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The miRNA-kallikrein axis of interaction: a new dimension in the pathogenesis of prostate cancer

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Abstract

Kallikrein-related peptidases (KLKs) are a family of serine proteases that were shown to be useful cancer biomarkers. KLKs have been shown to be dysregulated in prostate cancer (PCa). microRNAs (miRNAs) are short RNA nucleotides that negatively regulate gene expression and have been reportedly dysregulated in PCa. We compiled a comprehensive list of 55 miRNAs that are differentially expressed in PCa from previous microarray analysis and published literature. Target prediction analyses showed that 29 of these miRNAs are predicted to target 10 KLKs. Eight of these miRNAs were predicted to target more than one KLK. Quantitative real-time (qRT)-PCR demonstrated that there was an inverse correlation pattern in the expression (normal vs. cancer) between dysregulated miRNAs and their target KLKs. In addition, we experientially validated the miRNA-KLK interaction by transfecting miR-331-3p and miR-143 into a PCa cell line. Decreased expression of targets KLK4 and KLK10, respectively, and decreased cellular growth were observed. In addition to KLKs, dysregulated miRNAs were predicted to target other genes involved in the pathogenesis of PCa. These data show that miRNAs can contribute to KLK regulation in PCa. The miRNA-KLK axis of interaction projects a new element in the pathogenesis of PCa that may have therapeutic implications.

Keywords: cancer pathogenesis; kallikrein; KLK; miRNA; personalized medicine; prostate; prostate cancer; tumor markers.

Introduction

Prostate cancer (PCa) is the most common malignancy in the western world. In 2011, it is predicted that there will be over 240 000 newly diagnosed cases of PCa in the United States (American Cancer Society, 2011). The risk of developing PCa increases with age and is highest after the age of 60. The incidence rate of PCa has been on the rise over the past three decades, likely due to the recent advances in early detection of the disease. The mechanisms underlying prostate carcinogenesis is yet to be fully elucidated (Gu and Brothman, 2011).

Kallikrein-related peptidases (KLKs) are family of serine proteases well documented in the literature to be dysregulated in a number of cancers including ovarian, prostate, brain, and kidney cancers (Yousef et al., 2001, 2003). Many members of the KLK family have been reported to be dysregulated in PCa and thus are being investigated as possible biomarkers for the disease (Emami and Diamandis, 2008; Seiz et al., 2010).

Currently, prostate-specific antigen (PSA), which is encoded by the human KLK3 gene is the most widely adopted biomarker for early detection of PCa. However, accumulating evidence suggests the lack of sensitivity and specificity of PSA as a PCa tumor marker. Recent studies have shown that human KLK2 is upregulated during PCa progression and therefore may have use as a biomarker. Diagnostic tests that combine serum KLK2 with total PSA (Recker et al., 2000) and free PSA (Stephan et al., 2006) have improved the sensitivity and specificity of detection. In addition, KLK4 mRNA was shown to be upregulated in PCa tissues (Yousef et al., 1999; Obiezu et al., 2002; Xi et al., 2004; Avgeris et al., 2011) and may play a role in epithelial to mesenchymal transition (Veveris-Lowe et al., 2005). On the other hand, studies showed that KLK10, a potential tumor suppressor, was downregulated in more aggressive PCa s (Luo and Diamandis, 2000). In a PCa cell line, KLK10 mRNA and protein downregulation were suggested to be a result of CpG island hypermethylation (Sidiropoulos et al., 2005).

microRNAs (miRNAs) are small nonprotein-coding RNA nucleotides that have been widely described as having important biological and regulatory roles in cancer (Garofalo and Croce, 2011). miRNAs negatively regulate the expression of their target genes through complementary, sequence-specific binding, which leads to negative regulation of the target gene by either mRNA degradation or suppression of protein translation. miRNAs have been shown to be dysregulated in

a wide array of tumors including breast, lung, prostate, ovarian, and kidney cancers (Fendler et al., 2011; White et al., 2011), and their role in the pathogenesis of these cancers is being investigated (Schaefer et al., 2010b; White and Yousef, 2010).

The interaction between miRNAs and KLKs is emerging in the literature as a potential new dimension of regulation of carcinogenesis in many tumors including ovarian and kidney cancers (Chow et al., 2008; Yousef, 2008; White et al., 2010a,b). Recognizing this relationship will enhance our understanding of the mechanisms that control KLK dysregulation in PCa and will also shed the light on the downstream effector pathways through which miRNAs can affect tumor formation and progression.

In this study, we aimed to examine miRNA dysregulation in PCa and how these dysregulated miRNAs may contribute to KLK dysregulation. We performed target prediction analysis to determine potential KLK targets. We also correlated differential expression of miRNAs dysregulated in PCa with their target KLK expression using quantitative real-time (qRT)-PCR in PCa tissues. We experimentally verified the miRNA-KLK interaction in a PCa cell line model and examined the effect of this interaction on cellular growth. We also performed bioinformatics analyses to help understand the mechanism of miRNA dysregulation in PCa and how they affect KLKs.

Results

miRNAs dysregulated in PCa can target KLKs

A compiled total of 55 miRNAs were reported to be dysregulated in PCa by microarray analysis (Schaefer et al., 2010a) and multiple literature sources (Table 1). Twenty-three miRNAs were reported to be upregulated, while 25 were reported to be downregulated. There were conflicting reports for the dysregulation of seven miRNAs (i.e., reported to be upregulated and downregulated in different studies). We performed target prediction analysis on each of the 55 miRNAs and found that 29 (53%) of them are predicted to target KLKs (Table 1). Interestingly, eight of these miRNAs were predicted to target more than one KLK gene; e.g., miR-143 is predicted to target KLK2, KLK5, KLK10, and KLK13. Also, the same KLK can be targeted by more than one miRNA; e.g., KLK10 is predicted to be targeted by eight miRNAs including miR-1, miR-143, and miR-17-5p. The complexity of these potential interactions is shown in Figure 1. Three miRNAs, miR-24, miR-21, and miR-16 were predicted to target both KLK10 and KLK2.

KLKs and miRNAs show inverse correlation pattern of expression in PCa tissues

In order to validate the miRNA-KLK target interaction *in vivo*, we validated the expressions of miR-21, miR-24,

Table 1 A complied list of 55 miRNAs that are dysregulated in PCa. Twenty-nine miRNAs are predicted to target KLKs.

miRNA	Expression in PCa	KLK predicted target	miRNA	Expression in PCa	KLK predicted target
let-7a	Down	-	miR-21	UP	KLK2, KLK10
let-7i	Down	-	miR-221	Up/Down	-
miR-1	Up/Down	KLK10, KLK13	miR-222	Up/Down	-
miR-101	Down	-	miR-24	Up	KLK2, KLK3, KLK7, KLK10
miR-103	Up	KLK5	miR-30d	Down	-
miR-106b	Up/down	-	miR-31	Up	KLK10
miR-107	Up/down	KLK5	miR-32	Up	-
miR-125b	Up	-	miR-320	Up	-
miR-126	Down	-	miR-324-3p	Down	KLK2
miR-1296	Down	-	miR-330-3p	Down	KLK4
miR-133a	Up	-	miR-331-3p	Down	KLK2, KLK4
miR-133b	Down	-	miR-34a	Down	KLK13
miR-140	Down	KLK6, KLK9	miR-34c	Down	KLK13
miR-141	Up	-	miR-363	Down	-
miR-143	Down	KLK2, KLK5, KLK10, KLK13	miR-370	Down	KLK2
miR-145	Down	KLK7	miR-449	Down	-
miR-146a	Down	-	miR-449a	Down	KLK13
miR-148a	Up/Down	-	miR-485-3p	Up	-
miR-15	Down	KLK2	miR-486-5p	Up	KLK11
miR-16	Up/Down	KLK2, KLK10	miR-521	Up	-
miR-17-5p	Down	KLK5, KLK7, KLK10	miR-7	Down	KLK3
miR-193b	Up	KLK10	miR-766	Up	KLK3
miR-194	Up	-	miR-768-3p	Up	KLK11
miR-197	Up	-	miR-801	Down	-
miR-200a	Up	KLK13	miR-92	Up	-
miR-200b	Up	KLK7	miR-96	Up	-
miR-205	Down	KLK2	miR-99	Down	-
miR-20a	Up	-			

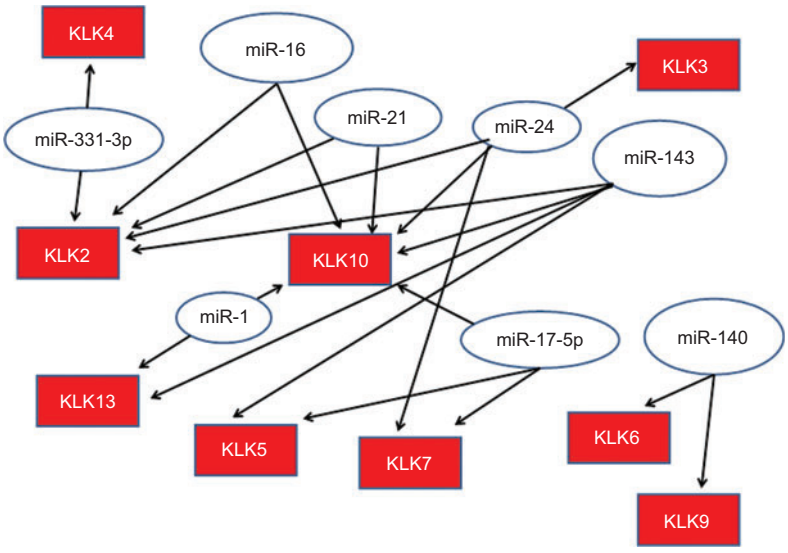

Figure 1 Dysregulated miRNAs in PCa are predicted to target more kallikreins.
The complex interaction between miRNAs and KLKs is shown in the interaction map. Only miRNAs that are predicted to target more than one KLK are shown. Two interesting phenomena are observed; one miRNA can target multiple KLKs, and the same KLK can be targeted by multiple miRNAs.

miR-143, and miR-331-3p and their targeting KLKs in PCa tissues and matched normal tissue from the same patients. We chose these miRNAs because (1) they were significantly dysregulated in PCa when compared to normal prostate tissue from the same patient; (2) target prediction analysis showed that these miRNAs could target KLKs that have been previously shown to be dysregulated in PCa; and (3) these miRNAs have been previously shown to be dysregulated in cancer.

When we measured the expression of miR-143 and its target KLK2 in 12 pairs of PCa and normal matched prostate tissue from the same patient, we found that 67% (8/12) patients showed increased expression of KLK2. Interestingly, when we examined the relationship between the expression of KLK2 and its targeting miRNA, miR-143, we identified an inverse correlation pattern of expression (Figure 2A and Supplementary Table 1), i.e., when there was decreased levels of miR-143 in cancer compared to normal, there was increased levels of KLK2 expression and vice versa. We also identified other miRNAs that are downregulated in PCa and are predicted to target KLK2. They include miR-15, miR-205, miR-324-3p, miR-370, and miR-331-3p. miR-143 (Clape et al., 2009; Peng et al., 2011) and miR-15 (Musumeci et al., 2011) have been shown by others to be downregulated in PCa.

KLK4 was upregulated in 58% (7/12) PCa cases studied (data not shown) and is predicted to be targeted by two miRNAs, miR-330-3p and miR-331-3p, that were downregulated in PCa by us and others (Epis et al., 2009; Lee et al., 2009a). We correlated the expression of miR-331-3p and its target KLK4 and found that there was an inverse correlation pattern of expression (Figure 2B and Supplementary Table 1), suggesting that dysregulated miRNAs can contribute to KLK dysregulation in PCa.

PSA, which was upregulated in 67% (8/12) PCa tissues (data not shown) examined, is predicted to be a putative target of miR-7, which was downregulated in PCa. Another study showed that miR-7 was downregulated in PCa and can target epidermal growth factor receptor (ERBB) (Giles et al., 2011). KLK3 was also predicted to be targeted by miR-24 and showed an inverse correlation pattern of expression (Supplementary Table 1).

KLK10, which was shown to be downregulated in 75% (9/12) PCa tissues (data not shown) when compared to normal matched prostate tissue, is predicted to be targeted by five miRNAs that are reported to be upregulated in PCa. These include miR-16, miR-193b, miR-21, miR-24, and miR-31. A number of studies have shown that miR-21 is upregulated and can effect cellular invasion and apoptosis in PCa cell lines (Li et al., 2009b; Ribas and Lupold, 2010). We found that the expression of miR-21 and its target KLK10 were inversely correlated (Supplementary Table 1).

miRNAs can target KLKs and affect cellular growth in a PCa cell line

In order to experimentally validate the miRNA-KLK interaction, we chose the DU-145 PCa cell line. This cell line model proved to be suitable as it has moderate expression of KLK4 and KLK10 and no expression of miR-331-3p or miR-143, respectively. This is ideal for validation experiments as we would expect to see decreased KLK expression after transfection with its targeting miRNA.

We first transfected DU-145 cells with miR-331-3p and compared the expression of its target, KLK4, before and after transfection (Figure 3A). We found that after transfection with miR-331-3p, there was decreased expression of KLK4 when compared to untransfected cells and control cells ($p<0.001$). We also experimentally validated the

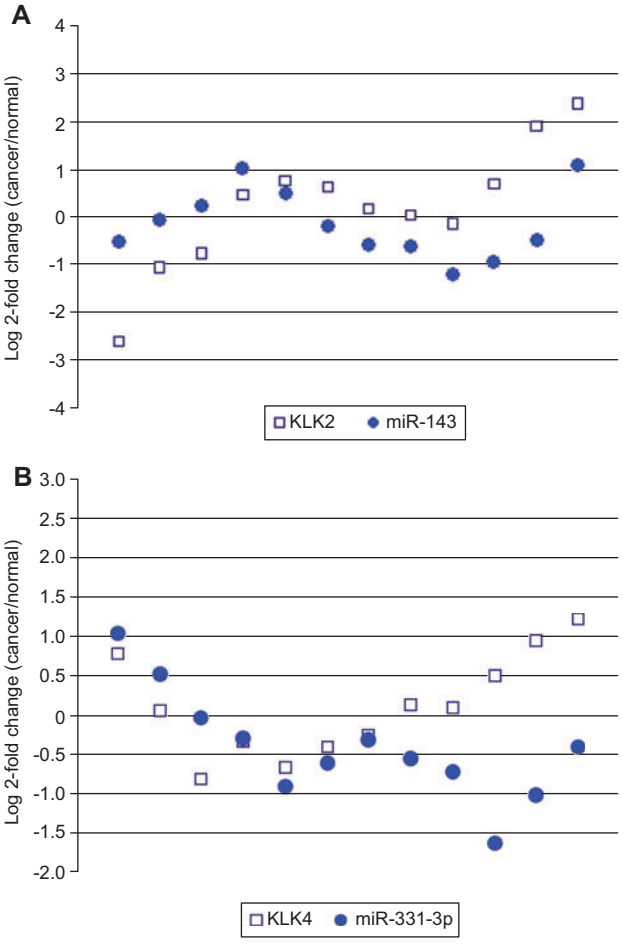

Figure 2 The inverse correlation relationship between miRNAs dysregulated in PCa and their target KLKs.
Expression changes (cancer/normal) were measured for both miRNAs and their target KLKs in pairs of cancer/normal tissues form the same patient. (A) Expression changes of miR-143 (cancer/normal) are inversely correlated to KLK2 expression in PCa tissues. (B) The expression changes of miR-331-3p are inversely correlated to KLK4 expression.

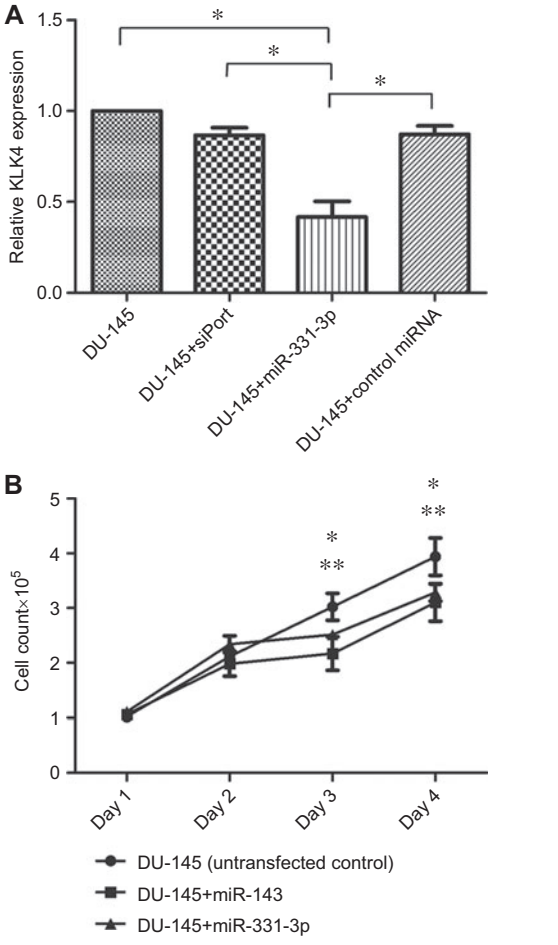

Figure 3 The effect of dysregulated miRNAs on kallikreins and cell proliferation in a PCa cell line model.
(A) KLK4 expression was significantly decreased in the DU-145 cell line after transfection with miR-331-3p when compared to untransfected controls and control cells. (B) After transfection with either miR-143 or miR-331-3p, cellular growth was decreased in the DU-145 cell line model when compared to untransfected controls after days 3 and 4 (*p<0.001, **p<0.05).

miR-143 and KLK10 interaction and found that after transfection with miR-143, KLK10 expression was decreased (p<0.005; data not shown).

In order to examine the biological effects of the miRNA-KLK interaction in PCa, we transfected DU-145 cells with either miR-143 or miR-331-3p and measured their effect on tumor cell growth. We found that cells transfected with either miR-143 (p<0.001) or miR-331-3p (p<0.05) showed a decrease in cellular growth when compared to untransfected cells after days 3 and 4 (Figure 3B), suggesting that the miRNA-KLK interaction can play a role in PCa pathogenesis.

Bioinformatics analysis

Identification of miRNA clusters located near the KLK cytogenetic locus Previous reports showed that approximately 50% of human miRNAs are organized into clusters within large polycistronic messages (Zhang et al., 2009). Using an inter-miRNA cutoff of a distance of 10 000 nucleotides, we identified four miRNA clusters that are located around the KLK locus (Table 2). The first cluster has two miRNAs, the second contains three, while the third cluster has 46 miRNAs (which is the largest human miRNA cluster), and the fourth has three miRNAs. Target prediction analysis determined that 72% (39/54) of miRNAs in the clusters were predicted to target KLKs. In order to confirm positive miRNA-KLK interactions, these targets need to be experimentally verified.

Correlation of miRNA with cytogenetic changes in PCa In order to explore the mechanism of miRNA dysregulation in PCa, we compiled a list of frequent chromosomal aberrations in PCa and correlated them with miRNA dysregulation in the malignancy. We found that there were 22 miRNAs that were reported to be dysregulated in PCa

Table 2 Many of the miRNAs that are located in the four clusters around the kallikrein locus are predicted to target KLK.

Cluster	miRNA	Cytoband	Inter-miRNA distance (nt)	Predicted KLK targets[a]
1	hsa-miR-330	19q13.31	-	KLK2, KLK3, KLK4, KLK5, KLK9, KLK10
	hsa-miR-642	19q13.31	35 841	KLK2, KLK3, KLK10
			Kallikrein locus	
2	hsa-miR-99b	19q13.32	-	
	hsa-let-7e	19q13.32	105	KLK2, KLK3, KLK6, KLK10, KLK15
	hsa-miR-125a	19q13.32	390	KLK2, KLK5, KLK6, KLK10, KLK5
3	hsa-miR-512-1	19q13.33	-	KLK2, KLK3, KLK7, KLK10
	hsa-miR-512-2	19q13.33	2395	KLK2, KLK3, KLK7, KLK10
	hsa-miR-1323	19q13.33	2714	
	hsa-miR-498	19q13.33	2157	KLK11
	hsa-miR-520e	19q13.33	1391	KLK2, KLK10
	hsa-miR-515-1	19q13.33	3206	KLK2, KLK3, KLK7, KLK10, KLK13
	hsa-miR-519e	19q13.33	855	KLK2, KLK3, KLK5, KLK10, KLK13
	hsa-miR-520f	19q13.33	2136	KLK3
	hsa-miR-515-2	19q13.33	2764	KLK2, KLK3, KLK7, KLK10, KLK13
	hsa-miR-519c	19q13.33	1378	KLK7
	hsa-miR-1283-1	19q13.33	1926	
	hsa-miR-520a	19q13.33	2314	KLK2, KLK3, KLK7, KLK10
	hsa-miR-526b	19q13.33	3428	KLK7, KLK10, KLK15
	hsa-miR-519b	19q13.33	738	KLK7
	hsa-miR-525	19q13.33	2240	KLK2, KLK6, KLK9
	hsa-miR-523	19q13.33	768	
	hsa-miR-518f	19q13.33	1544	
	hsa-miR-520b	19q13.33	1126	KLK10
	hsa-miR-518b	19q13.33	1450	
	hsa-miR-526a-1	19q13.33	3433	KLK9
	hsa-miR-520c	19q13.33	1117	KLK9, KLK10
	hsa-miR-518c	19q13.33	1196	
	hsa-miR-524	19q13.33	2167	
	hsa-miR-517a	19q13.33	1180	KLK2
	hsa-miR-519d	19q13.33	993	KLK5, KLK7, KLK10
	hsa-miR-521-2	19q13.33	3160	
	hsa-miR-520d	19q13.33	3416	KLK2, KLK10
	hsa-miR-517b	19q13.33	894	
	hsa-miR-520g	19q13.33	1024	KLK10
	hsa-miR-516b-2	19q13.33	3187	KLK2, KLK3, KLK10
	hsa-miR-526a-2	19q13.33	1396	KLK9
	hsa-miR-518e	19q13.33	2852	
	hsa-miR-518a-1	19q13.33	1081	
	hsa-miR-518d	19q13.33	3787	KLK2
	hsa-miR-516b-1	19q13.33	1882	KLK2, KLK3, KLK10
	hsa-miR-518a-2	19q13.33	2399	
	hsa-miR-517c	19q13.33	1894	KLK2
	hsa-miR-520h	19q13.33	1105	KLK10
	hsa-miR-521-1	19q13.33	6037	
	hsa-miR-522	19q13.33	2489	KLK3, KLK10
	hsa-miR-519a-1	19q13.33	1100	KLK7
	hsa-miR-527	19q13.33	1537	KLK12
	hsa-miR-516a-1	19q13.33	2639	KLK9
	hsa-miR-1283-2	19q13.33	1402	
	hsa-miR-516a-2	19q13.33	2815	KLK9
	hsa-miR-519a-2	19q13.33	1122	KLK7
4	hsa-miR-371	19q13.33	-	KLK2, KLK10
	hsa-miR-372	19q13.33	149	KLK5, KLK10
	hsa-miR-373	19q13.33	749	KLK2, KLK5, KLK10

[a]KLKs were only listed if they were predicted to be targeted by at least three programs.

that were located at sites of reported chromosomal aberrations in PCa. Fifty-nine percent (13/22) miRNAs were reported to be correlated with the direction of chromosomal change in PCa; e.g., miR-449 and miR-449a are downregulated in PCa and located at Ch 5q11.2, which has been reported to have chromosomal losses in PCa (Table 3). These data suggest that chromosomal changes may, in part, contribute to miRNA dysregulation in PCa.

Table 3 miRNAs that are dysregulated in PCa are located at sites of frequently reported chromosomal aberrations.

miRNA	miRNA dysregulation	Chromosomal location	Reported chromosomal changes
miR-1	Up	20q13.33	Gain
miR-106b	Up	7q22.1	Gain
miR-24	Up	9q22.32	Gain
miR-30d	Down	8q24.22	Gain
miR-32	Up	9q31.3	Gain
miR-96	Up	7q32.2	Gain
miR-107	Down	10q23.31	Loss
miR1296	Down	10q21.3	Loss
miR-140	Down	16q22.1	Loss
miR-15	Down	13q14.2	Loss
miR-324-3p	Down	17p13.1	Loss
miR-449	Down	5q11.2	Loss
miR-449a	Down	5q11.2	Loss

Involvement of dysregulated miRNAs in the pathogenesis of PCa To examine the role of miRNAs that are dysregulated in PCa and can target KLKs in PCa pathogenesis, we did target prediction analysis for these 29 miRNAs and examined their potential interaction with pathways that are frequently dysregulated in PCa (Table 4). We found that 16 of these miRNAs were validated to target components of pathways frequently dysregulated in PCa. One of these pathways that are hyperactivated in approximately 30%–50% of PCas is the phophatidylinositol 3-kinase/RAC-alpha serine/theronine-protein kinase (PI3K/Akt) signaling pathway (Sarkar et al., 2010). A major component of this pathway is the tumor suppressor phosphatase and tensin homolog (PTEN), which normally functions to block the activity of PI3K. Another pathway that is frequently dysregulated in PCa includes the androgen receptor (AR) pathway.

miR-331-3p, which is decreased in PCa and is predicted to target KLK2 and KLK4, has been shown to target two specific sites within the human ERBB2 mRNA 3′ untranslated region. Transfection of multiple PCa cell lines with miR-331 showed a reduction of ERBB2 mRNA and protein expression and a block of the downstream PI3K/Akt signaling pathway. miR-331-3p was also shown to block androgen signaling and reduce the activity of the PSA promoter, subsequently decreasing PSA expression (Epis et al., 2009).

miR-21, which is described as an oncomir, has been shown to have increased expression in almost all cancers including PCa. Validated targets for miR-21 in PCa include bone morphogenetic protein receptor, type II (BMPRII), myristoylated alanine-rich protein kinase C substrate (MARCKS), programmed cell death 4 (PDCD4), PTEN, acidic (leucine-rich) nuclear phosphoprotein 32 family, member A (ANP32A), SWItch/Sucrose NonFermentable (SWI/SNF) related, matrix-associated, actin-dependent regulator of chromatin, subfamily a, member 4 (SMARCA4), and sprouty 1 (SPRY1; Table 4). Yang et al. showed that when miR-21 was ectopically expressed in PCa cells, phosphorylation of Akt was induced by interferon but not induced in cells that do not express miR-21 (Yang et al., 2010). In addition, it has been shown that miR-21 can target the tumor suppressor PTEN (Liu et al., 2011a). Ribas et al. showed that AR can directly interact with miR-21 regulatory regions, indicating

Table 4 A complied list of 16 miRNAs that are dysregulated in PCa, predicted to target KLKs and have been validated to target other genes involved in prostate carcinogenesis.

miRNA	Expression in PCa	Validated targets	References
miR-107	Up/Down	GRN	(Wang et al., 2010)
miR-143	Down	MYO6, KRAS, ERK5, ICP4	(Clape et al., 2009; Lee et al., 2009a; Szczyrba et al., 2010; Xu et al., 2011)
miR-145	Down	MYO6, ICP4, SWAP70, FSCN1, TNFSF10	(Lee et al., 2009a; Szczyrba et al., 2010; Zaman et al., 2010; Chiyomaru et al., 2011; Fuse et al., 2011)
miR-15	Down	BCL2, CCND1, WNT3A	(Bonci et al., 2008)
miR-16	Up	BCL2, CCND1, WNT3A	(Bonci et al., 2008)
miR-200b	Up	ZEB2	(Kong et al., 2009)
miR-205	Down	BCL2	(Bhatnagar et al., 2010)
miR-21	Up	BMPRII, MARCKS, PDCD4, PTEN, ANP32A, SMARCA4, SPRY1	(Qin et al., 2009; Finlayson and Freeman, 2009; Shi et al., 2010; Yang et al., 2010; Darimipourain et al., 2011; Liu et al., 2011b; Schramedei et al., 2011)
miR-24	Up	FAF1	(Qin et al., 2010)
miR-31	Up	E2F6	(Bhatnagar et al., 2010)
miR-330-3p	Down	E2F1	(Lee et al., 2009b)
miR-331-3p	Down	ERBB2, PSA	(Epis et al., 2009)
miR-34a	Down	CD44, SIRT1, BCL2	(Fujita et al., 2008; Kojima et al., 2010; Liu et al., 2011a)
miR-34c	Down	E2F3, BCL2	(Hagman et al., 2010)
miR-449a	Down	HDAC-1, CCND1	(Noonan et al., 2009; Noonan et al., 2010)
miR-7	Down	EGFR	(Giles et al., 2011)

direct transcriptional induction (Ribas et al., 2009). They also found that overexpression of miR-21 enhanced tumor xenograft growth and was sufficient to support androgen-independent proliferation following surgical castration. Taken together, our data shows that KLKs can be involved in a network of interactions that are involved in PCa pathogenesis.

Discussion

Kallikreins have been shown to be involved in a number of different cancers, yet the mechanisms controlling their dysregulation are still uncertain for many cases. It is hypothesized that, in part, this regulation may be posttranscriptional through miRNAs (Chow et al., 2008; Yousef, 2008). We showed evidence indicating that miRNAs can contribute to KLK dysregulation in PCa through two independent lines of evidence. First, we observed a pattern of inverse correlation between dysregulated miRNAs and their target KLKs, miR-143 and its target KLK2, and miR-331-3p and its target KLK4 in PCa tissue (Figure 2), indicating that the miRNAs may control KLK expression in PCa. In addition, we showed that transfection of miR-143 into a PCa cell line model decreased KLK2 expression and cellular growth. This, however, must be interpreted with caution. It must be noted that miR-143 can have other targets that may contribute to this biological effect. Overexpression of miR-143 has been shown to suppress PCa proliferation and migration and increase sensitivity to docetaxel by targeting the mitogen-activated protein kinase signaling (EGFR/RAS/MAPK) pathway (Xu et al., 2011). Other targets that have been validated for miR-143 in PCa include extracellular signal-regulated kinase-5 (ERK5) (Clape et al., 2009) and genes involved in epithelial to mesenchymal transition (Peng et al., 2011).

The fact that miRNAs have multiple targets and can target more than one gene has important implications. On one hand, the targeting of a number of genes in the same pathway has potential therapeutic implications. In this case, the introduction of a miRNA can target or 'hit' the pathway at multiple points, which increases the chances of successful pathway suppression (Metias et al., 2009; White and Yousef, 2011). On the other hand, because miRNAs can have a number of targets in different pathways, it is experimentally challenging to control for off-target effects. This needs solid experimental design, combination of bioinformatics and experimental approaches, and the use multiple independent validation techniques.

We preformed bioinformatics analyses to explore the mechanism of miRNA dysregulation in PCa. The coordinated dysregulation of miRNA clusters, miRNAs that are located in close proximity, implies a coordinated regulation and function. Our analysis showed that the KLK locus, Ch 19q13, harbors a large number of miRNAs. This miRNA cluster, which has been termed C19MC, represents the largest cluster in the human genome (Bentwich et al., 2005). Interestingly, an amplicon of this region was found to be associated with an aggressive subgroup of primitive neuroectodermal tumors with distinct gene-expression profiles, characteristic histology, and dismal survival (Li et al., 2009a). Coordinated expression of miRNAs within clusters suggests a coordinated control mechanism and functions. Similarly, it has been suggested that KLKs share a similar control mechanism and function. It would be interesting to explore if a relationship exists between the two.

Other groups have examined the miRNA-KLK interaction in PCa by examining the relationship between miRNAs and PSA (KLK3) in PCa. Transfection of miR-99a, miR-99b, or miR-100 inhibited the growth of PCa cells and decreased the expression of PSA (KLK3) in PCa cell lines (Sun et al., 2011). PSA levels increased after inhibition of the miR-99 family, suggesting that miRNAs can contribute to PSA regulation in PCa.

miRNAs represent attractive useful clinical markers for PCa, and evidence for this use is emerging quickly. Hoa et al. reported an increase in the positive predictive value in PCa patients from 40% to 87.5% by integrating PSA blood levels with miR-21 and miR-141 profiles (Hao et al., 2011). It has also been reported that a panel of markers including 10 protein-coding genes and two miRNAs, miR-519d and miR-647, was identified to accurately separate patients with and without biochemical recurrence ($p<0.001$), as well as for the subset of 42 Gleason score of[FCP1] seven patients ($p<0.001$) (Long et al., 2011). More recently, circulating miR-141 levels in the plasma of PCa patients predicted clinical outcomes with an odds ratio of at least 8.3 (Gonzales et al., 2011). In the same study, miR-141 also had the highest correlation with temporal changes of PSA and demonstrated high correlation with changes of the other biomarkers. Circulating tumor markers represent attractive clinical markers as their non-invasive nature if superior to prostatectomy.

Through our study and others, it is now evident that the miRNA-KLK interaction is involved in PCa pathogenesis and has potential clinical utilities. However, this relationship is more complex than previously thought. One miRNA can target more than one KLK, and one KLK can be targeted by more than one miRNA leading to a complex network of interactions (Figure 1). In addition, these interactions may be direct or indirect, which adds to the complexity. Although miRNAs act posttranscriptionally, recent evidence supports that they also usually interfere with the stability of their target mRNAs (Cheng and Li, 2008; Gennarino et al., 2009; Guimbellot et al., 2009) Moreover, KLKs may also contribute to miRNA regulation through transcriptional regulation of miRNA promoters as suggested by KLK nuclear localization (Korkmaz et al., 2001; Klokk et al., 2007).

In conclusion, we provide bioinformatics and experimental evidence suggesting that miRNAs are involved in the regulation of KLKs and that both can actively contribute to PCa formation and progression.

Materials and methods

Sample collection

The study was approved by the Research Ethics Boards of St. Michael's Hospital, Toronto, Canada, and Charité-University Hospital, Berlin, Germany. Fresh prostate tissues from 12 pairs of tumor and normal tissues from the same patients were sampled directly after

surgical removal of the gland for treatment of PCa at Charité-University Hospital, Berlin, Germany. One full frontal section, which was grossly tumor suspicious, was deep frozen in liquid nitrogen. A diagnostic hematoxylin and eosin section was prepared to verify tumor content and margin status and to identify areas of normal and tumor tissue. These regions of interest were punch biopsied, and another frozen section was made to ascertain tumor content and to assign a Gleason score. Only cases with >90% tumor tissue were considered for further analysis.

miRNA dysregulation in PCa

In order to create a comprehensive list of miRNAs dysregulated in PCa, we combined data from our recently published miRNA microarray comparing PCa with normal matched prostate tissue from the same patient (Schaefer et al., 2010a) and did a literature search for additional reported miRNAs dysregulated in PCa when compared to normal prostate tissue (Sylvestre et al., 2007; Ambs et al., 2008; Shi et al., 2011; Sun et al., 2011).

Total RNA extraction and reverse transcription

Frozen matched malignant and non-malignant samples were collected in RNAlater Stabilization reagent (Qiagen GmbH, Hilden, Germany). RNA was extracted with the RNA miR Isolation Kit (Qiagen). Total RNA yields and A260/280 ratio were monitored with a NanoDrop 2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and RNA integrity numbers were measured with the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Only RNA extracts with RNA integrity number values >6 were included in further analysis. Samples were stored at -80°C until further use.

One microgram of total RNA was reverse transcribed using the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Indianapolis, IN, USA) following the manufacturer's recommendation.

qRT-PCR

qRT-PCR was performed using the Step One™ Plus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Primer/probe sets were purchased as premade TaqMan Assays on Demand for *KLK2* (Hs00428383_m1), *KLK3* (Hs02576345_m1), *KLK4* (Hs00191772_m1), and *KLK10* (Hs00173611_m1). Thermal cycling conditions were according to the manufacture's fast protocol, and all reactions were performed in triplicate. Relative expression was determined using the $\Delta\Delta C_T$ method, and expression values were normalized to hypoxanthine phosphoribosyltransferase 1 (HPRT1) (Hs01003267_m1).

miRNA target prediction and validation analysis

miRNA target prediction analyses were performed using TargetScan 5.1 (Lewis et al., 2005) and miRecords (Xiao et al., 2009). Experimental target validation of miRNA-KLK interactions were assayed by transfecting the PCa cell line DU-145 with hsa-miR-331-3p and hsa-miR-143 (Ambion, Foster City, CA, USA) and comparing the expression levels of target KLKs before and after transfection. Cells were transfected using the si-PORT™ NeoFX™ transfection system (Ambion) according to the manufacturer's protocol. miRNA precursors, hsa-miR-331-3p, hsa-miR-143, and a random sequence control (Applied Biosystems) were added at a final concentration of 5 nM. Transfection agent/RNAmolecule complex formation was carried out in Opti-MEM Reduced-Serum Media (1X; Invitrogen, Burlington, ON, Canada). The DU-145 cell line was maintained in RPMI 1640 supplemented with 10% fetal bovine serum (Invitrogen, Burlington, Canada) in 5% CO_2 and 37°C.

Cell proliferation assay

Cellular proliferation was measured by cell counting. DU-145 cells were plated at 8.0×10^4 cells per well in a 12-well plate and were either untransfected or transfected with miR-143, miR-331-3p, or a random sequence control miRNA. Cells were incubated for 1–4 days after which they were trypsinized and counted. Cells were counted in triplicate and repeated thrice.

In silico analysis

Chromosomal aberrations in PCa were extracted from the Progenetix database (www.progenetix.net). Average genetic changes from 687 cases of PCa were used for comparative genomic hybridization analysis. We also did a literature search for cytogenetic aberrations in PCa (Nupponen and Visakorpi, 2000; Brothman, 2002; Gu and Brothman, 2011). Results were compiled and were then compared to the chromosomal locations of dysregulated miRNAs in PCa.

miRNA clusters were defined as miRNAs, which lie within 10 000 nucleotides (inter-miRNA distance) from one another. The distance between miRNA and their chromosomal locations were determined using the latest release (Release 17) of miRBase and the UCSC's Genome Browser and Track feature.

Sequence homology and multiple alignment tools were performed using miROrtho (Faculty of Medicine, University of Geneva), which contains predictions of miRNA genes covering several animal genomes. Built into miROrtho is a T-COFFEE (version 6.92) output for the multiple sequence alignment. High conservation was defined as sequences having an alignment reliability core index >80.

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