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Original Article

A non-invasive miRNA based assay to detect bladder cancer in cell-free urine

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Abstract: RNA from cell-free urine was analyzed in an attempt to identify a microRNA (miRNA) profile that could be used as a non-invasive diagnostic assay to detect the presence of urothelial carcinoma of the bladder (UCB) and provide a discriminatory signature for different stages of progression. In addition, the presence of specific miRNAs co-isolating with urinary extracellular vesicles/exosomes was investigated. RNA was isolated from cell-free urine of patients diagnosed with UCB (TaG1, T1G3, ≥T2, CIS) and control patients (healthy control and UCB patients with no evidence of disease). MiRNAs were profiled by qRT-PCR array on pooled samples within each group. Validation of the miRNAs was performed on individual samples using qRT-PCR. Extracellular vesicles were isolated via ultracentrifugation. 236 miRNAs were detected in at least one of the pooled samples. Seven of the miRNAs validated on individual samples had significantly higher levels in the cancer group. A panel of miRNAs discriminated between cancer and cancer-free patients with a sensitivity of 88% and specificity of 78%, (AUC=88.8%). We recorded a sensitivity of 80% for TaG1, 95% for T1G3, 90% for ≥T2 with specificity of 77% for healthy controls and 80% for no evidence of disease. Select miRNAs were detected in extracellular vesicles of UCB patients and healthy controls, albeit at different levels. Utilizing this non-invasive assay, we identified miRNA capable of detecting UCB and distinguishing different stages of progression, providing evidence that miRNA profiling in cell-free urine holds promise for the development of valuable clinical diagnostic tools.

Keywords: microRNA, urine, urinary bladder neoplasm, cell-free system, exosomes

Introduction

Urothelial carcinoma of the bladder (UCB) is the fifth most commonly diagnosed cancer in the United States, with an estimated incidence of 74,000 and an estimated mortality of 16,000 in 2015 [1]. Transitional cell carcinoma, the most common form of UCB, can be divided into two groups, non-muscle invasive (NMI: Ta/T1) and muscle-invasive (MI: ≥T2), defined by distinct behaviors and different molecular profiles [2]. NMI tumors account for 75-80% of UCBs and can be effectively treated, although recurrence is common (2). These patients undergo regular surveillance by cystoscopy and urine cytology. Given that 50% of UCBs on average are fatal once they become MI [2], the goal of screening is to improve the prognosis by facilitating the detection and treatment of early-stage tumors before they become MI cancers. This may permit more bladder sparing approaches to treatment, thereby resulting in reduced morbidity and mortality.

Altered microRNA (miRNA) expression profiles have emerged as potential indicators of disease progression. MiRNA are small (18-22 bp) non-coding RNA that are involved in post-transcriptional gene regulation and have been shown to play a role in different cancers, including bladder, acting as major regulators of genes involved in oncogenesis and tumor suppression [3, 4]. As an example, we established differential expression of 9 miRNA between NMI and MI UCB and have investigated each of their roles in contributing to the invasive phenotype [5].

It is greatly anticipated that miRNA profiling may be useful as a new clinical tool. A number of studies have been reviewed [6, 7] investigating their potential in improving diagnosis and prognosis of UCB. There is much interest in developing non-invasive means of profiling these biomarkers in biofluids such as blood and urine. The use of whole urine as a substrate for miRNA profiling has been reported, demonstrating a miRNA ratio to detect UCB [8]. Several other recent investigations have included the use of miRNA profiles from urine sediment [9-12], or examined specific miRNA in cell-free urine [13, 14], demonstrating the feasibility of a clinical role for miRNA in patient stratification. In this study we profiled miRNA expression within cell-free urine supernatant as well as their potential presence within urinary extracellular vesicles (EVs).

EVs are a heterogeneous class of small membrane vesicles released by cells that serve a variety of functions including intercellular communication [15, 16]. They reportedly contain a variety of biomolecules including miRNA [17], and their utility as reservoirs for biomarkers of oncogenesis has recently been discussed [18, 19]. The presence of EVs in urine was first reported in 2004 [20], and have since been shown to be of interest in the Urologic arena as they harbor markers of UCB [21], prostate cancer [22, 23], and renal disease [24].

The goals of this work were to use cell-free urine, including EV isolates, to study miRNA as a biomarker to differentiate patients with and without UCB as well as to predict disease progression. Such a test could obviate the need for or lengthen the interval between surveillance cystoscopy for cancer detection as well as aide the urologist in decision making for operative intervention.

Materials and methods

Clinical samples and storage

Institutional review board approval was obtained for this prospective study. Urine samples were collected from 45 controls and 85 patients with UCB at the Lahey Clinic Hospital & Medical Center between 2008 and 2010. Samples were grouped into Healthy Control (HC), No Evidence of Disease (NED), TaG1, T1G3, ≥T2 or CIS categories based on disease status. Urine was centrifuged at 3000 rpm at 4°C for 15 minutes. Supernatant was decanted and stored at -20°C until further processing.

All patients with a suspicion of UCB seen at our institution were screened for this study. Patients were excluded from the study if they had an active cancer other than urothelial. Individual samples were excluded if a confirmed diagnosis by a pathologist was not available. Once enrolled in the study, all subsequent cystoscopic evaluation was recorded. Tumors resected were staged and graded according to the AJCC-UICC TNM classification system. Any intravesical or systemic treatments were noted.

Extracellular vesicle isolation

EV isolation, enriching for exosomes, was performed on 40 ml of urine from 4 health control pools (using 3 individuals per pool) and from 9 patients with ≥T2 UCB. Following a method described by Gonzales et al. [25], pre-processed urine (as described above) was subsequently spun at 17,000 g for 10 minutes at 4°C to pellet debris. The resulting cell-free supernatant was spun at 200,000 g for 1 hour at 4°C to isolate the EVs. An aliquot of the supernatant resulting from this high speed spin was retained. The pelleted EVs were resuspended and treated with DTT at 95°C for 2 minutes to remove Tamm-Horsfall protein and increase yield. The ultracentrifugation step was repeated and the resulting pellet was resuspended in ~200 ul of Cell Disruption Buffer (Life Technologies) and stored at -80°C until further processed for RNA or protein analysis.

RNA extraction

Total RNA was extracted from 600 µl of the cell-free and high speed supernatants as well as the isolated EVs (resulting from 40 ml of cell-free urine) using the *mir*Vana™ Paris and the *mir*Vana™ miRNA Isolation Kits respectively (Life Technologies), according to the manufacturer's protocol. Total RNA was quantified and purity was assessed using the Nanodrop® 1000 (Thermo Scientific).

qPCR array expression profiling

Extracted miRNA from each subgroup was pooled to create five groups (HC, TaG1, T1G3, ≥T2, and CIS) for qPCR expression analysis.

Table 1. Patient demographics

	No Cancer	Bladder Cancer
Sample size	45 including: HC=35, NED=10	85 including: TaG1=25, T1G3=20, ≥T2=30, CIS=10
Average age (range)	55 (26-77)	69 (47-87)
Female (%)	31%	27%
Smokers (%)	Unknown	72%
Follow up	1 year	1 year

HC=Healthy Control, NED=previous history of UCB but at time of sample collection there was No Evidence of Disease.

High-throughput RT-PCR was performed for 730 miRNAs as well as positive and negative controls. Briefly, the RNA was polyadenylated and converted to cDNA by reverse transcription. CDNA was then amplified by real-time PCR using the LNA™ UniversalRT microRNA PCR system (Human panel I and II). The amplified product was detected on the Roche Lightcycler 480 by Exiqon. Melting curve analysis was performed to evaluate assay specificity.

Real time quantitative PCR

Differentially expressed miRNAs were validated by qRT-PCR on individual samples, comprised of six groups: HC (n=35), NED (n=10), TaG1 (n=25), T1G3 (n=20), ≥T2 (n=30), and CIS (n=10), using Exiqon miRNA quantification kits (ExiLENT SYBR® Green Master Mix and LNA™ primer sets, Exiqon, Vedbaek, Denmark) following the manufacturer's instructions. Briefly, 4 ng of each RNA sample was converted to cDNA using the Universal cDNA synthesis kit in a 20 µl reaction volume. The resulting reverse transcriptase reactions were diluted 1:25, and 4 µl were used in a 20 µl qPCR using ExiLENT SYBR® Green master mix; each sample was tested in triplicate. Data were plotted as 1/Ct to reflect the inverse relationship of Ct and miRNA expression.

Western blot analysis

Samples were prepared in a final concentration of 1x SDS-Laemmli-DTT, boiled for 5 minutes, and loaded in each lane of a 7.5% or 12.5% polyacrylamide gel. Proteins were transferred overnight onto nitrocellulose. Membranes were blocked in 10% milk in TBS with 0.05% Tween (TBST) and placed on primary antibody overnight at 4°C. Antibodies to Alix (1:1000) (Cell Signaling Technology, Danvers, MA) and TSG101 (1:500) (Abcam, Cambridge, MA) were used in Western blot analysis. Following incubation with the primary antibody, blots were washed in TBST, three times for 15 minutes each, and secondary antibody linked to horseradish peroxidase was incubated with the blots for 1 hour at room temperature. Blots were then washed as described above and developed with an ECL kit (Amersham, Arlington Heights, IL).

Statistical analysis

MiRNAs with differential expression levels between cancer patients and healthy controls were determined via t-test. To identify miRNA that had differential expression between different cancer categories and healthy controls ANOVA was used. Pairwise post-hoc comparisons (with Tukey's adjustment) were used to identify miRNAs with significantly different expression levels between each pair of groups. To account for multiple comparisons we used Bonferroni correction. Two tailed *p*-values <0.05 were considered significant. Prior to analyses Ct values were inverted to obtain expression-levels. Stepwise selection in logistic regression was used to build a classifier discriminating between miRNA levels of all groups (HC, NED, TaG1, T1G3, ≥T2, and CIS), as well as a model excluding patients with only CIS disease. Statistical analysis was conducted using SAS v9.2 (Copyright (c) 2002-2010 by SAS Institute Inc., Cary, NC, USA) and statistical software R, packages *base* and *pROC*.

Results

Patient demographics

One hundred and thirty patients were enrolled in the study (**Table 1**). Samples were categorized into six groups according to pathologic diagnosis: HC (35), TaG1 (25), T1G3 (20), ≥T2 (30), NED (10), and CIS (10). The HC and NED groups were combined to create the control

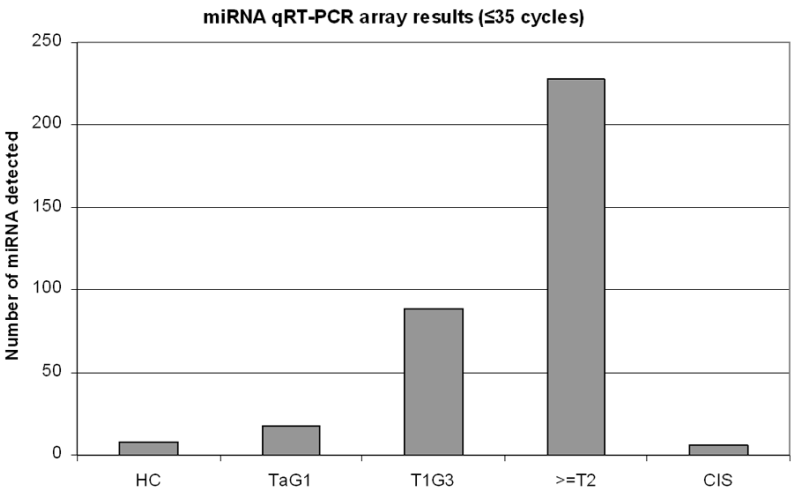

Figure 1. miRNA qRT-PCR array results. The number of microRNAs detected by qRT-PCR array analysis, per sample type, using a cycle threshold (Ct) cutoff of 35.

group and the TaG1, T1G3, ≥T2 and CIS comprised the cancer group.

Increased numbers of microRNA are detected in the urine of UCB patients

Cell-free RNA was isolated from urine of 35 healthy controls and 85 patients with UCB. The amount of total RNA extracted from cancer samples (mean; 1.38 ug) was significantly higher ($p<0.01$) than that from controls (mean; 0.74 ug). Pooled samples were used in a qRT-PCR miRNA array analysis. Of the 730 miRNA tested (Supplemental Table 1), 236 were detected in at least one of the pools using the Ct cutoff of ≤35. The number of miRNA detected in each pool exhibited a trend corresponding to disease progression where the number of miRNA detected in the cell-free extract from controls was 8, 18 in TaG1, 89 in T1G3, 228 in ≥T2, and 6 in CIS (**Figure 1**).

Differential expression of miRNA in urine of UCB patients

MiRNA detected across all cancer groups showed a trend toward increased expression throughout disease progression in the qRT-PCR array (Supplemental Table 1). A selection of differentially expressed miRNA was validated by qRT-PCR on individual samples. Evaluations of the residuals of these data were determined to be approximately normal. Mean miRNA levels in cancer and cancer-free samples demonstrated primarily increased expression in UCB patients (**Figure 2A**). Of the miRNA analyzed, seven had significantly higher levels in the cancer group ($p<0.05$) (**Figure 2B**). This increase in expression was highest for patients with T1G3 and ≥T2 disease (**Figure 3** and data not shown). For example, miR-940 was differentially expressed between invasive cancer and cancer-free samples, and its levels corresponded with disease progression which were highest in the more advanced cancers (T1G3 and ≥T2) and lowest in HC/NED samples (**Figure 3**). Likewise levels of miR-26a were higher in invasive cancers, relative to non-invasive tumors; however of critical importance to patient screening, its expression varied significantly between HC/NED and non-invasive UCB samples (**Figure 4**).

Levels of both individual miRNA and groups of miRNA were tested for their ability to detect cancer in the cell-free urine preps. A classifier based on four miRNA (miR-26a, miR-93, miR-191, and miR-940) was built using logistic regression with cancer presence being an outcome. It resulted in sensitivity of 70% with 95% CI [56%-84%] and specificity of 84% with 95% CI [74%-95%], (AUC=85.8%, **Figure 5A**). For the different stages it resulted in a sensitivity of 64% for the TaG1 group, 85% for T1G3, 87% for ≥T2, and 40% for CIS. The specificity was 83% for the HC and 90% for NED. When the CIS patients were removed from the analysis it resulted in an overall sensitivity of 88% with a 95% CI [81-95%] and specificity of 78% with a 95% CI [66-90%], (AUC=88.8%, **Figure 5B**). For the subgroups it resulted in sensitivity of 80% for TaG1, 95% for T1G3, 90% for ≥T2 with specificity of 77% for HC and 80% for NED.

Extracellular vesicle isolation confirmed via western blot analysis

We investigated whether or not the miRNA detected in cell-free urine of UCB and HC patients are primarily in EVs. EVs were isolated from urine via ultracentrifugation and their

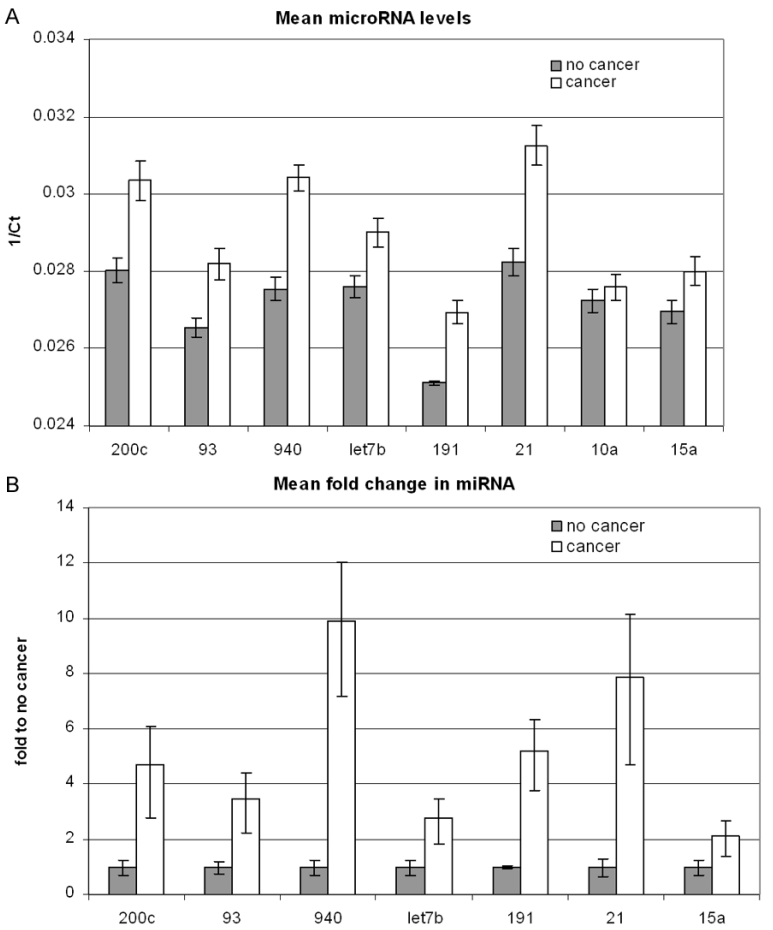

Figure 2. A: Mean miRNA levels in cancer and cancer-free samples. Error bars represent standard error above and below the mean. B: Mean fold change in cancer versus cancer-free samples. The 7 microRNAs with significantly higher levels in the cancer group ($p<0.05$) expressed as a mean fold increase relative to cancer-free samples. Error bars represent standard error above and below the mean.

presence in the pelleted sample was confirmed by the detection of Alix and TSG101 via Western Blot analysis (**Figure 6**). Both proteins are markers characteristic of exosomes [26], and their levels are markedly increased in the pelleted portion of the samples compared to the initial cell-free supernatants. Additionally, the amount of protein detected in the urine from ≥T2 UCB patients was greater than that observed in the urine from healthy controls indicating a greater number of exosomes were present in the urine of these patients.

Differential expression of microRNA within urinary EVs

A selection of four miRNAs (mir-21, miR-93, miR-200c, and miR-940) was tested by qRT-PCR on the EV pellets and supernatant, and all were found to be present in both the HCs and UCB patients (**Figure 7** and data not shown). However, the expression patterns varied between the two sample types. All of these miRNA were expressed at higher levels in the cell-free portion of UCB samples versus HCs. On average the level of miR-940 (**Figure 7A**) ranged from 7.7 fold higher in the cell-free supernatant of UCB samples compared to 12.2 fold higher in the EV pellets, when compared to HCs. In contrast, the level of miR-93 (**Figure 7B**) was only 1.6 fold higher in the EV pellet of UCB patients whereas it was 37.8 fold higher in the EV depleted high speed supernatant, as compared to HCs.

Discussion

A cost-effective, highly sensitive method of early stage UCB detection remains a major challenge in the prevention of cancer-related morbidity and death, and high-risk populations may derive the most benefit from this strategy. The costs associated with surveillance for recurrent disease make UCB the most expensive tumor to treat per patient per year as well as per lifetime [27]. A low-cost sensitive and specific screening assay could enable detection of a larger proportion of tumors at their earliest most treatable stage, as well as dramatically reduce health care expenditures. UCB tumors usually manifest abnormalities detectable in urine, including hematuria, as well as tumor-related antigens that are shed into urine or lie within exfoliated malignant cells. Urine cytology is the most specific test for disease recurrence, but is poorly sensitive, particularly for low grade disease. Detection strategies have been developed to improve sensitivity based on non-cellular protein assays, cell-based assays used in conjunction with cytolo-

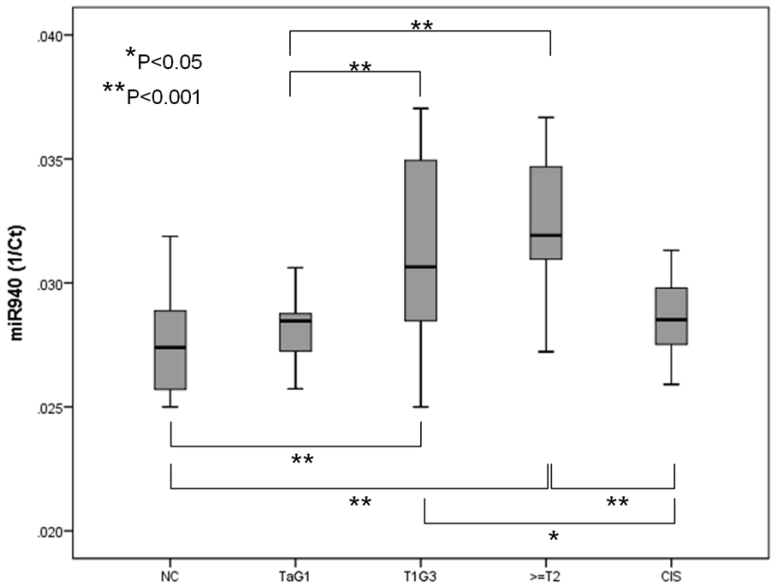

Figure 3. MiRNA-940 levels concordant with disease progression. The mean levels of miRNA-940 correlates with disease progression with significant differences between stages: as determined by one-way ANOVA [F(4,125)=26.936, p<0.001]. NC=no cancer.

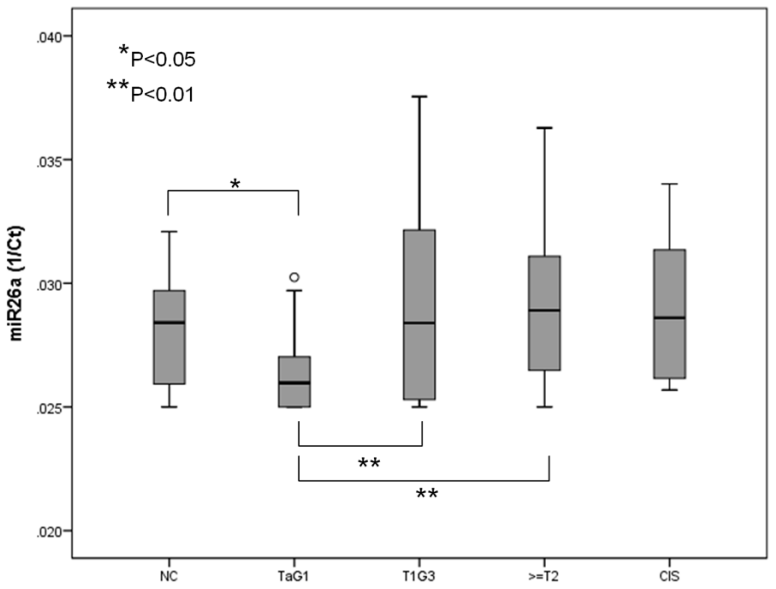

Figure 4. MiRNA-26a levels. Expression of miR-26a is lower in non-invasive UCB patients with significant differences between stages: as determined by one-way ANOVA [F(4,125)=4.902, p=0.001]. NC=no cancer.

gy, or fluorescence in-situ hybridization (FISH) for specific chromosomal alterations. Unfortunately, these assays are still skewed toward detection of high grade or specific subsets of disease, are expensive and have not had sufficient sensitivity or specificity to replace cystoscopy as a standard surveillance method.

This study describes a non-invasive diagnostic test utilizing cell-free urinary miRNA levels to detect UCB, including low and high-grade disease. Although the sensitivity for detecting CIS samples was poor, a status readily detected by cytology, it was able to make a critical distinction between MI and NMI disease. In addition, there was a correlation of the quantity of cell-free miRNA associated with presence of UCB and with disease progression. Relatively few miRNA were detected in the healthy control group (eight) whereas the ≥T2 group expressed 228 distinct miRNA. Expression levels of seven miRNA were increased in samples from patients with UCB compared to those from cancer-free patients. The best classifier was based on 4 miRNA, miR-26a, miR-93, miR-191, and miR-940 and discriminated disease-free patients from those with UCB with 88% sensitivity and 78% specificity. To our knowledge this is the first report of these miRNAs in the urine of cancer patients; although miR-26a, miR-93, miR-191 have been reported to have altered expression in other cell-free matrices of such patients [28, 29].

The function, composition, and extracellular distribution of EVs are of current interest throughout many fields and are of particular interest to researchers of oncogenesis. With respect to this study in cell-free urine, their co-isolation with miRNA discriminatory of UCB further highlights their importance and potential utility as reservoirs of diagnostic markers; especially in light of their apparent ability to shelter biomarkers, such as RNA, from degradation [23]. Interestingly, although the

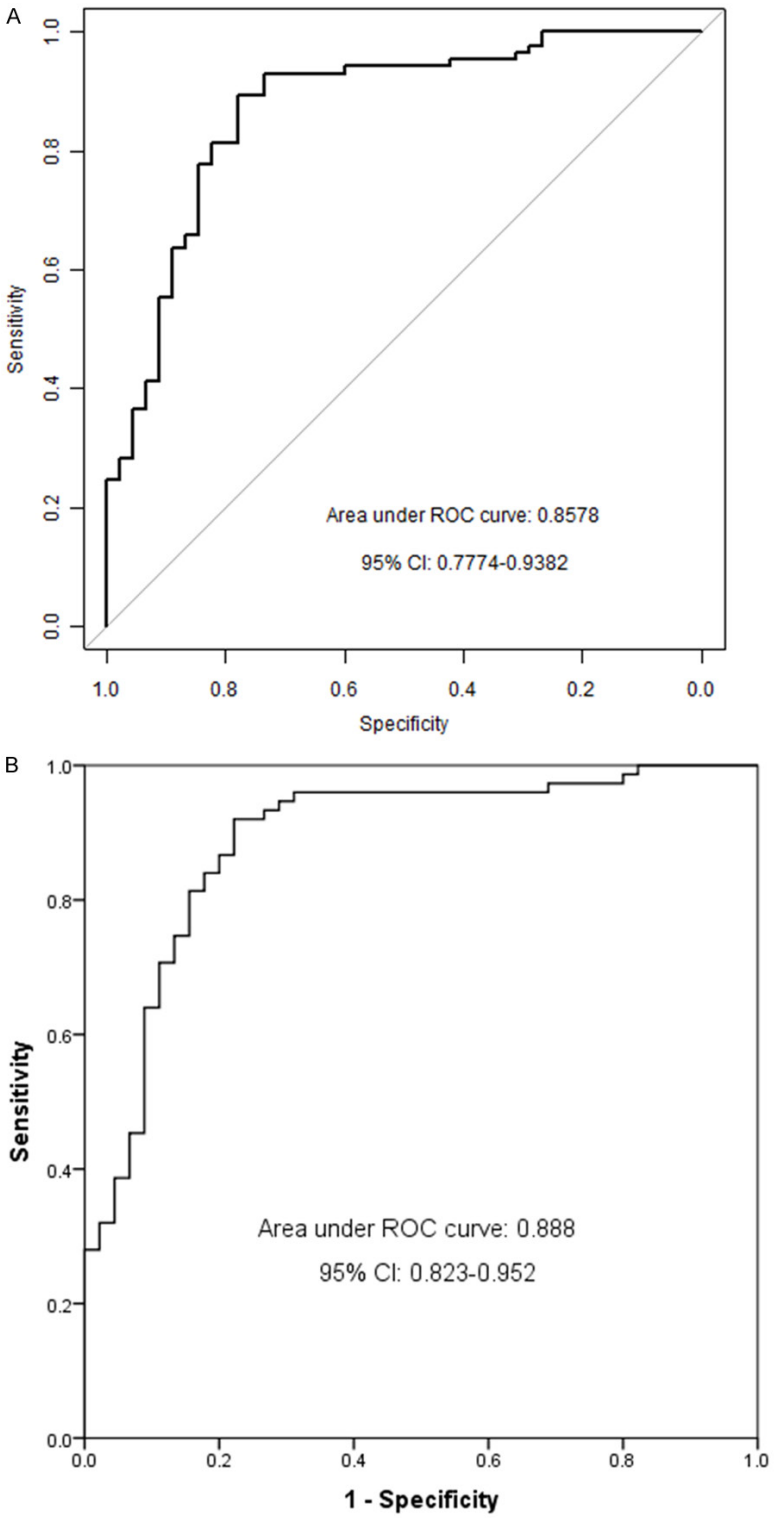

Figure 5. A: ROC Curve for the classifier using miR-26a, miR-93, miR-191, and miR-940: including CIS Samples. B: ROC curve for the classifier using miR-26a, miR-93, miR-191, and miR-940: excluding CIS samples.

data presented here are limited in this regard, the quantity and distribution of miRNA associated with EVs in UCB patients appears to differ from that of healthy controls. This is supported by the observation that the level of miR-93 was lower in the EV enriched pellet of UCB patients, relative to healthy controls, compared to the complementary depleted urine supernatant, and contrary to the relatively consistent results for miR-940. However, this observation is in alignment with studies recently reviewed by Hessvik et al indicating that while some extracellular miRNA are located within urinary EVs, others are not [30]. From a diagnostic perspective such differences in the distribution of biomarkers between samples has the potential for further refining their use in disease stratification. Their potential role facilitating communication between cancer cells and/or seeding normal cells with oncogenic factors, such as miRNA, warrants additional investigation. As do potential therapies targeting these mechanisms, especially within the bladder where intravesical treatment is an option.

The problem with the currently available urine-based molecular tests is that they aren't sufficiently accurate to replace cystoscopy, and can be particularly ineffective in the detection of low grade lesions. As our data reveal, a greater number of miRNA were detectable in cancer samples versus controls and this number correlated with disease progression. Given these results, the presence of increased levels of circulating miRNA in patients with UCB presents a simple and relatively inexpensive assay for cancer detection. A

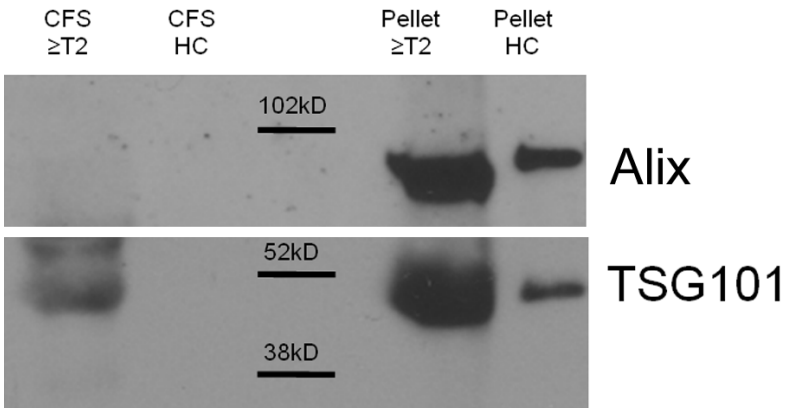

Figure 6. Western Blot Analysis of Alix and TSG101. Exosomal markers Alix and TSG 101 are concentrated in the EV pellets compared to the cell free urine supernatant (CFS) in both healthy control (HC) and cancer samples (≥T2). Increased levels of both markers are present in cancer samples compared to healthy control.

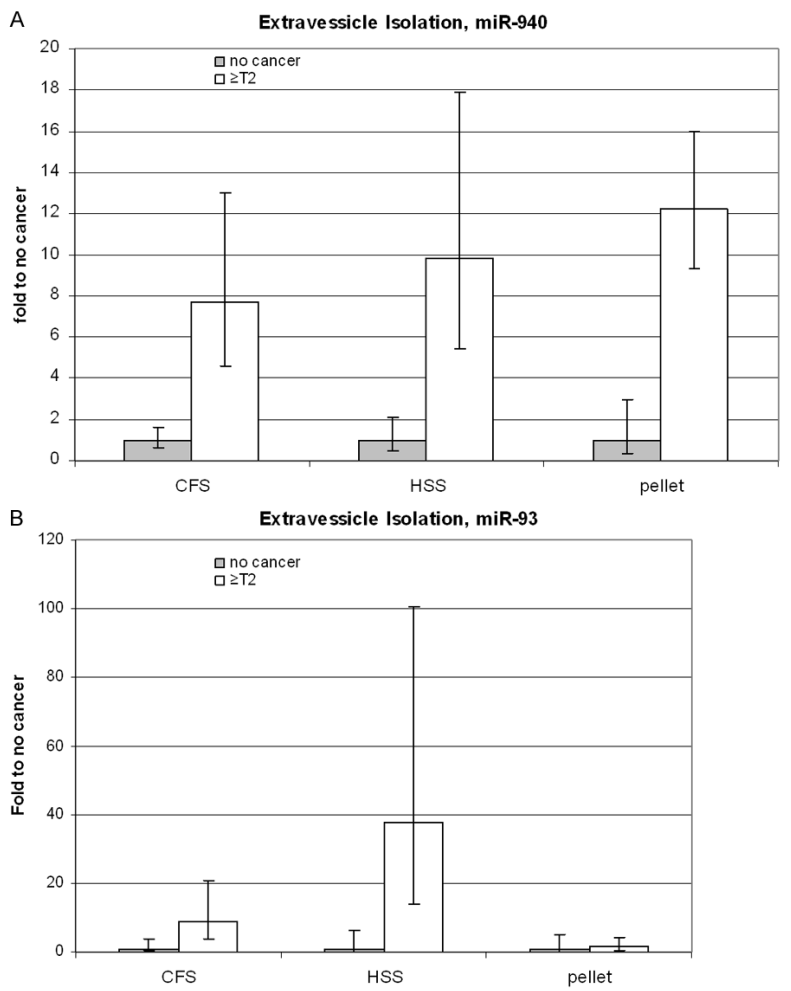

Figure 7. A: Mean fold level differences of miR-940 in urine samples before and after EV isolation. The levels of miR-940 are similar before (cell free supernatant (CFS)) and after (high speed supernatant (HSS) and pellet) EV isolation, expressed as a mean fold increase relative to healthy control. Error bars represent standard error above and below the mean. B: Mean fold level differences of miR-93 in urine samples before and after EV isolation. The relative level of miR-93 is considerably higher in the depleted high speed supernatant after EV isolation for cancer samples compared to healthy controls. This indicates a greater proportion of miR-93 is located outside of EVs in the urine of UCB patients. Error bars represent standard error above and below the mean.

low cost and accurate miRNA panel could be widely utilized for patients with UCB with several important advantages. Earlier detection may prevent progression to MI disease, with a corresponding reduction in cancer-specific morbidity and mortality. In addition, a highly accurate and affordable test could potentially replace cystoscopy in selected patients with a history of UCB who require this procedure on a routine basis. Finally, a test with a panel of biomarkers could provide a unique molecular signature for a patient's tumor which could potentially have clinical significance in terms of prognosis, and monitoring or predicting response to a specific treatment.

Conclusion

In summary, this study is the first to our knowledge to demonstrate the successful profiling of miRNAs from cell-free urine of bladder cancer patients. Utilizing noninvasive urine based assays; we identified a miRNA panel that has the potential to discriminate between cancer-free patients and patients with UCB. These findings

warrant a prospective validation study examining a larger cohort of patients from multiple institutions; however they provide evidence that profiling of miRNAs from cell-free urine holds the promise for the development of valuable clinical tools.

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Supplemental Table 1. qPCR Array Results

Ddesign_target_name	Layout	Healthy con	TaG1	T1G3	≥T2	CIS	Average	Count
hsa-miR-21	1		33.68	29.36	23.84	35.77	30.66	4
hsa-miR-200c	1	38.20	34.78	29.73	24.69	36.51	32.78	5
hsa-miR-141	1		37.08	30.77	25.30	39.18	33.08	4
hsa-miR-200b	1		37.75	31.21	25.79	38.64	33.35	4
hsa-miR-19a	1		36.52	32.55	25.96		31.68	3
hsa-miR-200a	1		35.84	31.19	26.00		31.01	3
hsa-miR-125a-5p	1		34.33	32.10	26.57	36.01	32.25	4
hsa-miR-375	1		35.07	36.39	26.98		32.81	3
hsa-miR-93	1	35.57	34.93	32.66	27.15	35.95	33.25	5
hsa-miR-210	1		35.74	33.79	27.19	37.20	33.48	4
hsa-miR-205	1		34.30	29.97	27.31	34.57	31.54	4
hsa-miR-20a	1		38.00	34.70	27.42		33.37	3
hsa-miR-16	1		38.82	33.57	27.45		33.28	3
hsa-miR-429	1		37.37	33.92	27.49		32.93	3
hsa-miR-27a	1		36.20	32.96	27.68		32.28	3
hsa-miR-103	2		36.55	32.25	27.75	36.01	33.14	4
hsa-miR-24	1		36.13	33.26	27.76		32.38	3
hsa-miR-103	1			31.71	27.81		29.76	2
hsa-miR-26a	1	36.47	34.79	31.75	27.82	35.83	33.33	5
hsa-let-7b	1		37.06	32.52	27.84	36.49	33.48	4
hsa-miR-451	1		38.64	31.44	27.86	36.65	33.65	4
hsa-miR-17	1			33.74	27.88		30.81	2
hsa-miR-181a	1			34.56	27.88		31.22	2
hsa-miR-106a	1			33.70	27.90		30.80	2
hsa-miR-27b	1		35.57	33.17	27.94		32.23	3
hsa-miR-23a	1		35.09	33.19	27.96	37.59	33.46	4
hsa-miR-34a	1			33.50	28.03		30.77	2
hsa-miR-30d	1		36.28	32.61	28.15	36.19	33.31	4
hsa-miR-940	1	37.02	34.69	30.95	28.21	35.46	33.27	5
hsa-miR-29c	1		37.02	32.29	28.33		32.55	3
hsa-miR-425	1			33.94	28.43	39.47	33.94	3
hsa-miR-320b	2	36.85		32.43	28.45		32.58	3
hsa-miR-99b	1			34.67	28.46		31.57	2
hsa-miR-320a	1		38.02	31.84	28.58	36.07	33.63	4
hsa-miR-107	1		36.14	32.70	28.61		32.49	3
hsa-miR-23b	1		37.33	32.90	28.61	36.65	33.87	4
hsa-miR-30e	1		36.28	33.79	28.63	37.27	33.99	4
hsa-let-7g	1		37.29	32.97	28.70		32.98	3
hsa-let-7a	1		35.79	32.72	28.79		32.43	3
hsa-miR-186	1		36.88	35.95	28.83		33.89	3
hsa-miR-342-3p	1		36.83	34.74	28.90		33.49	3
hsa-miR-223	1		33.58	30.92	29.16	34.78	32.11	4
hsa-miR-29b	1			33.73	29.18		31.45	2
hsa-miR-423-3p	1			33.82	29.29		31.56	2
hsa-miR-25	1			35.31	29.30		32.30	2
hsa-miR-10a	1	36.42	34.90	32.18	29.32	36.71	33.91	5
hsa-miR-30b	1	38.40	35.16	32.56	29.45	38.76	34.87	5
hsa-miR-15b	1			35.19	29.56		32.38	2

hsa-miR-193b	1			33.16	29.64		31.40	2
hsa-miR-934	1	33.18	33.85	32.94	29.66	32.98	32.52	5
hsa-miR-191	1	36.78	35.44	34.00	29.68	38.23	34.83	5
hsa-miR-191	2	36.18	37.91	33.85	29.68	35.71	34.67	5
hsa-miR-185	1		36.79	33.77	29.75		33.44	3
hsa-miR-216a	1				29.78		29.78	1
hsa-miR-30a	1		34.96	31.53	29.82	38.11	33.60	4
hsa-miR-148a	1			33.63	29.82		31.73	2
hsa-let-7i	1		37.98	35.49	29.82		34.43	3
hsa-miR-324-5p	1		37.03	35.14	29.83		34.00	3
hsa-miR-1979	1		33.87	32.60	29.84	34.99	32.82	4
hsa-miR-423-5p	2		37.18	33.35	29.86		33.46	3
hsa-miR-148b	1			35.58	29.86		32.72	2
hsa-miR-31	1		37.86	32.98	29.89		33.58	3
hsa-miR-96	1			38.13	29.90		34.01	2
hsa-miR-30c	1		36.72	32.80	29.90		33.14	3
hsa-miR-365	1		36.90	35.59	29.91		34.13	3
hsa-miR-33a	1		38.51	35.24	29.94		34.56	3
hsa-miR-217	1				29.95		29.95	1
hsa-miR-106b	1		38.37	34.84	30.04		34.42	3
hsa-miR-151-3p	1			35.67	30.04		32.86	2
hsa-miR-197	1	39.23		34.50	30.05	37.86	35.41	4
hsa-miR-423-5p	1		36.50	34.32	30.06		33.62	3
hsa-miR-1974	2		34.80	32.98	30.12	35.15	33.26	4
hsa-miR-151-5p	1		37.23	34.09	30.15		33.83	3
hsa-miR-10b	1		35.68	32.49	30.19		32.79	3
hsa-miR-149	1			35.86	30.25		33.05	2
hsa-miR-663	1	35.21	34.77	32.86	30.31	36.66	33.96	5
hsa-miR-497	1				30.33		30.33	1
hsa-miR-26b	1			34.01	30.34		32.17	2
hsa-miR-181d	1		39.51	36.45	30.44		35.47	3
hsa-miR-324-3p	1	36.86	37.04	35.00	30.44	37.87	35.44	5
hsa-let-7d*	1				30.45		30.45	1
hsa-miR-29a	1		36.90	35.50	30.46	39.60	35.62	4
hsa-miR-221	1		36.86	33.03	30.46		33.45	3
hsa-miR-484	1				30.55		30.55	1
hsa-miR-551b	1				30.57		30.57	1
hsa-miR-222	1	38.13	36.67	34.26	30.62	37.29	35.39	5
hsa-miR-192	1		37.04	35.50	30.63		34.39	3
hsa-let-7f	1		38.39	34.63	30.67		34.56	3
hsa-miR-652	1			36.45	30.75		33.60	2
hsa-miR-574-3p	1		36.76	32.84	30.78	36.71	34.27	4
hsa-miR-22	1		37.26	34.61	30.78		34.22	3
hsa-miR-28-3p	2			35.76	30.88	36.97	34.54	3
hsa-miR-378	1		39.96	33.80	31.01		34.92	3
hsa-miR-425*	1				31.03		31.03	1
hsa-miR-195	1				31.06		31.06	1
hsa-miR-338-3p	1			36.05	31.16		33.61	2
hsa-miR-17*	2		37.98		31.21		34.59	2

hsa-miR-196b	1				31.32		31.32	1
hsa-miR-361-3p	1		39.14		31.43		35.29	2
hsa-miR-187	1	35.56	37.39	33.86	31.43		34.56	4
hsa-miR-28-5p	1			35.46	31.48		33.47	2
hsa-miR-200b*	2			37.77	31.56		34.67	2
hsa-miR-424	1			33.43	31.58		32.51	2
hsa-miR-194	1		38.07	36.67	31.59		35.44	3
hsa-miR-449a	1				31.61		31.61	1
hsa-miR-181c	1			37.42	31.66		34.54	2
hsa-miR-135a	1		39.36	37.06	31.68		36.03	3
hsa-miR-125b	1	37.09	36.49	33.26	31.79	36.94	35.12	5
hsa-miR-20b	1			36.74	31.94		34.34	2
hsa-miR-326	1			36.23	31.94		34.09	2
hsa-let-7e	1				31.96		31.96	1
hsa-miR-93*	2				31.96		31.96	1
hsa-miR-18b	1			37.12	31.97		34.55	2
hsa-miR-181b	1			39.34	32.07		35.70	2
hsa-miR-128	1	36.69		35.65	32.11		34.82	3
hsa-miR-142-3p	1			38.03	32.15		35.09	2
hsa-miR-590-5p	1			35.63	32.23		33.93	2
hsa-miR-99a	1		37.68	33.43	32.23		34.45	3
hsa-miR-132	1				32.33	37.10	34.72	2
hsa-miR-335	1			37.07	32.36		34.71	2
hsa-miR-216b	1				32.43		32.43	1
hsa-miR-31*	1			35.26	32.48		33.87	2
hsa-miR-339-5p	1	38.20	37.26	35.93	32.50	37.36	36.25	5
hsa-miR-204	1		34.05	33.21	32.51	36.68	34.11	4
hsa-miR-143	1			37.39	32.52		34.96	2
hsa-miR-183	1				32.55		32.55	1
hsa-miR-140-3p	1			34.87	32.56		33.72	2
hsa-miR-339-3p	2				32.68		32.68	1
hsa-miR-532-3p	2			34.66	32.69		33.67	2
hsa-let-7c	1		38.25	35.44	32.70		35.46	3
hsa-miR-374b	1			36.56	32.70		34.63	2
hsa-let-7d	1			37.69	32.80		35.24	2
hsa-miR-29c*	2			37.47	32.81		35.14	2
hsa-miR-520c-3p	1				32.84		32.84	1
hsa-miR-769-5p	2				32.87		32.87	1
hsa-miR-486-5p	1			34.57	32.90		33.73	2
hsa-miR-215	1			36.40	32.90		34.65	2
hsa-miR-598	1	37.14	37.57	36.71	32.94		36.09	4
hsa-miR-517c	1			35.97	32.94		34.46	2
hsa-miR-99b*	2				32.94		32.94	1
hsa-miR-18a*	1				32.96		32.96	1
hsa-miR-660	1			37.21	32.99		35.10	2
hsa-miR-22*	1			35.24	33.02		34.13	2
hsa-miR-130b	1			36.93	33.04		34.98	2
hsa-miR-147b	1				33.07		33.07	1
hsa-miR-301a	1				33.09		33.09	1

hsa-miR-181c*	2				33.09		33.09	1
hsa-miR-141*	2			36.21	33.12	39.11	36.15	3
hsa-miR-193a-3p	1				33.12		33.12	1
hsa-miR-519a	1				33.16		33.16	1
hsa-miR-502-3p	2			36.65	33.19		34.92	2
hsa-miR-106b*	2				33.25		33.25	1
hsa-miR-30e*	1		37.81	34.85	33.25		35.31	3
hsa-miR-346	1	32.84	33.11	32.46	33.33	32.90	32.93	5
hsa-miR-584	1			35.15	33.46		34.31	2
hsa-let-7i*	2				33.47		33.47	1
hsa-let-7g*	2			36.71	33.48		35.09	2
hsa-miR-2110	2			37.00	33.52		35.26	2
hsa-miR-18a	1			38.45	33.53		35.99	2
hsa-miR-490-3p	1	33.74	33.94	33.77	33.65	34.15	33.85	5
hsa-miR-92b	1				33.65		33.65	1
hsa-miR-130a	1			37.73	33.69		35.71	2
hsa-miR-491-5p	1				33.70	37.96	35.83	2
hsa-miR-1260	2		37.12	35.11	33.73	35.66	35.40	4
hsa-miR-29b-2*	1			36.73	33.81		35.27	2
hsa-miR-877	1			36.92	33.81		35.36	2
hsa-miR-7	1			38.90	33.84		36.37	2
hsa-miR-138	1			38.74	33.85		36.29	2
hsa-miR-605	2		37.20	35.55	33.87	35.64	35.57	4
hsa-miR-663b	2		38.22	35.72	33.88		35.94	3
hsa-miR-95	1				33.92		33.92	1
hsa-miR-152	1				33.93		33.93	1
hsa-miR-29a*	2				33.98		33.98	1
hsa-miR-147	1				34.01		34.01	1
hsa-miR-421	1			37.03	34.02		35.53	2
hsa-miR-328	1			37.18	34.03		35.61	2
hsa-miR-200a*	2			38.07	34.04		36.05	2
hsa-miR-433	1			35.48	34.05		34.76	2
hsa-miR-218	1				34.05		34.05	1
hsa-let-7f-2*	2				34.07		34.07	1
hsa-miR-203	1			36.90	34.07		35.49	2
hsa-miR-500	1			35.88	34.12		35.00	2
hsa-let-7a*	2			38.85	34.14		36.49	2
hsa-miR-98	1				34.17		34.17	1
hsa-miR-643	2		36.99	39.29	34.18		36.82	3
hsa-miR-129-3p	1				34.30		34.30	1
hsa-miR-129-5p	1				34.31		34.31	1
hsa-miR-127-3p	1				34.31		34.31	1
hsa-miR-452	1				34.32	36.79	35.56	2
hsa-miR-501-3p	2			36.87	34.34		35.61	2
hsa-miR-455-3p	2				34.43		34.43	1
hsa-miR-941	2			37.19	34.45		35.82	2
hsa-miR-193b*	2		36.95		34.46		35.70	2
hsa-miR-612	2				34.50		34.50	1
hsa-miR-126	1				34.52		34.52	1

hsa-miR-708	1				34.52		34.52	1
hsa-miR-153	1				34.54		34.54	1
hsa-miR-29b-1*	2		37.14		34.55		35.84	2
hsa-miR-150	1		36.76		34.57	35.05	35.46	3
hsa-miR-30a*	2			34.78	34.59	36.98	35.45	3
hsa-miR-515-5p	2				34.59		34.59	1
hsa-miR-520b	2				34.63		34.63	1
hsa-miR-520f	2				34.64		34.64	1
hsa-miR-140-5p	1				34.65		34.65	1
hsa-miR-103-2*	2				34.66		34.66	1
hsa-miR-145	1				34.69		34.69	1
hsa-miR-558	2	38.40	37.48	33.86	34.69	35.52	35.99	5
hsa-miR-363	1			36.78	34.70		35.74	2
hsa-miR-622	1				34.72		34.72	1
hsa-miR-34a*	2			36.44	34.74		35.59	2
hsa-miR-582-5p	1			36.88	34.76		35.82	2
hsa-miR-105*	2		35.02	33.81	34.77	35.16	34.69	4
hsa-miR-516a-5p	1				34.77		34.77	1
hsa-miR-124	1	36.81			34.78	35.45	35.68	3
hsa-let-7b*	2				34.83		34.83	1
hsa-miR-182*	2	38.00	38.48	37.77	34.83		37.27	4
hsa-miR-16-2*	2			36.78	34.86		35.82	2
hsa-miR-376a	1				34.87		34.87	1
hsa-miR-146a	1			37.25	34.90		36.07	2
hsa-miR-224	1				34.90		34.90	1
hsa-miR-517b	2				34.93		34.93	1
hsa-miR-15b*	2				34.93		34.93	1
hsa-miR-501-5p	1			34.76	34.94		34.85	2
hsa-miR-651	1	39.30			34.96		37.13	2
hsa-miR-24-2*	2	36.81		36.02	34.96		35.93	3
hsa-miR-21*	1				34.97		34.97	1
hsa-miR-519b-3p	2				34.98		34.98	1
hsa-miR-125a-3p	2				35.00		35.00	1
hsa-miR-181a*	1				35.01		35.01	1
hsa-miR-512-5p	1				35.02		35.02	1
hsa-miR-199b-5p	1				35.05		35.05	1
hsa-miRPlus-A1031	2	36.95			35.10		36.03	2
hsa-miR-130b*	2				35.15		35.15	1
hsa-miR-30d*	2			36.69	35.15		35.92	2
hsa-miR-199a-5p	1				35.17		35.17	1
hsa-miR-20a*	2				35.19		35.19	1
hsa-miR-382	1				35.34		35.34	1
hsa-miR-196b*	2				35.43		35.43	1
SNORD38B	2	38.17	37.37		35.44	38.26	37.31	4
hsa-miR-181a-2*	2				35.47		35.47	1
hsa-miR-134	1	34.71	35.60	35.08	35.48	35.08	35.19	5
hsa-miR-9	1		37.02		35.54		36.28	2
hsa-miR-193a-5p	1				35.55		35.55	1
hsa-miR-342-5p	2				35.55		35.55	1

hsa-miR-200c*	2				35.56		35.56	1
hsa-miR-19b-1*	2				35.57		35.57	1
hsa-miR-664	2			39.23	35.61		37.42	2
hsa-miR-505*	2				35.63		35.63	1
hsa-miR-518f*	2			36.84	35.65		36.24	2
hsa-miR-224*	2				35.66		35.66	1
SNORD38B	1				35.66	39.38	37.52	2
hsa-miR-196a	1				35.66		35.66	1
hsa-miR-886-3p	1			36.71	35.67		36.19	2
hsa-miR-297	2	37.09	35.58	35.99	35.68	36.43	36.15	5
hsa-miR-886-5p	1	35.49		37.97	35.73	35.63	36.21	4
hsa-miR-524-5p	1	37.98	36.18	37.15	35.76		36.77	4
hsa-miR-139-5p	1	37.86	37.29	36.59	35.79		36.88	4
hsa-miR-373*	1				35.81	37.68	36.75	2
hsa-miR-199a-3p	1				35.85		35.85	1
hsa-miR-526b*	1				35.86		35.86	1
hsa-miR-744*	2		36.94		35.87		36.40	2
hsa-miR-769-3p	2				35.89		35.89	1
hsa-miR-519c-3p	2				35.89		35.89	1
hsa-miR-92a-1*	1				35.92		35.92	1
hsa-miR-135b	1			39.28	35.94		37.61	2
hsa-miR-629*	2		36.77	36.91	35.97	38.95	37.15	4
hsa-miR-615-3p	1				35.99		35.99	1
hsa-miR-1266	2	35.69	35.03	35.21	36.03	35.19	35.43	5
hsa-miR-455-5p	1				36.03		36.03	1
hsa-miR-378*	2				36.07		36.07	1
hsa-miR-518e*	2				36.07		36.07	1
hsa-miR-188-5p	1				36.08		36.08	1
hsa-miR-34b*	2	38.07		37.03	36.15		37.08	3
hsa-miR-542-5p	1		39.05		36.15	38.35	37.85	3
hsa-miR-32	1				36.16		36.16	1
hsa-miR-519e*	1				36.19		36.19	1
hsa-miR-154	1				36.19		36.19	1
hsa-miR-142-5p	1		39.47		36.21		37.84	2
hsa-miR-100	1			37.39	36.23		36.81	2
hsa-miR-520d-3p	2	36.39		38.98	36.23	35.59	36.80	4
hsa-miR-221*	2				36.23		36.23	1
hsa-miR-376b	1				36.24		36.24	1
hsa-miR-602	1				36.24		36.24	1
hsa-miR-877*	2	37.19			36.25	36.85	36.77	3
hsa-miR-582-3p	2				36.25		36.25	1
hsa-miR-765	1				36.27		36.27	1
hsa-miR-522	2				36.39		36.39	1
hsa-miR-340	1				36.40		36.40	1
hsa-miR-499-5p	1				36.42		36.42	1
hsa-miR-1224-3p	2				36.47	36.89	36.68	2
hsa-miR-520d-5p	1				36.53		36.53	1
hsa-miR-33a*	2				36.56		36.56	1
hsa-miR-520h	2	35.97			36.56	37.73	36.76	3
hsa-miR-132*	2				36.57		36.57	1

hsa-miR-767-5p	2				36.59		36.59	1
hsa-miR-362-3p	2				36.60		36.60	1
hsa-miR-99a*	1				36.60		36.60	1
hsa-miR-518c	1				36.62		36.62	1
hsa-miR-629	1				36.62		36.62	1
hsa-miR-488	1				36.64		36.64	1
hsa-miR-671-3p	2				36.65		36.65	1
hsa-miR-548c-5p	2				36.65		36.65	1
hsa-miR-370	1				36.69		36.69	1
hsa-miR-577	2	36.95		36.02	36.69	36.23	36.47	4
hsa-miR-625*	1				36.72		36.72	1
hsa-miR-192*	2				36.75		36.75	1
hsa-miR-1909	2			36.89	36.76	37.40	37.02	3
hsa-let-7f-1*	2				36.77		36.77	1
hsa-miR-1269	2			38.06	36.81	38.04	37.64	3
hsa-miR-376c	1				36.83		36.83	1
hsa-miR-524-3p	1			37.68	36.85		37.27	2
hsa-miR-942	2				36.86		36.86	1
hsa-miR-517a	1				36.88		36.88	1
hsa-miR-24-1*	2				36.89		36.89	1
hsa-miR-595	1		34.14	34.06	36.89	36.61	35.43	4
hsa-miR-149*	2				36.91		36.91	1
hsa-miR-875-3p	2				36.92		36.92	1
hsa-miR-19b-2*	2	38.65		33.96	36.92		36.51	3
hsa-miR-548b-3p	1				36.92		36.92	1
hsa-miR-411	1				36.92		36.92	1
hsa-miR-424*	2			36.89	36.94		36.91	2
hsa-miR-570	1				36.94		36.94	1
hsa-miR-549	1				36.96		36.96	1
hsa-miR-744	1				36.97		36.97	1
hsa-miR-9*	1				36.97		36.97	1
hsa-miR-876-3p	2				37.00		37.00	1
hsa-miR-1972	2		38.74		37.02		37.88	2
hsa-miR-521	1				37.08		37.08	1
hsa-miR-1537	2				37.08		37.08	1
hsa-miR-498	1				37.10		37.10	1
hsa-miR-27b*	2				37.12		37.12	1
hsa-miR-212	1	36.95		37.31	37.14	36.79	37.05	4
hsa-miR-662	1	39.99		37.96	37.17		38.37	3
hsa-miR-1267	2				37.18		37.18	1
hsa-miR-518e	1			37.25	37.20		37.22	2
hsa-miR-519d	1			36.90	37.21		37.06	2
hsa-miR-362-5p	1			38.22	37.21		37.72	2
hsa-miR-1296	2		36.47	36.15	37.22	38.73	37.14	4
hsa-miR-564	2	37.79	37.82	37.21	37.28	36.96	37.41	5
hsa-miR-454*	2				37.31		37.31	1
hsa-miR-518c*	1				37.36		37.36	1
hsa-miR-766	1				37.38		37.38	1
hsa-miR-519e	2				37.39		37.39	1

hsa-miR-143*	2				37.43		37.43	1
hsa-miR-571	2	38.49			37.47		37.98	2
hsa-miR-562	2		36.29	37.71	37.47	36.56	37.01	4
hsa-miR-7-1*	2				37.48		37.48	1
hsa-miR-596	1				37.57		37.57	1
hsa-miR-331-5p	2				37.63		37.63	1
hsa-miR-148a*	2				37.64		37.64	1
hsa-miR-550*	2				37.65		37.65	1
hsa-miR-454	1				37.67		37.67	1
hsa-miR-26b*	2				37.67		37.67	1
hsa-miRPlus-D1046	2			37.35	37.73		37.54	2
hsa-miR-25*	2				37.79		37.79	1
hsa-miR-450a	1				37.79		37.79	1
hsa-miR-518d-5p	2				37.82		37.82	1
hsa-miR-381	1			38.51	37.84	37.24	37.86	3
hsa-miR-642	1				37.89		37.89	1
hsa-miR-1244	2	38.12			37.89		38.00	2
hsa-miR-555	2				37.92	36.72	37.32	2
hsa-miR-374b*	1				37.99		37.99	1
hsa-miR-1247	2				38.03		38.03	1
hsa-miR-654-3p	2				38.03		38.03	1
hsa-miR-1270	2			39.07	38.08		38.57	2
hsa-miR-483-3p	1				38.13		38.13	1
hsa-miR-135a*	2				38.15		38.15	1
hsa-let-7c*	2			37.45	38.18	37.94	37.86	3
hsa-miR-566	2			36.87	38.22		37.54	2
hsa-miRPlus-D1033	2				38.22		38.22	1
hsa-miR-758	2				38.27		38.27	1
hsa-miR-23a*	2				38.34		38.34	1
hsa-miR-518f	1			36.79	38.37		37.58	2
hsa-miR-632	2				38.49		38.49	1
hsa-miR-1183	2	33.14	38.32	36.45	38.61	39.07	37.12	5
hsa-miR-190b	2				38.64		38.64	1
hsa-miR-15a*	2				38.67		38.67	1
hsa-miR-1912	2				38.71	39.02	38.87	2
hsa-miR-512-3p	2				38.86		38.86	1
hsa-miR-592	2				38.89		38.89	1
hsa-miR-628-3p	1				38.91		38.91	1
hsa-miR-1182	2				38.97		38.97	1
hsa-miR-133a	1				38.98		38.98	1
hsa-miR-552	2				39.00		39.00	1
hsa-miR-885-5p	1				39.01		39.01	1
hsa-miR-509-3p	1				39.03	37.33	38.18	2
hsa-miR-543	2	38.36			39.09	38.65	38.70	3
hsa-miR-616*	2				39.12	36.51	37.81	2
hsa-miR-608	1				39.19		39.19	1
hsa-miR-301b	1				39.19		39.19	1
hsa-miR-548o	2				39.19		39.19	1
hsa-miR-640	2				39.20		39.20	1

hsa-miR-377	1				39.22		39.22	1
hsa-miR-1538	2				39.33		39.33	1
hsa-miR-515-3p	2				39.43		39.43	1
hsa-miR-604	2	37.14			40.00		38.57	2
hsa-miR-19b	1			31.36			31.36	1
hsa-miR-331-3p	1			34.86			34.86	1
hsa-miR-15a	1		36.93	32.89			34.91	2
hsa-miR-1913	2		36.11	35.64			35.87	2
SNORD49A	1					36.09	36.09	1
hsa-miR-760	1			36.26			36.26	1
hsa-miR-937	2	35.89	36.70				36.30	2
hsa-miR-502-5p	1	37.21	36.73	36.27		36.51	36.68	4
hsa-miR-650	2	36.82	36.83	37.14		37.20	37.00	4
U6	1			36.72			36.72	1
hsa-miR-523	1					36.72	36.72	1
hsa-miR-1179	2			35.92		36.46	36.19	2
U6	2					36.75	36.75	1
hsa-miR-619	2	37.47		36.20			36.83	2
hsa-miR-218-1*	2			36.41		37.34	36.87	2
hsa-miR-518a-3p	1					36.90	36.90	1
hsa-miR-185*	1	36.57	37.24	36.56		37.22	36.90	4
hsa-miR-503	1			36.68			36.68	1
hsa-miR-1207-5p	2		38.83	35.27			37.05	2
hsa-miR-195*	2	36.63	37.59				37.11	2
hsa-miR-92b*	2			37.13			37.13	1
hsa-miR-330-5p	2					37.28	37.28	1
hsa-miR-329	1	37.41	36.84	37.69		37.82	37.44	4
hsa-miR-624*	2					35.65	35.65	1
hsa-miRPlus-C1076	2					37.43	37.43	1
hsa-miR-628-5p	2					37.44	37.44	1
hsa-miR-431*	2	37.46	39.43	37.90			38.26	3
hsa-miR-127-5p	1	38.34					38.34	1
hsa-miR-493	1			38.13		39.36	38.75	2
hsa-miR-194*	2		38.44				38.44	1
hsa-miR-631	1		38.47				38.47	1
hsa-miR-525-3p	2			38.53			38.53	1
hsa-miR-888	1	39.28					39.28	1
hsa-miR-639	2		38.88				38.88	1