

# MicroRNA Expression Profiling in Prostate Cancer

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## Abstract

**MicroRNAs (miRNA) are small, endogenously expressed non-coding RNAs that negatively regulate expression of protein-coding genes at the translational level. Accumulating evidence, such as aberrant expression of miRNAs, suggests that they are involved in the development of cancer. They have been identified in various tumor types, showing that different sets of miRNAs are usually deregulated in different cancers. To identify the miRNA signature specific for prostate cancer, miRNA expression profiling of 6 prostate cancer cell lines, 9 prostate cancer xenografts samples, 4 benign prostatic hyperplasia (BPH), and 9 prostate carcinoma samples was carried out by using an oligonucleotide array hybridization method. Differential expression of 51 individual miRNAs between benign tumors and carcinoma tumors was detected, 37 of them showing down-regulation and 14 up-regulation in carcinoma samples, thus identifying those miRNAs that could be significant in prostate cancer development and/or growth. There was a significant trend ( $P = 0.029$ ) between the expression of miRNAs and miRNA locus copy number determined by array comparative genomic hybridization, indicating that genetic aberrations may target miRNAs. Hierarchical clustering of the tumor samples by their miRNA expression accurately separated the carcinomas from the BPH samples and also further classified the carcinoma tumors according to their androgen dependence (hormone naive versus hormone refractory), indicating the potential of miRNAs as a novel diagnostic and prognostic tool for prostate cancer.** [Cancer Res 2007;67(13):6130–5]

## Introduction

MicroRNAs (miRNA) are a class of small, noncoding RNAs that are endogenously expressed in animal and plant cells. They regulate the expression of protein-coding genes at the translational level, either by triggering degradation or preventing translation of the target mRNAs (1). Primary transcripts of miRNAs (pri-miRNA) are generated by RNA polymerase II (2), after which they are sequentially processed by RNase III class enzymes, Drosha and Dicer, to first produce ~70-nt-long intermediate hairpin structures (pre-miRNAs) and finally the 17-24-nt-long, mature miRNAs (3). The single-stranded, mature miRNAs bind to their target sequences that most often are located in the 3'-prime untranslated regions of

the mRNA, usually showing only partial bp complementarity with the miRNA sequence. According to the current view, imperfect complementarity leads to repression of translation of the target mRNA and is the main mechanism of miRNA regulation in animals, whereas perfect complementarity induces degradation of the target mRNA and is mainly detected in plants (3).

At their discovery, miRNAs were shown to control fundamental cellular processes, such as differentiation of cells and timing of development of the organism (4, 5), suggesting that aberrations of miRNAs could be involved in various human diseases, including cancer. In the year 2002, the first piece of evidence connecting miRNAs and cancer was obtained when Calin et al. (6) showed that *miR-15* and *miR-16* are the target genes of the 13q14 deletion that is common in chronic lymphatic leukemia. After this initial finding, more than half of the known miRNAs have been reported to be located in cancer-associated genomic regions and to show copy number alterations in cancer (7, 8), and deregulation of several miRNAs, such as *miR-143*, *miR-145*, *miR-21*, *BIC/miR-155*, and the *let-7* family, has been detected in various cancers (9–16). Functional studies of individual miRNAs have shown that they can act as oncogenes (the so-called onco-miRs) or tumor suppressor genes (11, 17–20). Although targets of most miRNAs have not been identified, some have been shown to negatively regulate well-known oncogenes: *miR-15* and *miR-16* have been reported to repress the antiapoptotic factor BCL2 (21) and the *let-7* miRNA family to target the RAS oncogene (22).

Recently, miRNA expression profiling studies have been done to identify cancer-specific miRNA signatures (10, 12, 16, 23–31). Although there are some miRNAs, such as *miR-143* and *miR-145*, that have been reported to be aberrantly expressed in several different cancer types (9, 11, 12), the miRNA signatures of cancers of different cellular origin seem to be unique. In this study, expression profiling of 319 miRNAs in 6 prostate cancer cell lines, 9 prostate cancer xenograft samples, and 13 clinical prostate tissue samples was carried out, revealing the miRNA signature of prostate cancer. Furthermore, hierarchical clustering of the clinical tumor samples by their miRNA expression was done, showing the high accuracy of miRNA expression profiling in classifying prostate tumors.

## Materials and Methods

**Cell lines, xenografts, and tumor samples.** The prostate cancer cell lines PC-3, DU-145, LNCaP, and 22Rv1 were obtained from American Type Culture Collection, whereas LAPC4 and VCaP were kindly provided by Dr. Charles Sawyers (University of California at Los Angeles, Los Angeles, CA) and Dr. Jack Schalken (Radboud University Nijmegen Medical Center, Nijmegen, the Netherlands), respectively. All cell lines were cultured under the recommended conditions. Freshly frozen samples of prostate cancer xenografts (LuCaPs 41, 49, 69, 70, 73, 86.2, 92.1, 93, and 115) were made available for the analyses by one of the coauthors (R.L. Vessella). The characteristics of the xenografts have been reported previously (32, 33).

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

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Freshly frozen clinical tissue samples from 4 benign prostatic hyperplasias (BPH), 5 untreated prostate carcinomas, and 4 hormone-refractory prostate cancers were obtained from Tampere University Hospital (Tampere, Finland). The carcinoma samples were histologically examined for the presence of tumor tissue (>60%) using H&E-stained sections. The BPH samples were obtained from prostatectomy or cystoprostatectomy specimens from patients treated for BPH or bladder cancer. The specimens were histologically verified not to contain any prostate cancer cells. The clinicopathologic characteristics of the tissue specimens are given in Supplementary Table S1.

**miRNA array design and preparation.** Antisense oligonucleotide probes for all 319 different human miRNA sequences found from the miRNA database of the Sanger Institute<sup>5</sup> (34, 35) in October 2005 were designed and purchased from Invitrogen. The probes were 34-48 nt long, unmodified DNA oligonucleotides, in which the antisense sequence of the target miRNA was repeated twice. Antisense oligonucleotide probes for tRNA-Met and tRNA-Ala were used as positive controls. To confirm the specificity of the array hybridization signals, antisense oligonucleotide probes for *miR-16*, *miR-21*, and *miR-28* containing one, two, or three mismatching bases (G to C) per target sequence were used. The sequences and lengths of probes included in the array are summarized in Supplementary Table S2. All the probes were spotted in duplicate at 50  $\mu\text{mol/L}$  concentration onto nylon membranes (GeneScreen Plus, Perkin-Elmer) by using a 96-pin VP 409 replicator tool (V&P Scientific, Inc.) followed by immobilization of the probes on the membranes as described previously (36).

**RNA extraction and miRNA array hybridization.** RNA extraction, labeling, and hybridization were done essentially as described previously (36). Briefly, total RNA from the cell lines, xenografts, and clinical tissue samples was extracted by using the Trizol reagent (Invitrogen). Low-molecular weight (LMW) RNA (<300 nt) from 2  $\mu\text{g}$  total RNA was purified by using the Microcon YM-100 columns (Millipore), dephosphorylated with the calf intestine alkaline phosphatase (Fermentas International, Inc.), and 5' end labeled with [ $\gamma$ -<sup>32</sup>P]ATP (3,000 Ci/mmol; Amersham Biosciences/GE Healthcare) using T4 polynucleotide kinase (Fermentas). Unincorporated nucleotides were removed from the labeling reaction with the Microspin G-25 column (Amersham Biosciences/GE Healthcare). miRNA array membranes were prehybridized in MicroHyb hybridization solution (Invitrogen) for 1 h at +37°C, after which the labeled LMW RNA was added to the solution. Hybridization was carried out at +37°C overnight followed by washes. After exposing the membranes to the Storage Phosphor screen (GE Healthcare) for 24 h, signals were detected by using the Typhoon TRIO scanner (GE Healthcare).

The array hybridization signals were quantified with the ImageQuant TL software (GE Healthcare) using the spot edge average as a local background correction method. The background-corrected value of the mismatch miRNA spot giving the highest signal value in each hybridization was set for a cutoff value for truly positive hybridization signals. After that, normalization was carried out by using the average of all positive, background-corrected signal values of the array as a normalization factor.

Clustering analysis was done by using the Cluster software (37). Before the clustering, data points that were missing in >20% of the arrays, or did not show background-corrected and normalized expression values >0.2 in at least two of the samples, were excluded from the analysis. Subsequently, the values were adjusted by doing five cycles of median centering and normalizing the row values (miRNAs) and column values (arrays), after which hierarchical clustering was carried out using the average linkage clustering algorithm. Subsequently, the clustering results were visualized by using the TreeView software.<sup>6</sup> All array data were submitted using MIAMExpress to the ArrayExpress database (accession number to be received).

**Dot blot hybridization.** Total RNA was extracted from the cell lines followed by the isolation of the LMW RNA from 10  $\mu\text{g}$  total RNA as described

above. The LMW RNA was then pipetted onto a prewetted nylon membrane and immobilized at +80°C for 15 min, after which it was cross-linked by UV (IBI Ultralinker, Kodak). DNA oligonucleotide probes for *miR-99a* (5'-CACAAAGATCGGATCTACGGGT-3'), *miR-141* (5'-CCATCTTACCAGACAGTGTTA-3'), *miR-198* (5'-CCTATCTCCCTCTGGACC-3'), and *miR-205* (5'-CAGACTCCGGTGAATGAAGGA-3') were 5' end labeled with [ $\gamma$ -<sup>32</sup>P]ATP using T4 polynucleotide kinase. Unincorporated nucleotides were removed, and after the prehybridization, the dot blot membranes were hybridized with the labeled probe at +37°C overnight followed by washes. After exposing the membranes to the Storage Phosphor screen, signals were detected by using the Typhoon TRIO scanner. To normalize the miRNA hybridization signals, the membranes were stripped and rehybridized with a probe for the 5S rRNA (5'-CAGCCCCGACCTGCTTAGCTTCCGAGATCAGACGAGAT-3'). The dot blot hybridization signals were normalized by calculating the miRNA/5S rRNA expression ratios for each miRNA.

**Real-time quantitative PCR.** Total RNA from the clinical tissue samples was extracted by using the Trizol reagent. The quantitative real-time PCR (qRT-PCR) expression analysis of *miR-145* was done by using the miRNA-specific *mirVana* qRT-PCR Primer Set (Ambion Ltd.). First, 20 ng total RNA was reverse transcribed by using the *mirVana* RT Primer (Ambion) and the SuperScript III reverse transcriptase (Invitrogen) at +37°C for 30 min, after which the enzyme was inactivated at +95°C for 10 min. Subsequently, the reverse-transcribed miRNAs were used as templates in the qRT-PCR analysis using the LightCycler instrument (Roche Diagnostics) with the *mirVana* PCR primers (Ambion) and the LightCycler FastStart DNA Master SYBR Green I kit (Roche Diagnostics). After the activation of the polymerase enzyme at +95°C for 10 min, 45 cycles of +95°C for 1 s, +60°C for 10 s, and +72°C for 10 s were done. Both melting curve analysis and agarose gel run were used to confirm the specificity of the amplification reactions. For normalization, expression of the *TATA box binding protein* mRNA was measured from the each RNA sample as described previously (38).

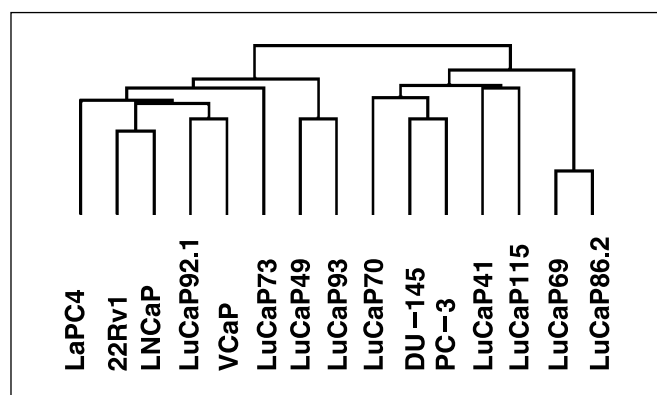
## Results

**miRNA expression in prostate cancer cell lines and xenografts.** An oligonucleotide array hybridization method was set up to study expression of 319 human miRNAs in prostate cancer. First, the method was set up and validated by hybridizing the arrays with radiolabeled LMW RNA from 6 prostate cancer cell lines and 9 prostate cancer xenograft samples. About 40% (128 of 319) of the miRNA probes gave detectable signal in at least one of the samples. To validate the array hybridization results, expression of four miRNAs (*miR-99a*, *miR-141*, *miR-198*, and *miR-205*) that showed expression differences between the cell lines was studied in five cell lines (PC-3, DU-145, LNCaP, 22Rv1, and LAPC4) using the dot blot hybridization. The dot blot hybridization results were in concordance with the array hybridization results (Supplementary Fig. S1).

After the validation of the array analysis, the samples were clustered by their miRNA expression profiles using the hierarchical clustering algorithm of the Cluster software (37). One major characteristic by which the cell line and xenograft samples differ from each other is their androgen receptor (AR) status: four of the samples studied (the cell lines PC-3 and DU-145 as well as the xenografts LuCaPs 49 and 93) are AR negative, whereas the others express AR at variable levels. In cluster analysis, the AR-positive cell lines (LNCaP, 22Rv1, LAPC4, and VCaP) were all clustered in the same branch together with one of the xenograft samples (LuCaP 92.1). On the other hand, the two AR-negative cell lines (PC-3 and DU-145) were clustered next to each other, distantly from the other cell lines. Similarly, the two AR-negative xenograft samples (LuCaPs 49 and 93) formed a separate node of their own (Fig. 1; Supplementary Fig. S2).

<sup>5</sup> <http://microrna.sanger.ac.uk/sequences/>

<sup>6</sup> <http://rana.lbl.gov/EisenSoftware.htm>



**Figure 1.** Hierarchical clustering of prostate cancer cell line and xenograft samples. The median-centered and normalized miRNA expression values were clustered by miRNAs (rows) and samples (columns) by using the average linkage clustering algorithm of the Cluster software. The complete TreeView image showing also the clustering of miRNAs is shown in Supplementary Fig. S1.

### Association of gene copy number and expression of miRNAs.

To study association between miRNA locus copy number and expression of miRNAs, previously published array comparative genomic hybridization (aCGH) data (32) of the cell lines (PC-3, DU-145, LNCaP, LAPC4, and 22Rv1) and seven of the xenografts (LuCaPs 49, 69, 70, 73, 86.2, 93, and 115) were used. For the analysis, the expression values of miRNAs were divided into three groups according to whether the samples showed deletions, amplifications, or no alterations of the corresponding miRNA loci according to the aCGH data (32). There was a moderate association ( $P = 0.0290$ , Kruskal-Wallis test) between the expression of the miRNAs and the copy number alterations of the loci. On average, the miRNAs located in the chromosomal regions of deletion showed the lowest level of expression, whereas the miRNAs located in the regions of amplifications showed the highest expression (Fig. 2).

**miRNA expression in clinical tissue samples.** After establishing the miRNA array hybridization system with the cell line and xenograft samples, the method was applied for miRNA expression profiling of clinical prostate tissue samples from 4 BPHs, 5 untreated prostate carcinomas, and 4 hormone-refractory prostate carcinomas. As shown in Fig. 3, the miRNA expression patterns in BPH, untreated prostate carcinoma, and hormone-refractory prostate carcinoma were clearly distinguishable from each other. To validate the array hybridization results, expression of miR-145 that showed clear expression differences between the tissue samples was studied by using the qRT-PCR, the result of which paralleled the array hybridization data (Supplementary Fig. S3).

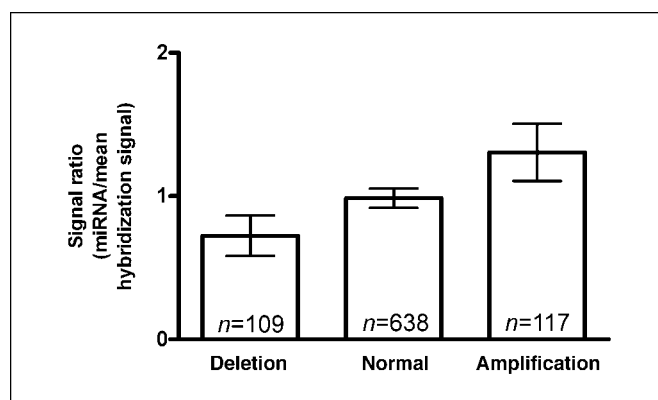
When the expression of miRNAs was compared between the BPH samples and the carcinoma samples, 51 miRNAs were found to be differentially expressed. Of these, 37 miRNAs were down-regulated (maximum expression value in the carcinoma sample group lower than minimum expression value in the BPH sample group). Twenty two of these 37 miRNAs showed decreased expression in all carcinoma samples (untreated and hormone refractory), whereas 15 of them were only down-regulated in the hormone-refractory carcinomas compared with BPH samples. In addition to these 37 down-regulated miRNAs, 14 miRNAs were up-regulated (minimum expression value in the carcinoma sample group greater than maximum expression value in the BPH sample group): 8 of them showed increased expression in all carcinoma

samples and 6 of them only in hormone-refractory carcinomas. The differentially expressed miRNAs are listed in Table 1.

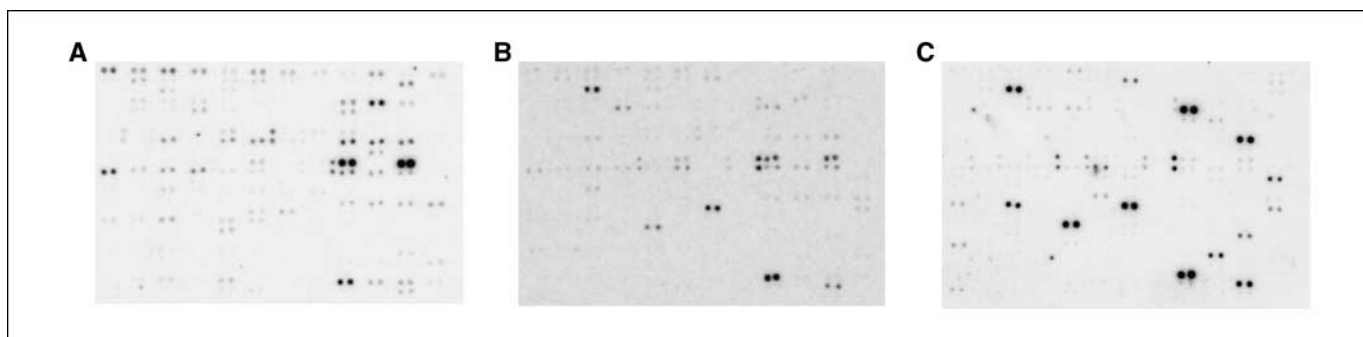
Subsequently, clustering analysis was done to classify the clinical tissue samples according to their miRNA expression patterns. The clustering analysis successfully separated the BPH samples from the carcinoma samples, as visualized by the hierarchical tree (Fig. 4; Supplementary Fig. S4). The samples were divided in two major subclusters, one of which consisted of all the four BPH samples and the other one containing all the nine carcinoma samples. In addition, the carcinoma samples were quite accurately further classified into two subclusters according to their clinical stage: all but one of the five untreated carcinoma samples were clustered in one branch of the carcinoma subcluster, whereas the four hormone-refractory carcinomas and the single remaining untreated carcinoma formed the other branch of the subcluster. In this subcluster, the single untreated carcinoma was separated to another branch from the hormone-refractory tumors.

## Discussion

Expression profiling of 319 miRNAs in 6 prostate cancer cell lines, 9 prostate cancer xenografts samples, and 13 clinical prostate tissue samples (4 BPH, 5 untreated prostate carcinomas, and 4 hormone-refractory prostate carcinomas) was carried out to identify the prostate cancer miRNA signature and to use the miRNA expression profiling in classifying prostate tumors. The antisense oligonucleotide array hybridization method was first set up and validated. The confirmation of the array data was done by dot blot hybridization as well as qRT-PCR. Expression of 128 of 319 (40%) miRNAs was detectable in, at least, one of the samples by array hybridization, indicating that the majority of the miRNAs were either not expressed in these samples or that the expression was too low to be detected by this method. Because the expression of miRNAs is highly tissue specific (39, 40), it was actually not expected that the expression of all the miRNAs would be detected in prostate cancer cells.



**Figure 2.** Association of the locus copy number (according to ref. 32) and average expression of miRNAs in 5 prostate cancer cell lines and 7 xenografts. Only miRNAs that showed signal values  $>0.5$  in, at least, one sample were included in the analysis. The expression values were normalized both row-wise (miRNAs) and column-wise (arrays). Columns, mean of the three groups; bars, SE.  $n$ , the number of data points in each group. Y axis, the signal ratio, calculated by normalizing the raw array hybridization signal value by the mean value of all signals within the hybridization. Kruskal-Wallis test for three data sets indicated significant association between the locus copy number and the expression ( $P = 0.0290$ ). Dunn's post test indicated significant ( $P < 0.05$ ) difference, specifically between the groups of amplification and deletion.



**Figure 3.** miRNA expression profiling of clinical prostate tissue samples. Examples of the array hybridization results of BPH (A), untreated prostate carcinoma (B), and hormone-refractory prostate carcinoma samples (C), showing the distinguishably different expression patterns of BPH and the two cancer types.

Surprisingly, clustering analysis of the cell lines and xenografts did not simply separate the cell lines and xenograft samples in their own subclusters, but instead they were mixed. They seemed to form subnodes according to their AR status: all AR-positive cell lines (LNCaP, 22Rv1, LAPC4, and VCaP) were located close to each other in the same subcluster, whereas the two AR-negative cell lines (PC-3 and DU-145) formed a separate subnode in the other subcluster. Similarly, the two AR-negative xenograft samples (LuCaPs 49 and 93), representing small-cell carcinomas of prostate, were clustered in their own subnode, separately from the other

xenografts. The results suggest that androgens might regulate the expression of some miRNAs. Very little is known about the regulation of the expression of miRNAs themselves, and no studies on their androgen regulation have been published thus far. Alternatively, the differential expression of miRNAs in AR-positive and AR-negative cells may reflect general fundamental differences between AR-positive and AR-negative prostate tumors that are not necessarily directly under the regulation of androgens.

When the expression of individual miRNAs between the clinical BPH and carcinoma samples was compared, 51 miRNAs were

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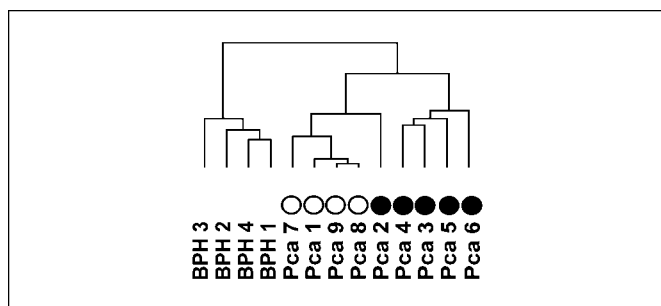
**Table 1.** miRNAs showing differential expression in the prostate carcinoma samples compared with the BPH samples

Down-regulated		Up-regulated	
In all carcinomas <sup>†</sup> (n = 22)	Only in hormone-refractory carcinomas <sup>‡</sup> (n = 15)	In all carcinomas <sup>†</sup> (n = 8)	Only in hormone-refractory carcinomas <sup>‡</sup> (n = 6)
let-7a	let-7f	miR-202	miR-184
let-7b	miR-19b	miR-210	miR-198
let-7c	miR-22	miR-296	miR-302c*
let-7d	miR-26b	miR-320	miR-345
let-7g	miR-27a	miR-370	miR-491
miR-16	miR-27b	miR-373*	miR-513
miR-23a	miR-29a	miR-498	
miR-23b	miR-29b	miR-503	
miR-26a	miR-30a_5p		
miR-92	miR-30b		
miR-99a	miR-30c		
miR-103§	miR-100		
miR-125a	miR-141		
miR-125b	miR-148a		
miR-143	miR-205		
miR-145			
miR-195			
miR-199a			
miR-199a*			
miR-221			
miR-222			
miR-497			

<sup>†</sup> P value of 0.0028 (Mann-Whitney U test) for all miRNAs in this category.

<sup>‡</sup> P value of 0.0286 (Mann-Whitney U test) for all miRNAs in this category.

<sup>§</sup> P value 0.0040 (Mann-Whitney U test) because of one missing value in the category of carcinomas.



**Figure 4.** Hierarchical clustering of clinical prostate tissue samples. The tree shows the accurate classification of the BPH samples in one subcluster and the carcinoma samples in the other. The carcinoma subcluster is further divided in two branches, one of which contains all but one of the five untreated carcinomas and the other one consisting of four hormone-refractory carcinomas and one untreated carcinoma. ●, untreated carcinoma samples; ○, hormone-refractory carcinomas. Pca, prostate carcinoma. The complete TreeView image showing also the clustering of miRNAs is shown in Supplementary Fig. S4.

detected to be differentially expressed. Interestingly, the number miRNAs that showed down-regulation ( $n = 37$ ) in carcinoma samples was three times higher than the number of up-regulated miRNAs ( $n = 14$ ). The data agree with the previously published miRNA profiling studies, most of which have shown down-regulation of miRNAs to be more common than up-regulation in cancer (9, 10, 16, 29). The global down-regulation of miRNAs in cancer cells has been suggested to reflect the lower differentiation stage of the tumor cells compared with normal cells (29). In accordance, here, 37 miRNAs were found to be down-regulated in hormone-refractory late-stage prostate carcinomas, whereas only 22 miRNAs were down-regulated in untreated early carcinomas. There were also more miRNAs that were up-regulated in hormone-refractory carcinomas ( $n = 14$ ) than in untreated carcinomas ( $n = 8$ ). Defects in the miRNA processing machinery that have been detected in cancer samples (41, 42) could explain the overall decreased expression of miRNAs, but also up-regulation of the components of the processing machinery in cancer has been reported (43), which is why the role of the miRNA maturation process in the aberrant expression of miRNAs in cancer still remains unclear. Other mechanisms suggested for the deregulation of miRNAs in cancer are alterations of the genomic regions, in which the miRNA genes are located (7, 8), and epigenetic regulation (44, 45). Our data support the suggestion that genetic alterations affect the expression of miRNAs. There was a significant positive association between the locus copy number and the expression of miRNAs. Previously, we have shown similar association between the expression and the locus copy number of protein coding mRNAs in prostate cancer (32).

The majority of the 51 miRNAs that were detected here to be differentially expressed in the carcinoma samples are the same ones that have also been identified in at least one of the three previously published miRNA profiling studies, in which prostate carcinoma samples have been included: 33 of them were shown by Lu et al. (29) to be among the differentially expressed miRNAs that distinguished malignant tissues from normal ones, and 11 of them were detected to be deregulated in prostate cancer also by Mattie et al. (30). However, when compared with the results of the third study by Volinia et al. (26), miRNAs showing differential expression were either not the same ones or showed the opposite results of up-regulation or down-regulation. This discrepancy may be due to the fundamental methodologic differences used in the studies.

Volinia et al. (26) actually measured the expression of both the primary miRNA transcripts (pri-miRNAs) and the active, mature miRNAs, simultaneously. Considering that the pri-miRNAs are transient products in the miRNA biogenesis and presumably are rapidly further processed after their synthesis (46), the expression status of the pri-miRNA and the mature miRNA is not necessarily the same. Therefore, the profiles of combined expression of the pri-miRNAs and the mature miRNAs and the profiles of expression of only the mature miRNAs probably should not be compared with each other.

Because of the tissue specificity of the miRNAs (39, 40), different sets of miRNAs are likely to be up-regulated or down-regulated in cancers of different cellular origins, although it has been reported that the miRNA signatures of different cancer types could share some individual miRNAs (26). Of the miRNAs that were detected here to be up-regulated or down-regulated in prostate carcinoma samples, some have been reported to be similarly deregulated in other cancers: down-regulation of *miR-125a* and *miR-125b* in breast cancer (12), the *let-7* miRNAs in lung cancer (15, 16), and *miR-143* and *miR-145* in several different cancer types (9, 11, 12). However, most of the differentially expressed miRNAs detected in this study have not been commonly reported to be deregulated in other cancers, suggesting that prostate cancer has a miRNA signature that is specific for this cancer type.

The differential expression of miRNAs in carcinoma samples suggests that miRNAs could be mechanistically involved in the development of cancer. To elucidate the role of the individual miRNAs in prostate cancer, genes and pathways that are regulated by the differentially expressed miRNAs should be studied. Unfortunately, only a few targets of miRNAs have been identified thus far. Of the differentially expressed miRNAs detected in this study, the *let-7* family has been shown to target the RAS oncogene (22), *miR-16* to repress the antiapoptotic factor BCL2 (21), and *miR-125a* and *miR-125b* to suppress the oncogenes ERBB2 and ERBB3 (47). Experimental evidence for targets of the other differentially expressed miRNAs is not available. Because of the imperfect complementarity of miRNAs and their target sequences, target prediction, even with the aid of special prediction programs, is very challenging and inaccurate. Therefore, targets of most of the miRNAs, and thereby also their functions, still remain to be studied.

Hierarchical clustering of the BPH, untreated, and hormone-refractory prostate carcinoma samples successfully separated the BPH samples from the carcinoma samples. Moreover, the carcinoma samples were quite accurately further classified according to their androgen dependence, with four of the five untreated tumors forming one subcluster and the four hormone-refractory, more aggressive tumors plus the remaining untreated tumor forming the other subcluster. In addition, the untreated tumor in the hormone-refractory subcluster was separated into its own branch. Therefore, it seems that the miRNA expression profiles of the hormone-refractory tumors were more similar to each other than those of the untreated ones. The surprisingly high accuracy of clustering suggests that, in spite of the relatively low number of miRNAs known by now, classifying tumors by their miRNA expression can be very informative. In fact, it has already been evidenced that miRNA profiling is more accurate in classifying poorly differentiated tumors than mRNA profiling (29). Therefore, miRNA expression profiling could be used, for example, in developing new diagnostic tools for cancers that currently are lacking good molecular markers, such as prostate cancer.

Individual miRNAs and miRNA signatures have already been shown to have prognostic value in some cancers, such as chronic lymphatic leukemia, diffuse large B-cell lymphoma, and lung carcinomas (14–16, 24). To identify miRNAs that could be used as prognostic or predictive markers in prostate cancer, more miRNA expression profiling studies with larger number of tumor samples are warranted.

In summary, expression profiling revealed several miRNAs that are underexpressed or overexpressed in prostate cancer, thereby identifying miRNAs that could be significant in prostate cancer development. The data also suggest that one mechanism for differential expression could be miRNA gene copy number alteration. Finally, hierarchical clustering of clinical prostate tissue

samples accurately separated carcinomas from BPH and also classified the carcinomas according to their clinical androgen dependence, suggesting that miRNAs could be used as diagnostic and prognostic tools in prostate cancer.

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