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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 - a microRNA gene located at chr5q14 region- in prostate cancer. Expression analyses of miR-3607 in a clinical cohort of prostate cancer (PCa) 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. Further, 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. Since 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.

Keywords

miR-3607; prostate cancer; deleted regions; SRC; LYN

INTRODUCTION

Prostate cancer (PCa) is the most common male malignancy and one of the leading causes of cancer death among men worldwide. Critical challenges plague the field of PCa hindering

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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 PCa (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 PCa 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 8p (67%), 5q (39%), 16q (37%), 6q (35%), 13q (33%), 10q (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 chromosome (chr) 5q is a frequent event in prostate cancer, particularly in advanced tumors (10). CGH analyses have identified that chr5q deletion is detected in ~28% cases of PCa and the common region of deletion is chr5q14-q23 (10–13). Loss of heterozygosity (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 was supported by large scale integrative analyses of transcriptomes and copy-number alterations (CNAs) (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). A microRNA gene, miR-3607, is located in this region. MicroRNAs (miRNAs) are small endogenous RNAs that suppress gene expression posttranscriptionally via sequence-specific interactions with the 3′-untranslated regions (UTRs) of cognate targets and play important regulatory roles in various cancers, including PCa (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 PCa clinical specimens and found that miR-3607 expression is frequently attenuated in PCa. Our analyses showed that lower miR-3607 expression levels are significantly associated with tumor progression and

poor survival outcome in PCa. Reconstitution of miR-3607 expression in PCa cell lines led to significantly decreased tumorigenicity of these cancer cell lines. Further, our data suggests that miR-3607 directly targets the SRC family of kinases (SFK). These kinases are non-receptor tyrosine kinases involved in signal transduction during key cellular processes (including proliferation, differentiation, apoptosis, migration) (17, 18) that are often augmented in PCa and correlate with disease severity/metastatic potential (17–20). Increasing evidence implicates these kinases in PCa 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 3 clinical trials for the treatment of PCa 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 PCa. Considering the fact that SFK inhibition is being exploited clinically as a therapeutic strategy for PCa patients, 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

Non-malignant 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 epidermal growth factor, 0.05 mg/mL bovine pituitary extract (Invitrogen). LNCaP, Du145, PC3 were maintained in RPMI 1640 media supplemented with 10% fetal bovine serum (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 ~24hrs before transfection. Transient transfection of miRNA precursor/anti-miR miRNA inhibitor (Ambion) was carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturers's protocol. All miRNA transfections were for 72h. miR-3607 precursor (AM17100), negative control (miR-CON) (AM17110), anti-miR-3607 inhibitor (MH19335), anti-miR-control inhibitor (4464076) were used for transfections.

Tissue samples and Ethics statement

Formalin-fixed, paraffin-embedded (FFPE) PCa samples were obtained from the SFVAMC. Written informed consent was obtained from all patients and the study was approved by the UCSF Committee on Human Research (Approval number: H9058-35751-01). All slides were reviewed by a board certified pathologist for the identification of PCa 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 hrs post-transfection, cells were counted and placed on control inserts or Matrigel inserts at 1×10^5 cells/ml in serum-free medium and were allowed to migrate for 20 h at 37°C. Cells were removed from the top of the inserts and cells that migrated/invaded though the polycarbonate/basement membrane were fixed, stained and quantified at OD 560nm after extraction. For clonogenicity assay, cells were counted, seeded at low density (1000 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, 72 hours by using the CellTiter 96 AQueousOne Solution Cell Proliferation Assay Kit (Promega), according to the manufacturer's protocol.

Flow Cytometry

Fluorescence-activated cell-sorting (FACS) analysis was done 72 hours post-transfection. The cells were harvested, washed with cold PBS, and resuspended in DAPI nuclear stain for cell cycle analysis. Cells were stained with 7-AAD 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).

Western blotting

Whole cell extracts were prepared in RIPA buffer [50 mmol/L Tris (pH 8.0), 150 mmol/L NaCl, 0.5% deoxycholate, 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, cat no. 2862), SRC (Cell Signaling, cat no. 3456), GAPDH (Santa Cruz Biotechnology, sc-32233).

Statistics

All quantified data represents an average of triplicate samples or as indicated. Data are represented as mean ± S.E.M. All statistical analyses were performed using StatView (version 5; SAS Institute Inc.) and MedCalc version 10.3.2. Two-tailed Student's t-test was used for comparisons between groups. Results were considered statistically significant at $P \leq 0.05$.

Supplemental data

The supplemental data includes supplemental materials and methods.

RESULTS

miR-3607 expression is attenuated in prostate cancer

Human miR-3607 gene is located at chromosomal position 5q 14.3 in the intron of a coding gene, COX7C (Cytochrome c oxidase subunit 7C) (Figure 1A), which is transcribed in the same direction as miR-3607. To evaluate the role of miR-3607 in PCa, we analyzed the relative expression of miR-3607-5p (major form of miR-3607, referred to as miR-3607) in a cohort of human PCa clinical specimens by real-time PCR (Figure 1B). Laser capture microdissected (LCM) PCa 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, high expression >1.25. While the expression of miR-3607 was unaltered in 22/100 cases (22%) and higher in 15/100 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 PCa 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 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, pathological stage, PSA levels and biochemical recurrence (Table 1). While 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 vs 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 pathological stage pT2, 79% of pT3 and 100% of pT4 cases. This trend indicates that miR-3607 expression tends to attenuate in higher stages of PCa (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 vs 32% of cases

with low PSA. miR-3607 was attenuated in 22/31 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 PCa.

miR-3607 is a potential prognostic and diagnostic marker in prostate cancer

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

Further, we determined the potential capability of miR-3607 as a diagnostic biomarker for PCa by performing ROC (Receiver Operating Characteristic) analyses on our cohort of PCa clinical samples (Figure 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% CI: 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 PCa.

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

In view of observed widespread downregulation of miR-3607 expression in PCa clinical specimens, we evaluated the potential tumor suppressive role of miR-3607 using PCa cell lines. We overexpressed miR-3607 in PCa cell lines (PC3, Du145 and LNCaP) followed by functional assays (Figure 2 and S1). Transient transfection of miR-3607 precursor led to overexpression of miR-3607 as determined by real-time PCR (Figure S1). Overexpression of miR-3607 significantly suppressed the proliferation of PCa cell lines as assessed by clonogenicity assay (Figure 2A). A significant decrease in cell viability was observed over time in PC3/Du145/LNCaP cells overexpressing miR-3607 as compared to cells expressing control miR (miR-CON) (Figure 2B).

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

Since miR-3607 overexpression led to reduced proliferation of PCa 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 (fluorescence activated cell sorting) analysis (Figure 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 to miR-CON. This suggests that miR-3607 overexpression induces a G0-G1 arrest in PCa 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-tranfected cells by flow cytometric analysis of Annexin-V-FITC-7-AAD stained PC3/Du145/LNCaP cells (Figure 2D). It was observed that the average apoptotic cell fractions (Early apoptotic + Apoptotic) were significantly increased upon miR-3607 overexpression compared to miR-CON/mock transfected cells with a concomitant decrease in the viable cell population. This suggests that miR-3607 induces apoptosis in PCa 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-tranfected PC3/Du145/LNCaP PCa cell lines (Figure 3). These assays showed that overexpression of miR-3607 significantly decreased the invasiveness (Figure 3A) and migratory abilities (Figure 3B) of all the PCa 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 (Figure 4). Basal level of miR-3607 expression in these normal immortalized prostate epithelial cell lines is higher than that of PC3 and Du145 (Fig. S2). miR-3607 knockdown was confirmed by RT-PCR (Figure 4A). Our results suggest that knockdown of miR-3607 increased the proliferation, invasiveness and motility of non-transformed epithelial cells (Figure 4B–D). Cell cycle analysis showed a significant increase in G2-M phase upon miR-3607 inhibition (Figure 4E). These results support a tumor-suppresseive role for miR-3607 in PCa.

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 while SRC has two potential miR-3607 binding sites (Figure 5A). While 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 (Figure 5B). Interestingly, miR-3607 overexpression led to decreased protein levels of LYN and SRC. Further, we investigated whether these nonreceptor tyrosine kinases are direct functional targets of miR-3607 in PCa. We transiently transfected PC3 cells with the control/LYN/SRC 3′UTR luciferase reporter plasmids along with miR-3607 precursor/miR-CON (Figure 5C). miR-3607 overexpression led to significant decreases in LYN/SRC luciferase reporter activity as compared to 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 PCa tissues by RT-PCR (n=15) and observed a negative correlation between the expression of these SRC kinases and miR-3607 in 14/15 tissues (93%) (Figure 5D–E). Clinical samples with low miR-3607 expression (relative to adjacent normal tissue) showed high levels of LYN and SRC expression (Figure 5D–E). These data support the concept that these SRC kinases are important targets of miR-3607 in PCa.

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

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

DISCUSSION

In this report, we define for the first time, a novel regulatory role for a miRNA gene located in frequently deleted region of PCa. Genomic studies have suggested that chromosomal region 5q deletions are associated with PCa, particularly in advanced tumors (8, 11–14). The common region of deletion is chromosome 5q14-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 5q 14.3, is frequently downregulated in human PCa clinical specimens. In view of its low expression, we assessed the potential for miR-3607 as a PCa 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 PCa. Low miR-3607 expression was significantly associated with PCa cases with higher stage and gleason score. These findings support the association of chromosome 5q losses with advanced prostatic tumors (10). Also, we observed that miR-3607 expression was significantly associated with serum PSA levels in PCa patients. Further, low miR-3607 expression was significantly correlated with poor survival outcome in PCa clinical specimens. These findings suggest that this novel miRNA may be a potential disease biomarker for PCa prognosis and diagnosis.

The observed downregulation of miR-3607 expression in PCa 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, Du145) human PCa 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 GO-G1 cell cycle arrest and induction of apoptosis in all the PCa cell lines tested. Further, miR-3607 overexpression also decreased invasiveness and migratory properties of PCa 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 suggests that miR-3607 is a tumor suppressive miRNA that is frequently downregulated in prostate cancer. Restoration of miR-3607 expression suppresses tumorigenicity in PCa cell lines. To our knowledge, this is the first report implicating a tumor suppressor role for this miRNA in prostate cancer.

Interestingly, our data suggests that miR-3607 regulates SRC family kinases- LYN and SRC. The SRC family of kinases (SFK) 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 PCa, and often correlates with disease severity/metastatic potential (17–20). Increased SFK activity has been reported in hormone-independent PCa leading to poor prognosis, hormone relapse and reduced overall survival (31). In PCa, 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 phosphatidyl inositol -3 (PI-3) kinase 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 PCa, 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 PCa (18, 34). LYN has been reported to mediate the effects of transforming growth factor β (39), a negative regulator of PCa growth (34, 40). Also LYN-mediated signaling mechanisms influences PCa cell migration (38). Infact, LYN inhibition by a specific sequence-based inhibitor decreased the proliferation of hormone-refractory PCa cell lines and significantly reduced tumor growth in prostatic cancer 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 suggests that miR-3607 inhibits LYN directly and its expression in clinical tissues is inversely correlated with miR-3607 levels. These data suggests a novel microRNA-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 suggests 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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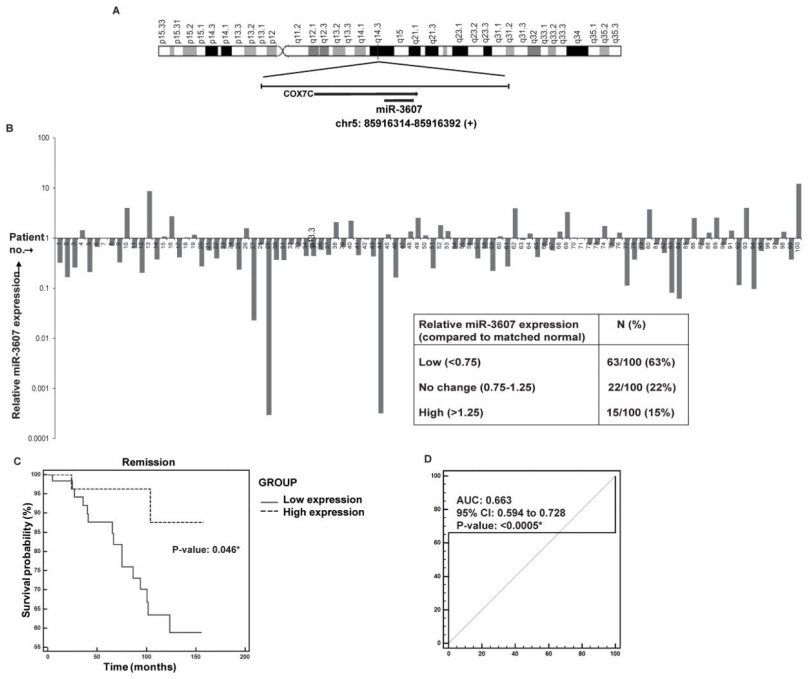

Fig. 1. miR-3607 expression is widely attenuated in prostate cancer

(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 laser capture microdissected (LCM) PCa 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 PCa patients, stratified based on miR-3607 expression (low and high). P-value based on log rank test (* P< .05).

(D) ROC curve analysis showing the ability of miR-3607 expression to discriminate between malignant and non-malignant prostate tissue samples.

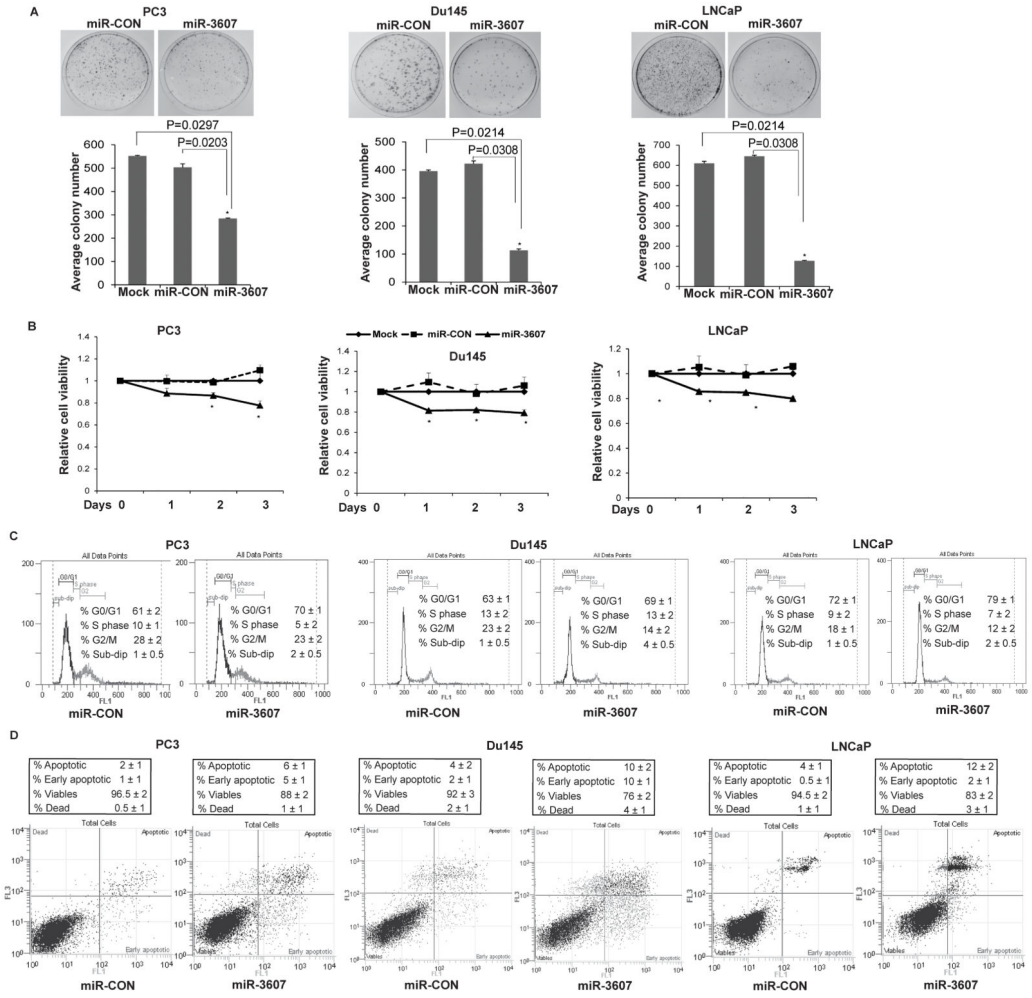

Fig. 2. Overexpression of miR-3607 suppresses proliferation and induces apoptosis in prostate cancer cell lines

To evaluate the functional significance of miR-3607 in PCa, miR-3607 precursor was overexpressed in PC3/Du145/LNCaP cell lines by transient transfections followed by functional assays (performed 72 hrs post-transfection). For comparison, mock transfections/ control miRNA (miR-CON) transfections were also carried out.

(A) Colony formation assay in PC3/Du145/LNCaP cells after miR-CON/miR-3607 transfection.

(B) Cellular viabilities in PC3/Du145/LNCaP cells after mock/miR-CON/miR-3607 transfection as assessed by MTS assay.

(C) Cell cycle analysis in PC3/Du145/LNCaP cells after miR-CON (left panel) or miR-3607 (right panel) treatments for 72 hrs. Cells were stained with DAPI for FACS analysis.

(D) Apoptosis assay in PC3, Du145, LNCaP cells after miR-CON (left panels) or miR-3607 (right panels) transfection for 72 hrs as assessed by ANNEXIN V-FITC/7-AAD staining.

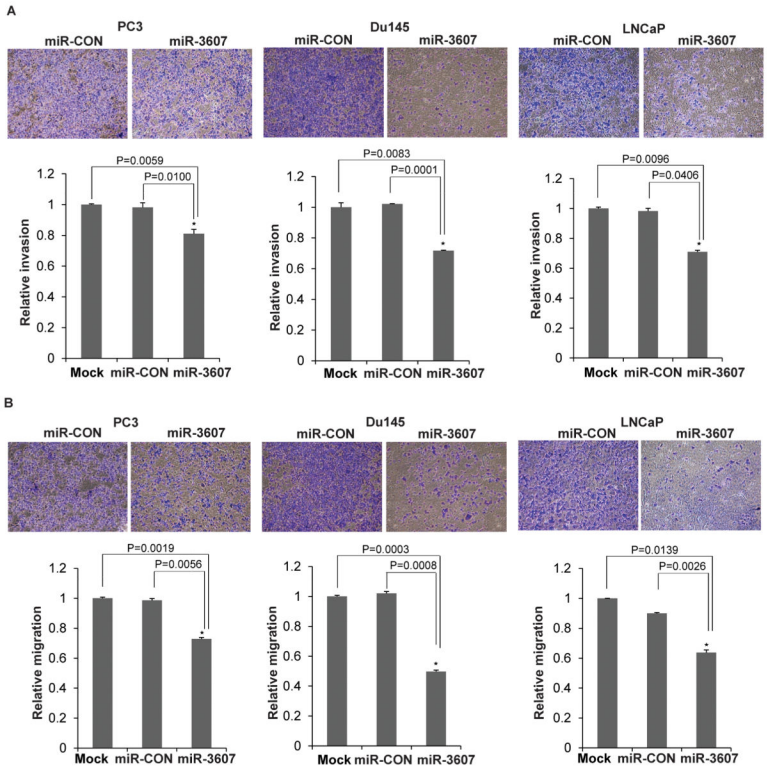

Fig. 3. Overexpression of miR-3607 reduces invasiveness of prostate cancer cell lines

(A) Transwell invasion assays and (B) Migration assays in control transfected and miR-3607-tranfected PC3/Du145/LNCaP PCa cell lines. These assays showed that overexpression of miR-3607 significantly decreased the invasiveness and migratory abilities of PCa cell lines (* P< .05).

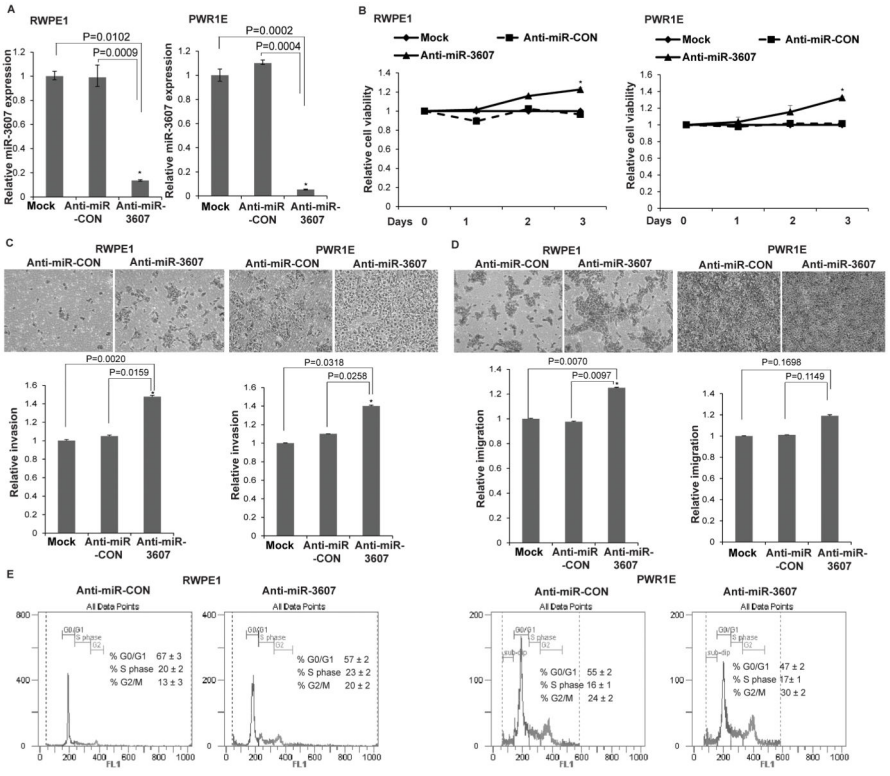

Fig. 4. miR-3607 knockdown increases invasiveness and proliferation of normal immortalized prostate epithelial cell lines

Normal immortalized prostate epithelial cell lines (RWPE1 and PWR1E) were treated with mock/miRVANA anti-miR-3607 inhibitor/control inhibitor (anti-miR-CON) followed by functional assays (performed 72 hrs post-transfection).

(A) Relative miR-3607 expression as assessed by real time PCR upon the indicated treatments.

(B) Cellular viabilities in RWPE1/PWR1E cells after mock/anti-miR-CON/anti-miR-3607 transfections as assessed by MTS assay.

(C) Transwell invasion assays (D) Migration assays (E) Cell cycle analysis in mock/anti-miR-CON/anti-miR-3607 tranfected cell lines (* P< .05).

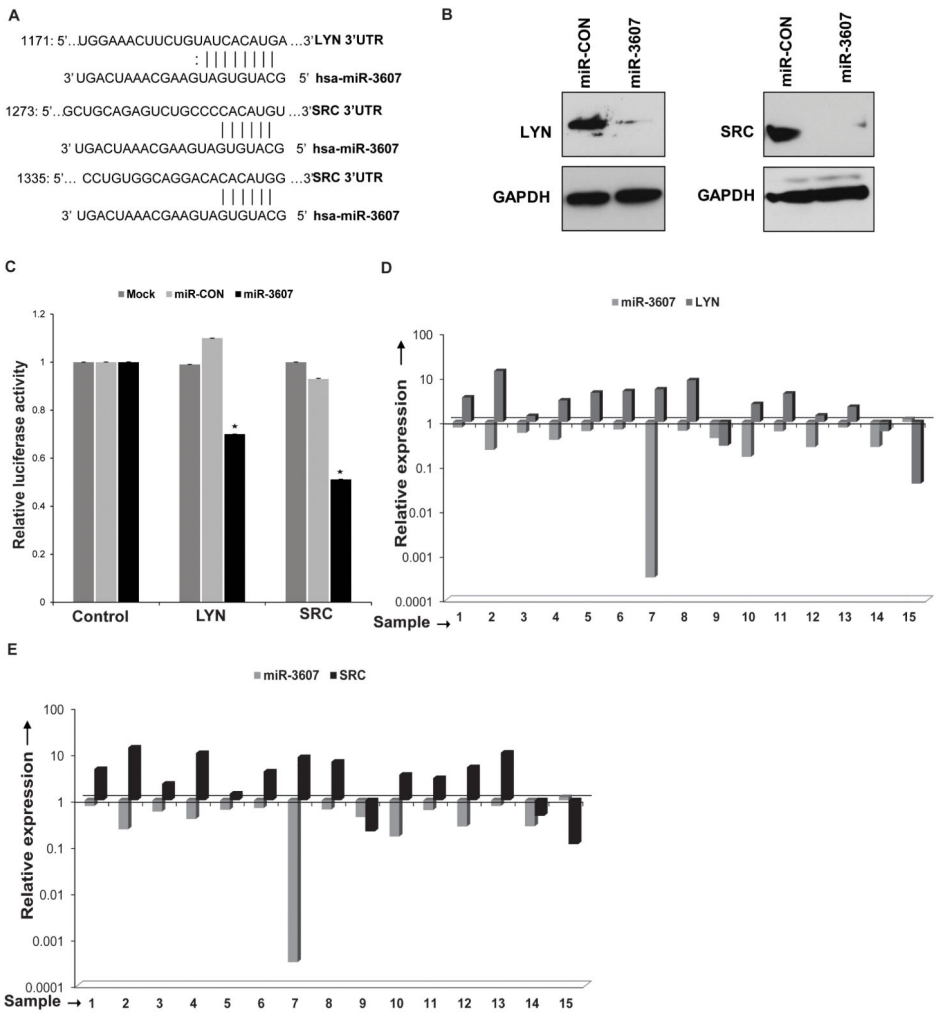

Fig. 5. miR-3607 directly targets SRC family kinases in prostate cancer

(A) Schematic representation of the LYN and SRC 3′-UTRs showing the relative positions of putative miR-3607 binding sites. LYN possesses one potential miR-3607 binding site within its 3′-UTR while SRC has two potential miR-3607 binding sites.

(B) Immunoblots of endogenous LYN and SRC in PC3 cells transfected as indicated. GAPDH was used a loading control.

(C) LYN/SRC 3′ UTR luciferase reporter construct encompassing miR-3607 binding site/sites or the control construct was cotransfected into PC3 cells with miR-3607 or miR-CON and assayed for relative luciferase activity (* P< .05).

(D) Correlation between miR-3607 and LYN expression in a clinical cohort subset (n=15). Relative expression levels of LYN and miR-3607 (as determined by RT-PCR) for the samples analysed are represented in the bar graph. Data were normalized to GAPDH control.

(E) Relative expression levels of SRC and miR-3607 (as determined by RT-PCR) for the samples analysed are represented in the bar graph. Data were normalized to GAPDH control.

Table 1

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

Correlation of miR-3607 expression with clinicopathological characteristics of PCa patients including age, gleason score, pathological stage, PSA levels and biochemical recurrence was examined (* P< .05).

Clinicopathological parameter**	Total n (%)	Relative miR-3607 expression			P-value
		Low	No change	High	
Age (in years)					
0–49	4/100 (4)	3/4 (75)	-	1/4 (25)	P=0.5065
50–59	34/100 (34)	21/34 (62)	6/34 (18)	7/34 (20)	
60–69	47/100 (47)	29/47 (62)	9/47 (19)	9/47 (19)	
70–79	14/100 (14)	9/14 (64)	-	5/14 (36)	
Gleason score					
6 (3+3)	48/100 (48)	26/48 (54)	10/48 (21)	12/48 (25)	P=0.0173*
7 (3+4)	29/100 (29)	16/29 (55)	4/29 (14)	9/29 (31)	
7 (4+3)	12/100 (12)	11/12 (92)	-	1/12 (8)	
8–10	9/100 (9)	8/9 (89)	1/9 (11)	-	
Pathological stage					
pT2	68/100 (68)	37/68 (54)	14/68 (21)	17/68 (25)	P=0.0360*
pT3	19/100 (19)	15/19 (79)	1/19 (5)	3/19 (16)	
pT4	1/100 (1)	1/1 (100)	-	-	
PSA levels					
≤ 4.7 (Median)	31/100 (31)	10/31 (32)	11/31 (36)	10/31 (32)	P=0.0001*
>4.7 (Median)	63/100 (63)	47/63 (75)	4/63 (6)	12/63 (19)	
Biochemical recurrence					
Yes	**31/100 (31)**	**22/31(71)**	**2/31 (6)**	**7/31 (23)**	P=0.2381
No	**63/100 (63)**	**36/63 (57)**	**12/63 (19)**	**15/63 (24)**	