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Original Article

Epithelial and stromal expression of miRNAs during prostate cancer progression

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Abstract: Global microRNA (miRNA) profile may predict prostate cancer (PCa) behaviors. In this study, we examined global miRNA expression by miRNA profiling as well as specific miRNA expression levels in PCa epithelium and stroma by in situ hybridization (ISH) and correlated with various clinicopathological features. We first performed comprehensive miRNA profiling on 27 macrodissected cases of PCa by miRNA microarray. A total of 299 miRNAs were significantly dysregulated in high grade and advanced stage PCa. We demonstrated that PCa can be readily classified into high grade/stage and low-grade/stage groups by its global miRNA expression profile. Next, we examined the expression of several selected dysregulated miRNAs, including *let-7c*, *miR-21*, *miR-27a*, *miR-30c*, and *miR-219*, in PCa by ISH. The levels of miRNA expression in epithelial and stromal cells were scored semiquantitatively and compared with clinicopathological features, including age, race, Gleason score, stage, PSA recurrence, metastasis, hormone resistance and survival. We found that the expression of *miR-30c* and *miR-219* were significantly down-regulated in PCa. *miR-21* and *miR-30c* were significantly down-regulated in PCa in African Americans compared to Caucasian Americans. In addition, down-regulation of *let-7c*, *miR-21*, *miR-30c*, and *miR-219* are associated with metastatic disease. Furthermore, down-regulation of *miR-30c* and *let-7c* are significantly associated with androgen-dependent PCa. In PCa stromal cells, *let-7c* downregulation is significantly associated with extraprostatic extension. Our data suggest that selected miRNAs may serve as potential biomarkers to predict cancer progression.

Keywords: miRNA, prostate cancer progression

Introduction

Prostate cancer (PCa) is the most common malignancy among United States men and the second leading cause of cancer related death in men. In particular, African Americans (AA) have the highest incidence rates of PCa in the United States, approximately 1.6 fold higher than Caucasian American (CA) men, moreover, the aged-adjusted death rate is 2.5 times higher than Caucasian American men from SEER data (http://seer.cancer.gov/statfacts/html/prost.html). Although cultural, social and psychological variables contribute to this disparity, they do not fully explain the differences observed among different ethnicities in the US. There is a biological basis for this racial disparity, including variations in levels of miRNA. Recently, chromosome 8q24 has been identified as the most frequently amplified chromosomal region in PCa and may contribute to the higher incidence rate of PCa in AA men compared to CA men [1]. Genomic studies have revealed distinct gene expression profiles between AA and CA PCa that may contribute to differences in tumor biology between AA and CA men [2]. These results implicate genetic and genomic factors that vary in populations between AA and CA men that may account for the differences in PCa risk between these groups.

MicroRNA (miRNA) is a class of small, non-coding RNAs that regulate the expression of miRNA specific target genes, including tumor suppressors and oncogenes, at the levels of transla-

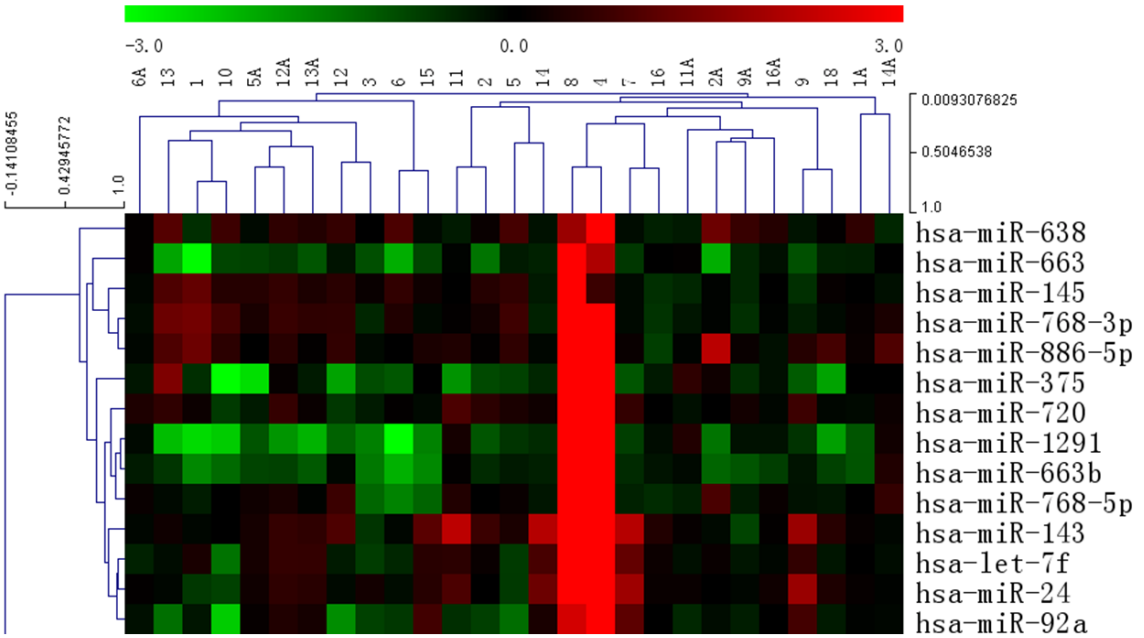

Figure 1. Unsupervised analysis of miRNA expression data (representative section). miRNA profiling data of 27 PCa samples were filtered and normalized for each feature. The data was then subjected to hierarchical clustering on both the samples (horizontally oriented) and the features (vertically oriented, with probe names on the left), with average linkage and Pearson correlation as a similarity measure. Sample names (staggered) are indicated on the top and miRNA names on the left.

tion, as well as transcription [3]. According to the most recent release in miRBase, a database for miRNA [4], 2264 miRNAs have been identified in humans and this number is steadily increasing. It is estimated that miRNAs regulate over 5,300 genes at the transcriptional and translational levels. Expression of miRNAs is tissue and tumor specific. miRNA genes are commonly located on chromosomal fragile sites, and are aberrantly expressed in certain types of solitary tumors [3].

Both increase and decrease in miRNA expression have been documented in PCa, regulating the expression of miRNA specific target genes involved in cell proliferation and growth inhibition. Jung et al. indicated that a fixed expression of a five miRNA combination correlated with Gleason score (GS) or pathological tumor stage in PCa [5]. *miR-100,* and *miR-218* were significantly overexpressed by localized high GS, pT3 PCa in comparison with prostate metastatic carcinoma [6], and miR-182-5p is proven to be a useful marker for high grade PCa [7]. Combined, these data show that miRNAs have potential to be useful biomarkers for PCa. *let-7c* has been consistently shown to be significantly down-regulated and *miR-30c* upregulated in PCa [3, 8, 9]. Further, there are distinct miRNA expression profiles between androgen-dependent and -independent PCa. Several studies on miRNA expression using tumor samples were able to accurately separate prostate carcinomas from benign prostate hyperplasia (BPH) samples and also to further classify tumors according to their androgen dependence (hormone naive versus hormone refractory), indicating the potential of miRNA expression profiles as a novel diagnostic and prognostic tool for PCa [9-11].

In this study, we examined global miRNA expression profiles by microarray. We further studied the expression of a group of commonly dysregulated miRNAs (*let-7c*, *miR-21, miR-27a, miR-30c,* and *miR-219*) by ISH and correlated with the clinicopathological parameters.

Material and methods

Patient cohort for miRNA array

The study cohort was archival PCa cases from New York University Langone Medical Center/VA hospital with Institutional Review Board approval. The pathological features were confirmed by consensus of two pathologists. We included 27 cases of radical prostatectomy

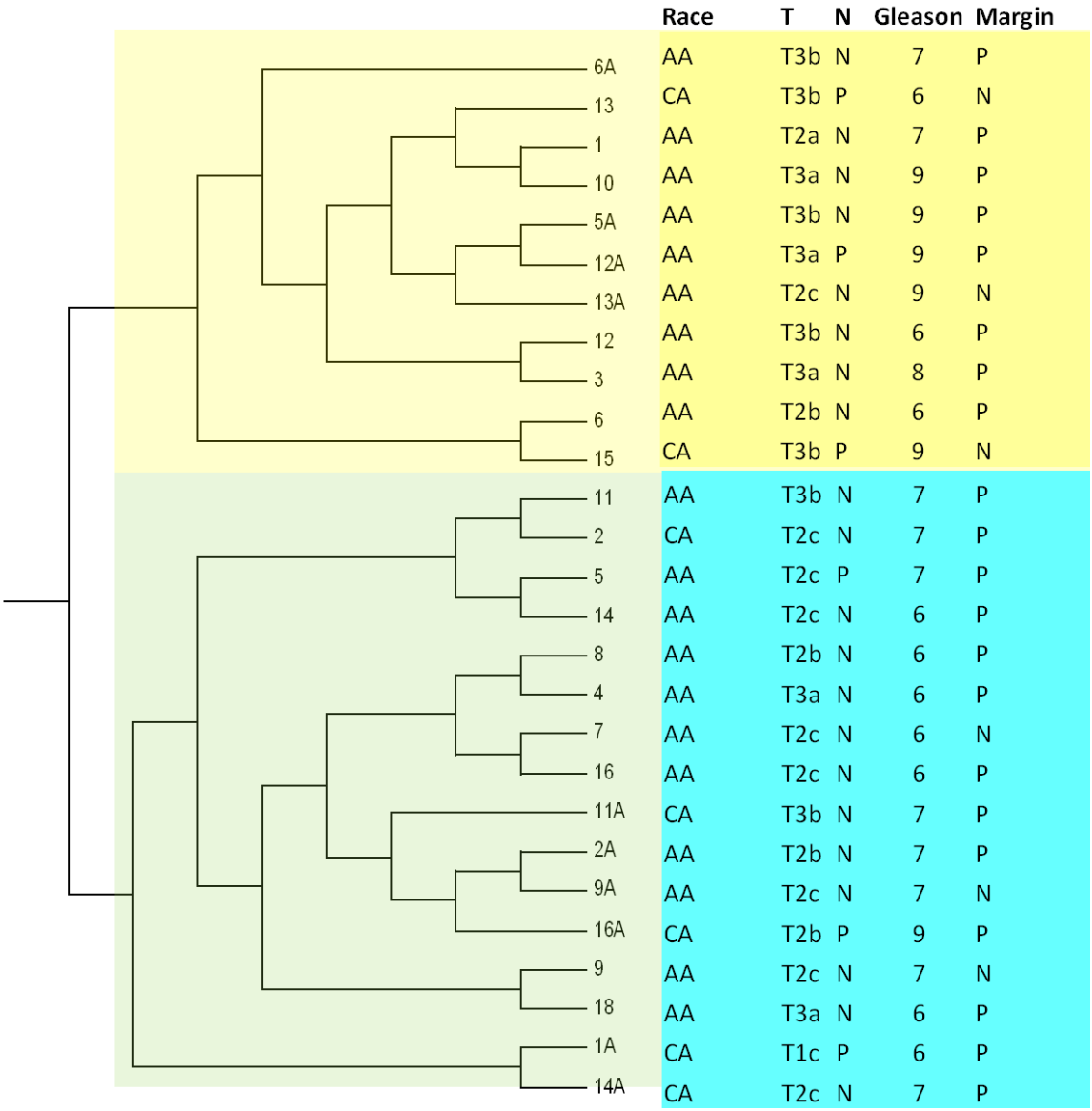

Figure 2. The PCa sample dendrogram generated by unsupervised clustering analysis of miRNA profiling. It was labeled according to each cancer sample's clinical annotation for race, Gleason score (GS), stage (T), margin and lymph node status (N). N = negative, P = positive.

PCa samples. The patients' ages ranged from 51 to 73 years old, 20 patients (75%) are AA and 7 (25%) are CA. 12 (44%) patients have high stage tumor (T3a and above); 7 (26%) patients have high grade cancer (Gleason score ≥ 8); 6 patients (22%) have lymph node metastasis and 21 (77%) patients have PCa with positive margin.

miRNA microarray

The formalin fixed paraffin embedded radical prostectomy PCa samples were macrodissected. The HTG Molecular qDiscovery miRNA Whole Transcriptome Array (WTA, including 687 human miRNAs, was used to compare the expression profiles of PCa. miRNA hybridization and scanning were performed by HTG [12]. Briefly, the proprietary lysis reagent from HTG containing a cocktail of nuclease protection probes is added to the tissue, incubated at 95°C for 10 min then cooled and incubated at 37°C overnight. S1 nuclease is added and incubated for 60 min at 37°C. Base is added and the sample heated at 95°C for 10 min to dissociate the probes from the target miRNA and

Table 1. Statistical analysis of the two most prominent dendrogram arms identified by unsupervised clustering of PCa samples' global miRNA expression profile in accordance with their clinicopathological features

Clinicopathological features	Group A	Group B	*p*-Value
AA	9 (33%)	11 (41%)	0.2232
CA	2 (7%)	5 (19%)	
GS ≥ 8	6 (22%)	1 (4%)	0.0498*
GS ≤ 6	3 (11%)	7 (26%)	
T ≥ 3	8 (30%)	4 (15%)	0.022*
T ≤ 2	3 (11%)	12 (44%)	
Margin+	8 (30%)	13 (48%)	0.3003
Margin-	3 (11%)	3 (11%)	
Node+	3 (11%)	3 (11%)	0.3003
Node-	8 (30%)	13 (48%)	

Fisher exact test. *indicates statistically significant.

destroy the released target miRNA. The solution is transferred into wells of the WT miRNA slide and incubated at 50°C overnight. The slide wells are washed and avidin-HRP is added and incubated 1 hr at 37°C. The wells of the slide are washed, frame removed, the slide is dried and scanned.

Patient cohort for TMA construction

The study cohort was archival PCa cases from New York University Langone Medical Center with Institutional Review Board approval. The pathological features were confirmed by consensus of two pathologists. Tissue microarray (TMA) was constructed as previously described [13]. For epithelial carcinoma cell study, TMA arrays consisted of a total 215 cases (4 cores per case) including 204 transurethral resections specimens of prostate (TURPs) from patients with PCa, 11 cases of non-tumor containing tissue from patients with benign prostatic hypertrophy (referred as normal). These specimens include 61 cases of hormone naïve PCa and 23 cases of castration resistant cancer. The determination of hormone naïve and hormonal resistance was as follows: 1) Tumor tissue from patients who had earlier undergone surgical orchiectomy (at least 6 months prior to the procedure) was considered hormone resistant PCa tissue. 2) Tumor tissue from patients who did not receive hormonal therapy prior to the TURP (all received hormonal therapy only after the TURP procedure) was considered hormone naïve PCa tissue. For stroma cell study, TMA arrays consisted of a total 146 cases (4 cores per case) of transurethral resections specimens of prostate (TURPs) from patients with PCa, among which 22 cases have data on the benign prostatic tissue.

miRNA in situ hybridization (ISH) with locked nucleic acid (LNA) modified probes

The miRCURY LNA modified miRNA probes for *let-7c*, miR30c, *miR-219*, miR27a, miR301, miR21 and *U6* were purchased from Exiqon (Vedbaek, Denmark). The detailed procedure for in situ hybridization was followed as per manufacturer's protocol [14]. In brief, 4 μm TMA slides were prepared. Following deparaffinization and deproteinization, the slides were prehybridized with 1x hybridization buffer without probe. The hybridization was carried out overnight in a 1 x hybridization buffer (30-70 μl) with predenatured miRCURY LNA-modified miRNA probes. After washing, the slides were blocked and incubated with AP conjugated anti-DIG Fab fragments (1:1500, Roche, Indianapolis, IN) and visualized for color detection.

Statistical analysis for miRNA array and ISH

The microarray data is normalized to U6 for each microarray. Hierarchical clustering was done to cluster the samples and miRNA expression patterns using MeV 4.0 software. Significance analysis of microarrays method was used to determine differentially expressed miRNAs between the high grade and stage PCa and low grade and stage groups. The ISH signal were scored semiquantitatively as 0 for negative, 1 for weak, 2 for moderate and 3 for strong staining for both epithelial and stromal cells. The scores of the selected miRNA expression from each tissue were normalized by relative intensity of U6 expression (miRNA/U6 score). We also compared the percent of patients with low, intermediate or high intensity levels of miRNA expression with tumor grade and metastasis. Intensity was graded as: +, miRNA/U6 < 0.4; ++, miRNA/U6 0.4-0.6; and +++, miRNA/U6 > 0.6). Paired student t-test was used for statistical analysis and the difference is considered significant with $p < 0.05$.

Results

miRNA expression by miRNA array analysis

We performed a miRNA array analysis using 687 human miRNAs in 27 samples of macro-

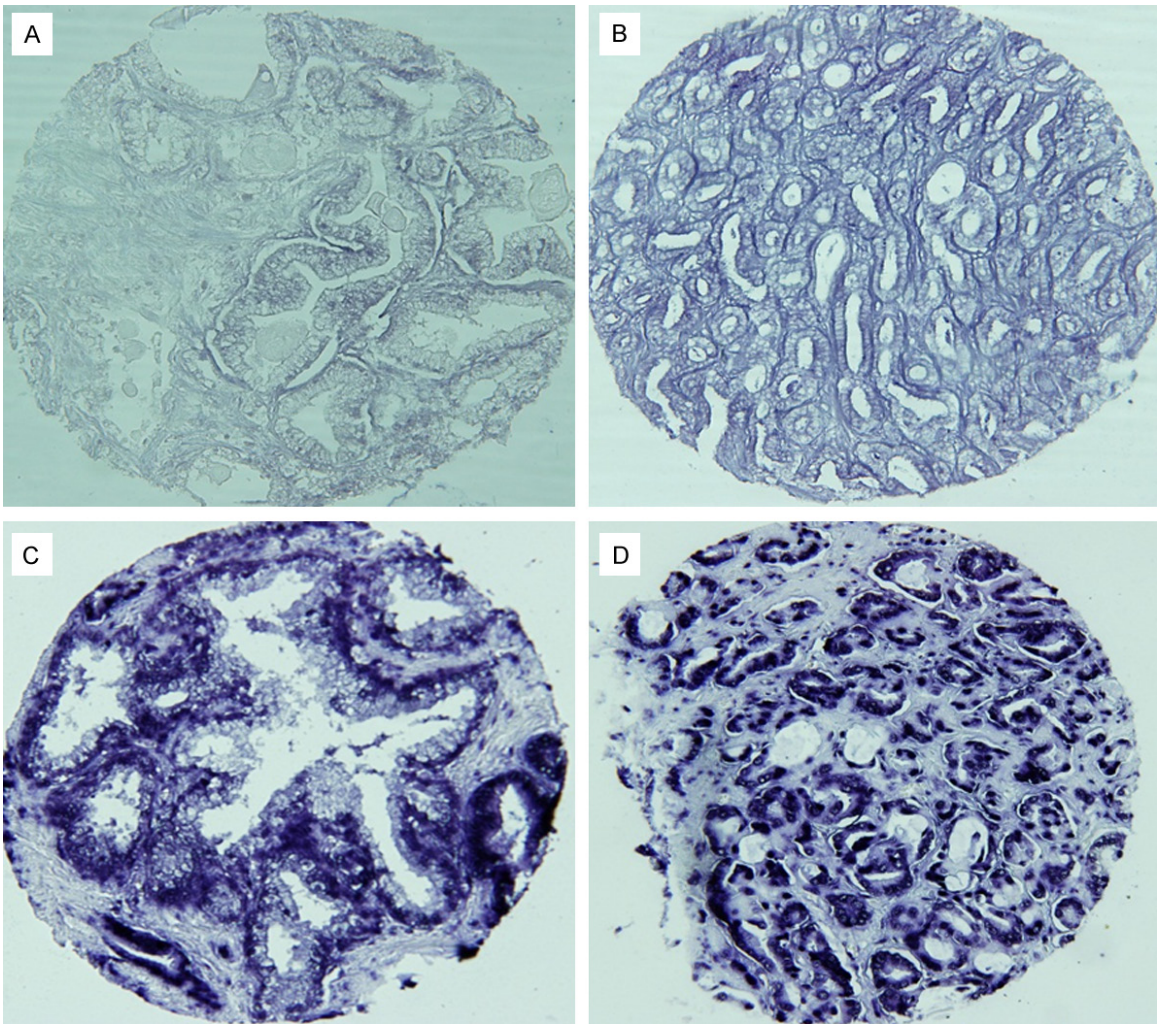

Figure 3. miRNA expression by LNA based in situ hybridization. A. Low levels of miR30c miRNA expression in PCa. B. High level of miR30c miRNA expression in benign prostate. C and D. Levels of U6 expression as internal control.

dissected human PCa and control tissues. Unsupervised hierarchical clustering was performed to cluster the samples and miRNAs based on their expression patterns (**Figures 1** and **2**; Supplementary Figure 1). PCa samples were distintly separated into two groups, group A (n = 11) and group B (n = 16). We further correlated miRNA expression with the clinical and pathological parameters, including race, tumor grade and stage (**Table 1**). Group A consists of PCa with significantly higher percentage (22%) of high grade PCa (GS ≥ 8) compared to Group B (7%) (p = 0.0498). Group A also contains a much higher percentage (30%) of high-stage PCa (T ≥ 3) compared to Group B (15%) (p = 0.022). There is no significant difference between the two groups in race, margin and lymph node status. These results suggest that global miRNA expression profile of tumors can potentially predict PCa progression.

Next, we performed a series of supervised microarray profiling of cellular miRNAs from high grade/stage PCa and low grade/stage PCa to identify systematic differences in miRNA expression patterns during PCa progression. A total of 299 human miRNAs (44%) were significantly differentially expressed between high grade/stage group and low grade/stage group, with false discovery rate lower than 0.05 and fold change higher than 1.3 (Supplementary Table 1). The supervised hierarchical clustering analyses revealed complete separation of the two groups based on the expression profiles of the differentially expressed miRNAs. The majority of the significant miRNA (294, 98%) were

Table 2. PCa related miRNA expression in the epithelial cells in association with clinicopathological parameters

Clinicopathological parameters	Sample Size (N)	let-7c (mean ± SEM)	miR-21 (mean ± SEM)	miR-27a (mean ± SEM)	miR-30c (mean ± SEM)	miR-219 (mean ± SEM)
Malignancy:						
Benign	11	0.545 ± 0.05	0.712 ± 0.119	0.727 ± 0.082	0.621 ± 0.06	0.773 ± 0.082
Malignant	204	0.440 ± 0.02	0.553 ± 0.024	0.560 ± 0.025	0.438 ± 0.022	0.467 ± 0.019
p-value		0.078	0.22	0.077	0.014*	0.004*
Age:						
< 70	147	0.490 ± 0.02	0.621 ± 0.029	0.619 ± 0.0291	0.486 ± 0.025	0.478 ± 0.023
≥ 70	60	0.353 ± 0.05	0.447 ± 0.039	0.478 ± 0.0463	0.361 ± 0.038	0.514 ± 0.035
p-value		0.009*	0.0004*	0.011*	0.214	0.402
Race:						
African American	11	0.348 ± 0.1	0.348 ± 0.097	0.485 ± 0.0965	0.212 ± 0.0748	0.485 ± 0.097
Caucasian	187	0.455 ± 0.02	0.574 ± 0.025	0.572 ± 0.0272	0.452 ± 0.0223	0.485 ± 0.021
p-value		0.309	0.045*	0.405	0.011*	1
GS:						
< 8	57	0.402 ± 0.03	0.636 ± 0.048	0.532 ± 0.031	0.478 ± 0.035	0.504 ± 0.031
≥ 8	147	0.455 ± 0.03	0.521 ± 0.027	0.571 ± 0.033	0.422 ± 0.027	0.452 ± 0.023
p-value		0.189	0.04*	0.39	0.202	0.184
Metastasis:						
No	111	0.491 ± 0.03	0.667 ± 0.037	0.614 ± 0.035	0.514 ± 0.029	0.532 ± 0.028
Yes	80	0.369 ± 0.033	0.463 ± 0.027	0.521 ± 0.036	0.354 ± 0.032	0.448 ± 0.029
p-value		0.007*	< 0.0001*	0.062	0.0003*	0.039*
Tissue Type:						
Hormone Naïve	61	0.340 ± 0.045	0.440 ± 0.048	0.515 ± 0.054	0.305 ± 0.04	0.460 ± 0.039
Hormone Refractory	23	0.521 ± 0.039	0.552 ± 0.033	0.638 ± 0.047	0.501 ± 0.035	0.455 ± 0.03
p-value		0.003*	0.056	0.09	0.0003*	0.909
Tissue Type:						
Neoadjuvant Radicals	36	0.405 ± 0.048	0.549 ± 0.063	0.495 ± 0.037	0.398 ± 0.049	0.465 ± 0.049
Radicals	37	0.484 ± 0.036	0.745 ± 0.048	0.550 ± 0.042	0.574 ± 0.044	0.502 ± 0.036
p-value		0.193	0.015*	0.333	0.009*	0.543
Survival:						
Alive	83	0.471 ± 0.0262	0.636 ± 0.039	0.536 ± 0.025	0.500 ± 0.031	0.523 ± 0.03
Dead	128	0.411 ± 0.0304	0.507 ± 0.03	0.566 ± 0.036	0.406 ± 0.028	0.458 ± 0.025
p-value		0.141	0.009*	0.497	0.024*	0.098
PSA Recurrence:						
None	36	0.419 ± 0.03	0.613 ± 0.048	0.551 ± 0.039	0.609 ± 0.036	0.525 ± 0.035
Yes	41	0.498 ± 0.048	0.661 ± 0.064	0.569 ± 0.042	0.415 ± 0.051	0.510 ± 0.052
p-value		0.163	0.556	0.754	0.003*	0.809
Hormone Therapy:						
Yes	151	0.429 ± 0.027	0.523 ± 0.027	0.552 ± 0.029	0.425 ± 0.025	0.469 ± 0.022
No	64	0.483 ± 0.036	0.652 ± 0.046	0.607 ± 0.047	0.499 ± 0.039	0.514 ± 0.036
p-value		0.229	0.0169*	0.322	0.113	0.284

Student t-test. *indicates statistically significant.

down-regulated in high-grade/stage PCa, suggesting their roles as tumor suppressors. Among the significantly down-regulated miRNAs are *let7-c* (1.6-fold), *miR-21* (1.6-fold), *miR-30c* (1.5-fold), and *miR-219* (2.2-fold).

miRNA expression in PCa epithelium by ISH on TMA

From the above miRNA array analysis, we selected and examined the expression levels of

Table 3. Differential categorization of PCa related miRNA expression in the epithelial cells in association with clinicopathological parameters

Clinicopathological parameters	miRNA/U6 ratio	let-7c	miR-21	miR-27a	miR-30c	miR-219
GS < 8	+ (< 0.4)	20/57 (35%)	11/57 (19%)	9/57 (16%)	15/57 (26%)	14/57 (25%)
	++ (0.4-0.6)	34/57 (60%)	23/57 (40%)	34/57 (60%)	30/57 (53%)	33/57 (58%)
	+++ (> 0.6)	3/57 (5%)	23/57 (40%)	14/57 (25%)	12/57 (21%)	10/57 (18%)
GS ≥ 8	+ (< 0.4)	58/147 (39%)	39/147 (27%)	36/147 (24%)	59/147 (40%)	51/147 (35%)
	++ (0.4-0.6)	57/147 (39%)	75/147 (51%)	71/147 (48%)	62/147 (42%)	75/147 (51%)
	+++ (> 0.6)	28/147 (19%)	33/147 (22%)	40/147 (27%)	26/147 (18%)	21/147 (14%)
Non-metastatic	+ (< 0.4)	35/111 (32%)	21/111 (19%)	19/111 (17%)	29/111 (26%)	31/111 (28%)
	++ (0.4-0.6)	52/111 (47%)	39/111 (35%)	54/111 (49%)	50/111 (45%)	50/111 (45%)
	+++ (> 0.6)	24/111 (22%)	51/111 (46%)	38/111 (34%)	32/111 (29%)	30/111 (27%)
Metastatic	+ (< 0.4)	35/80 (44%)	20/80 (25%)	19/80 (24%)	36/80 (45%)	25/80 (31%)
	++ (0.4-0.6)	37/80 (46%)	52/80 (65%)	43/80 (54%)	38/80 (48%)	47/80 (59%)
	+++ (> 0.6)	8/80 (10%)	8/80 (10%)	18/80 (23%)	6/80 (8%)	8/80 (10%)

Intensity score: + as low intensity; ++ as intermediate intensity; +++ as high intensity.

a panel of PCa related miRNAs (*let7-c*, *miR-21*, *miR-27a*, *miR-30c*, and *miR-219*) in the tumor epithelial cells and stromal cells separately using TMA of 215 cases by ISH analysis (**Figure 3**). The levels of miRNA were scored semiquantitatively and the average expression levels of these miRNAs were calculated and correlated with various clinicopathological factors, including age, race, Gleason score, metastasis, tissue type, survival, PSA recurrence, and hormone resistance (**Table 2**). The expression of two miRNA, *miR-21* and *miR-30c*, were significant different in most of the categories (7 out of 9 categories each). More importantly, both miRNAs were significantly lower in PCa with metastasis ($p < 0.0001$ and $p = 0.0003$ respectively), deceased patients ($p = 0.009$ and 0.02), tumor with neoadjuvant radicals ($p = 0.02$ and 0.01) and PCa of AA race ($p = 0.045$ and 0.01). The expression of *miR-21* was also significantly decreased in high grade tumor (GS ≥ 8) ($p = 0.04$) and slightly increased but not significantly in the androgen-independent tumor epithelial cells ($p = 0.06$). The expression of *miR-219* is significantly decreased in PCa with distant metastasis ($p = 0.04$). When we categorized the miRNA expression into low (+), intermediate (++) or high intensity (+++) groups (**Table 3**), *miR-21*, *miR-219* and *miR-30c* were highly expressed in the low-grade, non-metastatic tumors. The expression of *let-7c* was significantly decreased in PCa with distant metastasis ($p = 0.01$) and androgen-dependent PCa ($p = 0.003$). The expression of *miR-27a* was slightly decreased but not statistically significant in metastatic tumor ($p = 0.06$). There were also a higher percentage of cases with distant metastasis that show weaker *miR-27a* and *let-7c* expression (**Table 3**).

miRNA expression in PCa stromal cells by ISH on TMA

We further examined the expression of the selected miRNAs (*let-7c*, *miR-27a*, *miR-30c*, *miR-219* and *miR-301*) in the tumor-associated stroma in PCa. The correlation of the expression levels of miRNAs with various above clinicopathological factors in PCa was investigated (**Table 4**). The expression of *let-7c* in tumor-associated stroma was significantly decreased in PCa with extraprostatic extension ($p = 0.02$), and in patients with no PSA recurrence ($p = 0.03$). It was slightly decreased but not statistically significant in tumor with advanced tumor stage ($p = 0.08$). We observed no significant difference between the expression of *miR-27a*, *miR-30c*, *miR-219* and *miR-301* in tumor-associated stroma and any of the above mentioned clinicopathological factors.

Discussion

The role of miRNAs in cancer is a rapidly emerging area of investigation. Expression profiling has identified miRNA signatures in cancers that associate with diagnosis, staging, progression, and response to treatment [15]. Potential

Table 4. PCa related miRNA expression in the tumor-associated stromal cells in association with clinicopathological parameters

Clinicopathological parameters	Sample Size (N)	let-7c (mean ± SEM)	miR-27a (mean ± SEM)	miR-30c (mean ± SEM)	miR-219 (mean ± SEM)	miR-301 (mean ± SEM)
Age						
< 70	125	0.889 ± 0.051	0.757 ± 0.045	0.861 ± 0.049	0.850 ± 0.05	0.820 ± 0.053
≥ 70	21	1.03 ± 0.12	0.746 ± 0.092	0.762 ± 0.09	0.722 ± 0.12	0.817 ± 0.11
p-value		0.278	0.913	0.34	0.333	0.984
GS						
< 8	123	0.905 ± 0.05	0.769 ± 0.045	0.859 ± 0.047	0.839 ± 0.051	0.825 ± 0.054
≥ 8	23	0.935 ± 0.13	0.685 ± 0.093	0.779 ± 0.13	0.790 ± 0.11	0.790 ± 0.11
p-value		0.837	0.423	0.553	0.679	0.77
Malignancy						
Malignant	146	0.910 ± 0.047	0.756 ± 0.041	0.846 ± 0.044	0.832 ± 0.046	0.820 ± 0.048
Benign	22	0.758 ± 0.12	0.795 ± 0.13	0.886 ± 0.11	0.833 ± 0.098	0.727 ± 0.11
p-value		0.23	0.764	0.743	0.987	0.442
Extraprostatic Extension						
Yes	68	0.792 ± 0.068	0.728 ± 0.059	0.778 ± 0.066	0.766 ± 0.069	0.760 ± 0.07
None	78	1.01 ± 0.064	0.780 ± 0.056	0.906 ± 0.058	0.889 ± 0.062	0.872 ± 0.067
p-value		0.0184*	0.524	0.149	0.184	0.248
Survival						
Alive	111	0.890 ± 0.054	0.719 ± 0.045	0.821 ± 0.05	0.807 ± 0.053	0.814 ± 0.056
Dead	35	0.971 ± 0.094	0.871 ± 0.089	0.929 ± 0.09	0.910 ± 0.093	0.838 ± 0.096
p-value		0.46	0.134	0.3	0.341	0.827
Stage						
pT2	73	0.991 ± 0.065	0.763 ± 0.057	0.916 ± 0.061	0.886 ± 0.066	0.868 ± 0.068
pT3	72	0.826 ± 0.068	0.745 ± 0.059	0.774 ± 0.063	0.774 ± 0.065	0.769 ± 0.069
p-value		0.0824	0.834	0.11	0.229	0.31
PSA Recurrence						
None	37	0.703 ± 0.098	0.624 ± 0.083	0.847 ± 0.092	0.818 ± 0.1	0.685 ± 0.096
Yes	73	0.963 ± 0.06	0.755 ± 0.055	0.871 ± 0.066	0.833 ± 0.066	0.849 ± 0.067
p-value		0.0266*	0.192	0.831	0.895	0.164

Student t-test. *indicates statistically significant.

miRNA expression signatures specific to PCa have been previously reported [8, 9, 16, 17].

In this study, we performed miRNA expression profiling in PCa to identify its miRNA signature and to classify PCa subgroups. We demonstrated that PCa can be readily classified into high grade/stage and low-grade/stage groups by its miRNA expression profile. We identified 299 miRNAs (44%) that were significantly differentially expressed in the high grade/stage PCa group. Interestingly, the number miRNAs that showed down-regulation (n = 294) in high grade/stage carcinoma samples was much higher than the number of up-regulated miRNAs (n = 5). Previously published miRNA profiling studies have shown down-regulation of miRNAs to be more common than up-regulation in cancers, including PCa [9, 15]. We demonstrated the similar findings in the high-grade/stage PCa, supporting their roles as tumor suppressors. In general, tumor cells are at a reduced differentiation stage compared to normal cells, and the global down-regulation of miRNAs in cancer cells may reflect this difference [15]. Our list of miRNAs that were detected to be differentially expressed in carcinoma samples show overlaps with those previously reported in miRNA profiling studies using Pca samples. For example, 11 of them were reported by Porkka et al., including *miR-21*, *miR-30c* and *let-7c,* to be among the differentially expressed miRNAs that distinguished malignant tissues from normal ones [9], all of which were down-regulated.

We also compared the expression of six interesting miRNAs in the PCa epithelium and tumor-associated stroma from patients with a

wide variety of clinicopathological factors by ISH. Our data indicates that the expression levels of these miRNAs show significant differences in tumor epithelial cells in many clinicopathological categories. *miR-21* was noted in large-scale tumor survey and specific tumor studies to be up-regulated in a variety of tumors, including prostate [3], suggesting its role as oncogenic miRNA. *miR-21* was found to be elevated in PC3 and DU145 androgen-independent cell lines [18]. Our results revealed that *miR-21* expression is slightly but not significantly increased in androgen-independent tumor epithelial cells. Let-7 family miRNA functions as a master regulator of cell proliferation pathways involved in cell cycle functions [19]. Decreased let-7 expression is linked to increased tumorigenesis and poor prognosis [20]. *Let-7c* was found to be down-regulated in prostate carcinoma cells by Porkka and Ozen in the large-scale microarray studies [8, 9]. Our data on the FFPE tissue showed that *let-7c* is down-regulated in metastatic tumor epithelial cells, and is up-regulated in the androgen-resistant tumors. We further studied the expression pattern of *let-7c* in tumor-associated stroma cells and showed it is down-regulated in the stromal cells in tumors with extraprostatic extension. Together, these results support the role of *let-7c* as a general tumor suppressor gene. *miR-219* was reported as a brain-specific miRNA in mouse, functioning in the modulation of circadian clock located in the suprachiastic nucleus [21]. A large-scale study of miRNA expression in the lungs of rats exposed to environmental cigarette smoke shows significant down-regulation of *miR-219* [22]. We found that *miR-219* is down-regulated in metastatic prostatic tumor cells, suggesting it is also a tumor suppressor gene. *miR-30c* is found to be down-regulated in various chemoresistant cancer cell lines [23]. In PCa, *miR-30c* was found to be down-regulated in prostate carcinoma cells by Porkka and Ozen [8, 9], which is consistent with our data, but was reported as up-regulated by Volinia [3]. It is down-regulated in primary, recurrent, metastatic tumors, and tumors treated with neoadjuvant radicals, suggesting it functions as a tumor suppressor. *miR-27a* is an oncogenic miRNA which is overexpressed in gastric adenocarcinoma [24] and breast cancer cells [25]. *miR-27a* is an androgen-regulated oncogene in PCa, acting via targeting the tumour suppressor and AR corepressor [26]. Our data show it is slightly but not significantly down-regulated in metastatic tumors ($p = 0.06$) and slightly but not significantly down-regulated in androgen-sensitive tumors ($p = 0.09$).

To date, few articles have investigated the difference of miRNA expression profiles between AA and CA men. Jones et al. reported recently that differential expression of miRNAs contributes to aggressive PCa in AA [27]. *miR-99b* was significant down regulated in AA PCa as compared to CA and might be related to the aggressiveness associated with AA population [28]. In our study, we found no significant difference between two racial groups by global miRNA expression profiling. However, ISH of *miR-21* and *miR-30c* show significant down-regulation in AA with PCa gland epithelium, suggesting their role in the high-grade and stage tumors within this racial group. This discrepancy could be the result of limitations in our microarray study that the samples are macrodissected with mixed epithelial and stromal components, in stead of microdissected to separate the two components for microarray. miRNA expression in stroma can easily obscure the significant expression difference between the racial groups. Further supporting this observation is that we found a more significant difference between various clinicopathological groups in the ISH studies compared to the microarray.

Of note, a recent publication by Walter et al. [29] reported different sets of miRNA dysregulated in PCa compared to normal epithelium, cancer stroma compared to epithelium, and high-grade compared to low-grade PCa. This difference could be due to several reasons. The difference in study population could lead to the variation, as our sample collection contains a very high percentage of AA patients. Also, this study design is different than ours in that we define low-grade tumors as Gleason score 6 or less for the microarray study compared to them using Gleason score 7. In our opinion Gleason score 6 or less represents a more pure group of low-grade tumors. In addition, their study used very stringent criteria for selecting the miRNA candidates, which may potentially miss some miRNAs. For example, *let-7c*, a well-characterized miRNA dysregulated in PCa in many studies including ours, was not identified by Walter et al. In addition, we showed *miR-30c* is down-regulated in PCa in our study, consistent with most other published results [8, 9], while their group showed it to be up-regulated in PCa by 3.4-fold.

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Disclosure of conflict of interest

None to disclose.

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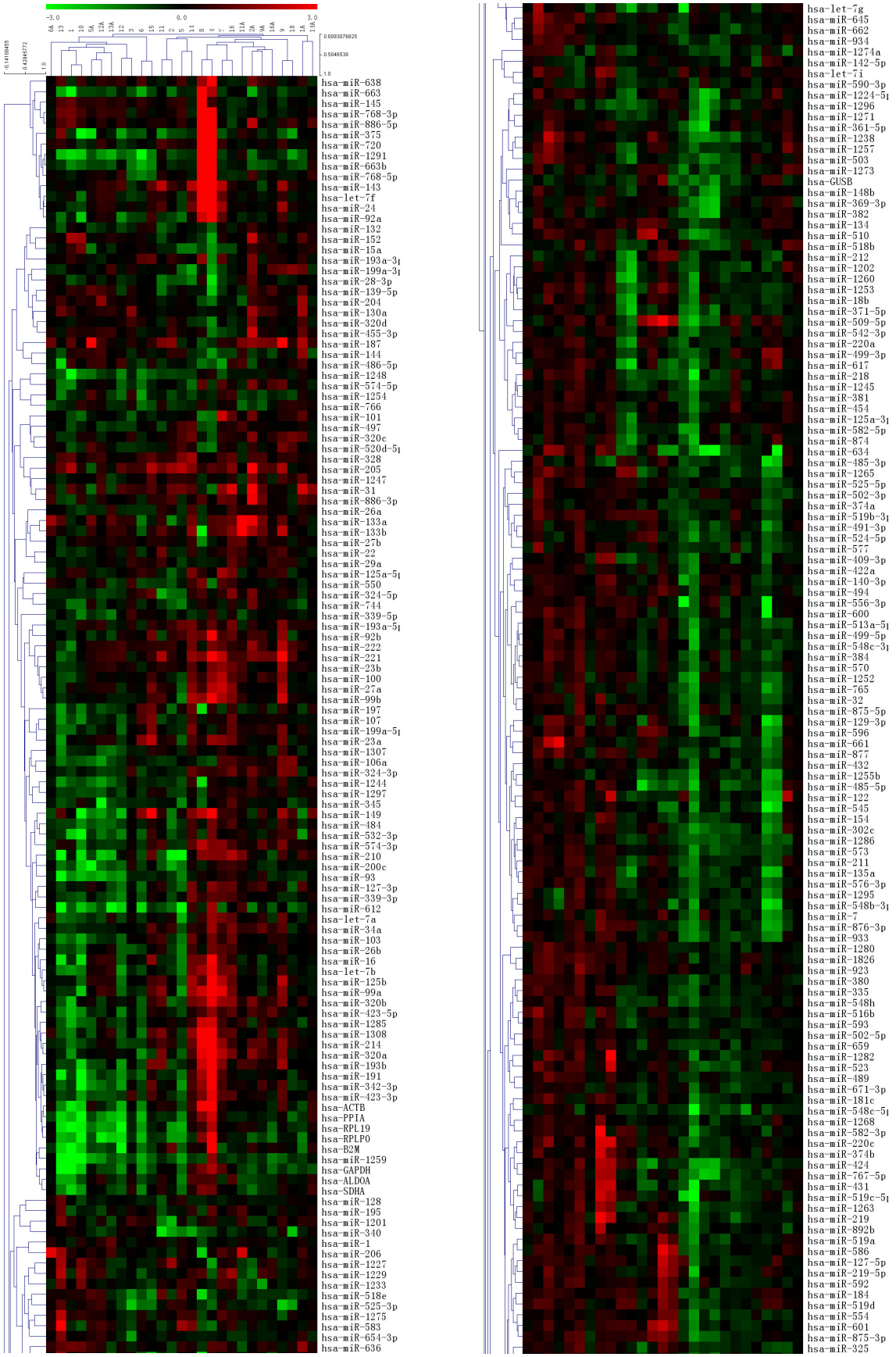
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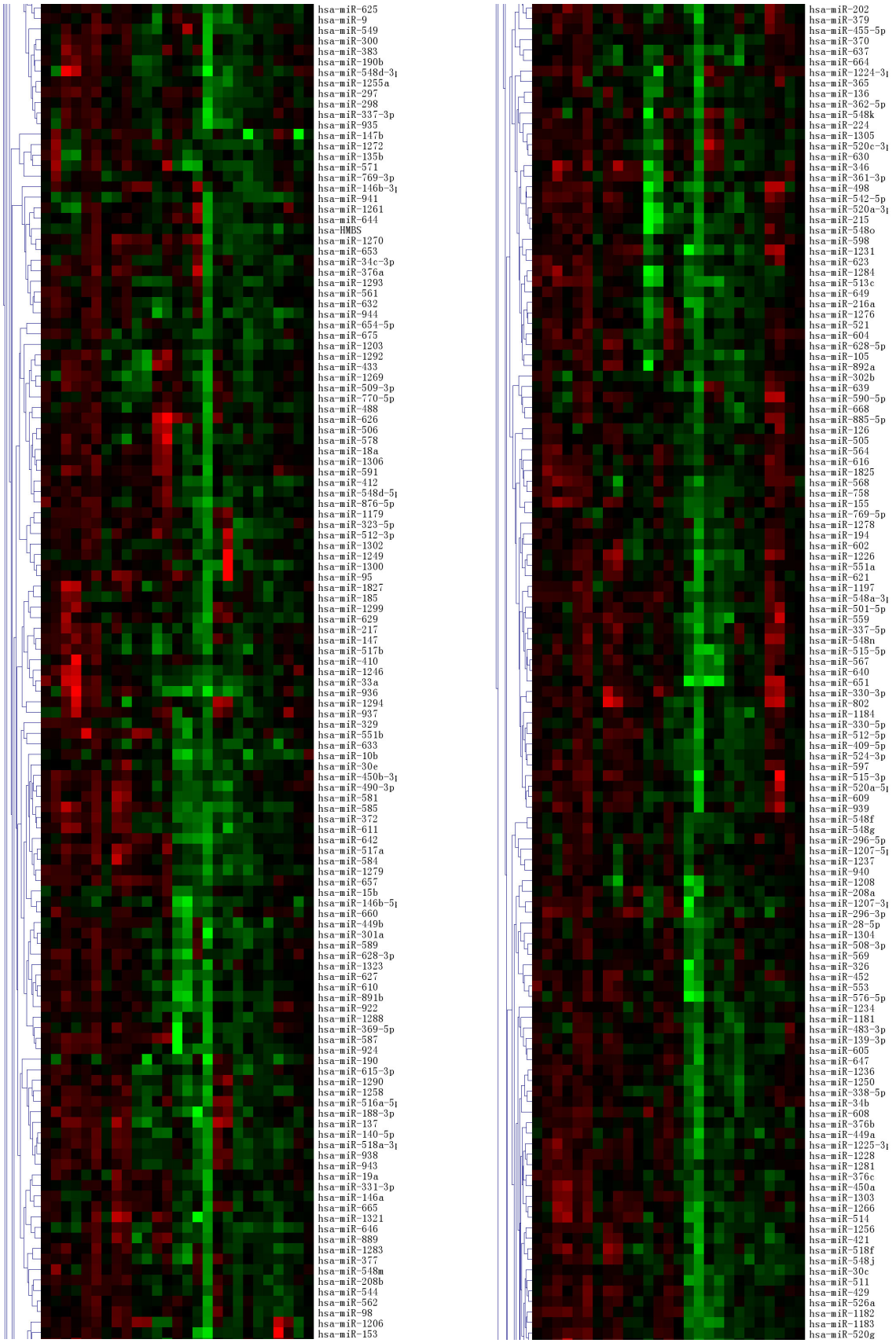
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hsa-miR-208a
hsa-miR-1207-3
hsa-miR-296-3p
hsa-miR-28-5p
hsa-miR-1304
hsa-miR-508-3p
hsa-miR-569
hsa-miR-326
hsa-miR-452
hsa-miR-553
hsa-miR-576-5p
hsa-miR-1234
hsa-miR-1181
hsa-miR-483-3p
hsa-miR-139-3p
hsa-miR-605
hsa-miR-647
hsa-miR-1236
hsa-miR-1250
hsa-miR-338-5p
hsa-miR-34b
hsa-miR-608
hsa-miR-376b
hsa-miR-449a
hsa-miR-1225-3
hsa-miR-1228
hsa-miR-1281
hsa-miR-376c
hsa-miR-450a
hsa-miR-1303
hsa-miR-1266
hsa-miR-514
hsa-miR-1256
hsa-miR-421
hsa-miR-518f
hsa-miR-548j
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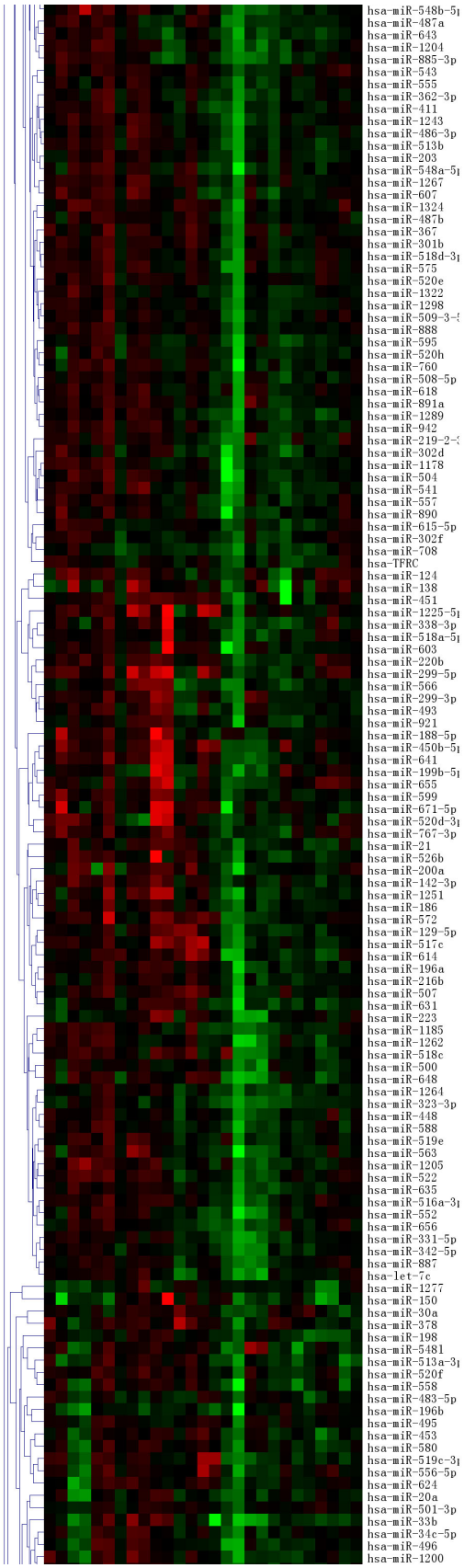

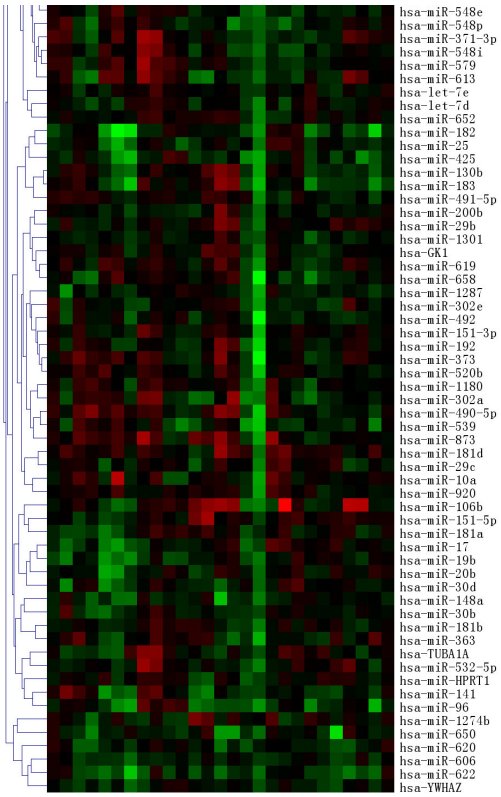

Supplementary Figure 1. Unsupervised analysis of miRNA expression data (complete).