MicroRNA-616 Induces Androgen-Independent Growth of Prostate Cancer Cells by Suppressing Expression of Tissue Factor Pathway Inhibitor TFPI-2

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Abstract

Expression of microRNA genes is profoundly altered in cancer but their role in the development of androgen-independent prostate cancer has received limited attention as yet. In this study, we report a functional impact in prostate cancer cells for overexpression of the microRNA miR-616, which occurred consistently in cells that were androgen-independent (AI) versus androgen-dependent (AD). miR-616 overexpression was confirmed in malignant prostate tissues as opposed to benign prostate specimens. Stable miR-616 overexpression in LNCaP cells by a lentiviral-based approach stimulated AI prostate cancer cell proliferation in vitro whereas concomitantly reducing androgen-induced cell growth. More importantly, miR-616 overexpressing LNCaP cells overcame castration resistance as shown by an enhanced ability to proliferate in vivo after bilateral orchectomy. Conversely, antagonizing miR-616 in AI prostate cancer cells yielded opposite effects. Microarray profiling and bioinformatics analysis identified the tissue factor pathway inhibitor TFPI-2 mRNA as a candidate downstream target of miR-616. In support of this candidacy, we documented interactions between miR-616 and the 3’UTR of TFPI-2 and determined TFPI-2 expression to be inversely correlated to miR-616 in a series of prostate cell lines and clinical specimens. Notably, reexpression of TFPI-2 in LNCaP cells with stable miR-616 overexpression rescued the AD phenotype, as shown by a restoration of androgen dependence and cell growth inhibition. Taken together, our findings define a functional involvement for miR-616 and TFPI-2 in the development and maintenance of androgen-independent prostate cancer. Cancer Res; 71(2); 583–92. ©2011 AACR.

Introduction

Prostate cancer is the most common malignancy occurring in men worldwide and is still the second leading cause of cancer deaths (1). Early-stage cancers can be managed by various treatments such as radical prostatectomy, radiation, or hormone ablation therapy. Although the cancer is temporarily manageable by hormone deprivation, it inevitably becomes refractory to hormonal therapy and no effective treatment has been developed for these insensitive cancers (2). Despite the magnitude of this problem, the lack of knowledge concerning the molecular alterations and mechanisms regulating androgen independence has prevented the development of more effective therapies for this lethal phenotype. Therefore, a better understanding of the molecular events by which androgen-dependent (AD) prostate cancer cells acquire the ability to resist androgen ablation may aid not only in the development of novel targets for the treatment of this deadly disease, but also in the improvement of strategies to prolong the survival of patients with androgen-independent (AI) prostate cancer.

Recently, the large class of small, nonprotein-coding RNAs termed microRNAs (miRNAs) has been found to be a new class of gene regulators (3). These molecules have been shown to control fundamental cell functions including proliferation, apoptosis, and differentiation, and consequently their alterations have been suggested to play a role in carcinogenesis (4–6). miRNAs exert their regulatory functions by binding to complementary regions in the 3’-untranslated region (UTR) of their target mRNA transcripts, which results in the posttranscriptional modification or degradation of its target RNA (3). miRNAs have been reported to be involved in prostate cancer (7–11), although only a few studies have been done on AI cancers (12–15). To better understand the role of miRNAs...
in the progression of AI prostate cancer, this study first identified comprehensive miRNA expression profiles in AD LNCaP and AI LNCaP-LNO, 2 sublines derived from the same parental prostate cancer cell line LNCaP but bearing different androgen-responsiveness. Among a number of differentially expressed miRNAs, the expression level of miR-616 had the most dramatic difference between AD and AI prostate cancer cells. Upregulation of miR-616 expression was also detected in malignant as compared with benign prostatic tissues, further supporting our initial finding. We subsequently completed a series of both in vitro and in vivo experiments to evaluate the contribution of miR-616 in AD and castration-resistant prostate cancer growth. Our results suggest the involvement of miR-616 and tissue factor pathway inhibitor 2 (TFPI-2) in the development and maintenance of AI prostate cancer.

Materials and Methods

Clinical samples and tissue microarray development

For immunohistochemistry (IHC) analysis of TFPI-2 expression, 113 formalin-fixed, paraffin-embedded prostate tissue specimens were selected from the Department of Pathology, Queen Mary Hospital, The University of Hong Kong. The tissue panel included 8 normal, 29 benign prostatic hyperplasia (BPH), and 76 prostate carcinoma specimens. Normal samples were collected from patients that were free from prostate cancer. Four of the normal samples were collected from radical prostatectomy specimens carried out due to bladder cancer or colon cancer invading the bladder. The other 4 normal samples were from autopsy materials of males dying of causes unrelated to prostate pathology. Tissue samples from patients who had undergone prior hormone deprivation or radiation therapy to prostate pathology. miRNA profiling was done using total RNA with the HT419 miRNA qPCR Array (System Biosciences), which simultaneously profiles 419 different human miRNAs. Human small nuclear RNU6B RNA was amplified as a normalization control. All miRNAs were registered in the Sanger miRBase database. Quantitative real-time PCR (qPCR) was carried out using SYBR Green as mentioned above. Analysis was done using the software provided by System Biosciences.

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miRCURY miR-616 (CTGCTCAAACCTCCTCAATGACT) 5′ – digoxigenin (DIG)-labeled probe, the scrambled-miR negative control (GTTAACACGTCTATAGCCCA) 5′ – DIG-labeled probe and the U6 positive control (CACGAATTTGCGTGTTATTCT) 5′ – DIG labeled, LNA-modified oligonucleotide detection probe were purchased from Exiqon, Denmark. Paraffin sections were deparaffinized in xylene and rehydrated in graded alcohol series and distilled water. Sections were then treated with proteinase K (10 μg/mL) for 10 minutes at 37°C, rinsed in 0.2% glycine for 1 minute, and fixed in 4% paraformaldehyde for 10 minutes at 25°C. Before hybridization, sections were prehybridized in hybridization buffer (50% formamide, 5× SSC, 0.1% Tween 20, 50 μg/mL heparin, 500 μg/mL yeast RNA) at 50°C for 2 hours. DIG-labeled LNA probe was diluted to 20 nmol/L in hybridization buffer. Slides were hybridized overnight with the diluted probe in a humidified chamber at 50°C. Sections were then washed with 2× SSC followed by 0.2× SSC at 50°C. After a blocking step (2% sheep serum, 2 mg/mL BSA in PBST) at room temperature for 1 hour, the slides were incubated with anti-DIG-AP Fab fragments (1:1000, Roche Diagnostics) at 4°C overnight. Signal was detected by incubating sections in NBT-BCIP solution (Roche Diagnostics) for up to 4 days. Sections were imaged by the Scanscope system (Aperio) and compared with the hematoxylin and eosin (H&E) image of the corresponding section. The abundance of miR-616 was categorized as high, moderate, or low based on the intensity of staining.

IHC

Paraffin sections were deparaffinized in xylene and rehydrated in a graded alcohol series and distilled water. The slides
were heated for antigen retrieval in 1 mmol/L EDTA (0.05% Tween 20, pH 8.0). The sections were incubated with polyclonal rabbit anti-human TFPI-2 (1:100; Santa Cruz) overnight at 4°C. The EnVision Plus System (Dako) was used for visualization according to manufacturer’s protocol. Staining was revealed by counter-staining with hematoxylin. The slides were then scanned by the ScanScope system (Aperio), and the staining intensity was quantified by the ImageScope positive pixel count algorithm (Aperio). All available cores of each sample were examined, and the core with the highest staining intensity was selected for later statistical analysis. Cores with staining intensities higher than the overall median intensity were classified as having high TFPI-2 expression, whereas those with intensities below the median were classified as having low TFPI-2 expression.

Results

miRNA expression profiling of AD LNCaP versus AI LNCaP-LNO cell lines

To investigate the involvement of miRNAs in AI prostate cancer development and growth, we compared the miRNA expression profiles of the AD LNCaP cell line and its derived AI counterpart, LNCaP-LNO. LNCaP is a widely used cell line model in prostate cancer research that was established almost 30 years ago (20). The cell line was initially isolated from a biopsy of a lymph node metastasis from a 50-year-old man. LNCaP-LNO cell line was derived from this parental line by continual maintenance in androgen-depleted medium (19). Therefore, the relationship between LNCaP and LNCaP-LNO on the tissue microarray resembles the progression of prostate cancer from the AD to the AI state. A total of 419 selected miRNAs known to be involved in all facets of cellular regulation (HT419 microRNA qPCR assay panel, System Biosciences) were analyzed by qPCR. miRNAs that displayed an initial >2-fold difference were tabulated, and the expression of 15 miRNAs was found to be significantly differentially regulated in LNCaP versus LNCaP-LNO cells. Of these, 10 were upregulated (miR-222, miR-331, miR-455, miR-520h, miR-542–5p, miR-569, miR-579, miR-616, miR-671, and miR-801) while 5 were downregulated (miR-135a, miR-181c, miR-365, miR-374, miR-587). From this list, we selected the 9 most highly differentially expressed miRNAs for validation by a second round of qPCR using the more precise TaqMan assay instead of the original SYBR Green method used for the first profiling screen (Fig. 1A). The results were

Figure 1. A, qPCR confirmation of the 9 most significantly deregulated miRNAs identified in the HT419 miRNA qPCR array comparing AI LNCaP-LNO versus AD LNCaP. B, ISH detection of miR-616 abundance in prostate cancer samples. Representative images of different stages are presented, including normal tissue, BPH, GS 7 prostate carcinoma, and GS 9 prostate carcinoma. The tissue sections were also stained with H&E as a reference. C, relative expression of miR-616 in a panel of prostate cell lines representing different AD and AI prostate cancer stages. The expression level was relative to LNCaP, which was arbitrarily set as 1.0. AD, androgen-dependent; AR, androgen-responsive; AI, androgen-independent.
Table 1. miR-616 expression in prostate cancer specimens

<table>
<thead>
<tr>
<th>Stage</th>
<th>N</th>
<th>Low</th>
<th>Moderate</th>
<th>Strong</th>
<th>P</th>
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<tr>
<td>Nontumor</td>
<td>13</td>
<td>8 (61.5%)</td>
<td>5 (38.5%)</td>
<td>0 (0%)</td>
<td>.001*</td>
</tr>
<tr>
<td>Tumor</td>
<td>12</td>
<td>4 (33.3%)</td>
<td>0 (0%)</td>
<td>8 (66.7%)</td>
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reproducible, confirming our initial screen, with the exception of miR-181c and miR-587, which both showed slightly less than a 2-fold change in the validation experiments (Fig. 1A). Because of the magnitude of the change in expression (~7.5-fold), we decided to focus on the significance of miR-616 in the development and maintenance of AI prostate cancer growth.

Detection of miR-616 in clinical prostate cancer samples and cell lines
To examine if our observation in overexpression of miR-616 in cell lines can be extended into clinical specimens, the expression of miR-616 in 25 human primary prostate normal and carcinoma samples was evaluated by ISH using DIG-labeled locked nucleic acid (LNA)–modified miRNA probes. Compared with normal and BPH, which were collectively classified as nontumor tissues, prostate tumor samples showed a relatively more abundant expression of miR-616 ($P = 0.001$; Table 1; Fig. 1B). No signal was detected when DIG-labeled LNA–scrambled miRNA was used as a negative control.

In addition to clinical prostate cancer specimens, the expression of miR-616 was also evaluated by qPCR in a panel of prostate cell lines representing different AD and AI prostate cancer stages. miR-616 was overexpressed in the more aggressive AI cell lines (LNO, C4–2B, 22rv1, and to a lesser extent, PC3 and DU145) when compared with the less aggressive AD cell lines (LNCaP) or the immortalized, normal prostate cell lines (HPrl and NPTX) (Fig. 1C). Taken together, these findings suggest that enhanced miR-616 expression is correlated with advanced stages of prostate cancer.

miR-616 stimulates AI growth in vitro
We hypothesized that elevated miR-616 may promote AI growth. To test this idea, we first tested the effect of miR-616 on the cell proliferation rate of cells grown in androgen-depleted medium. LNCaP cells were stably transduced with an empty vector (EV) control or a miR-616 expressing lentivirus. Similarly, LNCaP-LNO cells were stably transduced with anti-miR-616 (ZIP-616) or antisense scramble controls (ZIP-EV). The overexpression or downregulation of miR-616 after infection was confirmed by both qPCR (Fig. 2A) and the imaging of green fluorescent protein, which is also expressed upon successful transduction of the miRNA of interest (Supporting Information SI Fig. 1). Cell proliferation was measured using the XTT assay. Elevated miR-616 alone was sufficient to rescue LNCaP from androgen-ablated growth arrest and drive AI growth. In contrast, anti-miR-616, but not the corresponding antisense scramble control, significantly inhibited the growth of LNCaP-LNO cells when grown in androgen-depleted medium (Fig. 2B). Light microscopic analysis showed that the miR-616-transduced LNCaP cells retained typical morphological features when grown for 6 days in the absence of androgens, whereas those treated with the EV control acquired a neuroendocrine (NE) phenotype (21) morphologically characterized by the rounding of the cell bodies and neuron-like extensions. Similarly, the inhibition of miR-616 activity with anti-miR-616 in LNCaP-LNO cells resulted in a reduction in cell size and increased NE features compared with the antisense control–treated cells, LNCaP-LNO ZIP-EV (Fig. 2C).

The effect of androgens on the growth efficiency and expression of miR-616 was also evaluated in LNCaP cells that were stably transduced with miR-616 or in LNCaP-LNO cells with stable repression of miR-616. The addition of the synthetic androgen R1881 increased the growth rate of LNCaP by a factor of approximately 2. Interestingly, overexpression of miR-616 increased the growth rate of LNCaP in the absence of androgen approximately 2.5-fold, and further addition of R1881 in miR-616–transduced LNCaP cells only resulted in a moderate increase in cell growth. In contrast, the addition of R1881 did not significantly affect the growth rate of LNCaP-LNO cells. Transduction with antisense specifically against miR-616 in LNCaP-LNO reduced the cell growth by approximately 3-fold, and the growth of anti-miR-616–transduced LNCaP-LNO was increased by approximately 1.8-fold in response to R1881 treatment (Fig. 2D). In parallel experiments, the transduction of cells with nonspecific antisense scramble controls did not affect cell growth in the absence or presence of R1881.

miR-616 promotes castration resistance in vivo
To further confirm these in vitro observations, in vivo studies were then carried out in a prostate cancer xenograft model. The AI cell line 22rv1 was lentivirally transduced to stably repress miR-616 or an antisense scramble control and was subcutaneously implanted into the flanks of nude mice that have undergone bilateral orchiectomy. The 22rv1 cells with miR-616 repressed displayed a delay in palpable tumor onset and a significant decrease in tumor growth when compared with controls ($n = 6$, Figs. 3A–B). H&E staining of the xenografted tumors confirmed a prostate cancer phenotype (Fig. 3C). Similarly, AD LNCaP cells that were lentivirally transduced to stably express miR-616 or an EV control were subcutaneously implanted into the flanks of nude mice. The LNCaP miR-616 and EV controls had similar tumor intakes and tumor onset times. When the tumors reached an average volume of 300 mm$^3$, a bilateral orchectomy was carried out on the animals. The 22rv1 cells with miR-616 repressed displayed a delay in palpable tumor onset and a significant decrease in tumor growth when compared with controls ($n = 6$, Figs. 3A–B). H&E staining of the xenografted tumors confirmed a prostate cancer phenotype (Fig. 3C). Similarly, AD LNCaP cells that were lentivirally transduced to stably express miR-616 or an EV control were subcutaneously implanted into the flanks of nude mice. The LNCaP miR-616 and EV controls had similar tumor intakes and tumor onset times. When the tumors reached an average volume of 300 mm$^3$, a bilateral orchectomy was carried out on the animals. The 22rv1 cells with miR-616 repressed displayed a delay in palpable tumor onset and a significant decrease in tumor growth when compared with controls ($n = 6$, Figs. 3A–B). H&E staining of the xenografted tumors confirmed a prostate cancer phenotype (Fig. 3C). Similarly, AD LNCaP cells that were lentivirally transduced to stably express miR-616 or an EV control were subcutaneously implanted into the flanks of nude mice. The LNCaP miR-616 and EV controls had similar tumor intakes and tumor onset times. When the tumors reached an average volume of 300 mm$^3$, a bilateral orchectomy was carried out on the animals. The 22rv1 cells with miR-616 repressed displayed a delay in palpable tumor onset and a significant decrease in tumor growth when compared with controls ($n = 6$, Figs. 3A–B). H&E staining of the xenografted tumors confirmed a prostate cancer phenotype (Fig. 3C). Similarly, AD LNCaP cells that were lentivirally transduced to stably express miR-616 or an EV control were subcutaneously implanted into the flanks of nude mice. The LNCaP miR-616 and EV controls had similar tumor intakes and tumor onset times. When the tumors reached an average volume of 300 mm$^3$, a bilateral orchectomy was carried out on the animals. The 22rv1 cells with miR-616 repressed displayed a delay in palpable tumor onset and a significant decrease in tumor growth when compared with controls ($n = 6$, Figs. 3A–B).
TFPI-2 is a direct target of miR-616

The higher expression of miR-616 in AI prostate cancer may affect the expression of key components controlling the development of prostate tumorigenesis. Recent evidence indicates that miRNAs can make their target mRNAs unstable, thus reducing the target mRNA amounts to a level below the threshold of detection by standard microarray analysis (22). Therefore, we predicted that exogenously applied miR-616 might directly affect the mRNA levels of the genes that are naturally regulated by the miRNA and indirectly affect the expression of genes that are downstream of these direct targets, leading to measurable changes in the global expression profiles of the treated cells. Thus, in an effort to determine the potential downstream mRNA targets regulated by miR-616, oligonucleotide microarray analyses were done to compare the profiles of LNCaP cells that stably expressed either miR-616 or the EV control. Microarray analyses identified 5 downregulated genes with a greater than 2-fold difference (SI Table 2). Of these, only 2 were tumor-suppressor genes—TXNIP and TFPI-2. Preliminary analysis on the 3'UTR of TXNIP and the seed region of miR-616 failed to reveal any potential binding region. However, a computer search of the 3'UTR of TFPI-2 identified a putative binding region (nt 871–886) with significant complementarity to miR-616, suggesting that TFPI-2 may be a direct target of miR-616 (Fig. 4A). The 3'UTR sequence of TFPI-2 and miR-616 are extremely well conserved among different species, as shown by their identical sequence in different orthologs (Fig. 4A). To validate whether TFPI-2 is a bona fide target of miR-616, a human TFPI-2 3'UTR fragment was cloned downstream of the firefly luciferase reporter gene in both the sense and antisense directions. Fig. 4B schematically illustrates the reporter constructs used to evaluate the capacity of miR-616 to regulate TFPI-2 by binding to the putative target site. HEK293 cells expressing barely detectable levels of miR-616 were cotransfected with the TFPI-2 3'UTR reporter constructs in either the sense or antisense directions, the pRL-TK plasmid (to normalize transfection efficiency) and synthetic miR-616 precursor RNA or miR-control. Compared with mock experiments, luciferase activity was reduced by approximately 38% in the cells cotransfected with the miR-616 and the TFPI-2 3'UTR in the sense orientation. This inhibitory effect was abolished when an antisense TFPI-2 3'UTR construct was used in place of the sense construct, demonstrating specificity (Fig. 4B). In support of these results, a clear inverse pattern was observed in the level
of TFPI-2 expression at both the genomic and proteomic levels following lentivirally enhanced and reduced miR-616 expression in AD LNCaP and AI LNCaP-LNO cells, respectively (Fig. 4C–D). These findings indicate that miR-616 negatively regulates the expression of the tumor-suppressor TFPI-2.

**miR-616 induces AI growth of prostate cancer cells through suppression of TFPI-2**

To provide evidence that TFPI-2 is regulated by miR-616, we examined the expression of TFPI-2 in a series of prostate cell lines. Interestingly, TFPI-2 was found to be inversely correlated with miR-616. Abundant TFPI-2 expression was detected in the prostate cell lines HPr1 and NPTX, which both have low levels of miR-616 expression. Conversely, prostate cell lines that expressed high levels of miR-616 possessed low or no levels of TFPI-2. Interestingly, a stepwise inverse correlation was observed between the expression of miR-616 and TFPI-2 in the LNCaP sublines LNCaP, LNCaP-R, LNCaP-LNO and C4-2B, which have varying androgen responsiveness. Similarly, the AI cell line 22rv1 and immortalized normal HP1 and NPTX cell lines also displayed similar cumulative trends (Figs. 1C and 4E–F).

Next, the expression of TFPI-2 and the correlation of TFPI-2 and miR-616 expression were examined in clinical specimens. TFPI-2 expression was analyzed using a human prostate tissue microarray constructed from 8 normal, 29 BPH, and 74 prostate carcinoma specimens. Overall, normal tissues showed a significantly higher level of TFPI-2 expression than BPH (P = 0.027) and prostate carcinoma cases with GS < 7 (P = 0.007) and GS ≥ 7 (P = 0.002) (Fig. 5A). Specifically, strong TFPI-2 staining was detected in the normal prostate (Fig. 5B, upper left panel), while a less obvious but still significant TFPI-2 expression was observed in BPH (Fig. 5B, upper right panel). Each specimen in the prostate cancer group was classified based on its combined Gleason Score (GS) as either <7 or ≥7. Weak signals were detected in the more poorly differentiated cancerous glandular epithelia, with decreasing expression detected as the cancer progressed from low-grade to high-grade (Fig. 5B, lower panels). Most interestingly, in the subset of these samples in which ISH for miR-616 was also conducted (Fig. 1B), TFPI-2 was found to be significantly negatively associated with miR-616 expression (n = 25;...
Gamma = −0.758; *P = 0.011). For example, low miR-616 expression in BPH was accompanied by high TFPI-2 staining, whereas high miR-616 expression in advanced stage prostate cancer was accompanied by low TFPI-2 staining (Fig. 5C).

To examine the role of TFPI-2 in AI prostate cancer growth, TFPI-2 was stably transfected into 22rv1 AI Cells. After the efficiency of transfection was confirmed by immunoblot assay (Fig. 6A), these cells were tested for their responsiveness to androgen R1881. 22rv1 cells that stably expressed TFPI-2 or the
Figure 5. A, summary of TFPI-2 expression levels in normal, BPH, and prostate carcinoma (GS < 7 or GS ≥ 7) specimens by IHC on a human prostate tissue microarray. B, representative images of IHC staining of TFPI-2 proteins in normal prostate tissue, BPH, low-grade prostate carcinoma (GS 6) and high-grade prostate carcinoma (GS 9). Pictures were taken at 200 × magnification. C, representative images of the inversely correlated expression of miR-616 and TFPI-2 in BPH and advanced stage prostate carcinoma (tumor) by ISH and IHC, respectively. Tissue sections were also stained with H&E as a reference. Pictures were taken at 200 × magnification.

Figure 6. A, verification of TFPI-2 overexpression in 22rv1 cells by immunoblot. B, overexpression of TFPI-2 expression in 22rv1 cells inhibited cell growth and promoted dependence on androgen R1881. C, reexpression of TFPI-2 in LNCaP cells stably overexpressing miR-616 inhibited cell growth and promoted dependence on androgen R1881 (gray columns). LNCaP cells carrying the miR empty vector alone, miR-616 or miR-616 and a pcDNA3.1 empty vector were used as negative controls (white bars). Cells were counted after growth for 6 days in androgen-depleted medium (-R1881) or androgen-depleted medium supplemented with 1 nmol/L R1881 (+R1881).
EV control were grown in androgen-depleted growth medium with or without R1881. The 22rv1 TFPI-2 cells displayed delayed cell growth and greater androgen dependence than the 22rv1 control cells (Fig. 6B). To provide further evidence of the importance of the miR-616 and TFPI-2 interaction in the regulation of AI prostate tumor growth, a rescue experiment was carried out in which AD LNCaP cells that stably overexpressed miR-616 with repressed TFPI-2 expression were cotransfected with TFPI-2. As controls, LNCaP cells transfected with the miR-empty vector alone, miR-616 alone or miR-616 and the pcDNA3.1 empty vector were also tested. The cells were grown for 6 days in AD medium in the presence or absence of androgen R1881. Overexpression of TFPI-2 in LNCaP cells that stably expressed miR-616 inhibited the ability of the cells to grow and increased the dependence of the cells on androgens (Fig. 6C), thus confirming the importance of the interaction between miR-616 and TFPI-2 in the regulation of the AI growth of prostate cancer cells.

Discussion

In the past several years, extensive evidence has shown that miRNAs play important roles in controlling many fundamental cellular processes including those regulating carcinogenesis. A number of studies have now detected frequent alterations of miRNA expression in a variety of human tumors, suggesting that miRNAs may play a role as a novel class of tumor promoters or suppressor. Until recently, only a limited number of studies had been done to assess the effects of miRNA in prostate cancer, and only a few of these studies successfully identified any miRNA targets that were specifically modulated in tumors. In this study, we focused on the miRNA expression signatures of AI prostate cancer, which has the worst prognosis of all the disease forms. Our miRNA expression profiling identified 15 miRNAs that are differentially expressed in the AD LNCaP and AI LNCaP-LNO cell lines. Results from the first part of our study show that miR-616 acts as a tumor promoter and controls the expression of key factors involved in AI prostate cancer development and/or maintenance.

To our knowledge, the role of miRNAs in AI prostate cancer development has been reported in 4 studies thus far. In 2007, Shi et al. found miR-125b to be overexpressed in the AD LNCaP cells compared with their parental AD LNCaP cell line. Consistently, miR-125b was found to be overexpressed in a considerable number of prostate cancer specimens. In addition, transfection of synthetic miR-125b stimulated the AI growth of prostate cancer cells and downregulated the expression of Bak1, which was identified by the authors to be a direct target of miR-125b (15). In 2008, Lin and colleagues showed that miR-146a was downregulated in AI cell lines (PC3 and LNCaP C4–2B) compared with AD cell lines (PC3-AR9 and LNCaP). These authors showed that transfection of miR-146a markedly reduced cell proliferation, invasion, and metastasis via the modulation of ROCK-1 (12). However, no direct evidence was provided indicating the role of miR-146a in AI prostate cancer development. More recently, Sun et al. found that the levels of miR-221 and miR-222 were significantly increased in AI LNCaP-Abl compared with its parental AD LNCaP prostate cell line and that these miRNAs dictate the AR-mediated androgen sensitivity of prostate cancer cells (14). Ribas et al. identified miR-21 as an androgen receptor-regulated miRNA that promotes hormone-dependent prostate cancer growth. These authors showed that elevated miR-21 expression enhanced prostate cancer tumor growth in vivo and was sufficient for AD tumors to overcome castration-mediated growth arrest (13). Unfortunately, the authors failed to provide information on the possible mRNA targets of miR-21. Our miRNA expression profiling results partially agree with the findings from these earlier publications. We found miR-222 but not miR-125b, miR-146a, miR-221, or miR-21 to be differentially expressed between the AI LNCaP-LNO and AD LNCaP prostate cancer cells. The discrepancy between our findings and those of other groups could be explained by the different cell line models used. The biological significance of miR-125b, miR-146a, miR-221, and miR-21 in LNCaP and related prostate cancer cell lines needs to be further investigated.

The second part of our study identified TFPI-2 as a direct functional target of miR-616 as supported by 5 lines of evidence. First, there is a highly conserved miR-616 binding site in the 3′UTR of TFPI-2. Second, the stable overexpression or knockdown of miR-616 reduced and enhanced TFPI-2 levels, respectively, at both the miRNA and protein levels, in a series of prostate cell lines. Third, expression of miR-616 and TFPI-2 were found to be significantly inversely correlated in prostate clinical specimens. Fourth, TFPI-2 3′UTR-mediated luciferase activity is specifically responsive to the transduced expression of miR-616. Fifth, a rescue experiment, where AD LNCaP cells that stably overexpressed miR-616 with repressed TFPI-2 expression and dually cotransfected with TFPI-2, successfully inhibited the cells’ ability to grow and reverted the cells’ dependence on androgen. TFPI-2, also known as PP5 or MSPI, is a 32 kDa Kunitz-type serine protease inhibitor that negatively regulates the enzymatic activities of trypsin, plasmin, and the VIIa-tissue factor complex in a variety of human cells (23). TFPI-2, now considered to be a candidate tumor-suppressor gene, has been implicated in human carcinogenesis and metastasis (24) in association with epigenetic changes. Promoter methylation and downregulation of TFPI-2 is commonly observed in human cancers. For example, TFPI-2 has been reported to have a tumor-suppressive role in hepatocellular carcinoma (25), esophageal carcinoma (26), melanoma (27), glioma (28, 29), pancreatic cancer (30) and non—small-cell lung cancer (31). Specifically in prostate cancer, an early report from 2001 found that TFPI-2 regulates the invasive behavior of prostate cancer cells in vitro (32). Therefore, the downregulation of TFPI-2 by miR-616 may contribute to disease progression and resistance to treatment in prostate cancer. In support of this hypothesis, our prostate tissue microarray results found that TFPI-2 was significantly downregulated in prostate carcinoma compared with normal and BPH tissues.

In conclusion, we believe findings from this current study, on the interplay between miR-616 and TFPI-2, will lead to a
better understanding of the mechanism mediating the development and progression of AI prostate cancer. This will hopefully contribute to the development of more effective treatments that maybe used to prolong the survival of patients with the deadliest form of this disease.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


Grant Support

This work was generously supported by The University of Hong Kong Small Project Funding Program.

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Received July 15, 2010; revised October 21, 2010; accepted November 3, 2010; published online January 15, 2011.
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