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BIOMARKERS, GENOMICS, PROTEOMICS, AND GENE REGULATION

Elevated miR-615-3p Expression Predicts Adverse Clinical Outcome and Promotes Proliferation and Migration of Prostate Cancer Cells

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miR-615-3p has previously been described as up-regulated in prostate cancer (PC) tissue samples compared with nonmalignant controls; however, its prognostic potential and functional role in PC remain largely unknown. In this study, we investigated the clinical and biological relevance of miR-615-3p in PC. The expression of miR-615-3p was measured in PC tissue specimens from 239 men who underwent radical prostatectomy (RP), and it was investigated if miR-615-3p could predict postoperative biochemical recurrence (BCR). These findings were subsequently validated in three independent RP cohorts ($n = 222$, $n = 273$, and $n = 387$) and functional overexpression studies conducted in PC cells (PC3M). High miR-615-3p expression was significantly associated with BCR in four independent PC patient cohorts ($P < 0.05$, log-rank test). In addition, high miR-615-3p expression was a significant predictor of PC-specific survival in univariate (hazard ratio, 3.75; $P < 0.001$) and multivariate (hazard ratio, 2.66; $P = 0.008$) analysis after adjustment for the Cancer of the Prostate Risk Assessment Post-Surgical (CAPRA-S) nomogram in a merged RP cohort ($n = 734$). Moreover, overexpression of miR-615-3p in PC cells (PC3M) significantly increased cell viability, proliferation, apoptosis, and migration. Together, our results suggest that miR-615-3p is a significant predictor of postoperative BCR and PC-specific survival and has oncogenic functions in PC cells. *(Am J Pathol 2019, 189: 2377−2388; https://doi.org/10.1016/j.ajpath.2019.08.007)*

Prostate cancer (PC) is the most commonly diagnosed non−skin cancer and the third leading cause of cancer-related death among men in the Western world.[1] Although localized PC is curable by radical prostatectomy (RP), approximately one-third of the patients will experience postoperative biochemical recurrence (BCR), and some will further progress into advanced metastatic PC that remains incurable.[2] However, a large proportion of localized PCs are nonaggressive (indolent) and will not progress to advanced disease, even without treatment.[2] Currently, treatment decisions for newly diagnosed clinically localized PC are based on serum prostate-specific antigen levels, clinical tumor stage, and Gleason grade. However, this approach lacks prognostic accuracy, leading to overtreatment of many nonaggressive PCs as well as undertreatment or delayed treatment of aggressive PCs. Hence, there is an urgent need to uncover the molecular mechanisms that drive PC aggressiveness and to identify novel prognostic biomarkers that can help guide improved and more personalized treatment decisions.

miRNAs are small noncoding RNAs (approximately 22 nucleotides) that regulate posttranscriptional gene

expression by translational repression and mRNA degradation. It has been estimated that approximately 60% of all human transcripts are regulated by miRNAs and that miRNAs influence key cellular processes, such as differentiation, cell cycle progression, and apoptosis.[3] In addition, miRNAs have provided new insights into the understanding of human cancer biology, and evidence suggests that dysregulation of miRNA expression is involved in both PC development and treatment response.[4−9] Furthermore, miRNAs hold promising biomarker potential as they exhibit both tissue-specific expression profiles and associations to tumor, node, and metastasis stage and grade in many cancer types.[10,11] Moreover, miRNAs are highly stable molecules and are detectable also in archived tissue samples.[12]

Although up-regulation of miR-615-3p in PC compared with nonmalignant prostate tissue samples has been previously reported, the possible prognostic value and biological function of miR-615-3p in PC remain largely unknown.[13,14] In the present study, using four large independent PC patient cohorts (239, 222, 273, and 387 RP patients), it was found that high miR-615-3p expression in PC tissue samples was significantly associated with postoperative BCR and poor PC-specific survival (CSS). In addition, overexpression of miR-615-3p increased the viability, proliferation, and migration of PC cells, together indicating that miR-615-3p is an oncogenic driver in PC.

Materials and Methods

Clinical Samples and miRNA Profiling

Expression levels of miR-615-3p were measured in tumor tissue samples from four independent patient cohorts of men treated for clinically localized PC by curatively intended RP (Table 1): cohort 1/training, $n = 239$; cohort 2/validation, $n = 222$; cohort 3/validation, $n = 273$; and cohort 4/validation, $n = 387$.

PC tissue samples (formalin-fixed paraffin-embedded) from cohorts 1 and 2 were obtained from PC patients who underwent RP at the Department of Urology, Aarhus University Hospital (Aarhus, Denmark), between 1997 and 2005. These cohorts have also been previously described.[13,15] Before this study, patient follow-up information, including time to BCR, was updated for all patients in cohorts 1 and 2 (April 2018). PC tissue samples (formalin-fixed paraffin-embedded) from cohort 3 were obtained from PC patients who underwent RP from 2002 to 2005 at the Department of Urology, Rigshospitalet, Copenhagen University Hospital (Copenhagen, Denmark). For cohort 3, follow-up was updated in July 2017. Inclusion and exclusion criteria for cohorts 1 to 3 are reported according to the REporting Recommendations for Tumour MARKer Prognostic Studies (REMARK) guidelines in Supplemental Figure S1. Patients were excluded if they received preoperative/postoperative radiation/endocrine treatment, were lost to follow-up, and/or experienced BCR within 3 months after RP. Evaluation of tissue specimens, pathologic grading, and RNA extraction were performed, as previously described, for cohorts 1 and 2[16] and for cohort 3.[17] miR-615-3p and miR-151a-5p (for normalization) expression in cohorts 1 to 3 was profiled using the miRCURY LNA Universal RT microRNA PCR platform (Exiqon A/S, Vedbæk, Denmark), as previously described.[13] Missing or nondetected values were set to quantitation cycle (Cq) 42.

Written informed consent was obtained from all patients from Aarhus University Hospital (cohorts 1 and 2), and the study was approved by the regional scientific ethical committee and the Danish Data Protection Agency (file numbers 2000/0299 and 2013-41-2041). For patients from Rigshospitalet, Copenhagen University Hospital (cohorts 3), the Danish National Committee on Health Research Ethics (journal number H-6-2014-111) approved the use of archived tissue specimens for this research project.

For cohort 4, PC tissue samples were collected from RP patients by The Cancer Genome Atlas (TCGA) consortium at multiple centers in the United States and Europe and analyzed by small RNA sequencing.[18,19] Normalized miRNA profiling data and clinical data were retrieved from TCGA data portal[18] for 499 PC patients using the GDC Data Transfer Tool and Firehose from the Broad Institute Genome Data Analysis Center. Clinical information and miRNA data were available for 476 patients. Eight patients were excluded because of missing clinical/BCR data, and another 81 patients were excluded because the patient experienced BCR within 3 months of RP, leaving 387 patients eligible for the final analysis.

Statistical Analysis

Unless stated otherwise, statistical analyses were conducted in R version 3.5.1 using R Studio version 1.1.463.[20] Initially, miR-615-3p expression levels in cohorts 1 to 3 were normalized to a stably expressed miRNA (miR-151a-5p), previously identified by the NormFinder algorithm.[13,21] Normalization was performed according to the following: $\Delta Cq = Cq_{miR\text{-}151a\text{-}5p} - Cq_{miR\text{-}615\text{-}3p}$.

In cohort 4, normalized miRNA sequencing data were obtained from TCGA data portal and log2 transformed by the following: Reads per million $(RPM)^{log} = log2 (RPM + 1)$.[18]

Associations between miR-615-3p and routine clinicopathologic variables were assessed by the CAPRA-S risk nomogram, which includes preoperative prostate-specific antigen, pathologic Gleason score, surgical margin status, extracapsular extension, seminal vesicle invasion, and lymph node invasion, using nonparametric Wilcoxon rank-sum tests.[22] $P < 0.05$ was considered significant. To investigate prognostic potential, patients in cohort 1 (training) were dichotomized according to miR-615-3p expression levels based on the optimal cutoff identified by receiver operating characteristic curve analysis of BCR status and using Youden's J statistic in the pROC

Table 1 Clinicopathologic Characteristics for PC Patient Cohorts

Characteristics	Cohort 1: training set	Cohort 2: validation	Cohort 3: validation	Cohort 4: validation (TCGA)
Samples	RP ($n = 239$)	RP ($n = 222$)	RP ($n = 273$)	RP ($n = 387$)
Age in years, median (IQR)	64.2 (35.9—74.9)	63.5 (59.0—67.3)	62.5 (59.2—66.6)	61.0 (56.0—66.0)
PSA at diagnosis in ng/mL, median (IQR)	10.5 (7.1—15.7)	12.3 (9.1—19.0)	10.0 (6.8—14.0)	7.5 (5.2—11.1)
Gleason score, n (%)				
$\leq$6	77 (32.2)	75 (33.8)	111 (40.7)	37 (9.6)
7	126 (52.7)	104 (46.8)	139 (50.9)	194 (50.1)
8	22 (9.2)	34 (15.3)	12 (4.4)	55 (14.2)
>8	6 (2.5)	9 (4.1)	11 (4.0)	101 (26.1)
Unknown	8 (3.3)	0	0	0
Pathological T-stage, n (%)				
pT2a-c	173 (72.4)	137 (61.7)	182 (66.7)	149 (38.5)
pT3a	43 (18.0)	65 (29.3)	53 (19.4)	129 (33.3)
pT3b	17 (7.1)	20 (9.0)	38 (13.9)	96 (24.8)
Unknown	6 (2.5)	0	0	13 (3.4)
Surgical margin status, n (%)				
Negative	171 (71.5)	150 (67.6)	115 (42.1)	251 (64.9)
Positive	68 (28.5)	26 (26.1)	158 (57.9)	118 (30.5)
Unknown	0	14 (6.3)	0	18 (4.7)
Lymph node status, n (%)				
pN0	126 (52.7)	121 (54.5)	106 (38.8)	NA
pN1	14 (5.9)	12 (5.4)	3 (1.1)	NA
pNx	3 (1.3)	2 (0.9)	164 (60.1)	NA
Unknown	96 (40.2)	87 (39.2)	0	387 (100)
CAPRA-S risk groups, n (%)				
Low (CAPRA-S score 0—2)	63 (26.4)	73 (32.9)	79 (28.9)	99 (25.6)
Intermediate (CAPRA-S score 3—5)	123 (51.5)	86 (38.7)	119 (43.6)	139 (35.9)
High (CAPRA-S score 6—12)	46 (19.2)	51 (23.0)	75 (27.5)	110 (28.4)
Unknown	7 (2.9)	12 (5.4)	0	39 (10.1)
Recurrence status, n (%)				
No biochemical recurrence	138 (57.7)	105 (45.8)	152 (55.7)	356 (92.0)
Biochemical recurrence	101 (42.3)	117 (54.2)	121 (44.3)	31 (8.0)
Follow-up time in months, median (IQR)	106.5 (89.9—121.7)	139.4 (108.0—164.4)	136.4 (106.5—154.5)	20.9 (9.4—35.6)
Survival status, n (%)				
Alive	593 (80.8)			NA
Dead	92 (12.5)			NA
PC-specific death	33 (4.5)			NA
Survival status unknown	16 (2.2)			387 (100)

CAPRA-S, Cancer of the Prostate Risk Assessment Post-Surgical; IQR, interquartile range; NA, not available; PC, prostate cancer; PSA, prostate-specific antigen; RP, radical prostatectomy; TCGA, The Cancer Genome Atlas; T-stage, tumor stage.

package.[23] The cutoff fraction identified in cohort 1 was subsequently used and tested in cohorts 2 and 3. Because of the difference in expression data type (ΔCq values in cohorts 1 to 3; RPM^{log} in cohort 4), a separate cutoff was used in cohort 4, as identified by receiver operating characteristic curve analysis of BCR status. Univariate and multivariate Cox regression as well as Kaplan-Meier analyses were used to evaluate the prognostic potential of miR-615-3p expression using the survival package[24] with postoperative BCR (prostate-specific antigen $\geq$ 0.2 ng/mL) or CSS as end points. Patients not having experienced BCR were censored at their last normal prostate-specific antigen test result. Last known patient survival status was obtained using information from the Central Office of Civil Registration by personal identification number in May 2018 for cohorts 1 and 2 and in July 2017 for cohort 3. Predictive accuracy was determined using Harrell's concordance index (C-index).

Cell Line and Cell Culture

All functional studies of miR-615-3p overexpression were conducted in the human cell line PC3M, whereas miR-615-3p inhibition was performed in the human cell line PNT1A. The cell lines were selected because of a low and high endogenous expression of miR-615-3p, respectively (data not shown). PC3M was a kind gift from Prof. Raymond C. Bergan (Department of Medicine, Northwestern University, Chicago, IL), received in October 2011. PNT1A cells are immortalized prostate epithelial cells and are described by Degeorges et al.[25] The cell lines were authenticated by short

tandem repeat profiling with IdentiCell using the GenePrint 10 system (Promega, Madison, WI) in October 2012 and July 2014, respectively, and no cross contamination was observed. Before all experiments, cells were further confirmed negative for *Mycoplasma* contamination using the *Mycoplasma* qPCR Detection Kit (TaKaRa, Shiga, Japan), according to the manufacturer's instructions. Cells were cultured in RPMI 1640 medium with L-glutamine (Lonza, Basel, Switzerland) and supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Gibco, Grand Island, NY) at 37°C and 5% CO_2.

Transfection

To ectopically overexpress miR-615-3p, PC3M cells were reverse transfected using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA), according to the manufacturer's instructions. In brief, PC3M cells were transfected with mirVana miR-615-3p mimic (product MC117319) or negative control (mirVanaTM miRNA Mimic, Negative Control number 1; catalog number 4464058) (Ambion, Life Technologies, Carlsbad, CA) at a final miR-615-3p mimic or negative control concentration of 20 nmol/L, respectively.

To inhibit miR-615-3p expression, PNT1A cells were transfected using the same reverse protocol with mirVana miR-615-3p inhibitor (product MH11731) or negative control (mirVana miRNA Inhibitor, Negative Control number 1; catalog number 4464076) (Thermo Fisher Scientific) at an equal concentration of 20 nmol/L. For all experiments, Lipofectamine was diluted 1:1000. No evidence of Lipofectamine toxicity was observed using alamarBlue Cell Viability assays (Invitrogen, Carlsbad, CA) (data not shown). Before initiating functional experiments, transfection efficiency was assessed using a Cy3-labeled pre-miR Negative Control (catalog number AM17120; Ambion, Applied Biosystems, Waltham, MA) and was near to 100% at 48 hours after transfection for both PC3M and PNT1A.

Cell Viability and Proliferation Assay

Cell viability was assessed by a Rezasurin assay (alamarBlue Cell Viability Reagent) and measured using the Synergy HT reader (BioTek, Winooski, VT). In brief, PC3M cells were reverse transfected in a 96-well plate using 4000 cells per well. At 48 hours after transfection, PC3M cells were incubated with 10% alamarBlue Cell Viability Reagent for 3 hours before measurement of absorbance. All experiments were performed in four technical replicates and repeated three times.

Cell proliferation was estimated using the xCELLigence RTCA DP system (ACEA Biosciences, Inc., San Diego, CA). Cells were subjected to xCELLigence immediately after reverse transfection at 4000 cells per well and analyzed

for approximately 100 hours (two independent experiments with two and five technical replicates).

Caspase 3/7 Activity Assay

Cell apoptosis was assessed by a caspase 3/7 activity assay, as described previously.[26] Briefly, PC3M cells were reverse transfected with miR-615-3p mimic or scrambled control in 24-well plates at 40,000 cells per well. Forty-eight hours after transfection, cells were treated with the apoptosis-inducing agent staurosporine (1 μmol/L) for 2 hours before caspase 3/7 activity in cell lysates was measured by the liberation of 7-amino-4-trifluoromethyl coumarin (AFC) (excitation, 400 nm; emission, 489 nm) from the substrate Ac-DEVD-AFC (Enzo Life Sciences, Inc., Farmingdale, NY) using the Synergy HT reader (BioTek). All experiments were performed in triplicate and repeated twice.

Wound Healing Assay

To monitor cell migration, wound healing assays (scratch assays) were performed. PC3M cells were seeded and reverse transfected (miR-615-3p mimic or scrambled control) in 6-well plates at 300,000 cells per well. Forty-eight hours after transfection, four scratches were made per well using a 100-μL pipette tip. After scratching, the medium was changed to avoid reattachment of cells in the wound (gap area). Cell migration toward the wound was documented using a light-optical microscope (Axiovert40; Carl Zeiss, Oberkocken, Germany) with ×10 magnification. Images were taken at 0, 4, and 8 hours after performing the scratch. Three images were taken per well, and the width of the gap was measured at three locations per image using the ImageJ software version 1.51j8 (NIH, Bethesda, MD; *https://imagej.nih.gov/ij*) ($n = 9$ measurements/well).[27] For each individual well, all measurements of gap width were averaged. Migration was calculated as follows: % migration = (gap width after 4 or 8 hours)/(gap width after 0 hours).

All experiments were performed in triplicate and repeated at least twice.

Predicting Potential miR-615-3p Targets and Gene Set Enrichment Analysis

mRNA expression data were retrieved from TCGA data portal[18] for 297 PC patients and log2 transformed by the following: $RPM^{log} = log2 (RPM + 1)$.

Of these 297 patients, a total of 212 had matched miRNA-sequencing data (ie, overlapped with patients included in cohort 4) and were used for further analysis. Associations between potential target transcripts and miR-615-3p expression levels were further assessed using Pearson's product-moment correlation.

To investigate enriched gene sets in patients with high miR-615-3p expression compared with patients with low miR-615-3p expression, patients in cohort 4 with both

miRNA and mRNA data were dichotomized according to the median miR-615-3p expression level. Gene Set Enrichment Analysis was conducted using the gage package and the Gene Ontology (GO) terms.[28] To evaluate the strength of the analysis, a random split between the patients in cohort 4 was also performed using the sample function in R; and Gene Set Enrichment Analysis was performed on these two groups as well. Gene sets were considered significant if the false-discovery rate q < 0.05 (adjustment of the global *P* value using the Benjamini and Hochberg procedure).

Results

Prognostic Potential of miRNA-615-3p in Four Independent RP Cohorts

To investigate the prognostic potential of miR-615-3p, its expression level was initially measured in RP tissue specimens from patients with clinically localized PC (cohort 1, training set, n = 239); and these findings were subsequently tested and validated in three additional independent RP patient cohorts (cohorts 2, 3, and 4: n = 222, n = 273, and n = 387, respectively). Patient characteristics for all four cohorts are summarized in Table 1.

First, when compared with the established postoperative CAPRA-S nomogram, miR-615-3p was significantly up-regulated in high-risk versus low-risk tumors in cohorts 1, 2, and 4 (*P* < 0.05, Wilcoxon rank-sum test) (Figure 1), whereas this association was borderline significant in cohort 3 (Figure 1). This suggests that high miR-615-3p expression is associated with worse prognosis in PC.

To further evaluate this, patients in cohort 1 (training) were stratified into a high- versus low-risk group based on miR-615-3p expression. In cohort 1, high miR-615-3p expression was significantly associated with BCR in Kaplan-Meier analysis (*P* = 0.046) (Figure 2A) and in univariate Cox regression analysis [hazard ratio (HR) = 1.61; *P* = 0.048] (Table 2). Although miR-615-3p did not remain significant in multivariate Cox regression analysis after adjustment for CAPRA-S (*P* = 0.21) (Table 2), addition of miR-615-3p to CAPRA-S in a multivariate model increased Harrell's C-index from 0.675 to 0.684 in cohort 1 (Table 2), suggesting slightly improved predictive accuracy.

Next, for independent validation, patients in cohorts 2 and 3 were stratified into high- versus low-risk groups, using the cutoff fraction trained in cohort 1. Herein, high miR-615-3p expression was significantly associated with BCR in both Kaplan-Meier analysis (cohort 2/3: *P* = 0.010/*P* = 0.025) (Figure 2, B and C) and univariate Cox regression analysis (cohort 2: HR = 1.73, *P* = 0.011; cohort 3: HR = 1.61, *P* = 0.026) (Table 2). Consistent with our findings in cohort 1, miR-615-3p did not remain significant in multivariate Cox regression analysis after adjustment for CAPRA-S in
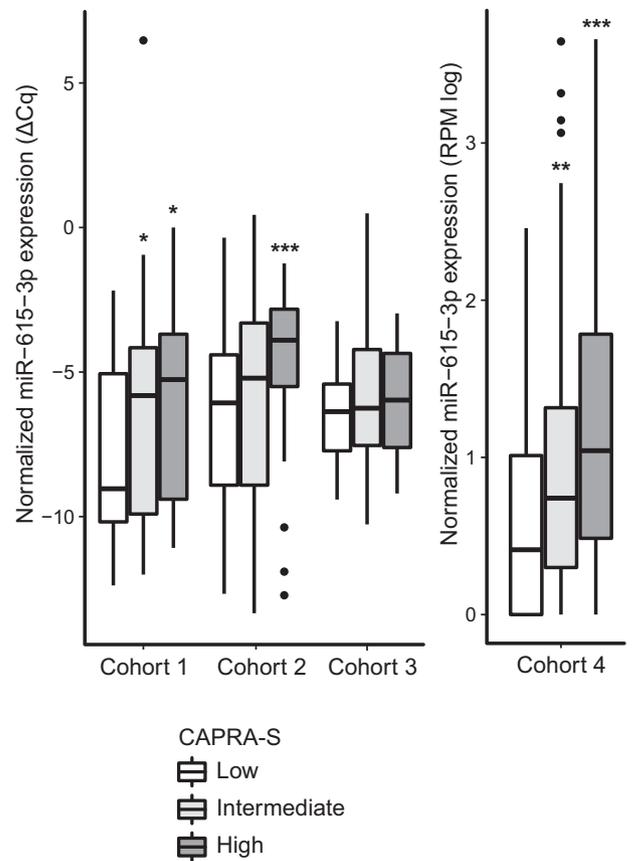

Figure 1 Box plot for miR-615-3p expression in CAPRA-S risk groups for four cohorts. The trend persisted in cohort 3 although not significant (*P* = 0.06). Boxes represent the 25th, 50th, and 75th percentiles. Whiskers illustrate highest and lowest values. Outlier cases, defined as >1.5 times the interquartile range from the median, are marked as individual dots. **P* < 0.05, **P < 0.01, and ***P < 0.001 CAPRA-S high-risk versus low-risk tumors (Wilcoxon rank-sum test). CAPRA-S, Cancer of the Prostate Risk Assessment Post-Surgical; Cq, quantitation cycle; RPM, reads per million.

cohorts 2 and 3 (*P* > 0.05) (Table 2) and had little effect on Harrell's C-index.

For additional validation, the prognostic potential of miR-615-3p was also examined in the publicly available RP patient data set from TCGA (cohort 4[18,19]). Again, high miR-615-3p expression was significantly associated with BCR in Kaplan-Meier analysis (*P* = 0.015) (Figure 2D) and univariate Cox regression analysis (HR = 2.09, *P* = 0.017) (Table 2), but failed in multivariate analysis after adjustment for CAPRA-S (*P* = 0.24) (Table 2). Still, addition of miR-615-3p to CAPRA-S in a multivariate model increased predictive accuracy (C-index) from 0.658 to 0.665 (Table 2), corroborating the results from cohort 1.

In summary, high miR-615-3p expression was significantly associated with postoperative BCR in four independent PC cohorts, including >1100 patients in total.

miRNA-615-3p Predicts PC-Specific Survival

Next, it was investigated if high miR-615-3p expression was predictive of CSS in a merged analysis of cohorts 1 to 3
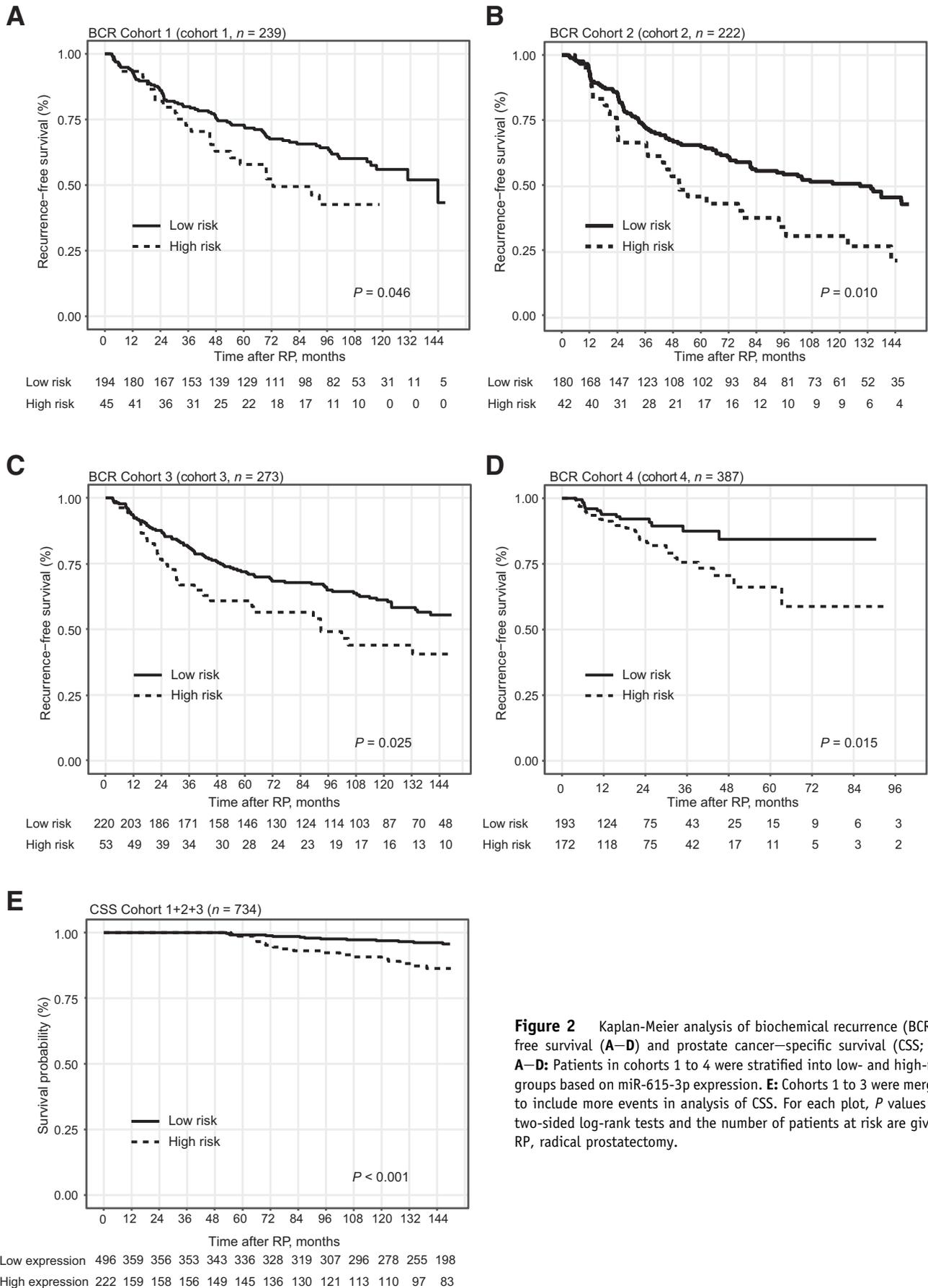
Figure 2 Kaplan-Meier analysis of biochemical recurrence (BCR)—free survival (**A**—**D**) and prostate cancer—specific survival (CSS; **E**). **A**—**D:** Patients in cohorts 1 to 4 were stratified into low- and high-risk groups based on miR-615-3p expression. **E:** Cohorts 1 to 3 were merged to include more events in analysis of CSS. For each plot, *P* values for two-sided log-rank tests and the number of patients at risk are given. RP, radical prostatectomy.

Table 2 Univariate and Multivariate Cox-Regression Analysis of BCR and CSS Using miR-615-3p in Four RP Cohorts

Variable	Characteristic	Univariate			Multivariate			
		HR (95% CI)	P value	C-index	HR (95% CI)	P value	C-index*	C-index†
Cohort 1, training set $n = 239$, 101 with BCR								
CAPRA-S	Low	Ref		0.68			0.675	0.684
	Intermediate	2.30 (1.22—4.34)	**1.02×10^{-2}**		2.28 (1.21—4.30)	**1.12×10^{-2}**		
	High	7.37 (3.81—14.25)	**3.06×10^{-9}**		7.11 (3.66—13.80)	**6.87×10^{9}**		
miR-615-3p	Low vs high	1.61 (1.01—2.57)	**4.77×10^{-2}**	0.54	1.38 (0.84—2.26)	0.21		
Cohort 2, $n = 222$, 117 with BCR								
CAPRA-S	Low	Ref		0.72			0.723	0.724
	Intermediate	3.06 (1.75—5.35)	**8.88×10^{-5}**		3.04 (1.73—5.33)	**1.00×10^{-4}**		
	High	10.80 (6.06—19.27)	**7.77×10^{-16}**		10.64 (5.87—19.28)	**6.37×10^{-15}**		
miR-615-3p	Low vs high	1.73 (1.14—2.64)	**1.07×10^{-2}**	0.54	1.05 (0.67—1.66)	0.82		
Cohort 3, $n = 273$, 121 with BCR								
CAPRA-S	Low	Ref		0.69			0.69	0.70
	Intermediate	2.60 (1.48—4.56)	**9.00×10^{-4}**		2.48 (1.41—4.38)	**1.7×10^{-3}**		
	High	6.66 (3.79—11.70)	**4.42×10^{-11}**		6.40 (3.63—11.30)	**1.52×10^{-10}**		
miR-615-3p	Low vs high	1.61 (1.06—2.45)	**2.63×10^{-2}**	0.54	1.31 (0.86—2.01)	0.21		
Cohort 4, $n = 387$, 31 with BCR								
CAPRA-S	Low	Ref		0.66			0.658	0.665
	Intermediate	5.11 (1.17—22.37)	**3.03×10^{-2}**		4.77 (1.09—20.98)	**3.86×10^{-2}**		
	High	10.70 (2.53—45.2)	**1.26×10^{-3}**		9.75 (2.29—41.51)	**2.07×10^{-3}**		
miR-615-3p	Low vs high	2.09 (1.14—3.84)	**1.73×10^{-2}**	0.57	1.46 (0.78—2.73)	0.24		
Cohort 1 + 2 + 3, $n = 734$, 33 with PC-specific death								
CAPRA-S	Low	Ref		0.70			0.702	0.751
	Intermediate	2.34 (0.64—8.52)	0.2		1.88 (0.51—6.94)	0.34		
	High	7.75 (2.29—26.2)	**9.8×10^{-4}**		5.53 (1.59—19.26)	**7.2×10^{-3}**		
miR-615-3p	Low vs high	3.75 (1.87—7.54)	**2.08×10^{-4}**	0.65	2.66 (1.29—5.49)	**8.4×10^{-3}**		

Univariate and multivariate Cox-regression analyses of BCR-free survival time in cohorts 1 to 4 and CSS in cohorts 1 to 3 combined. Significant P values are in bold.

*C-index based on CAPRA-S nomogram only.

†C-index based on final model, including miR-615-3p and CAPRA-S.

BCR, biochemical recurrence; CAPRA-S, Cancer of the Prostate Risk Assessment Post-Surgical; C-index, Harrell's concordance index; CSS, prostate cancer—specific survival; HR, hazard ratio; PC, prostate cancer; Ref, reference; RP, radical prostatectomy.

(because of the low number of events: $n = 6$, 13, and 14, respectively). High miR-615-3p expression was significantly associated with poor CSS in Kaplan-Meier analysis ($P < 0.001$) (Figure 2E) as well as in univariate Cox regression analysis (HR = 3.75, $P < 0.001$) (Table 2) and remained significant also after adjusting for CAPRA-S (multivariate Cox regression analysis: HR = 2.66, $P = 0.008$) (Table 2). Moreover, inclusion of miR-615-3p in a multivariate model together with CAPRA-S increased predictive accuracy from 0.702 to 0.751 (Table 2). Together, these results indicate that the level of miR-615-3p in PC tissue may refine prediction of CCS after RP beyond routine clinicopathologic variables.

miR-615-3p Influences Prostate Cancer Cell Viability, Proliferation, Apoptosis, and Migration

To investigate possible oncogenic functions of miR-615-3p in PC cells, miR-615-3p was transiently overexpressed in the PC cell line PC3M that has low endogenous miR-615-3p expression (data not shown). Furthermore, an miR-615-3p inhibitor was used in PNT1A prostate cells

that have high endogenous miR-615-3p expression (data not shown). Using a Rezasurin assay to monitor cell viability at 48 hours after reverse transfection, a significant increase in the viability of cells overexpressing miR-615-3p was found compared with the scrambled mimic-control ($P < 0.05$, t-test) (Figure 3A). Conversely, miR-615-3p inhibition significantly decreased cell viability compared with a scrambled inhibitor-control ($P < 0.05$, t-test) (Figure 3B). The phenotype of PC3M cells overexpressing miR-615-3p was further examined by evaluating cell proliferation, apoptosis, and migration. Cell proliferation was examined in real time using the xCELLigence RTCA DP system, and a significant increase in the proliferation of cells overexpressing miR-615-3p was found compared with the scrambled control (100 hours; $P < 0.001$, t-test) (Figure 3C). For cell apoptosis, caspase activity assays showed that overexpression of miR-615-3p significantly increased caspase activity at 48 hours after reverse transfection when cells were treated with staurosporine (1 μmol/L) for 2 hours compared with a scrambled control ($P < 0.01$, t-test) (Figure 3D).

In wound healing assays, overexpression of miR-615-3p significantly increased migration of PC cells compared with the scrambled control already at 4 and 8 hours after performing the scratch (4 hours: $P < 0.001$; 8 hours: $P < 0.01$; t-test) (Figure 4). Notably, there was no difference in cell proliferation rate between miR-615-3p and scrambled control transfected cells at 4 or 8 hours in the xCELLigence assay (Figure 3C), strongly indicating that the observed effect on cell migration is not due to differential proliferation.

In summary, overexpression of miR-615-3p significantly promoted cell viability, proliferation, apoptosis, and migration of PC3M prostate cancer cells, suggesting an oncogenic driver role for miR-615-3p in PC.

Potential Targets of miR-615-3p in PC and Gene Set Enrichment Analysis

As no validated target genes have been reported for miRNA-615-3p in PC, an integrative bioinformatic approach was used to identify top candidate targets for miR-615-3p of potential clinical relevance in PC. A subset of patients from cohort 4 for whom both miRNA and mRNA expression data were available ($n = 212$) and short-listed top candidate target genes that met the following criteria were used: i) included in top five list of predicted targets by either TargetScan or miRDB as sorted by cumulative weighted context++ score or target score, respectively, ii) genes identified as predicted targets by both TargetScan and miRDB, and iii) genes down-regulated in CAPRA-S high-risk versus CAPRA-S low-risk PC patients (log2 fold change ≤ -0.1).[29,30] Using this approach, three top candidate miR-615-3p target genes of potential clinical relevance in PC were identified: *LCOR* (ligand-dependent nuclear receptor corepressor), *USP44* (ubiquitin-specific peptidase 44), and *ITSN1* (intersectin 1) (Supplemental Table S1).

Expression levels for all three genes were significantly inversely correlated with miR-615-3p expression in cohort 4 ($\rho = -0.31$ to -0.16; $P < 0.05$, Pearson's product-moment correlation) (Supplemental Table S1), further supporting these genes as possible targets for miR-615-3p in PC. Further studies are warranted.

In the same subset of patients from cohort 4 with both miRNA and mRNA expression data, gene expression patterns among patients with high miR-615-3p expression compared with patients with low miR-615-3p expression were investigated. Gene Set Enrichment Analysis showed that the most highly enriched GO terms in patients with high miR-615-3p expression were cell cycle related ($q < 0.05$) (Figure 5), concordant with its promotion of cell proliferation in PC cells overexpressing miR-615-3p. In contrast, when doing a random split between the patients, no GO terms were significantly enriched ($q > 0.05$) (Figure 5).

In addition, the GO term DNA fragmentation during apoptosis was significantly enriched in patients with high miR-615-3p expression ($q < 0.001$) (Figure 5), in agreement with the increased level of apoptosis observed in PC cells overexpressing miR-615-3p.

Discussion

In the present study, a clear association was identified between high miR-615-3p expression and poor clinical outcome in PC patients. High miR-615-3p expression predicted postoperative BCR in univariate analysis in four independent RP cohorts, including >1100 patients in total. Furthermore, elevated miR-615-3p expression was a significant predictor of poor CSS, independent of routine clinicopathologic variables, as assessed by the CAPRA-S nomogram. These clinical findings were corroborated by functional studies *in vitro*, demonstrating that miR-615-3p overexpression in PC cells (PC3M) significantly increased
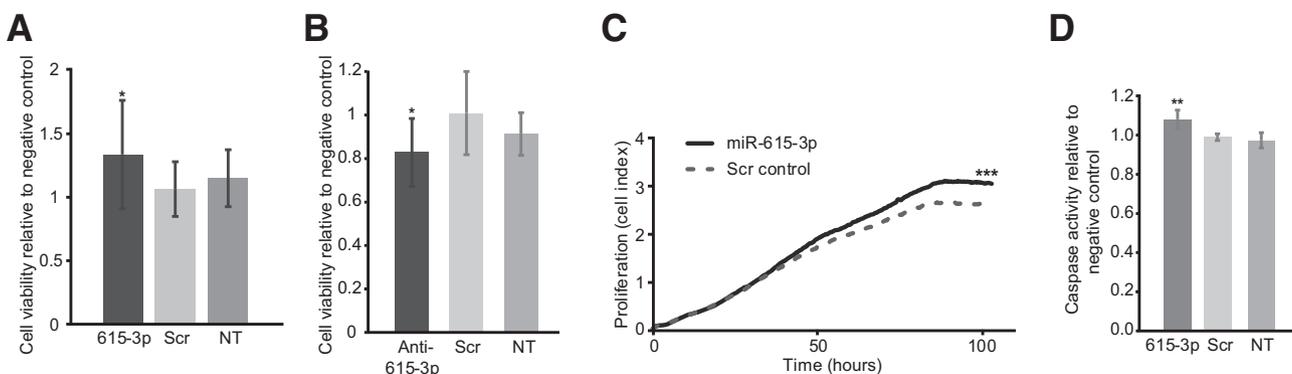

Figure 3   miR-615-3p promotes viability, proliferation, and apoptosis in prostate cancer cells. **A** and **B:** Cell viability, measured by alamarBlue cell viability assay 48 hours after reverse transfection of PC3M (**A**) or PNT1A (**B**) cells with miR-615-3p mimics (**A**) or anti-miR-615-3p (**B**), scrambled (Scr) control, or left nontransfected (NT). **A** and **B:** Error bars represent SD of three (**A**) or two (**B**) independent experiments performed in four technical replicates. **C:** Cell proliferation, measured using xCELLigence immediately after reverse transfection of PC3M cells with miR-615-3p mimics or Scr control. Representative example of two independent experiments in five technical replicates. **D:** Cell apoptosis, measured by caspase 3/7 activity assay, 48 hours after reverse transfection, measured with miR-615-3p mimics, Scr control, or NT and after 2 hours of incubation with 1 μmol/L staurosporine. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ miR-615-3p mimics/inhibitor versus scrambled control (independent t-test).
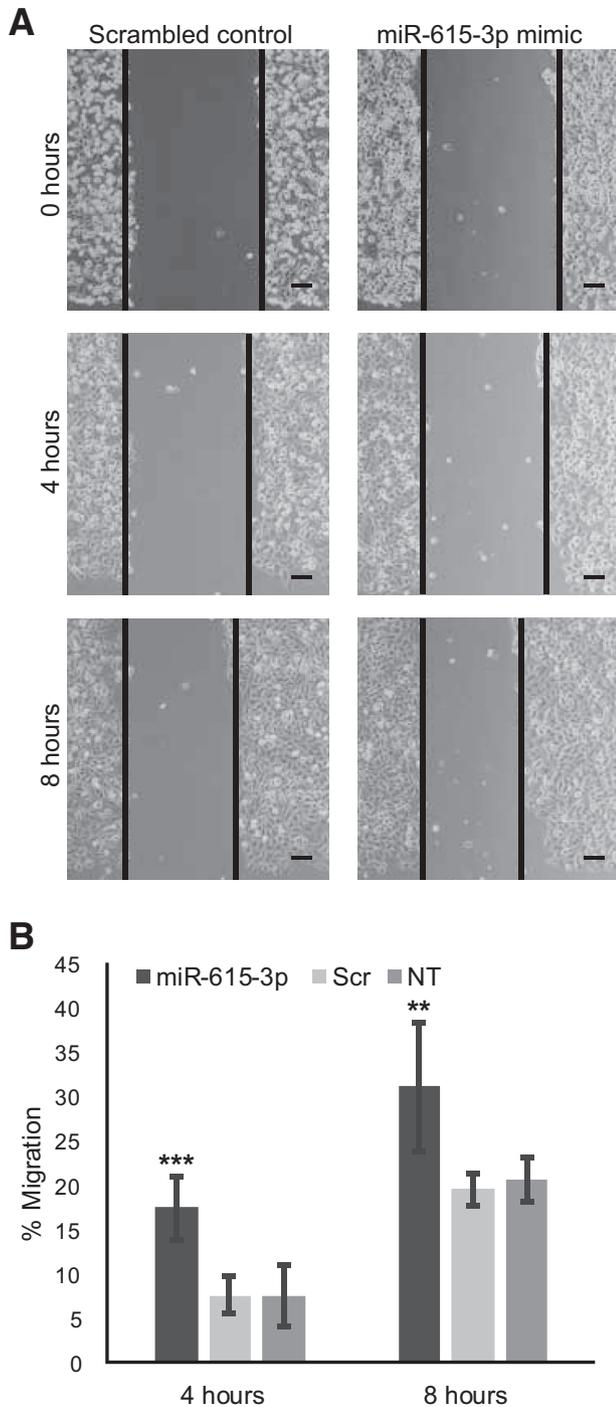
Figure 4 miR-615-3p promotes migration in prostate cancer cells. Wound healing assay after transfection of PC3M cells with scrambled (Scr) control, miR-615-3p mimic, or left nontransfected (NT). **A:** Scratch begins at 0 hours, and migration was measured after 4 and 8 hours. The migration front is highlighted by **vertical black lines**. **B:** Error bars represent SD of two independent experiments, performed in triplicate. ****P < 0.01, ***P < 0.001 miR-615-3p mimics versus scrambled control (t-test). Scale bars = 200 μm (A).

is a novel candidate biomarker for aggressive PC. To the best of our knowledge, this is the largest study to evaluate the prognostic potential of miR-615-3p (or any other single miRNA) in relation to PC.

This work confirms and expands on a few previous small−to−medium-scale PC studies that have reported significant up-regulation of miR-615-3p expression in PC tissue samples compared with nonmalignant controls.[13,14,31] Moreover, dysregulated miR-615-3p expression has previously been reported also in other human cancer types.[32,33] Thus, similar to the finding of elevated miR-615-3p levels in more aggressive PC, miR-615-3p has been found to be up-regulated in hepatocellular carcinoma patients with recurrence compared with patients without recurrence.[32] Moreover, miR-615-3p overexpression in hepatocellular carcinoma and gastric cancer cell lines has been demonstrated to promote cell proliferation and migration,[32,33] similar to the more aggressive phenotypes that were observed herein in PC cells after overexpressing miR-615-3p. In contrast, in non−small-cell lung cancer, miR-615-3p expression has been identified as down-regulated in tumor samples compared with normal tissue samples and to inhibit cell proliferation and migration *in vitro*.[34] The seeming discrepancy in regard to the role of miR-615-3p as either an oncomiRNA or a tumor suppressor in different malignancies indicates that miR-615-3p functions in a highly cell- and disease-type−specific manner. This may be explained, at least in part, by differences in target genes, as has previously been demonstrated for many other miRNAs.[35]

In addition, miR-615-3p overexpression increased apoptosis in PC3M cells. This is in contrast to an earlier finding in gastric cancer cells, where miR-615-3p over-expression inhibited cell apoptosis.[33] Although inhibition of apoptosis is generally viewed as a cancer hallmark, its role in cancer biology is more complex. Indeed, it is also well known that apoptosis can promote division of neighboring cells and drive tumor formation; and tumors often have elevated apoptosis rates, reflecting the increased cell turn-over compared with the nonmalignant counterparts.[36−38] The conflicting results between miR-615-3p over-expression and apoptosis in this study and the study in gastric cancer cells are most likely due to different cell- or disease-type−specific targets. Nevertheless, the observed increase in cell apoptosis in PC cells overexpressing miR-615-3p is in agreement with an enrichment of the GO term DNA fragmentation during apoptosis in patients with high miR-615-3p expression observed in cohort 4, where high miR-615-3p expression was also associated with poor outcome. Also, it may reflect the highly increased cell proliferation observed in PC cells overexpressing miR-615-3p.

The functional studies demonstrated that miR-615-3p promotes viability, proliferation, apoptosis, and migration of PC3M prostate cancer cells *in vitro*, indicating that miR-615-3p is an oncomiR in PC. Before our work, a single study reported that overexpression of miR-615 inhibited
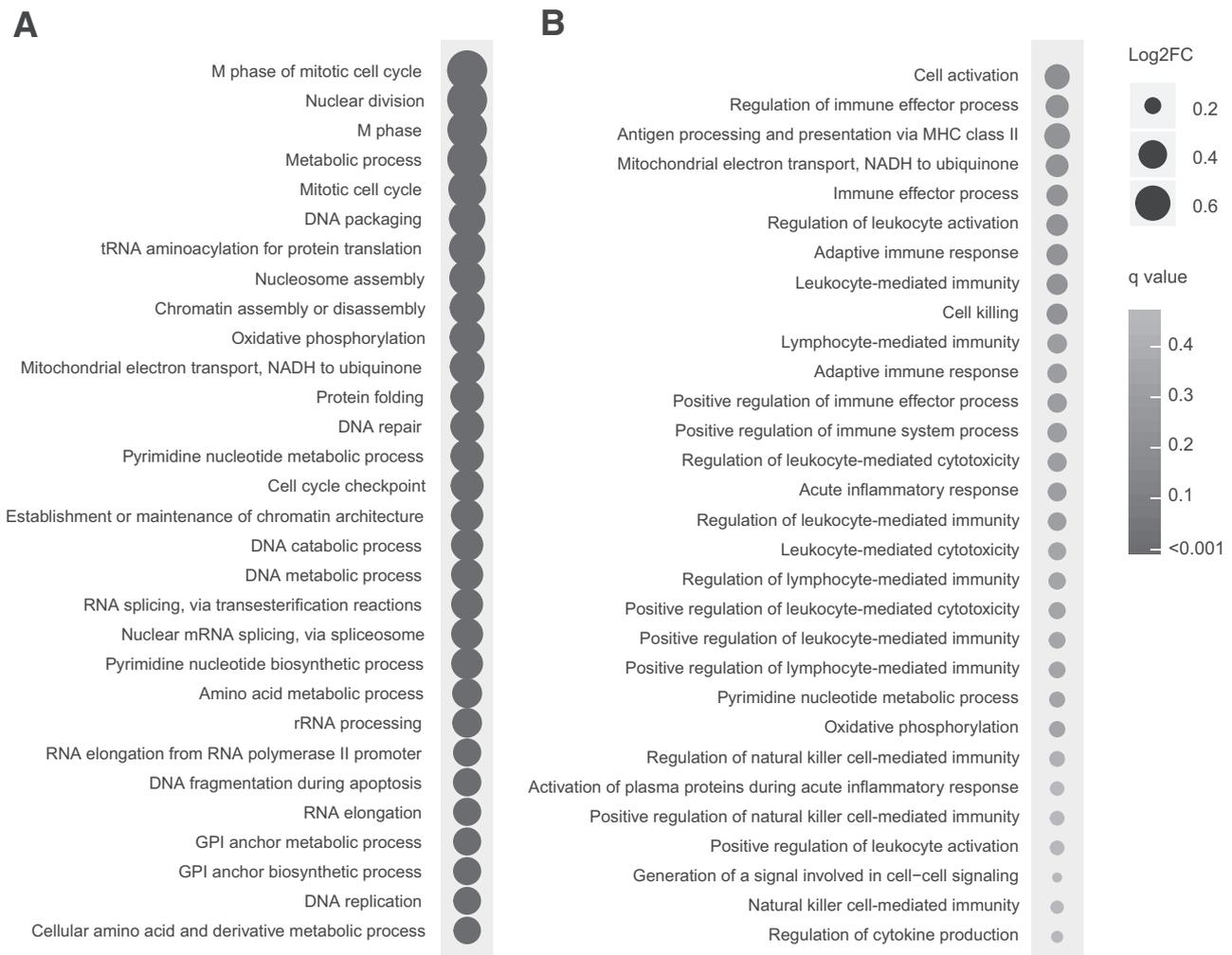
cell viability, proliferation, apoptosis, and migration. Together, these results indicate that miR-615-3p has oncogenic functions in PC and that high miR-615-3p expression

A

B

Figure 5 Cell-cycle—related genes are enriched in patients with high miR-615-3p expression. Patients in cohort 4 with matched miRNA and mRNA expression data available were dichotomized according to miR-615-3p expression (**A**) or a random split (**B**). Top 30 enriched Gene Ontology terms for patients with high miR-615-3p expression versus low miR-615-3p expression (**A**) or in the random split (**B**) are listed along with log2 fold change in expression (RPM^{log}) and false-discovery rate q value adjustment of P value from two-sample t-test using a Benjamini and Hochberg correction. n = 212 (**A** and **B**). GPI, glycosylphosphatidylinositol; MHC, major histocompatibility complex.

proliferation and migration of the related PC cell line PC3, but the authors did not specify whether they investigated the miR-615-3p or miR-615-5p isoform.[39] miR-615-5p has been reported as a tumor suppressor in other cancer types, but its function in PC is unclear.[40–42] Furthermore, it cannot be excluded that miR-615-3p has different target genes in PC3M and in PC3 cells. Future studies will clarify these findings.

Herein, by *in silico* analysis, three top candidate targets of miR-615-3p of potential clinical relevance were identified for PC patients: *LCOR*, *USP44*, and *ITSN1*. Previously, a study in splenic macrophages has identified an evolutionarily conserved target sequence for miR-615-3p in the 3′-untranslated region of *LCOR* and was able to validate this using a dual-luciferase assay and Western blot analysis experiments,[43] supporting our observation of *LCOR* as a potential target of miR-615-3p. However, further

investigation is needed in regard to the interaction between *LCOR* and miR-615-3p in PC.

Of the three identified candidates, *LCOR* has previously been related to PC; however, there are no reports of *USP44* or *ITSN1* in relation to PC.[44,45] Specifically, *LCOR* has been reported to repress PC growth in the human castration-resistant PC cell line C4-2 by colony formation assays and *in vivo* xenograft mouse models, consistent with a possible tumor suppressor role of *LCOR* in PC.[44] Future studies should investigate if a possible tumor suppressor function of *LCOR* is inhibited by high miR-615-3p expression in aggressive PC.

The present study has some potential limitations. Clinical follow-up for cohort 4 was relatively short (median, 21 months) in contrast to cohorts 1 to 3 (median, 107, 130, and 136 months, respectively). Also, a distinct miRNA platform was used for cohort 4 (small RNA sequencing), whereas

cohorts 1 to 3 were profiled using quantitative RT-PCR. Nevertheless, high miR-615-3p expression showed significant prognostic potential in univariate analysis in all four cohorts, indicating that our findings are robust. Another possible limitation is the use of BCR as a clinical end point. BCR is a surrogate end point for outcome after RP and is not necessarily associated with PC mortality.[46] Unfortunately, other more optimal end points, such as metastasis, CSS, and overall survival, require long follow-up time (>15 years).[47] Nevertheless, when cohorts 1 to 3 were merged, high miR-615-3p expression was a significant predictor of CSS in both univariate (HR = 3.75; $P < 0.001$) and multivariate (HR = 2.66; $P = 0.01$) analysis beyond routinely available postoperative clinicopathologic factors. Another limitation is the lack of miR-615-3p target gene validation, but this was considered to be beyond the scope of the present work. Nevertheless, three top candidate target genes of likely clinical relevance in PC were identified by an integrated bioinformatics approach and warrant further investigation in future studies. Additional clinical studies are also needed to assess the true clinical value of miR-615-3p as a potential prognostic marker for clinically localized PC.

In conclusion, high expression of miR-615-3p predicted BCR in four distinct RP cohorts; and miR-615-3p was significantly associated with CSS in a merged analysis of 734 RP patients. Furthermore, overexpression of miR-615-3p promoted an aggressive PC cell phenotype, characterized by increased cell viability, proliferation, apoptosis, and migration. Future studies may investigate if miR-615-3p knockdown/inhibition carries therapeutic value for PC.

Acknowledgments

Supplemental Data

Supplemental material for this article can be found at *http://doi.org/10.1016/j.ajpath.2019.08.007*.

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