed by the reduction of DHT induced PSA mRNA expression by 43% (miR-221 alone), 30% (miR-222 alone), and 48% (miR-221 and miR-222) respectively, and the control transfection by overexpressing a

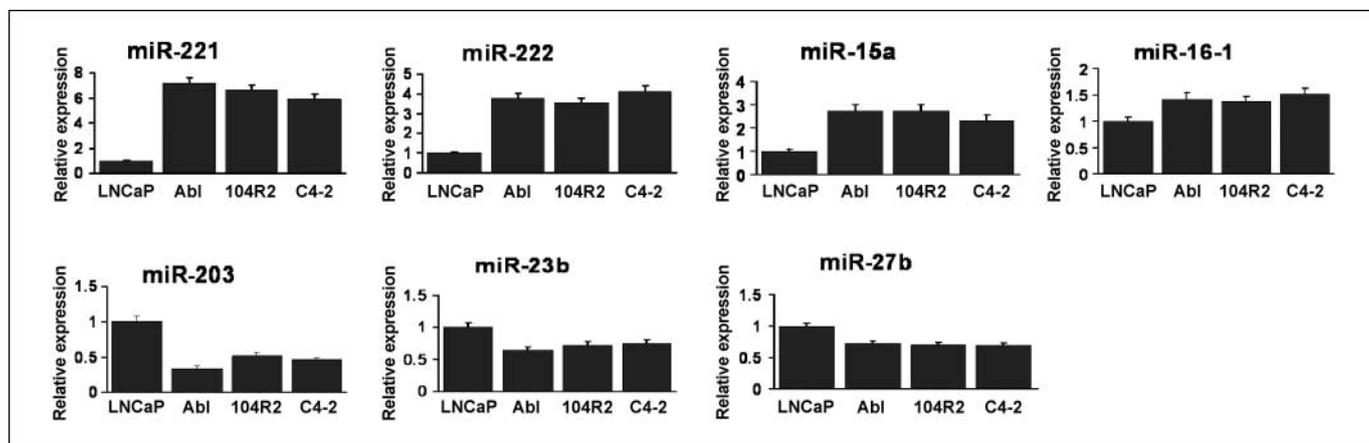


Figure 2. The relative expression of selected miRNAs in LNCaP and LNCaP-derived CRPC cell lines. Twenty-five nanograms of total RNA from each cell line were used to measure miR expression levels by qRT-PCR. All of the data were normalized by the U6 expression level and presented as the relative expression level. The relative expression level of each miR in LNCaP was arbitrarily set as 1.0.

commercially synthesized control miR (Neg RNA) did not affect the response of LNCaP to DHT (Fig. 3A, *middle*). The reduction of response to DHT was also observed in LNCaP cells that were transfected with the synthetic mature miR-221 duplex (data not shown). Because AR mediates PSA transcription, as a control, we determined the level of AR in each transfected cell line. Overexpression of miR-221 and miR-222 had no significant effect on the AR expression level in LNCaP (Fig. 3A, *right*).

LNCaP-Abl expressed miR-221 and miR-222 at a relatively high level. Transfection of LNCaP-Abl with anti-miR-221 and anti-miR-222 to knock down their expression significantly restored the sensitivity of LNCaP-Abl to DHT as indicated by the increased level of DHT induced PSA mRNA expression (Fig. 3B, *middle*). Transfection with a nonspecific anti-miR (Neg RNA) did not affect PSA transcription in response to DHT (Fig. 3B, *middle*), and the AR expression level in LNCaP-Abl cells was not affected by knocking down miR-221 and miR-222 expressions (Fig. 3B, *right*).

To further confirm the effect of miR-221 expression level on the response of CaP cells to the DHT treatment, we overexpressed miR-221 in another androgen-dependent CaP cell line, LAPC-4, which possesses a wild-type AR. LAPC-4 expressed a relatively low level of miR-221/-222, comparable with that in LNCaP (data not shown). Again, overexpression of miR-221 significantly reduced the DHT-induced PSA mRNA expression in LAPC-4 by ~39% (Fig. 3C). We also noticed that the basal level expression of PSA in LAPC-4 is much lower than that in LNCaP (Fig. 3C).

To assure that the influence of miR-221 and miR-222 expressions on the response to DHT in LNCaP and LNCaP-Abl was mediated by AR, we further examined the effect of antiandrogens [AR antagonists; flutamide (Eulexin) and bicalutamide (Casodex)] on the influence of miR-221. Treatment with antiandrogens completely blocked the restored DHT-induction of PSA expression (~2.5-fold) that was generated by knocking down miR-221, in LNCaP-Abl (Fig. 3D, *bottom*). As a control, we confirmed the inhibitory effect of flutamide and bicalutamide on the DHT response in LNCaP with or without miR-221 overexpression. As anticipated, antiandrogens reduced the DHT-induced PSA expression to ~52% in LNCaP control cells; treatment with antiandrogens further reduced the level of DHT induced PSA mRNA in Pre-miR-221-transfected LNCaP (Fig. 3D, *top*). In summary, these data indicate that the expression level of miR-221 and miR-222 did

not influence the expression of AR; however, they significantly affected the response of AR-mediated PSA expression in response to the DHT treatment in LNCaP and LNCaP-Abl, supporting the involvement of miR-221 and miR-222 in the development and/or maintenance of the androgen-independent phenotype.

The effect of miR-221 expression levels on the DHT dependent cell growth. To corroborate the hypothesis that miR-221/-222 are involved in the development of the androgen-independent phenotype, we further determined whether changing the expression level of miR-221 would influence the growth efficiency of LNCaP and LNCaP-Abl cells in the presence or absence of androgen. Cell proliferation was measured using the WST-1 assay. Figure 4A shows the growth of Pre-miR-221-transfected LNCaP with or without the DHT treatment, compared with those of the mock-transfected LNCaP and LNCaP-transfected with Pre-miR-negative control. A high level of overexpression of miR-221 in pre-miR-221-transfected LNCaP can be observed up to 3.5 days posttransfection (data not shown). As anticipated, the addition of DHT increased the growth of LNCaP by ~2.1-fold. Interestingly, overexpression of miR-221 increased the growth of LNCaP in the absence of androgen 2.6-fold, and further addition of DHT into Pre-miR221-transfected LNCaP only increased cell growth 1.4-fold.

In contrast, addition of DHT did not significantly affect the growth rate of LNCaP-Abl cells (Fig. 4B). However, transfection with anti-miR-221 to knock down the miR-221 expression in LNCaP-Abl reduced the cell growth by ~48%, and the growth of anti-miR-221-transfected LNCaP-Abl was increased by 1.8-fold in response to the DHT treatment (Fig. 4B). Transfection of cells with nonspecific anti-miR did not affect cell growth in the absence or presence of DHT, indicating that simply introducing small RNAs is not sufficient to affect cell growth or growth response to DHT. The cell growth analysis showed that increasing the miR-221 expression increases DHT-independent growth and reduces the cell response to the DHT stimulation in both LNCaP and LNCaP-Abl. This result further suggested that a high expression level of miR-221 and miR-222 is a requisite for maintaining the CRPC phenotype.

Role of known targets of miR-221 and miR-222 in CRPC development. Up-regulation of miR-221/-222 in CRPC cells presumably affects the expression of key components controlling the development of androgen independency. To further understand

the involved mechanisms, we analyzed the expression of some known targets of miR-221/-222. The tumor suppressor p27/kip1 and oncogene kit-1 are two known targets (20–22). However, kit-1 is not expressed in prostate cells (data not shown). P27/kip1 belongs to Cip/Kip family and possesses the ability to inhibit several cyclin-dependent kinase complexes (23). Expression level of p27/kip1 can be regulated by miR-221/-222 in CaP cells (20).

Loss of p27/kip1 expression could explain the function of miR-221/-222 that stimulates cell proliferation and survival by up-regulating G₁-S phase transition in the cell cycle (21, 24). We investigated the possibility of the involvement of p27/kip1 in CRPC development.

Western blot analysis of CaP cells with an anti-p27/kip1 antibody revealed that all three LNCaP-derived CRPC cell lines exhibit a

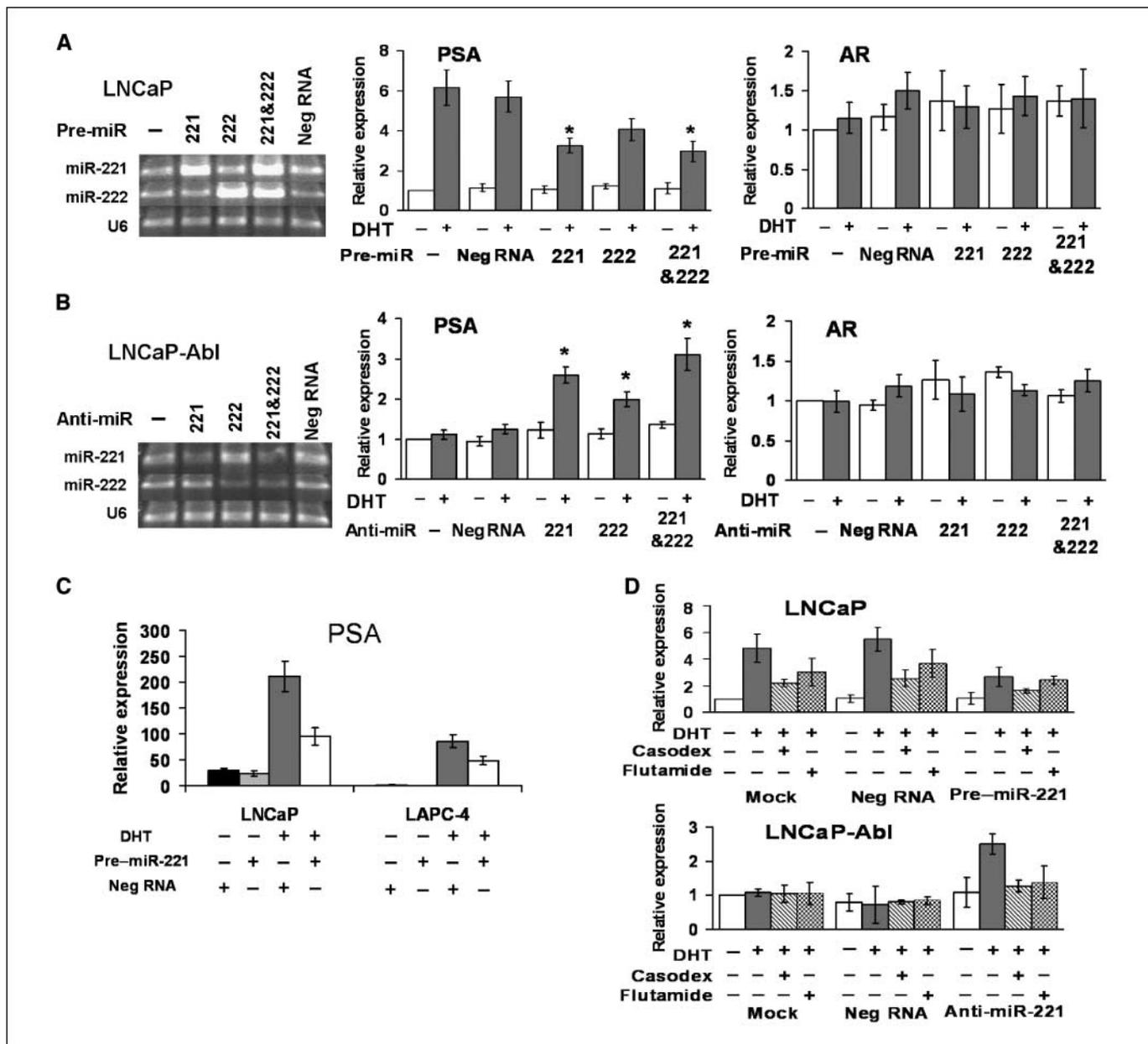


Figure 3. The effect of miR-221 and miR-222 expression levels on the AR-mediated transcription in response to the DHT treatment. *A* and *B*, quantitative analysis of the expression level of PSA and AR in LNCaP (*A*) and LNCaP-Abl (*B*) with (+) or without androgen, DHT (-). Total RNAs were isolated from LNCaP that were mock-transfected (-) and transfected with pre-miR-221 (221), pre-miR-222 (222), pre-miR-221&222 (221&222), or pre-miR-Neg RNA (Neg RNA), and from LNCaP-Abl that were mock-transfected (-) and transfected with anti-miR-221 (221), anti-miR-222 (222), anti-miR-221&222 (221&222), or anti-miR-Neg RNA (Neg RNA). *Left*, the expression levels of miR-221 and miR-222 by RT-PCR in each transfected LNCaP (*A*) and LNCaP-Abl (*B*) cell line. The expression of U6 was used as a control. *Middle*, the PSA expression levels analyzed by qRT-PCR. *Right*, the AR expression in each transfected cell line measured by qRT-PCR. *C*, comparison of the DHT-induced PSA expression in LNCaP and LAPC-4. Cells were transfected with (+) or without (-) pre-miR-221 or pre-miR-Neg. *D*, effect of antiandrogen treatment on the PSA expression induced by DHT. LNCaP and LNCaP-Abl were first transfected with Pre-miR-Negative (Neg RNA) or Pre-miR-221, and Anti-miR-Negative (Neg RNA) or Anti-miR-221, or without transfection (Mock) as indicated, respectively, and then treated with (+) or without (-) DHT, Casodex, and Flutamide. In all experiments, the relative expression levels of PSA and AR in each sample were normalized by the expression level of GAPDH. Values represent the fold differences relative to those in cells without any drug treatment or transfection (Mock), which were set as 1.0. *, the fold changes of those transfected samples compared with their corresponding negative controls show a *P* value of <0.05 in one-way ANOVA.

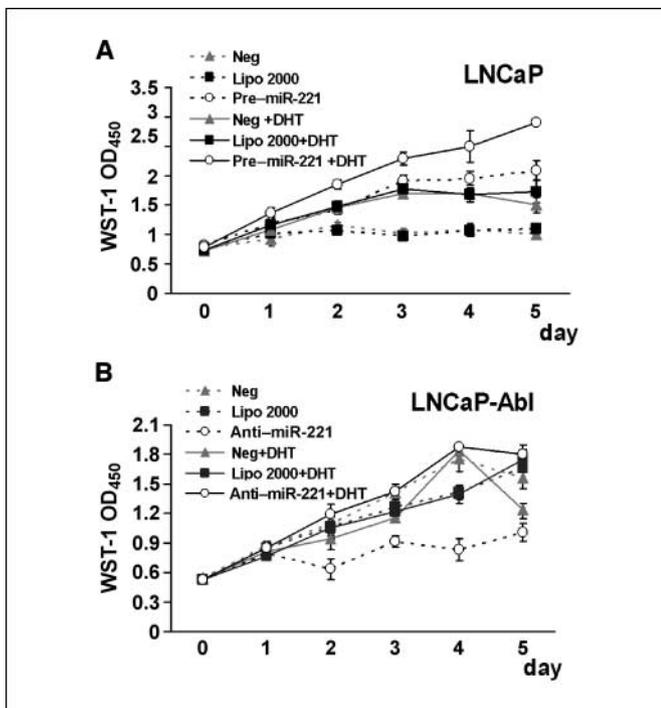


Figure 4. Effect of the expression level of miR-221 on the growth of LNCaP and LNCaP-Abl. **A**, WST-1 analysis of the growth of LNCaP cells that were transfected with Pre-miR-221, miRNA-precursor-negative control (*Neg*), mock transfected (*Lipo 2000*), and kept in medium with (+DHT, *solid lines*) or without (*broken lines*) androgen. **B**, WST-1 analysis of the growth of LNCaP-Abl cells that were transfected with Anti-miR-221, Anti-miRNA inhibitors negative control, mock transfected, and kept in medium with or without androgen. Triplicate experiments were performed for each set. *Points*, mean ($n = 3$); *bars*, SD.

similar p27/kip1 expression level to that in LNCaP, despite the fact that miR-221 and miR-222 are expressed at significantly higher levels in CRPC cells (Fig. 5A). This result indicated that p27/kip1 in LNCaP-derived CRPC cells is not the mechanistic target of miR-221/-222. Nevertheless, as reported by others (20, 21, 24), we also found that miR-221 and miR-222 can control the p27/kip1 expression level in LNCaP (Fig. 5B). Overexpression of miR-221 and miR-222 in LNCaP significantly suppressed the expression of p27/kip1 (Fig. 5B). The down-regulation of p27/kip1 by the overexpression of miR-221/-222 can be observed up to 5 days after transfection (data not shown). We postulated that the LNCaP-derived CRPCs that were developed via the activation of miR-221/-222 may require a certain amount of p27/kip1 to maintain their phenotype; as a result, mechanisms may be developed to allow p27/kip1 to escape from the control of miR-221/-222 in CRPCs.

To further address the significance of the expression level of p27/kip1 in the CRPC development in LNCaP, we determined the effect of the p27/kip1 expression level on cell growth and cell responses to the DHT treatment. As documented (20), we also found that 5 days after knocking down p27/kip1 by siRNA, the growth of LNCaP was increased by ~2.2-fold, compared with those were mock transfected or transfected with negative control siRNAs (Fig. 5C). Addition of DHT further increased the growth of p27/kip1-siRNA-transfected LNCaP by ~1.2-fold, suggesting that knocking down p27/kip1 was not sufficient to abolish the response of LNCaP to DHT (Fig. 5C). Furthermore, it seemed that either knocking down or overexpressing p27/kip1 had no significant effect on the DHT-induced PSA expression in LNCaP

(Fig. 5D). Thus, altering the expression level of p27/kip1 alone significantly affects cell growth, although it is not sufficient to completely eliminate the androgen response. In summary, it does not seem that p27/kip1 is the mechanistic target of miR-221/-222 during the development of LNCaP-derived CRPC cells; although p27/kip1 may be important for the maintenance of the CRPC phenotype.

Discussion

In the last few years, extensive evidence has shown that miRs play important roles in controlling many fundamental cellular

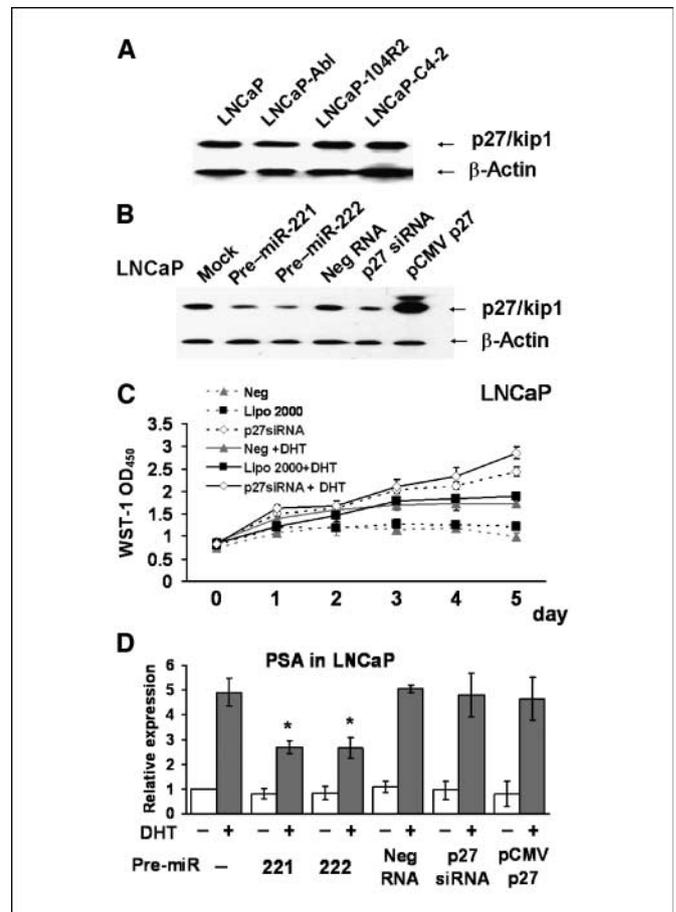


Figure 5. Effect of p27/kip1 expression on the DHT induction of the PSA expression in LNCaP. **A**, Western blot analysis of p27/kip1 in LNCaP and LNCaP-derived CRPC cell lines. The amount of β -actin in each lane is used as a loading control. **B**, Western blot analysis of p27/kip1 in LNCaP cells that were mock-transfected (*Mock*) or transfected with Pre-miR-221, Pre-miR-222, miRNA precursor-negative control (*Neg RNA*), p27/kip1 siRNA (*p27 siRNA*), or pCMV-SOPRT 6-p27/kip1 vector (*pCMV p27*). Total proteins were extracted from cells 48 h after transfection. **C**, the effect of p27/kip1 siRNA on the growth of LNCaP. WST-1 assay was used to measure the cell growth. LNCaP cells that were transfected with p27/kip1 siRNA (*p27siRNA*, *open squares*), anti-miRNA inhibitors negative control (*Neg*, *triangles*), and mock transfected (*Lipo 2000*, *black squares*) were cultured in medium with (+DHT, *solid lines*) or without (*broken lines*) DHT. Triplicate experiments were performed for each set. *Points*, mean ($n = 3$); *bars*, SD. **D**, QRT-PCR of the relative PSA expression level in transfected LNCaP cells as those described in **B**. The relative expression level of PSA in each sample was normalized by the expression level of GAPDH. Values represent the fold differences relative to that in mock-transfected cells without any drug treatment, which was arbitrarily set as 1.0. *, the fold changes of those transfected samples compared with their corresponding negative control exhibited a P value of <0.05 in one-way ANOVA analysis.

processes and in cancer gene regulation (7, 8). A number of miRs have been identified that function as oncogenes or tumor suppressors; so-called oncomirs (9). A profound understanding of the insight of biological mechanisms that a specific miR involved is a prerequisite for the potential use of miRs and miR-related machinery for therapeutic purposes. Despite the extensive studies carried out thus far, relatively little is known about the molecular details on how specific miRs regulate biological pathways.

In this study, we focused on miR expression signatures of CRPC, the lethal phenotype of CaP. Our miR expression profiling identified 7 miRs, miR-221, miR-222, miR-15a, miR-16-1, miR-203, miR-23b, and miR-27b, which are differentially expressed in androgen-dependent LNCaP and the CRPC LNCaP-Abl cell lines. We further validated the significance of *miR-221* and *miR-222* in the development of androgen resistance in CaP cells because these two miRs have the highest overexpression in CRPC cells. We showed that transfection of synthetic miR-221 or miR-222 reduced the DHT-induced growth and the AR-mediated transcription response in LNCaP; transfection of antisense miR-221 or miR-222 restored to a significant extent of the DHT-induced growth and the AR-mediated transcriptional response in LNCaP-Abl. Taken together, our results show that miR-221 and miR-222 dictate the AR-mediated androgen sensitivity of CaP cells, suggesting their function in controlling the expression of key factors involved in the CRPC development and/or maintenance.

The role of miRs in CaP development and progression has been explored by several other laboratories (25–28). Shi and colleagues (25) reported that miR-125b is differentially expressed in androgen-dependent (LNCaP) and androgen-independent (*cds1* and *cds2* cell lines) CaP cells and may be involved in the oncogenic process in CaP cells. However, no evidence indicating the role of miR-125b in CRPC development was presented. In contrast, our microarray profiling data reproducibly showed that miR-125b expressed at a relatively high and equal level in both LNCaP and LNCaP-Abl cell lines. Lin and colleagues (26) showed that miR-146a was down-regulated in androgen-independent cell lines (PC3 and LNCaP-C4-2B), compared with those in androgen-dependent cell lines (PC-3-AR9 and LNCaP), respectively. They showed that transfection of miR-146a markedly reduced cell proliferation, invasion, and metastasis, and they did not have direct evidence indicating the role of miR-146a in CRPC development. Based on our expression profiling data, we found that miR-146a is expressed at very low levels in LNCaP and its expression was not detectable in LNCaP-Abl. Thus, the biological significance of *miR-146a* in LNCaP and related CaP cell lines remains to be further investigated.

Recent reports revealed that the receptor tyrosine kinase kit-1 and the cyclin-dependent kinase inhibitors, p27/kip1 and p57/kip2, are the functional targets of both miR-221 and miR-222 (20–22, 24, 29–32). It has been shown that miR-221/-222 inhibit normal erythropoiesis and erythroleukemic cell growth via kit receptor down-regulation (22, 30). However, kit-1 is not expressed in prostate epithelial cells, thus eliminating the possibility of being a target of miR-221/-222 in CaP cell lines. Loss of p27/kip1 and p57/kip2 expression as a result of up-regulation of miR-221 and miR-222 may stimulate cell proliferation and provide a survival signal by up-regulating G₁-S phase transition in the cell cycle (21, 24, 32). Apparently, certain cancer cell lines require high levels of miR-221 and miR-222 to maintain low level of

p27/kip1 to promote cell proliferation. High levels of miR-221 and miR-222 occur in glioblastomas and in the CaP cell line PC3 and correlate with the expression of p27/kip1. Suppressing miR-221 level in PC3 cells resulted in a growth arrest, which coincided with an up-regulation of p27/kip1 protein, and inability to grow in soft agar. Based on this observation, Galardi and colleagues (20) speculated that miR-221/222 contributed to prostate oncogenesis and progression via down-regulating p27/kip1. More recently, Garofalo and colleagues (33) reported that high expression levels of miR-221 and miR-222 are needed to maintain the resistance to tumor necrosis factor-related apoptosis-inducing ligand in human non-small cell lung cancer, via the down-regulation of p27/kip1.

In our study, it seemed that the expression levels of p27/kip1 in LNCaP-derived CRPC cell lines was not significantly down-regulated by their high levels of miR-221 and miR-222, compared with those in LNCaP. Indeed, overexpression of miR-221 or miR-222 can efficiently down-regulate p27/kip1 expression in LNCaP. However down-regulation of p27/kip1 alone did not significantly affect the DHT induction of the AR-mediated expression of PSA in LNCaP. Together, we hypothesize that p27/kip1 may not be the primary factor leading to the development of LNCaP-derived CRPC. Currently, we do not know the exact miR-221/-222 targets that are involved in the CRPC development in LNCaP-Abl. Nevertheless, the fact that p27/kip1 escapes from the control by miR-221/-222 in LNCaP-derived CRPC is intriguing and it may suggest the requirement of p27/kip1 for the maintenance of CRPC. We do not know why and how the expression level of p27/kip1 is not responding to the high level of miR-221/-222 in LNCaP-Abl. It is possible that the 3' untranslated regions of the *p27/kip1* mRNA in LNCaP and LNCaP-Abl may be structurally different, thus potentially presenting different sequences at miRNA target sites. Such mechanisms were described for posttranscriptional control of miR-targets by Sanberg (34). Alternatively, the activity of the RNA-binding protein, such as Dnd1 that counteracts the function of miRNAs may be up-regulated in LNCaP-Abl (35). We are currently investigating these possibilities.

Studies have shown that CaP cells use a variety of AR-dependent and AR-independent pathways progressing to CRPC. Most miRs have multiple targets ranging from dozens to hundreds and target mRNAs may also be the target of multiple miRs. Thus, it is not surprised that different targets may be involved in the development of different CRPC cells via the same or different miRs. In addition to miR-221 and miR-222, we also found that miR-15a, miR-16-1, miR-203, miR-23b, and miR-27b are differentially expressed in LNCaP and LNCaP-derived CRPC cell lines. We are currently analyzing the role of these miRs in the CRPC development. We believe that our study on these differentially expressed miRs will lead to a better understanding of mechanisms mediating the development and progression of CRPC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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The Role of microRNA-221 and microRNA-222 in Androgen-Independent Prostate Cancer Cell Lines

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