Mol Biol Rep (2014) 41:8127–8135
DOI 10.1007/s11033-014-3713-5

Investigation of key miRNAs and target genes in bladder cancer using miRNA profiling and bioinformatic tools

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Received: 6 July 2014 / Accepted: 28 August 2014 / Published online: 5 September 2014

Abstract Despite the association of several miRNAs with bladder cancer, little is known about the miRNAs' regulatory networks. In this study, we aimed to construct potential networks of bladder-cancer-related miRNAs and their known target genes using miRNA expression profiling and bioinformatics tools and to investigate potential key molecules that might play roles in bladder cancer regulatory networks. Global miRNA expression profiles were obtained using microarray followed by RT-qPCR validation using two randomly selected miRNAs. Known targets of deregulated miRNAs were utilized using DIANA-TarBase database v6.0. The incorporation of deregulated miRNAs and target genes into KEGG pathways were utilized using DIANA-mirPath software. To construct potential miRNA regulatory networks, the overlapping parts of three selected KEGG pathways were visualized by Cytoscape software. We finally gained 19 deregulated miRNAs, including 5 ups- and 14 down regulated in 27 bladder-cancer tissue samples and 8 normal urothelial tissue samples. The enrichment results of deregulated miRNAs and known target genes showed that most pathways were related to cancer or cell signaling pathways. We determined the hub *CDK6, BCL2, E2F3, PTEN, MYC, RB,* and *ERBB3* target genes and hub *hsa-let-7c, hsa-miR-195-5p, hsa-miR-141-3p, hsa-miR-26a-5p, hsa-miR-23b-3p,* and *hsa-miR-125b-5p* miRNAs of the constructed networks. These findings provide new insights into the bladder cancer regulatory networks and give us a hypothesis that *hsa-let-7c, hsa-miR-195-5p*, and *hsa-miR-125b-5p,* along with *CDK4* and *CDK6* genes might exist in the same bladder cancer pathway. Particularly, hub miRNAs and genes might be potential biomarkers for bladder cancer clinics.

Keywords Bladder cancer · miRNA · Bioinformation · KEGG pathways · *CDK4* · *CDK6*

Introduction

Bladder cancer is the 9th most common cancer in the world with 430,000 new cases diagnosed in 2012 and

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Belgium had the highest rate of bladder cancer in both sexes (17.5 per 100,000), followed by Lebanon (16.6 per 100,000), Malta (15.8 per 100,000) and Turkey (15.2 per 100,000) [1]. In Turkey 10,757 new cases were diagnosed and 4,690 patients died from bladder cancer complications in both sexes in 2012 [1]. Both environmental and genetic factors contribute to carcinogenesis of bladder cancer; and tobacco smoking, occupational exposure to aromatic hydrocarbons, family history of cancer, chemotherapy and radiotherapy are major etiological factors [2, 3].

Micro ribonucleic acids (miRNAs) are short (19–22 nucleotides) non-protein coding RNAs and are found in all eukaryotic cells. They regulate gene expression epigenetically through complementarily binding to the 3′ UTR region of a target mRNA transcript, and resulting in translational repression and gene silencing. More than 60 % of mammalian mRNAs contain conserved regions that serve as targets for miRNAs. In addition, one mRNA can be the target of multiple miRNAs, and each individual miRNA has the capacity to target hundreds of genes, with an average of 500 targets per miRNA [4]. As of its most recent release in June of 2013, over 2,578 mature miRNAs were identified in humans and cataloged in the miRBase 20.0 databases [5].

Accumulating evidence suggests aberrant miRNA expression patterns in most human malignancies, and some highly expressed miRNAs might function as oncogenes by repressing tumor suppressors; conversely, miRNAs expressed at low levels might function as tumor suppressors by negatively regulating oncogenes [6, 7]. Based on reports describing miRNA signatures, several down regulated and up regulated miRNAs have been discovered in bladder cancer [8], [9] and [10]. Some of those miRNAs are thought to be potential biomarkers for bladder cancer in diagnosis and prognosis prediction, as well as a treatment target [11, 12]. Especially current studies mainly focus on miRNAs for providing prognostic information of related cancer [12], [13], and [14].

However, the exact roles of these miRNAs and their target genes in oncogenic pathways have not been clearly elucidated in bladder cancer. Insights into these molecular mechanisms of bladder cancer initiation and progression can provide targets for preventative and therapeutic approaches while providing reliable biomarkers.

In this study, we aimed to predict potential mechanisms of bladder-cancer related miRNAs and target genes by miRNA profiling and bioinformatics tools. By combining miRNA profiling and bioinformatics tools in our study, we sought not only to provide insights into the pathogenesis of bladder cancer but also to discover potential biomarkers for diagnosis, prognosis and treatment.

Matherials and methods

Tissue preparation and RNA isolation

The collection of samples and research protocols were reviewed and approved by the Ethics Committee of Eskisehir Osmangazi University, and the investigation was performed with written informed consent from all the patients. Tissue samples were taken from 30 bladder cancer patients from the central Anatolian part of Turkey who had undergone prospective cystectomy or transurethral resection of BCs, and 9 normal urothelial tissue samples were derived from patients who underwent control cystoscopy. All patients were males ranging from 35 to 65 years old (50.63 + 9.87). Hematoxylin eosin stained sections were examined for tumor cell percentage, and tumor tissues containing more than 85 % tumor cells were selected for microarray analysis.

The tissue samples were deposited in RNAlater solution (Qiagen, USA) and subsequently stored at −20 °C until the RNA extraction. Total RNA including miRNAs was extracted from frozen bladder cancer and normal urothelial tissues using Trizol reagent (Invitrogen, USA) according to the manufacture's protocol. Total RNA concentrations were measured using a spectrophotometer (NanoDrop Technologies, Wilmington, USA) and quantification analysis of total RNA was performed by a microfluidics-based platform (2100 Bioanalyzer, Agilent, Santa Clara, CA). Three tumor samples and one normal urothelial tissue sample were excluded from conducting miRNA array because of poor RNA quality.

MiRNA expression profiling

After quality assessment remaining 27 tumor samples (12 high grade and 15 low grade; 6 muscle invasive and 21 non-invasive) and 8 normal urothelial tissue samples were loaded onto Agilent's miRNA arrays with 723 human and 76 viral miRNA represented probes based on Sanger 10.1 (Agilent V 2.0). The input for the miRNA labeling system was 100 ng of total RNA. Following dephosphorylation and denaturation, total RNA was labeled with cyanine 3-pCp (Cy-3) and then hybridized on Human miRNA Microarray (Agilent V 2.0) using the miRNA Complete Labeling and Hyb Kit (Agilent). Each sample was hybridized at 55 °C for 20 h in an agitated hybridization

oven at 20 rpm. Then, the slides were washed using the Gene Expression Wash Buffer Kit (Agilent) and scanned by an Agilent Scanner (G2505C). The images were processed and analyzed with Feature Extraction Software (Agilent Technology Ver. 10.1). For genomic profiling, spots were background-corrected using the median foreground minus the median background signal intensities for dye, and log2 ratios were calculated. Following the microarray signal data transformation (linear to log values), a filtration process based on the flag value of spots was performed. Raw microarray data were normalized and analyzed using GeneSpring GX 11.5 (Agilent Technologies, San Francisco, USA). We performed quantile normalization for signals and used the median (50th percentile) method,which normalizes each chip on its median for "per chip" normalization.

Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis

Independent RNA samples prepared in parallel with the samples for the miRNA array were used to validate the miRNA array data. In the validation of microarray data, quantitative RT-PCR reactions were performed on randomly selected deregulated miRNAs including *hsa-miR-143* and *hsa-miR-145*. The reverse-transcription reactions were carried out according to the manufacturer's protocol by using a MiRCURY LNA Universal Reverse Transcriptase kit (Exiqon, Denmark). Initially, reverse transcription was performed with 5 ng/μl total RNA in a 20-μl volume. The assays were heated to 42 °C for 60 min and then 95 °C for 5 min.Before real-time assays, total RNAs were diluted 80 fold in nuclease-free water. Real-time PCR was carried out on a Roche Lifecycler 480 by using SYBR Green master mix and LNA primers of *hsa-miR-143, hsa-miR-145,* and housekeeping *5S RNA* according to the manufacturer's protocol. The transcription level of *5SRNA* was used as an internal control. Then, melting curve analysis was performed to evaluate the specificity of assays. Each sample was analyzed in triplicate.

Bioinformatic analysis

Mature sequences of miRNAs were loaded into the DIANA-Tarbase v 6.0 databases for target analysis [15]. We checked the mature sequences of our deregulated miRNAs from the Mirbase 20.0 databasebefore target analysis. Target genes of deregulated miRNAs were listed using DIANA-TarBase database v 6.0, which include experimentally validated miRNA targets in the literature. To explore the potential function of the whole miRNA and target gene signature, DIANA-mirPath, a web-based

Table 1 Differentially expressed miRNAs between bladder cancer and normal bladder urotelium. A value of $p < 0.05$ was considered as statistically significant and an absolute log fold change greater than 2 were set as significant cut-off

Mature sequence	Stem-loop sequence	Fold change	P value
miRNAs up-regulated in cancer			
hsa-miR-141-3p	hsa-miR-141	+6.93	5.21E–8
hsa-miR-193b-5p	hsa-miR-193b*	+2.19	8.07E–10
hsa-miR-370	hsa-miR-370	+2.57	0.002E–6
hsa-miR-200b-3p	hsa-miR-200b	+5.25	7.57E–9
hsa-miR-210	hsa-miR-210	+5.26	0.001E–7
miRNAs down-regulated in cancer			
hsa-let-7c	hsa-let-7c	−3.0	0.0015E–6
hsa-miR-125b-5p	hsa-miR-125b	−13.2	1.56E–11
hsa-miR-143-3p	hsa-miR-143	−9.16	6.06E–9
hsa-miR-145-5p	hsa-miR-145	−38.1	1.41E–13
hsa-miR-193a-3p	hsa-miR-193a-3p	−2.5	0.002E–7
hsa-miR-195-5p	hsa-miR-195	−4.8	1.21E–9
hsa-miR-195-3p	hsa-miR-195*	−2.79	0.001E–7
hsa-miR-23b-3p	hsa-miR-23b	−4.82	1.72E–10
hsa-miR-26a-5p	hsa-miR-26a	−2.43	0.001E–7
hsa-miR-27b -3p	hsa-miR-27b	−3.43	0.001E–6
hsa-miR-30a-5p	hsa-miR-30a	−4.01	8.09E–8
hsa-miR-572	hsa-miR-572	−2.89	6.72E–9
hsa-miR-57-3p	hsa-miR-574	−4.73	0.003E–7
hsa-miR-638	hsa-miR-638	−7.26	4.5E–11

Table 2 Number of known targets of deregulated miRNAs. Listed from Tarbase 6.0 database

Mature miRNA	Target gene number
hsa-let-7c	21
hsa-miR-125b-5p	116
hsa-miR-143-3p	20
hsa-miR-145-5p	100
hsa-miR-193a-3p	6
hsa-miR-195-5p	16
hsa-miR-195-3p	0
hsa-miR-23b-3p	30
hsa-miR-26a-5p	30
hsa-miR-27b-3p	17
hsa-miR-30a-5p	411
hsa-miR-572	1
hsa-miR-574-3p	0
hsa-miR-638	0
hsa-miR-141-3p	53
hsa-miR-193b-5p	0
hsa-miR-370	4
hsa-miR-200b-3p	44
hsa-miR-210	49

Table 3 The Enriched KEGG Pathways of miRNA Targets

	KEGG pathway	p-value	genes	miRNAs
1.	Hepatitis B (hsa05161)	<1e-16	22	8
2.	Chronic myeloid leukemia (hsa05220)	<1e-16	20	8
3.	Glioma (hsa05214)	<1e-16	15	9
4.	Bladder cancer (hsa05219)	<1e-16	15	9
5.	Pathways in cancer (hsa05200)	<1e-16	35	10
6.	Melanoma (hsa05218)	3.885781e-15	14	8
7.	Pancreatic cancer (hsa05212)	6.550316e-15	16	6
8.	Prostate cancer (hsa05215)	1.754152e-14	18	9
9.	Small cell lung cancer (hsa05222)	1.776357e-14	13	7
10.	Non-small cell lung cancer (hsa05223)	1.072475e-13	13	5
11.	Cell cycle (hsa04110)	9.709344e-12	16	6
12.	Transcriptional misregulation in cancer (hsa05202)	1.280638e-10	12	7
13.	Colorectal cancer (hsa05210)	4.78972e-10	17	7
14.	Endometrial cancer (hsa05213)	6.11459e-10	16	7
15.	ErbB signaling pathway (hsa04012)	2.406466e-09	13	7
16.	PI3 K-Akt signaling pathway (hsa04151)	1.183448e-08	15	4
17.	Thyroid cancer (hsa05216)	7.403071e-08	6	4
18.	HTLV-I infection (hsa05166)	4.897904e-07	16	6
19.	Viral carcinogenesis (hsa05203)	6.578508e-07	13	6
20.	Acute myeloid leukemia (hsa05221)	6.344504e-05	8	3
21.	p53 signaling pathway (hsa04115)	0.002359155	8	4
22.	Arrhythmogenic right ventricular cardiomyopathy (ARVC)(hsa05412)	0.006674703	9	1
23.	Focal adhesion (hsa04510)	0.009716456	19	4
24.	Neurotrophin signaling pathway (hsa04722)	0.03493194	7	2
25.	HIF-1 signaling pathway (hsa04066)	0.04751375	8	3

computational tool that was developed to identify molecular pathways that are potentially altered by the expression of multiple miRNAs, was used to incorporate miRNAs into KEGG molecular pathways [16]. The pathways were obtained with a p value < 0.05 and gene count >2. The overlapping parts of the "Pathways in cancer (hsa05200)", "Bladder cancer (hsa05219)", and "ErbB signaling pathway (hsa04012)" categories of enriched KEGG pathways and related miRNAs were visualized by Cytoscape software [17].

Statistical analysis

All miRNAs in the bladder cancer and normal samples were compared using a t-test to define differentially expressed miRNAs. Multiple testing corrections were done using the Benjamini-Hochberg method [18]. A false discovery rate (FDR) less than 0.05 and absolute log fold change (logFC) greater than 2 were set as the significant cut-offs. The expression levels of the transcripts were evaluated using the comparative CT method ($2^{-\Delta\Delta Ct}$) in qRT-PCR.

Table 4 Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) validation of microarray data

Bladder cancer/normal urotelium	Fold change Microarray	qRT-PCR
hsa-miR-143	−9.16*	−10.9*
hsa-miR-145	−38.1*	−27.02*

Negative fold change means down-regulated. * $p < 0.05$

Results

Identification of differentially expressed genes

According to the cut-off criteria of $|logFC| > 2$ and p value < 0.05, we finally gained 19 deregulated miRNAs, including 5 up- and 14 downregulated (Table 1).

Validation of differentially expressed miRNAs using qRT-PCR

Independent RNA samples that were prepared in parallel with the samples for the miRNA array were used to

Table 5 Kinds of enriched target genes and realted miRNAs in three selected KEGG pathways

Pathways in cancer (hsa05200)			Bladder cancer (hsa05219)		ErbB signaling pathway (hsa04012)	
GSK3B	SMAD4	hsa-let-7c	E2F1	hsa-let-7c	GSK3B	hsa-let-7c
STAT3	CCNE2	hsa-miR-125b-5p	ERBB2	hsa-miR-125b-5p	ERBB2	hsa-miR-125b-5p
E2F1	MMP1	hsa-miR-145-5p	NRAS	hsa-miR-143-3p	NRAS	hsa-miR-143-3p
TGFBR1	E2F3	hsa-miR-195-5p	RAF1	hsa-miR-145-5p	RAF1	hsa-miR-193a-3p
ERBB2	AKT1	hsa-miR-23b-3p	CDKN2A	hsa-miR-195-5p	EIF4EBP1	hsa-miR-23b-3p
NRAS	MYC	hsa-miR-26a-5p	KRAS	hsa-miR-572	ERBB3	hsa-miR-26a-5p
APC	GLI1	hsa-miR-572	TP53	hsa-miR-26a-5p	KRAS	hsa-miR-572
TCF7L1	RB1	hsa-miR-141-3p	CCND1	hsa-miR-23b-3p	STAT5B	
ETS1	TGFB2	hsa-miR-200b-3p	MMP1	hsa-miR-210	PTK2	
RAF1	EP300	hsa-miR-210	E2F3		AKT1	
BCL2	BCL2L1		MYC		MYC	
IGF1R	FN1		RB1		HRAS	
CDKN2A	CDKN1A		HRAS		CDKN1A	
CDK6	SMO		CDKN1A			
STAT5B	STAT1		VEGFA			
TPM3	VEGFA					
TP53	PTEN					
CCND1						

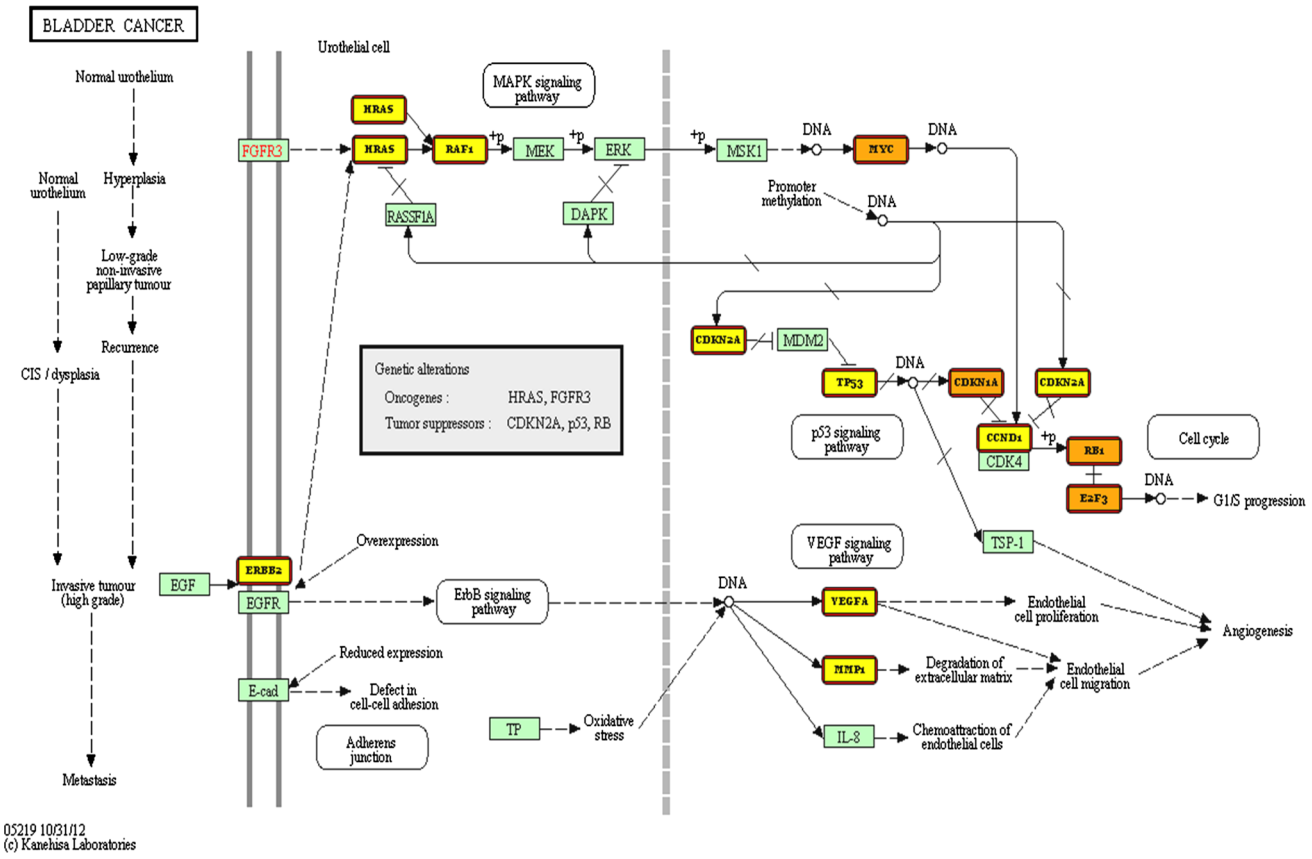

Fig. 1 Bladder cancer (hsa05219) pathway in KEGG pathways. *Yellow rectangles* represent genes targeted by only one miRNA, whereas orange rectangles represent genes targeted by one more miRNA in the bladder cancer pathway. *Green rectangles* represent genes which are not targeted by any miRNA

Table 6 Presentation of miRNA based target genes in three selected KEGG pathways

Pathways in cancer (hsa05200)		Bladder cancer (hsa05219)		ErbB signaling pathway (hsa04012)	
GENES	miRNAs	GENES	miRNAs	GENES	miRNAs
TGFBR1, NRAS,					
BCL2, CDK6,		MYC		NRAS	
MYC, BCL2L1	hsa-let-7c	NRAS	hsa-let-7c	MYC	hsa-let-7c
STAT3, ERBB2		ERBB2			
RAF1, CDKN2A		RAF1		RAF1	
SMO, TP53		E2F3		ERBB2	
E2F3, AKT1		TP53		AKT1	
GLI1, CDK6	hsa-miR-125b-5p	CDKN2A	hsa-miR-125b-5p	EIF4EBP1	hsa-miR-125b-5p
IGF1R, TPM3					
MMP1, MYC		HRAS		KRAS	
STAT1, CDKN1A	hsa-miR-145-5p	KRAS	hsa-miR-143-3p	NRAS	hsa-miR-143-3p
BCL2, CDK6		MMP1			
CCND1, VEGFA		MYC			
E2F3	hsa-miR-195-5p	CDKN1A	hsa-miR-145-5p	PTK2	hsa-miR-193a-3p
		CCND1			
E2F1, RB1		VEGFA			
STAT5B	hsa-miR-23b-3p	E2F3	hsa-miR-195-5p	STAT5B	hsa-miR-23b-3p
GSK3B, SMAD4					
CCNE2, PTEN				GSK3B	
RB1, MYC	hsa-miR-26a-5p	CDKN1A	hsa-miR-572		hsa-miR-26a-5p
		RB1			
CDKN1A	hsa-miR-572	MYC	hsa-miR-26a-5p	CDKN1A	hsa-miR-572
TCF7L1, PTEN		E2F1			
TGFB2, E2F3	hsa-miR-141-3p	RB1	hsa-miR-23b-3p		
TCF7L1, ETS1	hsa-miR-200b-3p	E2F3	hsa-miR-210		
FN1, EP300					
E2F3, APC	hsa-miR-210				

validate the miRNA array data. The expression of randomly selected deregulated miRNAs including *hsa-miR-143* and *hsa-miR-145* was validated using qRT-PCR (Table 4).

Target genes of miRNAs and their roles in the KEGG pathways

The experimentally validated target genes of deregulated miRNAs were obtained from DIANA-Tarbase v 6.0 databases. The results showthat *hsa-miR-30a-5p* had the highest number of targets (411), whereas *hsa-miR-370* had only 4 targets. *Hsa-miR-195-3p, hsa-miR-574-3p, hsa-miR-638,* and *hsa-miR-193b-3p* had no validated targets in the database (Table 2). To gain further insights into the function of deregulated miRNAs and their targets, DIANA-mirPath was applied to identify the significant KEGG pathways. Enrichment results showed that most pathways were related to cancer or cell signaling pathways (Table 3). Most target genes of miRNAs (35) were enriched in the "Pathways in cancer (hsa05200)" category (Table 5). Significantly,13 cancer-associated target genes and 7 related miRNAs were enriched in the "ErbB signaling pathway (hsa04012)" category (Table 5). *E2F1, ERBB2, NRAS, RAF1, CDKN2A, KRAS, TP53, CCND1, MMP1, E2F3, MYC, RB1, HRAS, CDKN1A,* and *VEGFA* target genes (Fig. 1) as well as the related *hsa-let-7c, hsa-miR-125b-5p, hsa-miR-143-3p, hsa-miR-145-5p, hsa-miR-195-5p, hsa-miR-572, hsa-miR-26a-5p, hsa-miR-23b-3p,and hsa-miR-210* were enriched in the "Bladder cancer (hsa05219)" category (Table 5).

MiRNA regulated gene networks associated with bladder cancer

The "Pathways in cancer (hsa05200)", "Bladder cancer (hsa05219)", and "ErbB signaling pathway (hsa04012)" categories from enriched KEGG pathways

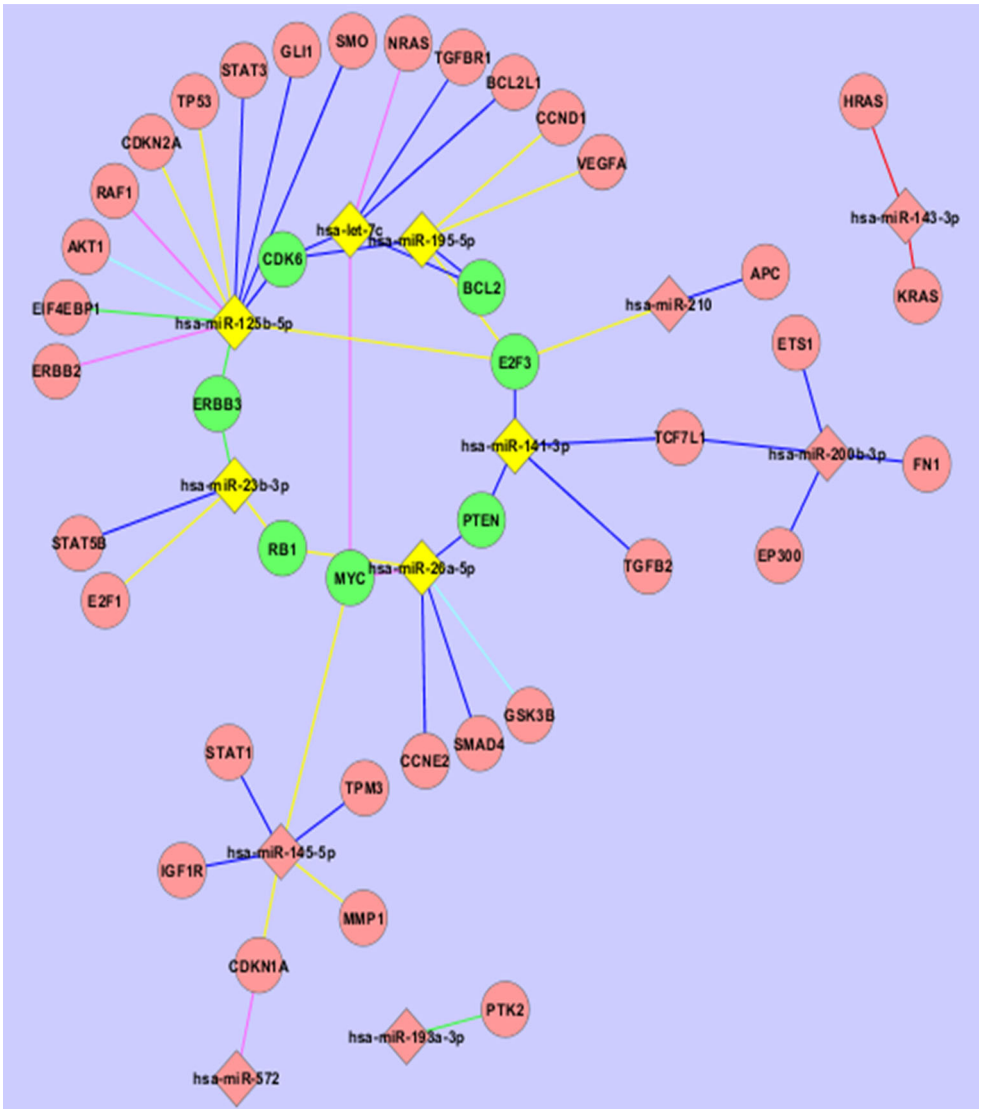

Fig. 2 Cytoscape representation of overlapping parts of three selected KEGG pathways and related miRNAs. *Pink circles* represent target genes of miRNAs, and *green circles* represent hub genes. *Pink diamonds* represent miRNAs, and *yellow diamonds* represent hub miRNAs. *Colored lines* represent target genes and miRNA interactions. We used "C", "B", and "E" abbreviations for "Pathways in cancer (hsa05200)", "Bladder cancer (hsa05219)", "ErbB signaling pathway (hsa04012)", respectively. *Blue lines* represent interactions in only C. *Black lines* represent interactions in only B. *Green lines* represent interactions in only E. *Pink lines* represent interactions of overlapping parts of C + B + E. *Yellow lines* represent interactions of overlapping parts of C + B. *Light blue lines* represent interactions of overlapping parts of C + E. *Red lines* represent interactions of overlapping parts of B + E

were selected for further investigation of the miRNA regulatory networks in bladder cancer. All of the interactions of enriched target genes and miRNAs of three pathways are listed in Table 6. Based on these data, the overlapping parts of three pathways were visualized by Cytoscape software (Fig. 2). We determined hub *CDK6, BCL2, E2F3, PTEN, MYC, RB,* and *ERBB3* target genes along with hub *hsa-let-7c, hsa-miR-195-5p, hsa-miR-141-3p, hsa-miR-26a-5p, hsa-miR-23b-3p,* and *hsa-miR-125b-5p* miRNAs for these interaction networks.

Discussion

In this study, we analyzed the expression profile in 27 tissue samples of histologically confirmed bladder cancer samples (12 high grade and 15 low grade; 6 muscle invasive and 21 non-invasive) and 8 non-malignant tissue samples of the bladder. A total of 19 miRNAs, including 5 ups- and 14 down regulated, displayed significant differential expression in cancerous tissue compared to non-cancerous tissue.To date, differential expressions of miRNAs in bladder cancer have been published in several studies [19, 20]. Although the results of these studies are not the same, the down regulation of *hsa-miR-143, hsa-miR-145, hsa-miR-125b, and hsa-miR-26a* and the up regulation of *hsa-miR-141* are in agreement with most studies, including our study [19, 20].

Known targets of 19 deregulated miRNAs and their roles in biological processes were demonstrated via KEGG pathway analysis. The KEGG enrichment analysis results in this study confirmed the reliability of our findings, and many of them have been implicated in various cancers and related cell signaling pathways. We considered that the

"Pathways in cancer (hsa05200)", "Bladder cancer (hsa05219)", and "ErbB signaling pathway (hsa04012)" categories from KEGG pathways were highly related to bladder cancer initiation and progression [21].Studies suggest that more centralized genes in the network are more prone to be key deliverers to proper cellular function than peripheral genes [22, 23]. Therefore; we constructed miRNA regulatory networks based on overlapping parts of these three pathways. Our results showed that *CDK6, BCL2, E2F3, PTEN, MYC, RB, and ERBB3* hub genes along with *hsa-let-7c, hsa-miR-195-5p, hsa-miR-141-3p, hsa-miR-26a-5p, hsa-miR-23b-3p,* and *hsa-miR-125b-5p* hub miRNAs might play key roles in miRNA regulatory networks in bladder cancer.

We also reviewed some of the literature and reconfirmed the roles of some hub genes in bladder cancer.Bladder cancer has been reported to be associated with loss of function, affecting tumor-suppressor genes such as *Tp53, RB,* and *PTEN* [24, 25]. Oncogenes, especially *E2F* genes (e.g.*E2F1*and *E2F3*), which affect the *RB* pathways, have also been reported to be involved in the progression of this neoplasm [26]. Huang et al. [27] previously reported on the association between *hsa-miR-125b* and *E2F3*. Similar to previous studies, we demonstrated that *PTEN, RB1*, and *E2F3* were hub genes in our network, and their regulatory hub miRNAs, including *hsa-miR-141-3p, hsa-miR-26a-5p, hsa-miR-23b-3p,* and *hsa-miR-125b-5p*, might play a central role in bladder cancer development in our regulatory networks (Fig. 2). Accumulating evidence suggests that aggressive bladder tumors are associated with the overexpression of some oncogenes [28]. The overexpression of *MYC, BCL2,* and*ERBB3* hub oncogenes might be related to the downregulation of *hsa-let-7c, hsa-miR-195-5p, hsa-miR-26a-5p, hsa-miR-23b-3p,* and *hsa-miR-125b-5p* in our study. These gene network associations have to be confirmed with further comprehensive functional studies.

The *CDK6* gene is a member of the cyclin-dependent protein kinase (CDK) family.This kinase is a catalytic subunit ofthe protein kinase complex that is important for cell cycle G1 phase progression and G1/S transition.This kinase, along with *CDK4*, has been shown tophosphorylate and thus regulate the activity of tumor-suppressor protein *RB*. Although increased expression of this oncogene has rarely been reported in bladder cancer, we speculate that *CDK6* might have a significant effect on bladder cancer development [29].

Lin et al. [30] reported that *CDK4* is a novel target in has-miR-195-mediated cell cycle arrest in bladder cancer cells. As mentioned, *CDK4* and *CDK6* had similar functions in the cell cycle. In our networks, *CDK6* was related to *hsa-let-7c, hsa-miR-195-5p,* and *hsa-miR-125b-5p* (Fig. 2). From this point, we speculated that these miRNAs, *CDK4*, and *CDK6* genes might exist in the same bladder cancer pathway. However, the exact regulatory networks remain elusive, and it is hard to estimate the actual false-positive rate of bioinformatic tools. Therefore, further experimental studies should be carried out in order to confirm our results.

Conclusion

Overall, deregulated miRNAs and known target genes might play key roles in the pathogenesis of bladder cancer according to the pathway enrichment analysis. Particularly, hub genes and miRNAs of our constructed network might be central actors of molecular alterations in bladder cancer and candidate biomarkers for diagnostic, prognostic and therapeutic purposes. Of course, further research is needed to confirm their exact roles.

Acknowledgments This research was supported by the Eskisehir Osmangazi University Research Fund with Grant no. 200811031. We are also thankful to Ece TURKMEN, the Product Manager at SEM Laboratuar Cihazlari Paz. San. Tic. A. S, (Istanbul-Turkey), who provided facilities to carry out this study.

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