Genomic Profiling of MicroRNA and Messenger RNA Reveals Deregulated MicroRNA Expression in Prostate Cancer

Stefan Ambs,[1] Robyn L. Prueitt,[1] Ming Yi,[2] Robert S. Hudson,[1] Tiffany M. Howe,[1] Fabio Petrocca,[3] Tiffany A. Wallace,[1] Chang-Gong Liu,[3] Stefano Volinia,[3] George A. Calin,[4] Harris G. Yfantis,[5] Robert M. Stephens,[2] and Carlo M. Croce[3]

[1]Laboratory of Human Carcinogenesis, Center for Cancer Research, National Cancer Institute, NIH, Bethesda, Maryland; [2]Advanced Biomedical Computing Center, National Cancer Institute-Frederick/Science Applications International Corporation-Frederick, Inc., Frederick, Maryland; [3]Department of Molecular Virology, Immunology, and Medical Genetics, Comprehensive Cancer Center, Ohio State University, Columbus, Ohio; [4]Experimental Therapeutics Department, MD Anderson Cancer Center, Houston, Texas; and [5]Pathology and Laboratory Medicine, Baltimore Veterans Affairs Medical Center, Baltimore, Maryland

Abstract

MicroRNAs are small noncoding RNAs that regulate the expression of protein-coding genes. To evaluate the involvement of microRNAs in prostate cancer, we determined genome-wide expression of microRNAs and mRNAs in 60 primary prostate tumors and 16 nontumor prostate tissues. The mRNA analysis revealed that key components of microRNA processing and several microRNA host genes, e.g., *MCM7* and *C9orf5*, were significantly up-regulated in prostate tumors. Consistent with these findings, tumors expressed the *miR-106b-25* cluster, which maps to intron 13 of *MCM7*, and *miR-32*, which maps to intron 14 of *C9orf5*, at significantly higher levels than nontumor prostate. The expression levels of other microRNAs, including a number of *miR-106b-25* cluster homologues, were also altered in prostate tumors. Additional differences in microRNA abundance were found between organ-confined tumors and those with extraprostatic disease extension. Lastly, we found evidence that some microRNAs are androgen-regulated and that tumor microRNAs influence transcript abundance of protein-coding target genes in the cancerous prostate. In cell culture, E2F1 and p21/WAF1 were identified as targets of *miR-106b*, Bim of *miR-32*, and exportin-6 and protein tyrosine kinase 9 of *miR-1*. In summary, microRNA expression becomes altered with the development and progression of prostate cancer. Some of these microRNAs regulate the expression of cancer-related genes in prostate cancer cells. [Cancer Res 2008;68(15):6162–70]

Introduction

Recently, a new class of small RNAs has been described, termed microRNAs, which was found to regulate mRNA function by modulating both mRNA stability and the translation of mRNA into protein (1, 2). MicroRNA genes are expressed as large precursor RNAs, called pri-mRNAs, which may encode multiple microRNAs in a polycistronic arrangement (3). These precursors are converted into a mature microRNA of 19 to 25 nucleotides by the nuclear RNase III enzyme, Drosha, and the cytosolic RNase III enzyme, Dicer. These two enzymes and their cofactors, e.g., DGCR8/Pasha, TRBP, and EIF2C2/argonaute-2, are key components of microRNA processing. Changes to their expression levels can alter cell function and induce cellular transformation (4).

A crucial role of microRNAs in cancer has been shown (5). Their expression is commonly altered in solid human tumors (6). MicroRNA expression profiles also classify tumors by developmental lineage and differentiation state (6, 7). Multiple microRNAs have been shown to have oncogenic properties or act like tumor suppressor genes (5, 8). These microRNAs have been termed oncomiRs. An alteration in their expression is causatively linked to cancer development.

We investigated the microRNA profiles of 60 prostate tumors and 16 nontumor tissues to evaluate the relationship between microRNA expression and prostate cancer. We also studied the global expression of mRNAs. That approach was used to find alterations in the expression of genes that regulate prostatic microRNA processing and to identify candidate mRNAs that are posttranscriptionally repressed by microRNAs in the prostate. Lastly, we studied the influence of an androgen on microRNA transcript abundance and used precursor and antisense microRNAs and luciferase reporter constructs to show that microRNAs regulate the expression of cancer-related genes in human prostate cancer cells.

Materials and Methods

Clinical samples. Sixty fresh-frozen prostate tumors (macrodissected) and patient's clinicopathologic information were received from National Cancer Institute (NCI) Cooperative Prostate Cancer Tissue Resource (CPCTR) and University of Maryland. Written informed consent was obtained from all donors. The tumors had not received any therapy before prostatectomy. Surrounding nontumor prostate tissue was collected from 16 patients with prostate cancer.

RNA isolation, expression analysis by microarray, and quantitative real-time PCR. See detailed information in supplementary materials.

Regulation of protein expression by microRNAs. LNCaP and PC3 human prostate cancer cells (American Type Culture Collection) were grown to 50% confluency and transfected with either microRNA precursor or antisense microRNA inhibitor (Ambion) at 100 nmol/L final concentration using Lipofectamine 2000 reagent (Invitrogen). After 48 h, cells were harvested by scraping and protein was extracted with radioimmunoprecipitation assay buffer (Pierce Biotechnology). The following primary antibodies were used to visualize protein expression by Western blot analysis: polyclonal rabbit anti–exportin-6 antibody, 1:200 (ProteinTech Group; 11408-1-AP); monoclonal mouse anti-PTK9 antibody, 1:500 (Abnova Corp.; clone 1E2); monoclonal mouse anti-E2F1 antibody, 1:200 (Santa Cruz

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: Stefan Ambs, Laboratory of Human Carcinogenesis, National Cancer Institute, Building 37/Room 3050B, Bethesda, MD 20892-4258. Phone: 301-496-4668; Fax: 301-496-0497; E-mail: ambss@mail.nih.gov or Carlo Croce, Department of Molecular Virology, Immunology and Medical Genetics, Comprehensive Cancer Center, Ohio State University, Columbus, OH 43210. Phone: 614-292-3063; E-mail: carlo.croce@osumc.edu.

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Biotechnology); monoclonal mouse anti-p21/WAF1 antibody, 1:200 (Santa Cruz Biotechnology); and polyclonal rabbit anti-BIM antibody, 1:1,000 (Cell Signaling). A quantification of protein expression was obtained with the AIDA Biopackage, 2D-Densitometry (raytest Isotopenmessgeraete GmbH).

Luciferase assays of reporter constructs containing the 3′ untranslated region of *E2F1*, *BCL2L11*, and *CDKN1A*. The *E2F1*, *BCL2L11*, and *CDKN1A* 3′ untranslated regions (UTR) containing the predicted *miR-106b* and *miR-32* target sequence, respectively, were amplified from genomic DNA (293T cells) and cloned into the pGL3 firefly luciferase control vector (Promega) at the *Xba*I restriction site immediately downstream of the luciferase reporter gene. To generate *E2F1* and *BCL2L11* 3′UTRs with a mutant target sequences, a deletion of the first three nucleotides was inserted into the *miR-106b* and *miR-32* seed region complementary sites using the QuikChange-site-directed mutagenesis kit (Stratagene). Translational inhibition of the luciferase reporter gene by either *miR-106b* or *miR-32* was assayed in LNCaP prostate cancer cells. Briefly, 1.2×10^5 LNCaP cells per well were seeded in 24-well plates. The next day, cells were transfected with 500 ng of reporter plasmid, 2 ng *Renilla* reporter and either microRNA negative control or precursor microRNA at a 100 nmol/L final concentration using the Lipofectamine 2000 reagent according the manufacturer's instructions (Invitrogen). Transfections were performed in triplicates. After 24 h, cells were lysed according to a Promega standard protocol, and the relative luciferase activity was determined using a DYNEX Technologies MLX luminometer. Reporter activity was normalized to the protein concentration in the cell extracts.

Apoptosis assay. We used the Caspase-Glo^R apoptosis assay, as described by the manufacturer (Promega). For more details, see supplementary materials.

Regulation of microRNA expression by androgen. Experiments are described in supplementary materials.

Results

Up-regulation of Dicer in prostate tumors. Prostate tumors were collected from African-American and European-American patients with localized disease (Table 1). After isolation of total RNA from these tumors and from 16 nontumor tissues, the expression of ~13,000 protein-coding genes and 329 unique human microRNAs was determined with microarrays.

Initially, the gene expression profiles of these samples were searched for cancer-related alterations in the expression of those mRNAs that have been shown to regulate the processing of microRNAs, e.g., mRNAs that encode Drosha or Dicer, among others. Our analysis revealed that *Dicer* is significantly higher expressed in prostate tumors [1.6-fold; false discovery rate (FDR) < 1%] when compared with nontumor tissue. *DGCR8*, which encodes an essential cofactor for Drosha, was also up-regulated in tumors (1.2-fold; FDR < 1%). The increased expression of *Dicer* and *DGCR8* in tumors was confirmed by quantitative reverse transcription–PCR (qRT-PCR), which revealed a larger fold difference than indicated by the microarray (Supplementary Fig. S1). Further analysis showed that *Dicer* and *EIF2C2*, both components of the RISC complex, were more highly expressed in tumors with a high Gleason sum score (score, 7–9) than in tumors with a low Gleason sum score (score, 5 and 6). However, these expression differences were rather modest (*Dicer*, 1.2-fold; *EIF2C2*, 1.3-fold). Because a frequent coexpression of host genes and intronic microRNAs has been found in human cells (9), we also investigated the expression of microRNA host genes in prostate tumors. Among those, the

Table 1. Clinical characteristics of the study population

	All cases (n = 60)	African-American (n = 30)	European-American (n = 30)	P* (t test)
Age at prostatectomy				
Median (range) n = 60	60 (47–73)	61 (48–72)	60 (47–73)	0.91
PSA at diagnosis				
Median (range) n = 44 †	6.1 (1.3–47.7)	6.0 (1.3–47.7)	6.1 (4.0–20.0)	0.67
Largest individual nodule (g)				
Median (range) n = 51 †	1.6 (0.2–2.9)	1.5 (0.8–2.9)	1.6 (0.2–2.8)	0.78
	N (%)	N (%)	N (%)	Fisher's exact test
Source of tissue				
NCI CPCTR	52 (87)	27 (90)	25 (83)	
University of Maryland	8 (13)	3 (10)	5 (17)	0.71
Gleason sum score				
<7 (5–6)	15 (25)	8 (27)	7 (23)	
≥7 (7–9)	45 (75)	22 (73)	23 (77)	1.0
Extraprostatic extension †				
No	35 (67)	19 (70)	16 (64)	
Yes	17 (33)	8 (30)	9 (36)	0.77
Surgical margin status †				
Negative	30 (59)	17 (63)	13 (54)	
Positive	21 (41)	10 (37)	11 (46)	0.58
Seminal vesicle invasion †				
No	43 (83)	20 (74)	23 (92)	
Yes	9 (17)	7 (26)	2 (8)	0.14

*P value for difference between African-Americans and European-Americans.
† Cases with unknown status are not included.

Table 2. MicroRNAs differentially expressed between tumor and nontumor tissue

Up-regulated in tumors			Down-regulated in tumors		
Gene name	Fold change	Chromosomal location	Gene name	Fold change	Chromosomal location
miR-32	2.1	9q31.3	*miR-520h*	0.3	19q13.42
miR-182	1.9	7q32.2	*miR-494*	0.4	14q32.31
miR-31	1.8	9p21.3	*miR-490*	0.4	7q33
miR-26a-1/2	1.8	3p22.3/12q14.1	*miR-133a-1*	0.5	18q11.2
miR-200c	1.7	12p13.31	*miR-1-2*	0.6	18q11.2
miR-375	1.6	2q35	*miR-218-2*	0.6	5q34
miR-196a-1/2	1.6	17q21.32/12q13.13	*miR-220*	0.6	Xq25
miR-370	1.6	14q32.31	*miR-128a*	0.6	2q21.3
miR-425	1.6	3p21.31	*miR-221*	0.7	Xp11.3
miR-194-1/2	1.5	1q41/11q13.1	*miR-499*	0.7	20q11.22
miR-181a-1/2	1.5	1q31.3/9q33.3	*miR-329*	0.7	14q32.31
miR-34b	1.5	11q23.1	*miR-340*	0.7	5q35.3
let-7i	1.5	12q14.1	*miR-345*	0.7	14q32.2
miR-188	1.4	Xp11.22	*miR-410*	0.7	14q32.31
miR-25	1.4	7q21.11	*miR-126*	0.7	9q34.3
miR-106b	1.4	7q21.11	*miR-205*	0.8	1q32.2
miR-449	1.4	5q11.2	*miR-7-1/2*	0.8	9q21.33/15q26.1
miR-99b	1.4	19q13.41	*miR-145*	0.8	5q32
miR-93	1.3	7q21.11	*miR-34a*	0.8	1p36.22
miR-92-1/2	1.3	13q31.3/Xq26.2	*miR-487*	0.8	14q32.31
miR-125a	1.3	19q13.41	*let-7b*	0.8	22q13.31

NOTE: FDR < 5% and P (t-test) < 0.01 for all microRNAs. Fold change, reference is nontumor tissue.

expression of five was found to be altered in prostate cancer (all FDR < 1%). Of those, *C9orf5* (2.1-fold up-regulation), which is the host for *miR-32*, and *MCM7* (1.7-fold up-regulation), which is the host for the *miR-106b-25* cluster (*miR-106b/miR-25/miR-93*), were most highly overexpressed in tumors. *NFYC* (host of *miR-30c-1*), *SMC4L1* (host of *miR-15b* and *miR-16-2*), and *PTPRN2* (host of *miR-153-2*) showed a more moderate 30% to 40% increased expression in tumors when compared with nontumor tissue.

MicroRNA gene signature of prostate cancer. We first searched for the microRNAs that showed differential expression between tumor and nontumor tissue. As shown in Table 2, the expression of multiple microRNAs was altered in prostate tumors. Among the microRNAs with lower transcript levels in tumors than nontumor tissues, *miR-520h, miR-494*, and *miR-490* were most highly decreased. Two other notable microRNAs in this list were *miR-1(-2)* and *miR-133a(-1)*. These two microRNAs are encoded by the same pri-mRNA. *miR-32* was the most significantly up-regulated tumor microRNA, followed by *miR-182, miR-31*, and *miR-26a*. The list of more highly expressed tumor microRNAs also contained all members of the *miR-106b-25* cluster (*miR-106b/miR-93/miR-25*) and two members of the *miR-99b* cluster, *miR-99b* and *miR-125a*. The up-regulation of both *miR-32* and the *miR-106b-25* cluster is consistent with the increased expression of their respective host genes, *C9orf5* and *MCM7*, in prostate tumors. Statistical analysis of the microarray data confirmed that tissue transcript levels of *C9orf5* and *miR-32* are statistically significantly correlated (P = 0.0003). The Pearson coefficient indicated that this correlation was moderately strong across all samples [0.39; 95% confidence interval (95% CI), 0.18–0.57; n = 76]. Similar data were obtained for the correlation between *MCM7* and *miR-106b-25* cluster transcript levels [*miR-106b*, 0.37 (Pearson coefficient), P = 0.001; *miR-93*, 0.35, P = 0.002; *miR-25*, 0.23, P = 0.04].

We corroborated the microarray data by qRT-PCR analysis of selected microRNAs in a random subset of the tumor and

Table 3. MicroRNAs associated with extraprostatic disease

Gene name	Fold change*	FDR	Chromosomal location
miR-101-1/2	1.6	<1%	1p31.3/9p24.1
miR-200a	1.6	10–15%	1p36.33
miR-200b	1.6	10–15%	1p36.33
miR-196a-1/2	1.3	10–15%	17q21.32/12q13.13
miR-30c-1/2	1.3	10–15%	1p34.2/6q13
miR-484	1.3	10–15%	16p13.11
miR-99b	1.3	10–15%	19q13.41
miR-186	1.3	10–15%	1p31.1
miR-195	1.3	10–15%	17p13.1
let-7f-2	1.3	10–15%	Xp11.22
miR-34c	1.2	10–15%	11q23.1
miR-371	0.7	15–20%	19q13.42
miR-373	0.7	10–15%	19q13.42
miR-410	0.7	15–20%	14q32.31
miR-491	0.7	15–20%	9p21.3

NOTE: P (t-test) < 0.01 for all microRNAs.
*Comparing tumors with and without (reference) extraprostatic extension.

nontumor tissues. Consistent with the microarrays, we found that mature *miR-32* (average, 3.2-fold) and *miR-106b* (average, 3.0-fold) were higher expressed in tumors than nontumor tissues (Supplementary Fig. S2). We also found that mature *miR-1* was down-regulated (average, 0.44-fold), and *miR-106a*, a *miR-106b* homologue, was overexpressed (average, 3.7-fold) in the tumors when compared with nontumor tissues (Supplementary Fig. S2).

Lastly, we performed a paired analysis of the microarray data for those 10 tumors in our study whose surrounding nontumor tissue was available. The paired analysis corroborated our previous findings. At an FDR of <10%, *miR-26a, miR-30c-1, miR-32, miR-146b, miR-181a, miR-182, miR-196a, miR-200c, miR-375*, and all microRNAs of the *miR-106b-25* cluster were found to be up-regulated in tumors (1.5-fold to 2.5-fold). The most significantly down-regulated tumor microRNAs were *miR-494* (0.4-fold) and *miR-126* (0.6-fold). However, the *miR-1-133a* cluster was not found to be significantly differently expressed in this tumor subset.

Association of microRNAs with extraprostatic extension. We next analyzed our dataset for differences in microRNA expression associated with extraprostatic extension of the tumors. At a FDR < 20%, we found 15 microRNAs with a difference in expression between tumors that showed an extraprostatic extension of the disease (n = 17) and those that did not (n = 35; Table 3). *miR-101* was the most consistently overexpressed microRNA in localized prostate tumors that spread out of the prostate gland (FDR < 1%). Extraprostatic extension shared a portion of its microRNA signature with the tumor signature. Two microRNAs, *miR-99b* and *miR-196a*, are common to both signatures. Two other microRNAs of the extraprostatic extension signature, *miR-200a* and *miR-200b*, have an extensive homology with *miR-200c* in the tumor signature. We could not identify a robust microRNA signature associated with Gleason score in our dataset.

Because the tumors in our study were collected from African-American and European-American patients that were well matched on clinicopathologic variables (Table 1), we compared the tumor microRNA signatures between African-Americans (n = 30) and European-Americans (n = 30). Few microRNAs were differently expressed (P < 0.01). At an FDR of <20%, *miR-129, miR-196b*, and *miR-342* were found to be less abundant (20–30% lower) in tumors of African-Americans than in tumors of European-Americans. From this analysis, it does not seem that tumor microRNAs are very differently expressed by race/ethnicity.

Relationship between transcript abundance of microRNAs and their target mRNAs in prostate tissue. MicroRNAs regulate the expression of protein-coding genes by target-specific translational inhibition. However, it has recently been shown that some microRNAs, e.g., *miR-1*, can down-regulate the transcript levels of a large number of target genes in mammalian cells (1, 2). Because *miR-1* was among the down-regulated microRNAs in prostate tumors, we performed a correlation analysis between *miR-1* expression levels and the expression levels of predicted *miR-1* target genes in these tumors. This test was performed to identify candidate *miR-1* target genes that become overexpressed in prostate tumors because of diminished *miR-1* expression. The analysis yielded putative target mRNAs that were found to be up-regulated in prostate tumors (FDR < 1%) and inversely correlated with *miR-1* expression (Supplementary Table S1). Among those, transcripts for *WDR6, XPO6*, and *SMARCA4* showed the most significant inverse correlation with tumor *miR-1* expression (each $P < 1 \times 10^{-10}$). The relationship between XPO6 and *miR-1* transcript levels in prostate tumors is shown in Supplementary Fig. S3. We also found that XPO6 protein levels in the tumors are inversely correlated with *miR-1* (−0.29, Spearman correlation coefficient; n = 8). However, not all predicted targets of *miR-1* showed an inverse relationship with *miR-1* transcript levels in the tumors. For example, *TWF1* (also termed *PTK9*) was positively correlated with *miR-1*, suggesting that binding of microRNAs to its target sequence may sometimes lead to mRNA sequestration and cellular accumulation of the inhibited mRNA (10).

Our analyses were extended to other microRNAs that were either up-regulated or down-regulated in prostate tumors. Here, we initially determined the global distribution of the Pearson correlation coefficients between the microRNA of interest and either all mRNAs that are probed by the HG-U133A 2.0 array or only those mRNAs that are predicted targets of the microRNA. For two microRNAs, *miR-106b* and *miR-181a*, the distribution of the correlation coefficients was notably different between all mRNA and those mRNA that are the predicted targets of *miR-106b* and *miR-181a* (Fig. 1). The distribution curves for predicted target mRNAs of *miR-106b* and *miR-181a* showed a distinct shoulder that extended toward negative Pearson correlation coefficients. This

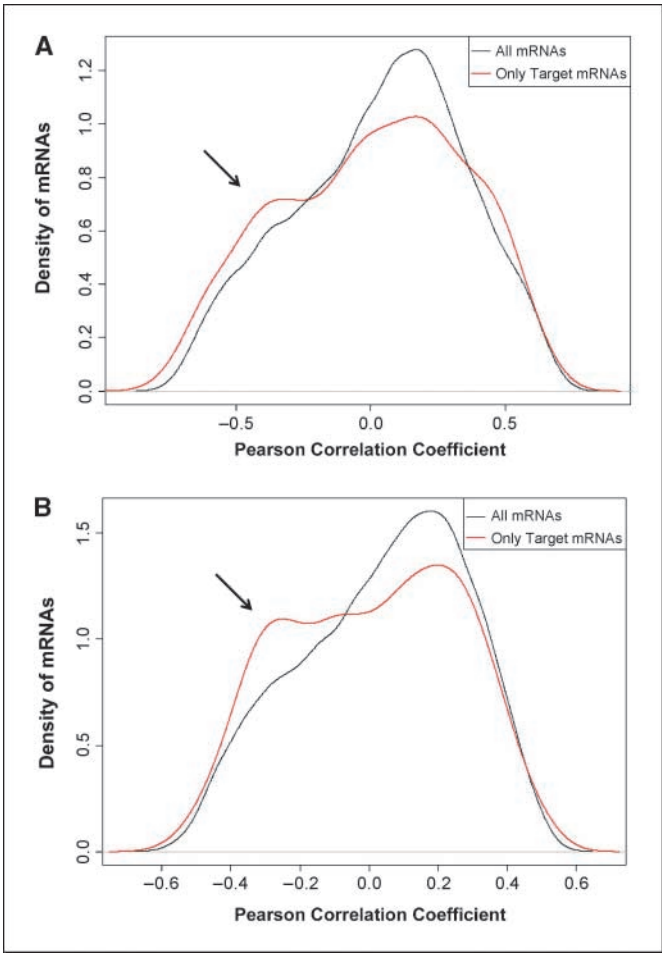

Figure 1. Analysis of the relationship between transcript levels of microRNAs and their respective target mRNAs in prostate tissue. Global distribution of the Pearson correlation coefficients between mRNAs and *miR-106b* (*A*) or *miR-181a* (*B*). The black-lined curves show the distribution of the correlation coefficients for all mRNAs. The red-lined curves show the correlation coefficient distribution for only those mRNAs that are predicted target of either *miR-106b* or *miR-181a*. The red-lined curves have an additional shoulder (*arrow*) indicating an enrichment of target mRNAs, whose transcript levels are negatively correlated with the transcript levels of the microRNA.

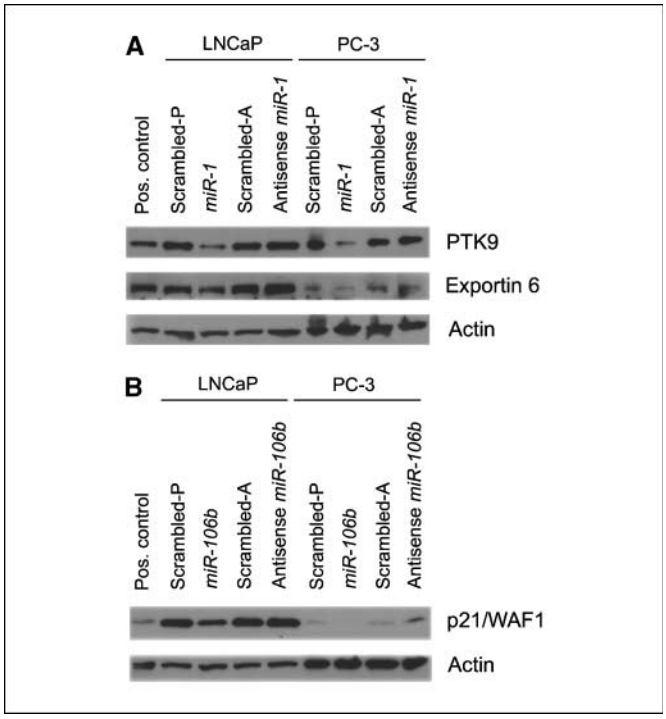

Figure 2. Inhibition of protein expression by *miR-1* (*A*) and *miR-106b* (*B*). LNCaP and PC-3 human prostate cancer cells were transfected with either microRNA precursor (*miR-1* and *miR-106b*) or antisense microRNA (antisense *miR-1* and antisense *miR-106b*), or their respective vector controls, scrambled precursor microRNA (*Scrambled-P*), and scrambled antisense microRNA (*Scrambled-A*). Protein extracts were prepared 48 h after transfection, and protein expression was examined by Western blot analysis. Loading, 50 μg protein/lane.

pattern is a departure from a normal distribution and indicates that tissue transcript levels of a subset of mRNAs, which have a predicted microRNA target sequence in the 3′ UTR, are reduced by *miR-106b* and *miR-181a*. A list of target genes that were significantly down-regulated in tumors (FDR < 1%) and whose transcript level inversely correlated with *miR-181a* expression is shown in Supplementary Table S2. A comparison of these target genes with a list of genes that correlated with *miR-181a* transcript levels in leukemia samples (11) showed that several, e.g., *SLC9A6, RIN2, KLHL2*, and *GHITM*, were negatively correlated with *miR-181a* in both lists.

Inhibition of protein expression by candidate oncomiRs in prostate cancer cells. Our results from the tumor studies suggest that *miR-1, miR-32*, and the *mir-106b-25* cluster are oncomiRs in prostate cancer. *miR-32* and *miR-25* share a high degree of homology, and their predicted target genes are the same.[6] Moreover, the *mir-106b-25* cluster is highly homologous to a known oncomiR, the *miR-17-92* cluster (12), and the predicted targets of *miR-17-5p* and *miR-106b* are identical. A target of the *miR-17-92* cluster is E2F1 (12).

We transfected two human prostate cancer cell lines, LNCaP and PC-3, with precursor and antisense microRNAs to examine whether *miR-1, miR-32*, and *miR-106b* regulate the protein expression of cancer-related genes in these cells. The endogenous expression of these microRNAs in the cell lines was *miR-106b* > *miR-32* > *miR-1* by qRT-PCR. *miR-1* expression was at the detection limit. For *miR-1*, we tested whether the relationship between transcript abundance of microRNAs and their target mRNAs in tumor tissue is useful to identify microRNA targets and examined whether the protein expression of exportin-6 (*XPO6*) and protein tyrosine kinase 9 (*TWF1*) is regulated by *miR-1*. Transfection of the prostate cancer cells with *miR-1* confirmed that it represses both exportin-6 and protein tyrosine kinase 9 on the protein level in both prostate cancer cell lines (Fig. 2*A*). Neither *miR-32* nor *mir-106b* altered the expression of these proteins (data not shown).

We next investigated the regulation of E2F1 and p21/WAF1 protein levels by *miR-106b*. Both proteins are encoded by mRNAs that have a predicted target sequence of *miR-106b* in their 3′ UTRs. Whereas E2F1 did not correlate with *miR-106b* on the transcript level in prostate tumors, a significant inverse correlation existed between the expression of *CDKN1A* (encodes p21/WAF1) and *miR-106b* in these tumors (−0.34; 95% CI, −0.09 to −0.55; $P = 0.003$). As shown in Fig. 2*B*, transfected precursor *miR-106b* decreased p21/WAF1 protein levels and antisense *miR-106b* increased p21/WAF1 protein levels in the two cell lines. We obtained the same results for E2F1 after transfection of the prostate cancer cells with precursor and antisense *miR-106b* (Fig. 3*A*). Next, we studied the effect of *miR-32* on Bim protein expression. Bim is encoded by *BCL2L11* and a predicted target of *miR-32. BCL2L11*, and *miR-32* transcript levels did not correlate in the tissue samples suggesting that *miR-32* may regulate this target mostly by translation inhibition. Transfection of prostate cancer cells with precursor *miR-32* decreased Bim protein levels, whereas antisense *miR-32* increased Bim protein levels (Fig. 3*C*). The protein expression of E2F1 and p21/WAF1 was not influenced by *miR-32* nor was the protein expression of Bim influenced by *miR-106b* in these cell lines (data not shown).

E2F1 and Bim are direct targets of *miR-32* and *miR-106b*. To further corroborate our findings and provide evidence that these proteins are direct targets of *miR-32* and *miR-106b*, LNCaP cells were cotransfected with precursor microRNA and pGL3 luciferase reporter constructs containing either wild-type or mutant 3′ UTR of two genes, *E2F1* and *BCL2L11*, respectively. Mutant 3′ UTRs contained a deletion of the first three nucleotides in the *miR-106b* and *miR-32* seed region complementary sites. The 3′ UTRs were placed at a position that would lead to a translational inhibition of the luciferase reporter when the microRNA binds to the target sequence. As shown in Fig. 3*B* and *D*, cotransfection of either *miR-106b* with the reporter construct containing the wild-type 3′ UTRs of *E2F1* or *miR-32* with the reporter construct containing the wild-type 3′ UTRs of *BCL2L11* resulted in a significant inhibition of the luciferase reporters when compared with the precursor microRNA negative control. There was no inhibition of the reporter by the microRNAs in the absence of the 3′ UTR. The presence of a mutant 3′ UTR either abolished or attenuated the effect of the microRNAs. The results are consistent with a direct effect of the microRNAs on protein translation by binding to their 3′ UTR target sequence. Such a mechanism has also been established for the regulation of p21/WAF1 by *miR-106b* in human colon and gastric cancer cells (13, 14). Accordingly, we observed that *miR-106b* inhibits a luciferase reporter by a *CDKN1A* 3′ UTR-mediated mechanism in LNCaP cells (Supplementary Fig. S4).

Inhibition of caspase activation by the *miR-106b-25* cluster in 22Rv1 human prostate cancer cells. Our previous data indicated that *miR-32, miR-106b,* and their homologues (e.g., *miR-25*) may act as oncogenes, because they target the proapoptotic

[6] www.targetscan.org

function of Bim and E2F1. To evaluate the effect of the *miR-106b-25* cluster on apoptosis induced by doxorubicin and etoposide, we infected 22Rv1 cells, a nonmetastatic human prostate cancer cell line, with a lentiviral expression construct encoding the *miR-106b-25* cluster. Using the Caspase-Glo apoptosis assay, we observed a significant inhibition of caspase-3/caspase-7 activation by this cluster in anticancer drug-treated cells (Supplementary Fig. S5). The data are consistent with an antiapoptotic function of the *miR-106b-25* cluster in prostate cancer cells.

Identification of androgen-regulated microRNAs. Androgens play a key role in physiology and tumor biology of the prostate. We examined the regulation of microRNAs by androgens in DU145 and LNCaP cells. Treatment of the androgen-insensitive DU145 cells with R1881 did not yield any significant changes in microRNA expression. In contrast, expression of several microRNAs was significantly changed (FDR < 5%) in androgen-sensitive LNCaP cells after the R1881 treatment (Supplementary Table S3). One microRNA, *miR-338*, was significantly up-regulated. The other microRNAs were down-regulated, including *miR-126-5p, miR-146b, miR-219-5p*, and all members of the *miR181b-1, miR-181c*, and *miR-221* clusters. An analysis of the baseline microRNA expression in cultured DU145 and LNCaP cells showed that all members of the three microRNA clusters had a significantly higher expression in the androgen-insensitive DU145 cells than in the androgen-responsive LNCaP cells (FDR < 5%). Using a motif search in the Genomatix transcription factor binding site database, we found that the aforementioned microRNAs have putative androgen receptor binding sites in their flanking regions (Supplementary Table S4). We further corroborated the microarray results in experiments with LNCaP cells that were treated with either 1 or 10 nmol/L R1881 for 12, 24, and 48 hours. qRT-PCR analysis of mature *miR-338* and *miR-221* showed that their expression level is androgen-regulated (Supplementary Fig. S6).

Discussion

The present study revealed a distinct microRNA expression signature in prostate tumors and alterations in the expression of genes that regulate tumor microRNA processing. Furthermore, we found evidence that the deregulation of microRNAs influences transcript abundance and protein expression of target mRNAs in the prostate. The results are consistent with a pathogenic role of altered microRNA expression in human prostate carcinogenesis.

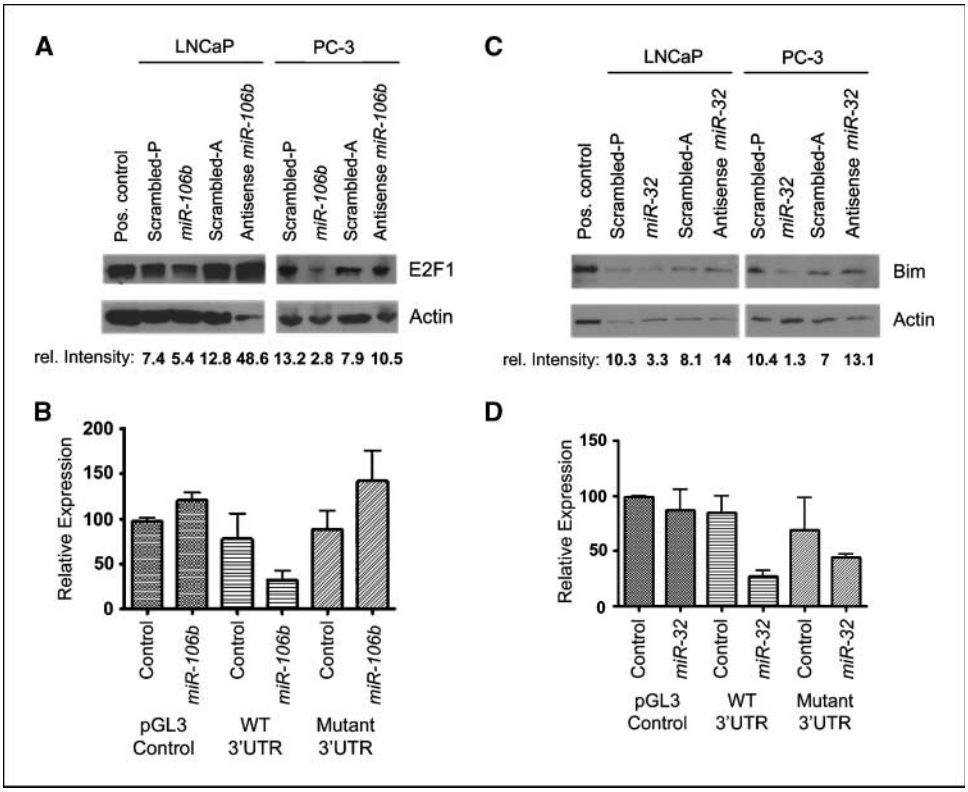

Figure 3. *miR-106b* and *miR-32* inhibit expression of E2F1 and Bim, respectively, by a 3′ UTR–mediated mechanism. *A* and *C,* LNCaP and PC-3 human prostate cancer cells were transfected with either microRNA precursor (*miR-106b* or *miR-32*) or antisense microRNA (antisense *miR-106b* or antisense *miR-32*), or their respective vector controls, scrambled precursor microRNA (*Scrambled-P*) and scrambled antisense microRNA (*Scrambled-A*). Protein extracts were prepared 48 h after transfection and protein expression was examined by Western blot analysis. To obtain relative intensity values, E2F1 and Bim expressions were normalized to β-actin. *B,* pGL3 luciferase reporter constructs containing either the wild-type or mutant 3′ UTR target sequence of *miR-106b* in the *E2F1* gene were cotransfected into LNCaP cells with either precursor microRNA-negative control or *miR-106b* precursor (each $n = 3$; mean ± SD). For comparison, cells were also transfected with the pGL3 control vector that did not contain the 3′ UTR. After 24 h, luciferase activity was determined in the cell extracts. In the presence of the wild-type *E2F1* 3′ UTR, transfection with precursor *miR-106b* lead to a significant inhibition of the luciferase reporter when compared with the vector control ($P = 0.045$; two-sided *t* test). This inhibition was not observed if the reporter construct contained a mutant 3′ UTR target sequence of *miR-106b. D,* pGL3 luciferase reporter constructs containing either the wild-type or mutant 3′ UTR target sequence of *miR-32* in the *BCL2L11* (Bim) gene were cotransfected into LNCaP cells with either precursor microRNA negative control or *miR-32* precursor (each $n = 3$). In the presence of the wild-type *BCL2L11* 3′ UTR, transfection with *miR-32* lead to a significant inhibition of the luciferase reporter when compared with the vector control ($P = 0.003$, two-sided *t* test). This inhibition was attenuated if the reporter construct contained a mutant 3′ UTR target sequence of *miR-32*.

This is the first study to use large-scale gene expression profiling of both microRNAs and protein-encoding RNAs to identify alterations in microRNA function that occur in human prostate tumors. We found an increased expression of *Dicer* and *DGCR8* in prostate tumors and of *Dicer* and *EIF2C2*, which encodes argonuate-2, in tumors with a high Gleason score. The observation that *Dicer* and other genes involved in microRNA processing are up-regulated in prostate cancer is consistent with a recent report (15) and may indicate that prostate tumors are more efficient than normal prostate tissue in processing microRNA precursors into mature microRNA. Nonetheless, Dicer has functions independent of microRNA processing, and future research is needed to address the relationship between Dicer expression and microRNA processing in prostate tumors.

MicroRNA expression profiles classify human cancers (6, 7). Distinct signatures for several epithelial cancers, including breast, lung, pancreatic, and gastric cancers, have been reported (14, 16–18). Other studies explored microRNA expression in prostate cancer (19, 20). Consistent with these studies, we observed that *miR-145* and *miR-221* are significantly down-regulated in prostate tumors. However, the previous studies were rather small and examined only few tumors when compared with our study. We identified a tumor gene signature that contained up-regulated and down-regulated microRNAs. The most highly up-regulated microRNA was *miR-32*, followed by *miR-182, miR-31, miR-26a, miR-200c*, and *miR-196a*. The list of overexpressed tumor microRNAs also contained the *miR-106b-25* cluster, which is consistent with the observed gain in copy number for *mir-25, mir-93*, and *mir-106b* in several human malignancies (21). The most significantly down-regulated microRNAs included *miR-520h, miR-494, miR-490*, and the *miR-1-133a* cluster.

Altered expression of microRNAs in human cancer has been observed in numerous studies. Up-regulation of microRNAs in tumors is common (5, 6, 8), and it is consistent with the known oncogenic activity of many microRNAs (22–25). Mechanisms of up-regulation include transcriptional activation and the increase in gene copy numbers. A decrease in the abundance of mature microRNA may result from altered processing, as shown recently (26), which would lead to an indiscriminate lower expression of mature microRNAs. We did not observe that in the present study. Alternatively, microRNA expression could be lost because of mutations or genomic alterations (21) or epigenetic silencing of microRNA loci (27, 28). Epigenetic silencing is an important mechanism in prostate cancer (29), and future studies will have to address whether this mechanism impedes microRNA expression in prostate tumors.

Little is known about the function of most of the deregulated tumor microRNAs that we identified. *miR-32* is a homologue of *miR-25, miR-92, miR-363*, and *miR-367*. Several of them were also up-regulated in the prostate tumors, suggesting a particular significance of this microRNA family in prostate cancer development. *miR-32* is increased in colon and pancreatic cancer (6) and is a mediator of the antiviral defense of human cells (30). It is this function of *miR-32* that could be the causal link between its altered expression and prostate cancer development because several of the known prostate cancer susceptibility genes are also involved in host defense (31). As shown for other microRNAs, *miR-32* should regulate protein expression of target genes. We made the novel observation that *miR-32* inhibits the expression of Bim, a proapoptotic member of the BCL-2 family. This result is consistent with the observation that the *miR-32* homologue, *miR-25*, suppresses Bim in gastric cancer cells (14). Bim has key roles in the apoptosis of epithelial tumors and mediates antitumor effects of chemotherapy (32). Thus, down-regulation of Bim by *miR-32* may contribute to the resistance of tumor cells to apoptotic stimuli in the tumor environment.

Other notable microRNAs with a known function include *miR-1, miR-133a*, and *mir-196a*. The *miR-1-133a* cluster has been shown to regulate cell differentiation (33). *miR-1* is a homologue of *miR-206*, which is a suppressor of metastasis in breast cancer (34). Our discovery that *miR-1* is down-regulated in prostate tumors is consistent with the tumor suppressor function of its homologue. We observed that expression of *miR-1* inhibits the expression of exportin-6 and protein tyrosine kinase 9 (also termed A6/twinfilin) in prostate cancer cells. Not much is known about the function of these two genes, but recent data suggest that both regulate cellular actin dynamics (35, 36). *miR-196a* was identified as a repressor of *HOXB8* (37), and elevated expression of *miR-196a* predicts poor survival in pancreatic cancer (18). This microRNA was common to both the tumor signature and the extraprostatic extension signature in our study, indicating that up-regulation of *miR-196a* in prostate cancer could be a factor in disease progression.

The analysis of the genomic location of microRNAs can provide clues about their putative function and the mechanisms that cause altered microRNA expression in tumors (38). Recent studies have shown that microRNAs are frequently located within introns of protein-coding genes and are coexpressed with these host genes (39). We investigated host gene expression in prostate tumors and found that several of them were increased in prostate tumors. *C9orf5* and *MCM7* were the two most highly up-regulated host genes, and their expression correlated with the expression of the intronic microRNAs, *miR-32* and the *miR-106b-25* cluster, respectively. The data suggest a common mechanism that leads to the up-regulation of the host gene and cotranscripted microRNA in prostate tumors.

Whereas the role of *C9orf5* in cancer is unknown, *MCM7* amplifications have previously been associated with prostate cancer. The *MCM7* locus was found to be amplified in 88% of cases with cancer relapse (40). *MCM7* overexpression is not restricted to prostate cancer and has been observed in other malignancies. Further studies will have to address if the *miR-106b-25* cluster is overexpressed in these and other cancers, and whether the oncogenic effect of *MCM7* locus amplification in human cancer is due to either *MCM7* or *miR-106b-25* cluster expression, or both. We examined whether *miR-106b* targets *E2F1* and *CDKN1A* in prostate cancer cells and found that protein expression of these genes is inhibited by *miR-106b*. The *miR-106b-25* cluster has extensive homologue with two other microRNA clusters that are candidate human oncogenes, the *miR-17-92* cluster and the *miR-106a-363* cluster (22, 24). *E2F1* is also a target of *miR-17-5p* and *miR-20a* in the *miR-17-92* cluster (12), and it has both oncogene and tumor suppressor functions (41). Like Bim, translated E2F1 can be proapoptotic and cooperates with the tumor suppressor p53 to mediate apoptosis (42). Its overexpression induces apoptosis in LNCaP cells (43), which indicates that inhibition of *E2F1* translation by *miR-106b* may protect prostate cancer cells from apoptosis in the tumor environment. p21/WAF1 is another mediator of p53-induced tumor suppression (44). The growth inhibitory effect of p21/WAF1 in prostate cancer has been shown (45), and it mediates cell cycle arrest in prostate carcinoma cells in response to anticancer agents (46, 47). We tested whether the *miR-106b-25* cluster has antiapoptotic activity and found that it

inhibits caspase activation by doxorubicin and etoposide in 22Rv1 cells. These data are consistent with an oncogenic function of the *miR-106b-25* cluster in prostate cancer, in part, because of its ability to suppress E2F1 and p21/WAF1 protein expression.

Neither *miR-1, miR-32*, nor the *miR-106b-25* cluster was regulated by androgen stimulation of LNCaP cells. However, we identified several other microRNAs that were up-regulated or down-regulated by androgen treatment. Those included *miR-338* and *miR-126* and the *miR-181b-1, miR-181c, miR-221* clusters, among others. A motif search showed that these microRNAs have putative androgen receptor binding sites in their flanking regions. *miR-338* was the only significantly up-regulated microRNA. There are no reports on the function of this microRNA, but it is located in a region with frequent copy number gains in three epithelial cancers (21). *miR-181* family members influence hematopoietic lineage differentiation (48), and their expression is altered in leukemia and several solid tumors (6, 11). The *miR-221* cluster has been found to regulate the $p27^{Kip1}$ tumor suppressor and may have oncogenic properties in prostate cancer (49). However, this cluster also inhibits the oncogene c-Kit and angiogenesis (50).

The identification of protein-coding genes that are regulated by a specific microRNA has been proved difficult despite the development of computational approaches to predict microRNA targets. The ability to find target mRNAs is further complicated by the fact that target selectivity of microRNAs may depend on the cellular microenvironment. We used an exploratory approach and conducted a correlation analysis between microRNA expression and mRNA expression in prostate tissue. This approach can be successful if the microRNA of interest affects transcript abundance of target mRNAs, but it will fail if the target genes are regulated only by translational inhibition. We found that the expression of *miR-1* is inversely correlated with a number of computationally predicted target genes in prostate tumors, e.g., *XPO6*. However, we also found that tumor *miR-1* expression correlated positively with the transcript level of predicted targets, e.g., *TWF1*. Subsequent validation of these observations in cell culture confirmed that *XPO6* and *TWF1* are both regulated by *miR-1* in prostate cancer cells. The data provide new evidence that binding of microRNAs to 3′ UTR sequences can lead to both degradation and accumulation of the targeted mRNA in mammalian cells and that both an inverse and a positive correlation between a microRNA and an mRNA in a human tissue can be predictive of a microRNA target gene. Thus, correlation analysis of microRNA and mRNA expression in human tissue may prove useful in identifying mRNAs that are regulated by microRNAs.

In conclusion, our study identified alterations in microRNA expression that occur in human prostate tumors and correlate with expression variations of protein-coding genes in these tissues. Experiments in cell culture showed that tumor microRNAs regulate the expression of cancer-related genes in human prostate cancer cells. These results indicate a pathogenic role of microRNAs in prostate cancer biology.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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