Companion Diagnostics and Cancer Biomarkers

Regulation of SRC Kinases by microRNA-3607 Located in a Frequently Deleted Locus in Prostate Cancer

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Abstract
Genomic studies suggest that deletions at chromosome (chr) 5q region (particularly chr5q14-q23) are frequent in prostate cancer, implicating this region in prostate carcinogenesis. However, the genes within this region are largely unknown. Here, we report for the first time the widespread attenuation of miR-3607, an miRNA gene located at chr5q14 region, in prostate cancer. Expression analyses of miR-3607 in a clinical cohort of prostate cancer specimens showed that miR-3607 is significantly attenuated and low miR-3607 expression is correlated with tumor progression and poor survival outcome in prostate cancer. Our analyses suggest that miR-3607 expression may be a clinically significant parameter with an associated diagnostic potential. We examined the functional significance of miR-3607 in prostate cancer cell lines and found that miR-3607 overexpression led to significantly decreased proliferation, apoptosis induction, and decreased invasiveness. Furthermore, our results suggest that miR-3607 directly represses oncogenic SRC family kinases LYN and SRC in prostate cancer. In view of our results, we propose that miR-3607 plays a tumor-suppressive role in prostate cancer by regulating SRC kinases that in turn regulates prostate carcinogenesis. To our knowledge, this is the first report that: (i) identifies a novel role for miR-3607 located in a frequently deleted region of prostate cancer and (ii) defines novel miRNA-mediated regulation of SRC kinases in prostate cancer. Because SRC kinases play a central role in prostate cancer progression and metastasis and are attractive targets, this study has potential implications in the design of better therapeutic modalities for prostate cancer management. Mol Cancer Ther; 13(7); 1952–63. ©2014 AACR.

Introduction
Prostate cancer is the most common male malignancy and one of the leading causes of cancer death among men worldwide. Critical challenges plague the field of prostate cancer hindering the development of effective diagnostic, prognostic, and therapeutic options for disease management (1). One of the major challenges is the limitation of current methods used for screening and predicting disease course (PSA screening, histopathological grading) in prostate cancer (2, 3). These methods cannot readily distinguish indolent from aggressive prostate tumors, emphasizing the critical need of novel disease biomarkers with better diagnostic and predictive potential. Another major challenge is disease recurrence, progression, and metastasis. Although significant gains have been made in early prostate cancer management when the disease is largely hormone dependent, limited therapeutic options exist for hormone-independent castration-resistant/advanced stage disease (4). Advanced prostate cancer is usually associated with metastatic dissemination, typically to bones, causing significant morbidity and mortality (5). At present, there is no effective therapy for advanced prostate cancer, with the most effective standard chemotherapeutic regimens resulting in a marginal increase in survival time (1, 6). Thus, there is a critical need to understand the molecular mechanisms underlying prostate cancer progression and metastasis that will translate into developing better therapeutic modalities for the disease.

Complex genomic alterations underlie prostate cancer (1). Characterization of genomic alterations associated with prostate cancer offers the potential to increase the efficacy of current targeted therapies for prostate cancer (7). Integrative genomic techniques including array comparative genomic hybridization (CGH), exome sequencing, and methylation profiling have yielded information on the genomic landscape of prostate cancer (8). These studies have identified several conserved genomic regions that are deleted, amplified, mutated, or translocated. Studies suggest that deleted regions of recurrent genomic loss in prostate cancer are located at the following chromosomal locations: Chromosome (chr) 8p (67%), 5q (39%), 16q (37%), 6q (35%), 13q (33%), 18q (33%),...
17p (30%), 12p (24%), and 2q (20%), whereas frequent copy number gains are observed at 8q (30%), 7 (22%), and 3q (13%) (9). Several of these genomic studies suggest that deletion at chr5q is a frequent event in prostate cancer, particularly in advanced tumors (10). CGH analyses have identified that chr5q deletion is detected in approximately 28% of cases of prostate cancer and the common region of deletion is chr5q14-q23 (10–13). LOH analysis suggest that LOH at chr5q is frequent and is particularly associated with higher tumor stage (14). Frequent deletions at chr5q locus in prostate cancer were supported by large-scale integrative analyses of transcriptomes and copy number alterations (8). This evidence suggests that chr5q region may play an important role in prostate carcinogenesis. However, the potential tumor suppressor genes within this region are not fully defined (9). An miRNA gene, miR-3607, is located in this region. miRNAs are small endogenous RNAs that suppress gene expression posttranscriptionally via sequence-specific interactions with the 3′-untranslated regions (UTR) of cognate targets and play important regulatory roles in various cancers, including prostate cancer (15). miR-3607 is a recently discovered miRNA (16) that has not been well studied. Considering the important role of chr5q in prostate cancer, the primary objective of the present study was to explore the role of this novel miRNA gene located within this deleted region in prostate cancer development and progression.

We examined the expression of miR-3607 in a cohort of human prostate cancer clinical specimens and found that miR-3607 expression is frequently attenuated in prostate cancer. Our analyses showed that lower miR-3607 expression levels are significantly associated with tumor progression and poor survival outcome in prostate cancer. Reconstitution of miR-3607 expression in prostate cancer cell lines led to significantly decreased tumorigenicity of these cancer cell lines. Furthermore, our data suggest that miR-3607 directly targets the SRC family of kinases (SFK). These kinases are nonreceptor tyrosine kinases involved in signal transduction during key cellular processes (including proliferation, differentiation, apoptosis, and migration; refs. 17, 18) that are often augmented in prostate cancer and correlate with disease severity/metastatic potential (17–20). Increasing evidence implicates these kinases in prostate cancer progression, transition to an androgen-independent state and metastasis (21–23). SRC kinases represent attractive therapeutic targets and several SFK inhibitors are currently being tested clinically. For example, dasatinib (BMS-354825), a SFK inhibitor (24), is currently in phase III clinical trials for the treatment of prostate cancer bone metastasis (25–27). Here, we demonstrate for the first time, that two key SRC family members, SRC and LYN, are directly negatively regulated by miR-3607 that is associated with a frequently deleted region in prostate cancer. Considering the fact that SFK inhibition is being exploited clinically as a therapeutic strategy for patients with prostate cancer, this study may have important implications for prostate cancer treatment. To our knowledge, this is the first study that demonstrates miR-3607-mediated inhibition of the clinically important therapeutic targets of SRC family.

**Materials and Methods**

**Cell lines and cell culture**

Nonmalignant epithelial prostate cell lines (RWPE-1 and PWR-1E) and prostate carcinoma cell lines (LNCaP, Du145, PC3) were obtained from the American Type Culture Collection (ATCC) and cultured under recommended conditions as described previously (28). RWPE-1 and PWR-1E cells were cultured in keratinocyte growth medium supplemented with 5 ng/mL human recombinant EGF, 0.05 mg/mL bovine pituitary extract (Invitrogen). LNCaP, Du145, and PC3 were maintained in RPMI-1640 media supplemented with 10% FBS (Atlanta Biologicals) and 1% penicillin/streptomycin. Cell lines were maintained in an incubator with a humidified atmosphere of 95% air and 5% CO2 at 37°C. Cell lines were authenticated by DNA short tandem repeat analysis by ATCC. The experiments with cell lines were performed within 6 months of their procurement/resuscitation.

**miRNA transfections**

Cells were plated in growth medium without antibiotics approximately 24 hours before transfection. Transient transfection of miRNA precursor/anti-miR miRNA inhibitor (Ambion) was carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. All miRNA transfections were for 72 hours. miR-3607 precursor (AM17100), negative control (miR-CON; AM17110), anti-miR-3607 inhibitor (MH19335), and anti-miR-control inhibitor (4464076) were used for transfections.

**Tissue samples and ethics statement**

Formalin-fixed, paraffin-embedded (FFPE) prostate cancer samples were obtained from the SFVAMC. Written informed consent was obtained from all patients and the study was approved by the University of California San Francisco (San Francisco, CA) Committee on Human Research (Approval number: H9058-35751-01). All slides were reviewed by a board certified pathologist for the identification of prostate cancer foci as well as adjacent normal glandular epithelium.

**RNA and miRNA extraction**

Total RNA was extracted from microdissected FFPE tissues using a miRNeasy FFPE Kit (Qiagen), and an miRNeasy Mini Kit (Qiagen) was used for miRNA extraction from cultured cells following the manufacturer’s instructions.

**Migration, invasion, and clonogenicity assays**

Cytoselect cell migration and invasion assay kit (Cell Biolabs, Inc.) was used for migration and invasion assays, according to the manufacturer’s protocol. Briefly, 48 hours posttransfection, cells were counted and placed on...
control inserts or Matrigel inserts at 1 × 10⁵ cells/mL in serum-free medium and were allowed to migrate for 20 hours at 37 °C. Cells were removed from the top of the inserts and cells that migrated/invaded through the polycarbonate/basement membrane were fixed, stained, and quantified at OD 560 nm after extraction. For clonogenicity assay, cells were counted, seeded at low density (1,000 cells/plate), and allowed to grow until visible colonies appeared. Then, cells were stained with Giemsa and colonies were counted.

**Cell viability assays**
Cell viability was determined at 24, 48, and 72 hours by using the CellTiter 96 AQueousOne Solution Cell Proliferation Assay Kit (Promega) according to the manufacturer’s protocol.

**Flow cytometry**
FACS analysis was done 72 hours posttransfection. The cells were harvested, washed with cold PBS, and resuspended in 4′, 6-diamidino-2-phenylindole (DAPI) nuclear stain for cell-cycle analysis. Cells were stained with 7-

**Western blotting**
Whole-cell extracts were prepared in RIPA buffer [50 mmol/L Tris (pH 8.0), 150 mmol/L NaCl, 0.5% deoxycytolate, 0.1% SDS, and 1.0% NP-40] containing protease inhibitor cocktail (Roche). Total protein was electrophoresed by SDS-PAGE and Western blotting was carried out according to standard protocols. The following antibodies were used for Western blotting: LYN (Cell Signaling Technology, cat no. 3456), and Annexin-V-FITC using ANNEXIN V-FITC/7-AAD KIT (Beckman Coulter) for apoptosis analysis according to the manufacturer’s protocol. Stained cells were immediately analyzed by FACS (Cell Lab Quanta SC; Beckman Coulter, Inc).

**Statistical analysis**
All quantified data represent an average of triplicate samples or as indicated. Data are represented as mean ± SEM. All statistical analyses were performed using StatView (version 5; SAS Institute Inc.) and MedCalc version 10.3.2. Two-tailed Student t test was used for comparisons between groups. Results were considered statistically significant at P ≤ 0.05.

**Supplementary data**
The supplementary data include Supplemental Materials and Methods.

**Results**

**miR-3607 expression is attenuated in prostate cancer**
Human miR-3607 gene is located at chromosomal position 5q14.3 in the intron of a coding gene, COX7C (Cytochrome c oxidase subunit 7C; Fig. 1A), which is transcribed in the same direction as miR-3607. To evaluate the role of miR-3607 in prostate cancer, we analyzed the relative expression of miR-3607-5p (major form of miR-3607, referred to as miR-3607) in a cohort of human prostate cancer clinical specimens by real-time PCR (Fig. 1B). Laser capture microdissected (LCM) prostate cancer tissues (n = 100) and matched adjacent normal regions were used for this analysis. For each tissue sample, tumor/normal ratios were calculated. The following thresholds were used for dichotomizing samples based on relative miR-3607 expression in tumor/normal tissues: low expression <0.75 and high expression >1.25. Although the expression of miR-3607 was unaltered in 22 of 100 cases (22%) and higher in 15 of 100 cases (15%), a major fraction of tissue samples (63/100, ~63%) showed lower miR-3607 levels relative to matched adjacent normal tissues. The differences were statistically significant with the Wilcoxon signed rank test (P < 0.0001). This suggests that miR-3607 expression is attenuated in prostate cancer and that miR-3607 may be a potential tumor-suppressive miRNA. Clinicopathological characteristics of the patients used for miR-3607 expression analysis are summarized in Supplementary Table S1.

**Downregulation of miR-3607 expression is associated with prostate cancer progression**
We determined whether miR-3607 expression in clinical tissues was correlated with clinicopathological characteristics such as age, Gleason score, pathologic stage, PSA levels, and biochemical recurrence (Table 1). Although there was no significant correlation with age, decreased miR-3607 expression was observed in 54% of cases with low Gleason score (6), 66% of cases with Gleason 7, and in 89% of cases with high Gleason score (8–10). For cases with Gleason score 7, decreased miR-3607 expression was observed in 92% cases with grade 4+3 tumors versus 55% with grade 3+4 tumors (Table 1), suggesting that decreased miR-3607 expression is particularly associated with higher grade tumors (P = 0.0173). Similarly, decreased miR-3607 expression was observed in 54% of cases with pathologic stage pT2, 79% of pT3, and 100% of pT4 cases. This trend indicates that miR-3607 expression tends to attenuate in higher stages of prostate cancer (P = 0.0360). Interestingly, miR-3607 expression was significantly associated with serum PSA levels in our clinical cohort (P = 0.0001). Low miR-3607 expression was observed in 75% of cases with high PSA levels versus 32% of cases with low PSA. miR-3607 was attenuated in 22 of 31 cases (71%) with PSA failure within this cohort of samples though no statistically significant correlation was observed between miR-3607 expression and biochemical recurrence. These analyses suggest that downregulation of miR-3607 expression is associated with tumor progression in prostate cancer.

**miR-3607 is a potential prognostic and diagnostic marker in prostate cancer**
In view of the observed widespread downregulation of miR-3607 in prostate cancer clinical specimens, we evaluated the potential clinical significance of miR-3607...
expression. We examined the correlation between miR-3607 expression and overall survival of patients with prostate cancer. For this analysis, we stratified our prostate cancer clinical cohort based on miR-3607 expression (high vs. low) and performed Kaplan–Meier survival analysis (Fig. 1C). This analysis showed that survival probability was significantly reduced in patients with low miR-3607 expression compared with those with high expression (Fig. 1C; $P = 0.0464$). These results indicate that downregulation of miR-3607 expression is associated with poor survival outcome in prostate cancer.

Furthermore, we determined the potential capability of miR-3607 as a diagnostic biomarker for prostate cancer by performing ROC analyses on our cohort of prostate cancer clinical samples (Fig. 1D). ROC analyses showed that miR-3607 expression is a significant parameter to discriminate between normal and tumor tissues with an area under the ROC curve (AUC) of 0.663 (95% confidence interval, 0.594–0.728, $P = 0.0005$).

The preceding analyses suggest that miR-3607 expression may be a clinically significant parameter that has associated prognostic and diagnostic potential for prostate cancer.

**Overexpression of miR-3607 suppresses proliferation of prostate cancer cell lines**

In view of observed widespread downregulation of miR-3607 expression in prostate cancer clinical specimens, we examined the effect of miR-3607 overexpression on cell line proliferation. We transfected two prostate cancer cell lines with low natural miR-3607 expression levels with miR-3607 mimic and measured cell proliferation at 24, 48, and 72 hours. Overexpression of miR-3607 significantly reduced cell proliferation by 15% at 24 hours, 30% at 48 hours, and 40% at 72 hours compared with control cells (Fig. 2A).

**Figure 1.** miR-3607 expression is widely attenuated in prostate cancer. A, schematic representation showing the chromosomal location of miR-3607. miR-3607 is located at chromosomal position 5q 14.3 within the intron of a coding gene, COX7C (Cytochrome c oxidase subunit 7C). B, quantitative RT-PCR analysis of relative miR-3607 expression levels in LCM prostate cancer tissues ($n = 100$) and patient-matched adjacent normal regions. Data were normalized to RNU48 control. Table below summarizes the relative 3607 expression levels in these specimens. C, Kaplan–Meier survival curves for patients with prostate cancer, stratified on the basis of miR-3607 expression (low and high). $P$ value based on log-rank test ($^* P < 0.05$). D, ROC curve analysis showing the ability of miR-3607 expression to discriminate between malignant and nonmalignant prostate tissue samples.
we evaluated the potential tumor suppressive role of miR-3607 using prostate cancer cell lines. We overexpressed miR-3607 in prostate cancer cell lines (PC3, Du145, and LNCaP) followed by functional assays (Fig. 2 and Supplementary Fig. S1). Transient transfection of miR-3607 precursor led to overexpression of miR-3607 as determined by real-time PCR (Supplementary Fig. S1). Overexpression of miR-3607 significantly suppressed the proliferation of prostate cancer cell lines as assessed by clonogenicity assay (Fig. 2A). A significant decrease in cell viability was observed over time in PC3/Du145/LNCaP cells overexpressing miR-3607 as compared with cells expressing control miR (miR-CON; Fig. 2B).

Table 1. Correlation of miR-3607 expression with clinicopathological parameters of patients with prostate cancer

<table>
<thead>
<tr>
<th>Clinicopathological parameter</th>
<th>Total n (%)</th>
<th>Low</th>
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<th>High</th>
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<td>40</td>
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<td>50</td>
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<td>48/100 (48)</td>
<td>26/48 (54)</td>
<td>10/48 (21)</td>
<td>12/48 (25)</td>
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<tr>
<td>7 (3+4)</td>
<td>29/100 (29)</td>
<td>16/29 (55)</td>
<td>4/29 (14)</td>
<td>9/29 (31)</td>
<td>P = 0.0173b</td>
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<td>7 (4+3)</td>
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<td>11/12 (92)</td>
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<td>1/12 (8)</td>
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<td>8–10</td>
<td>9/100 (9)</td>
<td>8/9 (89)</td>
<td>1/9 (11)</td>
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<td>14/68 (21)</td>
<td>17/68 (25)</td>
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<td>PSA levels</td>
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<td>31/100 (31)</td>
<td>10/31 (32)</td>
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<td>47/63 (75)</td>
<td>4/63 (6)</td>
<td>12/63 (19)</td>
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<td>Biochemical recurrence</td>
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</table>

*aCorrelation of miR-3607 expression with clinicopathological characteristics of patients with prostate cancer including age, Gleason score, pathologic stage, PSA levels, and biochemical recurrence was examined.

*bP < 0.05.

miR-3607 overexpression induces G0–G1 arrest in prostate cancer cell lines

Because miR-3607 overexpression led to reduced proliferation of prostate cancer cell lines, we also evaluated its effects on the cell cycle. After 72 hours of transfection of miR-3607 precursor/miR-CON PC3/Du145/LNCaP cells were stained with nuclear stain DAPI followed by FACS analysis (Fig. 2C). Our analyses showed that miR-3607 overexpression led to a significant increase in the number of cells in the G0–G1 phase of the cell cycle compared with miR-CON. This suggests that miR-3607 overexpression induces a G0–G1 arrest in prostate cancer cell lines.

miR-3607 overexpression induces apoptosis in prostate cancer cell lines

We measured apoptosis in control (mock or miR-CON transfected) and miR-3607–transfected cells by flow-cytometric analysis of Annexin-V-FITC-7-AAD stained PC3/Du145/LNCaP cells (Fig. 2D). It was observed that the average apoptotic cell fractions (early apoptotic + apoptotic) were significantly increased upon miR-3607 overexpression compared with miR-CON/mock–transfected cells with a concomitant decrease in the viable cell population. This suggests that miR-3607 induces apoptosis in prostate cancer cell lines.

Overexpression of miR-3607 expression reduces invasiveness of prostate cancer cell lines

We performed transwell migration and invasion assays in control (mock or miR-CON transfected) and miR-3607–transfected PC3/Du145/LNCaP prostate cancer cell lines (Fig. 3). These assays showed that overexpression of miR-3607 significantly decreased the invasiveness (Fig. 3A) and migratory abilities (Fig. 3B) of all the prostate cancer cell lines tested.
miR-3607 knockdown increases invasiveness and proliferation of normal immortalized prostate epithelial cell lines

In a reciprocal approach, we knocked down miR-3607 expression in normal immortalized prostate epithelial cell lines (RWPE1 and PWR1E) using miRVANA anti-miRNA inhibitor (Ambion) followed by functional assays (Fig. 4). Basal level of miR-3607 expression in these normal immortalized prostate epithelial cell lines is higher than that of PC3 and Du145 (Supplementary Fig. S2). miR-3607 knockdown was confirmed by RT-PCR (Fig. 4A). Our results suggest that knockdown of miR-3607 increased the proliferation, invasiveness, and motility of nontransformed epithelial cells (Fig. 4B–D). Cell-cycle analysis showed a significant increase in G2–M phase upon miR-3607 inhibition (Fig. 4E). These results
miR-3607 directly targets SRC family of kinases in prostate cancer

In silico analysis identified that SRC family kinases, LYN and SRC, are putative miR-3607 targets. LYN possesses one potential miR-3607–binding site within its 3′-UTR, whereas SRC has two potential miR-3607–binding sites (Fig. 5A). Although other miRNAs are predicted to target SRC/LYN, the potential ability of miR-3607 to simultaneously bind to 3′-UTRs of both SFK family members makes it unique. To validate these SRC kinases as target genes for miR-3607, we performed Western blot analysis for these kinases in PC3 cells that were either mock transfected or transfected with miR-3607/miR-CON (Fig. 5B). Interestingly, miR-3607 overexpression led to decreased protein levels of LYN and SRC. Furthermore, we investigated whether these nonreceptor tyrosine kinases are direct functional targets of miR-3607 in prostate cancer. We transiently transfected PC3 cells with the control/LYN/SRC 3′-UTR luciferase reporter...
miR-3607 precursor/miR-CON (Fig. 5C). miR-3607 overexpression led to significant decreases in LYN/SRC luciferase reporter activity as compared with miR-CON/mock–transfected cells suggesting that miR-3607 directly represses these genes.

Expression of LYN and SRC is inversely correlated with miR-3607 expression in prostate cancer

To confirm LYN and SRC as functionally relevant targets of miR-3607 in vivo, we examined the correlation between miR-3607 and LYN/SRC expression in a subset of our clinical cohort. We examined LYN/SRC expression in prostate cancer tissues by RT-PCR (n = 15) and observed a negative correlation between the expression of these SRC kinases and miR-3607 in 14 of 15 tissues (93%; Fig. 5D and E). Clinical samples with low miR-3607 expression (relative to adjacent normal tissue) showed high levels of LYN and SRC expression (Fig. 5D and E). These data support the concept that these SRC kinases are important targets of miR-3607 in prostate cancer.

miR-3607 expression is altered by docetaxel treatment in prostate cancer cell lines

We further examined whether miR-3607 expression is altered by docetaxel treatment in prostate cancer cell lines. Although androgen deprivation therapy is used for initial treatment of localized prostate cancer, chemotherapeutic drug docetaxel is the first line of
treatment for castration-resistant prostate cancer (6). Prostate cancer cell lines (LNCaP, PC3, and Du145) were treated with docetaxel at varying concentrations and time periods (6 and 24 hours) followed by miR-3607 expression analysis by real-time PCR (Supplementary Fig. S3). Androgen-dependent LNCaP cells were treated with 2 and 4 nmol/L docetaxel. Androgen-independent prostate cancer cell lines (PC3 and Du145)
were treated with 1 and 2 nmol/L docetaxel as these cell lines have been reported to be more sensitive to the drug (29, 30). Significant increases in miR-3607 expression were observed in all cell lines particularly with longer treatment. These results suggest that docetaxel treatment upregulates this tumor-suppressive miRNA in prostate cancer.

Discussion

In this report, we define for the first time, a novel regulatory role for a miRNA gene located in frequently deleted region of prostate cancer. Genomic studies have suggested that chromosomal region 5q deletions are associated with prostate cancer, particularly in advanced tumors (8, 11–14). The common region of deletion is chr5q14-q23 (10). Despite a large body of evidence suggesting genomic loss of this chromosomal region, genes within this region are largely unknown (9). We found that miR-3607, an intronic miRNA located at chromosomal position 5q14.3, is frequently downregulated in human prostate cancer clinical specimens. In view of its low expression, we assessed the potential for miR-3607 as a prostate cancer biomarker. Our analyses suggest that low miR-3607 expression can be a significant parameter to discriminate between normal prostate and tumor tissues. Correlation with clinicopathological parameters suggest that downregulation of miR-3607 expression is associated with tumor progression in prostate cancer. Low miR-3607 expression was significantly associated with prostate cancer cases with higher stage and Gleason score. These findings support the association of chr5q losses with advanced prostatic tumors (10). Also, we observed that miR-3607 expression was significantly associated with serum PSA levels in patients with prostate cancer. Furthermore, low miR-3607 expression was significantly correlated with poor survival outcome in prostate cancer clinical specimens. These findings suggest that this novel miRNA may be a potential disease biomarker for prostate cancer prognosis and diagnosis.

The observed downregulation of miR-3607 expression in prostate cancer clinical samples also suggested that this miRNA may possess tumor-suppressive activity. To test this, we performed functional studies using both androgen-dependent (LNCaP) and androgen-independent (PC3 and Du145) human prostate cancer cell lines. We overexpressed miR-3607 in these cell lines followed by functional assays. miR-3607 overexpression led to significant decreases in cell growth and clonability. FACS analysis showed that miR-3607 promotes G0-G1 cell-cycle arrest and induction of apoptosis in all the prostate cancer cell lines tested. Furthermore, miR-3607 overexpression also decreased invasiveness and migratory properties of prostate cancer cell lines. In a reciprocal approach, miR-3607 knockdown in normal immortalized prostate epithelial cell lines, RWPE1 and PWR1E, led to increased proliferation, invasiveness, and motility. Collectively, these data suggest that miR-3607 is a tumor-suppressive miRNA that is frequently downregulated in prostate cancer. Restoration of miR-3607 expression suppresses tumorigenicity in prostate cancer cell lines. To our knowledge, this is the first report implicating a tumor suppressor role for this miRNA in prostate cancer.

Interestingly, our data suggest that miR-3607 regulates SRC family kinases, LYN and SRC. The SFKs are non-receptor tyrosine kinases that are responsible for signal transduction during key cellular processes, including proliferation, differentiation, apoptosis, migration, and adhesion (17, 18). The levels of SFK are often augmented in various human cancers, including prostate cancer, and often correlate with disease severity/metastatic potential (17–20). Increased SFK activity has been reported in hormone-independent prostate cancer leading to poor prognosis, hormone relapse, and reduced overall survival (31). In prostate cancer, two SFKs (LYN and SRC) have been specifically implicated in tumor growth and progression (32).

LYN, originally identified as a hematopoietic-specific kinase (33), is expressed in various other tissues and has been implicated in numerous signaling cascades including the PI3K pathway (18, 33, 34). It has been reported that LYN is a negative regulator of apoptosis (35, 36) and has been shown to control cellular proliferation (37) and migration (38). LYN expression is upregulated in solid tumors of various organs including prostate, glioblastoma, colon, and aggressive breast cancer and is a promising therapeutic target (18, 34). In prostate cancer, LYN is overexpressed in cancer cell lines and primary prostatic tumors (18, 34, 38). LYN+/− mice manifest prostate gland morphogenesis defects suggesting an important role of LYN in normal prostate development and implications in prostate cancer (18, 34). LYN has been reported to mediate the effects of TGFβ (39), a negative regulator of prostate cancer growth (34, 40). Also LYN-mediated signaling mechanisms influence prostate cancer cell migration (38). In fact, LYN inhibition by a specific sequence-based inhibitor decreased the proliferation of hormone-refractory prostate cancer cell lines and significantly reduced tumor growth in prostatic xenografts along with induction of apoptosis (18, 34). These studies suggest that LYN inhibition may be an effective strategy for treatment of hormone refractory prostate cancer. Our data suggest that miR-3607 inhibits LYN directly and its expression in clinical tissues is inversely correlated with miR-3607 levels. These data suggest a novel miRNA-mediated regulation of this important kinase in prostate cancer.

SRC, the prototypical member of SRC family kinases (41–43), is aberrantly activated in prostate cancer (17). SRC signaling is implicated in androgen-induced proliferation of prostate cancer cells (17, 44), progression to an androgen-independent state, and metastasis (21–23). Studies have shown that SRC inhibition in prostate cancer cell lines leads to significantly decreased proliferation, invasion, and migration in vitro (17, 45–48). In vivo studies report that SRC inhibition led to decreased...
prostate cancer growth and metastasis in xenografts (17, 32) and orthotopic (32) prostate mouse models. This kinase also plays a role in positively regulating osteoclast physiology and thus is implicated in prostate bone metastasis as well (49, 50). Our data suggest that SRC kinase is directly regulated by miR-3607 in prostate cancer. Thus, we provide first evidence that a novel miRNA located in a frequently lost genomic region plays a crucial role in prostate cancer via its ability to repress SRC family members, LYN and SRC.

In conclusion, our study suggests that miR-3607 is a crucial tumor-suppressive miRNA in prostate cancer that regulates SRC kinases that in turn regulates proliferation, apoptosis, invasion, and migration of prostate cancer cells. Frequent downregulation of miR-3607 in prostate cancer leads to upregulation of SRC and LYN proto-oncogenes that culminates in increased proliferation, invasion, and decreased apoptosis of prostate cancer cells. Thus, we have identified a novel miRNA-mediated regulatory loop that controls these important kinases in prostate cancer. Considering the critical role of SRC kinases in prostate cancer development, progression, and metastasis, these kinases are important therapeutic targets. SRC kinase inhibitors are in phase III clinical trials for treatment of advanced prostate cancer. A study suggests that SRC inhibitor, dasatinib, inhibited phosphorylation of SRC and LYN and the downstream substrate FAK in hormone-sensitive and hormone-refractory prostate cancer cell lines (31). In view of our present results, we suggest that restoration of miR-3607 levels may represent a novel therapeutic modality for prostate cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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 Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Saini, V. Shahryari, Z.L. Tabatabai, R. Dahiya
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