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MicroRNAs and their Potential for Translation in Prostate Cancer

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Abstract

Objective—Patients die of prostate cancer (CaP) because predictably after a period of response to androgen withdrawal, their CaP becomes castrate resistant. In this paper we discuss the role that microRNA's (miRNAs) may play in this process.

Methods—miRNAs are a group of endogenous, small non-coding RNA molecules that are thought to be responsible for the regulation of up to 30% of gene expression. The miRNA expression profile between androgen responsive and castrate resistance CaP cell lines is compared. Functional studies were carried out to identify the importance of the microRNA's targets in controlling this process.

Results—There were 17 differentially expressed miRNAs found, 10 up-regulated and 7 down-regulated. Among these, miRNA-126b was found to have the ability of rendering LNCaP cells resistant to androgen withdrawal. It was found to be androgen regulated and one of its targets, BAK1, was identified as being involved in how these CaP cells undergo apoptosis functionally.

Conclusion—miRNA-125b, at least in the CaP cell lines tested, is involved in the development of castrate resistance. While clearly, this miRNA is only part of the answer, miRNA's may lead us in a new direction in trying to solve the central problem in CaP.

Keywords

MicroRNA; Prostate Cancer; Androgen-Independent

Introduction

MicroRNAs (miRNAs) are a group of endogenous, small, non-coding RNA molecules that are thought to be responsible for the regulation of up to 30% of gene expression [1]. Recent investigations have revealed that miRNAs have unique expression profiles in different cancer types at different stages and play an important role in the initiation and progression of many diseases. These features make miRNAs ideal candidates for use as both biomarkers and therapeutic targets. MiRNAs are initially transcribed in the nucleus as long primary miRNAs (pri-miRNAs) that are then processed to produce hairpin-like miRNA precursors (pre-miRNAs) that are ~70 nt in length. The pre-miRNAs are transported to the cytoplasm where they are further processed into mature miRNAs with a final length of ~22 bases [2,3]. A

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multitude of publications have demonstrated that miRNAs regulate gene expression. Mature miRNAs bind to their target mRNAs by complete or incomplete complementation of their 5'-end nucleotides 1-8 (seed sequences) with a binding site in the 3'- or 5'-untranslated regions (UTRs) of target transcripts. This process results in direct cleavage of the targeted mRNAs or inhibition of translation [4,5]. Currently, nearly 500 human miRNAs have been identified. It is postulated that up to ~1000 miRNAs exist [6]. Bioinformatic analyses have predicted that each miRNA recognizes on average about 100 different mRNA targets [6-8] meaning that each miRNA has the potential to mediate the regulation of a great number of protein-coding genes. Unsurprisingly studies have shown that miRNAs play a role in almost all biological processes in mammals, including cell proliferation, differentiation, stress response, apoptosis, immunity, and transcriptional regulation [9].

Over the last few years, miRNA-mediated post-transcriptional or translational regulation of protein-coding genes has emerged as a promising area of research. MiRNA can mediate rapid changes in protein synthesis without the need for transcriptional activation and subsequent mRNA processing steps [10]. This regulation provides the cell with a more precise, immediate, and energy-efficient mechanism for controlling the expression of proteins.

miRNA's and human cancer

When human miRNAs were discovered, it was noticed that many miRNAs are located in cancer-associated genomic regions that are commonly amplified or deleted in human cancer [11,12], suggesting a linkage between miRNAs and carcinogenesis. Indeed, later studies verified that aberrant expression of some miRNAs occurs in many different types of human cancer [13,14]. The aberrant expression of several miRNAs has recently been reported to promote cell survival and tumor growth [15-19]. These observations strongly suggest that miRNAs play a critical role in the pathogenesis of cancer. In cancer cells, many factors affect the cellular abundance of miRNAs, including miRNA gene copy gain or loss [20], mutation of precursor miRNAs [21], hypermethylation and histone deacetylation of the miRNA promoter [22,23], and aberrant miRNA processing [24]. In the past years, differential expression of miRNAs has been reported in benign and malignant tissues, in different types of cancer, and in different stages of disease progression [25-27]. Both increased and decreased expression of miRNA's can contribute to cancer pathogenesis. In the case of "tumor suppressor" miRNAs, their downregulation would result in increased expression of oncogene or proliferation-related genes. An example is *miR-15/16* that acts as a tumor suppressor by targeting translation of *BCL-2* mRNA, but is diminished in >50% of cases of chronic lymphocytic leukemia (CLL) [28].

miRNA in Prostate Cancer—Thus it is reasonable to ask if studies of miRNAs can help in our understanding and treatment of prostate cancer (CaP). Aberrant expression of several miRNAs has been found in CaP cell lines and in CaP clinical specimens. The table below is constructed as a summary of four recent papers that analyzed miRNA expression comparing in each case normal prostate versus prostate cancer tissue [29-32].

One of the most promising aspects of analyzing miRNAs in patient specimens is that the miRNAs are extremely stable. It is concerning that there is so little agreement on the miRNAs found in each of these studies. This limitation has been noted previously and was indeed addressed in the above papers. The discussion of the technical issues that may have led to these differences is beyond the scope of this paper. However, it does need to be recognized and addressed otherwise a great deal of time, effort and funds may be spent on chasing false leads.

Having found the aberrantly expressed miRNAs, it is then necessary to find the CaP-related targets of these miRNAs. Considering that a given miRNA may have up to 100 mRNA targets, the issue is how to decide which of these targets may be biologically active and important.

Without a bioinformatics method to do this, again a tremendous amount of time may be spent on false leads.

With the above issues in mind, we decided to study if miRNAs might be differentially expressed in androgen dependent (AD) and androgen independent (AI) CaP cell lines.

Differential expression of miRNAs in androgen dependent (AD) and androgen independent (AI) prostate cancer (CaP) cells

In order to explore the role of miRNAs in CaP, we investigated the differential expression of 132 miRNAs in AD LNCaP cells and the related AI subline LNCaP-cds1 (named cds1 [33]) using miRNA microarray. Using a 3-fold difference as cut-off, 17 differentially-expressed miRNAs were identified, 10 being upregulated and 7 downregulated in cds1 cells related to LNCaP cells (Table 1). Northern blot analysis of five miRNAs (red in Tab. 1) validated the microarray results (Fig. 1A). As shown in Figure 1B, it was found that: 1) when compared to two benign prostate lines, most CaP lines had increased expression of *miR-125b*; 2) Five androgen receptor (AR)-positive cell lines (CWR22R, PC-346C, LNCaP, cds1 and cds2) expressed more *miR-125b* than four AR-negative lines (DU145, PC3, pRNS-1-1 and RWPE-1); 3) When compared to the AD/S parental LNCaP cells, the AI cds1 and cds2 cells expressed 5-fold increased levels of *miR-125b*. These findings suggest that *miR-125b* may be related to prostatic tumorigenesis and AI growth, and the AR may regulate the expression of this miRNA in CaP cells.

As we have previously published [34], we showed that transfection of miRNA-125b into LNCaP cells allows them to grow in an androgen depleted media.

- Antisensing miRNA-125b in cds cells in which it is elevated decreased cell growth.
- miR-125b was expressed in CaP clinical specimens. The above data suggests that this miRNA could have an oncogenic role in CaP.
- The next question was what were the targets of miR-125b. Database searches showed that the most likely target to have biological activity was BAK1 [35,36].
- By western blot analysis we showed that miR-125b reduced BAK1 expression while antisensing miRNA-125b upregulated BAK1.
- In Northern Blotting, both LNCaP [low miRNA-125b] and cds1 [high miRNA-125b], showed that androgen up-regulated miRNA-125b.
- By CHIP analysis, we showed that AR loaded to the 5' prime DNA region of miRNA-125b.
- In keeping with AR up-regulating miRNA-125b, we showed that Casodex repressed expression of miRNA-125b.
- When we blocked BAK1, we did not get the same growth reduction in cds1 cells as had been achieved by blocking miRNA-125b. This was not unexpected and showed other targets of miRNA-125b were helping to sustain the AI growth of our CaP cells.

We have also developed a bioinformatics strategy to determine the most likely targets of *miR-125b* that would be biologically relevant in sustaining the AI growth of our CaP cells. This approach essentially enabled us to extract the predicted *miR-125b* target genes from AI-specific gene expression profiles and subsequently define their associations within signal transduction networks and with biological processes. It was readily apparent that a prominent action of *miR-125b* might be to decrease apoptosis susceptibility through disruption of the mitochondrial apoptosis pathway, specifically via suppression of pro-apoptotic Bcl-2 family proteins which have distinct functions. In addition to *BAK1* (described above), the

bioinformatics search identified *PUMA/BBC3* as another potential *miR-125b* target. Bak1 is pro-apoptotic, but is usually kept inactive by binding to Bcl-2. Puma's pro-apoptotic activity is attributed to its ability to bind and displace Bcl-2 from Bak1, thereby rendering the latter active. While validation studies are ongoing, they certainly imply that blocking *miRNA-125b* would be more effective, and possibly more feasible, than attempting to reactivate just one of its targets (e.g., *BAK1*). This also points to the complexity of miRNA actions in cells. We are hopeful that the identification of further miRNAs and subsequent miRNA-based therapies (blocking or restoration) will have real translational use in managing CaP.

Detection of circulating microRNAs—Cancers effect the levels of microRNAs in the blood stream where they are very stable in both plasma and serum (ref 37 and 38) which has led to the idea of utilizing them as blood based biomarker for cancer detection. Mitchell et al, recently reported that there is a 6.35 fold increase in *miR-125b* in the sera of patients with metastatic CaP compared to sera from healthy men (37). In the case of *miR-141* the differential expression was 46 fold. This study involved 25 patients with metastatic disease. The authors reported a 60% sensitivity and 100% specificity utilizing *miR-141*. Clearly, a 65% sensitivity in patients with metastatic CaP is unsatisfactory in terms of using this as a potential marker. However, no information is given in this article regarding the treatment status of the patients, or if on therapy, whether they were responding. These results should not deter us from persuing further investigation of the potential use of circulating microRNA's as a biomarker in CaP. This could be persued by comparing the positivity of microRNA's in the patient's tumor and serum. In patients who have blood available prior to and after a radical retropubic prostatectomy, comparisons could be made between the level of microRNA in the prostate specimen as compared to levels found in the pre and post surgical serum samples. Similarly, serum from patients with metastatic disease could be tested prior to and after the initiation of therapy. In such a manner, could the role of microRNAs as biomarkers in CaP be determined.

In conclusion, the exact role that microRNAs play in the pathogenesis and particularly in the conversion of androgen independent CaP to castrate resistance prostate cancer remains to be determined. However, results in other cancers and the early results in CaP are encouraging. Clearly, there is reason to hope that understanding the roles that microRNAs play in this process may lead to the development of new biomarkers and new therapies for this disease.

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Summary of miRNA array data from studies of normal prostate versus prostate cancer tissue

Study	# CaP/ Normal	RNA isolation method	miRNA array platform	miRNA array data analysis	Common differentially expressed miRNA (shared in 2 of 3 data sets)	Validated differentially expressed miRNA	Target ID prediction method	Validated miRNA targets
Amb's et al., 2008 + [29]	60/16*	TRIzol	OSU-CCC_hsa-miRNA-chip_v3	RMA, significance analysis of microarrays (SAM)	miR-26a-1 miR-370 let-7i miR-92-1/2 miR-125a miR-218-2 miR-128a miR-221 miR-145 miR-34a let-7b	miR-106b miR-32 miR-1	TargetScan, Whole Pathway Scope database	miR-106b=E2F1 miR-106b=p21 miR-32=Bim miR-1=PTK9
Ozen et al., 2008 [30]	16/10**	TRIzol, FlashPAGE Fractionator	miRvana miRNA bioarrays	Normalization, 2- sided t-test on log transformed array intensity values	-	miR-125b, miR-145 let-7c	miRanda, TargetScan, PicTar	miR-125b=RAS, miR-125b=E2F3 miR-125b=BCL-2 miR-125b=MCL-1
Porkka et al, 2007 + [31]	9/4***	TRIzol, Microcon YM-100	Array made in house.	Normalization, Mann-Whitney U Test	let-7b let-7d miR-125a miR-145 miR-195 miR-221 miR-370	-	-	-
Volinia et al., 2006 [32]	56/7****	TRIzol	Array made in house.	Normalization, significance analysis of microarrays (SAM)	let-7d miR-128a miR-34a miR-195 miR-218-2 let-7i miR-92-2 miR-26a-1	-	-	-

* 'normal' = tissue taken from patients with prostate cancer, non-cancerous areas selected.

** 'normal' = tissue taken from benign peripheral zone tissues of patients with prostate cancer.

*** 'normal' = 4 benign prostatic hyperplasias (BPH), (CaP = 5 untreated prostate carcinomas, and 4 hormone-refractory prostate cancers).

**** source not stated.

+ array data submitted to ArrayExpress database.

^ shared between 2 out of 3 data sets (Amb's, Ozen and Volini data sets, complete list of differentially expressed miRNA not available for Ozen).