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A Four miRNAs Signature as a Potential Biomarker for Predicting Survival Using Bioinformatics Analysis in Bladder Cancer

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Abstract

Increasing evidences showed that a huge number of miRNAs were abnormally expressed in bladder cancer tissues and played vital roles in tumorigenesis, progression and metastasis. The aim of our study was to identify the differential miRNAs expression between bladder cancer and normal bladder tissues by analyzing the high-throughput miRNA data downloaded from TCGA database. Additionally, we evaluated the prognostic values of the differentially expressed miRNAs and constructed a four-miRNA signature that could effectively predict patient survival. According to the cut-off criteria ($P<0.05$ and $|\log_2 FC>1.2$), a total of 348 differentially expressed miRNAs were identified between bladder cancer tissues and matched normal tissues, including 264 up-regulated miRNAs and 84 down-regulated miRNAs. The Kaplan-Meier survival method revealed the prognostic function of the four miRNAs (miRNA-217, miRNA-378c, miR-33b and miRNA-615). Univariate and multivariate Cox regression analysis showed that the four-miRNA signature was an independent prognostic factor in bladder cancer. The functional enrichment analysis showed that the target genes of four miRNAs may be involved in various pathways related to cancer, including Notch signaling pathway, PI3K-Akt signaling pathway, p53 signaling pathway, cGMP-PKG signaling pathway, FOXO and signaling pathway. Taken together, our study suggested that four-miRNA signature could be used as a prognostic marker in bladder cancer.

Keywords: Bladder cancer; miRNA profiles; miRNA signature; Prognosis; TCGA; Bioinformatics analysis

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Introduction

As one of the most common urogenital cancers, Bladder Cancer (BCa) caused approximately 386,300 new BCa cases and 150,200 deaths in 2008 [1]. The incidence of BCa varies greatly among different geographic regions with the highest incidences in countries where the dominant population is Caucasoid [2]. The risk of developing BCa is associated with many factors such as smoking which is a major risk factor for developing BCa and the relative risk of death from BCa among smokers is 2.75 for current smokers while 2.0 for former smokers and with exposure to some other carcinogens [3-5]. Moreover, BCa accounts for 3.1% and 1.8% of the overall cancer mortality in males and females, respectively and BCa occurs more frequently in the elderly. The median ages of men and women diagnosed with BCa are 72 and 74 years, respectively [6]. Many chemicals are thought to be carcinogens for BCa, including aniline dyes and aromatic amines [7]. Urinary tract infection, chronic irritation from catheters or bladder stones, and a non-functioning bladder are also associated with an increased risk of Squamous Cell Carcinoma (SCC) of the bladder [8]. Bladder infection by Schistosoma Haematobium carries an increased risk of BCa, especially SCC, and is endemic in Egypt; inflammation is thought to play an important role in carcinogenesis associated with this parasite [9]. Exposure to pelvic radiation, for example in men with prostate cancer, appears to increase the risk of BCa [10]. Strong epidemiologic evidence does not exist for a hereditary cause of most BCa [11]. Current prognostic factors, namely Tumor Node Metastasis (TNM) stage and

pathological grade, are insufficient to predict individual clinical outcome [12]. These clinicopathological risk factors do not clearly distinguish between patients who have a high or low risk of disease recurrence, chemotherapy response and Overall Survival (OS). Thus, understanding of the molecular mechanisms of bladder cancer development and identification of novel biomarkers are required for the early detection and treatment of bladder cancer. Many of molecular peculiarities may serve as diagnostic and/or prognostic markers of tumor growth, as well as signs of disease progression. BCa diagnostics can be also based on the detection of molecular markers, which can provide detailed molecular insight into the progression and metastasis of disease and the clinical use of molecular markers can lead to more accurate and surveillance and patient-specific prognoses. In addition, the use of biomarkers has the potential to improve the quality of life of BCa patients compared with the traditional limiting invasive and painful procedures used to diagnose tumor growth. Nowadays, the most commonly used molecular markers of BCa are protein-coding genes and their products, which show differential expression in tumor cells versus normal cells. However, non-coding small RNA molecules may be more useful alternative BCa biomarkers. There are several classes of small RNAs like miRNAs, including PIWI Interacting RNAs (piRNAs), Small Interfering RNAs (siRNA), and others that are each characterized by their different targets, mechanisms of maturation, and action. Small non-coding RNAs take part in the regulation of major biological processes such as cell division, apoptosis, differentiation, growth, migration, etc [13]. The miRNAs can be used as biomarkers for many types of cancers [14]. Some miRNAs may help trace the tissue of origin of cancers whose primary origin is unknown [15]. In addition, miRNA molecules are advantageous for molecular diagnostics due to their greater stability *in vitro* compared to mRNA molecules [16]. The best studied non-coding RNAs are microRNAs (miRNAs). Furthermore, different cancer types, stages, and differentiation grades may have unique miRNA expression profiles, which make miRNAs potent biomarkers for cancer diagnosis [17-20]. The miRNAs are a group of small non-coding RNAs that negatively regulate the translation and stability of partially complementary target mRNAs. In that way, they play important roles in a wide array of biologic processes, including cell proliferation, differentiation, and apoptosis [21]. It has been shown that miRNAs are aberrantly expressed in various types of malignancies and function either as oncogenes or tumor suppressors [22]. Increasing evidence suggests that dysregulation of miRNA expression contributes to the initiation and progression of human cancer [23,24]. What's more, in mammals, miRNAs are necessary for normal development, cell growth, differentiation, apoptosis, and the regulation of many other processes [25]. The miRNAs are also known to play significant roles in tumorigenesis. More than 50% of miRNA genes are located in cancer-associated genomic regions or in fragile sites of the genome. Many types of cancer are associated with aberrantly expressed miRNAs. Both losses and gains of miRNA function contribute to cancer development and continued tumor growth [26]. Altered miRNA expression is thought to play an important role in the pathogenesis of Bladder Cancer (BCa) and in certain tumor phenotypes. Therefore, miRNAs have a large potential to serve as promising markers in the diagnosis, prognosis, and personalized targeted therapies.

Several previous researches used miRNA microarrays with limited and varied probes to profile the miRNA expression in bladder cancer [27-30]. However; their results did not always indicate consistent results and recent micro array data sets for miRNA showed inconsistent results between the studies due to different technological platforms and small sample size application. To date, only a few reports have described miRNA expression and its association with survival in bladder cancer. The miRNA profiling data sets were emerging rapidly with the employ of high-throughput technologies. Considering the inconsistent annotation and ongoing discovery of new miRNAs, different detection methods used by different technological platforms and various methods for data processing and analysis, we used the Cancer Genome Atlas Project (TCGA) database which is a National Cancer Institute (NCI) effort to profile at least 20 different tumor types using genomic platforms and to make raw and processed data available to all researchers [31]. The TCGA released a large number of miRNA sequencing data for bladder cancer patients. The aim of the present study was to identify the differential miRNAs expression between bladder cancer tissues and matched normal bladder tissues by analyzing the high-throughput miRNA data downloaded from TCGA database. Additionally, we evaluated the prognostic value of the differential expressed miRNAs and constructed a four-miRNA signature that could effectively predict patient survival.

Materials and Methods

Data processing

The raw sequencing data and clinical information were downloaded from TCGA database (https://cancergenome.nih.gov/). The inclusion bladder was set as follows: (1) The sample with both miRNA sequencing data and clinical information; (2) The sample with prognosis information. Finally, a total of 407 samples were enrolled in this study, including 388 bladder cancer tissues and 19 matched normal tissues. The miRNA sequencing data were processed using R language package. The differentially expressed miRNAs between bladder cancer and normal tissues were analyzed by limma package in R. The Folds Changes (FCs) in the expression of individual miRNA were calculated and differentially expressed miRNAs with $\log_2|FC|>1.2$ and $P<0.05$ were considered to be significant.

Association of differentially expressed miRNAs and patient prognosis

The differentially expressed miRNA profiles were normalized by $\log_2$ transformed. The prognostic value of each differentially expressed miRNA was evaluated using Kaplan-Meier curve and Log-rank method. The miRNAs that were significantly associated with overall survival were identified as prognostic miRNAs, and then subjected to a binary logistic regression analysis. Subsequently, a prognostic miRNA signature was constructed, and the miRNA signature could calculate a risk score for each bladder cancer patient. With the miRNA signature, bladder cancer patients were classified into high risk and low risk groups using the median risk score. Then, the differences in patients' survival between the high risk group and low risk group were evaluated by Kaplan-Meier method.

The target gene prediction of prognostic miRNA signature

The target genes of prognostic miRNAs were predicted using Target Scan (http://www.targetscan.org/), miRDB (http://www.mirdb.org/miRDB/), PicTar (http://pictar.mdc-berlin.de/), and miRanda (http://www.microrna.org/) online analysis tools. To further enhance the bioinformatics analysis reliability, the overlapping target genes were identified using Venn diagram. Then, the overlapping genes were analyzed by The Database for Annotation, Visualization and Integrated Discovery (DAVID) bioinformatics tool (https://

Table 1: Clinical characteristics of cervical cancer patients.

Variables	Case, n (%)
Gender	
Female	106 (26.0%)
Male	301 (74.0%)
Age at diagnosis	
<60	88 (21.6%)
≥60	319 (78.4%)
Metastasis	
M0	195 (47.9%)
M1	11 (2.7%)
MX	198 (47.7%)
NA	3 (0.7%)
Lymph node status	
N0	236 (58.0%)
N1-2	122 (30.0%)
N3	7 (1.7%)
NX	36 (8.8%)
NA	6 (1.5%)
Stage	
I+II	132 (32.4%)
III+IV	273 (67.1%)
NA	2 (0.5%)
T stage	
T0+T1+T2	123 (30.2%)
T3+T4	251 (61.7%)
TX	1 (0.2%)
NA	32 (7.9%)
Grade	
High Grade	383 (94.1%)
Low Grade	21 (5.2%)
NA	3 (0.7%)
Smoking History Category	
<3	199(48.9%)
≥3	195(47.9%)
NA	13(3.2%)

david.ncifcrf.gov/). DAVID is a web-based online bioinformatics resource that aims to provide a comprehensive set of functional annotation tools for the investigators to understand the biological mechanisms associated with large lists of genes/proteins 41. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were then performed for the target genes. The P-value <0.05 and gene count ≥ 3 were set as the cut-off criteria.

Statistical analysis

The data were expressed as mean ± Standard Deviation (SD). The expression levels of miRNAs in bladder cancer and matched normal tissues were analyzed by unpaired t test. The chi-square and t tests were performed to assess the relationship between miRNA expression and clinical features. Kaplan-Meier survival analysis and univariate/multivariate Cox proportional hazard regression analysis were carried out to compare each miRNA (low *vs.* high level) and prognostic miRNA signature (low *vs.* high risk). P value less than 0.05 was considered as statistical significant. The statistical analysis was performed using IBM SPSS Statistics software program version 22.0 (IBM Corp., NY and USA).

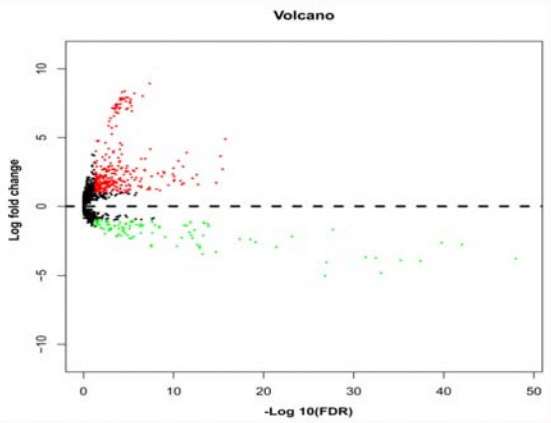

Figure 1: Volcano plot of differentially expressed miRNAs. The red dot represents up-regulated miRNA, and green dot represents down-regulated miRNA.

Results

Identification of differentially expressed miRNAs in bladder cancer

In the present study, a total of 407 samples were enrolled in this study, including 388 bladder cancer tissues and 19 matched normal tissues. The detailed clinical characteristics include gender, diagnosis at age, metastasis, lymph node status, stage, T stage, grade and smoking history category (Table 1). According to the cut-off criteria ($P<0.05$ and $|\log_2 FC|>1.2$), a total of 348 differentially expressed miRNAs were identified between bladder cancer tissues and matched normal tissues, including 264 up-regulated and 84 down-regulated miRNAs. In order to prove the P value and $|\log_2 FC|$ whether conform to logic with different test, we present the result as Volcano plot (Figure 1).

Identification of four miRNAs associated with os in bladder cancer

To identify the miRNAs which would be potentially associated with overall survival of bladder cancer patients, we evaluated the association between miRNAs expression and patients' survival using Kaplan-Meier curve and Log-rank test. The results showed that one miRNA (miR-378c) was negatively correlated with Overall Survival (OS), and three miRNAs (miR- 217, miR-33b and miR-615) were positively related to OS (Figure 2). The association between four miRNAs and clinical features was evaluated in bladder cancer patients (Table 2). The results showed that miR-378c was significantly associated with stage (P=0.001), grade (P<0.001) and T stage (P=0.005); miR-33b was associated with stage (P=0.022); miR-217 was significantly associated with lymph node status (P=0.013), grade (P=0.001) and T stage (P=0.002). Except gender, no significant difference was found between miR-615 and other clinical features (P>0.05).

Prognostic value of four miRNAs signature risk score in bladder cancer

We constructed a prognostic signature by integrating the

Table 2: Association of four miRNAs and clinical features.

Variables	Numbers	miR-378c	P value	miR-33b	P value	miR-217	P value	miR-615	P value
Age at diagnosis									
<60	88	5.25 ± 1.35	0.085	3.12 ± 1.67	0.817	5.57 ± 2.36	0.342	2.19 ± 1.98	0.598
>=60	319	4.98 ± 1.10		3.07 ± 1.78		5.84 ± 2.09		2.29 ± 1.84	
Gender									
Female	106	5.01± 1.11	0.763	2.94 ± 1.82	0.347	6.05 ± 2.29	0.15	2.70 ± 1.87	0.003
Male	301	5.05± 1.18		3.13 ± 1.74		5.69 ± 2.10		2.12 ± 1.85	
Metastasis									
M0	195	5.16 ± 1.16	0.295	3.28 ± 1.61	0.49	5.53 ± 2.23	0.304	2.31 ± 2.03	0.274
M1	11	4.59 ± 1.68		3.70 ± 1.90		6.91 ± 3.93		3.34 ± 2.53	
Lymph node status									
N0	236	5.10 ± 1.18	0.121	3.00 ± 1.59	0.475	5.67 ± 2.10	0.013	2.23 ± 1.90	0.569
N1-2	122	4.91 ± 1.06		2.85 ± 2.04		6.26 ± 2.15		2.43 ± 1.84	
Stage									
I+II	133	5.32 ± 1.21	0.001	3.34 ± 1.45	0.022	5.25 ± 2.28	0.001	1.97 ± 1.85	0.156
III+IV	273	4.90 ± 1.11		2.95 ± 1.88		6.05 ± 2.03		2.41 ± 1.87	
T stage									
T0+T1+T2	123	5.27 ± 1.14	0.005	3.21 ± 1.39	0.136	5.35 ± 2.26	0.002	1.87 ± 1.81	0.062
T3+T4	251	4.92 ± 1.11		2.95 ± 1.80		6.11 ± 2.00		2.44 ± 1.87	
Grade									
High Grade	383	4.97 ± 1.14	0	3.08 ± 1.79	0.926	5.78 ± 2.15	0.493	2.35 ± 1.86	0.05
Low Grade	21	6.19 ± 0.87		3.10 ± 1.30		6.11 ± 2.10		1.10 ± 1.61	
Smoking History Category									
<3	199	5.09 ± 1.14	0.425	3.02 ± 1.69	0.464	5.84 ± 2.35	0.562	2.32 ± 1.92	0.8
>=3	195	4.99 ± 1.19		3.15 ± 1.87		5.71 ± 1.99		2.22 ± 1.81	

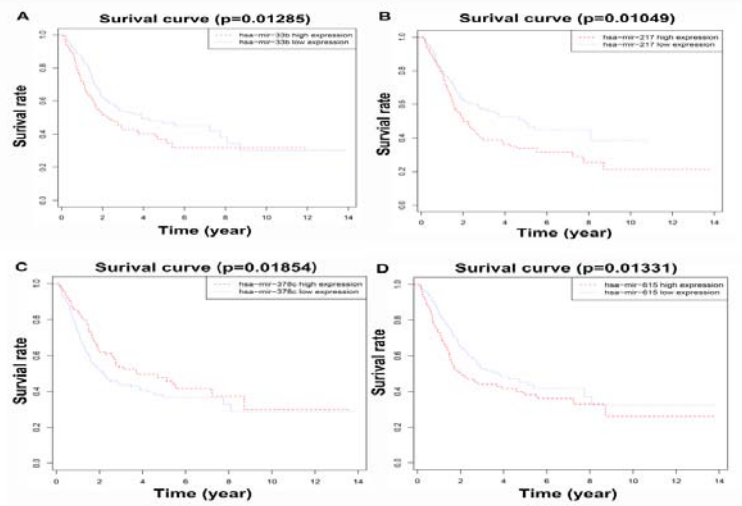

Figure 2: Four miRNAs were associated with overall survival in bladder cancer patients by using Kaplan-Meier curve and Log-rank test. The patients were stratified into high level group and low level group according to median of each miRNA. (A) miR-33b; (B) miR-217; (C) miR-378c and (D) miR-615.

expression profiles of four miRNAs and corresponding estimated regression coefficient. Then, we calculated a risk score for each patient, and ranked them according to increased score. Thus, a total of 407 patients were classified into a high risk group (n=203) and a low risk group (n=204) according to the median risk score. Cox regression analysis validated the panel of four miRNAs signature as a potential prognostic biomarker (HR: 28.7, P=8.97e-06). Survival analysis and Receiver Operating Characteristic curve (ROC) was performed using the Kaplan-Meier method with a Log-rank statistical test. The result showed that patients in high risk group have significantly worse OS than patients in low risk group (P=5e-05, Figure 3A) and ROC are presented for prediction models (AUC= 0.64, Figure 3B).

Target prediction and function analysis

The target genes of four miRNAs (miR-378c, miR-217, miR-33b

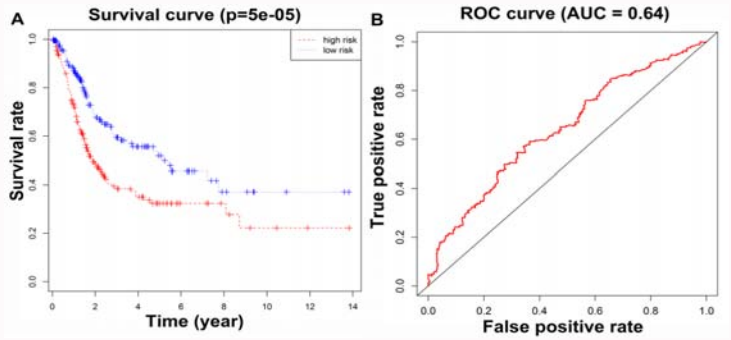

Figure 3: Kaplan-Meier curve for the four-miRNA signature in bladder cancer patients. (A) The patients were stratified into high risk group and low risk group based on median. (B) ROC curve analysis for the detection of four miRNAs.

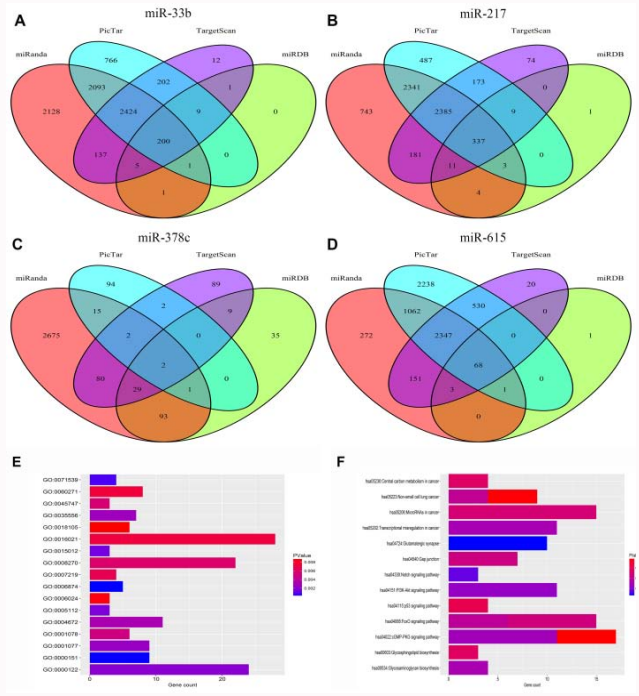

Figure 4: The target gene prediction and function analysis. The overlapping target genes were predicted using Target Scan, miRDB, PicTar, and miRanda online analysis tools. (A) miRNA-33b; (B) miRNA-217; (C) miR-378c; (D) miR-615; (E) The significant enriched GO biological processes of target genes. (F) The significant enriched KEGG pathways of target genes.

and miR-615) were predicted using Target Scan, miRDB, PicTar, and miRNA online analysis tools. A total of 200 overlapping genes of miR-33b, 337 overlapping genes of miR-217, 2 overlapping genes of miR-378c and 68 overlapping genes of miR-615 were identified (Figure 4). Then, enrichment analysis was performed to elucidate the biological function of consensus target genes. The KEGG pathways were significantly enriched in Notch signaling pathway, PI3K-Akt signaling pathway, p53 signaling pathway, cGMP-PKG signaling pathway, FOXO and signaling pathway. In addition, the GO Biological Process (BP) terms were mainly enriched in signal transduction, protein kinase activity, regulation of cell migration, and regulation of transcription.

Discussion

Recently, miRNAs, the master modulators of multiple biological and pathological processes, are a hot research topic in the area of cancer development. Increasing evidence has demonstrated that miRNAs established a complex combinatorial system of gene expression and pathway regulation, as well as prognostic indicators and therapeutic targets in different cancers, including bladder cancer [32,33]. Other studies have also demonstrated that many miRNAs are crucial for the initiation, progression and metastasis of bladder cancer by regulating various processes, including cancer cell proliferation, differentiation, apoptosis, adhesion, cell cycle arrest, migration and invasion [34]. In the present study, a total of 388 differentially expressed miRNAs were identified, and four of them were associated with overall survival in bladder cancer patients. The four-miRNA (miR-217, miR-378c, miR-615 and miR-33b) signature was established and was identified to be an independent prognostic factor for bladder cancer patients. Furthermore, we screened the target genes of these four miRNAs, and predicted the enrichment pathways and biological functions of target genes using bioinformatics methods to explore their potential functions. To date, several studies had identified a number of miRNAs with prognostic values in bladder cancer, such as miR-125, miR-29c, miR-96, miR-210 and miR-182-5p and so on [35-39]. However, previous studies were based on small sample size, sample types, different detection platforms, various assay methods, and relatively limited numbers of miRNAs. In our study, three up-regulated miRNAs (miR-217, miR-33b and miR-615) and one down-regulated miRNA (miR-378c) were analyzed by high-throughput data and were associated with clinical outcome of bladder cancer patients. Brown MS et al., [40] reported that increased miR-33b levels in the liver could thus potentially contribute to both high VLDL levels as well as low HDL levels found in individual suffering from metabolic syndrome. Sheng-Qing Lv et al., [41] demonstrated that both deletion and amplification were detected in miR-33b in Medulla blastomas. These results are in agreement with combined data on >350 medulla blastomas using array CGH (http://www.progenetix.net/progenetix/), which show that chromosomal locus at 17p11.2 (locus of miR-33b) showed both loss (18% to 25%) and gain (30% to 32%) [42]. The miR-33b was confirmed to be a tumor suppressor in human gastric cancer, lung cancer and colorectal cancer, suggesting its complexity role in cancer as it can act either as oncogene or tumor suppressor depending on the origin of cancer [43-45]. Our results showed that miR-33b was up-regulated in bladder cancer and miR-33b was significantly correlated with overall survival rate of bladder cancer patients. Few studies were found on miR-378c. But the family of miR-378, a tumor suppressor, was strongly down-expressed and related to proliferation, migration and invasion of colon cancer [46]. Moreover, the analysis of human prostate cancer and prostate control specimens confirmed the deregulated expression of miR-378 in primary tumors. Forced expression of the miRNAs mentioned above

affected tumorigenic properties, such as proliferation, migration and invasion [47]. Xuedong Chen et al., [48] report altered expression of miR-378 in human lung adenocarcinoma cell lines with varying sensitivities to cisplatin, and have shown that miR-378 can restore cisplatin chemosensitivity in the human lung adenocarcinoma cells. The expression levels of hsa-miR-615-3p was significantly higher in urine samples of patients with PCa than in those of BPH controls and the same results were in the bladder cancer [49,50]. In hepatocellular carcinoma, miR-615 was restrictedly expressed and its overexpression alleviates the tumorigenic effects [51]. Moreover, miR-615-5p was epigenetically inactivated and functions as a tumor suppressor in pancreatic ductal adenocarcinoma [52]. Obviously, miR-615 was closely associated with urinary tract tumors, especially bladder and prostate cancer. Combined with our analysis, we believed that miR-615 has a good predictive effect in survival of bladder cancer. The expression of miR-217 was markedly increased in hepatocellular carcinoma tissues and cells. Overexpression of miR-217 promoted, while silencing miR-217 suppressed, the fraction of the side population and the expression of cancer stem cell factors *in vitro* and tumorigenicity *in vivo* in hepatocellular carcinoma cells [53]. Compared to that in normal breast samples, the expression of miR-217 was significantly upregulated in breast cancer tissues. High level of miR-217 was notably correlated with highly histological grade, the triple negative subtype and advanced tumor stage. Moreover, the expression of miR-217 was negatively correlated with the expression of DACH1 [54]. In contrast, miR-217 acted as a tumor suppressor role in human epithelial ovarian cancer, cardiac myxoma and colorectal cancer [55-57]. Our results showed that miR-217, miR-33b, miR-615 was up-regulated in bladder cancer, and may be as an oncogene in development of bladder cancer. Furthermore, miR-217 was significantly associated with stage, lymph node status and T stage; miR-378c was associated with stage, T stage, and grade, miR-33b was associated with lymph node status indicating miR-217, miR-33b and miR-378c were involved in the progression of bladder cancer. But, no significant difference was found between miR-615 and clinical features. Maybe, miRNA-615 was related to other factors. The future study will focus on this point, and investigate the function of miRNA-615 in bladder cancer. In the present study, we found that miR-217, miR-33b, miR-615 and miR-378c were differentially expressed, and significantly associated with overall survival in bladder cancer patients. While efficacy of a single marker was limited, multi-markers based model may provide more powerful information for the prognosis prediction of patients. We constructed four-miRNA signature, and the results suggested that the four-miRNA signature (high risk and low risk) predicted survival well, and was an independent prognostic factor in bladder cancer. To gain a deep insight into the molecular functions of four miRNAs, we predicted the target genes and analyzed the related pathways and GO annotations. Abnormal signaling pathways play crucial roles in the pathogenesis and progression of bladder cancer. We found that four miRNAs could regulate several key signaling pathways, including Notch signaling pathway, PI3K-Akt signaling pathway, p53 signaling pathway, FOXO signaling pathway, and PKG signaling pathway. Accumulating evidence has demonstrated that activation of the NOTCH pathway stabilizes the epithelial phenotype through its effector HES1 and, consequently, loss of NOTCH activity favors the process of epithelial-mesenchymal transition. Evaluation of human bladder cancer samples revealed that tumors with low levels of HES1 present mesenchymal features and are more aggressive [58]. Li Y et al., [59] reported that activation of PI3K-Akt signaling pathway promotes cellular proliferation in bladder cancer. Tang M et al., [60] reported that the degradation of p-FOXO protein expression decreased the activated effect on cell proliferation, viability. Moreover, it has been well established that the PI3K-Akt signaling pathway plays a crucial role in bladder cancer development, and inhibition of FOXO protein activity suppress tumor growth. Therefore, further molecular investigations are needed to confirm these predictions, and it can provide new therapeutic interventions in bladder cancer. Taken together, we identified four-miRNA signature as a potential prognostic predictor for bladder cancer patients. Further studies are needed to validate our findings in large sample size, and further function investigation are also required to explore the molecular mechanism of these miRNAs in bladder cancer progression.

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