Genistein Up-Regulates Tumor Suppressor MicroRNA-574-3p in Prostate Cancer

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Abstract

Genistein has been shown to inhibit cancers both in vitro and in vivo, by altering the expression of several microRNAs (miRNAs). In this study, we focused on tumor suppressor miRNAs regulated by genistein and investigated their function in prostate cancer (PCa) and target pathways. Using miRNA microarray analysis and real-time RT-PCR we observed that miR-574-3p was significantly up-regulated in PCa cells treated with genistein compared with vehicle control. The expression of miR-574-3p was significantly lower in PCa cell lines and clinical PCa tissues compared with normal prostate cells (RWPE-1) and adjacent normal tissues. Low expression level of miR-574-3p was correlated with advanced tumor stage and higher Gleason score in PCa specimens. Re-expression of miR-574-3p in PCa cells significantly inhibited cell proliferation, migration and invasion in vitro and in vivo. miR-574-3p restoration induced apoptosis through reducing Bcl-xL and activating caspase-9 and caspase-3. Using GeneCodis software analysis, several pathways affected by miR-574-3p were identified, such as 'Pathways in cancer', 'Jak-STAT signaling pathway', and 'Wnt signaling pathway'. Luciferase reporter assays demonstrated that miR-574-3p directly binds to the 3' UTR of several target genes (such as RAC1, EGFR and EP300) that are components of 'Pathways in cancer'. Quantitative real-time PCR and Western analysis showed that the mRNA and protein expression levels of the three target genes in PCa cells were markedly down-regulated with miR-574-3p. Loss-of-function studies demonstrated that the three target genes significantly affect cell proliferation, migration and invasion in PCa cell lines. Our results show that genistein up-regulates tumor suppressor miR-574-3p expression targeting several cell signaling pathways. These findings enhance understanding of how genistein regulates with miRNA in PCa.

Introduction

The most commonly diagnosed type of cancer among men in 2012 is prostate cancer (PCa) that is expected to account for 29% (241,740) of all new cancer cases. PCa ranks second to lung cancer in cancer-related deaths and is expected to account for 9% (28,170) of all male cancer deaths in 2012 [1]. Metastatic PCa is not curable and continues to be the major cause of cancer deaths [2]. Palliation can be achieved by hormone deprivation therapy however after an excellent initial response, in approximately 2 to 3 years most of these PCas will relapse to the castration resistant form of the disease [3] with death usually occurring within several years [4]. There are no successful treatments for androgen-independent PCa. A better understanding of biological mechanisms of androgen-independent PCa may lead to novel approaches to treat unresponsive PCa more successfully.

Development of chemotherapeutic agents with low patient toxicity is currently being investigated by many scientists. Many of these agents are derived from natural plant products. Genistein is a phytoestrogenic isoflavonoid that has pleiotropic biological effects in a wide variety of cancers without any visible toxicity to normal cells [5]. Genistein is a protein tyrosine kinase inhibitor and affects cell proliferation, apoptosis, tumor angiogenesis, metastasis and attenuates multidrug resistance involving key components of signal transduction pathways [6,7,8].

microRNAs (miRNAs) are small non-coding RNAs (21–23 nucleotides) that mainly bind imperfectly to the 3’ untranslated region (UTR) of target miRNAs and negatively regulate gene expression post-transcriptionally by translational repression and degradation of target mRNA [9,10]. Since the identification of miRNAs in 1993 over 1500 human miRNAs have been registered in the miRBase database (http://microrna.sanger.ac.uk/). Bioinformatics indicate that more than 60% of protein-coding genes may be targeted by miRNAs [11]. miRNAs play an important part in many biological processes, such as development, differentiation,
proliferation, apoptosis, angiogenesis and metabolism. In addition, they are key regulators in many diseases including cancer [12] and miRNAs may function as oncogenes or tumor suppressor genes [13,14]. The effects of genistein on the regulation of several miRNAs have been reported [15,16,17]. Our laboratory has shown that genistein inhibits cancer cell growth targeting oncogenic miRNAs such as miR-21, miR-151, miR-221 and miR-222. In this study, we focused on tumor suppressor miR-574-3p that is regulated by genistein and investigated its function in PCa and target pathways.

Results

Genistein Treatment Increases miR-574-3p Expression which is Down-regulated in PCa

To determine relative expression levels of miR-574-3p in prostate cells, we performed quantitative real-time PCR using PC3 and DU145 cell lines and compared them with normal prostate epithelial cells (RWPE-1). We observed that miR-574-3p expression was significantly down-regulated in PCa cell lines compared to RWPE-1 cells (PC3 0.68-fold, DU145 0.65-fold) (Fig. 1A).

To identify miRNAs regulated by genistein, we conducted a miRNA microarray using PC3 cells after genistein treatment (Table 1). The expression of 33 miRNAs was significantly up-regulated using two concentrations of genistein (25 μM and 50 μM) with miR-574-3p being the most affected. Previous miRNA expression studies from our lab also showed that miR-574-3p was significantly down-regulated in PCa samples compared with non-cancerous prostate tissues [18]. To confirm expression of miR-574-3p, we performed TaqMan quantitative real-time PCR analysis and observed that miR-574-3p expression was significantly up-regulated with genistein treatment (1.31 to 1.50-fold) (Fig. 1B).

miR-574-3p is Significantly Down-regulated in PCa Tissue Specimens

We evaluated expression levels of miR-574-3p in human PCa tissues (n = 48) and adjacent non-cancerous tissues (n = 48). The expression level of miR-574-3p was significantly lower in PCa compared with normal tissues (P < 0.0001; Fig. 1C). To determine if the levels of miR-574-3p in tumor tissues correlates with clinicopathological factors, we analyzed miR-574-3p expression levels in human tumor samples. Clinical demographics of the study cohort are summarized in Table 2. Correlation of 574-3p expression with clinicopathological variables such as pathological stage (pT) and Gleason score is shown in Fig. 1D. These results reveal that cases with low miR-574-3p expression increase from low grade, low pathological stage to high grade and high pathological stage.

Figure 1. Effect of genistein treatment and expression of miR-574-3p in PCa cells and specimens. (A) Expression of miR-574-3p in PCa cell lines (DU145 and PC3) and normal prostate epithelial cells (RWPE-1). Real-time PCR showed that the expression levels of miR-574-3p were down-regulated in PCa cell lines (DU145 and PC3). miR-574-3p expression was normalized to RNU48. Data are presented as mean ± SE. *, P < 0.05. (B) Effect of genistein on miR-574-3p expression in PC3 cells. (C) Expression of miR-574-3p in prostate tissues. (D) Correlation between miR-574-3p and clinicopathologic parameters.
These results suggest that miR-574-3p is significantly down-regulated in PCa and may be a putative tumor suppressor in PCa.

**Effect of miR-574-3p Over-expression on Cell Proliferation, Migration, and Invasion in PCa Cell Lines in vitro and in vivo**

To examine the functional roles of miR-574-3p, we performed gain-of-function studies using a pre-miR-574-3p miRNA precursor transfected into PC3 and DU145 cells. The expression of miR-574-3p was markedly up-regulated in Pre-miR miRNA precursor transfectants (Fig. 2A; PC3 316.8-fold, DU145 307.5-fold). Cell proliferation assay (MTS) and wound healing assay showed significant inhibition in miR-574-3p transfectants in both the PC3 and DU145 cells compared to the control transfectants (Fig. 2B and 2C). Invasion assay (Matrigel) also showed that the number of invading cells was significantly decreased in miR-574-3p transfectants compared with their counterparts (Fig. 2D). To confirm the effect of miR-574-3p on tumorigenicity in vivo, miR-574-3p and miR-control-transfected DU145 cells were subcutaneously injected into nude mice. We observed that miR-574-3p over-expression inhibited DU145 cell tumor formation in vivo (Fig. 2E). These results suggest that miR-574-3p plays an important role in tumor cell progression.

**miR-574-3p Influences Cellular Apoptosis in PCa Cells**

Since miR-574-3p restoration significantly inhibited cell proliferation in PCa cell lines, we hypothesized that its restoration may induce apoptosis. Fig. 3A and 3B showed that the apoptotic and early apoptotic fractions (upper right and lower right in the quadrant images, respectively) were greater in miR-574-3p transfectants compared to control. This points to a pro-apoptotic

| Table 1. Effect of genistein treatment for microRNA profiles in prostate cancer cells (PC3). |
|-----------------------------------------------|
| **Normalized Intensity** | **Ratio** | **P value** |
| non-treat | 25 µM | 50 µM | 25 µM/non | 50 µM/non | Average | 25 µM | 50 µM |
| hsa-miR-574-3p | 291.88 | 825.70 | 794.68 | 2.83 | 2.72 | 2.78 | 0.0000629 | 0.0000743 |
| hsa-miR-29a | 1839.50 | 3294.02 | 5835.99 | 1.79 | 3.17 | 2.38 | 0.0008651 | 0.000297 |
| hsa-miR-29b | 1197.37 | 1878.11 | 4315.52 | 1.57 | 3.6 | 2.38 | 0.0031300 | 0.000131 |
| hsa-miR-1234 | 253.86 | 805.18 | 439.51 | 3.17 | 1.73 | 2.34 | 0.0000002 | 0.000018 |
| hsa-miR-4700-3p | 190.06 | 474.90 | 346.86 | 2.5 | 1.83 | 2.14 | 0.0000049 | 0.000050 |
| hsa-miR-4732-3p | 162.91 | 387.93 | 307.66 | 2.38 | 1.89 | 2.12 | 0.0001546 | 0.000130 |
| hsa-miR-5096 | 166.98 | 493.47 | 250.64 | 2.96 | 1.5 | 2.11 | 0.0000680 | 0.0090343 |
| hsa-miR-1972 | 149.33 | 258.95 | 378.93 | 1.73 | 2.54 | 2.1 | 0.0000051 | 0.000303 |
| hsa-miR-3663-3p | 162.91 | 343.96 | 323.10 | 2.11 | 1.98 | 2.05 | 0.0002100 | 0.000106 |
| hsa-miR-3194-5p | 166.98 | 341.03 | 329.04 | 2.04 | 1.97 | 2.01 | 0.0000023 | 0.001121 |
| hsa-miR-4436b-5p | 362.47 | 941.99 | 538.10 | 2.6 | 1.48 | 1.96 | 0.0000694 | 0.001283 |
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Table 2. Prostate cancer patient information.

| Characteristics | (%) |
|-----------------|-----|
| Age (years)     |     |
| Median (range)  | 62 (47-81) |
| PSA (ng/ml)     |     |
| Median (range)  | 7.0 (0.2-90) |
| Total number    | 48 (100.0) |
| Gleason Score   |     |
| GS 6            | 23 (47.9) |
| GS 7            | 15 (31.3) |
| GS 8            | 6 (12.5)  |
| GS 9            | 3 (6.2)   |
| unknown         | 1 (2.1)   |
| Pathological tumor stage |     |
| pT2a            | 8 (16.7) |
| pT2b            | 10 (20.8)|
| pT2c            | 13 (27.1)|
| pT3a            | 8 (16.7) |
| pT3b            | 1 (2.1)  |
| unknown         | 8 (16.7) |

Abbreviations: PSA = prostate-specific antigen; GS = Gleason Score.

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Role of miR-574-3p in Apoptosis and Tumor Suppression

In the context of prostate cancer, the role of miR-574-3p has been extensively studied due to its ability to regulate various apoptotic pathways. For instance, it has been shown to target genes involved in the Wnt signaling pathway, which plays a crucial role in tumor suppression. The downregulation of miR-574-3p has been associated with increased expression of several tumor suppressor genes, including ARHGDIA, EGFR, and EP300. This suggests that miR-574-3p has a significant role in the regulation of apoptotic pathways and tumor suppression.

Search for miR-574-3p Target Genes in Silico Analysis

To identify potential target genes of miR-574-3p, computational approaches have been utilized. By searching the TargetScan database, we found that miR-574-3p targets genes involved in the Wnt signaling pathway, as well as other pathways. Among these, AGK, AKT2, and EP300 were identified as potential target genes.

Luciferase Reporter Assays Using Vectors Containing 3'UTR Binding Sites of Putative Target Genes

To confirm the interaction between miR-574-3p and its predicted target genes, we performed luciferase reporter assays. These assays were carried out using vectors containing the 3'UTR binding sites of the putative target genes. The results showed that miR-574-3p could bind directly to the 3'UTR of these genes, thereby regulating their expression.

Regulation of Target Gene Expression in PCa Cell Lines by miR-574-3p

Quantitative real-time PCR analysis showed that the expression levels of several target genes were reduced in miR-574-3p transfected cells compared to controls. This indicates that miR-574-3p can downregulate the expression of these genes, potentially interfering with their proliferation and survival, thereby contributing to tumor suppression.

Effect of Target Gene siRNA Knockdown on Cell Proliferation, Migration, and Invasion Activity in PCa Cell Lines

To further explore the functional role of the target genes, we performed loss-of-function studies using siRNA knockdown with PC3 and DU145 cells. The knockdown of AGK, AKT2, and EP300 resulted in a significant inhibition of cell proliferation and migration, suggesting that these genes are crucial for tumor cell growth and metastasis.

Discussion

Many studies have shown that genistein, a natural compound found in plants, regulates cancer cell proliferation and metastasis by targeting several genes. For example, genistein has been shown to downregulate the expression of MPP2 and MMP9, which are involved in tumor progression and angiogenesis, respectively. Moreover, genistein has also been shown to inhibit the Wnt/β-catenin signaling pathway, which is known to promote tumor growth and survival.

In summary, miR-574-3p has been identified as a regulator of the Wnt signaling pathway, which is critical for tumor suppression. Through its binding to target genes, miR-574-3p can downregulate the expression of crucial genes involved in tumor cell proliferation and migration, thereby contributing to the suppression of prostate cancer.
Genistein Regulates MiR-574-3p in Prostate Cancer

A. Real time PCR

B. MTS cell proliferation assay

C. Wound healing assay

D. Invasion assay

E. Tumor growth assay
Figure 2. Effect of miR-574-3p overexpression on PCa cell proliferation, migration and invasion in vitro and in vivo. (A) miR-574-3p expression levels in PCa cell lines (PC3 and DU145) were determined by real-time PCR at 72 hours after transfection of Pre-miR miRNA precursor. miR-574-3p expression was normalized to RNU48. Data are presented as the mean ± SE. (B) Overexpression of miR-574-3p significantly inhibits cell viability. Cell viability was analyzed by the MTS cell proliferation assay 1, 2 and 4 days after transient transfection. *, P<0.05. (C) Over-expression of miR-574-3p significantly inhibits cell migration. After transfection (48 hours), a wound was formed by scraping and measured after 6, 12 and 24 hours. Representative images of wound healing assay are shown at 200× magnification. **, P<0.0001. *, P<0.005. (D) Over-expression of miR-574-3p significantly decreased cell invasion. Representative images of invasion assay are shown at 200× magnification. *, P<0.005. (E) Representative images of tumors in nude mice 5 weeks after subcutaneous injection of transfected miR-574-3p DU145 cell lines or control cell lines and time course of tumor growth.
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Figure 3. Effect of miR-574-3p overexpression on apoptosis. (A) Apoptosis assay using flow cytometry. Representative quadrant figures of miR-control and miR-574-3p transfectants in PC3 (upper) and DU145 (lower) cells. (B) The bar chart indicates the ratio of apoptotic cell fractions (early apoptotic plus apoptotic cells) in miR-574-3p transfectants compared with controls. Data for the apoptotic cell fractions are expressed as the relative value for the average expression of the miR-control transfectant. *, P<0.05. (C) Immunoblots analysis for apoptotic markers in miR-control and miR-574-3p transfected DU145 cells. GAPDH was used as a loading control.
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markedly down-regulated by miR-574-3p. Therefore genistein may down-regulate RAC1 and EP300 by up-regulating miR-574-3p.

Over-expression of Notch1 leads to induction of the EMT phenotype and increased expression of miR-21 [31]. Genistein has been shown to inactivate Notch and hedgehog signaling [31,32] and we previously reported that genistein inhibited tumor cell growth by reducing miR-21 expression in renal cell carcinoma [15]. Thus genistein has a tumor suppressor function, regulating ‘Notch signaling’ by down-regulation of miR-21. Genistein can also reduce cell proliferation by regulating ‘Wnt signaling’ [33,34,35] through miR-574-3p in cancer. In this study EGFR, a putative target gene for miR-574-3p, was up-regulated in PCa and increased in advanced cancer [36,37]. EGFR expression was correlated with a high Gleason score, disease relapse and hormone-refractory status [37,38]. Researchers have reported that EGFR is regulated by several miRNAs such as miR-7, miR-128b, miR-133, miR-145, miR-146a, miR-146b-5p, miR-331-3p, miR-542-5p [39–47]. Genistein up-regulated miR-146a expression in pancreatic cancer cells and functions as a tumor suppressor in castration-resistant PCa [44,45]. Genistein might down-regulated EGFR levels by up-regulating miR-574-3p.

Genistein induces apoptosis by regulating intrinsic and extrinsic signaling pathways. Pro- and anti-apoptotic Bcl-2 family proteins play a crucial role in regulating the mitochondrial apoptotic pathway [48] and down-regulation of Bcl-xL by genistein induces apoptosis in PCa cells [49]. In this pathway activated caspase-9 accelerates executioner caspase activation, including caspase-3, and successively cleaves signaling molecules and cellular proteins [49,50]. In our study, miR-574-3p induced apoptosis and regulated expression of Bcl-xL, caspase-9 and caspase-3. Therefore this study shows that genistein induced apoptosis in PCa cells occurs by increased miR-574-3p expression.

In this study, we focused on miR-574-3p that was up-regulated by genistein and was a significantly down-regulated miRNA specific to PCa in the miRNA profile [18]. Our previous studies indicated that miR-574-3p might be a tumor suppressor miRNA in PCa and bladder cancer [18,51,52]. miR-574-3p is located on chromosome 4p14, a frequently deleted chromosomal region in PCa and bladder cancer cell lines [51,53]. Su et al reported that the expression of miR-574-3p was reduced in gastric cancer and cell proliferation, migration and invasion were significantly inhibited in miR-574-3p-transfected gastric cancer cells [54]. They found the CUL2 gene to be a target of miR-574-3p using by computational prediction and experimental validation. Our previous study demonstrated that miR-574-3p has tumor suppressor function and that the oncogenic MESDC1 gene is targeted by miR-574-3p in bladder cancer [52]. In this study, we have demonstrated that miR-574-3p is down-regulated in clinical PCa samples and androgen-independent PCa cell lines (PC3 and DU145). Down-regulation of miR-574-3p expression in tumors is related to high tumor stage and Gleason score indicating that miR-574-3p may be used as a biomarker for tumor progression in PCa.

In conclusion, our results show that genistein up-regulates miR-574-3p expression which targets several cell signaling pathways. These findings enhance our understanding of how genistein regulates miRNA expression in PCa.

**Materials and Methods**

**Clinical Prostate Specimens**

All tissue slides were reviewed by a board certified pathologist for the identification of PCa foci as well as adjacent normal glandular epithelium. All cancer patients had elevated levels of prostate specific antigen (PSA) and had undergone radical
A) Putative miR-574-3p binding and mutated sites in the 3′ UTR

| Position | miR-574-3p | RAC1 3′ UTR (Wild) | RAC1 3′ UTR (Mutated) | EGFR 3′ UTR (Wild) | EGFR 3′ UTR (Mutated) | EP300 3′ UTR (Wild) | EP300 3′ UTR (Mutated) |
|----------|------------|---------------------|-----------------------|---------------------|-----------------------|---------------------|-----------------------|
| 551-558  | 3′ ACAACCACACAGU--ACUCGAC | 5′ UGUACUGUAUAGGACGUA... | 3′ ACAACCACACAGU--ACUCGAC | 5′ UGUACUGUAUAGGACGUA... | 3′ ACAACCACACAGU--ACUCGAC |
| 718-724  | 3′ ACAACCACACAGU--ACUCGAC | 5′ AGUGGUUCCAGUCGUAGCCGUU... | 3′ ACAACCACACAGU--ACUCGAC | 5′ AGUGGUUCCAGUCGUAGCCGUU... | 3′ ACAACCACACAGU--ACUCGAC |
| 653-660  | 3′ ACAACCACACACGUAUCGCAC | 5′ UCUAAUUCGUAUGACGUA... | 3′ ACAACCACACACGUAUCGCAC | 5′ UCUAAUUCGUAUGACGUA... | 3′ ACAACCACACACGUAUCGCAC |

B) Luciferase reporter assay

C) Realtime PCR

D) Western blot

*P < 0.05
miR-control and miR-574-3p transfected PC3 cells. GAPDH was used as a loading control.

change. TaqMan probes and primers for RAC1 (assay ID: Hs01090242_m1), EGFR (assay ID: Hs01076078_m1), EP300 (assay ID: Hs00914223_m1), GAPDH (assay ID: Hs00978991_g1), miR-574-3p (assay ID: 002349), RNU48 (Assay ID: 001006) were obtained from Applied Biosystems. GAPDH and RNU48 were used as internal controls.

Western Analysis
At 72 hours after transfection, cells were lysed with RIPA buffer (Pierce, Brevieres, France) containing protease inhibitors (Sigma). Protein quantification was done using a BCA protein assay kit (Pierce). Protein lysate (30 μg) was separated on 4% to 20% SDS polyacrylamide gels and transferred to a PVDF membrane. Antibodies to EP300 and Bcl-xL were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against RAC1, EGFR and GAPDH were purchased from Cell Signaling Technology (Danvers, MA, USA). After incubation with primary antibody the membrane was washed and then incubated with secondary antibodies conjugated to horseradish peroxidase (Cell Signaling Technology, Danvers, MA, USA). Specific complexes were visualized with an enhanced chemiluminescence (ECL) detection system (GE Healthcare, Little Chalfont, UK). The membrane was stripped using ReBlot Plus Strong Antibody Stripping Solution (Millipore, Billerica, MA, USA). The expression level of genes was then evaluated by using ImageJ software (ver. 1.43; http://rsbweb.nih.gov/ij/index.html).

Transfection
Pre-miR miRNA precursor and negative control (Applied Biosystems) were used in the gain-of-function experiments. RAC1, EGFR and EP300 siRNA (Sigma) and negative control siRNA (D-001810-10; Thermo Fisher Scientific, Waltham, MA, USA) were used in the loss-of-function experiments. PC3 and DU145 cells were transiently transfected using Lipofectamine 2000 transfection reagent (Invitrogen), according to the manufacturer’s recommendations.

Cell Proliferation, Migration, and Invasion Assays
Cell proliferation was measured using a CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS) (Promega, Madison, WI, USA) performed according to the manufacturer’s instructions. Cell proliferation was determined by absorbance measurements at 490 nm using SpectraMAX 190 (Molecular Devices Co., Sunnyvale, CA, USA). Cell migration activity was evaluated by a wound-healing assay. Cells were plated in six-well dishes, and the cell monolayers were scraped using a P-20 micropipette tip. The width of the initial gap (0 h) and the residual gap 6, 12 and 24 hours after wounding were calculated from photomicrographs. A cell invasion assay was carried out using modified Boyden Chambers consisting of transwell-precoated Matrigel membrane filter inserts with eight micron pores in 24-well tissue culture plates (BD Biosciences, Bedford, MA, USA). Minimum essential medium containing 10% FBS in the lower chamber served as the chemottractant, as described previously [55]. All experiments were performed in triplicate.
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A. Real-time PCR

PC3

- si-Control
- si-RAC1
- si-EGFR
- si-EP300

DU145

- si-Control
- si-RAC1
- si-EGFR
- si-EP300

B. Western blot

RAC1

- EGFR

GAPDH

- si-Cont
- si-RAC1
- si-EGFR
- si-EP300

C. MTS cell proliferation assay

PC3

- Absorbance at 490 nm

DU145

- Absorbance at 490 nm

D. Wound healing assay

PC3

- 0hr
- 6hr
- 12hr
- 24hr

DU145

- 0hr
- 6hr
- 12hr
- 24hr

E. Invasion assay

PC3

- Invasion cell (% of si-Cont)

DU145

- Invasion cell (% of si-Cont)
In vivo Tumor Growth
All animal care was in accordance with the guidelines of the San Francisco Veterans Affairs Medical Center and the study was approved by the San Francisco VA IACUC (Protocol number: 11-006-01). Animal users have completed training programs to handle and work with mice through AAALAS (American Association for Laboratory Animal Science) prior to animal experiments. For the subcutaneous xenograft mouse model, DU145 cells (2.5 x 10^5) that were transiently transfected with miR-574-3p or miR-control were suspended in 50 µL RPMI 1640 medium and were subcutaneously injected into female nude mice (strain BALB/c nu/nu; Charles River Laboratories, Inc., Wilmington, MA, USA, 5 weeks old). A total of 6 nude mice (4-miR-574-3p, 4-miR-control) were used and tumor growth was examined over the course of 35 days. Tumor volume was calculated on the basis of width (x) and length (y): x^2y/2, where x < y.

Apoptosis Assays
Fluorescence-activated cell-sorting (FACS) analysis for apoptosis was done 96 hours post-transfection, using Annexin V-FITC/7-AAD Kit (Beckman Coulter, Brea, CA, USA), according to the manufacturer’s protocol. Stained cells were immediately analyzed with a flow cytometer (Cell Lab Quanta SC; Beckman Coulter).

Identification of miR-574-3p Regulated Target Genes and Bioinformatic Analysis
To search for genes regulated by miR-574-3p, we used TargetScan algorithm (release 6.2, http://www.targetscan.org/). To identify the biological processes or pathways potentially regulated by miR-574-3p, we performed GeneCodis analysis (http://geneCodis.dacya.ucm.es/) using all of the candidate genes. Then, to identify networks among the miRNAs and their target genes, we analyzed and characterized those genes in KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway categories. These data were used to examine miRNA-regulated molecular networks in human cells. We performed gene expression analyses of all candidate genes involved in each of the pathways using microarray data approved by the GEO and were assigned GEO accession numbers (GSE29079). In the Affymetrix Human Exon 1.0 ST Array (Affymetrix, Santa Clara, CA, USA) datasets, we examined 47 PCa tissues and 47 normal prostate tissues, all of which were collected from patients who had not been exposed to neo-adjuvant radio-, cytotoxic- or endocrine therapy before the operation. The data was normalized and analyzed with the GeneSpring (Agilent Technologies, Santa Clara, CA, USA). Statistical analyses were conducted using the Mann Whitney U-test with cut-off P<0.05.

Plasmid Construction and Dual-luciferase Reporter Assays
For 3' UTR luciferase reporter assay, PmirGLO Dual-Luciferase miRNA Target Expression Vector was used (Promega). The oligonucleotide sequences (wild-type) used are shown in Table S1. We also constructed mutated oligonucleotides for each of the wild-type oligonucleotides (Table S1). In a total volume of 25 µL, 1 µL each of 100 µM forward and reverse oligonucleotide, 2.5 µL of 10X annealing buffer (100 mM Tris–HCl, pH 7.5, 1 M NaCl and 10 mM ethylenediaminetetraacetic acid) and 20.5 µL water were incubated at 95°C for 3 min and then placed at 37°C for 15 min. The oligonucleotides were ligated into the PmeI-XbaI site of pmirGLO Dual-Luciferase miRNA Target Expression Vector. For 3' UTR luciferase assay, PCa cells were co-transfected with Pre-miR miRNA precursor and pmirGLO Dual-Luciferase miRNA Target Expression Vectors using Lipofectamine 2000 (Invitrogen) and X-tremeGENE HP DNA Transfection Reagent (Roche Diagnosis, Basel, Switzerland, USA) according to the manufacturer’s instructions. Luciferase reporter assay was performed using the Dual-Luciferase Reporter Assay System (Promega) 24 hours after transfection. Firefly luciferase activities were normalized with Renilla luciferase. We also included basic vector containing no insert as a mock control.

Statistical Analysis
The relationship between two variables and the numerical values obtained by real-time RT-PCR were analyzed using the nonparametric Mann-Whitney U test. All analyses were performed using Expert StatView (version 4, SAS Institute Inc., Cary, NC, USA). Data are shown as mean values ± standard error. P values of <0.05 were regarded as statistically significant.

Supporting Information
Table S1 Primer oligonucleotide sequences (wild-type and mutated).

(DOC)

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Author Contributions
Conceived and designed the experiments: TC HE NS MN RD. Performed the experiments: TC SY HH. Analyzed the data: TC HH NS. Contributed reagents/materials/analysis tools: SF SM SS SA GD VS IC YT ZLT NS. Wrote the paper: TC RD.

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