

# MicroRNA expression profiles in prostate cancer cell lines

## Abstract

MicroRNAs (miRNAs), a group of small non-coding RNAs, can regulate gene expression by triggering translation repressing and/or RNA degradation. Recent studies proved that they were deeply involved in tumorigenesis. Here we show that, compared with the two normal prostate cell lines, eighteen miRNAs in the array were over-expressed and 18 showed lower levels of expression in at least 2 of 3 cancer cell lines using miRNA microarray. MiR-99a, miR-335, miR-375 and miR-625 were the most significantly over-expressed miRNAs, whereas miR-155, miR-205, miR-224, miR-422a, miR-422b, miR-452, and miR-452\* (\* refer to miR-452-3p) were among the most down-regulated miRNAs. Expressions of several of the most significantly differential expression miRNAs were confirmed by Northern blot analysis and real-time RT-PCR. In this study, the expression of miR-10a, miR-99a and miR-125b were also up-regulated in prostate cancer tissues compared with their own normal tissues. For miR-99a, its expression was up-modulated in prostate cancer in comparison with all three normal tissues. Whereas miR-27a and miR-205 have lower level expression in cancer tissues than that in normal tissues. Some miRNAs are aberrantly expressed in human prostate cancer suggesting their involvement in the development and progression of this malignancies. Further studies of these deregulation miRNAs will help clarify their role in tumorigenesis and detect their potential clinical usefulness for early diagnosis, prognosis and therapy of prostate cancer.

**Keywords:** prostate cancer, mirna, microarray

## Introduction

MicroRNAs (miRNAs) are a class of small non-coding RNAs that negatively regulate gene expression at the protein level by either perfect or nearly perfect complementary binding to mRNA sequences.<sup>1</sup> MiRNAs are 19-25 nucleotide (nt) long molecules cleaved from 70-100nt hairpin pre-miRNA precursors. The precursor is cleaved by cytoplasmic RNase III Dicer into  $\approx$ 22nt miRNA duplex and finally to a single-strand mature miRNA. miRNAs can decrease the levels of many of their target transcripts as well as the amount of protein encoded by these transcripts.<sup>1,2</sup> Previous studies suggest that miRNAs play important roles in various processes, such as cell differentiation, apoptosis, proliferation, development, metabolism and so on.<sup>3</sup> There is emerging evidence that miRNAs are involved in tumorigenesis. Aberrant miRNAs expression has been detected in different human cancers.<sup>4,5</sup> MiRNAs might function as a novel class of tumor suppressor genes or oncogenes.<sup>6,7</sup> Specific overexpression or under expression has been correlated with particular tumor types.<sup>8,9</sup>

Prostate cancer (PCa) is the most commonly diagnosed malignancy and the second leading cause of cancer-related deaths in the western world.<sup>10</sup> Despite great efforts made in the past few years to clarify prostate tumorigenesis, the molecular mechanisms involved in its initiation and progression are still poorly understood.

In this study, to investigate the specific miRNAs for prostate cancer, miRNA microarray was performed with 3 prostate cancer cell lines and 2 normal prostate cell lines. The results were confirmed using Northern Blot analysis and real-time RT-PCR. Several miRNAs are aberrantly expressed in human prostate cancer suggesting their involvement in the development and progression of these malignancies.

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## Materials and methods

### Cell lines and prostate cancer tissues

The tumor cell lines DU145, LNCaP, PC-3, and normal cells RWPE-1, WPE1-NB26 were purchased from American Type Culture Collection (ATCC, Manassas, VA). All tumor cell lines were maintained according to the recommended culture conditions provided by ATCC supplemented with 100 $\mu$ g/ml streptomycin and 100 $\mu$ g/ml penicillin. Tumor tissues obtained at the time of diagnosis of prostate cancer were used for this study. Pure normal and compared cancer cells were obtained by laser capture microdissection (LCM). Written informed consent was obtained from these subjects and approved by the institutional Review Board of Georgia's Health Sciences University.

### RNA extraction

Total RNA isolation from prostate cell lines and tissues was performed with mirVanaTM miRNA Isolation Kit (Ambion, Austin, Texas) according to the instructions provided by the manufacturer. The quality of the RNA was assessed with 15% denaturing polyacrylamide gel electrophoresis and spectrophotometric (Eppendorf BioPhotometer, Eppendorf, Hamburg, Germany).

### MicroRNA microarray

miRNA microarray analysis was done by LC Sciences (<http://www.lcsciences.com/>; Houston, TX). In brief, poly-A tails were added to the RNA sequences at the 3'-ends using a poly(A) polymerase, and nucleotide tags were then ligated to the poly-A tails. For each dual-sample experiment, two-sets of RNA sequences were added with tags of two different sequences. The tagged RNA sequences were

then hybridized to the miRNA microarray chip (Atactic  $\mu$ ParaFlo<sup>TM</sup> microfluidics chip, LC Sciences, Houston, Texas) containing 313 human mature miRNA transcripts listed in Sanger miRBase Release 7.0 (<http://www.sanger.ac.uk/Software/Rfam/mirna>).

The probe sequences are available upon request. The labeling reaction was carried out during the second hybridization reaction using tag-specific dendrimer Cy3 and Cy5 dyes. Total RNAs from prostate cancer cell lines and normal prostate cell lines were labelled with Cy3 and Cy5, respectively. The human miRNA chip includes nine repeats for each miRNA. Multiple control probes were included in each chip, which were used for quality control of chip production, sample labelling and assay conditions. Hybridization signals were detected by Axon Genepix 4000B Microarray Scanner (Molecular Devices, Sunnyvale, CA) and saved as TIFF files. Numerical intensities were extracted for control, background, and miRNA probes and converted into Microsoft Excel spreadsheets.

## Data analysis

The data were corrected by subtracting the background and normalizing to the statistical median of all detectable transcripts. Background was calculated from the median of 5% to 25% of the lowest-intensity cells. The data normalization balances the intensities of Cy3- and Cy5-labeled transcripts so that differential expression ratios can be correctly calculated. Statistical comparisons were performed by ANOVA (Analysis of Variance) using the Benjamini and Hochberg correction for false-positive reductions. Differentially detected signals were generally accepted as true when the ratio of the P value was less than 0.01. Clustering analysis was made with a hierarchical method and visualized using the TIGR MeV (Multiple Experimental Viewer) (the Institute for Genomic Research) microarray program.

## Real-time RT-PCR for miRNA precursors

miRNAs expression was analyzed with mirVana<sup>TM</sup> qRT-PCR miRNA Detection Kit (Ambion, Austin, Texas) as recommended by the manufacturer. Briefly, 20ng total RNA was reverse transcribed by using mirVana<sup>TM</sup> RT primer and Superscript II reverse transcriptase at 37°C for 30mins, after which the enzyme was deactivated at 95°C for 10mins, then cDNA was generated. The PCR reaction consisting of appropriate number of cycles (95°C for 15s, 60°C for 30s) was performed in iCycler (Bio-Rad, Hercules, CA) after an initial denaturation step (95°C for 3min) by use of mirVana<sup>TM</sup> qRT-PCR Primer Sets (Ambion, Austin, Texas). Moreover, the real-time PCR products were detected on 3.5% agarose gel and visualized with ethidium bromide on the ChemiImager Imaging System 5500 (Alpha Innotech, San Leandro, CA). The 5S RNA was used to quantify the amount of RNA loaded in individual samples.

## Northern blotting

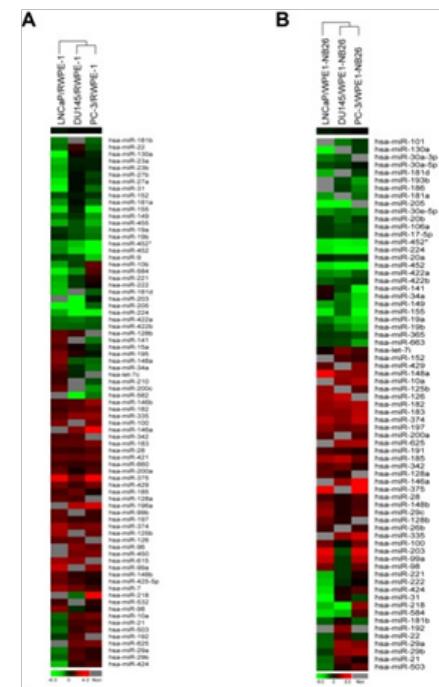
Northern blot analysis was performed as previously described.<sup>11</sup> Briefly, total RNA (10 $\mu$ g) from each sample was resolved on 15% polyacrylamide gel with 8M urea and transferred onto BrightStar<sup>®</sup>-Plus positively charged nylon membrane (Ambion, Austin, Texas) with semi-dry transfer apparatus (Bio-Rad, Hercules, CA). Blots were prehybridized at 65°C for 1h in prehybridization buffer (200mM Na<sub>2</sub>HPO<sub>4</sub>, pH7.0, 5% SDS) and subjected to hybridization with 32P-labeled miR-10a, -27a, -99a, -125b, -205 or -224 probe which is complementary to the mature miRNA overnight. Normalization was done with 32P-labeled U6 probe. Following hybridization, membranes were washed twice for 10min each at

25°C with low-stringency buffer (25mM Na<sub>2</sub>HPO<sub>4</sub>, pH7.5, 5% SDS, 3×SSC), and once for 10min at 42°C with high-stringency buffer (1% SDS, 1×SSC). Finally, the blots were exposed to X-ray film (PIERCE, Rockford, IL). The Oligonucleotides used as probes are as follows (miR Registry, <http://www.sanger.ac.uk/Software/Rfam/mirna>): miR-10a: 5'-cacaatccggatctacaggta-3'; miR-27a: 5'-gccaacttagccactgtgaa-3'; miR-99a: 5'-ctcaagatccggatctacgggt-3'; miR-125b: 5'-tcacaagttgggtctcaggga-3'; miR-205: 5'-cagactccggtgaaatgaagg-3' and miR-224: 5'-taaacggaccactgtgacttg-3'. Oligonucleotides complementary to the U6 RNA (5'-gcaggggccatgctaattttctgtatcg-3') were used to normalize.

## Results

### miRNA profiles revealed between prostate cancer cell lines and normal prostate cell lines

Total RNAs from prostate cancer cell lines and normal prostate cell lines were isolated and sent to LC Sciences for miRNA microarray analysis after assessing the quality. MiRNA profiles were revealed between prostate cancer cell lines and normal prostate cell lines, and correlation analysis was performed (Figure 1A & 1B). By correlating the results from 2 chips, most of the labelling, handling, and system related biases can be eliminated and therefore calls can be narrowed down to the true biological differences. A list of differentially expressed miRNAs (at P<0.01) between prostate cancer cell lines and normal prostate cell lines was shown in Table 1. We just show the up-regulated or down-regulated miRNAs with more than 2 fold differences in the prostate cancer cell lines compared with the two normal prostate cell lines respectively.



**Figure 1** MiRNA microarray analysis for prostate cancer cell lines compared with the prostate normal lines.

- Comparison of miRNAs expression in DU145, LNCaP, PC3 and RWPE-1 by microarray analysis.
- Comparison of miRNAs expression in DU145, LNCaP, PC3 and WPEI-NB26 by microarray analysis.

**Table 1** miRNAs showing differential expression in the prostate cancer cell lines compared with the normal prostate cell lines

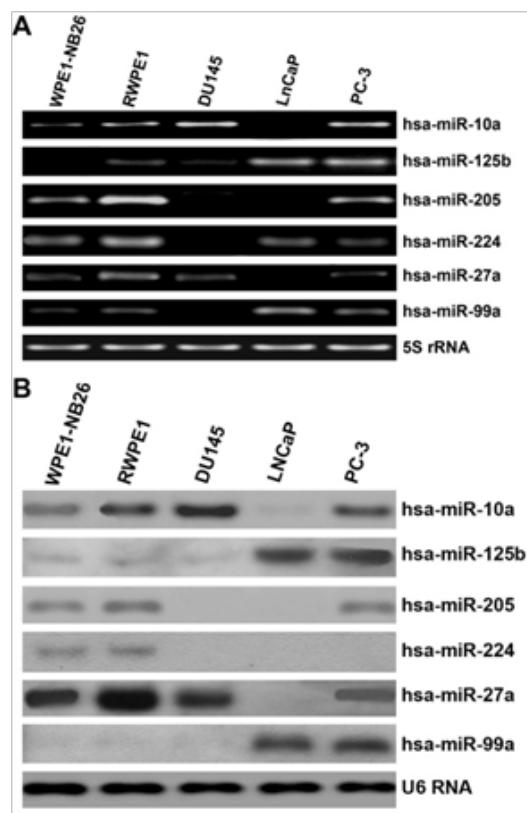
Up-regulated miRNAs	Down-regulated miRNAs
hsa-miR-100	hsa-miR-130a
hsa-miR-125b	hsa-miR-149
hsa-miR-126	hsa-miR-155
hsa-miR-146a	hsa-miR-181a
hsa-miR-182	hsa-miR-181b
hsa-miR-183	hsa-miR-181d
hsa-miR-185	hsa-miR-205
hsa-miR-191	hsa-miR-20b
hsa-miR-197	hsa-miR-19a
hsa-miR-200a	hsa-miR-19b
hsa-miR-28	hsa-miR-224
hsa-miR-29c	hsa-miR-30a-3p
hsa-miR-335	hsa-miR-30e-5p
hsa-miR-342	hsa-miR-422a
hsa-miR-375	hsa-miR-422b
hsa-miR-429	hsa-miR-452

### Validation of the miRNA microarray by RNA blot analysis

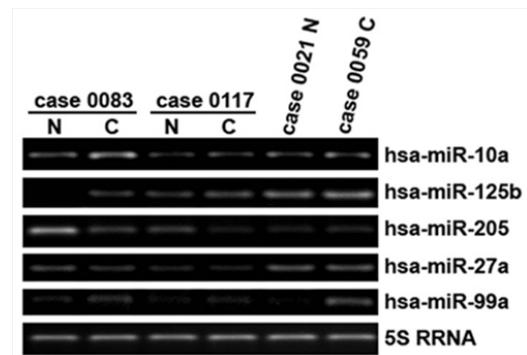
To validate the array hybridization results, expression of six miRNAs candidates miR-10a, -27a, 99a, 125b, -205 or -224 that showed differential expression between the cancer and normal cell lines were determined using Northern blot and real-time PCR. MiR-10a, miR-99a and miR-125b were up-modulated in 2 of 3 prostate cancer cell lines, and the miR-27a, miR-205, and miR-224 showed lower level expression in 2 or 3 cancer cell lines (Figure 2). The Northern blot and real-time PCR results were in accordance with the miRNA microarray analysis results.

### Real-time RT-PCR analysis of miRNAs expression in prostate cancer tissues

The expression of five miRNAs candidates miR-10a, -27a, 99a, 125b, -205 were detected in 2 pairs prostate cancer tissues and 1 normal and 1 cancer tissues using real-time PCR (Figure 3). The expression of miR-10a, miR-99a and miR-125b were up-regulated in prostate cancer tissues compared with their own normal tissues. For miR-99a its expression was also up-modulated in cancer in comparison with all three normal tissues. Whereas miR-27a and miR-205 have lower level expression in cancer tissues than that in normal tissues.

**Figure 2** Validation of miRNA microarray results.

- Real-time RT-PCR of the prostate cancer cell lines and the normal prostate cell lines cancer cells. The 5S rRNA was used as an internal control.
- Northern blot of the prostate cancer cell lines and the normal prostate cell lines cancer cells. The U6 RNA was used as a loading control.

**Figure 3** miRNAs expression in prostate LCM tissue samples by qRT-PCR. N: normal tissue; C: tumor tissue. 5S rRNA was used as a loading control.

### Discussion

Expression profiling of 313 human mature miRNAs between prostate cancer cell lines and normal prostate cell lines was carried out to identify the prostate specific miRNA. The expression of several miRNAs including miR-10a, -27a, 99a, 125b, -205 or -224 were detected with Northern blot as well as real-time PCR to confirm the array data. We also analyze the expression of these miRNAs in a few prostate tissues by real-time PCR. The results showed they have similar expression pattern in prostate cancer lines to that in prostate cancer

tissues. When the expression of individual miRNAs between the prostate cancer cell lines and normal prostate cell lines was compared, eighteen miRNAs in the array were up-modulated and 18 showed lower levels of expression in at least 2 of 3 cancer cell lines. Three out of thirty-six differentially expressed miRNAs was not reported to be deregulated in cancer so far. Most of the differentially expressed miRNAs which we have found altered in prostate cell lines showed behavior consistent with previous published data. For example, miR-200a was increased here and in ovarian cancer;<sup>12</sup> miR-181b which was decreased in the present study and in prostate cancer and glioblastoma.<sup>13</sup> We detected miR-100 and miR-191 as over-expressed and miR-181a and miR-224 as under-expressed in prostate cancer cell lines, in agreement with Volinia et al.'s study.<sup>5</sup> MiR-100, also showed up-modulated in other tumors, such as hepatocellular carcinoma.<sup>14</sup> In acute myeloid leukemia, high expression of miR-191 was correlated with survival.<sup>15</sup> MiR-181a was down-regulated in glioblastoma.<sup>13</sup> MiR-155, which was regarded the oncogenic miRNA,<sup>16</sup> appears as down-regulated in the prostate cancer cell lines compared with the normal prostate cell lines in this study, so did Volinia et al.'s study.<sup>5</sup> The almost exclusively down-modulation of miRNA in cancer reported by Lu et al.<sup>9</sup> was not shown in our study. When compared with the results of Porkka et al.'s study,<sup>17</sup> four differentially expressed miRNAs were also detected in our study showing the opposite results of up-regulation or down-regulation.

MiR-205, which was found to be down-regulated only in hormone-refractory carcinomas by Porkka et al.<sup>17</sup> was the most down-modulated miRNA (up to 9 folds) in both AR-negative and AR-positive cell lines of our study suggesting loss of miR-205 might contribute to the progression of prostate carcinogenesis. We also detected down-regulation of miR-205 in prostate cancer tissues in comparison with their own prostate normal tissues. Our results were supported by the recent studies which showed that miR-205 exerts a tumor suppressive effect in human prostate by counteracting epithelial-to-mesenchymal transition and reducing cell migration/invasion in part through the downregulation of protein kinase CE.<sup>18</sup> Given the effect of miR-205 on cell migration/invasion, another potential target- MMP16 (matrix metalloproteinase 16), which is one of the downstream targets of miR-146b being involved in glioma cell migration and invasion,<sup>19</sup> might be of interest for future studies. Moreover, it has been reported that miR-205 were expressed 10-fold lower in esophageal squamous cell carcinoma and adenocarcinomas than in normal epithelium,<sup>20</sup> and it served as a tumor suppressor in breast cancer suggesting the loss of miR-205 in cancer might be a common event.<sup>21,22</sup> Further studies are needed to test its clinical significance in prostate cancer therapy especially for the metastasis cancer.

MiR-99a, up-regulated in Acute Megakaryoblastic Leukemia cell lines compared with that of CD34 in vitro-differentiated megakaryocytic,<sup>23</sup> was found significantly increased in prostate cancer cell lines, also its expression up-modulated in prostate cancer tissues in comparison with all the three normal tissues. It has been reported that mir-99a was down regulated in serous ovarian cancer and squamous cell carcinoma of the tongue.<sup>24,25</sup> By searching the potential targets of mir-99a, B lymphocyte induced maturation protein 1 (Blimp1), a zinc finger transcriptional repressor, is an interesting potential targets of mir-99a. It has been found Blimp1 plays an important role as a tumor suppressor gene in blood cells. Given its expression in a wide variety of tissues, Blimp1 may also serve as a tumor suppressor gene in non-hematopoietic cell types, however, this has not yet been explored. Moreover, several other PRDM family members also show properties of tumor suppressor genes, suggesting

that this may be a conserved property of this family.<sup>26-28</sup> It is possible that mir-99a might be involved in prostate carcinogenesis through down regulating the expression of Blimp1. Obviously, this hypothesis needs further investigation. Future studies will surely shed light on the following questions: what is the role of mir-99a in prostate carcinogenesis? Is Blimp1 a directly target of mir-99a involved in such process? Does mir-99a have any potential clinical usefulness as diagnostic and prognostic tool?

In some cancers, miR-126 was lost and over expressing miR-126 can inhibit cancer cell growth. Aberrant Expression of Oncogenic and Tumor-Suppressive MicroRNAs in Cervical Cancer Is Required for Cancer Cell Growth. For human breast cancer, miR-126 and miR-335 were identified as metastasis suppressor microRNAs<sup>29</sup> and miR-375, a islet-specific miRNA, which was a regulator of insulin secretion, was down-modulated in pancreatic cancer.<sup>30</sup> On the contrary, they were found up-regulated in prostate cancer cell lines compared with the normal prostate cell lines in our study, suggesting the same microRNAs can exert opposite effects in different organs.

Among the down-modulated genes, notably, miR-224, miR-452 and miR-452\* were expressed 3- to 7-fold lower in all the prostate cancer cell lines than in the two normal prostate cell lines. In prostate tumors with perineural invasion (PNI) 19 microRNAs were higher expressed in PNI tumors than in non-PNI tumors, among which the most differentially expressed microRNA was miR-224. In hepatocellular carcinoma, miR-224 was revealed up-regulation and apoptosis inhibitor-5 as a miR-224-specific target.<sup>31</sup> MiR-224 was also the most differentially overexpressed miRNA in thyroid tumors.<sup>32</sup> Further study of biological effect of miR-224 will be helpful to determine its role in prostate cancer initiation and progression. For miR-452 and miR-452\*, recent data show that they are overexpressed in node positive tumors of urothelial carcinomas.<sup>33</sup>

Differential expression of these miRNA genes in cancer including miR-422a, miR-422b and miR-625 hasn't been described so far to our knowledge. Profiling of prostate cancer versus normal tissues would be necessary to evaluate these findings. Further studies to reveal the underlying mechanisms of why and how miRNAs become deregulated are also very important.

## Conclusion

In conclusion, the focus of our study was to investigate the specific miRNAs expression pattern in prostate cancer cell lines. What roles the miRNAs of interest play in prostate cancer remains largely unknown, and the clinical usefulness of these miRNAs needs to be determined. However, we believe that our data provide a starting point for future studies of miRNAs in prostate cancer.

## Acknowledgements

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## Conflict of interest

The author declares no conflict of interest.

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