Signatures Containing miR-133a identified from Large Scale Micro RNA Expression Profiling in Bladder Cancer Tissue

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Abstract:

1.1. Objective: According large scale miRNA expression profiling of bladder cancer (BC) tissue samples and adjacent normal tissue samples from patients with bladder cancer, we discovered several miRNA expression signatures containing miR-133a correlated with BC diagnosis, that has shed light inprospective molecular mechanism of miRNA-133a and other miRNAs in the tumorigenesis of BC.Two miRNA clusters of miR183-96-and miR200 are significantly up-regulated in bladder cancer tissue samples compared with those in normal tissue samples, suggesting theses miRNAs may be involved in bladder cancer development.

1.2. Methods: According expression data of large scale miRNA profiling in BC tissue sample pool and in adjacent normal bladder tissue sample pool, we selected differential expressed miRNAs between two pools, and generated one 384-well panel consists of primers to detect the selected miRNAs and controls. This panel was used to profile miRNA expression in each individual sample in training group and in double blind test group. Data analysis was performed using a machine learning approach of a Support Vector Machine classifier with a Student's t-test feature selection procedure.

1.3. Results: We identified signatures consisting of three or four miRNAs with optimum classification performance; all identified signatures containing hsa-miR-133a could distinguish BC cases from normal controls with an accuracy of 100% in training model and accuracy over 95% in double blinding test.

1.4. Conclusions: miRNA signatures containing hsa-miR-133a may be used as biomarkers in BC diagnosis and serves as a complement to cystoscopy.

1. Introduction

Bladder cancer (BC) is the second most common urological malignancy in humans globally with an estimated 430, 000 new cases diagnosed and 165,000 deaths in 2016 **[1-3]**. In China, BC is the most common genitourinary cancer **[4].**The gold standards for the initial diagnosis of BC are combination of cystoscopy and biopsy. However, these methods are invasive, uncomfortable, and costly **[5,6]**. Urinary cytology is of high specificity (90%−95%), but the sensitivity is relative low (~30%). Furthermore, several new urine-based tests for BC, such as BC antigen stat, nuclear matrix protein 22 (NMP22, Sysmex Corp., Kobe, Japan), and FISH (UroVysion, Abbott Molecular Inc, Des Plaines, IL, USA), have been approved for clinical application. However, these new urinary markers have not been widely applied because they have relatively low specificities (60%−80%) compared with urinary cytology, despite of their higher sensitivities (50%−70%) **[7]**. Accordingly, the development of highly accurate BC diagnostic biomarkers is urgently needed.Since first miRNA was discovered in 1993, miRNAs have been shown to regulate gene expression at post-transcriptional level by binding through partial sequence homology to the 3' untranslated region or through complete sequence homology to the coding region of target mRNAs to cause repression of translation or mRNA degradation **[8-10].** The accumulating data indicate that miRNAs play important roles in tumorigenesis, metastasis, and drug responsiveness in BC and in other urological cancers **[11-14].**

In this study, we have applied Biovue'sqRT-PCR miRNA assay platform to profile large scale miRNAs (1900 miRNAs) in a set of BC tissue samples and in adjacent normal tissue samples and have identified signatures of three or four-miRNA that can distinguish BC patients from normal controls with high sensitivity and specificity. These identified signatures all contain hsa-miRNA-133a thus indicating the importance of hsa-miRNA-133a in tumorigenesis and diagnosis of BC.

2. Materials and Methods

2.1. Collection of bc Tissue Samples

The study comprised 30 patients at Department of Urology, Zhongshan Hospital (Fudan University, Shanghai, China) during the period May-November 2016. All the tissue samples in our study were divided into two groups, training group and double-blind test group. Training group contains 19 BC tissues samples and 19 adjacent normal tissue samples as normal controls. Double blinding test group contains 11 BC tissue samples and 10 adjacent normal tissue samples. All tissue samples were immediately frozen in liquid nitrogen after they were being removed from body and stored at-80°C. The demographics and clinical features of the patients are listed in **(Table 1).**

Demographic features		Diagnostic Model		Validation	
		Adjacent (n=19)	Cancer (n=19)	Adjacent (n=10)	Cancer (n=11)
Average age (range)		73.0 (47-90)	73.0 (47-90)	61.4 (46-77)	66.1 (46-88)
Gender: male/females, n		16/3	16/3	8/1	10/1
Stage	Tis	1			
	I	15		10	
	II	2			
	III	1			
	IV			1	

Table 1: Demographic and Clinical Characteristics of Participants.

Abbreviation: Tis   :   Tumor in situ.

2.2. miRNA Isolation and cDNA Synthesis

Total RNA was isolated from < 50 mg of frozen tissue sample with miRNeasy Mini Kit (Qiagen, 217004) according to the manufacturer's instructions. First strand cDNA was generated from total RNA sample in reverse transcription by using Sharp vue[TM]miRNA First Strand Kit (Biovue, 9000004) following manufacturer's protocol.The reactions were incubated at 37 °C for 60 minutes, and then were inactivated by incubation at 95 °C for 10 minutes by using Gene Amp PCR 9700 Thermocycler (Applied Biosystems).

2.3. Quantitative Real-Time PCR Assay

Single tube miRNA assays were used to detect miRNAs by Sharpvue[TM]2x Universal qPCR Master Mix High Rox (Biovue, 9000008) and Sharpvue[TM] Human miRNA Primer Array (Biovue) according to manufacturer's protocol. The level of miRNA expression was quantified using ABI 7900HT Fast Real-Time PCR System (Applied Biosystems). The reactions were incubated in a 384-well optical plate at 95°C for 10 minutes, following by 3 cycles of 96°C for 5 seconds and 60°C for 1 minute, then 37 cycles of 96°C for 5 seconds and 60°C for 30 seconds and run melting curve at last. SYBR was set as reporter and Rox was set as reference respectively.

2.4. BC miRNA Panel

Expressions of miRNAs in BC tissue samples pool and in adjacent normal tissue samples pool were detected by primers for 1900 miRNAs and controls in five 384-well plates. Each plate contains primers to detect 380 miRNAs, 2 endogenous controls, one spiking control and one no template control. We selected differential expressed miRNAs between BC

tissue samples pool and adjacent normal tissue samples pool; and generated one 384-well panel to detect 380 selected miRNAs, two endogenous controls, one spiking control and one no template control. This panel will be used to profile miRNA expression of each individual sample in training group and in double blind test group.

3. Result

3.1. Clinical and Pathological Findings

The clinical features of samples collected from epithelial of patients (30 BC tissue samples; 29 adjacent normal tissue samples) in our study are summarized in Table 1. 30 bladder cancer tissue samples include 1 case of stage Tis (in training group), 25 cases of stage I (15 cases in training group and 10 cases in double blind group), 2 cases of stage II (including one of low-lever urothelial carcinoma in training group), 1 cases of stage III (in training group) and 1 cases of stage IV (in double blind group). 29 adjacent normal tissue samples include 19 cases in training group and 10 cases in double blind test group.

3.2. Biomarker Selection Based on qRT-PCR

The miRNA expression data from real-time PCR was performed by using R and package e1071 with some modifications as the following: First, set Ct value as 32 to any miRNAs if their measured Ct values were greater than 32. Then, we compared mean Ct-value of each miRNA between tumor tissue and control tissue for 365microRNAs (15 miRNAs without expression were removed from analysis), 2 endogenous controls. There are many miRNAs with significant higher Ct-value in cancer tissue were shown in (**Figure 1**). Afterwards, we did T-test for

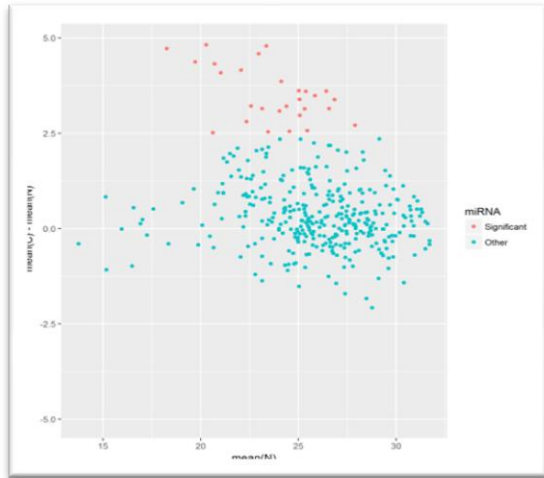each miRNA. 70 miRNAs with P-value < 0.001 were displayed in **(Figure 2).**

Fig. 1: Schematic of dysregulation miRNAs in BC (red).
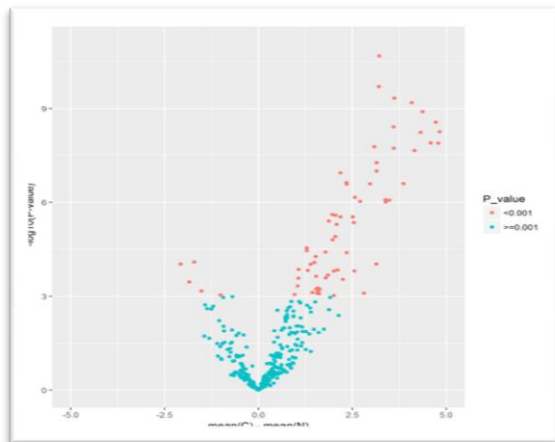
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Figure 2: Schematic of dysregulation miRNAs in BC (red). The volcanoplot of miRNAs with P-value.

In order to prove there are tumor related markers and to find out these markers, we trained a SVM model, and predicted 20 other samples. The model was built as the following: a). we produced 66430 new variables by computing the difference between Ct of each tow miRNAs and got 66796 total variables with the original 366 variables. b). we did T-test on all these variables and took 2 variables with the

greatest P-value and some variables with smallest P-value. Until we had got 20 candidate miRNAs those variables included. c). for each miRNA subset with markers less than or equal to 12 of the 20 candidate miRNAs, we trained a SVM model with default parameters by function SVM and evaluated the accuracy by 50 times 10-fold cross validation. d) Then 10 subsets with the best accuracy and AUC were selected and 10 SVMs based on these 10 marker subsets were trained by total 38 samples and the stat of 21 blind samples was predicted. They all showed good performances on blind samples with accuracy greater than 0.95. The best one was selected for further analysis.

3.3. miRNA Expression Profiling in BC And Normal Controls

To identify BC specific miRNA expression signatures as biomarkers to diagnose BC, we applied the panel to profile miRNA expression of 38 tissue samples in training group, including 19 BC tissue samples and 19 matched adjacent normal tissue samples. The 365 dysregulated microRNAs and two endogenous controls in BC tissue samples and in adjacent normal tissue samples were presented in the **(Supplemental Table 1).** There were 121 microRNAs, including miR-182, miR-431, miR-183, miR-429 and miR-425 etc., with a higher expression levels in the BC tissues compared with adjacent normal tissues. In contrast, 245 microRNAs, such as miR-1, miR-133a, miR-133b miR-125b miR-143 and miR-145 etc, had a lower expression level in the BC tissues relative to adjacent normal tissues. The aberrant expression levels of miRNAs were summarized in **(Table 2).**

miRNA name	mean Normal	Log$_2$FC	T-value	P-value
hsa-miR-1	26.12782155	4.8691869	9.809440884	5.82E-12
hsa-miR-30a	22.60102595	3.08240765	9.287222774	5.48E-11
hsa-miR-133a	24.4354524	3.10808475	8.931966341	1.19E-10
hsa-miR-4328	21.0968902	3.91722335	8.532184649	8.06E-10
hsa-miR-143#	25.0985113	3.42840055	8.006753062	1.29E-09
hsa-miR-143	19.7855096	4.1667027	7.83836364	2.59E-09
hsa-miR-145	18.30939145	4.5212111	8.084599629	4.80E-09
hsa-miR-376c	25.36776165	3.4553682	7.558748119	9.56E-09
hsa-miR-125b	20.29446115	4.6285196	7.775000198	1.13E-08
hsa-miR-100	23.00215805	4.40793215	7.77647622	1.58E-08
hsa-miR-199b-3p	20.7305781	4.1213263	7.973725661	1.61E-08
hsa-miR-99a	23.36811305	4.5790223	7.746394335	3.30E-08
hsa-miR-199a-5p	22.08252775	3.9837205	7.058102887	4.01E-08
hsa-miR-497	24.0621254	2.91987705	7.134383565	4.94E-08
hsa-miR-376a	26.4500204	3.4226842	7.246161876	5.76E-08
hsa-miR-195	23.15245275	3.00588535	6.595963782	1.09E-07
hsa-miR-154	26.59476795	3.0009218	6.989134323	1.72E-07
hsa-miR-139-5p	26.58507925	2.0498703	6.217934445	3.22E-07
hsa-miR-127-3p	25.1306052	2.2363153	6.219988897	3.39E-07
hsa-miR-379	29.15859985	2.23430305	6.093823941	4.29E-07
hsa-miR-152	25.1137449	2.8155216	6.427856879	4.53E-07
hsa-miR-495	27.95195155	2.5630645	6.082969797	1.32E-06
hsa-miR-133b	27.0639709	2.0207833	5.912748166	1.33E-06
hsa-miR-377	25.89236105	3.2962058	6.39185024	1.48E-06
hsa-miR-136	25.0941168	3.2224151	5.970151312	1.51E-06
hsa-miR-136#	26.87362035	3.20967815	5.7729383	1.57E-06
hsa-miR-140-3p	25.4251528	2.45418445	5.679138579	1.60E-06
hsa-miR-150	24.11973495	3.6107569	5.717103638	1.87E-06
hsa-miR-574-3p	23.36441105	1.79655005	5.469180968	3.79E-06
hsa-miR-337-3p	26.6682857	2.07009815	5.594781395	4.07E-06
hsa-miR-130a	23.46221215	2.4440971	5.745567912	4.11E-06
hsa-miR-29a	20.6555566	2.386061	5.668752022	4.33E-06
hsa-miR-101	21.5610767	1.8347093	5.299070261	5.43E-06
hsa-miR-342-3p	23.18032995	1.9355929	5.237219218	1.45E-05
hsa-miR-126#	22.86494995	1.9387173	4.937661612	1.96E-05
hsa-miR-222	23.4050734	1.85868785	4.942452211	2.20E-05
hsa-miR-365b-3p	24.1221182	1.22701895	4.847253766	3.32E-05
hsa-let-7c	24.06564905	2.2533086	4.646655955	3.98E-05
hsa-miR-28-3p	25.65795785	1.2129748	4.651457821	4.09E-05
hsa-miR-27b	22.46738325	1.6925897	4.621989619	4.53E-05
hsa-miR-431	27.33753895	-1.7050862	-4.620089861	4.77E-05
hsa-miR-451	21.8264741	2.1363373	4.468465735	6.98E-05

hsa-miR-551b	25.33340815	3.03985395	4.508074422	7.56E-05
hsa-miR-320c	23.24441335	1.41644265	4.478917155	8.59E-05
hsa-miR-543	29.7072064	1.3195017	4.36105705	0.000101719
hsa-miR-182	28.69664615	-1.9300384	-4.272721358	0.000151213
hsa-miR-193b	23.1125698	1.0128699	4.18768074	0.000168706
hsa-miR-342-5p	29.4783259	1.38146835	4.183250867	0.000177017
hsa-miR-1247	22.3717192	1.23820375	4.138444581	0.000193647
hsa-miR-328	25.83411935	1.024637	4.067152197	0.000235068
hsa-miR-126	21.15470095	1.7589386	4.016215178	0.000283073
hsa-miR-10b	24.7410833	1.4424195	4.065717946	0.000290896
hsa-miR-338-3p	25.78045215	2.148419	3.955388217	0.000322289
hsa-miR-140-5p	28.2960132	1.8576039	3.943014528	0.000337715
hsa-miR-369-3p	28.4255048	1.6673728	4.001696311	0.000376451
hsa-miR-183	28.4274269	-1.72647045	-3.866474124	0.0004299
hsa-miR-429	25.01997015	-1.48664115	-3.844260434	0.000447105
hsa-miR-142-5p	24.57882015	2.3212409	3.844958064	0.000481869
hsa-miR-30e#	25.2996504	1.54164295	3.780538	0.000554659
hsa-miR-425	27.31893245	-0.98565215	-3.711287716	0.00066976
hsa-miR-24	21.9793475	1.45237895	3.696846761	0.000700445
hsa-miR-532-5p	27.0696411	0.96784885	3.645865378	0.000797014
hsa-miR-132	26.4796401	1.51286615	3.655198409	0.000893664
hsa-miR-191#	30.9403011	-0.66744925	-3.598585801	0.000910332
hsa-miR-23b	21.7122885	1.83916365	3.598870952	0.000931943
hsa-miR-221	25.57643955	1.47041835	3.619747281	0.00094584
hsa-miR-409-3p	28.0150883	1.45061025	3.643228525	0.000970027
hsa-miR-186	24.5825067	0.9065164	3.568946078	0.001015458
hsa-miR-383	26.53589485	0.8543973	3.541428863	0.001103413
hsa-miR-4457	25.88232885	1.52570425	3.548862709	0.001120198
hsa-let-7i#	25.55904145	1.32723705	3.526383583	0.001199008
hsa-miR-4787-3p	24.5164283	-0.8828859	-3.414370137	0.00153735
hsa-miR-142-3p	22.3634303	2.57962	3.410067456	0.001554499
hsa-miR-4515	23.11883335	-1.3588254	-3.426627311	0.00160518
hsa-miR-365	24.77483395	1.019026	3.453564069	0.001667241
hsa-miR-323-3p	30.3736771	1.06185165	3.416645217	0.001729454
hsa-miR-141#	30.42505505	-1.36608445	-3.333558522	0.001993619
hsa-miR-3610	22.76108095	-1.15089165	-3.340350091	0.002009853
hsa-miR-210	26.5849375	-1.2365398	-3.316575134	0.002099143
hsa-miR-340	27.5344029	1.40211665	3.359967783	0.002116023
hsa-miR-148a	22.63442665	1.222918	3.314373401	0.002204698
hsa-miR-320b	23.2907638	0.75952705	3.280475204	0.002228309
hsa-miR-23a	21.59763405	1.3045986	3.28322114	0.0023896
hsa-miR-155	27.46101985	1.8160122	3.235218869	0.002520089
hsa-miR-196b	25.9830149	1.7902629	3.195800366	0.002824899
hsa-miR-656	28.94392765	0.89953625	3.063244039	0.004027979

hsa-miR-660	27.185685	0.7636651	3.066741473	0.004091327
hsa-let-7i	22.68492035	1.3000338	3.05140275	0.004167271
hsa-miR-3529	30.1631947	0.70401995	3.001770901	0.004748783
hsa-let-7b#	27.279987	0.732047	2.993056875	0.004871397
hsa-miR-144#	30.8821511	0.8829912	3.067629297	0.004952988
hsa-miR-548q	31.32132105	0.45014005	2.957800399	0.006153716
hsa-miR-493	30.0825144	0.6951375	2.897813094	0.006210302
hsa-miR-223	23.44406645	1.97853525	2.919699876	0.006528151
hsa-miR-3614-3p	29.59248965	-0.984494	-2.86694845	0.006724513
hsa-miR-532-3p	26.78098585	0.65564045	2.862324396	0.006863026
hsa-miR-5001-5p	25.71753005	1.05425915	2.866148314	0.006888698
hsa-miR-505	27.19126775	0.67007905	2.855420334	0.00694156
hsa-miR-362-3p	27.6832793	0.82100295	2.840258198	0.007204649
hsa-miR-3135b	24.97284205	0.6417884	2.777500699	0.008481823
hsa-miR-592	31.13443675	0.66345465	2.738041497	0.0095927
hsa-miR-212	29.5486406	1.0008928	2.729779462	0.009597216
hsa-miR-196a	27.38908385	1.10877205	2.72659871	0.009653931
hsa-miR-26a	21.32681625	1.69057045	2.713278386	0.009958922
hsa-miR-361-3p	28.12515965	0.9541058	2.711820581	0.009994861
hsa-miR-432	28.36620585	0.81835125	2.714243032	0.0101904
hsa-miR-1273	31.47666495	-0.525494	-2.679633285	0.011156313
hsa-miR-1470	24.80158215	-0.87661205	-2.667075277	0.011180228
hsa-miR-449b	26.96619415	-0.66588595	-2.647380932	0.012146338
hsa-miR-4646-3p	22.8328203	0.82195005	2.687707151	0.012173562
hsa-miR-1179	22.78256425	-0.3981945	-2.615021723	0.012733052
hsa-let-7b	21.12472435	1.1289838	2.610223587	0.012894397
hsa-miR-1260	23.5983059	-0.87562655	-2.59199796	0.013496655
hsa-miR-3193	26.76498135	0.83129875	2.606812743	0.013614125
hsa-miR-27a	20.92300265	0.9000644	2.575819125	0.014270306
hsa-miR-26b	22.07461815	1.29933495	2.539189591	0.015339391
hsa-miR-30d	25.2785083	0.564175	2.520050052	0.016276762
hsa-miR-3907	22.6478882	0.91337795	2.511927544	0.016814744
hsa-miR-30e	23.58118445	0.6111057	2.490614312	0.017472739
hsa-miR-424	24.251826	1.15479345	2.491208423	0.018049261
hsa-miR-770-5p	26.96190865	-1.3695525	-2.446231884	0.019913924
hsa-miR-146b-5p	25.450884	1.33221555	2.453154447	0.01994029
hsa-miR-1181	22.0476171	-0.766908	-2.428060122	0.020262004
hsa-miR-3162-3p	24.53635555	-0.55586185	-2.426201684	0.020573687
hsa-miR-29c	19.6668291	1.0106059	2.424665936	0.020633033
hsa-miR-206	31.35934975	0.3786274	2.424188071	0.021099703
hsa-miR-3187-5p	24.84167505	-0.8962384	-2.366808186	0.023474191
hsa-miR-146a	25.09430145	1.4475159	2.399573529	0.023936978
hsa-miR-517#	31.1082896	0.4856575	2.360789774	0.024120909
hsa-miR-130b	29.3360632	-0.69554485	-2.357316617	0.024287788

hsa-miR-200a	24.30833635	-0.95845155	-2.338242734	0.02492053
hsa-miR-299-5p	25.3406319	0.40735705	2.314080763	0.026169736
hsa-miR-483-3p	27.4458182	-0.80552955	-2.310682326	0.026862126
hsa-miR-200b	24.43627245	-1.0711679	-2.296911695	0.027584516
hsa-miR-34a	23.32164945	1.11229155	2.306203211	0.028765759
hsa-miR-363	28.08783975	0.9611428	2.283438761	0.02887056
hsa-miR-21#	27.65452255	-0.85893615	-2.227477144	0.031921518
hsa-miR-625	28.70441055	0.98901795	2.201772292	0.033876062
hsa-miR-934	28.9376091	-1.1932694	-2.181842067	0.035431306
hsa-miR-144	26.57500715	1.28001735	2.175975202	0.036636816
hsa-miR-28-5p	26.6206536	0.9144506	2.149840334	0.038976013
hsa-miR-374b	25.6924562	1.07738825	2.129072287	0.039896843
hsa-miR-1287	30.7139527	0.5464396	2.120721248	0.040598869
hsa-miR-559	25.37516275	-0.9639269	-2.093759505	0.043051703
hsa-miR-16	20.7079641	0.61409515	2.090024936	0.043385631
hsa-miR-422a	31.73138245	-0.43618595	-2.030613352	0.051080563
hsa-miR-22	23.21079415	0.7015379	2.013561587	0.051512891
hsa-miR-31	24.2055013	1.36200945	2.012972117	0.051808029
hsa-miR-9	29.86422025	0.98867865	2.008900607	0.052376647
hsa-miR-2682	31.71242595	-0.29300055	-1.996650461	0.053350767
hsa-let-7a#	26.47691995	0.5758533	1.976738187	0.055395972
hsa-miR-29c#	25.9761347	0.8367708	1.977295206	0.055994364
hsa-miR-483-5p	29.8680788	-0.7170832	-1.942458281	0.059520735
hsa-miR-423-3p	24.3024306	0.5268758	1.880717317	0.067695148
hsa-miR-4516	15.1663808	0.7559816	1.87169274	0.069106072
hsa-miR-331-5p	29.75397915	0.54540195	1.861309505	0.070467054
hsa-miR-486-5p	25.29719555	0.49366285	1.850955216	0.072390574
hsa-miR-339-3p	27.45386695	0.42801395	1.813789201	0.077909463
hsa-miR-378b	27.3473778	-0.4910585	-1.807974627	0.078633532
hsa-miR-33b	27.5739101	0.5511337	1.809235704	0.079145316
hsa-miR-30c	22.3352712	0.88364805	1.787936387	0.081810396
hsa-miR-18a	29.6545935	-0.7482391	-1.78560926	0.082864009
hsa-miR-566	26.2098305	-0.55491275	-1.786615375	0.083512205
hsa-miR-3944	26.6809559	-0.5481203	-1.781717884	0.083812955
hsa-miR-423-5p	24.26223985	0.3969786	1.76031078	0.086460263
hsa-miR-33a	24.5977212	0.56275095	1.752026931	0.087844695
hsa-miR-219-2-3p	31.25709005	0.30535645	1.740985155	0.089870468
hsa-miR-125a-5p	23.55308655	0.79933375	1.718049039	0.093928347
hsa-miR-1285	29.1246945	-0.50452625	-1.714001351	0.095052799
hsa-miR-15b#	29.45490595	-0.4796401	-1.711580877	0.095296274
hsa-miR-193a-5p	27.88657425	0.46327125	1.704456152	0.097065419
hsa-miR-1273d	25.55828555	-0.6731021	-1.699354427	0.097518893
hsa-miR-4454	15.2017796	-0.98903195	-1.690625947	0.099260855
hsa-miR-373#	25.7436403	-0.18420255	-1.666448123	0.104029387

hsa-miR-1268	21.1941402	0.85487755	1.649408005	0.107755216
hsa-miR-93	25.3874974	-0.66449435	-1.639808116	0.110163099
hsa-miR-605	29.2925058	-0.6245109	-1.614872778	0.114984963
hsa-miR-425#	26.63673985	-0.4792545	-1.604445581	0.116946305
hsa-miR-4286	16.49819445	-0.90876835	-1.599542667	0.118081575
hsa-let-7a	21.1478772	0.92555255	1.589114877	0.120344398
hsa-miR-224	26.93167345	0.91555935	1.603333073	0.121186155
hsa-let-7f-1#	28.4389334	0.380989	1.587978482	0.121732096
hsa-miR-650	27.8012068	-0.8874117	-1.566605666	0.126039181
hsa-miR-590-5p	27.11414205	0.3327394	1.567640662	0.126578009
hsa-miR-296-3p	25.74392935	-0.44644515	-1.542520097	0.131552252
hsa-miR-374a	25.30958305	0.86202575	1.518348862	0.137264061
hsa-miR-19b	22.3236621	0.40619995	1.51236616	0.138812082
hsa-miR-361-5p	26.31010785	0.60508395	1.507141503	0.140046769
hsa-miR-146b-3p	30.7919129	0.38168175	1.472416161	0.149151616
hsa-miR-1306-5p	29.3238386	0.38521345	1.470100793	0.149786464
hsa-miR-30b	22.4946482	0.83752655	1.461738506	0.152044836
hsa-miR-4251	26.24347615	-0.4066147	-1.454992365	0.154775248
hsa-miR-554	26.9298151	-0.40861715	-1.45199967	0.155812974
hsa-miR-29b	22.4214774	0.7732205	1.444473218	0.157019582
hsa-miR-4685-5p	26.56914765	0.7128942	1.413234747	0.165903946
hsa-miR-324-3p	23.49381115	0.2036009	1.414088233	0.166525221
hsa-miR-769-5p	28.93151495	0.4491276	1.398633999	0.170099467
hsa-miR-99b	24.79095835	0.4699214	1.38802164	0.173403385
hsa-miR-3182	22.0917711	0.87863845	1.369014165	0.180092033
hsa-miR-21	19.89994595	-0.47206045	-1.357314949	0.182944616
hsa-miR-3162	27.52056975	0.27188335	1.356724807	0.183236798
hsa-miR-3622b-5p	29.84767925	-0.341081	-1.346601703	0.187425209
hsa-miR-500a#	28.988497	-0.3800054	-1.332282675	0.191417956
hsa-let-7g	22.5160448	0.6208779	1.288815201	0.20528729
hsa-miR-16-2#	28.9569723	0.3507927	1.275855156	0.210050585
hsa-miR-639	22.70162175	0.5668435	1.258599219	0.216595312
hsa-miR-3184-3p	27.1632112	-0.42711215	-1.251088663	0.218889234
hsa-miR-92b	22.3066823	0.3910935	1.246320525	0.221177981
hsa-miR-324-5p	24.3754331	0.4253259	1.226410457	0.228249243
hsa-miR-190	28.3002212	0.68841005	1.222898687	0.229465423
hsa-miR-1296	27.4683343	0.31583935	1.213811544	0.232310267
hsa-miR-5096	24.2779367	0.46403835	1.215719567	0.232383243
hsa-miR-629	30.4329805	0.2767291	1.212709864	0.232758375
hsa-miR-4492	19.08022115	0.6325124	1.207992999	0.23451776
hsa-miR-181a	25.3775098	0.5237116	1.205679978	0.235489098
hsa-miR-92a	22.29835705	0.3137744	1.181328726	0.245156721
hsa-miR-421	29.46268685	-0.38368865	-1.180455185	0.245379254
hsa-let-7e	24.28009255	0.7446999	1.179494837	0.245553482

hsa-miR-3646	24.6028077	0.46447895	1.176789037	0.248227461
hsa-miR-371b-5p	30.71878265	-0.43535225	-1.169666136	0.250437128
hsa-miR-32	25.02813385	-0.36665765	-1.166781534	0.250819927
hsa-miR-149	27.83458655	-0.60014675	-1.155921134	0.255196798
hsa-miR-138	28.02253835	-0.5825292	-1.146044004	0.261386111
hsa-miR-937	28.3246925	0.33720585	1.139310241	0.261714167
hsa-miR-671-3p	27.9938128	-0.2949438	-1.126925869	0.268504783
hsa-miR-151b	25.71631315	0.3004291	1.118739089	0.270347491
hsa-miR-92b#	20.65776195	-0.5388198	-1.108847598	0.274473184
hsa-miR-3663-3p	26.73863275	-0.4288733	-1.101674933	0.277994139
hsa-miR-4284	18.3304225	-0.37322	-1.098648887	0.278840814
hsa-miR-4532	16.5986575	0.5003781	1.085063268	0.285893938
hsa-miR-191	24.70332185	-0.6153625	-1.080614366	0.286991717
hsa-miR-1277	30.82314515	0.31158625	1.038872865	0.305508588
hsa-miR-20b	29.82143965	0.36721185	1.038761116	0.305558152
hsa-miR-4635	25.95357065	-0.40219675	-1.038598271	0.306115543
hsa-miR-1260b	22.4532214	-0.4510095	-1.031106897	0.309023332
hsa-miR-124	29.18081985	0.54780785	1.027852462	0.314013189
hsa-miR-141	23.17711065	-0.7161285	-1.022710498	0.315071895
hsa-miR-1180	26.0773021	0.3620099	0.988714866	0.329822994
hsa-miR-885-5p	29.4275937	-0.31758155	-0.982636826	0.332068742
hsa-miR-642b	27.25053295	0.4879913	0.979463207	0.333822884
hsa-miR-148b	25.54500595	0.2281798	0.979304764	0.333839677
hsa-miR-762	17.59962505	0.46696045	0.97683529	0.334868614
hsa-miR-4698	26.5296455	0.2940295	0.974212547	0.337135312
hsa-miR-4263	23.76872745	-0.2930882	-0.97414815	0.337635105
hsa-miR-378d	24.6378642	0.27294275	0.962214586	0.342030016
hsa-miR-339-5p	27.75511755	0.33064545	0.959533798	0.343383228
hsa-miR-22#	27.92357525	0.30962125	0.941339142	0.352745552
hsa-miR-197	25.4805562	0.22025195	0.939742699	0.354983429
hsa-miR-379#	26.18597855	0.1408204	0.936471092	0.355649127
hsa-miR-584-3p	26.8685574	-0.61018395	-0.930093043	0.358438609
hsa-miR-1183	26.98519235	0.2903467	0.921756672	0.362497576
hsa-miR-335	28.5332077	0.37705105	0.920353413	0.363800157
hsa-miR-320a	21.04389085	0.29208245	0.903684028	0.372040786
hsa-miR-448	28.23364305	0.30251855	0.902264886	0.372642657
hsa-miR-375	26.4133213	-0.3852335	-0.89618551	0.375873877
hsa-miR-7	28.77767115	-0.45543415	-0.895889826	0.377980919
hsa-miR-323b-5p	27.40918575	-0.3787318	-0.887247451	0.380620946
hsa-miR-1274b	13.75481125	-0.3756949	-0.885158549	0.381927799
hsa-miR-181c#	30.50849865	0.2356556	0.874434976	0.38740319
hsa-miR-744	27.20480145	0.3506853	0.869253007	0.390475117
hsa-miR-1234	24.93561275	-0.293629	-0.860221249	0.395251938
hsa-miR-5095	26.5877565	-0.28790035	-0.85357325	0.399280773

hsa-let-7d	25.0008752	0.48898075	0.843168971	0.404632823
hsa-miR-1307	25.38789105	0.3200131	0.824910203	0.414760055
hsa-miR-494	25.32735315	-0.34495905	-0.82434122	0.414970658
hsa-miR-98	27.57443425	-0.3996101	-0.823926665	0.415170358
hsa-miR-3180-5p	17.0559855	0.21824015	0.806429554	0.425238312
hsa-miR-185	26.1815539	0.189505	0.754060576	0.455806273
hsa-miR-612	29.63939555	-0.26334875	-0.746922502	0.4599483
hsa-miR-766	26.87308365	-0.21527685	-0.72760122	0.471317294
hsa-miR-3651	25.76222025	-0.2232142	-0.727400218	0.47152996
hsa-miR-3191	24.8191096	-0.22560725	-0.727094849	0.471673478
hsa-miR-4695-3p	26.3316455	-0.23	-0.72431449	0.474482712
hsa-miR-106b	26.61006545	-0.3564899	-0.721599514	0.475066425
hsa-miR-320d	28.5826086	0.19390255	0.721857936	0.475397793
hsa-let-7g#	28.09309415	0.19231945	0.72164345	0.475469554
hsa-miR-216a	30.94009165	-0.2079436	-0.705383566	0.485429561
hsa-miR-93#	27.5081386	-0.1523531	-0.698294251	0.489639785
hsa-miR-5585-3p	25.4169486	0.1774127	0.697320391	0.489854914
hsa-miR-551b#	28.78316675	-0.1843535	-0.693770869	0.492655007
hsa-miR-1972	26.7827905	-0.22763265	-0.689941928	0.494424772
hsa-miR-326	24.25290355	-0.1539566	-0.68939688	0.495514013
hsa-miR-1224-3p	28.53325345	0.2251597	0.676649703	0.50310755
hsa-miR-3679-5p	26.4020032	0.2771728	0.674941659	0.504354724
hsa-miR-151-5p	25.1502141	0.22582085	0.659305932	0.513933309
hsa-miR-3619-3p	28.58486465	-0.2143546	-0.655142748	0.516554414
hsa-miR-625#	25.9377285	0.1554537	0.625559393	0.535343552
hsa-miR-654-5p	31.52516395	0.11648045	0.623917691	0.536423734
hsa-miR-302a	30.84744205	0.15741805	0.580578711	0.565002131
hsa-miR-1268b	26.0974757	-0.19846125	-0.579862391	0.567010382
hsa-miR-25	25.29295555	-0.1389799	-0.562271552	0.577249138
hsa-miR-323-5p	25.196418	0.15207035	0.551788961	0.584428268
hsa-miR-4290	30.2469358	-0.1581287	-0.544861615	0.589035576
hsa-miR-17	24.96355105	-0.24533295	-0.529715731	0.599870904
hsa-miR-20b#	27.6140579	0.17891275	0.524923087	0.603200452
hsa-miR-202	27.629297	0.12079885	0.523675137	0.604147683
hsa-miR-1299	31.63629935	0.0845147	0.522010887	0.6050627
hsa-miR-19a	23.68422655	0.1386444	0.50851605	0.614071319
hsa-miR-205	23.1873946	-0.2737087	-0.507709419	0.614891442
hsa-miR-642a-3p	27.61033735	0.2242457	0.504667643	0.61685273
hsa-miR-3141	24.13523625	0.09131945	0.504959479	0.617345024
hsa-miR-1207-5p	23.82764015	0.35541785	0.485376317	0.63019722
hsa-miR-550a#	29.96839845	-0.15557395	-0.48075854	0.633444623
hsa-miR-4664-3p	27.9946398	-0.1505473	-0.478845988	0.635584039
hsa-miR-17#	25.7515808	0.0976056	0.474246322	0.63818301
hsa-miR-2277-3p	24.84175595	-0.15218825	-0.462732031	0.646370519

hsa-let-7f	23.6711256	0.2959151	0.461866988	0.646877126
hsa-miR-564	27.23760085	0.23435625	0.457184877	0.65020731
hsa-miR-106a	25.39988795	-0.2100676	-0.444123366	0.65981449
hsa-miR-15a	23.1720913	0.17049295	0.442231218	0.660863024
hsa-miR-300	28.623326	-0.1314028	-0.414758253	0.680740147
hsa-miR-382	28.3467667	-0.1000463	-0.412582616	0.682451735
hsa-miR-1915	16.9399378	0.1236714	0.406935	0.686576961
hsa-miR-3689d	27.98357805	-0.07652995	-0.396705564	0.694457682
hsa-RNU48-1	21.95787005	0.3272244	0.393264742	0.696650292
hsa-miR-574-5p	25.41036955	0.13404955	0.380278301	0.70585643
hsa-miR-4725-5p	26.949534	-0.11933515	-0.378061612	0.707500641
hsa-miR-103-2#	28.4360144	0.1036946	0.372475071	0.711608704
hsa-miR-1246	17.30316425	-0.17320005	-0.372342033	0.711741213
hsa-miR-3181	20.5008378	-0.1995124	-0.363505146	0.718250828
hsa-miR-20a	25.0810381	-0.1477488	-0.35863641	0.722024089
hsa-miR-4306	27.22936405	-0.08594795	-0.338895232	0.736966577
hsa-miR-378g	27.46283575	0.09311515	0.337927878	0.737281911
hsa-miR-433	26.9782495	-0.2073267	-0.336341808	0.738607559
hsa-miR-301a	29.73122385	-0.1412877	-0.321838932	0.749338801
hsa-miR-652	27.8164068	0.09792065	0.320862128	0.750088077
hsa-miR-15b	23.37290045	0.1003191	0.320445035	0.750394543
hsa-miR-129-5p	28.8794664	0.10297905	0.315500916	0.754186863
hsa-miR-378	26.36678715	0.0988369	0.314487233	0.755068288
hsa-miR-665	20.0920677	0.10787025	0.283461241	0.778378911
hsa-miR-10a	25.6904664	-0.14084815	-0.283563401	0.778868098
hsa-miR-4484	28.00819605	0.09548285	0.269841541	0.788761755
hsa-miR-15a#	28.2211569	-0.0777877	-0.257849524	0.797924792
hsa-miR-1233	25.59041025	0.1306009	0.246570649	0.807044501
hsa-miR-484	24.9432688	-0.0504117	-0.245186001	0.807725846
hsa-miR-1202	26.86419775	0.0332515	0.228171891	0.82095575
hsa-miR-103	24.32553745	0.13688355	0.22647014	0.822051185
hsa-miR-941	27.8593266	0.05860165	0.224363525	0.823677058
hsa-miR-330-3p	28.72115875	-0.0720646	-0.196959293	0.844916079
hsa-miR-1254	28.54717835	0.058288	0.1896801	0.850581949
hsa-miR-3132	27.4959067	-0.0570482	-0.185182972	0.8545818
hsa-miR-501-5p	25.8247457	-0.0753557	-0.173588229	0.863151249
hsa-miR-454	28.7386076	0.07162315	0.17352609	0.86317055
hsa-miR-16-1#	29.34863545	-0.05301645	-0.166348808	0.86882912
hsa-miR-365b-5p	30.38777815	0.04380215	0.158426752	0.874978772
hsa-miR-331-3p	25.5062669	0.0446007	0.148997565	0.882573582
hsa-miR-2355-3p	30.95855525	0.06435495	0.140128443	0.889626869
hsa-miR-151-3p	25.15628685	0.04211665	0.13352093	0.894486117
hsa-let-7d#	24.00656225	-0.0395099	-0.133460794	0.894544296
hsa-miR-346	28.6858022	-0.04241905	-0.126901935	0.899723074

hsa-miR-3185	25.2378073	-0.07098325	-0.122512599	0.903142174
hsa-miR-4323	24.9424088	-0.04437345	-0.115628473	0.908570377
hsa-miR-3198	27.40352145	0.0345949	0.107008127	0.915472693
hsa-miR-3613-5p	25.59744405	-0.0395708	-0.106765334	0.915639947
hsa-miR-4499	24.6237142	-0.03937785	-0.105615673	0.916465316
hsa-miR-192	27.774702	0.04120985	0.104152241	0.917683785
hsa-miR-1274a	24.02371645	0.0386535	0.082935026	0.934344551
hsa-miR-1909#	25.54695015	-0.02103895	-0.078541132	0.937870115
hsa-miR-194	27.88834705	-0.0250198	-0.065966097	0.947785094
hsa-miR-545	29.5629911	-0.0156341	-0.050727576	0.95983062
hsa-miR-345	27.08308965	-0.01217395	-0.04508245	0.964278115
hsa-miR-1301	29.32401765	0.0079466	0.031756097	0.974832864
hsa-miR-3663-5p	28.97261735	-0.0061759	-0.022203476	0.982407235
hsa-miR-874	26.11694465	0.00386435	0.017253037	0.986327137
hsa-miR-663	22.2101501	0.0027166	0.007669137	0.993921278
hsa-7SL-scRNA	15.94935815	-0.00258635	-0.003819258	0.996977373

Supplemental Table 1: The expression levels of 365 dysregulated miRNAs and two endogenous controls in the BC tissues compared with those in adjacent normal tissues.

Mean Normal ct = qPCR ct value of miRNA in bladder adjacent tissues; FC = fold change; FC > 0 means decreased expression level in cancer tissues compared with adjacent tissues, FC < 0 means increased expression level in cancer tissues compared with adjacent tissues.

miR name	Mean Cancer ct	Mean Normal ct	Log$_2$ FC	Fold Change	T-value	P-value
hsa-miR-1	31.11	26.06	5.05	33.14	10.0188	6.09E-12
hsa-miR-30a	25.79	22.57	3.21	9.28	9.7216	2.11E-11
hsa-miR-133a	27.60	24.39	3.21	9.26	8.9519	2.00E-10
hsa-miR-143#	28.65	25.03	3.62	12.28	8.4715	4.67E-10
hsa-miR-4328	25.12	21.03	4.09	16.99	8.7692	6.55E-10
hsa-miR-143	24.10	19.72	4.37	20.75	8.1777	1.27E-09
hsa-miR-145	22.99	18.26	4.73	26.46	8.3910	2.74E-09
hsa-miR-376c	28.97	25.37	3.60	12.13	7.9053	3.84E-09
hsa-miR-125b	25.11	20.28	4.82	28.32	8.0892	5.55E-09
hsa-miR-199b-3p	25.03	20.71	4.32	19.97	8.4551	5.84E-09
hsa-miR-100	27.55	22.96	4.59	24.08	7.9632	1.27E-08
hsa-miR-99a	28.15	23.36	4.79	27.71	8.1981	1.28E-08
hsa-miR-497	27.11	24.03	3.08	8.48	7.5693	1.69E-08
hsa-miR-376a	30.03	26.43	3.61	12.17	7.7034	1.90E-08
hsa-miR-199a-5p	26.23	22.07	4.16	17.87	7.3058	2.21E-08
hsa-miR-195	26.30	23.15	3.15	8.86	6.8696	5.41E-08
hsa-miR-154	29.72	26.57	3.15	8.86	7.2751	9.96E-08
hsa-miR-139-5p	28.73	26.55	2.18	4.54	6.6091	1.13E-07

hsa-miR-127-3p	27.45	25.11	2.35	5.09	6.3966	2.33E-07
hsa-miR-150	27.99	24.13	3.86	14.55	6.3678	2.52E-07
hsa-miR-152	28.05	25.07	2.97	7.84	6.7012	2.57E-07
hsa-miR-379	31.49	29.14	2.35	5.11	6.3446	2.63E-07
hsa-miR-140-3p	28.02	25.45	2.57	5.94	6.0036	6.91E-07
hsa-miR-136#	30.24	26.85	3.39	10.45	6.0200	8.19E-07
hsa-miR-377	29.34	25.85	3.49	11.22	6.7306	8.48E-07
hsa-miR-495	30.61	27.90	2.71	6.54	6.3051	9.28E-07
hsa-miR-136	28.45	25.06	3.39	10.49	6.1732	9.73E-07
hsa-miR-101	23.47	21.50	1.97	3.91	5.6021	2.47E-06
hsa-miR-133b	29.08	27.03	2.06	4.16	5.7448	2.57E-06
hsa-miR-337-3p	28.82	26.64	2.18	4.54	5.7657	2.90E-06
hsa-miR-29a	23.15	20.63	2.52	5.72	5.8648	2.91E-06
hsa-miR-574-3p	25.23	23.35	1.88	3.67	5.5423	3.96E-06
hsa-miR-130a	25.99	23.45	2.54	5.81	5.7732	4.45E-06
hsa-miR-342-3p	25.23	23.15	2.08	4.24	5.6557	5.05E-06
hsa-miR-126#	24.90	22.86	2.05	4.13	5.1135	1.24E-05
hsa-miR-222	25.34	23.36	1.97	3.92	5.0994	1.58E-05
hsa-miR-28-3p	26.90	25.61	1.29	2.44	4.7988	2.87E-05
hsa-miR-365b-3p	25.38	24.10	1.29	2.44	4.8883	3.49E-05
hsa-miR-27b	24.19	22.41	1.79	3.45	4.7126	3.89E-05
hsa-let-7c	26.41	24.06	2.35	5.10	4.6752	4.04E-05
hsa-miR-342-5p	30.97	29.44	1.53	2.88	4.6693	5.39E-05
hsa-miR-431	25.68	27.39	-1.71	-3.27	-4.4787	8.06E-05
hsa-miR-320c	24.70	23.21	1.49	2.81	4.5258	8.33E-05
hsa-miR-182	26.70	28.78	-2.08	-4.22	-4.4854	9.35E-05
hsa-miR-551b	28.47	25.33	3.14	8.81	4.4619	9.38E-05
hsa-miR-543	31.08	29.69	1.39	2.61	4.4079	9.56E-05
hsa-miR-193b	24.15	23.08	1.07	2.10	4.2799	1.39E-04
hsa-miR-451	24.01	21.90	2.11	4.32	4.2547	1.45E-04
hsa-miR-1247	23.67	22.36	1.31	2.48	4.2604	1.50E-04
hsa-miR-140-5p	30.30	28.29	2.01	4.01	4.2463	1.56E-04
hsa-miR-142-5p	27.09	24.54	2.55	5.87	4.2429	1.56E-04
hsa-miR-126	23.02	21.17	1.85	3.61	4.1344	2.10E-04
hsa-miR-10b	26.22	24.68	1.54	2.90	4.1934	2.28E-04
hsa-miR-369-3p	30.14	28.35	1.80	3.47	4.1931	2.57E-04
hsa-miR-328	26.89	25.82	1.07	2.10	4.0529	2.64E-04
hsa-miR-338-3p	28.04	25.79	2.25	4.75	4.0157	2.89E-04
hsa-miR-183	26.64	28.48	-1.84	-3.57	-3.9608	3.49E-04
hsa-miR-532-5p	28.10	27.06	1.04	2.06	3.8482	4.68E-04

hsa-miR-409-3p	29.55	27.97	1.58	2.99	3.8801	5.41E-04
hsa-miR-24	23.50	21.96	1.54	2.90	3.7752	5.87E-04
hsa-miR-132	28.07	26.45	1.63	3.09	3.8190	5.97E-04
hsa-miR-30e#	26.88	25.28	1.60	3.02	3.7311	6.74E-04
hsa-miR-429	23.52	25.04	-1.51	-2.86	-3.7180	6.81E-04
hsa-let-7i#	26.96	25.52	1.44	2.70	3.7037	7.65E-04
hsa-miR-142-3p	25.15	22.34	2.81	7.01	3.6692	7.95E-04
hsa-miR-221	27.09	25.53	1.56	2.96	3.7036	7.97E-04
hsa-miR-4457	27.52	25.90	1.62	3.07	3.6927	8.31E-04
hsa-miR-186	25.53	24.57	0.96	1.95	3.6399	8.80E-04
hsa-miR-425	26.33	27.34	-1.01	-2.02	-3.6236	9.07E-04
hsa-miR-155	29.47	27.46	2.01	4.03	3.6076	9.51E-04

Table 2: 70 aberrant miRNAs with P-value < 0.001.

Mean Normal ct = qPCR ct value of mirna in bladder adjacent tissues; Mean Cancerct = qPCR ct value of mirna in bladder cancer tissues; FC = fold change; $Log_2FC > 0$ means decreased expression level in cancer tissues compared with adjacent tissues, $Log_2FC < 0$ means increased expression level in cancer tissues compared with adjacent tissues.

3.4. Developing miRNAs Expression Signatures In Diagnosis of BC

An unpaired T-test ($p < 0.05$) with a Benjamini Hochberg FDR multiple testing corrections was used to identify significantly dysregulated miRNAs that distinguish BC from normal controls. Accurate classification of BC patients from normal controls is crucial for successful BC treatment; we investigated the diagnostic value of the miRNA-expression profile in BC patients. Among the 70 significant miRNAs (P-value < 0.001) checked (these miRNAs listed in **(Table 2)** with t-value and p-value),several

expression signatures of three or four-miRNAs were developed as predictors of BC from normal controls. These signatures were selected based on a machine learning approach of support vector machine (SVM).

The best 10 groups of miRNA signatures were listed in Table 3. Thereby, in the group 1, the expression levels of miR-133a ($log_2FC = 9.258$; P < 0.000000001) were significant down regulated in BC patients. However, expression of miR-431 ($log_2FC = -3.268$; $P < 0.0001$) was significant higher in BC tissues than in adjacent normal tissues. In spite of level of miR-4251 ($P > 0.1$) was slightly higher in BC tissues than in adjacent normal tissues. The results showed that the use of improved comparative Ct method seems to be an easily applicable method with potential for general clinical use that avoids the need for large-scale, high-throughput profiling analyses and was therefore used to develop clinically useful signatures based on tissues biomarkers **(Supplemental Figure 1).**
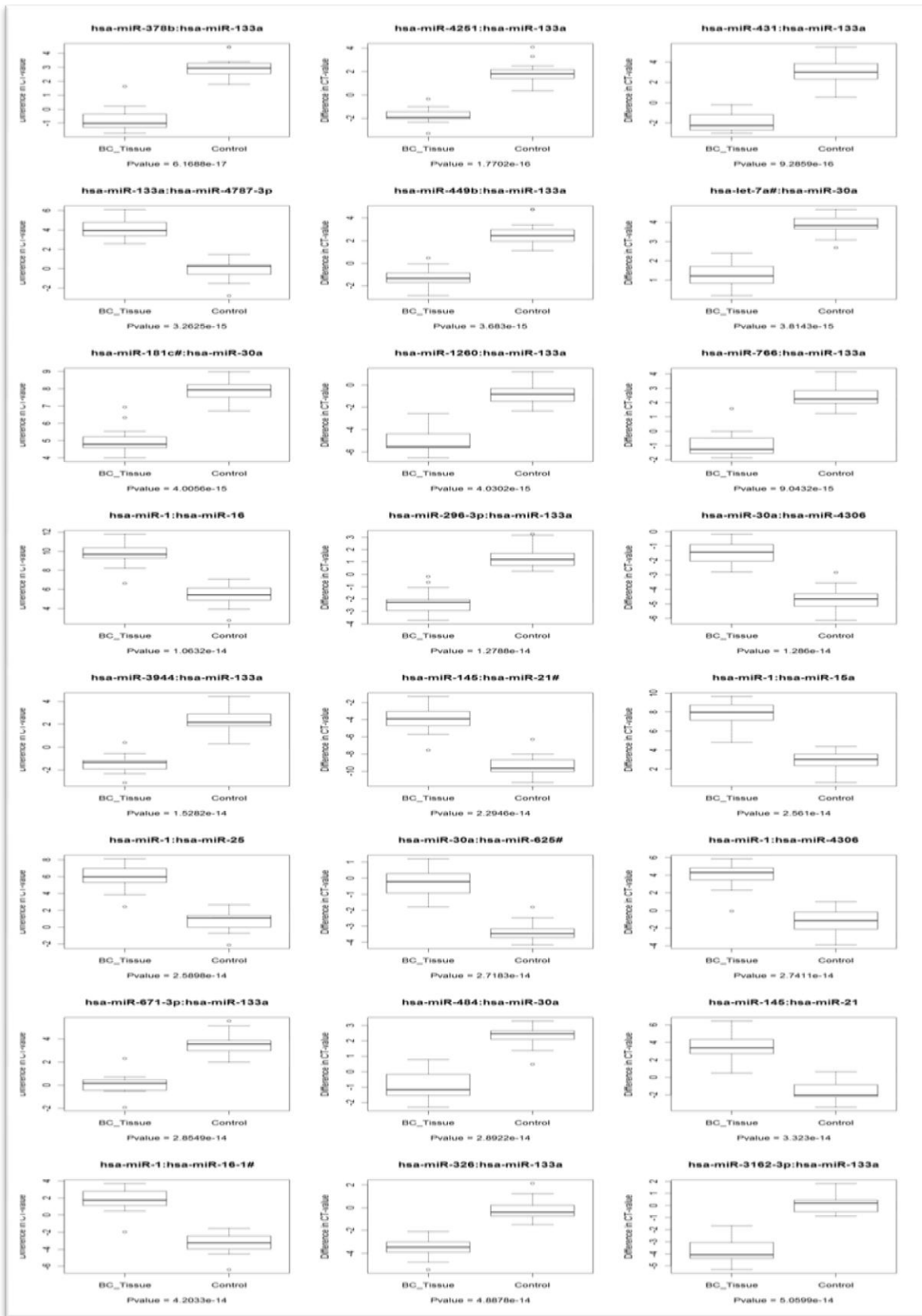
Supplemental Figure 1: Supply the results showed that the use of improved comparative Ct method seems to be an easily applicable method with potential for general clinical use that avoids the need for large-scale, high-throughput profiling analyses and was therefore used to develop clinically useful signatures based on tissues biomarkers.

3.5. Prediction of BC and Control Subjects by Risk Score Analysis

To verify the accuracy and specificity of these three or four miRNA signatures to be used as BC biomarkers, we further assessed the 3 miRNAs in the former set consisting of 38 samples, including of 19 BC tissue samples and 19 matched adjacent normal tissue samples **(Figure 3)**. The areas under the ROC curve (AUC) were 1 with 100% sensitivity and 100% specificity was respectively **(Figure 4)**.
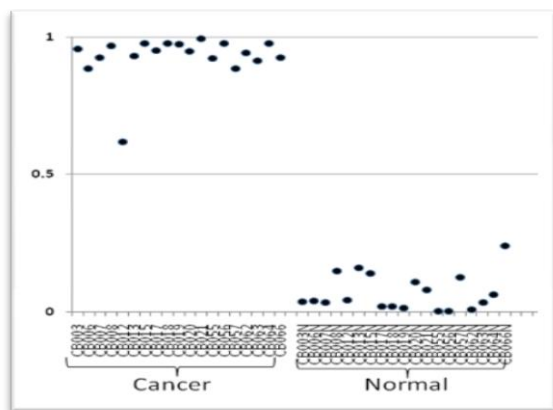
Figure 3: The classification performance of the three selected miRNAs on 19 bladder cancer tissues compared with the 19 matched patients' adjacent tissues. SVM prediction probability for 38 samples with an error of 0.
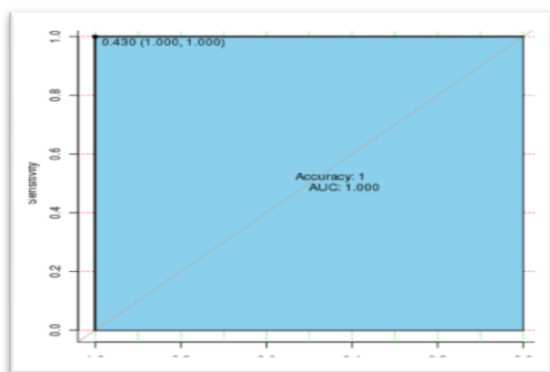
Figure 4: The classification performance of the three selected miRNAs on 19 bladder cancer tissues compared with the 19 matched patients' adjacent tissues. The receiver operating characteristic curve (area under the curve = 1) estimation for the miRNA panel in the BC and the matched adjacent tissue groups.

3.6. Double Blind Test

To verify the accuracy and specificity of identified miRNA signatures to be used as BC biomarkers, another 21 samples (including 11 BC tissue samples and 10 adjacent normal bladder tissue samples) were tested in a double-blind fashion to validate the predictive ability of the miRNA-based signatures for BC diagnosis. The accuracy 95.2% of the signature consisting of hsa-miR-133a, hsa-miR-431 and hsa-miR-4251 with 100% sensitivity and 90% specificity was respectively **(Figure 5)**. Especially, 10 of stage I BC tissues were confirmed by cytology.
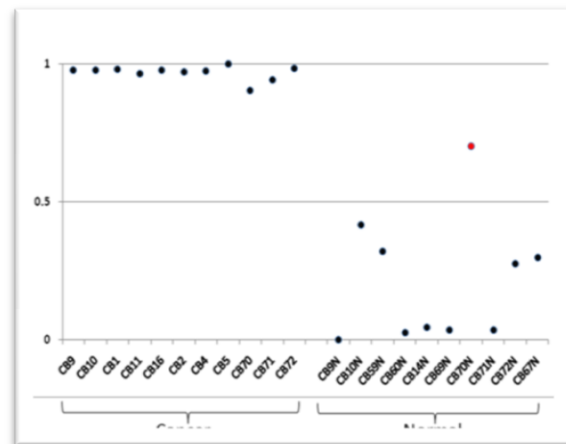
Figure 5: Validation ofBC miRNA Expression Signature Model 1 in another 21 samples. 11 bladder cancer tissues and 10 matched adjacent controls were separated by SVM score, Cancer > = 0.5, Normal < 0.5.

4. Discussion

In order to find specific signatures of BC tissue-based miRNAs, a comparative study was performed using a qRT-PCR array platform to profile 19 BC tissue samples from patients with BC and 19 adjacent normal tissue samples from matched patients.The study revealed that 70 aberrant miRNAs **(Table 2)**hsa-miR-96, hsa-miR-182, hsa-miR-183, hsa-miR-

429, hsa-miR-425, hsa-miR-431 are overexpressed in bladder cancer comparing to the normal. Among these miRNAs, miR-96, miR-182 and miR-183 are clustered at one locus of the chromosome 7 [15]. miR-429 belongs to the miR-200 family, which is clustered on the chromosomes 12, we further found that other members (200a/b/c) of miR200 family are also overexpressed in bladder cancer comparing to the normal **(Supplement table 1).** Both two miRNA clusters are well-known oncogenic miRNA clusters that have been extensively reported to involve in tumor genesis in ovarian cancer and other types of cancer **[16-19]** suggesting theses miRNAs may be involved in bladder cancer and ovarian cancer development.

The ten groups of miRNAs signatures have been selected to discriminate the BC from the normal controls with 100% sensitivity and 100% specificity, suggesting their potential value for diagnosis of BC **(Table 3, Figure 5).** These selected signatures all contain hsa-miR-133a that means hsa-miR-133a may play a key role in discriminating BC from normal controls. The number one signature includes three miRNAs, hsa-miR-133a, hsa-miR-431 and hsa-miR-4251. Hsa-miR-133a was significantly down regulated and hsa-miR-431 was significantly up-regulated in BC. However, the expression of hsa-miR-4251 wasn't changed between BC and normal controls. Furthermore, these signatures can predict BC with statistically significant high accuracy over 95% in double blind test. More importantly, these miRNA signatures could effectively distinguish early stage (25 cases in stage I) of BC tissues from normal controls, suggesting their potential valuein detection of BC at early stage.

miRNA signatures groups	marker1	marker2	marker3	marker4	Accuracy	AUC
1	hsa-miR-133a	hsa-miR-431	hsa-miR-4251	-	1	1
2	hsa-miR-133a	hsa-miR-296-3p	hsa-miR-4251	-	1	1
3	hsa-miR-133a	hsa-miR-10b	hsa-miR-4251	-	1	1
4	hsa-miR-133a	hsa-miR-449b	hsa-miR-484	-	1	1
5	hsa-let-133a	hsa-miR-7a#	hsa-miR-431	hsa-miR-4251	1	1
6	hsa-let-133a	hsa-miR-449b	hsa-miR-7a#	hsa-miR-4251	1	1
7	hsa-miR-133a	hsa-miR-449b	hsa-miR-1260	hsa-miR-4251	1	1
8	hsa-miR-133a	hsa-miR-449b	hsa-miR-296-3p	hsa-miR-4251	1	1
9	hsa-miR-133a	hsa-miR-449b	hsa-miR-484	hsa-miR-4251	1	1
10	hsa-miR-133a	hsa-miR-181c#	hsa-miR-326	hsa-miR-4251	1	1

Table 3: 10 best groups of miRNA signatures.

A previous study**[20]** has reported the expression level of miR-133a, miR-133b, miR-1 and miR-99a have down-regulated, however, miR-182 has up-regulated in BC by using microarray in large number of samples. These miRNAs might be involved in the tumorigenesis and deterioration of BC. Our results confirmed the previous findings, and further demonstrated that the signatures containing miR-133a can diagnose BC even at early stage with high sensitivity and specificity.
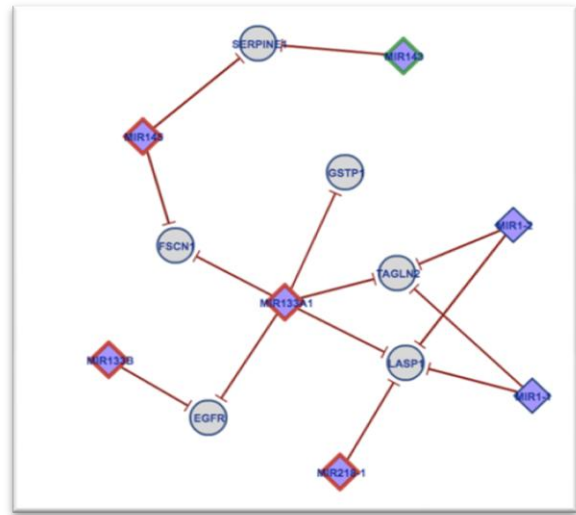
miR-133a is involved in numerous pathways related with different biological processes, cellular components, molecular functions, and some cell signaling, such as caspase signaling, insulin/IGF signaling, and EGFR signaling pathways **(supplemental Figure 2).** The connecting between importance of miR-133a in tumorigenesis and significantly down regulation of miR-133a expression in BC has shed light in prospective molecular mechanism of miRNA-133a and other miRNAs in the tumorigenesis of BC.
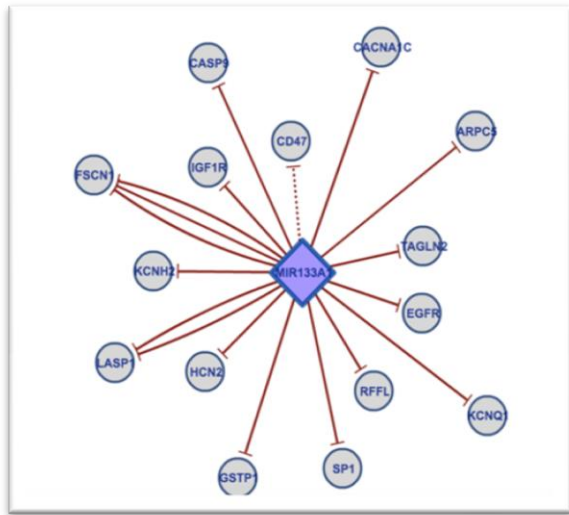
Supplemental Figure 2: miR-133a is involved in numerous pathways related with different biological processes, cellular components, molecular functions, and some cell signaling, such as caspase signaling, insulin/IGF signaling, and EGFR signaling pathways.

Supplemental Figure 3: Genes encoding miR-133 (miR-133a-1, miR-133a-2 and miR-133b) are transcribed as bicistronic transcripts together with miR-1-2 and miR-1-1. We had analyzed potential target gene and function of miR-133a by using www.mirnet.ca.

As reported from other investigators **[21,22]**, our results also showed that, miR-1, miR-133a, miR-133b, miR-143, miR-145 and miR-10b, are frequently downregulated in BC tissues, suggesting a major role of these tumor suppressor miRNAs in bladder carcinoma. Chief of all frequently downregulated miRNAs, the miR-133a was the only one important miRNA shown up in all ten BC diagnosis signatures **(Table 3).** miR-133a was one member of the miR-133 family, which was first experimentally characterized in mice**[23]**. miR-133 and miR-1 are clustered on the same chromosomal locus in the human genome (18q11.2) and share the same transcriptional unit, which have shown their essential functions in controlling skeletal muscle proliferation and differentiation**[25]**. Genes encoding miR-133 (miR-133a-1,miR-133a-2 and miR-133b) are transcribed as bicistronic transcripts together with miR-1-2 and miR-1-1. We had analyzed potential target gene and function of miR-133a by using www.mirnet.ca**(supplemental Figure 3).** We recognized that there is a correlation between miR-133a and miR-1 by target on TAGLN2 and LASP1 just like two references are reported **[22,25]**.In agreement with our study, miR-133a has been reported to be frequently down regulation in several types of cancer, including BC**[22, 26-28]**. miR-133a appears to act as tumor suppressors by inhibiting cell proliferation, invasion, migration, and apoptosis **[22,25]**.

References

1. Antoni S, Ferlay J, Soerjomataram I, Znaor A, Jemal A, et al. (2017) Bladder Cancer Incidence and Mortality: A Global Overview and Recent Trends. Eur Urol 71: 96-108.
2. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, et al. (2015) Global cancer statistics, 2012. CA Cancer J Clin 65: 87-108.
3. Siegel RL, Miller KD, Jemal A (2016) Cancer statistics, 2016. CA Cancer J Clin 66: 7-30.
4. Chen W, Zheng R, Baade PD, Zhang S, Zeng H, et al. (2016) Cancer statistics in China, 2015. CA Cancer J Clin 66: 115-132.
5. Avritscher EB, Cooksley CD, Grossman HB, Sabichi AL, Hamblin L, et al. (2006) Clinical model of lifetime cost of treating bladder cancer and associated complications. Urology 68: 549-553.
6. Lotan Y, Svatek RS, Sagalowsky AI (2006) Should we screen for bladder cancer in a high-risk population?: A cost per life-year saved analysis.

Cancer 107: 982-990.

7. Schmitz-Drager BJ, Todenhofer T, van Rhijn B, Pesch B, Hudson MA, Chandra A, et al. (2014) Considerations on the use of urine markers in the management of patients with low-/intermediate-risk non-muscle invasive bladder cancer. Urol Oncol 32: 1061-1068.

8. Saxena S, Jonsson ZO, Dutta A (2003) Small RNAs with imperfect match to endogenous mRNA repress translation. Implications for off-target activity of small inhibitory RNA in mammalian cells. J Biol Chem 278: 44312-44319.

9. Wu L, Belasco JG (2008) Examining the influence of microRNAs on translation efficiency and on mRNA deadenylation and decay. Methods Enzymol 449: 373-393.

10. Wu L, Fan J, Belasco JG (2006) MicroRNAs direct rapid deadenylation of mRNA. Proc Natl Acad Sci U S A 103: 4034-4039.

11. Adam L, Wszolek MF, Liu CG, Jing W, Diao L, et al. (2013) Plasma microRNA profiles for bladder cancer detection. Urol Oncol 31: 1701-1708.

12. Hanke M, Hoefig K, Merz H, Feller AC, Kausch I, et al. (2010) A robust methodology to study urine microRNA as tumor marker: microRNA-126 and microRNA-182 are related to urinary bladder cancer. Urol Oncol 28: 655-661.

13. Jiang X, Du L, Wang L, Li J, Liu Y, et al. (2015) Serum microRNA expression signatures identified from genome-wide microRNA profiling serve as novel noninvasive biomarkers for diagnosis and recurrence of bladder cancer. Int J Cancer 13: 854-862.

14. Wu P, Cao Z, Wu S (2016) New Progress of Epigenetic Biomarkers in Urological Cancer. Dis Markers 2016: 9864047.

15. Landgraf P, Rusu M, Sheridan R, Sewer A, Iovino N, et al. (2007) A mammalian microRNA expression atlas based on small RNA library sequencing. Cell 129: 1401-1414.

16. Hu X, Macdonald DM, Huettner PC, Feng Z, El Naqa IM, et al. (2009) A miR-200 microRNA cluster as prognostic marker in advanced ovarian cancer. Gynecol Oncol 114: 457-464.

17. Liu Z, Liu J, Segura MF, Shao C, Lee P, et al. (2012) MiR-182 overexpression in tumourigenesis of high-grade serous ovarian carcinoma. J Pathol 228: 204-215.

18. Segura MF, Hanniford D, Menendez S, Reavie L, Zou X, et al. (2009) Aberrant miR-182 expression promotes melanoma metastasis by repressing FOXO3 and microphthalmia-associated transcription factor. Proc Natl Acad Sci U S A 106: 1814-1819.

19. Wang L, Zhu MJ, Ren AM, Wu HF, Han WM, et al. (2014) A ten-microRNA signature identified from a genome-wide microRNA expression profiling in human epithelial ovarian cancer. PLoS One 9: e96472.

20. Wei Y, He R, Wu Y, Gan B, Wu P, et al. (2016) Comprehensive investigation of aberrant microRNA profiling in bladder cancer tissues. Tumour Biol 37: 12555-12569.

21. Pignot G, Cizeron-Clairac G, Vacher S, Susini A, Tozlu S, et al. (2013) microRNA expression profile in a large series of bladder tumors: identification of a 3-miRNA signature associated with aggressiveness of muscle-invasive bladder cancer. Int J Cancer 132: 2479-2491.

22. Yoshino H, Chiyomaru T, Enokida H, Kawakami K, et al. (2011)The tumour-suppressive function of miR-1 and miR-133a targeting TAGLN2 in bladder cancer. Br J Cancer 104: 808-818.

23. Lagos-Quintana M, Rauhut R, Yalcin A, Meyer J, Lendeckel W, et al. (2002) Identification of tissue-specific microRNAs from mouse. Curr Biol 12: 735-739.

24. Chen JF, Mandel EM, Thomson JM, Wu Q, Callis TE, et al. (2006) The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation. Nat Genet 38: 228-233.

25. Chiyomaru T, Enokida H, Kawakami K, Tatarano S, Uchida Y, et al. (2012) Functional role of LASP1 in cell viability and its regulation by microRNAs in bladder cancer. Urol Oncol 30: 434-443.

26. Catto JW, Abbod MF, Wild PJ, Linkens DA, Pilarsky C, et al. (2010) The application of artificial intelligence to microarray data: identification of a novel gene signature to identify bladder cancer progression. Eur Urol Mar 57: 398-406.

27. Catto JW, Miah S, Owen HC, Bryant H, Myers K, et al.(2009) Distinct microRNA alterations characterize high- and low-grade bladder cancer. Cancer Res 69: 8472-81.

28. Song T, Xia W, Shao N, Zhang X, Wang C, et al. (2010) Differential miRNA expression profiles in bladder urothelial carcinomas. Asian Pac J Cancer Prev 11: 905-911.