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Original Article

Genetic alteration and prospective signaling pathways of miR-517a-3p in bladder cancer: a study based on miRNA sequencing data and bioinformatics methods

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Abstract: Purpose: To explore the clinicopathological value and prospective function of microRNA-517a-3p (miR-517a-3p) in bladder cancer (BC). Methods: The clinical importance of miR-517a-3p expression and genetic alteration in BC was unraveled by using The Cancer Genome Atlas (TCGA), cBioPortal, Gene Expression Omnibus (GEO) and ArrayExpress. The potential target genes of miR-517a-3p were obtained via the combination of predicted genes and down-regulated genes post miR-517a-3p transfection *in vitro* from microarray (GSE39093). The probable signaling pathways were further evaluated with multiple bioinformatics approaches. Results: MiR-517a-3p expression was significantly higher in the samples of pathologic N stage (N1-N3), pathologic stage (III-IV) and patients with lymphovascular invasion than that of their counterparts ($P<0.05$) based on data from TCGA. The amplification was the only alteration type which counted for 3% (14/412) in BC as indicated by cBioPortal. A total of 858 genes were gained by prediction from at least two predicting programs. Compared to control cells, there were 4844 genes down-expressed after BC cells (BOY and T24) were transfected with miR-517a-3p. Only "hsa05200: Pathways in cancer" was significantly enriched via Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis ($P<0.05$). Three significant pathways were discovered in PANTHER pathway analysis ($P<0.05$). Hub genes, such as CREB1, MAPK1, RPS6KA5, SMAD3 and PPARA, were identified by protein-protein interaction. Conclusion: The results in this study indicate that miR-517a-3p may play a potential role in the occurrence and development of BC, especially its genetic alteration, via various signal pathways. However, the exact mechanism still needs to be ascertained by *in vitro* and *in vivo* experiments.

Keywords: Bladder cancer, miR-517a-3p, amplification, pathways, hub genes

Introduction

Bladder cancer (BC) ranks the second most common urogenital canal malignant tumor. Despite breakthrough advances in treatment, including surgical operation and adjuvant therapies, BC continues to be one of the most common diseases with high mortality and 70% recurrence rate in the world [1]. Among recurrent tumors of BC, 10-15% cases keep developing into muscle invasion and metastasis. The disease is divided into non-muscle-invasive and muscle-invasive cancers according to the status of invasiveness [2-5]. Diagnosis delay can cause a poor prognosis. Therefore, it is urgent to have a better understanding of the exact mechanism of bladder carcinogenesis, hence to improve the diagnostic strategies of BC. Recently, evidence accumulated through bioinformatics and molecular biology creates the possibility of understanding deeply of BC phenotype, genotype and predicting the risk of cancer, including the application of microRNAs (miRNAs) [6-9].

The finding of miRNAs, which contain ~22 nucleotide RNAs that suppress protein synthesis based a sequence-specific mode has brought

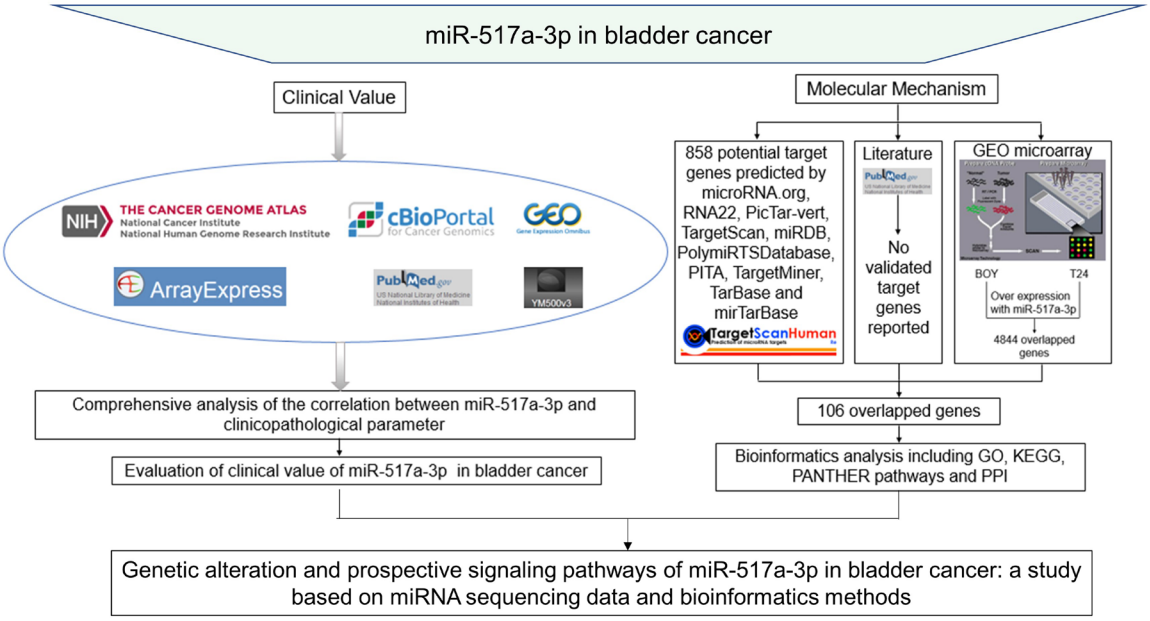


Figure 1. The work flow of the bioinformatical analysis for miR-517a in bladder cancer.

courage for innovative diagnostic and therapeutic strategies for cancers [10-13]. It has been discovered that miRNAs can modulate the expression of oncogenes or tumor suppressive genes involved in the occurrence and progress of malignant tumors, including BC [14-17]. The abnormal expression and genetic alteration of various miRNAs are closely related to BC, such as miR-34, miR-100, miR-146b, miR-9 and miR-193a-3p [18-20].

MiR-517a-3p, located on chromosome 19q-13.42, has been investigated in several cancers, including neuroblastoma [21], colorectal cancer [22], lung cancer [23] and hepatocellular carcinoma [24]. However, only two studies have been carried out so far to explore the function of miR-517a-3p in BC. MiR-517a-3p was identified as one of the hypoxia-regulated miRNAs (HRMs) in BC [25]. But the clinical role, biological function or molecular mechanism of miR-517a-3p in BC was not studied by the research group of Blick et al [25]. MiR-517a-3p was hypothesized to be tumor-suppressive miRNA in BC. The miR-517a-3p (former name as miR-517a) restoration revealed a remarkable suppression of cell growth in two BC cell lines of BOY and T24. Additionally, transfection of miR-517a-3p prominently induced cells apoptosis in BC cells. Moreover, oligo microarray analysis indicated 35 lower and 19 higher expressed genes after transfection of miR-517a-3p into the BC cells [26]. However, the clinical significance, as well as the potential target genes of miR-517a-3p, was not explored by the group of Yoshitomi et al [26]. Thus, an in-depth analysis is urgently required to define whether miR-517a-3p participates in the occurrence and development of BC, and to comprehensively investigate the prospective target genes and regulation networks of miR-517a-3p in BC.

Therefore, in the current investigation, we first attempted to explore the clinical role of the expression level and genetic alteration of miR-517a-3p in BC with data from the cancer genome atlas (TCGA, https://cancergenome.nih.gov/), cBioPortal for Cancer Genomics (http://www.cbioportal.org/), Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/), and YM500v3 (http://driverdb.tms.cmu.edu.tw/ym500v3/). Second, we gathered the potential target genes via predicting platforms and gene profiling post miR-517a-3p overexpression *in vitro*. Further signaling pathway analyses were performed with enrichment of functional annotation and biological pathway analyses to explore the prospective role of miR-517a-3p in the carcinogenesis and progress of BC (**Figure 1**).

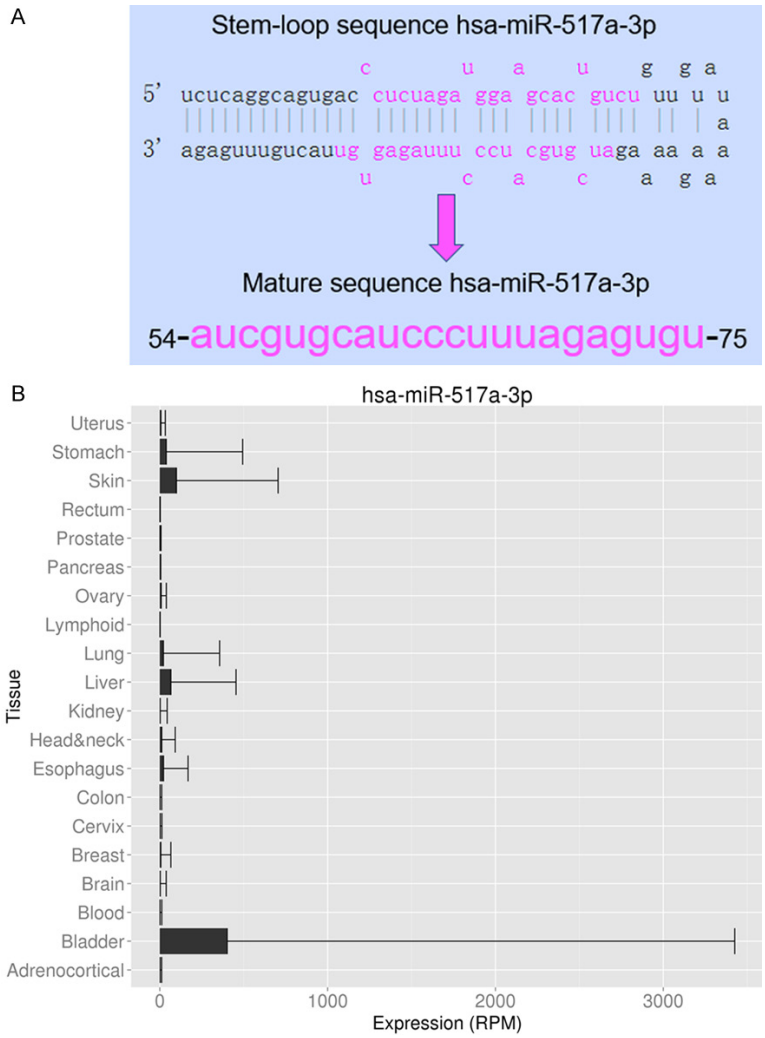


Figure 2. Expression level of miR-517a-3p provided from YM500v3. A. Sequence of hsa-miR-517a-3p (www.miRbase.org); B. Expression level of miR-517a-3p in different cancers (YM500v3, http://driverdb.tms.cmu.edu.tw/ym500v3/).

Materials and methods

Data extraction and assessment from TCGA

Since TCGA did not provide the expression data of mature miRNA, we extracted the data of miR-517a and clinical information of BC patients. The dataset contained 429 samples including 410 cases and 19 controls. After the exclusion of those samples whose log2 scale of miR-517a expression was lower than 1, only 106 cases and no controls were left. The clinical information and follow-up data of these patients, including gender, Body Mass Index (BMI), neoplasm histologic grade, clinical T stage, lymphovascular invasion, pathologic T stage, pathologic N stage, pathologic M stage, pathologic stage, tobacco smoking history, and primary therapy outcome were also obtained to evaluate the potential relationship between expression levels of miR-517a and clinical parameters. The genetic alteration of miR-517a was downloaded from cBioPortal with 412 BC patients being involved.

MicroRNA microarray searching and data analysis from GEO and ArrayExpress

To further collect the information of miR-517a-3p expression in BC, public microarray data from GEO and ArrayExpress were searched with the following keywords: (bladder OR urothelial OR urinary OR urogenital) AND (cancer OR carcinoma OR tumor OR neoplasm* OR malignant*). Two authors (Xing-Gu Lin and Rong-Quan He) performed the initial blind screening, data extraction and data re-calculation. A third author (Gang Chen) re-examined and ensured the correctness of all steps. The expression data of miR-517a-3p was extracted from BC and relevant controls.

Altogether, 12 microarrays were obtained from both GEO and ArrayExpress, including GSE20414, GSE20418, GSE31616, GSE31617, GSE36121, GSE39067, GSE39093, GSE40355, GSE48008, GSE50894, GSE81201 and GSE86411. Microarrays without normal tissue controls or expression data were excluded. Finally, only data from GSE39093 could be re-calculated, which led to the failure to perform a meta-analysis.

Prediction of the prospective target genes of miR-517a-3p

The prediction of miR-517a-3p target genes was conducted with different bioinformatics tools, including microRNA.org, RNA22, PicTarvert, TargetScan, miRDB, PolymiRTS Database, PITA, TargetMiner, TarBase and mirTarBase.

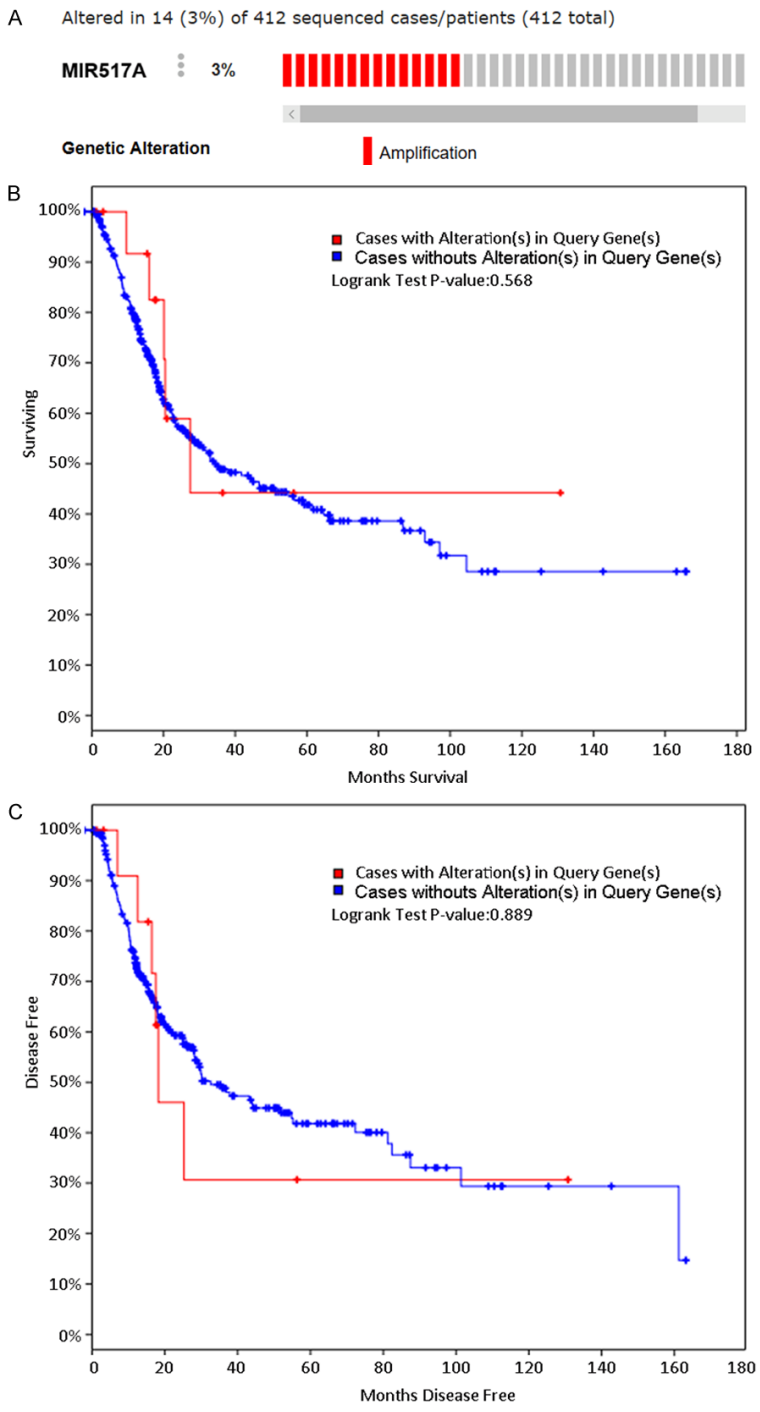


Figure 3. Alteration and prognostic value of miR-517a provided by cBioPortal. A. Genetic alterations of miR-517a in bladder cancer. B. The relationship between amplification of miR-517a and overall survival. C. Disease free survival.

Only genes appearing for over two times among 10 platforms were regarded as potential target genes of miR-517a-3p.

The validated target genes of miR-517a-3p were also obtained from literatures. We searched PubMed, Web of Science, as well as Chinese datasets of CNKI, Wanfang to identify validated target genes of miR-517a-3p up to 1st Feb, 2017. The following terms were used for searching: (bladder OR urothelial OR urinary OR urogenital) AND (cancer OR carcinoma OR tumor OR neoplasm* OR malignant*) AND (MicroRNA517a OR miRNA517a OR miR517a OR miR-517a OR miRNA-517a OR microRNA-517a OR "microRNA517a" OR "miRNA517a" OR "miR517a" OR miR-517a-3p OR miRNA-517a-3p OR microRNA-517a-3p).

Correlative genes of miR-517a-3p in BC as assessed by microarray

Previously, a microarray with correlative genes of post miR-517a-3p transfection *in vitro* was achieved from GEO (GSE-24782) with two BC cell lines of BOY and T24. The down-regulated genes from both two cell lines were gathered and integrated with the predicting genes mentioned above.

Gene ontology (GO) and pathway analysis

To explore the prospective biological effects of miR-517a-3p in BC, target genes of miR-517a-3p were sent for GO, Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses, which were performed via The Database for Annotation, Visualization and Integrated Discovery (DAVID, version 6.8) and PANTHER analysis. Furthermore, protein-protein interaction (PPI) was conducted to identify the hub genes.

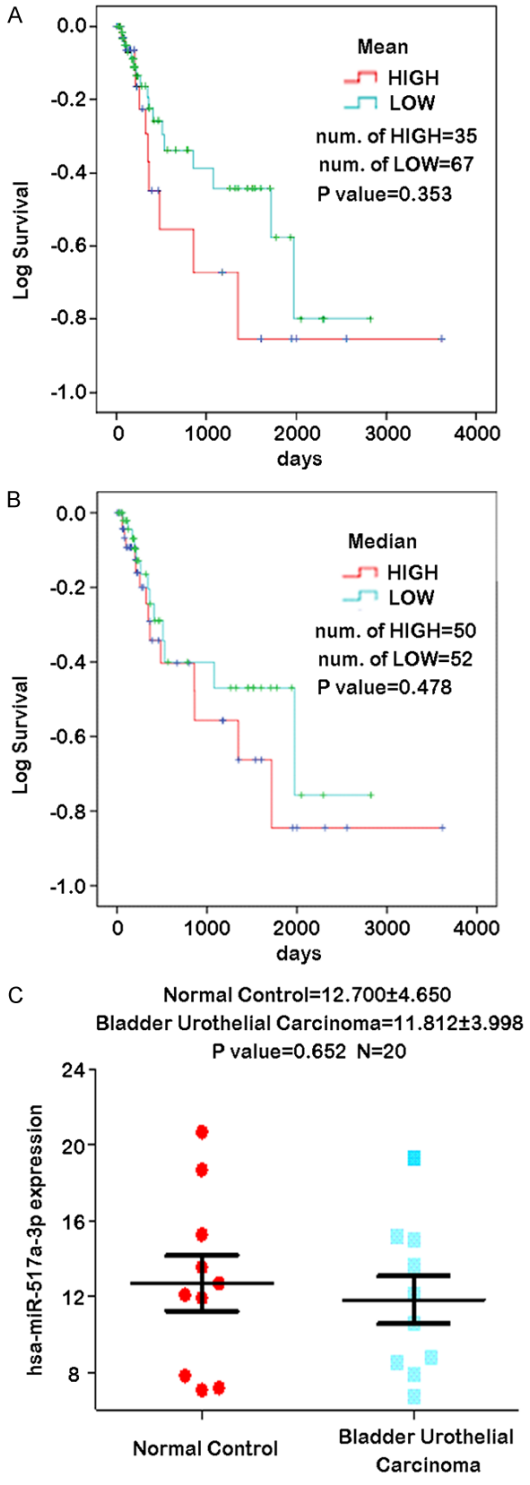


Figure 4. Clinical role of miR-517a-3p expression in bladder cancer based on data from TCGA and GEO. Relationship between miR-517a expression and patient survival based on TCGA data: (A) The groups of high and low were divided based on mean (A) or median (B) of miR-517a-3p level. (C) MiR-517a-3p expression level between normal control and bladder cancer tissues from GSE39093. Note: TCGA, the Cancer Genome Atlas; GEO, Gene Expression Omnibus.

Statistical analysis

Statistical analysis was performed by the software of Statistical Product and Service Solutions (SPSS, IBM Corporation, NY, USA). The data of miR-517a expression were exhibited as mean ± standard deviation (SD). Student's t-test for independent-samples was used to assess the clinical role of miR-517a. Survival curves were drawn by the Kaplan-Meier (K-M) analysis. *P* value <0.05 was considered significant in the current study.

Results

Clinical role of miR-517a in the data of TCGA

The expression level of miR-517a in different cancers could be obtained from YM500v3 (**Figure 2**) and BC tissues exhibited clearly a high level of miR-517a compared to other cancers. Due to the lack of data in controls, it was not possible to compare the difference of miR-517a level between BC and non-cancerous groups. As for the genetic alterations of miR-517a, 14 among 412 cases were amplified in BC (**Figure 3A**). The amplification was the only one alteration type identified for miR-517a and no mutation, upregulation or downregulation was observed. When concerning the relationship between miR-517a amplification and the outcome of BC patients, no significant relationship was found, including overall survival or disease free survival (**Figure 3B**, **3C**). We were also interested in the influence of miR-517a level on the prognosis of BC patients. Patients were divided into high and low group by the mean or median of miR-517a expression data. K-M curves showed that patients with lower level of miR-517a tended to have a slightly better survival as compared to those with higher level; however, both of the *P* values did not reach to be significant (P_{mean}=0.353, **Figure 4A**; P_{median}=0.478, **Figure 4B**). However, remarkable correlations were found between miR-517a expression and three clinical parameters respectively, including lymphovascular invasion (t=-1.201, P=0.023), pathologic N stage (t=-2.007, P=0.048) and pathologic stage (t=-1.993, P=0.049) (**Table 1**).

MiR-517a level from GEO and ArrayExpress

The relative expression level of miR-517a-3p was 11.812±3.998 in BC tissues, slightly lower than that in the controls (12.700±4.650, P=0.652). However, only 10 cases of BC patients

Table 1. Correlation between miR-517a expression and clinicopathological parameters in BC

Clinicopathological parameters	N	MiR-517a expression		
		Mean ± SD	t	P
Gender			0.988	0.325
Male	82	3.625±2.578		
Female	24	3.059±2.038		
BMI			1.786	0.077
≤25	39	4.051±2.772		
>25	60	3.127±2.337		
Neoplasm histologic grade			0.190	0.850
Low grade	5	3.684±2.019		
High grade	99	3.469±2.485		
Clinical T stage			0.503	0.618
T1-T2	36	3.062±2.177		
T3-T4	10	2.695±1.390		
Lymphovascular invasion			-1.201	0.023
No	37	3.168±2.660		
Yes	45	3.854±2.501		
Pathologic T stage			-1.139	0.258
T0-T2	31	3.249±2.180		
T3-T4	64	3.881±2.689		
Pathologic N stage			-2.007	0.048
N0	57	3.140±2.436		
N1-N3	36	4.199±2.548		
Pathologic M stage			0.271	0.787
M0	52	3.173±2.381		
M1	2	2.712±0.024		
Pathologic stage			-1.993	0.049
I-II	33	2.787±1.989		
III-IV	71	3.803±2.593		
Tobacco smoking history			-0.446	0.657
<2.46	50	3.380±2.475		
≥2.46	55	3.597±2.503		
Primary therapy outcome			-0.423	0.674
CR+PR+SD	57	3.385±2.402		
PD	10	3.746±2.957		

Abbreviations: BMI, Body Mass Index; CR, Complete Remission/Response; PR, Partial Remission/Response; SD, Stable Disease; PD, Progressive Disease.

and 10 controls were enrolled in this microarray (**Figure 4C**). We also attempted to perform a meta-analysis to study the clinical role of miR-517a in BC with data from literatures. But no sufficient data could be achieved.

Prediction of the potential target genes of miR-517a-3p

Since miRNA regulation depends on the influence upon their target-protein-coding genes, we screened out the predicted targets of the miR-517a-3p based on multiple platforms. Among the 10 predicting programs, PicTar-vert and PolymiRTS Database provided no results. Thus, based on the results of the leaving eight prediction databases, including TargetScan, microRNA.org, RNA22 tool, miRDB, PITA, TargetMiner, TarBase and mirTarBase, a total of 858 genes, each of which was predicted by at least two databases, were selected for further analysis. Unfortunately, no validated target genes could be found in all literatures by far.

Correlative genes of miR-517a-3p in BC as assessed by microarray

GSE24782 microarray was performed by Agilent whole genome microarrays with several human cancer cell lines (BOY, T24, A498, PC3, DU145, FaDu, SAS, HSC3 and IMC3) transfected with different miRNAs (miR-517a-3p, miR-218, miR-145, miR-1 and miR-874). Among all the cell lines, BOY and T24 are BC cells. Due to the potential inverse relationship between miRNA and target genes, only those down-regulated genes were regarded as prospective target genes of miR-517a-3p. Compared to control cells, there were 4844 genes down-expressed with a fold change (FC) <0.75 after BOY and T24 cells were transfected with miR-517a-3p. These genes were chosen for further analysis.

GO and Pathway analyses of target genes of miR-517a-3p

Both of the predicted targets of miR-517a-3p and correlative genes by microarray inevitably contain a certain false positivity. To improve the accuracy of the prospective target genes, we further overlapped the genes from prediction and microarray. A number of 106 overlapped genes that were more prone to be the targets of miR-517a-3p were achieved (**Table 2**), and categorized in GO, KEGG and PANTHER analyses. There were 65 pathways in biological process-

Table 2. A total of 106 overlapped genes from predicted targets of miR-517a-3p and correlative genes by microarray

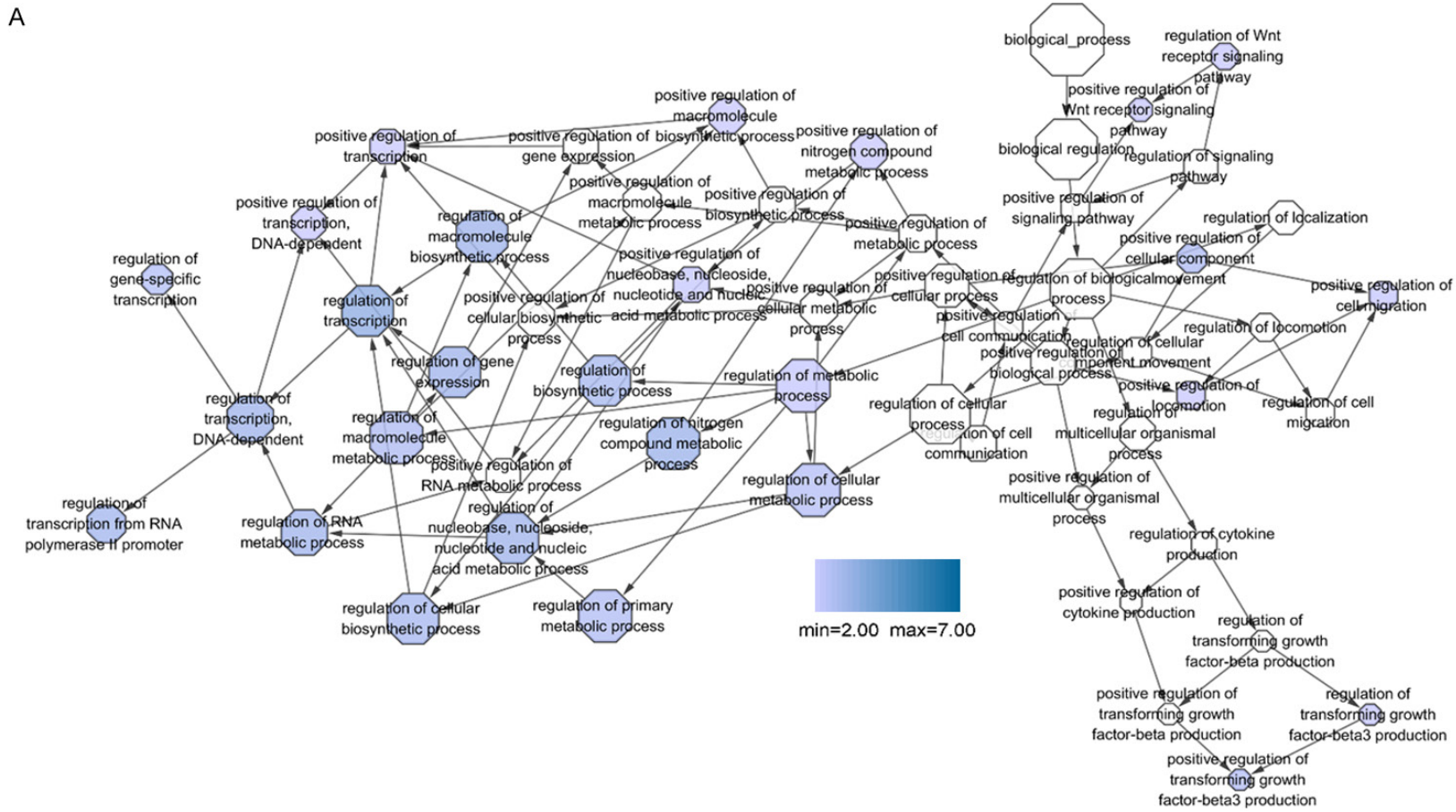
TMCC1	IGF1	SF3B1	SP4	MAGI1	ETS1	SMAD3	TNIP3
SPN	DENND1B	SYNJ2BP	APC	DCX	SPOPL	NTRK3	PRDM2
KIAA2018	OPCML	SLTM	SP3	MAEA	PODXL	ADAMTSL1	CEP170
CCNT2	EIF1AX	DBT	NPAT	MAPK1	RAP2B	CLIP1	UBE2N
SLC4A7	WASL	SPAG9	BPTF	RPS6KA5	CLOCK	GEM	MAP1B
TCERG1	ELF2	KLF12	TBXA2R	SEC63	ZZEF1	TNS1	RIF1
PGS1	SPTLC1	PCDH11Y	PIK3IP1	PPM1A	STK4	CDADC1	CRX
PFKFB4	FOXP1	CLIP4	TROVE2	CREB1	CLCC1	USP6	SYNE1
TMEM108	DOCK5	GPR107	SACS	DGKE	MAML1	DISC1	COX18
USP54	ZNF440	MTX3	BTBD9	PPARA	ZNF431	SRGAP1	BRD2
MPPE1	IRF2BP2	ZC3H12C	HAPLN4	HELZ	SH3BGRL2	DCP1A	PCF11
HYAL1	RBM33	KLF9	EEA1	EXOC5	ATRX	RAD51AP1	CCDC150
TRAF1	BCAP29	LRRC58	CUL5	RAD21	SRCAP	ELAVL2	NUPL1
FOXK1	MUC5AC						

Table 3. The most strongly enriched pathways of potential target genes of miR-517a-3p in bladder cancer from GO analysis (*P* Value <0.05)

Term (Biological Process)	Count	*P* Value
GO:0006357~regulation of transcription from RNA polymerase II promoter	17	8.49E-06
GO:0045449~regulation of transcription	33	4.25E-05
GO:0045935~positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	14	1.15E-04
GO:0006355~regulation of transcription, DNA-dependent	25	1.35E-04
GO:0051173~positive regulation of nitrogen compound metabolic process	14	1.58E-04
GO:0045893~positive regulation of transcription, DNA-dependent	12	1.70E-04
GO:0045941~positive regulation of transcription	13	1.78E-04
GO:0051254~positive regulation of RNA metabolic process	12	1.82E-04
GO:0010557~positive regulation of macromolecule biosynthetic process	14	1.84E-04
GO:0051252~regulation of RNA metabolic process	25	1.92E-04
GO:0010604~positive regulation of macromolecule metabolic process	16	2.27E-04
GO:0010628~positive regulation of gene expression	13	2.35E-04
GO:0031328~positive regulation of cellular biosynthetic process	14	2.89E-04
GO:0009891~positive regulation of biosynthetic process	14	3.32E-04
GO:0051272~positive regulation of cell motion	6	3.54E-04
GO:0006350~transcription	26	7.08E-04
GO:0051270~regulation of cell motion	7	0.00126068
GO:0032583~regulation of gene-specific transcription	6	0.00146061
GO:0010638~positive regulation of organelle organization	5	0.00175119
GO:0043193~positive regulation of gene-specific transcription	5	0.0020818
GO:0045944~positive regulation of transcription from RNA polymerase II promoter	9	0.00210604
GO:0030335~positive regulation of cell migration	5	0.00226227
GO:0040017~positive regulation of locomotion	5	0.00320887
GO:0030334~regulation of cell migration	6	0.00400028
GO:0006310~DNA recombination	5	0.00410968
GO:0051130~positive regulation of cellular component organization	6	0.00534255
GO:0040012~regulation of locomotion	6	0.00682873
GO:0050678~regulation of epithelial cell proliferation	4	0.0097452
GO:0019827~stem cell maintenance	3	0.01041824
GO:0042981~regulation of apoptosis	12	0.0107028
GO:0045596~negative regulation of cell differentiation	6	0.01103444
GO:0033043~regulation of organelle organization	6	0.01124082
GO:0048864~stem cell development	3	0.01124129
GO:0043067~regulation of programmed cell death	12	0.01147102

GO:0010941~regulation of cell death	12	0.01176982
GO:0032916~positive regulation of transforming growth factor-beta3 production	2	0.01238059
GO:0045597~positive regulation of cell differentiation	6	0.01392659
GO:0007346~regulation of mitotic cell cycle	5	0.01476604
GO:0048863~stem cell differentiation	3	0.01674905
GO:0051726~regulation of cell cycle	7	0.01689543
GO:0043065~positive regulation of apoptosis	8	0.0173286
GO:0043068~positive regulation of programmed cell death	8	0.01793244
GO:0010942~positive regulation of cell death	8	0.01834319
GO:0032910~regulation of transforming growth factor-beta3 production	2	0.01851396
GO:0010551~regulation of specific transcription from RNA polymerase II promoter	4	0.02065052
GO:0008285~negative regulation of cell proliferation	7	0.0246773
GO:0030098~lymphocyte differentiation	4	0.02618972
GO:0006974~response to DNA damage stimulus	7	0.02837028
GO:0051094~positive regulation of developmental process	6	0.0293097
GO:0051495~positive regulation of cytoskeleton organization	3	0.03174387
GO:0031346~positive regulation of cell projection organization	3	0.03438775
GO:0006351~transcription, DNA-dependent	6	0.0351275
GO:0046649~lymphocyte activation	5	0.03525592
GO:0032774~RNA biosynthetic process	6	0.03691246
GO:0009896~positive regulation of catabolic process	3	0.03711437
GO:0008361~regulation of cell size	5	0.03924512
GO:0043353~enucleate erythrocyte differentiation	2	0.04267335
GO:0042110~T cell activation	4	0.04359366
GO:0051783~regulation of nuclear division	3	0.04727491
GO:0007088~regulation of mitosis	3	0.04727491
GO:0002521~leukocyte differentiation	4	0.04798168
GO:0006917~induction of apoptosis	6	0.0487915
GO:0034330~cell junction organization	3	0.04880096
GO:0010552~positive regulation of specific transcription from RNA polymerase II promoter	3	0.04880096
GO:0012502~induction of programmed cell death	6	0.04933039
Term (Molecular Function)	Count	*P* Value
GO:0016563~transcription activator activity	11	2.24E-04
GO:0008134~transcription factor binding	11	0.001285
GO:0030528~transcription regulator activity	20	0.002025
GO:0003702~RNA polymerase II transcription factor activity	7	0.004154
GO:0003700~transcription factor activity	14	0.007031
GO:0008017~microtubule binding	4	0.010245
GO:0008092~cytoskeletal protein binding	9	0.013126
GO:0016564~transcription repressor activity	7	0.013981
GO:0003690~double-stranded DNA binding	4	0.022675
GO:0015631~tubulin binding	4	0.024542
GO:0003712~transcription cofactor activity	7	0.025716
GO:0003704~specific RNA polymerase II transcription factor activity	3	0.029432
GO:0043565~sequence-specific DNA binding	9	0.035161
GO:0003713~transcription coactivator activity	5	0.044698
GO:0003677~DNA binding	22	0.04643
Term (Cellular Component)	Count	*P* Value
GO:0005654~nucleoplasm	11	0.017607
GO:0031981~nuclear lumen	15	0.018885
GO:0015630~microtubule cytoskeleton	8	0.02622
GO:0043233~organelle lumen	17	0.026996
GO:0044451~nucleoplasm part	8	0.02761
GO:0031974~membrane-enclosed lumen	17	0.031766
GO:0070013~intracellular organelle lumen	16	0.044557
GO:0043228~non-membrane-bounded organelle	21	0.047171
GO:0043232~intracellular non-membrane-bounded organelle	21	0.047171

Abbreviations: GO, Gene Ontology.


A
biological_process
regulation of Wnt receptor signaling pathway
positive regulation of Wnt receptor signaling pathway
biological regulation
regulation of signaling pathway
positive regulation of signaling pathway
regulation of localization
positive regulation of cellular component
regulation of biological movement
positive regulation of cell migration
regulation of locomotion
regulation of cellular component movement
positive regulation of locomotion
regulation of cell migration
regulation of multicellular organismal process
positive regulation of multicellular organismal process
regulation of cytokine production
positive regulation of cytokine production
regulation of transforming growth factor-beta production
positive regulation of transforming growth factor-beta production
regulation of transforming growth factor-beta3 production
positive regulation of transforming growth factor-beta3 production
positive regulation of macromolecule biosynthetic process
positive regulation of transcription
positive regulation of gene expression
positive regulation of nitrogen compound metabolic process
positive regulation of macromolecule metabolic process
positive regulation of biosynthetic process
positive regulation of transcription, DNA-dependent
regulation of macromolecule biosynthetic process
positive regulation of metabolic process
regulation of gene-specific transcription
positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process
positive regulation of cellular process
regulation of transcription
positive regulation of cellular biosynthetic process
positive regulation of cellular metabolic process
positive regulation of cell communication
regulation of gene expression
regulation of biosynthetic process
regulation of metabolic process
regulation of transcription, DNA-dependent
regulation of cellular process
regulation of macromolecule metabolic process
regulation of nitrogen compound metabolic process
positive regulation of RNA metabolic process
regulation of cell communication
regulation of cellular metabolic process
regulation of transcription from RNA polymerase II promoter
regulation of RNA metabolic process
regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process
regulation of cellular biosynthetic process
regulation of primary metabolic process
min=2.00 max=7.00

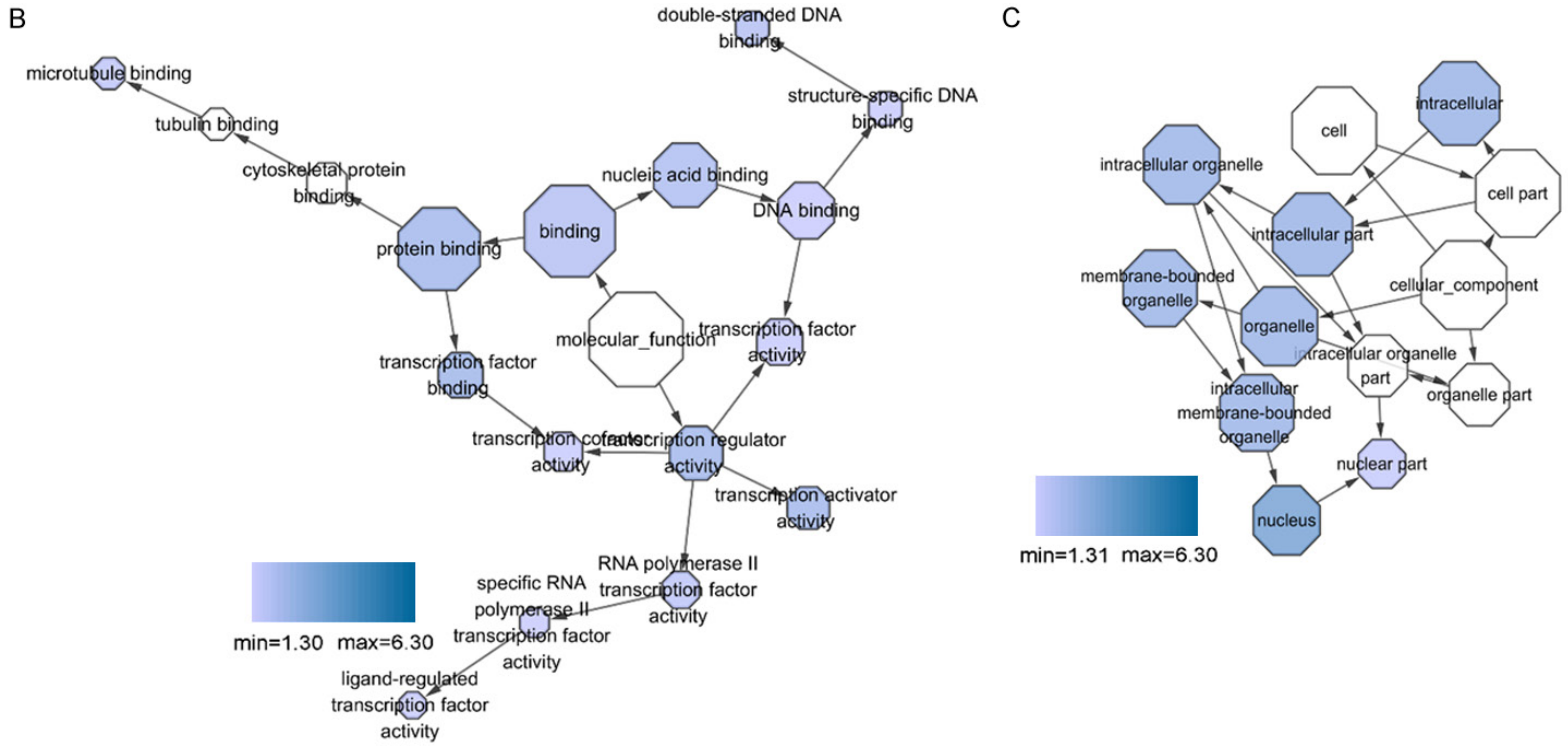


Figure 5. Gene network analysis with the overlapped target genes of miR-517a-3p for GO analysis. Cytoscape was used to picture the network. The octagons represented various terms of BP (A), MF (B) and CC (C). Arrows displayed the correlation among terms. The color gradient of octagons exhibited the significance of relative terms. (A) Terms of BP were selected with the remarkable value of 0.01 for the current DAG, which possessed 54 nodes and 98 edges. (B) Members of MF were picked with the notable level of 0.05 for the DAG with 18 nodes and 19 edges. (C) Components of CC were gathered with the standard of 0.05 for the DAG containing 13 nodes and 20 edges. Note: GO, Gene Ontology; BP, biological processes; MF, molecular function; CC, cellular component; DAG, Direct Acyclic Graph.

Table 4. The top 3 KEGG pathways of potential target genes of miR-517a-3p in bladder cancer

Term	Count	*P* Value	Bonferroni	Benjamini	FDR	Genes
hsa05200: Pathways in cancer	7	0.01494	0.681486221	0.681486221	14.65014	TRAF1, MAPK1, ETS1, SMAD3, IGF1, STK4, APC
hsa04520: Adherens junction	3	0.08387	0.998715456	0.964159462	60.21901	MAPK1, SMAD3, WASL
hsa05210: Colorectal cancer	3	0.09728	0.999581167	0.925180689	65.93677	MAPK1, SMAD3, APC

Abbreviations: KEGG, Kyoto Encyclopedia of Genes and Genomes.

Table 5. The top 3 PANTHER pathways of potential target genes of miR-517a-3p in bladder cancer

Term	Count	*P* Value	Bonferroni	Benjamini	FDR	Genes
P00047: PDGF signaling pathway	6	0.006854584	0.213951248	0.213951248	5.910559	RPS6KA5, MAPK1, TCERG1, ELF2, ETS1, SRGAP1
P00052: TGF-beta signaling pathway	5	0.01955633	0.499052004	0.292223202	16.04916	MAPK1, FOXK1, DCP1A, SMAD3, FOXP1
P00032: Insulin/IGF pathway-mitogen activated protein kinase kinase/MAP kinase cascade	3	0.041699544	0.774804702	0.391603875	31.42765	RPS6KA5, MAPK1, IGF1

es (BPs) (P<0.05), such as regulation of transcription from RNA polymerase II promoter, regulation of transcription. In addition, 15 pathways of molecular functions (MFs) and nine pathways in cellular component (CC) were also noted (P<0.05) (**Table 3**; **Figure 5**). Via the KEGG pathway analysis, we discovered that only "hsa05200: Pathways in cancer" was significantly enriched (P<0.05) (**Table 4**). We also discovered three significant pathways, including platelet derived growth factor (PDGF) signaling pathway; transforming growth factor (TGF)-beta signaling pathway; Insulin/IGF pathway-mitogen activated protein kinase kinase/MAP kinase cascade in PANTHER pathway analysis (P<0.05) (**Table 5**). Furthermore, PPI was conducted to identify the hub genes, including CAMP responsive element binding protein 1 (CREB1), Mitogen-activated protein kinase 1 (MAPK1), Ribosomal protein S6 kinase alpha-5 (RPS6KA5), SMAD family member 3 (SMAD3) and peroxisome proliferator activated receptor alpha (PPARA) (**Figure 6**).

Discussion

In the current study, the clinical significance of miR-517a-3p in BC was first investigated based on data from miRNA sequencing (TCGA, cBioPortal and YM5003v). Gene amplification was the only genetic alteration of miR-517a-3p in BC. Furthermore, pathway analyses revealed that miR-517a-3p could play its roles via targeting multiple pathways and "hsa05200: Pathways in cancer" was the most significant one as defined by KEGG with contained several genes: TNF receptor associated factor 1 (TRAF1), MAPK1, ETS proto-oncogene 1 (ETS1), SMAD3, insulin like growth factor 1 (IGF1), serine/threonine kinase 4 (STK4) and APC, WNT signaling pathway regulator (APC), among which, MAPK1 and SMAD3 were also identified as hub genes of miR-517a-3p in BC.

It has been well documented that miRNA expression levels could become favorable markers for diagnosis, prognosis, and treatment outcome prediction in cancers. MiR-517a-3p is one of the attention-grabbing cancer-relevant miRNAs which has been involved in different cancers. A prominently lower expression of miR-517a-3p was detected in seminomas than that in non-seminomas, in both tissue and serum samples, which pointed out that miR-517a-3p could serve as a prospective biomarker for the testicular germ cell tumor [27]. MiR-517a-3p expression was identified to be remarkably up-regulated in colorectal cancer tissues than that in adjacent non-tumor tissues. Up-regulation of miR-517a-3p was also closely related to poorer prognosis of colorectal cancer patients. Furthermore, miR-517a-3p can act as an independent prognostic biomarker for colorectal cancer patients [22]. Two groups investigated the clinical role of miR-517a-3p in hepatocellular carcinoma (HCC) and inconsistent discoveries were reported. Liu et al [24] disclosed that miR-517a-3p was down-regulated in HCC samples. On the contrary, Toffanin et al [28] documented that miR-517a-3p was an oncogenic miRNA and could assist tumor progression. Thus, miR-517a-3p could play different roles in various cancers. By far, only two studies have mentioned miR-517a-3p

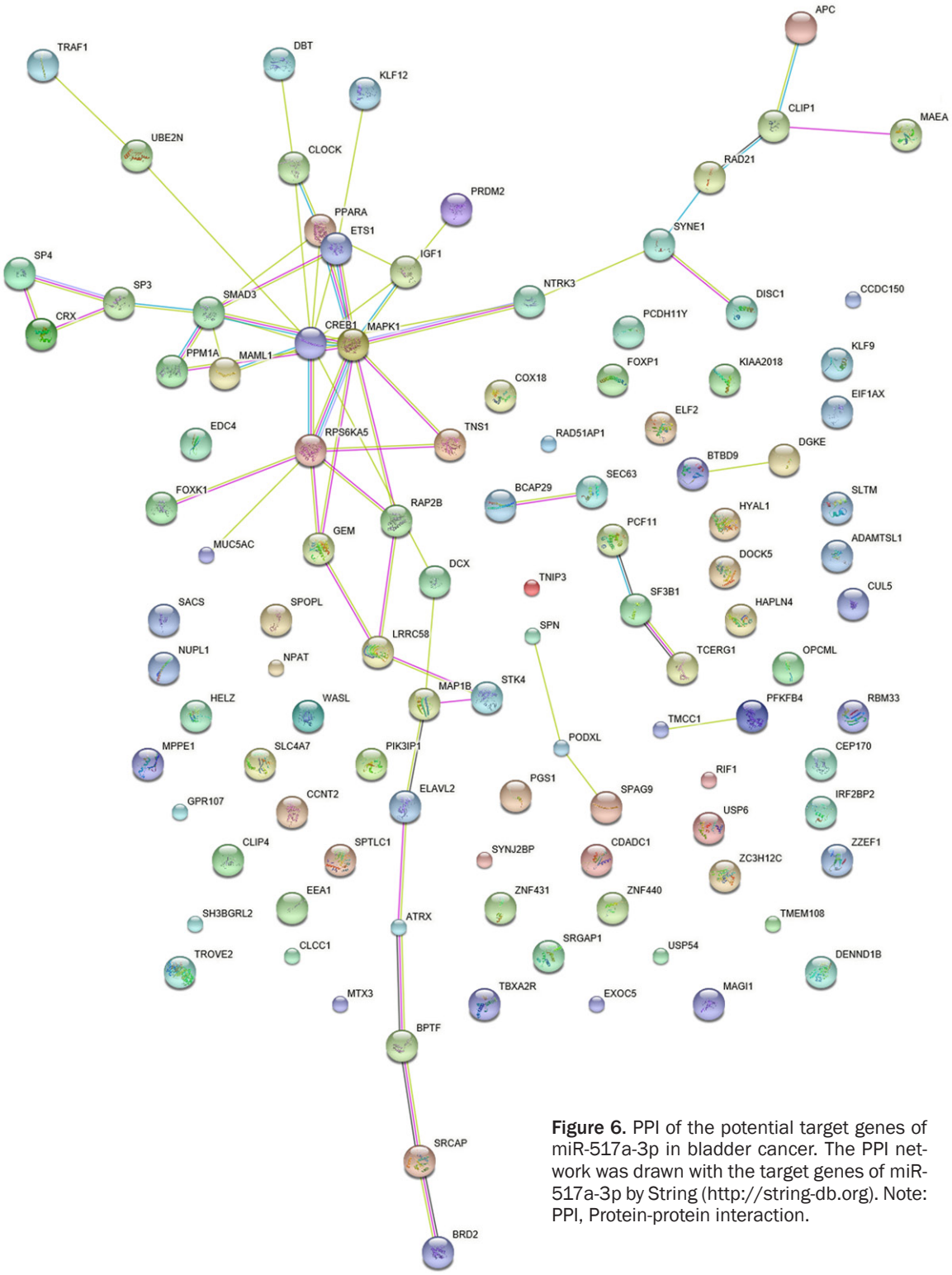


Figure 6. PPI of the potential target genes of miR-517a-3p in bladder cancer. The PPI network was drawn with the target genes of miR-517a-3p by String (http://string-db.org). Note: PPI, Protein-protein interaction.

in BC. MiR-517a-3p was proposed as one of the hypoxia-regulated miRNAs (HRMs) in BC [25]. Furthermore, miR-517a-3p was hypothesized to act as a tumor-suppressor miRNA in BC. The miR-517a-3p overexpression led to an obvious inhibition of cell growth in two BC cell lines of BOY and T24. Additionally, transfection of miR-517a-3p clearly induced cells apoptosis in BC

cells. But the clinical role of miR-517a-3p in BC was not studied by the above two studies [25, 26]. In the current study, we attempted to analyze the clinical role of miR-517a-3p based on the high throughput miRNA sequencing data. However, due to the lack of data of the non-tumorous controls, we could not assess the alteration of miR-517-3p expression between BC and non-cancerous bladder tissues with data from TCGA. The only data we could evaluate was from a GEO microarray (GSE39093), which indicated a slightly lower level of miR-517a-3p in BC tissues versus normal controls. However, the sample size was too small to draw any convincing conclusions. Moreover, no significant correlation between miR-517a-3p and clinical prognostic features was observed based on data from TCGA. Hence, the clinicopathological value of miR-517a-3p remains to be investigated with larger sample size in the future. Interestingly, we found the gene amplification was the only type of genetic alterations of miR-517a-3p in BC tissues. However, the clinical relevance of this amplification in BC also requires in-depth exploration.

When concerning the molecular mechanism of miR-517a-3p in diseases, only several studies have reported its possible target genes. Forkhead box J3 (FOXJ3) has been confirmed to be a target of miR-517a-3p both in colorectal cancer [22] and lung cancer [23], and miR-517a-3p could directly modulate FOXJ3 expression by binding to FOXJ3 promoter. Pyk2 was verified to a target of miR-517a-3p in HCC [24]. Moreover, clear inverse correlation of miR-517a-3p with CDKN2A was exhibited in glioblastoma multiforme [29]. Additionally, miR-517a-3p could induce the expression of endogenous NF-κB targets and stimulate the nuclear localization of p65 and the degradation of IκB. TNFAIP3 interacting protein 1 (TNIP1) was demonstrated as a target and characterized a functional SNP in the miR-517a-3p binding site [30]. To the best of our knowledge, only one study has investigated the probable mechanism of miR-517a-3p in BC. After transfection of miR-517a-3p into BC cells, oligo microarray analysis indicated 35 down-regulated genes and 19 up-regulated genes [26]. Among these genes, amphiregulin (AREG) and BCL2-associated transcription factor 1, transcript variant 1 (BCLAF1) were highlighted by the authors, but no validating experiments were performed to ensure whether these two genes were direct targets of miR-517a-3p in BC [26].

In the current study, the putative target genes of miR-517a-3p were unveiled using the combination of publicly available predicting databases and microarray data after miR-517a-3p mimic transfection. Altogether, 858 genes were predicted by various online platforms. We also re-assessed the data from GSE24782 which covered a large range of correlative genes of miR-517a-3p in BC cells and achieved 4844 down-expressed genes with a fold change (FC) <0.75 from both BOY and T24 cells. Eventually 106 overlapped genes were achieved, which were more prone to be the target genes of miR-517a-3p in BC. Next, functional analyses revealed that these genes were enriched in different pathways involved in the tumorigenesis and worsening of BC. The most significant pathway indicated in biological process (BP) was the pathway of regulation of transcription from RNA polymerase II promoter. And the pathway of nucleoplasm was the top one in CC, while the pathway of transcription activator activity ranked the first in MF. PDGF signaling pathway was identified to be the most substantial pathway by PANTHER analysis. Most importantly, "hsa-05200: Pathways in cancer" was the most enriched pathway as defined by KEGG analysis, which contained seven genes. They were TRAF1, MAPK1, ETS1, SMAD3, IGF1, STK4 and APC. Among these seven target genes, MAPK1 and SMAD3 were also presented as hub genes of miR-517a-3p in BC. Since both of MAPK1 [31, 32] and SMAD3 [33] have been well documented as pivotal genes in the tumorigenesis and progression of BC, it could be hypothesized that miR-517a-3p exerts its function via direct targeting MAPK1 and SMAD3 in BC. However, this hypothesis needs further confirmation with *in vitro* and *in vivo* experiments.

Collectively, miR-517a-3p may play critical roles in the occurrence and progression of BC; however, the clinical function of miR-517a-3p in BC needs to be validated. Furthermore, we predict several key pathways for miR-517a-3p in BC with *in silico* investigation. But further functional experiments and well-designed translational studies are requisite to unveil the molecular mechanism of miR-517a-3p in BC.

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Disclosure of conflict of interest

None.

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