miR-24 Regulates CDKN1B/p27 Expression in Prostate Cancer

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BACKGROUND. MicroRNAs (miRNAs) are small, non-coding RNA molecules that regulate gene expression by interacting with messenger RNAs (mRNAs). In prostate cancer, several miRNAs are expressed abnormally, raising the possibility that miRNAs will be useful for diagnosis, prognosis, and potential therapeutic intervention in this disease. However, the contribution of individual miRNAs to the development and progression of this disease remains poorly understood. This study investigated the role of miR-24, which has not been extensively studied in relation to prostate cancer.

METHODS. We used PCR to investigate the expression of miR-24 in a panel of prostate cancer cell-lines and in a series of clinical prostate biopsy specimens. The biological significance of miR-24 expression in prostate cancer cells was assessed by a series of in vitro bioassays and the effect on proposed targets p27 (CDKN1B) and p16 (CDK2NA) was investigated.

RESULTS. We showed that miR-24 expression was significantly lower in prostate cancer cell lines compared to a normal prostate epithelial cell line. Decreased expression of miR-24 was also more frequently observed in both needle core and prostatectomy tumor tissue relative to matched normal tissue. Low miR-24 expression correlated with high PSA serum levels and other markers of increased prostate cancer progression. Importantly, over-expression of miR-24 inhibited cell cycle, proliferation, migration, and clonogenic potential of prostate cancer cells, as well as inducing apoptosis. p27 and p16 were confirmed as targets of miR-24 in prostate cancer cells and a significant inverse correlation between miR-24 and p27 was revealed in clinical prostatectomy specimens.

CONCLUSIONS. These findings provide evidence that miR-24 has a tumor suppressor role in prostate cancer and also targets p27 and p16 in prostate cancer cells. We propose that it may be a useful progression biomarker or focus of therapeutic intervention for this disease.

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KEY WORDS: microRNA; miR-24; prostate cancer; p27; p16

BACKGROUND

MicroRNAs (miRNAs) are small, non-coding RNA molecules that regulate gene expression by interacting with messenger RNAs (mRNAs). In prostate cancer, several miRNAs are expressed abnormally, raising the possibility that miRNAs will be useful for diagnosis, prognosis, and potential therapeutic intervention in this disease [1–3]. Several characteristics of miRNAs make them ideal biomarkers for prostate cancer. They are much more stably preserved than mRNA in clinical samples, including formalin-fixed paraffin-embedded
miR-24 is a microRNA which has been studied in a range of disorders, including several cancers. Results from various studies reveal that it appears to play a dual role, depending on the specific disease being investigated. For example, in some settings it apparently promotes cell proliferation and an up-regulation in its expression has been associated with breast cancer [6–7], oral carcinoma [8], non-small cell lung cancer [9], and glioma [10], with elevated serum levels of circulating miR-24 also reported for lung cancer [9,11,12], breast cancer [13], and acute myeloid leukemia [14]. In contrast, other studies have suggested miR-24 has a tumor suppressor role, since it is frequently down-regulated in colorectal [15], laryngeal [16], bladder [17], gastric [18], and hepatocellular [19] carcinoma, with low serum levels reported in acute lymphoblastic leukemia patients [14]. These contrasting reports are likely to reflect the fact that miR-24, like most miRNAs, demonstrates promiscuous binding to target mRNAs, and is known to directly regulate expression of several genes, including c-myc [20], XIAP [21], FOXM1 [17], RegIV [18], and p27 [22]. Moreover, several dozen other predicted targets remain to be validated in a biological setting, so it seems likely that the precise balance of all these interactions will ultimately determine the effect of miR-24 on cells in different settings.

To date, however, very few studies have investigated miR-24 in relation to prostate cancer. A study by Szczyrba et al. suggested miR-24 expression was reduced in a panel of 26 prostate tumor samples relative to matched controls, which led to the up-regulation of miR-24 targets ZNF217, hnRNP-K, VEGF-A, and IPO7 in these tissues [23]. Goto et al also reported that expression of the miR-23b/27b/24-1 cluster was significantly reduced in prostate cancer tissue, but did not explore any targets of miR-24 specifically [24]. A third in vitro study demonstrated that over-expression of miR-24 in DU145 prostate cancer cells induced apoptosis by targeting FAF1 [25]. Thus, given the relative lack of research on miR-24 in this disease, we wished to investigate its functionality further by investigating how miR-24 action upon other targets might contribute to the disruption of normal molecular pathways in prostate cancer cells. In particular, we were interested in its relationship with the cyclin-dependent kinase inhibitors p27 (CDKN1B) and p16 (CDKN2A), which we had previously demonstrated as targets of miR-24 in human keratinocytes [22]. Both p27 and p16 are known to be deregulated in prostate cancer [26,27], but there is conflicting evidence about the clinical significance of this, since their expression is highly variable in prostate tissue, depending on the stage, or type of tumor being investigated. For example, at earlier stages of neoplastic transformation, induction of p27 has been reported in association with markers of senescence and the development of prostatic intraepithelial neoplasia (PIN) [28], whereas loss of p27 has been linked to cancer progression in a specific subset of ERG-negative, low-grade tumors [29]. Similarly, high expression of p16 in prostate tumor tissue has been reported as being a useful marker for higher risk of biochemical relapse [30,31], while others showed that low levels of p16 were associated with an increased risk of distant metastasis and shorter disease-specific survival [32,33]. We hypothesized that this variability may be dependent in part on control by miR-24 and that measuring miR-24 expression might give further insight into how p27 and p16 expression becomes deregulated. Linked to this, we were also interested to see if miR-24 had any value as a genetic marker for identifying different patient subgroups, with a view to stratifying patients into high- and low-risk categories based on their miR-24 profile.

We therefore proceeded to investigate miR-24 expression in prostate cell-lines and clinical prostate samples, as well as examining how manipulation of miR-24 levels affect cell behavior and levels of p27 and p16 expression in prostate cancer cells. This is the first study to describe the impact of miR-24 upon these targets in relation to prostate cancer.

**MATERIALS AND METHODS**

**Cell Culture and Transfections**

All cell-lines were obtained from American Type Culture Collection (ATCC). Non-malignant prostate epithelial cell-lines RWPE1 and PWR-1E were cultured in keratinocyte growth medium supplemented with 5 ng/ml human recombinant epidermal growth factor and 0.05 mg/ml bovine pituitary extract (Life Technologies, Paisley, UK). Human prostate cancer cell-lines PC3, 22RV1, LNCaP, and DU145 were cultured in RPMI-1640 supplemented with 10% FBS (Life Technologies). All cells were grown in an incubator with a humidified atmosphere of 95% air and 5% CO2 at 37°C and routinely passaged.

For transient transfections, PC3 or 22RV1 cells were seeded at 100,000 cells/well in a six-well
plate. After 24 hr, cells were transfected with miR-24 (Pre-miR-24) and non-targeting negative control (Pre-neg) (both Life Technologies) at a final concentration of 25 nM using Lipofectamine 2000 (Life Technologies). After 72 hr, cells were harvested for RNA or protein extraction.

RNA Extraction From FFPE Human Prostate Tumor Samples

FFPE prostate cancer samples were obtained from Altnagelvin Area Hospital, Derry. Use of patient material and information, as well as research protocols, were approved by ORECNI (Ref. 10/NIR02/13). Anonymized patient data for prostatectomy samples is presented in Supplementary Table S1. For preparation of RNA from FFPE needle core biopsies, five 10 μM sections were prepared for each case (n = 14). Following examination by certified pathologist, tumor tissue, and normal adjacent tissue were identified and separated by dissection from the slides, before RNA extraction was performed. For preparation of RNA from FFPE prostatectomy biopsy samples (n = 22), five 10 μM sections containing >50% tumor were cut for RNA extraction. Sections of matched normal prostate tissue from the unaffected lobe of the same patient were similarly cut. RNA extraction on all FFPE tissue was performed using the RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE (Life Technologies) following manufacturer’s instructions.

Luciferase Reporter Assay

Luciferase reporter constructs based on the pMIRTarget firefly luciferase plasmid were purchased from OriGene Technologies (Rockville, MD). One construct contained the wild-type p27 3′UTR region with the miR-24 binding site intact (p27-3′UTR). A matched control construct (p27-MUT) contained two mutated bases in the miR-24 binding site (cataCTGAGCCAagtat changed to cataCTGTACCAagtat). Cells were seeded at a concentration of 100,000 cells/well in 12-well plates and transfected with 300 ng of either p27-3′UTR plasmid of p27-MUT, together with either pre-miR-24, or control pre-neg at a concentration of 25 nM. The total of 30 ng Renilla luciferase vector was included in each well to control for transfection efficiency. After 48 hr, cells were lysed in lysis buffer (Promega, Southampton, UK) and luciferase activity measured using the Dual-Glo™ Luciferase Assay Kit (Promega) on a Fluostar Omega plate reader (BMG LabTech, Aylesbury, UK). Transfections were carried out in triplicate, measurements within experiments were performed in duplicate, and firefly luciferase readings were normalized against renilla luciferase readings before analysis.

PCR Analysis

RNA extraction was carried out using Trizol (Life Technologies) according to manufacturer’s instructions. One microgram RNA was used for first strand cDNA synthesis using random primers with transcriptor high-fidelity cDNA synthesis kit (Roche, Sussex, UK) according to manufacturer’s instructions. For quantitative Real-time PCR (qRT-PCR), amplification of PCR products was quantified using FastStart SYBR Green Master (Roche) on a Roche LC480 Lightcycler, using primer sets for p27 (Forward 5′-TTTGACCTTGCAAGAAGAGC-3′; Reverse 5′-AGCTGTCTCTGAAGGACCATT-3′), p16 (Forward 5′-GTGACCTGGCTGAGGAG-3′; Reverse 5′-CTTTC AACGGGAGTGTCTG-3′), XIAP (Forward 5′-TGGGAAAGCCATGGAAGACC-3′; Reverse 5′-AGTTCTTACACACACTCCTCA-3′) FAF1 (Forward 5′-TGGGACCTGGCTGAGGAG-3′; Reverse 5′-CTTTC AACGGGAGTGTCTG-3′), E2F1 (Forward 5′-TCCGATTCAAGGCGACT -3′; Reverse 5′-TTACACACCCGAGCAAGGC-3′), MYC (Forward 5′-AGTCGAGCCAagat changed to cataCTGTACCAagtat). Expression was normalized to HPRT and graphs represent the combined results of three independent biological replicates.

qRT-PCR of miRNAs was performed using the miRCURY LNA™ microRNA PCR system (Exiqon, Vedbaek, Denmark). The total of 50 ng (clinical samples) or 20 ng (cell-line samples) template RNA was used in each first strand cDNA synthesis reaction. PCR was performed over 40 amplification cycles and fluorescence monitored on the Roche LC480 Lightcycler. For all qRT-PCR miRNA analysis, normalization was against U6snRNA and graphs represent the combined results from three independent biological replicates, unless otherwise indicated.

Western Blot Analysis

Protein lysates were electrophoresed and transferred to nitrocellulose membrane. Primary antibodies used for blotting were anti-p16 (BD Biosciences, Oxford, UK), anti-human p27Kip1 (DAKO, Cambridgeshire, UK) with anti-β-Actin (Sigma, Poole, UK) as loading control. Secondary antibodies were goat anti-mouse- and anti-rabbit-HRP (Santa Cruz). Luminescence was revealed by incubation with enhanced chemiluminescent reagent (Pierce, Rockford, UK) and
signal detected on a ChemiDoc™ XRS+ imaging system (Bio-Rad, Hertfordshire, UK).

**Flow Cytometry**

Cell cycle analysis was performed on cells transfected with pre-miR-24 and negative control, as well as untreated cells. After 72 hr cells were harvested, washed in ice-cold PBS, and fixed in 90% ethanol overnight at 4°C. Following a further wash with ice-cold PBS, cells were resuspended in 1 ml PBS containing propidium iodide (PI) (10 μg/ml), RNAse A (0.1 mg/ml), FBS (5%), NaN3 (0.02%), NP40 (0.1%), and tri-sodium citrate (50 μg/ml). After 30 min incubation at room temperature, cells were analyzed on a Gallios™ Flow Cytometer (Beckman-Coulter, High Wycombe, UK). For apoptotic analysis, transfected cells and controls were harvested after 72 hr treatment and dual-stained with PI and Alexa Fluor 488-Annexin V (Life Technologies) following manufacturer’s instructions. Stained cells were immediately analyzed on the same Gallios™ Flow Cytometer (Beckman-Coulter).

**Proliferation and Viability Assays**

A cell proliferation XTT assay (Roche) was carried out to measure cell viability. Transfected and control cells were seeded in a 96-well plate at 5,000 cells/well and absorbance measured at 24 hr intervals at 495 nm and 650 nm using a FLUCOstar Omega microplate reader (BMG Labtech). Eight replicates were performed for each experiment and results represent the combined results of multiple biological replicates for each treatment on the microplate reader. Following staining, the Allred IHC scoring system was employed for qualitative and semi-quantitative analysis of the slides. Scoring analysis of both staining proportion and staining intensity was carried out by a registered state pathologist. Staining proportion was measured on scale on 1–5 (1=low proportion and 5=high proportion) and staining intensity on a scale of 1–3 (1=weak and 3=strong). For each slide these two scores were multiplied to generate a cumulative score.

**Statistics**

Experiments were carried out at least three times, unless otherwise indicated. Two-tailed Student’s t-test or two tailed Spearman’s rank correlation were used to calculate P-values where appropriate, with thresholds of ***P < 0.001, **P < 0.01, and *P < 0.05.

**RESULTS**

**Expression of miR-24 in Prostate Cancer Cells and Tissues**

Our results demonstrate that miR-24 expression is significantly decreased in all the prostate cancer cell-lines examined in comparison to normal prostate epithelial cell-line RWPE1 (Fig. 1A). It was also interesting to note that it was down-regulated in PWR-1E cells, which are derived from a benign prostatic hyperplasia, suggesting loss of miR-24 may be an early occurrence in the development of abnormal prostate cells, even before they take on more cancerous characteristics. In clinical samples, it was clear that a large variation in miR-24 expression existed between individual samples, making the interpretation of such data more complicated. This is not uncommon as most studies profiling miRNAs in clinical samples observe a range of expression levels across individual patient samples. Hence, rather than view the absolute loss or gain of any given miRNA expression as an event associated with a disease, it is probably more useful to examine if these differences provide prognostic information about patient outcome. We therefore adopted two approaches in this study to help probe this suggestion further.

Firstly, within individual cases, we compared miR-24 levels in tumor tissue relative to normal tissue to reveal how frequently up- or down-regulation of miR-24 occurs across the cohort. In a small set of FFPE needle core biopsy samples (n = 14), miR-24 was...
Overall, miR-24 levels were found to be significantly less transcribed in tumor tissue compared to matched adjacent normal regions (Fig. 1B). Viewed individually, miR-24 levels were reduced in 78.5% (11/14) of the samples (Fig. 1C). In a larger set of prostatectomy biopsy samples (n = 22), overall expression of miR-24 was less in tumor tissue compared to the patient-matched normal tissue, although this result fell just short of significance (P = 0.06). Viewed on a case-by-case basis, it was clear that miR-24 levels varied greatly between individuals, with 31.8% (7/22) up-regulated, and 59% (13/22) down-regulated in tumor tissue compared to paired normal tissue. However, we concluded that loss of miR-24 expression is a more common event in tumor tissue specimens relative to matched controls.

**Tumors With High Levels of PSA Show Lowest miR-24**

Secondly, since loss of miR-24 expression is not consistently observed across all prostate tumor tissue specimens, we also divided all the samples into two separate groups with “high” or “low” expression of miR-24. The 22 prostatectomy tumor cases were ranked according to the expression of miR-24 levels, normalized to U6snRNA. Cases with miR-24 levels up to and including the median were categorized as “low” miR-24 expression, while the remainder were categorized as “high” miR-24 expression. For this type of stratification to be accepted clinically, it is important to correlate miRNA expression with

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**Fig. 1.** Expression of miR-24 in prostate cancer cells and tissues. (A) qRT-PCR analysis demonstrates that miR-24 expression is significantly decreased in a panel of prostate cancer cell lines relative to normal prostate epithelial cell line RWPE1. (B) qRT-PCR analysis of RNA isolated from needle core biopsy clinical specimens (n = 14) shows that miR-24 expression is significantly down-regulated in tumor tissue relative to matched adjacent normal tissue. (C) Fold change expression of miR-24 in individual tumor cases relative to matched normal tissue (D) qRT-PCR analysis of RNA isolated from prostatectomy biopsy clinical specimens (n = 22) shows a trend towards down-regulation of miR-24 in tumor tissue relative to paired normal tissue. (E) Fold change expression of miR-24 in individual tumor cases relative to paired normal tissue. (F) Correlation of miR-24 expression in prostatectomy biopsy cases with individual PSA levels (ng/μl). All miR-24 data were normalized to housekeeping control U6snRNA and are mean ± SE of triplicate (A) or duplicate (B–E) experiments. (Student’s t-test P-values: *P < 0.05, **P < 0.01, and ***P < 0.001).
Currently used measures of prostate cancer progression. Serum PSA is a common marker for detection of prostate cancer and our analysis showed that patients expressing low levels of miR-24 had significantly higher PSA levels (Fig. 1F). Similarly, among patients who showed markers of more aggressive prostate cancer, such as Gleason grade and extent of spread, the number of cases showing low levels of miR-24 was almost twice as many as those showing high levels (Supplementary Fig. S1). This is encouraging data, although we acknowledge that a larger cohort study would be necessary to definitively conclude if miR-24 levels correlate with other important parameters. For example, most of our cases were stage pT2 so we were unable to correlate miR-24 expression with different stages of prostate cancer, or with castrate-resistant prostate cancer (CRPC) progression free survival and biochemical recurrence, which would be valuable to know. A larger set of patient samples, with a greater variety in different clinico-pathological measurements and outcomes, would allow further informative correlations of miR-24 to be performed.

The Cyclin-Dependent Kinase Inhibitor p27 is a Direct Target of miR-24

Identifying the targets of any given miRNA is crucial to establishing its function within the cell. Due to the link between low miR-24 levels and increased PSA levels described above, we hypothesized that androgen receptor (AR) might be a target of miR-24. However, this was not borne out by any of our target prediction data-mining analyses. On the other hand, using three separate miRNA target prediction algorithm programs, miR-24 was consistently predicted to target p27, which is an important regulator of the G1-S phase progression (Fig. 2A and B), so we proceeded to investigate this further. To test this relationship in vitro, miR-24 was over-expressed by transient transfection in PC3 cells (Fig. 2C). qRT-PCR (Fig. 2D) and western blotting (Fig. 2E) demonstrated that p27 levels were significantly decreased as a result. As a control, expression of p16, a more established target of miR-24 [20,34] and another important regulator of the G1-S phase progression, was examined and was also found to be decreased by miR-24 over-expression. Similar findings were also observed when experiments were performed on another prostate cancer cell-line, 22RV1, (Supplementary Fig. S2). Taken together with the findings of our previous study in keratinocytes [22], this provides further confirmation that miR-24 does indeed directly target p27 and p16. We then confirmed that p27 was a direct target of miR-24 in PC3 cells with a luciferase reporter assay (Fig. 2F). The luciferase activity of a reporter construct containing the wild-type p27 3’UTR region (p27-3’UTR) showed significant reduction when miR-24 was over-expressed in the same cells, suggesting that miR-24 was binding to the target region in the 3’UTR of p27 mRNA (shown in Fig. 2B). However, when miR-24 was over-expressed with a reporter construct which had mutated residues in the miR-24 binding site of p27 3’UTR (p27-MUT), no reduction in luciferase activity was observed, therefore indicating that p27 is a direct target of miR-24 in these cells. Hence, through this mechanism, the abnormal expression of miR-24 has the potential to impact upon control of cell proliferation.

Over-Expressing miR-24 Inhibits Cellular Proliferation and Increases Apoptosis in Prostate Cancer Cells

We carried out a number of functional bioassays to further explore the effect of miR-24 on prostate cell behavior. Since we had demonstrated that miR-24 targeted p27 and p16, we first examined effect on cell cycle. Flow cytometry revealed that miR-24 over-expression did indeed result in a significant decrease of cells in G0-G1 phase and concomitant increase in S-phase compared to control transfectants (Fig. 3A and Supplementary Fig. S3), which would be expected if p27 and p16 were being inhibited. However, we did not see a significant increase in numbers of cells in G2-M phase, suggesting arrest of cell cycle in S-phase. Importantly, XTT proliferation assays demonstrated that over-expression of miR-24 in PC3 cells actually led to significant inhibition of cell proliferation compared to control cells (Fig. 3B and C). Moreover, cell migration as measured with a wound healing assay demonstrated that cells transfected with miR-24 has reduced migratory capacity compared to control and untreated cells (Supplementary Fig. S4A). Hence, although its action upon p27 and p16 might be expected to result in increased proliferative capacity, the opposite appears to be the case.

Although we observed an increase in Sub G0 fraction of miR-24 transfectant cells, we surmized that an apoptotic pathway may instead be activated by miR-24 and proceeded to test this by using an Annexin V flow cytometry apoptosis assay. The percentage of apoptotic cells (both apoptotic and early apoptotic cells) was significantly increased in miR-24 transfectants compared to control cells (Fig. 3D and Supplementary Fig. S3B). Quantification of crystal violet colony assays indicated that over-expressing miR-24 in cells resulted in significantly decreased clonogenic capacity of PC3 cells (Fig. 3E and Supplementary Fig. S4B).
Together these results provide evidence that miR-24 inhibits proliferation and induces apoptosis in prostate cancer cells, in agreement with previous findings investigating miR-24 over-expression on cell cycle in prostate [23,24], bladder [17], colorectal [15], and gastric [18] cancer cell lines. This is despite its action upon p27 and p16, which might otherwise lead one to predict that miR-24 might promote cell proliferation. However, it is worth remembering that miR-24 also targets several other genes involved in cell cycle [18,20], DNA repair [35,36], and apoptosis [21,25], which will also contribute to the regulation of cell growth. We also confirmed that miR-24 over-expression down-regulated a selection of these genes (Fig. 3F), which demonstrates how miR-24 could help promote a transition from proliferation to apoptosis in these cells. This also illustrates how the overall functionality of miR-24 in any given cell-type or setting will be mediated through effects on a complex regulatory network of targets, which we suggest explains the apparent ability of miR-24 to act in contrasting fashions in different cell types and cancers, as summarized in our introduction.

**Increased Levels of p27 and p16 in Prostate Tissue**

Previous studies have reported variable expression of both p16 and p27 in prostate cancer [26–33]. To establish any link with miR-24 expression, we needed
to examine p27 and p16 levels in our clinical samples. Immunohistochemical staining of prostatectomy biopsy tissues (n = 22) was carried out and all samples were quantitatively assessed using the Allred system. Scoring for both intensity and proportion was performed to account for staining heterogeneity across samples. This revealed that both p16 and p27 expression were significantly increased in prostate tumor tissue compared to normal prostate tissue (Fig. 4 A–C). Images from two representative cases are shown in panels 4B and C. p16 staining was generally absent in normal tissue (Fig. 4A and B), whereas p27 staining was apparent in normal tissue of several cases (Fig. 4A and C). Similar scoring of needle core biopsies (n = 14) revealed that p16 and p27 protein staining was increased in tumor tissue relative to adjacent normal tissue (Fig. 4D–F). Images from two representative cases are shown in panels 4E and F. The increase observed in p27 staining in tumor tissue presumably reflects increased proliferation and/or check-point events in a proportion of the tumor cells, which varies from case to case, resulting in heterogeneity in staining between cases, and across individual tumor samples. These observations are supported by a recent tissue microarray study on a much larger cohort of prostate samples, which reported similar variation in p27 staining and therefore concluded that p27 was not a particularly reliable marker of tumor progression because of this variability [29]. Since we also see p27 staining variability in our cases, we would support that view, even though we see a clear difference between overall scores for p27 in normal
and tumor tissue. Interestingly, using Gene Expression Omnibus (GEO) profiling, a similar variation in p27 gene expression is observed across the largest dataset of prostate cancer sample analysis (n = 171) in the GEO repository (Supplementary Fig. S5). p27 was generally expressed at a higher level than p16 in these samples, which agrees with our findings, but only in metastatic tissues was there a trend towards increased expression of both genes in comparison to normal prostate tissue. It is therefore tempting to speculate that increased p27 expression we observe in our samples may be occurring in a sub-set of tumor cells which may go on to develop PIN, as previously suggested [28], or is associated with cells which have the potential to become metastatic. In any event, it seems clear that the expression of p27 in isolation is unlikely to be a useful clinical measurement in prostate cancer, emphasising the need for other potential novel biomarkers, such as miR-24.

**p27 Expression is Significantly Increased in Cell and Clinical Samples With Low miR-24**

Although a large inter-individual variation existed for both p27 and miR-24 expression across our patient cohort, we nevertheless hypothesized that if miR-24 was targeting p27, we might expect to see an inverse correlation between the expression of these two markers in our samples. Starting with our cell-lines, we observed an inverse correlation between the levels of miR-24 and p27 mRNA expression in the panel of cell-lines examined (Fig. 5A and B), although the
Performing the same correlation in FFPE-fixed clinical tissues is less easy to perform, as it is difficult to obtain reliable expression levels of p27 at the mRNA level in such specimens due to the extent of RNA fragmentation. However, we could correlate the miR-24 expression with p27 protein expression, by using the p27 immunostaining score in clinical specimens. Taking this approach, we demonstrated a significant inverse correlation exists between the two measurements (Fig. 5C). A similar inverse correlation was noted in the same samples between miR-24 expression and p16 immunostaining, although this fell just short of significance (Fig. 5D). Together, these clinical observations support our in vitro findings that p27 is regulated by miR-24.

**DISCUSSION**

Currently, the diagnosis and prognosis of prostate cancer are still reliant on traditional measurements of serum PSA, Gleason score, and tumor staging. However, it is recognized that even in patients with similar clinicopathological profiles, the outcome varies widely. There is therefore a need for more sensitive biomarkers which can improve the detection and management of this disease. Profiling miRNA expression in prostate cancer represents an attractive strategy for this, but it is clear that identification of relevant miRNAs and their function is required to develop such a strategy into the basis for a clinically feasible test. With this in mind, this study focused on miR-24, which has not been extensively investigated in prostate cancer, although it has been implicated as playing an important role in several other tumor types [6–14]. We hypothesized that it might play a similarly crucial role in prostate cancer and could be a useful diagnostic or prognostic biomarker for this disease if its expression and functionality in this setting is better understood.

Our study is the first to show that miR-24 expression is down-regulated in a panel of prostate cancer cell lines in comparison with normal prostate epithelial RWPE1 cells. In clinical prostate specimens, we show that loss of miR-24 expression is more common event in tumor tissue specimens relative to matched controls. Our in vitro assays show that over-expression of miR-24 inhibited cell cycle, proliferation, migration, and clonogenic potential of prostate cancer cells, as well as inducing apoptosis. We therefore conclude that...

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**Fig. 5.** Correlation of miR-24 and p27 expression in cell and clinical samples. (A) qRT-PCR analysis demonstrates that p27 mRNA expression is significantly increased in all cell lines tested, except PC3, relative to normal prostate epithelial cell line RWPE1. Data in graph is mean ± SE from triplicate experiments (Student’s t-test P-values: *P < 0.05, **P < 0.01, ***P < 0.001). (B) Correlation of miR-24 and p27 in the six cell lines indicated a trend towards an inverse relationship. (C) In prostatectomy cases, correlation of miR-24 expression with p27 protein levels, as measured by immunostaining, revealed a significant inverse relationship. (D) Similar analysis of miR-24 expression with p16 protein levels also revealed an inverse correlation that fell just short of significance. P-values were generated using Spearman’s two-tailed test.
miR-24 plays a predominantly tumor suppressor function in prostate cancer. Further evidence is provided by the observation that loss of miR-24 expression inversely correlates with serum PSA levels and other indicators of increased prostate cancer spread, suggesting it could be an important genetic marker for this disease. We propose that stratifying patients into groups which show high or low miR-24 levels could be a useful tool to identify low- and high-risk cohorts, respectively for prostate cancer progression, as others have done for miR-24 expression in bladder cancer [17], oral carcinoma [8], lung cancer [9], colorectal cancer [15], and acute leukaemia [14].

Although several targets of miR-24 have been biologically verified, very few have been proven in prostate cancer [23,25]. This is also the first study to show that p27 and p16 are regulated by miR-24 in prostate cancer cells in vitro. Furthermore, even though there was a large inter-individual variation for p27 and miR-24 expression across our small sample set, we were also able to demonstrate an inverse correlation existed between these two measurements in prostate tissue, suggesting that p27 is influenced by miR-24 levels in vivo as well. We therefore conclude that miR-24 is one of the factors contributing to the regulation of p27 expression in prostate cancer and may explain why altered expression of both p27 (and p16) has been reported in prostate cancer [26–33]. This is significant because it means miR-24 plays an integral role in control of cell cycle and its aberrant expression is therefore likely to disrupt normal cell growth. However, the effect of miR-24 on p27 alone is not likely to fully explain the phenotypic effects observed and it is worth remembering that miR-24 will undoubtedly exert an effect on several other targets that control cell growth as well (Fig. 3F and Supplementary Fig. S6). It is important to emphasize that it is the precise balance of the interactions discussed here, as well as several others, which will determine the overall functionality of miR-24 in the cell and which explains the apparent ability of miR-24 to act in contrasting fashions in different cancerous settings.

CONCLUSIONS

Our findings provide evidence that miR-24 is likely to act as a tumor suppressor in prostate cancer. This is the first study to show that miR-24 targets p27 and p16 in prostate cancer cells, thereby contributing to the control of their expression and therefore playing a key role in regulation of the cell cycle. From a clinical perspective, further investigation on a larger number of clinical samples would help establish whether miR-24 will be a useful measurement to help stratify patients into low- or high-risk categories for prostate cancer, either alone, or as a panel of miRNA markers. We also propose that targeting miR-24 itself may be an attractive strategy for a therapeutic intervention for this disease.

AUTHORS’ CONTRIBUTIONS

SML and DJM carried out the majority of the experimental work and data analysis. MMM provided expertise for the selection, preparation, and immunohistochemical analysis of clinical samples. CPW gained ethical approval for clinical samples and was involved in the discussion and interpretation of the data. DJM, the principal investigator, was responsible for planning, designing, analysis of the data and overall supervision of the work and final preparation of the manuscript. All authors read and approved the final manuscript.

REFERENCES


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