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Screening biomarkers of bladder cancer using combined miRNA and mRNA microarray analysis

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Abstract. Biomarkers, such as microRNAs (miRNAs) may be useful for the diagnosis of bladder cancer. In order to understand the molecular mechanisms underlying bladder cancer, differentially expressed miRNAs (DE-miRNAs) and their target genes in bladder cancer were analyzed. In the present study, miRNA and mRNA expression profiles (GSE40355) were obtained from the Gene Expression Omnibus. These consisted of healthy bladder samples (n=8) and urothelial carcinoma samples (low-grade, n=8 and high-grade, n=8). DE-miRNAs and differentially expressed genes (DEGs) were identified using the limma package and the Benjamin and Hochberg method from the multtest package in R. Target genes of DE-miRNAs were screened. Associations between DEGs were investigated using STRING, and an interaction network was constructed using Cytoscape. Functional and pathway enrichment analyses were performed for DEGs from the interaction network. 87 DE-miRNAs and 2058 DEGs were screened from low-grade bladder cancer samples, and 40 DE-miRNAs and 2477 DEGs were screened from high-grade bladder cancer samples. DE-target genes were significantly associated with the regulation of cell apoptosis. Bladder cancer, non-small cell lung cancer and pancreatic cancer biological pathways were found to be enriched. The results of the present study demonstrated that E2F transcription factor 1, which is targeted by miR-106b, and cyclin-dependent kinase inhibitor 2A (CDKN2A) and V-Erb-B2 avian erythroblastic leukemia viral oncogene homolog-2, which are targeted by miR-125b, participate in the bladder cancer pathway. In conclusion, DE-miRNAs in bladder cancer tissue samples and DE-targeted genes, such as miR-106b and CDKN2A, which were identified in the present study, may provide the basis for targeted therapy for breast cancer and enhance understanding of its pathogenesis.

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Key words: bladder cancer, differentially expressed microRNA, differentially expressed gene, interaction network

Introduction

Bladder cancer is an important issue globally. Its incidence ranks ninth among malignant tumors, of which >90% of cases are transitional cell carcinomas (TCCs) (1). In China, bladder cancer is the most common type of urinary system tumor. Furthermore, the recurrence rate of bladder cancer is 60-70%, and 11% of patients who relapse progress to metastatic cancer (2).

The occurrence of bladder cancer is the result of the interaction between genetic and environmental factors, and is associated with a number of different genes (3). Two of the most significant risk factors for bladder cancer are smoking and exposure to chemicals, such as aromatic amines (3). The formation of DNA adducts in response to aromatic amine chemical exposure, results in transitional mutations, which may be associated with the development of bladder carcinogenesis (4). Liver enzymes, such as cytochrome p450 1A2 (CYP1A2), N-acetyltransferase 2 (NAT2) and glutathione S-transferase M1 (GSTM1) affect the formation of these DNA adducts (5). Cigarette smokers with slow NAT2/rapid CYP1A2 phenotypes are more likely to develop bladder cancer than those with rapid NAT2/slow CYP1A2 phenotypes (6). Smokers who are homozygous for deletions in the detoxifying enzyme, GSTM1, are at a 1.8 fold greater risk of developing bladder cancer than those with 1-2 copies of GSTM1 (7).

Recent studies have demonstrated that a number of microRNAs (miRNAs), which are involved in the biological regulation process of tumor cells, indirectly act as proto-oncogenes and anti-oncogenes, and are associated with tumor initiation and development (8). Using oligonucleotide microarray analysis, Chiyomaru *et al* (9) transfected a bladder cancer cell line with miRNA-145/miRNA-133a, and demonstrated that the expression of ~200 genes decreased. The most marked downregulation was observed in beam protein 1 (FSCN1) gene. Ichimi *et al* (10) demonstrated that keratin 7 expression was greater in a bladder cancer cell line transfected with miRNA-30a-3p/-133a/-199a, which inhibited tumor cell growth, compared with a control cell line. A separate study showed that the ratios of miRNA-126:miRNA-152 and miRNA-182:miRNA-152 in urine, may be useful markers of bladder cancer in asymptomatic patients, with high sensitivity and specificity (11).

A number of tumor suppressor genes have been detected in bladder cancer tissues. The retinoblastoma tumor suppressor gene encodes a nuclear phosphoprotein (pRb), which acts

as a cell cycle regulator. Unphosphorylated pRb influences tumors by negatively regulating and binding with E2F, which is a protein transciption factor (12). Phosphorylated pRb is not able to bind with E2F, and Rb gene mutations were shown to be <30% in bladder cancer samples, compared with control samples (13). The p53 tumor suppressor gene, which encodes a 53kDa transcription factor, is associated with DNA repair and apoptosis. p53 expression was found to be upregulated in 50% of locally invasive TCCs (14), resulting in a 2-fold increase in bladder cancer mortality rates (15). Chromosome 9 is the only chromosomal aberration at the initiation of the disease, and is not associated with disease progression (16). Multiple tumor suppressor 1 on chromosome 9 encodes for p16 and cyclin-dependent kinase inhibitor 2A (CDKN2), which have been previously identified to inhibit cyclin-dependent kinase 4 (17). Although a number of studies have been conducted in order to investigate bladder cancer, the mechanisms underlying this disease remain unclear.

In the present study, DE-miRNAs and DEGs associated with bladder cancer were identified. Target genes of DE-miRNAs were screened. The associations of the DEGs were analyzed using Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) and interaction networks were constructed using Cytoscape. Furthermore, functional and pathway enrichment analyses were conducted for the DEGs from the interaction network.

Materials and methods

Microarray data. The GSE40355 expression profile was obtained from the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/), which was based on the platforms of GPL8227-Agilent-019118 human miRNA microarray 2.0 G4470B (miRNA ID version) and GPL13497-Agilent-026652 whole human genome microarray 4x44K v2 (probe name version). This dataset was deposited by Hecker *et al* (18). Using healthy bladder tissue samples (n=8) and urothelial carcinoma samples (low-grade, n=8; high-grade, n=8), miRNA and mRNA microarray expression profiling analyses were performed.

DE-miRNAs and DEGs screening. miRNAs and genes in the probe-level data from Affymetrix CEL files were matched and a Log2 conversion was then conducted (19). Probes without corresponding gene symbols were filtered. The limma package in R was used to identify DEGs of low- and high-grade bladder cancer samples, compared with healthy bladder tissue samples (20). The Benjamin and Hochberg method in multtest package was used to adjust the raw P-values into false discovery rate (FDR) values (21). FDR <0.01 and |logFC| >1 were used as cut-off criteria.

Comparison of DE-miRNAs and DEGs. DE-miRNAs were compared with DEGs screened from low- and high-grade bladder cancer samples, compared with healthy bladder tissue samples. The results are represented using a Venn diagram.

Obtaining DE-miRNA target genes. Each miRNA has a number of corresponding target genes. In order to elucidate reliable predicted target genes, two miRNA databases, including miRDB (http://mirdb.org/miRDB/) and microRNA.org (http://www.microrna.org), were used. Only the target genes that were identified by both databases were used as target genes in the present study. MiRDB is an online database for animal microRNA target prediction and functional annotations (22,23). microRNA.org is a software and database developed by researchers from the Memorial Sloan-Kettering cancer research center (24). Following target gene prediction, DEGs were mapped to the target gene set and target genes with significantly differential expression, were screened.

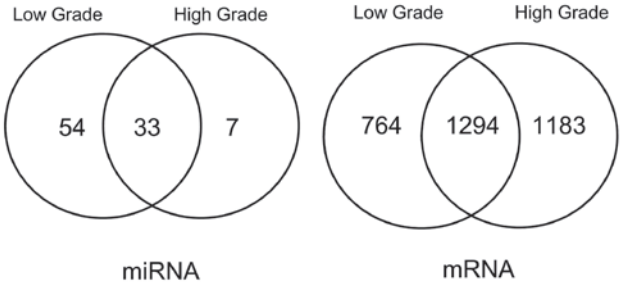

Figure 1. Venn diagrams for differentially expressed miRNAs (left) and mRNAs (right) in low- and high-grade bladder cancer samples. miRNA, microRNA.

Network analysis. STRING online software was used to investigate associations between the DEGs and to construct the interaction network (25). Based on the interactions between DE-miRNAs and DEGs, an interaction network was constructed and visualized using Cytoscape (26).

Functional and pathway enrichment analysis. WebGestalt is used for gene set enrichment pathway analysis and consists of four modules: Information retrieval; gene set management; statistics; and organization/visualization (27,28). Functional and pathway enrichment analyses were performed for the genes in the interaction network. P<0.05 was considered to indicate a statistically significant difference. The raw P-values were adjusted using the Benjamin and Hochberg method in multtest package.

Results

DE-miRNAs and DEGs. By performing the transformation of probe IDs into miRNAs or mRNAs, a total of 721 effective human miRNAs (those converted from corresponding probe IDs) and 29,833 genes were obtained. In the miRNA and mRNA expression profiles, the following were screened: 87 DE-miRNAs and 2,058 DEGs in low-grade bladder cancer samples compared with healthy bladder samples, and 40 DE-miRNAs and 2,477 DEGs in high-grade bladder cancer samples compared with healthy bladder samples

Comparison of DE-miRNAs and DEGs. DE-miRNAs and DEGs screened from low- and high-grade bladder cancer samples were compared (Fig. 1). 33 DE-miRNAs identified in both low- and high-grade bladder cancer samples are listed in Table I, while 1,294 DEGs were identified in both low- and

Table I. DE-miRNAs in low- and high-grade bladder cancer.

miR	Low-grade	High-grade
hsa-miR-133b	-10.2175	-10.2837
hsa-miR-133a	-9.05375	-10.12
hsa-miR-1	-8.865	-9.7912
hsa-miR-490-3p	-8.99875	-9.095
hsa-miR-139-5p	-8.4075	-8.6137
hsa-miR-204	-8.42	-8.5162
hsa-miR-490-5p	-7.065	-7.3625
hsa-miR-381	-6.3325	-7.3325
hsa-miR-127-3p	-6.7525	-7.1162
hsa-miR-502-3p	-5.64375	-6.655
hsa-miR-28-3p	-5.41	-6.225
hsa-miR-145	-5.265	-5.8162
hsa-miR-143	-4.885	-5.4862
hsa-miR-125b	-4.4375	-4.8337
hsa-miR-338-3p	-6.56375	-4.78
hsa-miR-199a-5p	-3.1025	-3.4625
hsa-miR-497	-2.68	-3.0137
hsa-miR-30a	-2.325	-2.4425
hsa-miR-28-5p	-1.8975	-1.9475
hsa-miR-23b	-2.0875	-1.9138
hsa-miR-26a	-1.49375	-1.1363
hsa-miR-106b	2.37875	1.5037
hsa-miR-19a	3.00625	1.6963
hsa-miR-425	2.8275	2.2575
hsa-miR-494	3.20875	2.2887
hsa-miR-210	3.04	3.2862
hsa-miR-200c	3.97375	4.0837
hsa-miR-200b	4.20375	4.415
hsa-miR-200a	4.15375	4.7762
hsa-miR-429	4.68	4.8925
hsa-miR-141	4.6225	4.9112
hsa-miR-183	9.90375	10.3038
hsa-miR-96	9.84	10.4275

miR, microRNA; DE-miRNA, differentially expressed miRNA.

Table II. DEGs targeted by DE-miRNAs.

miR	Target gene(s)
hsa-miR-1	NETO2, AXL, BDNF, LASP1 GNPDA2, TDP1, POLR2K, POGK OAT, CHST11, CNN3, TPM4 ANKRD29, TIMP3, FBLN2 RABGAP1L
hsa-miR-106b	E2F1
hsa-miR-125b	ERBB2, ERBB3, CDKN2A
hsa-miR-133a	KRT7
hsa-miR-143	DNMT3A
hsa-miR-145	KRT7
hsa-miR-19a	ESR1
hsa-miR-19a	NR4A2
hsa-miR-200a	ZEB1, ZEB2
hsa-miR-200b	ZEB2, ZEB1
hsa-miR-200c	ZEB1
hsa-miR-26a	EZH2
hsa-miR-338-3p	UBE2Q1

DE, differentially expressed; miR, microRNA; DEG, differentially expressed gene; NETO2, neuropilin and tolloid (TLL)-like 2; BDNF, brain-derived neurotrophic factor; LASP1, LIM and SH3 Protein 1; GNPDA2, glucosamine-6-phosphate deaminase 2; TDP1, tyrosyl-DNA phosphodiesterase 1; POLR2K, polymerase (RNA) II (DNA directed) polypeptide K; POGK; pogo transposable element with KRAB domain; OAT, ornithine aminotransferase; CHST11, carbohydrate (chondroitin 4) culfotransferase 11; CNN3, calponin 3, acidic; TPM4, tropomyosin 4; ANKRD29, ankyrin repeat domain 29; TIMP3, tissue inhibitor of metalloproteinases 3; FBLN2, fibulin 2; RABGAP1L, RAB GTPase activating protein 1-like; E2F1, E2F transcription factor 1; ERBB, V-Erb-B2 avian erythroblastic leukemia viral oncogene homolog; CDKN2A, cyclin-dependent kinase inhibitor 2A; KRT7, keratin 7; ESR1, estrogen receptor 1; NR4A2, nuclear receptor subfamily 4, group A, member 2; ZEB, zinc finger E-box binding homeobox; EZH2, enhancer of zeste homolog 2; UBE2Q1, ubiquitin-conjugating enzyme E2Q family member 1.

high-grade bladder cancer samples, compared with healthy samples. The trends of expression for the 33 DE-miRNAs in low and high-grade bladder cancer samples were similar, and the differences in the high-grade bladder cancer samples were more marked than those in the low-grade bladder cancer samples.

DE-miRNA target genes. Predicted target genes were obtained from the miRDB and microRNA.org databases. In order to improve the reliability of the predicted target genes, only the target genes predicted from both databases were selected for further analysis. Subsequently, DEGs were mapped to the target genes. The DE-target genes and their corresponding DE-miRNAs are listed in Table II.

Interaction network construction. Using the STRING online software, the associations between DEGs were analyzed, and an interaction network was constructed. The regulatory associations between miRNAs and target genes combined with interactions of the DE-target genes were used for network construction using Cytoscape software (Fig. 2).

Functional and pathway enrichment analysis of genes in the interaction network. WebGestalt was used in order to conduct functional and enrichment pathway enrichment analysis of DEGs in the interaction network. The 10 most significantly enriched functions are listed in Table III, which includes function of negative regulation of apoptosis [adjusted P-value (adj p)=4.12E-04], negative regulation of programmed cell death (adj p=4.39E-04) and negative regulation of cell death (adj p=4.44E-04). Three Kyoto encyclopedia of genes and genomes biological pathways were enriched: hsa05219 bladder cancer (adj p=0.003494), hsa05223 non-small cell lung cancer (adj p=0.005726) and hsa05212 pancreatic cancer (adj p=0.010013), which included three DE-target genes [E2F transcription factor 1 (E2F1), CDKN2A and V-Erb-B2 avian

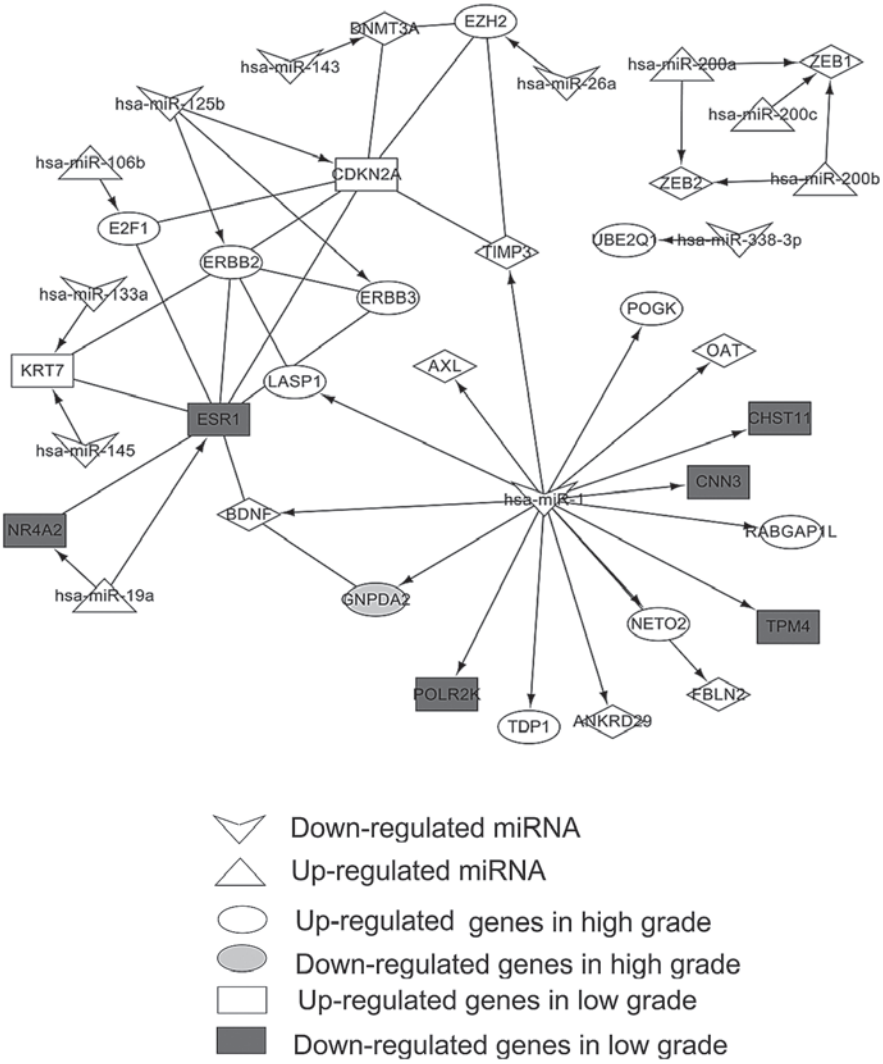

Figure 2. Interaction network of differentially expressed miRNAs and their differentially expressed target genes. Diamonds represent common differential genes from the low- and high-grade bladder cancer groups. miRNA, microRNA; NETO2, neuropilin and tolloid (TLL)-like 2; BDNF, brain-derived neurotrophic factor; LASP1, LIM and SH3 Protein 1; GNPDA2, glucosamine-6-phosphate deaminase 2; TDP1, tyrosyl-DNA phosphodiesterase 1; POLR2K, polymerase (RNA) II (DNA directed) polypeptide K; POGK; pogo transposable element with KRAB domain; OAT, ornithine aminotransferase; CHST11, carbohydrate (chondroitin 4) culfotransferase 11; CNN3, calponin 3, acidic; TPM4, tropomyosin 4; ANKRD29, ankyrin repeat domain 29; TIMP3, tissue inhibitor of metalloproteinases 3; FBLN2, fibulin 2; RABGAP1L, RAB GTPase activating protein 1-like; E2F1, E2F transcription factor 1; ERBB, V-Erb-B2 avian erythroblastic leukemia viral oncogene homolog; CDKN2A, cyclin-dependent kinase inhibitor 2A; KRT7, keratin 7; ESR1, estrogen receptor 1; NR4A2, nuclear receptor subfamily 4, group A, member 2; ZEB, zinc finger E-box binding homeobox; UBE2Q1, ubiquitin-conjugating enzyme E2Q family member 1; EZH2, enhancer of zeste homolog 2.

erythroblastic leukemia viral oncogene homolog 2 (ERBB2); Table IV]. E2F1 was targeted by hsa-miR-106b, and CDKN2A and ERBB2 were targeted by hsa-miR-125b.

Discussion

In the present study, using bioinformatic methods, significant DE-miRNAs in bladder cancer tissue samples and DE-target genes from mRNA expression profiles were screened. According to the functional analysis, the DEGs were most significantly associated with cell apoptosis. Results of pathway analysis suggested that E2F1, which is targeted by hsa-miR-106b, and CDKN2A and ERBB2, which are targeted by hsa-miR-125b, were shown to be involved in the bladder cancer pathway.

Neely *et al* (29) investigated the expression levels of 343 miRNAs in bladder cancer cells, and demonstrated that there were 9 DE-miRNAs (miR-21, -31, -200a, -200c, -205, -373, -487b, -498 and -503) between invasive bladder cancer and noninvasive bladder cancer. Similarly, 94 DE-miRNAs were screened in the present study, of which 33 were differentially expressed in low- as well as high-grade bladder cancer samples. These included miR-200a and -200c. Furthermore, studies have suggested that miR-200c expression is associated with early stage T1 bladder tumor progression. Furthermore, miR-200 and -205 loci are associated with coordinated epigenetic repression in bladder cell lines and bladder tumors (30). Therefore, miR-200a and-200c may be involved in bladder cancer.

According to the present study, hsa-miR-125b targets CDKN2A and ERBB2, which were differentially expressed in

Table III. Top 10 significantly enriched functions for the differentially expressed target genes in the interaction network.

ID	Function	Count	Adjusted P-value	Genes
GO:0043066	Negative regulation of apoptosis	6	4.12E-04	BDNF, ERBB3, ERBB2, CHST11, NR4A2, ESR1
GO:0043069	Negative regulation of programmed cell death	6	4.39E-04	BDNF, ERBB3, ERBB2, CHST11, NR4A2, ESR1
GO:0060548	Negative regulation of cell death	6	4.44E-04	BDNF, ERBB3, ERBB2, CHST11, NR4A2, ESR1
GO:0042981	Regulation of apoptosis	8	4.77E-04	BDNF, CDKN2A, ERBB3, ERBB2, CHST11, NR4A2, ESR1, TIMP3
GO:0043067	Regulation of programmed cell death	8	5.06E-04	BDNF, CDKN2A, ERBB3, ERBB2, CHST11, NR4A2, ESR1, TIMP3
GO:0010941	Regulation of cell death	8	5.18E-04	BDNF, CDKN2A, ERBB3, ERBB2, CHST11, NR4A2, ESR1, TIMP3
GO:0043523	Regulation of neuron apoptosis	4	5.89E-04	BDNF, ERBB3, NR4A2, ESR1
GO:0030155	Regulation of cell adhesion	4	0.001985	CDKN2A, ERBB3, FBLN2, ERBB2
GO:0006355	Regulation of transcription, DNA-dependent	10	0.003174	E2F1, DNMT3A, CDKN2A, POGK, POLR2K, EZH2, NR4A2, ESR1, ZEB2, ZEB1
GO:0033084	Regulation of immature T cell proliferation in the thymus	2	0.003693	CDKN2A, ERBB2

miRNA, microRNA; NETO2, neuropilin and tolloid (TLL)-like 2; BDNF, brain-derived neurotrophic factor; LASP1, LIM and SH3 Protein 1; GNPDA2, glucosamine-6-phosphate deaminase 2; TDP1, tyrosyl-DNA phosphodiesterase 1; POLR2K, polymerase (RNA) II (DNA directed) polypeptide K; POGK, pogo transposable element with KRAB domain; OAT, ornithine aminotransferase; CHST11, carbohydrate (chondroitin 4) culfotransferase 11; CNN3, calponin 3, acidic; TPM4, tropomyosin 4; ANKRD29, ankyrin repeat domain 29; TIMP3, tissue inhibitor of metalloproteinases 3; FBLN2, fibulin 2; RABGAP1L, RAB GTPase activating protein 1-like; E2F1, E2F transcription factor 1; ERBB, V-Erb-B2 avian erythroblastic leukemia viral oncogene homolog; CDKN2A, cyclin-dependent kinase inhibitor 2A; KRT7, keratin 7; ESR1, estrogen receptor 1; NR4A2, nuclear receptor subfamily 4, group A, member 2; ZEB, zinc finger E-box binding homeobox; UBE2Q1, ubiquitin-conjugating enzyme E2Q family member 1; EZH2, enhancer of zeste homolog 2.

Table IV. Enriched KEGG pathways for differentially expressed target genes in the interaction network.

ID	KEGG	Adjusted P-value	Genes
hsa05219	Bladder cancer	0.003494	E2F1, CDKN2A, ERBB2
hsa05223	Non-small cell lung cancer	0.005726	E2F1, CDKN2A, ERBB2
hsa05212	Pancreatic cancer	0.010013	E2F1, CDKN2A, ERBB2

Counts for each pathway = 3. KEGG, kyoto encyclopedia of genes and genomes; E2F1, E2F transcription factor 1; CDKN2A, cyclin-dependent kinase inhibitor 2A; ERBB2, V-Erb-B2 avian erythroblastic leukemia viral oncogene homolog.

low- and high-grade bladder cancer samples. The CDKN2A locus encodes the tumor suppressor gene p16 and $p14^{ARF}$, which induce cell cycle arrest (31,32). Using RT-qPCR in order to detect homozygous deletions, Berggren *et al* (33) demonstrated that CDKN2A/ARF inactivation occurs in the early stages of TCC. ERBB2 is closely associated with bladder cancer (34). In ~40% studies, membranous ERBB2 expression was found to be greater in bladder cancer samples compared with healthy samples (35). miRNA-10a is overexpressed in noninvasive bladder cancer, and miRNA-222 and-125b are overexpressed in invasive bladder cancer (36). Therefore, hsa-miR-125b may be used as a biomarker for the diagnosis of bladder cancer (36). Huang *et al* (37) found that miRNA-125b inhibits the E2F3-cyclinA2 signaling pathway and suppresses bladder

tumor cells growth during G phase via the reduction of E2F3 expression. Therefore, hsa-miR-125b downregulation may lead to bladder cancer via the upregulation of CDKN2A and ERBB2.

In the present study, E2F1 targeted by hsa-miR-106b was shown to participate in the bladder cancer pathway. E2F1 and E2F3 are members of E2F transcription factors family, and they combine with the retinoblastoma protein (pRB) tumor suppressor (36). Additionally, miR-106a is involved in the regulation of a number of genes, such as TGFBR2 and RB, and controls cell cycle regulation, migration, angiogenesis and apoptosis via a number of different pathways (38). Ivanovska *et al* (39) demonstrated that miR-106b promotes cell cycle progression via the TP53-p21 pathway, via p21 suppression. Hershko *et al* (40) found that E2F1 may regulate the expression of the pro-apoptotic BH3-only proteins, Noxa, Hrk/DP5, p53-upregulated modulator of apoptosis and bcl-2 interacting mediator of cell death. Furthermore, increased PUMA and Noxa expression is associated with E2F1-induced apoptosis. E2F1 expression may induce oncogenic stress and promote premalignant cell apoptosis, thereby inhibiting tumor development (41). The majority of the functions enriched in the present study were associated with cell apoptosis. Therefore, overexpression of E2F1, which is targeted by hsa-miR-106b, may be one of the possible mechanisms underlying the development of bladder cancer.

In conclusion, a number of DE-miRNAs and target DEGs involved in bladder cancer were identified. In particular, hsa-miR-106b and 125b may be involved in bladder cancer by targeting E2F1, CDKN2A and ERBB2. Therefore, these miRNAs and genes may be useful biomarkers of bladder cancer.

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