miRNA-708 Control of CD44 \(^+\) Prostate Cancer–Initiating Cells

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Abstract

Tumor recurrence in prostate cancer has been attributed to the presence of CD44-expressing tumor-initiating cells. In this study, we report that miR-708 is a key negative regulator of this CD44 \(^+\) subpopulation of prostate cancer cells, with important implications for diagnosis and prognosis of this disease. miR-708 was underexpressed in CD44 \(^+\) cells from prostate cancer xenografts. Reconstitution of miR-708 in prostate cancer cell lines or CD44 \(^+\) prostate cancer cells led to decreased tumorigenicity \textit{in vitro}. Intratumoral delivery of synthetic miR-708 oligonucleotides triggered regression of established tumors in a murine xenograft model of human prostate cancer. Conversely, miR-708 silencing in a purified CD44 \(^-\) population of prostate cancer cells promoted tumor growth. Functional studies validated CD44 to be a direct target of miR-708 and also identified the serine/threonine kinase AKT2 as an additional target. Clinically, low miR-708 expression was associated significantly with poor survival outcome, tumor progression, and recurrence in patients with prostate cancer. Together, our findings suggest that reduced miR-708 expression leads to prostate cancer initiation, progression, and development by regulating the expression of CD44 as well as AKT2. miR-708 therefore may represent a novel therapeutic target or diagnostic and prognostic biomarker in prostate cancer. 

Introduction

Despite many advances, clinical management of prostate cancer remains a major challenge and this disease continues to represent a leading cause of cancer-related morbidity and mortality (1). A major challenge in prostate cancer diagnosis is posed by the inability of current diagnostic methods, including prostate-specific antigen (PSA) screening and histopathologic grading, to distinguish between indolent and aggressive tumors. Recent changes in recommendations suggest later and less frequent PSA screenings (2, 3). In view of this, there is an urgent need to identify alternate prostate disease biomarkers with better prognostic and diagnostic potential. Another challenging aspect of prostate cancer is the high rates of recurrence associated with the disease. About 40% of men with localized prostate cancer suffer from disease recurrence, as monitored by rising PSA and tumor progression to hormone-refractory/castration-resistant prostate cancer (1, 5) that is essentially untreatable (1, 6). Therefore, a second major clinical challenge is the elucidation of pathways of tumor recurrence and metastasis of prostate cancer, which could lead to the design of better therapeutic strategies.

Tumor recurrence and progression in prostate cancer has been associated with the existence of tumor-initiating cells (TIC) or prostate cancer stem cells within the bulk of the tumor that are refractory to current therapies (7). Several putative TICs have been identified in human prostate cancer and are characterized by various cell surface markers (8–12). Cell adhesion molecule CD44 has been identified as a predominant cell surface marker of prostate cancer TICs (7–12). It has been reported that CD44 \(^+\) prostate cancer cells are more proliferative, clonogenic, tumorigenic, and metastatic than the isoegenic CD44 \(^-\) prostate cancer cells (10). Here, we report that CD44 \(^+\) prostate cancer cells are regulated by a miRNA (miR), miR-708. Analysis of an array of human prostate cancer specimens showed the widespread attenuation of miR-708 expression, suggesting that miR-708 downregulation is a common event in prostate cancer. We also found that this molecular alteration has significant diagnostic and prognostic potential. Consistent with the functional role of miR-708 in regulating prostate cancer TIC marker CD44, a positive correlation was found between reduced miR-708 expression and tumor progression, biochemical recurrence, and poor survival outcome in prostate cancer.
Materials and Methods

Cell lines and cell culture
NOMInonmalignant epithelial prostate cell lines (RWPE-1 and PWR-IE) and prostate carcinoma cell lines (LNCaP, Du145, PC3, VCaP, MDA-PCa-2b) were obtained from the American Type Culture Collection (ATCC) and cultured under recommended conditions as described previously (ref. 13; also described in Supplementary Methods). These human-derived cell lines were authenticated by DNA short tandem repeat analysis by ATCC. The experiments with cell lines were conducted within 6 months of their procurement/resuscitation.

Prostate cancer xenografts
Xenograft human prostate tumors LAPC-4 and LAPC-9 were courtesy of R. Reiter (14, 15), PC3 xenograft tumors were established using early-passage cells and maintained in non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice. NOD/SCID mice (Charles River Laboratories) were maintained under standard conditions. All animal care was in accordance with the guidelines of the San Francisco Veterans Affairs Medical Center (VAMC) and the study was approved by the San Francisco Veterans Affairs Institutional Animal Care and Use Committee.

Purification of CD44+ subpopulations of xenograft tumors
Basic procedures previously described were followed (10, 16, 17). Single-cell suspensions obtained from xenograft tumors were stained for CD44 with human-specific fluorescein isothiocyanate (FITC)-conjugated anti-CD44 antibody (Miltenyi Biotec, 130-095-195) as per manufacturer’s protocol. Stained cells were purified into CD44+ and CD44− cells with fluorescence-activated cell sorting (FACS). Post-sort analysis revealed purities of both populations being more than 95%.

miRNA/siRNA transfections
Cells were plated in growth medium without antibiotics for approximately 24 hours before transfections. Transient transfections of miRNA precursor (Ambion)/siRNA (Origene) were carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. All miRNA/siRNA transfections were done for 72 hours.

Tissue samples
Formalin-fixed, paraffin-embedded prostate cancer samples were obtained from the San Francisco VAMC. Written informed consent was obtained from all patients and the study was approved by the University of California San Francisco Committee on Human Research. All slides were reviewed by a board-certified pathologist for the identification of prostate cancer foci as well as adjacent normal glandular epithelium.

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 after transfection, 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 though the polycarbonate basement membrane were fixed, stained, and quantified at optical density of 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.

Apoptosis assays
FACS analysis for apoptosis was done 72 hours after transfection, using Annexin V-FITC/7-AAD Kit (Beckman Coulter, Inc.), according to the manufacturer’s protocol. Stained cells were immediately analyzed with a flow cytometer (Cell Lab Quanta SC; Beckman Coulter, Inc.).

Luciferase assays
For CD44 and AKT2, the corresponding 3′-untranslated region (UTR) reporter constructs (catalog no., HmiT022972, HmiT004350-MT01) were obtained from GeneCopoeia along with control construct (CmiT000001-MT01). All these target clones were in SV-40 promoter–based vector pEZX-MT01 (GeneCopoeia). Control constructs and various 3′-UTR reporter constructs (0.2 µg) were cotransfected into PC3/LNCaP cells cultured in 24-well plates along with 50 nmol/L miR-708 or miR-CON (Ambion) using Lipofectamine 2000 (Invitrogen). 48 hours after transfection, firefly and Renilla luciferase activities were measured by using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer’s protocol. Firefly luciferase was normalized to Renilla luciferase activity.

In vivo intratumoral delivery of miR-708
We examined the antitumor effects of miR-708 by local administration in established tumors. Nude mice (4- to 5-week-old, Charles River Laboratories; n = 12) received subcutaneous injections of 3 × 106 PC3 cells in the right flank area in a volume of 100 µL. Once palpable tumors developed, caliper measurements were taken twice a week and tumor volume was calculated on the basis of width (x) and length (y): x2y/2, where x < y. When tumors reached an average volume of 100 to 150 mm3, 6.25 µg of synthetic miRNA (miR-708/miR-CON) complexed with 1.6 µL siPORT Amine transfection reagent (Ambion) in 50 µL PBS was delivered intratumorally in 3-day intervals. The dosage was selected on the basis of previous studies done by our group and others (18, 19). Synthetic miRNAs are double-stranded, ready-to-use miRNA mimics and were purchased from Ambion (pre-miR, cat. no. AM17100). Mice were killed 2 days after the last treatment (day 58), tumors were collected, and total RNA and protein was extracted.

Statistics
All quantified data represent an average of at least triplicate samples or as indicated. Data are represented as mean ± SEM. All statistical analyses were conducted 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 $P \leq 0.05$.

Full Methods are available in the Supplementary Materials and Methods.

Results

CD44 is a functional target of miR-708 in prostate cancer

Because CD44 is an important marker that determines tumorigenic behavior of prostate cancer cells, it is essential to elucidate regulatory mechanisms that converge on this molecule. miRNAs constrain gene expression by binding to the 3'UTRs of cognate mRNA targets (20). To explore the potential regulation of this TIC marker by miRNAs, we used 2 algorithms that predict the mRNA targets of a miRNA, miRAN-DA (21) and TargetScan (22). From these analyses, miR-708 stood out for the presence of 2 binding sites, suggesting cooperative binding and interaction (Fig. 1A). To test the potential regulation of CD44 by miR-708, we purified CD44+ and CD44− subpopulations of prostate cancer cells from 2 prostate cancer xenograft mouse models (PC3 and LAPC9) followed by miR-708 expression profiling (Fig. 1B). miR-708 expression was significantly downregulated in CD44+ versus CD44− cells suggesting that miR-708 regulates the CD44+ subpopulation in prostate cancer. To validate this further, we overexpressed miR-708 in prostate cancer cell lines (PC3 and LNCaP). Transient transfection of miR-708 precursor led to overexpression of miR-708 as determined by real-time PCR.

Figure 1. CD44 is a functional target of miR-708 in prostate cancer. A, schematic representation of CD44 3'-UTR showing the relative positions of putative miR-708 target sites. B, relative miR-708 levels in purified CD44+ and CD44− subpopulations of prostate cancer xenografts (PC3 and LAPC9). Data were normalized to RNU48 control and are represented as mean ± SEM. C, relative miR-708 expression in PC3/LNCaP cells transfected with control miR/miR-708/mock-transfected cells as assessed by RT-PCR (left). Immunoblots for endogenous CD44 protein in transfected PC3/LNCaP cells (right). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used a loading control. D, CD44 3'-UTR construct or the control construct was cotransfected into PC3/LNCaP cells along with miR-708/miR-CON/mock-treated cells and assayed for luciferase activity. Firefly luciferase values were normalized to Renilla luciferase activity and plotted as relative luciferase activity (*, $P < 0.05$).
miR-708 expression is widely attenuated in prostate cancer

To evaluate the role of miR-708 in prostate cancer, miR-708 expression was assayed in human prostate cell lines, which included normal epithelial prostate cell lines (RWPE-1 and PWR-1E) and prostate carcinoma cell lines (LNCaP, LAPC4, Du145, PC3, VCaP, MDA-PCa-2b, LAPC9). Relative miR-708 expression was specifically attenuated in all the examined prostate carcinoma cell lines compared with normal epithelial prostate cell lines (Fig. 2A). To examine the clinical relevance of

Figure 2. miR-708 expression is widely attenuated in prostate cancer. A, qRT-PCR analysis of relative miR-708 expression in normal epithelial prostate cell lines (RWPE-1 and PWR-1E) and prostate carcinoma cell lines (LNCaP, LAPC4, Du145, PC3, VCaP, MDA-PCa-2b, LAPC9). Relative miR-708 expression levels in normal epithelial prostate cell lines and patient-matched adjacent normal regions as assessed by RT-PCR. Table below summarizes the relative miR-708 expression levels in these specimens. C, in situ hybridization analysis of relative miR-708 expression levels in prostate cancer tissues and normal prostate tissues. Tissues were hybridized with digoxigenin-labeled locked nucleic acid–based probes for miR-708 and U6 (control). miR-708 expression was scored and normalized to U6 scores. The average value for each group is represented by the horizontal line. D, correlation of miR-708 expression with clinicopathologic characteristics of patients with prostate cancer. E, ROC curve analysis showing the ability of miR-708 expression to discriminate between malignant and nonmalignant tissue samples. F, Kaplan–Meier survival curves for patients with prostate cancer, stratified on the basis of miR-708 levels. *P value is based on a log-rank test. (*, P < 0.05).
this finding, we extended our analysis to human clinical prostate samples \((N = 136); \) Fig. 2B and C, Supplementary Fig. S1). Clinicopathologic characteristics of the patients are summarized in Supplementary Table S1. We examined the expression levels of miR-708 in laser capture microdissected (LCM) prostate cancer tissues \((n = 96)\) and matched adjacent normal regions by RT-PCR (Fig. 2B). While the expression of miR-708 was unaltered in 13 of 96 cases \((13\%)\) and higher in 16 of 96 cases \((17\%)\), a major fraction of tissue samples \((67 of \; 96, \; \sim70\%)\) showed lower miR-708 levels relative to matched normal tissues. The differences were statistically significant with the Wilcoxon signed rank test \((P < 0.0001)\). We extended our analysis of clinical specimens by assessing miR-708 levels in 40 additional cases of prostate adenocarcinoma, 8 of which had matched normal adjacent tissue by in situ hybridization analysis and again observed attenuated expression of miR-708 in cancer tissues compared with normal tissues (Fig. 2C, Supplementary Fig. S1). This confirms that miR-708 is widely down-regulated in prostate cancer and suggests that miR-708 is a potential tumor suppressor.

**Downregulation of miR-708 expression is associated with tumor progression and biochemical recurrence in prostate cancer**

We also looked to see whether miR-708 expression in clinical tissues correlated with clinicopathologic characteristics such as age, Gleason score, pathologic stage, and biochemical recurrence of prostate cancer (Fig. 2D). While there was no significant correlation with age, decreased miR-708 expression was observed in 63% of cases of low Gleason score \((4–6)\), 74% of cases of Gleason score \(7\), and in 90% of cases of higher Gleason score \((8–10)\). This trend suggests that miR-708 expression tends to attenuate in higher grade prostate cancer \((P = 0.0098)\).

Similarly, decreased miR-708 expression was observed in 72% of cases of pathologic stage pT2, 83% of cases of pT3, and 100% of pT4 cases, though no statistically significant correlation was observed between miR-708 expression and pathologic stage. Importantly, miR-708 was specifically attenuated in 18 of 22 cases \((82\%)\) of PSA failure within this cohort of samples suggesting that downregulation of miR-708 is associated with biochemical recurrence.

Furthermore, we stratified the tissue cohort, based on age and PSA and used age-adjusted PSA ranges to examine the correlation of miR-708 expression with serum PSA levels (Supplementary Table S2). Within our dataset, 23 of 85 \((21\%)\) samples had age-adjusted PSA within the normal range. Analysis of miR-708 expression in these cases showed that 16 of 23 cases \((\sim70\%)\) exhibited attenuated miR-708 expression suggesting that miR-708 expression has a better disease predictive value than PSA in these cases.

**miR-708 as a diagnostic and prognostic marker in prostate cancer**

In view of the observed widespread downregulation of miR-708 in prostate cancer cell lines and clinical malignancies, we evaluated the potential clinical significance of miR-708 expression. To evaluate the potential capability of miR-708 as a diagnostic biomarker for prostate cancer, we conducted receiver operating characteristic (ROC) analyses on the cohort of clinical samples (Fig. 2E). ROC analyses showed that miR-708 expression can be a single significant parameter to discriminate between normal and tumor tissues with an area under the ROC curve \((AUC)\) of \(0.937\) \([95\%\; confidence\; interval\; (CI), \; 0.901–0.963; \; P < 0.0001]\). Furthermore, Kaplan–Meier survival analysis for patients with prostate cancer, stratified on the basis of miR-708 levels, showed that survival was significantly reduced in patients with low miR-708 expression (Fig. 2F). The HR between the cases with low or high miR-708 expression was 6 with a 95% CI from 2.2 to 16.4 and with an associated \(P\) value of 0.0223. This analysis suggests that miR-708 has significant potential to be used as a diagnostic and prognostic marker for prostate cancer.

**miR-708 reexpression suppresses tumorigenicity in vitro**

To assess the tumor-suppressive role of miR-708, we reexpressed miR-708 in both prostate cancer cell lines (PC3 and LNCaP; Fig. 1C, left) followed by functional assays (Fig. 3). A significant decrease in cell viability was observed over time in PC3/LNCaP cells overexpressing miR-708 (Fig. 3A) as compared with cells expressing control miR \((\text{miR-CON})\). miR-708 reexpression decreased the clonogenicity (Fig. 3B), migration (Fig. 3C), and invasiveness (Fig. 3D) of PC3/LNCaP cells. Reexpression of miR-708 also led to marked morphologic changes in both the cell lines (Supplementary Fig. S2). Specifically, there was a decrease in the fraction of elongated, spindle-shaped cells paralleled by an increase in rounded, apoptotic cells. Apoptosis assay (Fig. 3E–G) showed that the average apoptotic cell fractions were significantly increased upon miR-708 reexpression compared with miR-CON/mock-transfected cells with a concomitant decrease in the viable cell population. As shown in Fig. 3G, miR-708 introduction led to a 3.15-fold and 3.73-fold increase in apoptosis in PC3 and LNCaP cells, respectively, relative to mock/miR-CON controls. These observations suggest that miR-708 reexpression suppresses the tumorigenicity of prostate cancer cells in vitro.

**Intratumoral delivery of miR-708 leads to tumor regression in prostate cancer xenografts and attenuates CD44 expression**

We further examined the therapeutic potential of synthetic miR-708 mimics in vivo in a mouse prostate cancer xenograft model. Toward this, we subcutaneously injected PC3 cells into nude mice and maintained them until the animals developed solid, palpable tumors, following which miR-708 or miR-CON was administered intratumorally every 3 days. Intratumoral delivery of synthetic miR-708 induced a specific inhibitory response and significantly inhibited tumor growth compared with control mice (Fig. 4A and B). To correlate the therapeutic response with delivery of miR-708, RNA was extracted from harvested control \((n = 2)\) or miR-708 tumors \((n = 5)\), and miR-708 expression was assessed by quantitative RT-PCR \((qRT-\text{PCR}; \) Fig. 4C). Tumors injected with miR-708 mimic contained significantly more miR-708 than control tumors. Furthermore, as our in vitro data suggested that CD44 is a direct functional target of miR-708, we surmised that if biologically meaningful, this mechanism might also be found in these xenograft tumors.
Figure 3. Restoration of miR-708 expression suppresses tumorigenicity in vitro. To evaluate the role of miR-708 in prostate cancer, miR-708 precursor was reintroduced in PC3/LNCaP cell lines by transient transfections followed by functional assays after 72 hours. Cell viability assay (A), colony formation assay (B), Transwell migration assay (C), and invasion assay (D) with PC3/LNCaP cells mock-transfected/transfected with miR-CON or miR-708. E, apoptosis assay in PC3 and LNCaP (F) cells. Seventy-two hours after transfection, cells were stained with Annexin V-FITC/7-AAD and immediately analyzed with a flow cytometer. G, representation of relative apoptotic fractions (early + late apoptotic) in each group for PC3/LNCaP cells. Apoptosis induced by miR-CON/miR-708 was normalized to mock control (\( * \), \( P < 0.05 \)).
models. To validate this, we extracted protein from these xenografts and gauged whether intratumoral administration of miR-708 altered CD44 expression (Fig. 4D). Indeed, CD44 protein levels were repressed in miR-708 injected xenografts compared with the control tumors. This finding reinforces the idea that CD44 is a direct target of miR-708.

CD44 knockdown partially phenocopies miR-708 reexpression in prostate cancer cells

To determine whether CD44 is a functionally relevant target of miR-708 in prostate cancer, we inhibited CD44 expression using siRNA to see whether CD44 knockdown functionally mimics the effects of miR-708 overexpression in prostate cancer. We treated PC3 cells with CD44 siRNA followed by in vitro functional assays. We initially tested 3 sets of siRNA against CD44 to achieve efficient knockdown, as assessed by RT-PCR and immunoblot analysis (Fig. 5A). si1 produced the most efficient knockdown and was used in subsequent experiments. siRNA inhibition of CD44 led to decreased cell viability, migration, and invasion of PC3 cells (Fig. 5B–D). Apoptosis assay showed that apoptotic cell fractions were significantly increased upon CD44 knockdown compared with control, an effect similar to that observed upon miR-708 reintroduction in PC3 cells (Fig. 5E and F). These results suggest that CD44 is an important determinant of the tumorigenic properties of prostate cancer cells and that CD44 inhibition partially phenocopies the antitumorigenic effects of miR-708 in prostate cancer. Furthermore, in view of our present results showing that CD44+ cells from prostate cancer xenografts presented lower miR-708 expression, we also examined whether CD44 knockdown in PC3 cells altered miR-708 expression (Fig. 5G). Interestingly, siRNA-mediated CD44 knockdown led to augmentation of miR-708 expression. This observation reinforces the regulatory interplay between miR-708 and CD44.

miR-708 inhibits tumor-initiating capacity of prostate cancer cells in vitro

Because CD44 has been identified as a major molecular determinant of prostate cancer TICs (7–12), and in view of our present results suggesting that miR-708 regulates CD44, we
Figure 5. CD44 knockdown partially phenocopies miR-708 reexpression in prostate cancer cells. PC3 cells were transfected with CD44 siRNA/nonspecific (NS) control siRNA for 72 hours, followed by various assays. A, relative CD44 mRNA expression and protein expression after siRNA transfections as assessed by RT-PCR and immunoblotting, respectively. Cell viability assay (B), Transwell migration assay (C), invasion assay (D), and apoptosis assay (E) in PC3 cells after NS siRNA (left) or CD44 siRNA (right) treatment. F, representation of average apoptotic fractions in each group (\( P < 0.05 \)). G, relative miR-708 levels after siRNA transfections as assessed by RT-PCR. Data were normalized to RNU48 control and are represented as mean ± SEM.
asked whether miR-708 has the potential to alter the tumor-initiating capacity of prostate cancer cells. To this end, we purified CD44\(^+\) and CD44\(/\)0 subpopulations from PC3 xenografts. miR-708 was overexpressed in CD44\(^+\) cells (A–D) and inhibited in CD44\(/\)0 cells (E–G), followed by in vitro assays. A, validation of miR-708 overexpression in CD44\(^+\) cells by qRT-PCR. B, phase contrast images of CD44\(^+\) cells transfected with miR-CON/miR-708. Sphere formation assay (C) and clonogenicity assays (D) in miR-CON/miR-708–transfected CD44\(^+\) cells. E, assessment of miR-708 inhibition in purified CD44\(/\)0 PC3 cells by qRT-PCR. F, phase contrast images of CD44\(/\)0 cells transfected with anti-miR-CON/anti-miR-708 oligos. G, sphere formation assay in anti-miR-CON/anti-miR-708–transfected CD44\(/\)0 cells (*, P < 0.05).
AKT2 is also a functional target of miR-708 in prostate cancer

Using miRNA target prediction algorithms miRanda (21) and TargetScan (22), we found that AKT2 is another putative miR-708 target as it possesses 3 potential miR-708-binding sites within its 3'-UTR (Fig. 7A). miR-708 reconstitution in PC3/LNCaP cells led to reduced endogenous protein levels of AKT2 (Fig. 7B). Luciferase reporter assays with control/AKT2 3'- UTR reporter constructs in miR-708/miR-CON—expressing or mock-transfected PC3/LNCaP cells showed that miR-708 represses AKT2 directly (Fig. 7C). To validate this further, we examined AKT2 levels from xenograft mouse model study and found that intratumoral administration of miR-708 led to reduced AKT2 expression compared with control (Fig. 7D). We also examined AKT2 regulation in CD44− and CD44+ subpopulations of PC3 xenografts (Supplementary Fig. S3) and found increased AKT2 expression in CD44+ subpopulation. This observation lends support to our hypothesis that AKT2 levels are directly regulated by miR-708.

To explore whether AKT2 is a biologically relevant mediator of antitumorigenic effects of miR-708 in prostate cancer, we conducted phenocopy experiments, where we inhibited AKT2 by siRNA and determined its effects on the tumorigenicity of prostate cancer cell line PC3 (Fig. 7E–I). Out of 3 sets of siRNAs tested, si2 produced the most efficient knockdown of AKT2 as assessed by RT-PCR and immunoblot analysis (Fig. 7E). Subsequent experiments using this siRNA showed that inhibition of AKT2 led to decreases in cellular viability (Fig. 7F), migration (Fig. 7G), and invasion (Fig. 7H) of PC3 cells and increased apoptosis compared with control siRNA–treated cells (Fig. 7I). This suggests that AKT2 is another biologically relevant target of miR-708 in prostate cancer and miR-708–mediated repression of AKT2 may partially determine its effects on proliferation, survival, apoptosis, and migration in prostate cancer.

Discussion

Here, we identify a novel miRNA alteration that is widely represented in prostate cancer. miR-708 was consistently downregulated in all prostate cancer cell lines tested suggesting that miR-708 may have tumor-suppressive activity in prostate cancer. The widespread attenuation of miR-708 in prostate cancer was further validated in a cohort of human prostate cancer tissue specimens. We evaluated the potential for low miR-708 expression as a diagnostic biomarker for prostate cancer, and our analyses suggest that miR-708 expression can be a significant parameter to discriminate between normal and tumor tissues in prostate cancer. Furthermore, low miR-708 expression was significantly correlated with poor survival outcome, tumor progression, and recurrence in clinical specimens. Taken together, these findings suggest that this widely observed miRNA alteration has significant potential as a disease biomarker for diagnosis and prognosis in prostate cancer. Furthermore, our in vitro and in vivo data suggest that reconstitution of miR-708 in prostate cancer suppresses tumorigenicity, confirming the tumor-suppressive role of miR-708 in prostate cancer. Moreover, intratumoral delivery of synthetic miR-708 oligonucleotides was sufficient to trigger in vivo regression of established tumors in a murine xenograft model of human prostate cancer, supporting the therapeutic potential of this novel miRNA in prostate cancer.

The existence of tumor-initiating subpopulations within the bulk of tumor has been described in murine and human prostate cancer. These cells are endowed with high tumorigenic capacity and drive tumor growth, metastasis, cause treatment resistance, and recurrence (23, 24). Several studies have identified CD44 as the predominant marker of prostate cancer TICs (7–12) in addition to other cell surface markers (7, 9–12, 23). CD44, a transmembrane receptor for hyaluronan, plays critical roles in cell–cell and cell–matrix adhesion, migration, signaling, and tumor metastasis (25). Significantly, this study documents an intricate interplay between miR-708 and CD44. Several lines of evidence support this regulatory interplay: (i) We isolated CD44+ prostate cancer cells from prostate cancer xenografts and found that miR-708 expression is consistently downregulated in this subpopulation compared with CD44− subset purified from xenograft tumors. This observation suggests that miR-708 expression is specifically attenuated in prostate cancer TICs and also points to a possible miR-708–mediated regulation of CD44. (ii) Furthermore, miR-708 reexpression in prostate cancer cell lines led to decreased endogenous CD44 protein expression, and (iii) reporter assays suggest that miR-708 directly regulates CD44 expression. (iv) Intratumoral administration of miR-708 in prostate cancer xenografts led to reduced CD44 protein levels suggesting that CD44 is a direct target of miR-708. (v) CD44 inhibition partially phenocopies the antitumorigenic effects of miR-708. (vi) Also, siRNA-mediated CD44 knockdown led to increased miR-708 expression. In addition, we also introduced miR-708 in CD44− subpopulation of prostate cancer xenografts and observed growth inhibitory effects in vitro. Conversely, miR-708 inhibition in the purified CD44− population from PC3 xenografts led to increased growth. Collectively, this data suggests that miR-708 is an important regulator of CD44 and the tumor-initiating capacity of prostate cancer. The positive correlation of miR-708 downregulation with prostate cancer recurrence lends further support to this interplay. In view of these results, we suggest that the tumor-inhibitory effects of miR-708 observed in bulk prostate cancer cells in vitro and in vivo might be due to its effects on prostate cancer TICs.

Recent evidence suggests that TICs are intricately regulated by networks of miRNAs (26). Bioinformatical analysis and experimental evidence suggest that the 3'-UTR of a single gene is frequently targeted by several different miRNAs (27, 28), suggesting that miRNAs might cooperate to regulate specific gene expression (29, 30). CD44 seems to exemplify such a combinatorial control as it has been reported that miR-34a (17), miR-373, and miR-520c (31) regulate CD44 expression in
Figure 7. AKT2 is also a functional target of miR-708 in prostate cancer. A, schematic representation of the AKT2 3′-UTR showing the relative positions of 3 putative miR-708 target sites. B, immunoblots for endogenous AKT2 protein in PC3/LNCaP cells transfected as indicated. GAPDH was used as a loading control. C, luciferase activity assay with the AKT2 3′ UTR construct/control construct co-transfected with miR-CON/miR-708. Firefly luciferase values were normalized to Renilla luciferase activity and plotted as relative luciferase activity (\( P < 0.05 \)). D, AKT2 expression in harvested tumors from mouse prostate cancer xenografts intratumorally injected with either miR-CON or miR-708 as assessed by immunoblot analysis (T1–T5 are individual tumors). E, PC3 cells were transfected with AKT2/NS siRNA for 72 hours. Relative AKT2 mRNA expression and protein expression after siRNA transfections as assessed by RT-PCR and immunoblotting, respectively. Cell viability assay (F), Transwell migration assay (G), invasion assay (H), and apoptosis assay (I) in PC3 cells after NS siRNA (left) or AKT2 siRNA (right) treatment. J, representation of average apoptotic fractions in each group (\( P < 0.05 \)).
miR-708 is a key negative regulator of CD44 and AKT2, which is associated with poor outcome in prostate cancer (38, 39). AKT2 inhibition suppresses the growth of human prostate cancer cells in vitro and in vivo (40). AKT2/AKT pathways also play a role in the maintenance and viability of stem-like cell populations in prostate cancer (41). In view of this and our present results, we propose that miR-708-mediated repression of CD44 and AKT2 may underlie its role in potentially affecting prostate tumor xenografts, we envision that restoring miR-708 may provide a novel therapeutic modality for prostate cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


Correction: miRNA-708 Control of CD44⁺ Prostate Cancer–Initiating Cells

In the original version of this article (1), there are errors in Fig. 3. The first author inadvertently submitted an incorrect version of Fig. 3, containing an incorrect representative figure for the migration assay for LNCaP cell line in the right panels of Fig. 3C, while correcting the proof to incorporate better contrast for the Fig. 3C panels. The error has been corrected in the latest online HTML and PDF versions of the article. The authors regret this error.

Reference

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