Identifying the miRNA biomarker associated with survival time in patients with bladder cancer using miRNA expression profiles

Abstract

Bladder cancer has a complex and multifactorial pathophysiology. Numerous environmental, molecular, or genetic factor are involved in the development of urothelial cell cancers. In recent years, extraordinary progress has been made in terms of identifying the origin and exact functions of miRNA, MicroRNA (miRNA) expression profiles can used for discovering potential prognostic biomarkers of bladder cancer. This work adopted optimized support vector regression(SVR) method called SVR-IBCGA based on a identified miRNA signature for estimating the survival time of bladder cancer patients. SVR-IBCGA use inheritable bi-objective combinatorial genetic algorithm to identify a small set of informative miRNAs cooperating with SVR by maximizing prediction accuracy. In this work SVR-IBCGA identified 33 out of 884 miRNAs using 10-fold cross-validation and got a correlation coefficient of 0.86, R-squared of 0.74 and mean absolute error of 0.56 year prediction model, The identified miRNA signature which is a set highly related to the bladder cancer patient survival time, these 33 miRNAs are hsa-mir-323a, hsa-mir-659, hsa-mir-498, hsa-mir-181d, hsa-mir-3913-2, has-mir-4768, hsa-mir-548ba, hsa-mir-10a, hsa-mir-424, hsa-mir-1283, hsa-mir-3173, has-mir-939, hsa-mir-105-1, hsa-mir-4446, hsa-mir-4636, hsa-mir-5008, hsa-mir-539, hsa-mir-3664, hsa-mir-579, hsa-mir-4777, hsa-mir-4691, hsa-mir-629, hsa-mir-1229, hsa-mir-185, hsa-mir-4733, hsa-mir-6882, hsa-mir-4755, hsa-mir-455, hsa-mir-4727, has-mir-519a-2, hsa-mir-3680-1, hsa-mir-4501, hsa-mir-6781. To confirm and extend this signature, we use literature mining, Kaplan-Meier curve analysis and also building the miRNAs target gene network and then perform Gene ontology and KEGG pathway functional enrichment analysis of each miRNA and miRNA signature. After that, summarizing its biological significance in bladder cancer and cancer related pathways. Totally, our study not only identifying the bladder cancer biomarker, but also contracted a new survival estimate model. We consider the identification of miRNA and discovering their role in cancer are important for bladder cancer diagnostics and therapeutics and the estimation of survival time in bladder cancer patient can be useful for diagnosis, prognosis and monitoring the efficacy of treatment.

Introduction

Bladder cancer (BC) is the seventh most common cancer among men and the eleventh most common cancer in the world. The incidence rate (per 100,000 people / year) is 9.0 for men and 2.2 for women. Worldwide, the BC age -standardized mortality rate (per 100,000 person / years) was 3.2 for men vs. 0.9 for women in 2012. Survival rate depends on tumor grade, tumor stage, tumor invasion depth and genetic characteristics at the time of diagnosis. According to AJCC 8th edition, BC stage is based on the degree of invasion of the tumor, the lymph node or distant metastasis. Therefore, BC is staged into stage one to four. Non muscle invasive bladder cancer(NMIBC) can be treated with transurethral bladder tumor resection and intravesical chemotherapy or BCG installation. The muscle-invasive bladder cancer(MIBC), radical cystectomy and radiation may be required, with or without neoadjuvant chemotherapy and adjuvant chemotherapy. Moreover, treatment response is related to clinical and biological factors. Recently, BC has also used molecular classifications about four major subtypes as basal BC with the basal and claudin low-type group, luminal BC with luminal and p53-like subtype. The basal group shows an over-expression of epidermal growth factor receptor 3 (EGFR3) and is chemo-sensitive. The luminal type can display an over-expression of fibroblast growth factor receptor 3 (FGFR3), epidermal growth factor receptors (ERBB2 and ERBB3), and is chemotherapy resistant. According to a multi-institutional database, 888 consecutive BC patients undergone radical cystectomy, with a 5-year cancer specific survival of 66%. External validation of BC-specific mortality after operative nomograms showed similar results, with a bladder cancer specific survival of 62%. In another large single-center study including 1,054 patients, overall survival rate were 66% at 5 years, and 43% at 10 years. According to the data published by ACS in 2020, 5 year survival of stage 1 patient is 90%, and stage 2 is 70%. With Stage 3, and the 5-year survival rate is 36% and 5%. Judgment of the most appropriate treatment in BC patients is important to prolong survival in those high stage patients but the clinical behavior and molecular mechanism of tumor growth are still unclear due to the heterogeneity of BC.

Recent evidence indicates that molecular marker-based targeted therapy has potential for the prognosis of various diseases. Research based on molecular targets focuses on the progress of microRNA (miRNA) expression profiling because they play

an important role in tumor development and metastasis. MiRNA is a small non-coding RNA that regulates gene expression and is involved in human carcinogenesis. In the past few years, many studies have reported the important role of miRNAs in the molecular pathogenesis of bladder cancer tumors. MiRNA analysis studies have identified miRNAs that are abnormally expressed in bladder cancer tumors and their functions. In patients with BC, the miR-200 family members in urine are expected to become non-invasive, diagnostic and prognostic indicators. In another study, miRNA levels were measured in blood of patients with or without BC. Comparing miRNAs in non-malignant and malignant bladder tissues, the researchers identified 7 significantly up-regulated miRNAs and 8 down-regulated miRNAs. Highly expressed miRNAs -21 was associated with poorer overall survival and miR-21 and 378 can be used as independent prognostic factors for relapse. A general downregulation of miRNA has been observed in low-grade, non-muscle invasive bladder cancer (NMIBC), including FGFR3 pathway. In contrast, compared to adjacent normal bladder urothelial cells, increased expression of miRNAs was observed in high-grade muscle-invasive bladder cancer (MIBC), including miRNAs predicted to target p53.

MiRNA expression profiles are helpful to identify survival-related variants of bladder cancer. The miRNA signature associated with patient survival is also helpful for the development and evaluation of gene target based therapy. Some machine learning methods have been studied to diagnose bladder cancer. Some people use the degree of image invasion to predict the prognosis of bladder cancer. Some people use clinical data plus the p53 mutation to establish a machine learning model to predict the 10-year survival rate[86]. There is no currently uses miRNA to predict bladder cancer patient survival time base on machine learning by a miRNA signature. This work proposes a support vector regression (SVR) based predictor, SVR-IBCGA, to identify the miRNA signature associated with the survival time of patients with bladder cancer. Estimating survival time is a important thing in bladder cancer patient prognosis, Identification of miRNA signature associated with survival time will help to further understand the miRNA mechanism in bladder cancer. In addition, through this new bladder cancer Biomarker it can take the opportunity to give the new research direction of bladder cancer mechanism and the role of these miRNAs.

Result and Discussion

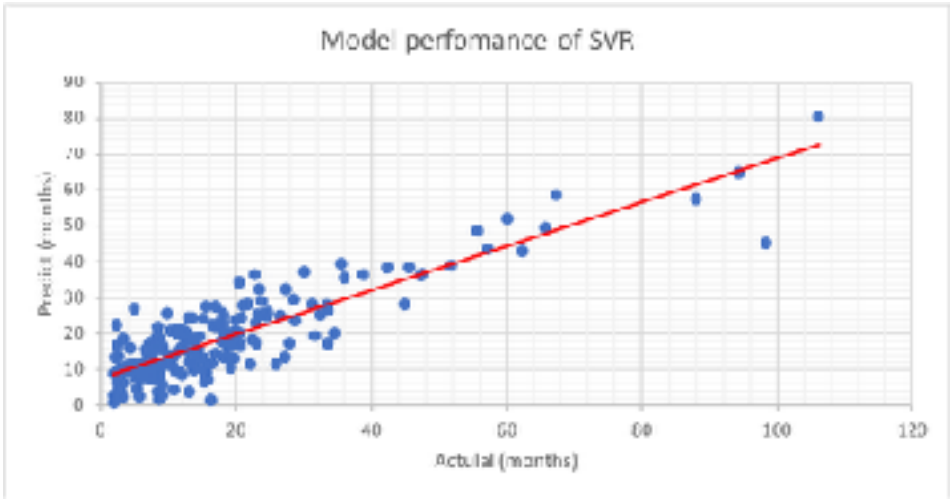
SVR-IBCGA identified the miRNA signature and estimated the survival time of bladder cancer patients using miRNA expression profiles. Through this miRNA signature, we can further analysis these miRNAs individually. SVR-IBCGA identify a set of 33 informative miRNAs (referred to a miRNA signature) associated with the estimation of bladder cancer survival time. Since the optimal feature selection method IBCGA is a non-deterministic method, we employed 30 independent runs to select one robust feature set. We compare the SVR-BLCA method with penalized regression methods, such as LASSO, Elastic net, and linear regression. The Elastic net is a combination of both methods LASSO and Ridge regression. The correlation plots of the real and estimated survival time for SVR-BLCA, Elastic net, and Multiple linear regression are shown below.

BLCA Features: 33 #ranked by MED score, listed up to down, left to right

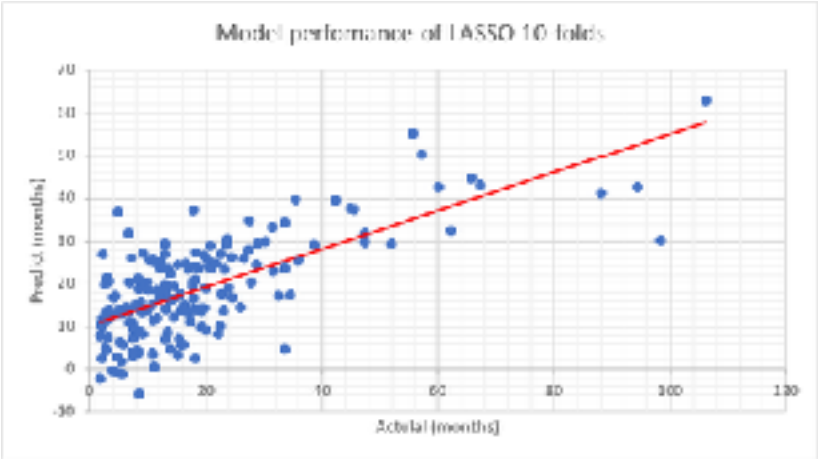
- Used an optimal feature selection algorithm IBCGA to identify more informative miRNAs.

TOP 10			
hsa-mir-323a	hsa-mir-3173	hsa-mir-4691	hsa-mir-3680-1
hsa-mir-659	hsa-mir-939	hsa-mir-629	hsa-mir-4501
hsa-mir-498	hsa-mir-105-1	hsa-mir-1229	hsa-mir-6781
hsa-mir-181d	hsa-mir-4446	hsa-mir-185	
hsa-mir-3913-2	hsa-mir-4636	hsa-mir-4733	
hsa-mir-4768	hsa-mir-5008	hsa-mir-6882	
hsa-mir-548ba	hsa-mir-539	hsa-mir-4755	
hsa-mir-10a	hsa-mir-3664	hsa-mir-455	
hsa-mir-424	hsa-mir-579	hsa-mir-4727	
hsa-mir-1283	hsa-mir-4777	hsa-mir-519a-2	

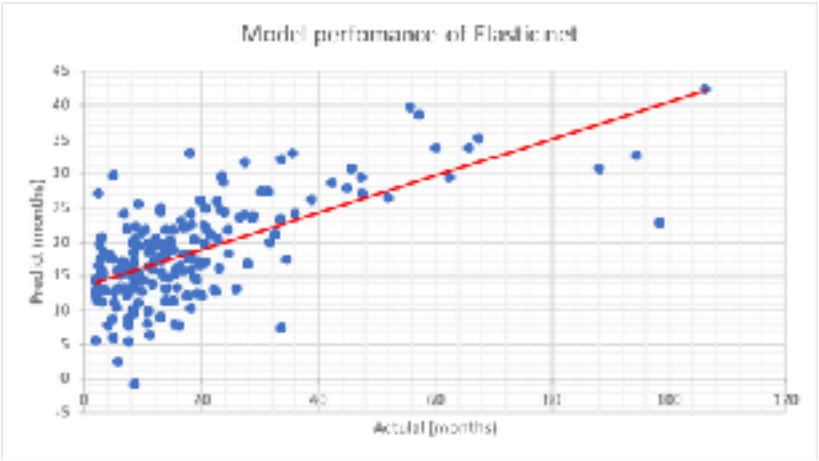
Bladder Cancer



- 10-Fold SVR survival time prediction result Correlation coefficient : 0.86

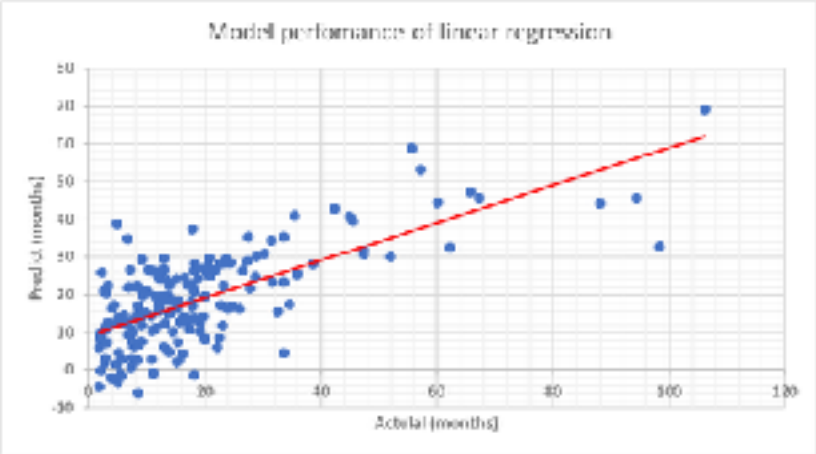


- 10-Fold Lasso survival time prediction result Correlation coefficient : 0.70



- Elastic net survival time prediction result Correlation coefficient : 0.63

Bladder Cancer



- Linear regression survival time prediction result Correlation coefficient : 0.70

Based on these identified 33 miRNAs features the highest correlation coefficient of different kind model is SVR. The estimation accuracy of SVR was the correlation coefficient of 0.86 and mean absolute error of 0.56 year using 10-CV. The LASSO method achieved a correlation coefficient and mean absolute error of 0.70 and 0.73 year using 10-CV, and ridge regression achieved a correlation coefficient and mean absolute error of 0.70 and 0.75 year, respectively. The Elastic net method with 33 miRNAs achieved a correlation coefficient and mean absolute error of 0.66 and 0.77 year, and Multiple linear regression with 33 miRNAs obtained a correlation coefficient and mean absolute error of 0.70 and 0.75 year, respectively. Comparing fairly SVR with these methods is better than these compared methods.

Roles of the identified miRNAs

This part of the thesis discusses the findings from the IBCGA features selection, To confirm and extend these results, I analyzed the individual roles of the top-10 miRNAs in the 33-miRNA signature. First, I perform literature mining checking whether there were existed study shown the miRNAs related to bladder cancer. As a miRCancer[99] literature mining result, I sort these 10 miRNAs to 2 parts, one part are those had been indicated that regulate bladder cancer already, I will briefly describe the role of this miRNA, another part are those no involved in cancer or it had been mentioned in others cancer studies but no bladder cancer. Among the top-10 miRNAs, five miRNAs which are hsa-mir-323a, hsa-mir-659, hsa-mir-3913, hsa-mir-10a and hsa-

mir-424 have been experimental demonstrated the involvement of bladder cancer regulation. The others miRNAs hsa-mir-498, hsa-mir-181d, hsa-mir-4768, hsa-mir-548ba and hsa-mir-1283 exist some previous studies have been shown that was related to others cancer type but no bladder cancer and there were also some study indicated that associated with the survival rate of bladder cancer using the statical method, we focused on second part which are hsa-mir-498, hsa-mir-181d, hsa-mir-4768, hsa-mir-548ba and hsa-mir-1283, to find out the possible function of each new finding miRNAs, we use bioinformatic analysis method to annotate the most significance biological process/pathway[5]. Additionally, I also use kmplot[44] to did a Kaplan–Meier analysis to validate whether the identified miRNAs are survival biomarker in bladder cancer.

Table(1)

Top 10 miRNA involved in various cancer (miRCancer[#99], dbDEMC2,0)

miRNA ID	Cancer Type	Profile(down, up, overexpression)	Reference/ dbDEMC2.0 [#102]
hsa-mir-323a	bladder cancer	down	#33
	glioblastoma cancer	down	#57
	osteosarcoma cancer	down	#10
hsa-mir-659	bladder cancer	down	#51
	colorectal cancer	down	#37
hsa-mir-498	breast cancer	down up	#9 #40
	cervical cancer	down	#54
	colorectal cancer	down	#22
	gastric cancer	down	#82
	non-small cell lung cancer	down	#63
	ovarian cancer	down	#29
	liver cancer	down	#79
hsa-mir-181d	glioma	down	#64
	prostate cancer	up	#61
	ovarian cancer	down	#35

Bladder Cancer

miRNA ID	Cancer Type	Profile(down, up, overexpression)	Reference/ dbDEMC2.0 [#102]
	non-small cell lung cancer	down	#26
hsa-mir-3913	bladder cancer	down	#46
hsa-mir-4768	larynx cancer	down	#8
	Pancreatic ductal adenocarcinoma	up	#75
hsa-mir-548ba	bladder cancer	down	#81
hsa-mir-10a	bladder cancer	up, down	#15,17,31
	breast cancer	down	#30
	cervical cancer	up	#38
	colorectal cancer	down up	#36 #52
	gastric cancer	up	#12
	lung cancer	up	#13
hsa-mir-424	bladder cancer	down	#66,67
	cervical cancer	down	#72
	colorectal caner	down	#19
	non-small cell lung cancer	up	#78
	gastric cancer	up	#65
	liver cancer	down	#47
	pancreatic cancer	up	#68
hsa-mir-1283	glioma cancer	down	#11
	serrated adenoma	up	GSE73487

1. **hsa-mir-323a:**

There is a study shows the expression of hsa-mir-323a is decreased in bladder cancer tissues. In this study the Kaplan–Meier analysis revealed that downregulation of miR-323a-3p was significantly associated with overall survival rate of BLCA(**p-value<0.01)[33](Li, Xu et al. 2017).

hsa-mir-323a regulate cell migration and invasion in bladder cancer via to its target gene(MET, SMAD2, SMAD3, STAT3), when hsa-miR-323a-3p was overexpressed in T24 and UM-UC3 cell lines, the relative activities of MET and SMAD3 were decreased. MET activated the AKT/GSK-3β/SNAIL signal to induce EMT and migration. Migration and invasion repressed by miR-323a-3p in T24 and UM-UC3 cell lines. To summarize, we demonstrated that SAMD3 as an important protein targeted by miR-323a-3p was involved in the progression of BLCA. Experiments revealed a significant inhibition of migration and invasion by regulating a MET/SMAD3/SNAIL circuit. All the findings highlighted the tumor suppressor role of miR-323a-3p in BLCA[33](Li, Xu et al. 2017).

EMT(epithelial-mesenchymal transition), which is a key role for cancer metastasis, EMT can induced by TGFb, EGFR signaling pathway or Wnt signaling pathway, when EMT happen in tumor cell, transcription factor Snail or Twist decrease the connection between cell and cell in tumor via E-cadherin expression, make tumor cell possess strong invasive and mobility, promote tumor metastasis.

2. hsa-mir-659:

Genetic studies on GRN in neurodegenerative disease proved that hsa-miR-659 [51](Rademakers, Eriksen et al. 2008) is a critical negative regulator of PGRN mRNA levels, PGRN promotes tumor cell proliferation, migration and survival, and induces drug resistance. Increasing or decreasing PGRN production enhances or inhibits respectively the growth of PGRN-sensitive tumors in vivo. PGRN activity is associated with p44/42 mitogen-activated protein kinase as well as phosphatidylinositol 3-kinases signaling pathways.

PGRN expression was detectable in both bladder cancer cells and normal bladder urothelium, and suggested that PGRN may play important roles in carcinogenesis as well as regulation of normal physiological activity. Significantly higher immunostaining of PGRN was observed in invasive bladder tumors as compared with normal bladder tissues. PGRN mRNA expression in bladder cancer using microarray database and found that overexpression of PGRN was observed in primary bladder cancers. PGRN mRNA expression levels was higher in high-grade bladder cancer than

that of low-grade bladder cancer, that PGRN level was significantly higher in patients with malignant lesions compared to healthy individuals[4](Bateman 2012).

Regarding the clinical outcome, BLCA-Kaplan–Meier analysis revealed that downregulation of hsa-mir-659 was significantly associated with the overall survival rate of BLCA(**p-value<0.01).

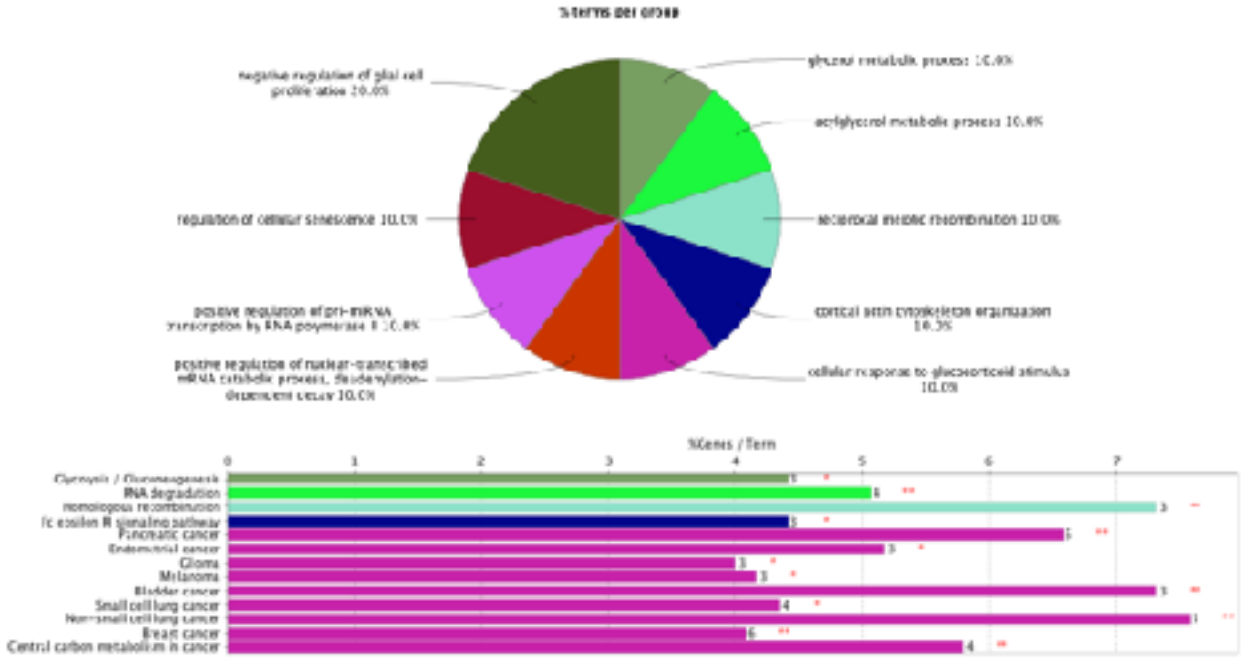
SPHK1 which targeted by hsa-mir-659(miRDB[100] target score:83, miRTarBase[25]: strongly evidence), is a master kinase conservative enzyme that modulates the balance between S1P and ceramide/sphingosine levels and is involved in a lot of cellular behaviors such as cell proliferation, cycle, migration, apoptosis, invasion, and metabolism. Experiment revealed SPHK1 expression was upregulated in the bladder cancer and was correlated with histologic gradeand tumor stage. Patients with high expression of SPHK1 had reduced overall 5-year survival rates, but there is no study demonstrate hsa-miR-659 regulate SPHK1 in bladder cancer cell[42,76].(Meng, Zhou et al. 2014)

3. hsa-mir-498:

There wasn't previous study revealed that hsa-mir-498 was related to bladder cancer, but there were other study have found hsa-mir-498 downregulate others tumor cell, like the expression of has-mir-498 was decreased in liver cancer cell lines compared to normal liver cell line. hsa-mir-498 overexpression markedly inhibited liver cancer cell proliferation, migration and invasion by targeting ZEB2 which inactivates the TGF-β/SMAD and Wnt/β-catenin pathways. hsa-mir-498 overexpression induced cell cycle arrest and apoptosis while it suppressed epithelial-mesenchymal transition (EMT) in liver cancer cells[23,69,73,79,83].(Gupta, Hau et al. 2016, Xia, Zeng et al. 2018, Yang, Liu et al. 2018, Zhuo, Li et al. 2018, Zhang, Xu et al. 2019)

To get a thorough understanding of the way hsa-mir-498 regulate bladder cancer, I use miRTarbase[25] chose target score>=0.6 target gene extended the target gene network, then preformed a functional enrichment by clueGO[5](cytoscape plug-in application), there were 9 groups GO Biological process and 13 KEGG pathways had been enriched.

Bladder Cancer



In GO biological pathway deadenylation dependent decay(p-value=7.4E-4) might involved in bladder tumor cell growth, migration[59](Shen, Wu et al. 2015). In KEGG pathway, besides the cancer pathway, the Homologous recombinations(p-value=8.5E-3) have long been known to play a role in the tumor development, progression[43](Mouw 2017).

Moreover, regarding the clinical outcome, BLCA Kaplan–Meier analysis revealed that the low expression group of hsa-mir-498 in the interval 25 months to 100 months which HR(hazard ratio)=0.77 show survival is better in the high expression group.

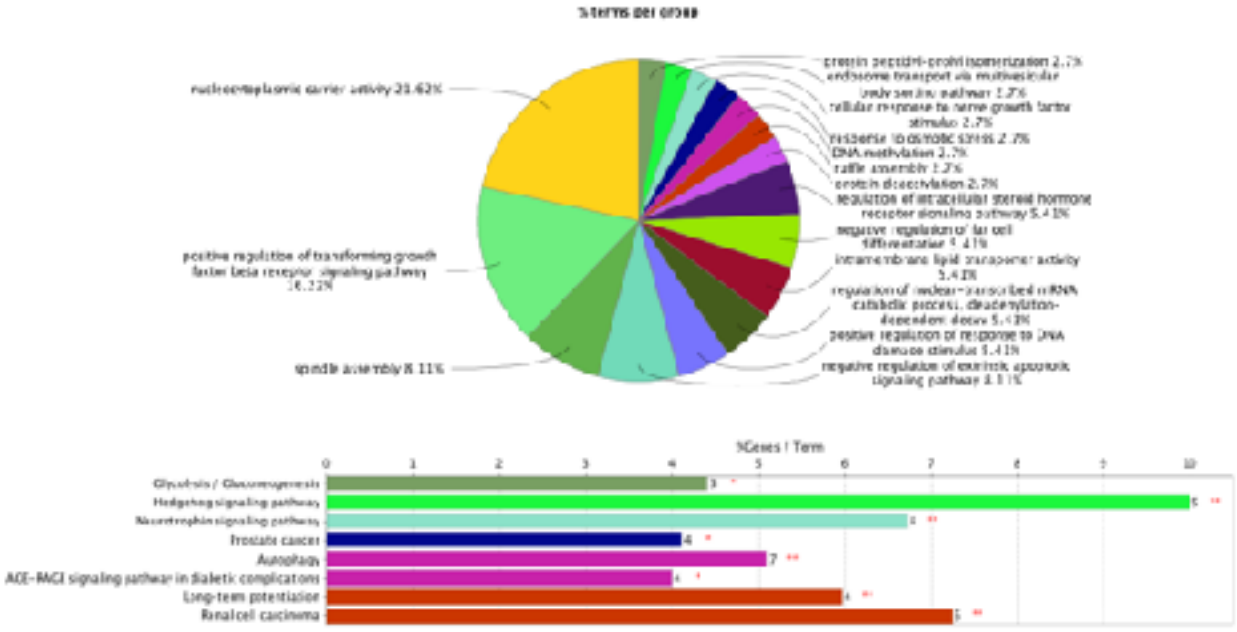
4. hsa-mir-181d:

Some studies have been found hsa-mir-181d was a tumor suppressor by targeting K-ras and Bcl2 in non-small cell lung cancer, ovarian cancer and glioblastoma cancer…. But there was no study show that hsa-mir-181d regulate bladder cancer[64,48]. (Wang, Shi et al. 2012, Pignot, Cizeron-Clairac et al. 2013)

Then, to find up the possibly function of hsa-miR-181d, I performed enrichment analyses(cytoscape clueGO[5]) to elucidate the biological function of target genes(miRTarbase[25] score>=0.6) of hsa-miR-181d, there are 16 groups GO biological process and 8 KEGG pathways were enriched.

Bladder Cancer

In GO biological process, Necleocytoplamic carrier activity(p-value=3.2E-4) might related to apoptosis, so the Necleocytoplamic carrier protein KPNA2 is over expressed in bladder cancer cell and inhibit apoptosis[39](Martinez-Olivera, Datsi et al. 2018). In KEGG pathway, besides cancer pathway, the cancer-restraining effect of Hedgehog pathway activity(p-value=2.6E-4) is associated with stromal expression of BMP signals, which stimulate urothelial differentiation. Progression is dramatically reduced by pharmacological activation of BMP pathway activity[60](Shin, Lim et al. 2014).



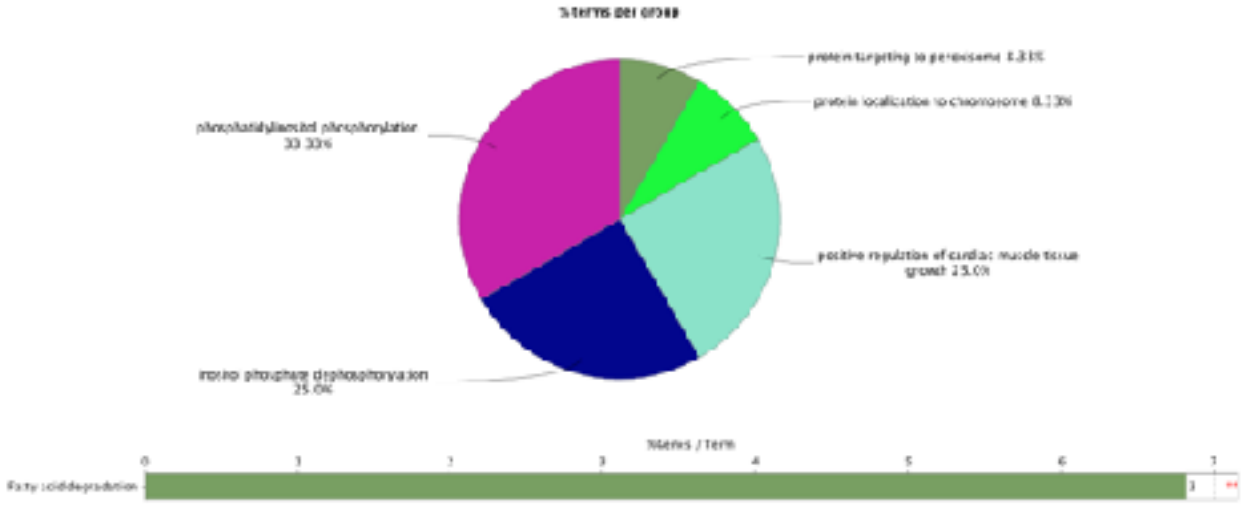
Furthermore, regarded the clinical outcome, BLCA Kaplan–Meier analysis revealed that expression of hsa-mir-181d was highly significant and downregulation the survival rate of BLCA(**p-value<.01). According to exist research and our study, I consider hsa-mir-181d can be see as new bladder cancer biomarker.

5. hsa-mir-3913:

There is a previous research about bladder cancer prognostic model, hsa-mir-3913 also been selected a prognostic feature in their predict model[46](Peng, Li et al. 2017).

On the wet-lab experimental side, there was no experiment proved that hsa-mir-3913 regulate bladder cancer. To check the role of hsa-mir-3913, I extended the hsa-mir-3913 target gene network via miRTarbase[25](score>=0.6) and performed enrichment

analyses by clueGO[5] to find out the biological processes/pathways which hsa-mir-3913 was involved, there are 5 groups GO biological process and 1 KEGG pathway were



enriched in the criteria of p-value<.05.

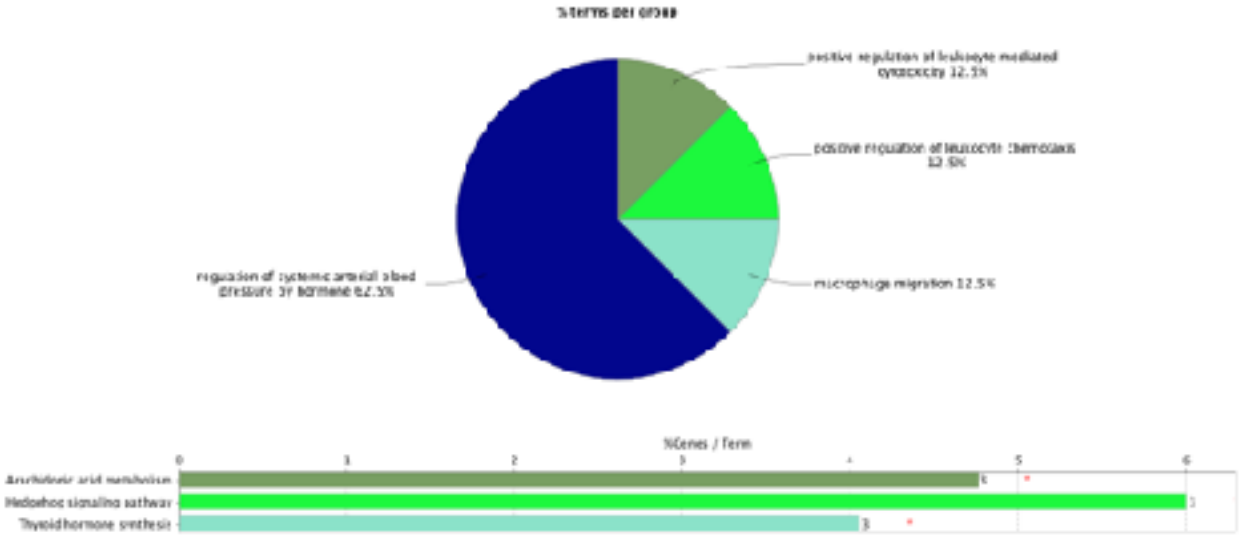
In GO biological process, Phosphatidylinositol phosphorylation(p-value=3.8E-3) is related to cell apoptosis, in bladder cancer is regulated by PI3K/Akt pathway[45](Oka, Tanimoto et al. 2006). In KEGG pathway, the fatty acid degradation(p-value=9.4E-3) can inhibit the malignant of bladder cancer[32](Li, Yao et al. 2019).

Moreover, regarding the clinical outcome, BLCA Kaplan–Meier analysis revealed that expression of hsa-mir-3913 was extremely significance and downregulate the survival rate of BLCA(***p-value<.001).

6. hsa-mir-4768:

According to the previous study about Ovarian cancer, their research found overexpression of hsa-miR-4768-5p reduces the level of Cyclin D1 by 20%, and the Cyclin D1 has lots of paper show it act a important role on tumor cell progression in many cancer type also include bladder cancer[1,56](Alao 2007, Seiler, Thalmann et al. 2014).

In the case of bladder cancer, the cell line between bladder cancer and ovarian cancer are not same, it need more experimental evidence to prove hsa-miR-4768—Cyclin D1 regulation whether happen in bladder cancer cell line(Karedath, Ahmed et al. 2019).



Because there wasn't any previous study found hsa-miR-4768 associated with bladder cancer, I use miRTarbase[25](target score>=0.6) to extend a hsa-miR-4768 target gene network and use Cytoscape plug-in application clueGO[5] perform a functional enrichment, there are 4 group GO biological process and 3 KEGG pathway were enriched.

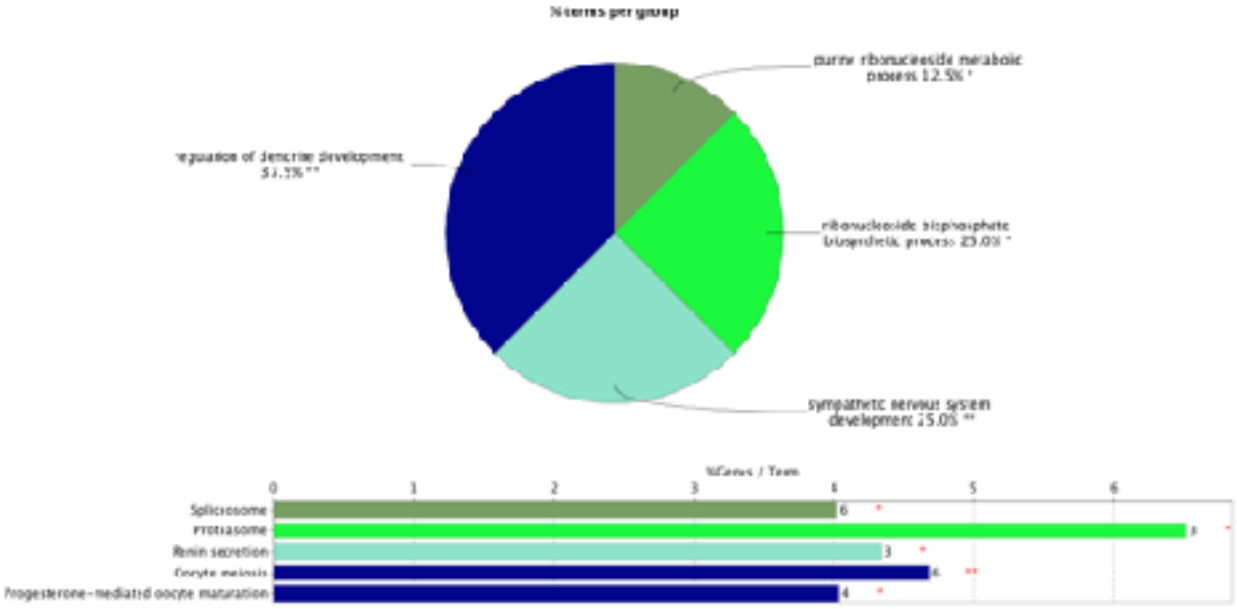
In GO biological process, leukocyte chemotaxis(p-value=6.0E-4) is the phenomenon by which the movement of cells is directed in response to an extracellular chemical gradient and it is involved in tumor cell migration[24,55](Hausman and Brosman 1976, Roussos, Condeelis et al. 2011). In KEGG pathway, the Hedgehog signaling pathway(p-value=1.4E-2) is involved in tumor cell migration and the Arachidonic acid metabolism(p-value=2.6E-2) would related to eicosanoids, which have been implicated in the pathogenesis of bladder cancer, and are considered important in tumor promotion, progression, and metastasis[41,6](Matsuyama and Yoshimura 2009, Biswas, Das et al. 2014). Furthermore, regarding the clinical outcome, BLCA Kaplan–Meier analysis revealed that expression of hsa-miR-4768 was significant and downregulate the overall survival rate of BLCA(*p-value<.05).

7. hsa-mir-548ba:

A study shows hsa-mir-548ba is negative factor for the survival of BLCA patients[23], but there isn't any experimental evidence demonstrated how hsa-mir-548ba regulate bladder cancer, to further realize hsa-mir-548ba, we can see hsa-

mir-548ba is under-expression in tumor cell, so it might downregulated in bladder tissue.

After that, we want to get the thorough understanding the way of hsa-mir-548ba regulate bladder cancer, I use miRDB[100] database extended the target gene(target score>=80) network, then preformed a functional enrichment by clueGO[5](cytoscape plug-in application), there were 4 groups GO biological processes and 5 KEGG pathway been enriched.



In GO biological process, the regulation of dendrite development(p-value=1.2E-3) is critical role of human body anti-tumor response, The dendritic-based strategies not only prevent cancer recurrence, but also reduce its progression and metastasis(Xiu, Ma et al. 2016). In KEGG pathway, the Oocyte meiosis(p-value=7.3E-3) was mainly enriched in others bladder cancer researches by different analysis strategy and have shown the fact that promoting the proliferation[34](Li, Liu et al. 2017).

Furthermore, regarding the clinical outcome, BLCA Kaplan–Meier analysis revealed that the survival is better in high hsa-mir-548ba expression group HR=0.77 and in the high group the survival rate are higher within the survival time 20 months to 90 months.

8. hsa-mir-10a:

There have been several investigation into hsa-miR-10a on bladder cancer, these studies have shown hsa-miR-10a can separate two genetically distinct bladder

tumor groups, which are, low-grade non-invasive pTa and high-grade invasive pT2-3 UC, hsa-miR-10a is over-expression in low-grade and under-expression in high-grade. According to the exist researches, hsa-miR-10a to be up-regulated in the majority of low-grade pTaUC act a FGFR3 regulator in 5637 cell line, FGFR3 stimulus to PI3K/AKT/ STAT pathway and low-grade non-invasive tumor development.

In high-grade invasive pT2-3 UC(EJ and 253j cell lines), hsa-miR-10a is under-expression and the expression levels of ITGB1(lncRNAs) and hsa-miR-10a were negatively correlated. ITGB1 may promote the progression of BLCA.

ITGB1 expression was remarkably upregulated in BLCA tissue, which was related to the pathological stage of Bladder cancer. ITGB1 might promote BLCA cell proliferation via regulating hsa-miR-10a. Notably, hsa-miR-10a was can inhibit the regulatory effect of ITGB1 on the proliferation of BLCA cells[31,16,17,18,15](Köhler, Bryk et al. 2013, Dip 2014, Dip, Reis et al. 2014, Dip, Reis et al. 2018, Dai, Chai et al. 2019).

9. hsa-mir-424:

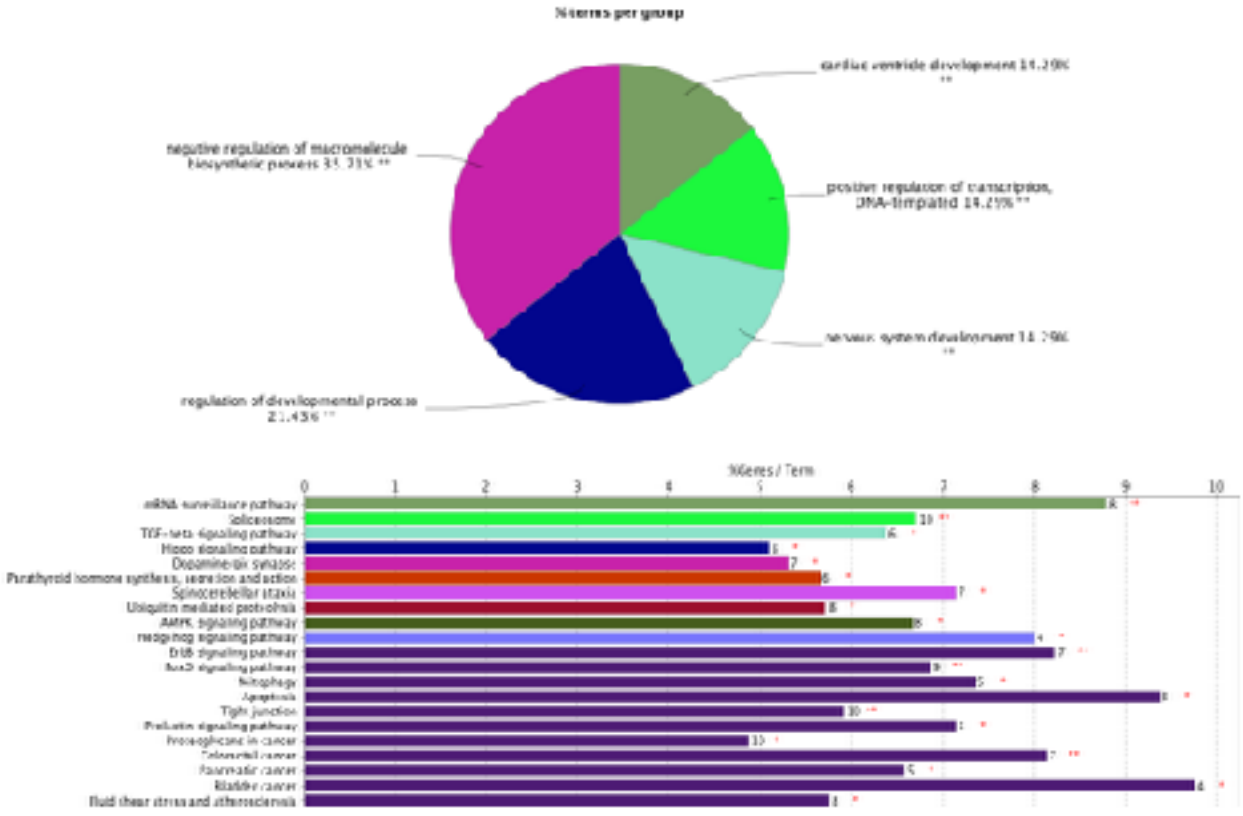
According a study, it shows the lack of hsa-miR-424 expression is significantly linked to aggressive bladder tumor growth, that is to say increased hsa-miR-424 suppressed the tumor growth rate and invasion ability. The EMT is a key event in the invasion process, EMT may induce a number of invasion-related factors, including VEGF and MMP-9. As determined through IHC analysis, the hsa-miR-424 expression vector resulted in lower expression levels of VEGF and MMP-9.

Another role of hsa-mir-424[66] (Wu, Lin et al. 2015). Urothelial cancer cell lines, including HT1376 and HT1197, overexpress EGFR, the EGFR pathway plays a role in the transmission of the hsa-miR-424 signal that regulates cell growth and the epithelial-to-mesenchymal transition. The hsa-miR-424 expression vector decreased the levels of EGFR and the major downstream mediators, including p-EGFR and p-AKT. Moreover, a significant negative correlation was observed in the clinical specimens between EGFR and hsa-miR-424, and EGFR-PI3K-AKT pathway may be the mechanism responsible for the aggressive tumor behavior in DNMT1-positive bladder cancer.

In summary, that increased hsa-miR-424 production plays an important tumor-suppressive role in bladder cancer.

10. hsa-mir-1283:

There were no previous experimental evidence about the regulation of hsa-mir-1283 in bladder cancer, yet there was a research indicated hsa-mir-1283 act a tumor suppressor by targeting ATF4 in the gliomas, they found the level of hsa-mir-1283 was dramatically decreased in glioma tissues and its cell lines, the expression of ATF4 was significantly increased, and the low level of hsa-miR-1283 was closely associated with high expression of ATF4 in glioma tissues. In summary hsa-miR-1283 significantly inhibited proliferation and invasion of glioma cells[11](Chen, Zhang et al. 2019). Then, to get a thorough understanding of the way hsa-mir-1283 regulate bladder cancer, I use miRDB[100] database extended the target gene(target score>=80) network, then preformed a functional enrichment, there were 10 groups GO biological processes and 11 groups KEGG pathway been enriched.



In GO biological process, can not finding the previous study related to these term. In KEGG pathway, lots of cancer pathway been enriched include bladder cancer, ErbB signaling pathway(p-value=5.1E-3) also a significance pathway, it is driven growth pathway has been implicated in human epithelial malignancies(Amsellem-Ouazana, Bièche et al. 2006). Finally, regarding to the clinical data, BLCA Kaplan–Meier analysis

revealed that downregulation of hsa-miR-1283 was significantly associated with the survival rate of BLCA(*p-value<.05).

Table(2)

Functional annotation of unknown function miRNA

miRNA	GO/ KEGG	Term	P-value adjusted with Bonferroni step down	Relationship of term and Bladder cancer
hsa-mir-498	GO	negative regulation of glial cell proliferation	5.2E-04	
		positive regulation of nuclear-transcribed mRNA catabolic process	7.4E-04	#49
	KEGG	Non-small cell lung cancer	5.7E-04	#cancer
		Homologous recombination	8.5E-03	#43
hsa-mir-181d	GO	nucleocytoplasmic carrier activity	3.2E-04	#39
		regulation of nuclear-transcribed mRNA catabolic process	5.4E-05	#49
	KEGG	Neurotrophin signaling pathway	6.5E-05	#14
		Hedgehog signaling pathway	2.6E-04	#60
hsa-mir-3913	GO	phosphatidylinositol phosphorylation	3.8E-03	#45
		inositol phosphate dephosphorylation	7.5E-04	#3
	KEGG	Fatty acid degradation	9.4E-03	#32
hsa-mir-4768	GO	regulation of systemic arterial blood pressure by hormone	1.2E-03	
		positive regulation of leukocyte chemotaxis	6.0E-04	#55,24
	KEGG	Hedgehog signaling pathway	1.4E-02	#60
		Arachidonic acid metabolism	2.6E-02	#6,41
hsa-mir-548ba	GO	regulation of dendrite development	1.2E-03	#71
		sympathetic nervous system development	1.2E-02	#71
	KEGG	Oocyte meiosis	7.3E-03	#34, 85
		Spliceosome	1.2E-02	#50
hsa-mir-1283	GO	Nervous system development	1.4E-05	
		Cardiac ventricle development	2.5E-05	

Bladder Cancer

miRNA	GO/ KEGG	Term	P-value adjusted with Bonferroni step down	Relationship of term and Bladder cancer
	KEGG	mRNA surveillance pathway	1.8E-03	#84
		ErbB signaling pathway	5.1E-03	#2

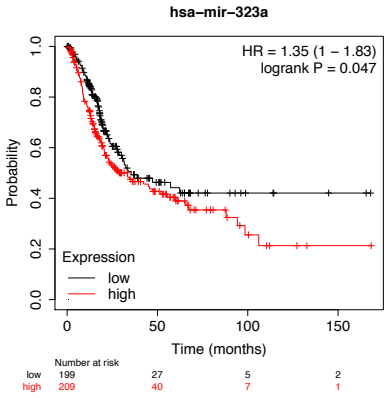
Table(3)

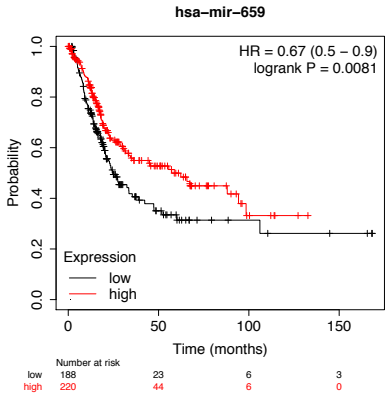
Kaplan-Meier analysis results

Top10 miRNA Biomarker	Low-High expression group BLCA Survival curve log rank test p-value	Low/High expression in lower survival probability group	OcomiRs / Tumor suppressor	Reference
hsa-mir-323a	**0.047**	**high**	**Tumor suppressor**	**#33**
hsa-mir-659	**0.0081**	**low**	**Tumor suppressor**	**#51**
hsa-mir-498	0.076	**low**	**-**	
hsa-mir-181d	**0.0059**	**low**	**-**	
hsa-mir-3913	**0.00034**	**low**	**Tumor suppressor**	**#46**
hsa-mir-4768	**0.017**	**low**	**-**	
hsa-mir-548ba	0.053	**low**	**-**	
hsa-mir-10a	0.088	**high low**	**OcomiR(5637) Tumor suppressor(EJ and 253j)**	**#31,15-18**
hsa-mir-424	0.29	**low**	**Tumor suppressor**	**#66**
hsa-mir-1283	**0.047**	**low**	**-**	

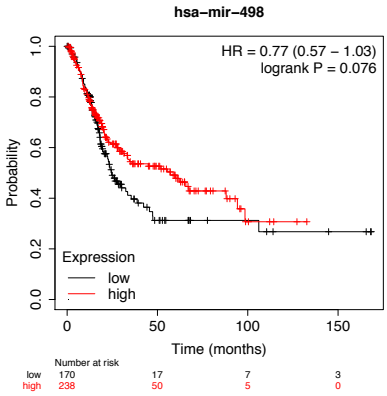
red : Log rank test *p-value<.05

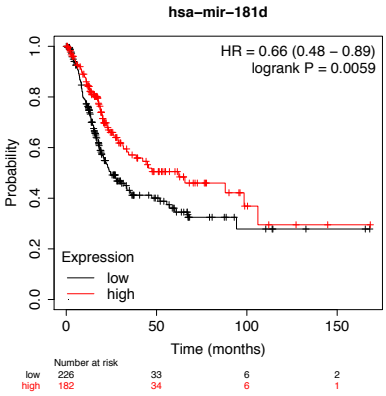
Analysis by Kmplot[44]

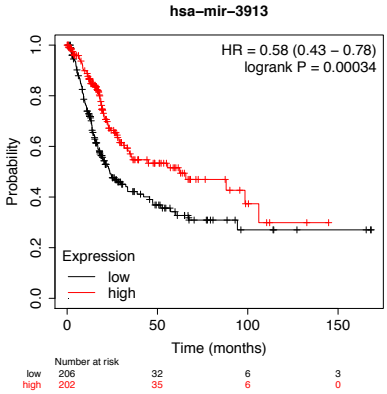


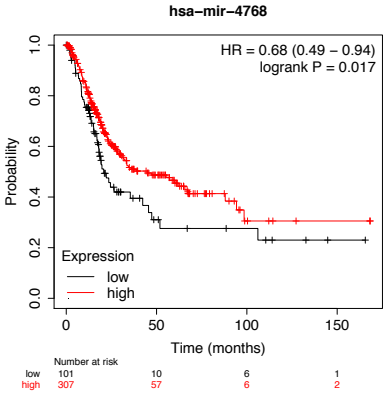


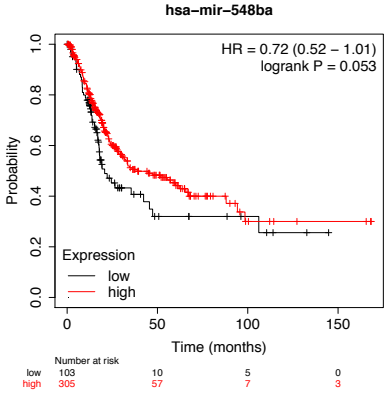
Bladder Cancer

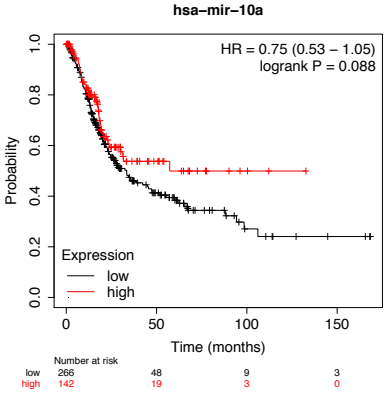


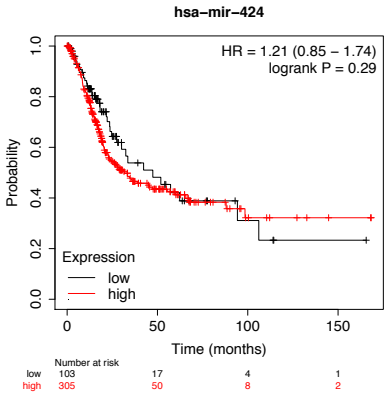


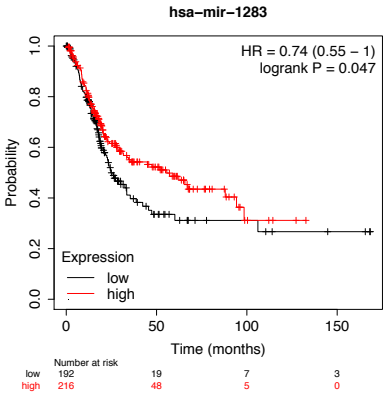




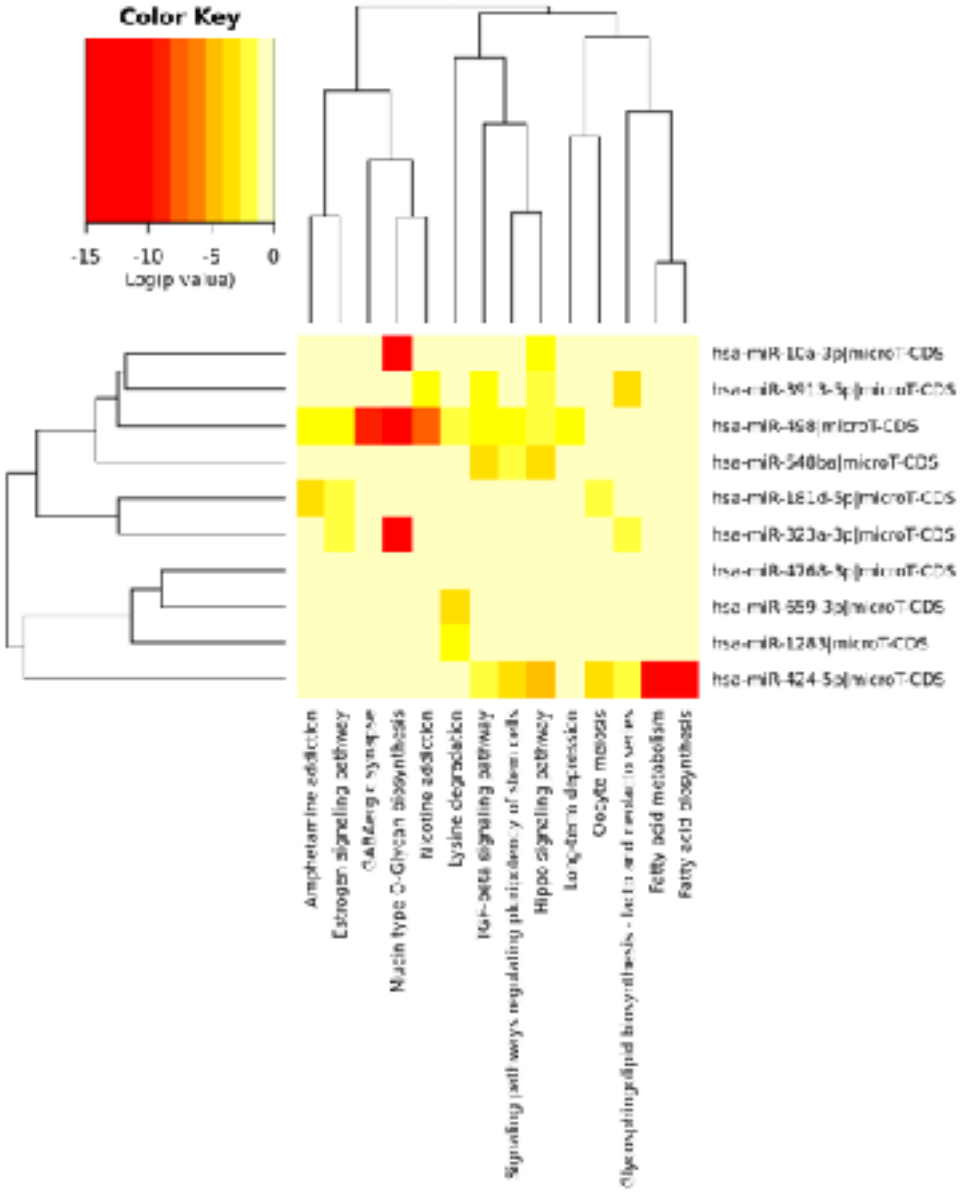








- **Biological significance of the top-ranked miRNAs**
  - **Heapmap of KEGG pathway**

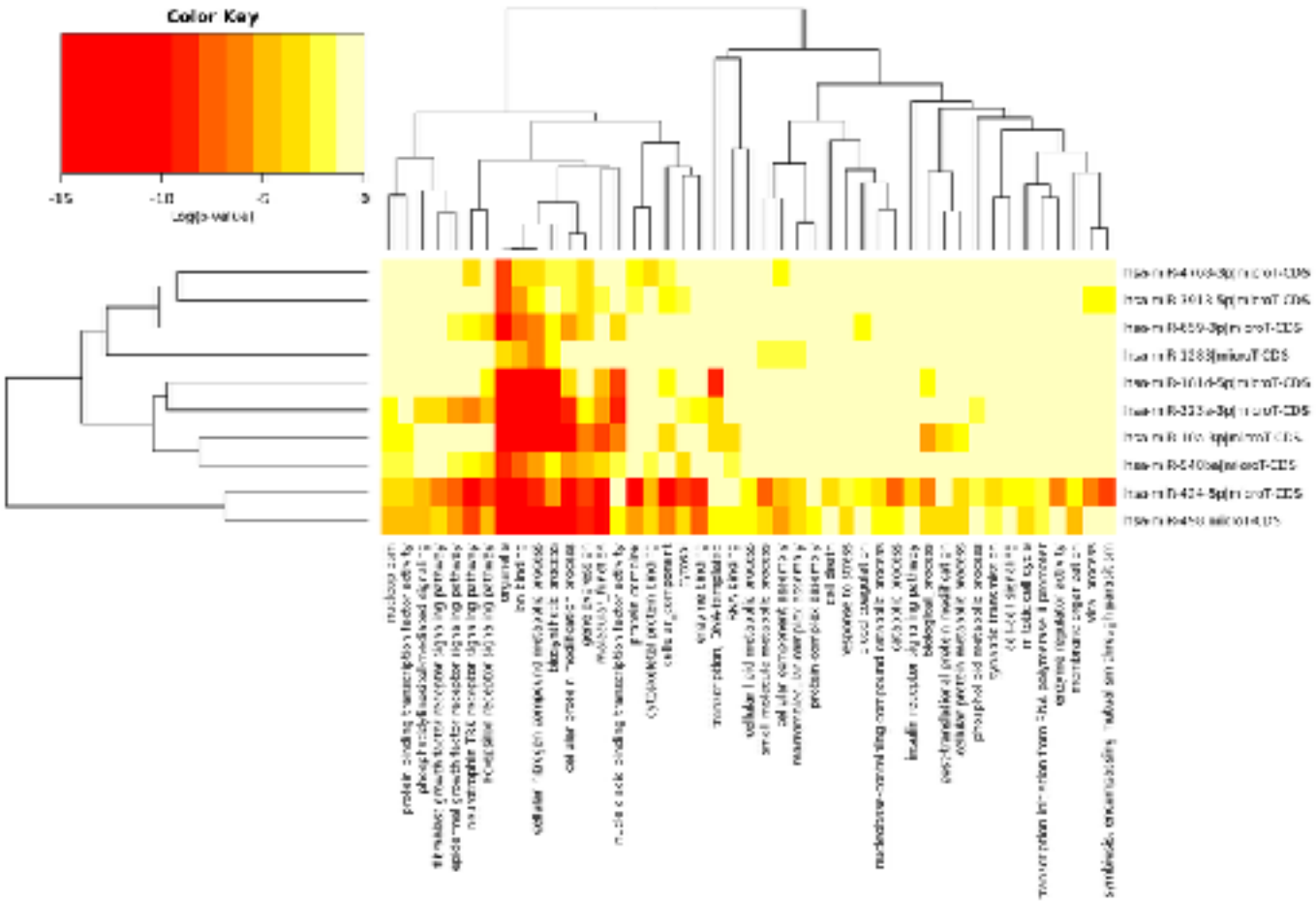


In the miRPath[62](Vlachos, Zagganas et al. 2015) miRNA target gene KEGG heat map, there were 4 pathway annotated most significance(p-value<1E-10), which are Fatty acid biosynthesis, Fatty acid metabolism(hsa-miR-424), Mucin type O-Glycan biosynthesis(hsa-miR-323a, hsa-miR-498, hsa-miR-10a) and GABAergic synapse(hsa-miR-498) and we also found some studies that shown the relationship between the term and Bladder cancer.

Bladder Cancer

A study indicated that blocking the Fatty acid biosynthesis can inhibit the malignant of bladder cancer[32](Li, Yao et al. 2019). We also found Mucin type O-Glycan biosynthesis was related to Bladder tumor metastasis, Glycans play an important role in cell-to-cell interactions and signal transduction. Glycans bind to proteins, lipids, and other glycosyl groups through glycosylation, As a key regulatory mechanism, glycosylation regulates the physiological, and pathological processes of some cells. (Brockhausen 1999, Quidville, Alsafadi et al. 2013, Gao, Chen et al. 2018, Jian, Xu et al. 2020). Final, GABAergic synapse pathway was been mentioned in another study which were also bioinformatic analysis based on DEG and core modules analysis in the PPI network, they suggests hypermethylated/low-expression genes related to GABAergic synapse might play an important role in BC development[80](Zhang, Fang et al. 2018).

2. Heapmap of GO gene ontology



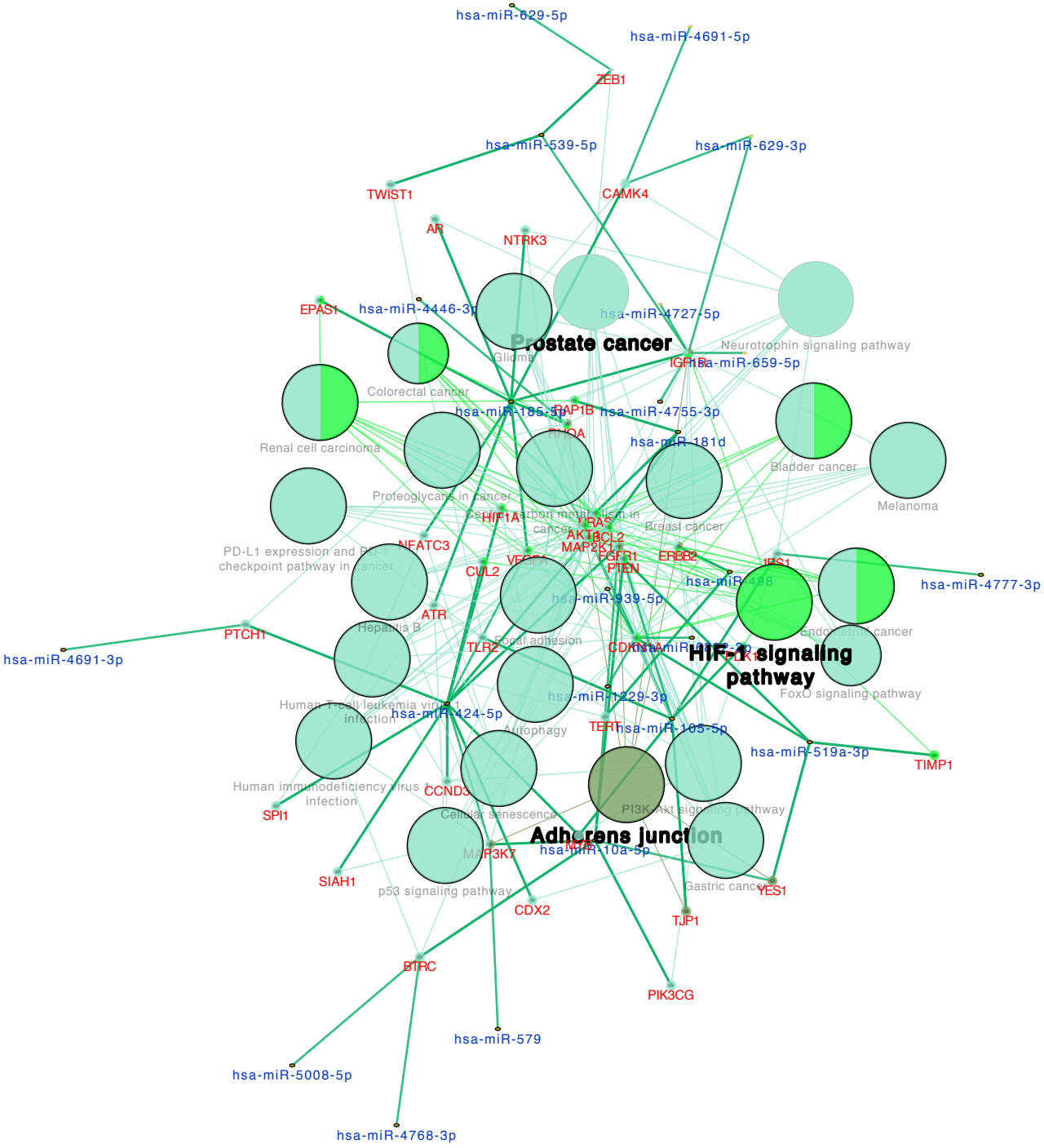
Bladder Cancer

Secondly, we employed a miRPath[62] GO annotation for the top10 miRNA signature. GO annotation results showed that almost all top10 miRNAs are highly enriched in specific molecular functions, biological processes and cell components, such as nucleic acid binding transcription factor activity, Fc-epsilon receptor signaling pathway, neurotrophin TRK receptor signaling pathway, gene expression, cellular protein modification process, cellular nitrogen compound metabolic process, ion binding, organelle, protein complex, enzyme binding, epidermal growth factor receptor signaling pathway, transcription, DNA-templated, fibroblast growth factor receptor signaling pathway, small molecule metabolic process, cellular component assembly, cytoskeletal protein binding, phosphatidylinositol-mediated signaling, protein binding transcription factor activity and symbiosis, encompassing mutualism through parasitism. There are top20 enriched GO term all P-value<1E-6.

- **All 33 miRNAs features target gene network functional enrichment**

First, Use all 33 miRNAs built a target gene network(miRTarbase[25]), the target gene selected criteria was top100 in whole miRNAs target genes ranked by validated score. Second, perform a functional enrichment(KEGG) and labeling the gene which are shared with pathway and miRNA. Finding out the important miRNAs target genes in this bladder cancer miRNA signature. As the enrichment result, Adherens junction, HIF-1 signaling pathway, Colorectal cancer, Renal cell carcinoma, Endometrial cancer, Prostate cancer, Central carbon metabolism in cancer, Proteoglycans in cancer, PI3K-Akt signaling pathway have been enriched and it is reveals that the intervention of one miRNA may have complex effects on cancer-related genes. To further validate whether the expression levels of potential target genes in bladder cancer patient are significance difference to normal sample, we did analysis using Ualcan databases[101]. After being compared with normal samples, the target gene ERBB2, TERT, HRAS, BCL2, FGFR1, PTEN, YES1, CDX2, FGFR1, MYB, MAP2K1, PTCH1, SIAH1, VEGFA, CCND3, CDKN1A, IRS1, PDK1, TJP1, TLR2, ZEB1, TWIST1, PTCH1, AR, NTRK3, ATR, VEGFA, TIMP1 are statistically significant(*p-value<0.05) in normal sample and bladder tumor sample, we considered these target genes in all the annotated pathways which the expression are statistically significant in normal samples and tumor samples can identified as candidate target genes for further analysis.

Bladder Cancer



Table(4)

The shared gene between KEGG pathway and miRNA target gene which the expression are statistical significance in normal sample and bladder tumor sample

miRNA	**Gene *p-value<0.05**
hsa-miR-498	ERBB2, TERT
hsa-miR-181d	HRAS#52, BCL2
hsa-miR-10a	FGFR1, PTEN, YES1
hsa-miR-424	CDX2, FGFR1, MYB, MAP2K1, PTCH1, SIAH1, VEGFA, CCND3
hsa-miR-939	CDKN1A
hsa-miR-105	IRS1, MYB, PDK1, PTEN, TJP1, TLR2
hsa-miR-539	ZEB1, TWIST1
hsa-miR-4777	IRS1
hsa-miR-4691	PTCH1
hsa-miR-1229	CDKN1A
hsa-miR-185	AR, NTRK3, ATR, VEGFA
hsa-miR-6882	CDKN1A
hsa-miR-519a	CDKN1A, PTEN, TIMP1, YES1

Conclusions

The dysregulated miRNAs have been considered as a new terms of “ocomiRs” or “tumor suppressor”, it playing critical roles in cancer initiation and progression. Hence, miRNAs represent a novel feature to help in the development of prediction, managing, and treating agents to improve low survival rates. The therapeutic potential of miRNAs can be implicated after investigating downstream regulatory mechanisms observed among different molecular pathways.

We devote to find out the important “ocomiRs” or “tumor suppressor” in bladder cancer based on the miRNA expression profiles and patients survival time and then we first proposed a support vector regression based method cooperated with an optimal feature selection algorithm IBCGA(SVR-IBCGA) to identify the miRNA signature associated with survival time and we also first constructed the SVR survival time predictor in bladder cancer study.

As SVR-IBCGA result, it identified 33 most informative miRNAs which are strongly correlated with the survival time of bladder cancer patient. Then, we analysis the role of the top-10 miRNA ranked by the MED value. Among the top-10 miRNA signatures hsa-mir-498, hsa-mir-181d, hsa-mir-4768, hsa-mir-548ba and hsa-mir-1283 were previously unreported for the involvement with bladder cancer. As the target gene functional enrichment results, it revealed their functional role in bladder cancer and others major cancer types. Through the functional enrichment and Kaplan-Meier analysis we can say these miRNAs might be new Bladder cancer biomarkers and provide a new insight into molecular therapeutic approaches to improving the therapies of bladder cancer patients.

Besides, the performance of the survival estimation model: correlation coefficient of 0.86 and mean absolute error of 0.56 year using 10-fold cross validation. We consider this estimate model can be useful for diagnosis, prognosis and monitoring the efficacy of treatment. In summary, our study not only identifying the bladder cancer biomarker, but also contracted a new survival estimate model for cancer diagnosis.

We hope that the identified miRNA signature will contribute to further discovering their pathway mechanism in bladder cancer and developing the new therapeutic strategies for the treatment of bladder cancer patients in the future, the restoration of these miRNAs levels to that of healthy tissue could therefore be beneficial in maintaining the endogenous anti-tumour regulatory mechanisms.

Materials and Methods

Dataset

We download the bladder cancer miRNA expression quantification and clinical data from TCGA portal, there were 412 patients in TCGA-BLCA project, the data category is transcriptome profiling, data type is miRNA quantification, the type of analysis workflow is BCGSC miRNA Profiling, the miRNA profiling was implemented on the Illumina HiSeq. 2000 miRNA sequencing platform. Then, we filtered some datas use following criteria. We deleted the patients with a survival period of less than one month and delete the miRNA label which the counts of expression quantification read-count=0 higher than 75% of all patients. Final, we merged the remaining miRNA quantification

and clinical data into a single dataset. As a result, there were 179 patients with expression profiles of 884 miRNAs.

Method

SVR-IBCGA

SVR-IBCGA which is a an integrated method combining support vector regression(v-SVR) and feature selection algorithm IBCGA(Inheritable bi-objective genetic algorithm), it is designed to identify a set of informative miRNA signature based on the its expression correlation with the survival time and also estimate the bladder cancer patient survival time.

Support vector regression

Support vector machine (SVM) is a state-of-the-art method for solving classification and regression problems. SVM has extensively been used in solving biological problems[87]. SVR is one of type of SVM. Because of the regression ability, SVR also been used in biological problems, such as estimation of survival time in glioblastoma cancer patients[88], The v-SVR is a new regression method of SVM which presents good performance depending on the number of support vectors and training error[89]. Given a set of *N* data points, $\{(x_1, y_1), (x_2, y_2), \ldots, (x_N, y_N)\}$, where $x_i \in R^m$ is an input sample (patient) and $y_i \in R_1$ is a target label. In this study, y_i is the survival time. The optimization problem of the v-SVR can be described as follows.

$$min\ \frac{1}{2}w^T w + b + C(\nu\varepsilon + \frac{1}{N}\sum_{i=1}^{N}(\xi_i + \xi_i^*)) \qquad (1)$$

Subject to

$$(w^T \varnothing(x_i) + b) - y_i \leq \varepsilon + \xi_i, \qquad (2)$$

$$y_i - (w^T \varnothing(x_i) + b) \leq \varepsilon + \xi_i^* \quad (3)$$

$$\xi_i, \xi_i^* \geq 0,\ i = 1, \ldots . N, \varepsilon \geq 0 \quad (4)$$

where $0 \leq v \leq 1$. C is a regularization parameter and b is a constant. The ε-insensitive loss function means that if $w\ T\varnothing(xi)$ is in the range of y±ε, no loss is considered. The y^T is known as the soft margin where v is an upper bound on the fraction of margin errors and a lower bound of the fraction of support vectors.

Fitness function

The fitness function of the IBCGA is the only guide to search for an optimal solution. In this study, the fitness function is to maximize the Pearson's correlation coefficient (*CC*) of 10-CV as follows:

$$max\ CC = \frac{\sum_{i=1}^{M} \left(y_i - \overline{y}\right)\ \left(z_i - \overline{z}\right)}{\sqrt{\left[\sum_{i=1}^{M} \left(y_i - \overline{y}\right)^2\right]\ \left[\sum_{i=1}^{M} \left(z_i - \overline{z}\right)^2\right]}} \quad (5)$$

where y_i and z_i are real and predicted survival time of the i^{th} patient, and $\bar{y}$ and $\bar{z}$ are their corresponding means. *M* is the total number of patients (*M*=179 in this study). The mean absolute error (*MAE*) is also used for measuring prediction performance:

$$MAE = \frac{1}{M} \sum_{i=1}^{M} \left| y_i - z_i \right|^2 \quad (6)$$

Inheritable bi-objective combinatorial genetic algorithm

SVR-IBCGA used the optimal feature selection method IBCGA to identify a small set of m informative miRNAs from n=884 miRNAs cooperating with v-SVR by maximizing estimation accuracy of survival time. The IBCGA uses an intelligent evolutionary algorithm[90] for solving the large combinatorial optimization problem C(n, m) to obtain an optimized v-SVR where n is a given large constant and the best value of the variable m is not known beforehand. The intelligent evolutionary algorithm uses an orthogonal array crossover with a systematic reasoning ability to reproduce better offspring instead of random recombination in the crossover operation. The intelligent evolutionary algorithm can obtain a good solution $S\,k$ to C(n, k) if k is a given constant. The IBCGA can obtain a set of solutions, Sr, where $r=r_{\text{start}}, r_{\text{start}+1}, \ldots, r_{\text{end}}$ in a single run to efficiently search for a solution S_{r+1} to C(n, $r+1$) by inheriting a good solution S_r to C(n, r). The S_m is the best solution among the solutions S_r. In this work, the LibSVM package[91] was used for implementation of v-SVR.

The chromosome of the IBCGA consists of 884 genes for encoding the 884 miRNAs and three 4-bit genes for encoding the three variables γ, C, andvof thev-SVR. The parameter tuning of IBCGA was same with the previous study[92,93]. The customized IBCGA for obtaining the m-miRNA signature where $r_{\text{start}} \le m \le r_{\text{end}}$ is described below.

Step 1) (Initialization) Randomly generate an initial population with $N\,pop$ individuals. Each individual has $r_{1's}$ and n-$r_{0's}$ encoded into the n binary genes fi, where $r=r_{\text{start}}$.

Step 2) (Evaluation) Evaluate all individuals in the population using the fitness function.

Step 3) (Selection) Use a tournament selection method that selects the winner from two randomly selected individuals to form a mating pool.

Step 4) (Crossover) Select $P\,c \cdot N\,pop$ parents from the mating pool to perform the orthogonal array crossover[90], where $P\,c$ is the crossover probability.

Step 5) (Mutation) A traditional mutation operator is applied to the randomly selected $P\,m \cdot N\,pop$ individuals except the best individual, where $P\,m$ is the mutation probability.

Step 6) (Termination) If the stopping condition of performing G_{max} generations is satisfied, output the best individual in the population as S_r. Otherwise, go to Step 2.

Step 7) (Inheritance) If $r<r_{end}$, randomly change one bit in the binary genes f_i from 0 to 1 for each individual; increase the number r by one, and go to Step 2. Otherwise, stop the algorithm.

Step 8) (Output) Let m be equal to the value of r that S_r is the best solution in the population. Output the m miRNAs and the corresponding v-SVR model.

Appearance score

Since the IBCGA is a non-deterministic algorithm that the solutions of multiple runs are not always the same, selection of a robust solution is necessary. SVR-IBCGA automatically identifies a robust solution (miRNA signature) from R (R=30 in this study) independent runs for estimating the survival time of patients with bladder cancer. The robust set of features (miRNAs) has the highest appearance score obtained using the following procedure.

Step 1: Prepare the training dataset for 10-CV.

Step 2: Perform R independent runs of SVR-IBCGA by maximizing CC of 10-CV for obtaining R miRNA signatures. There are mt features in the t-th signatures, t=1, …, R.

Step 3: Appearance score is calculated as follows:

1. Calculate the appearance frequency f(p) for each feature p that ever presents in the R sets of miRNAs.

2. Calculate the score S_t, t=1, …, R where p_i is the i-th feature in the t-th solution:

$$S_t = \sum_{i=1}^{m_t} f(p_i)/m_t \qquad (7)$$

Step 4: Output the t-th feature set with the highest appearance score S_t.

Multiple regression analysis

We employed the Multiple linear regression method[94] to estimate the survival time in lung adenocarcinoma patients. The Multiple linear regression method is formulated as

$$yi = \beta 0 + \beta 1 xi1 + \beta 2 xi2 + \cdots + \beta m xim + \varepsilon, \qquad (8)$$

where *y i* is a dependent variable (survival time of the *i*-th patient in this study); *xi1*, *xi2*, …, and *xim* are independent variables (miRNA expression); *β0* is a regression constant; *β1 , β2*, *…*, and *βm* are the regression coefficients; *m* is the number of terms in the model, and ε is the error term. In this study, *m* is the number of selected miRNAs. A stepwise feature addition method was used for feature selection[95].

Elastic net

Elastic net is a regularization with an automatic feature selection technique[96], which is a combination of ridge regression[97] and least absolute shrinkage and selection operator (LASSO)[98]. The objective function of the Elastic net method using 10-CV is formulated as follows:

$$Min_{\beta_0,\beta} \left(\frac{1}{2M} \sum_{i=1}^{M} (y_i - \beta_0 - x_i^T \beta)^2 + \lambda P_\alpha(\beta) \right) \qquad (9)$$

where *y* i is the sample response (survival time) at observation *i* (patient); $x_i \in R^m$ is the vector of *m* miRNA expression values for the *i*-th observation, λ is a regularization parameter, *β0* and *β* are regression coefficients, and *M* is the total number of observations.

KEGG pathway and Gene ontology annotation analysis

I used the miRTarbase[25], miRDB[100], microT-CDS miRNA target gene prediction database extended miRNA target gene network, then performed functional enrichment(GO Biological process, KEGG Pathway) to see the significance pathway

that miRNA involved[53](Rivals, Personnaz et al. 2007) and shown its biological significance in cancer and cancer related pathways. The functional enrichment was use one-sided hypergeometric test to annotate significance(p-value<0.05) pathway. In the part of GO term enrichment analysis. I adopted the GO fusion[5](Bindea, Mlecnik et al. 2009)algorithm to chose representative GO biological process via the hierarchical relationship of GO term and I also adopted GO grouping[5](Bindea, Mlecnik et al. 2009) algorithm which was considering the shared gene between each term, that will group the functional groups via their Cohen's kappa coefficient which is define by term-term interrelation and functional groups calculated by shared gene between the term. To know the role of miRNAs, I make the pie chart for each miRNA which shows the percentage of GO biological processes category in whole annotated GO term, let we can understand the main role that miRNA acted in human body, I also listed most significance annotated processes and pathways in the Table(2) and listed the relationship of annotated term and bladder cancer. (The significance threshold was set at .05 is all that is required.)

Kaplan-Meier survival curve analysis

Kaplan-Meier[44](Nagy, Lánczky et al. 2018) is a non-parametric static used to estimate the survival function from survival time, we adopted Kaplan-Meier method to analysis the relationship of survival probability-miRNA expression, the miRNAs and clinical data were also from TCGA database, as the Kaplan-Meier curve, we can validate whether the identified miRNAs are survival biomarker in bladder cancer. Besides observing the Kaplan-Meier curve, we usually use Log-Rank Test to compare two expression groups it is a test of statistical significance, if the Log-Rank<0.05 we said the the difference in survival between the Low and High group was statically significant.(Table(3))

Reference

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Supplementary analysis:

Table(5)

Top-10 miRNA target gene functional enrichment analysis result

KEGGID	KEGGTerm	Term PValue	Nr. Genes	Associated Genes Found
KEGG:04520	Adherens junction	4.55E-07	7	[ERBB2, FGFR1, IGF1R, MAP3K7, RHOA, TJP1, YES1]
KEGG:04066	HIF-1 signaling pathway	1.27E-10	11	[AKT1, BCL2, CDKN1A, CUL2, ERBB2, HIF1A, IGF1R, MAP2K1, PDK1, TIMP1, VEGFA]
KEGG:05210	Colorectal cancer	2.42E-05	6	[AKT1, BCL2, CDKN1A, HRAS, MAP2K1, RHOA]
KEGG:05211	Renal cell carcinoma	6.98E-10	9	[AKT1, CDKN1A, CUL2, EPAS1, HIF1A, HRAS, MAP2K1, RAP1B, VEGFA]
KEGG:05213	Endometrial cancer	2.41E-06	6	[AKT1, CDKN1A, ERBB2, HRAS, MAP2K1, PTEN]
KEGG:05219	Bladder cancer	7.81E-06	5	[CDKN1A, ERBB2, HRAS, MAP2K1, VEGFA]
KEGG:04068	FoxO signaling pathway	2.78E-05	7	[AKT1, CDKN1A, HRAS, IGF1R, IRS1, MAP2K1, PTEN]
KEGG:04115	p53 signaling pathway	8.66E-06	6	[ATR, BCL2, CCND3, CDKN1A, PTEN, SIAH1]

Bladder Cancer

KEGGID	KEGGTerm	Term PValue	Nr. Genes	Associated Genes Found
KEGG:04140	Autophagy	3.10E-07	9	[AKT1, BCL2, HIF1A, HRAS, IGF1R, IRS1, MAP2K1, MAP3K7, PTEN]
KEGG:04151	PI3K-Akt signaling pathway	8.26E-09	15	[AKT1, BCL2, CCND3, CDKN1A, ERBB2, FGFR1, HRAS, IGF1R, IRS1, MAP2K1, MYB, PIK3CG, PTEN, TLR2, VEGFA]
KEGG:04218	Cellular senescence	1.15E-06	9	[AKT1, ATR, BTRC, CCND3, CDKN1A, HRAS, MAP2K1, NFATC3, PTEN]
KEGG:04510	Focal adhesion	7.63E-08	11	[AKT1, BCL2, CCND3, ERBB2, HRAS, IGF1R, MAP2K1, PTEN, RAP1B, RHOA, VEGFA]
KEGG:04722	Neurotrophin signaling pathway	9.18E-08	9	[AKT1, BCL2, CAMK4, HRAS, IRS1, MAP2K1, NTRK3, RAP1B, RHOA]
KEGG:05161	Hepatitis B	1.26E-05	8	[AKT1, BCL2, CDKN1A, HRAS, MAP2K1, MAP3K7, NFATC3, TLR2]
KEGG:05166	Human T-cell leukemia virus 1 infection	1.86E-06	10	[AKT1, ATR, CCND3, CDKN1A, HRAS, MAP2K1, NFATC3, PTEN, SPI1, TERT]
KEGG:05170	Human immunodeficiency virus 1 infection	1.17E-05	9	[AKT1, ATR, BCL2, BTRC, HRAS, MAP2K1, MAP3K7, NFATC3, TLR2]
KEGG:05205	Proteoglycans in cancer	7.63E-10	13	[AKT1, CDKN1A, ERBB2, FGFR1, HIF1A, HRAS, IGF1R, MAP2K1, PTCH1, RHOA, TLR2, TWIST1, VEGFA]
KEGG:05210	Colorectal cancer	2.42E-05	6	[AKT1, BCL2, CDKN1A, HRAS, MAP2K1, RHOA]
KEGG:05211	Renal cell carcinoma	6.98E-10	9	[AKT1, CDKN1A, CUL2, EPAS1, HIF1A, HRAS, MAP2K1, RAP1B, VEGFA]
KEGG:05213	Endometrial cancer	2.41E-06	6	[AKT1, CDKN1A, ERBB2, HRAS, MAP2K1, PTEN]

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KEGGID	KEGGTerm	Term PValue	Nr. Genes	Associated Genes Found
KEGG:05214	Glioma	6.65E-07	7	[AKT1, CAMK4, CDKN1A, HRAS, IGF1R, MAP2K1, PTEN]
KEGG:05215	Prostate cancer	3.49E-11	11	[AKT1, AR, BCL2, CDKN1A, ERBB2, FGFR1, HRAS, IGF1R, MAP2K1, PTEN, ZEB1]
KEGG:05218	Melanoma	5.01E-07	7	[AKT1, CDKN1A, FGFR1, HRAS, IGF1R, MAP2K1, PTEN]
KEGG:05219	Bladder cancer	7.81E-06	5	[CDKN1A, ERBB2, HRAS, MAP2K1, VEGFA]
KEGG:05224	Breast cancer	6.16E-06	8	[AKT1, CDKN1A, ERBB2, FGFR1, HRAS, IGF1R, MAP2K1, PTEN]
KEGG:05226	Gastric cancer	6.81E-06	8	[AKT1, BCL2, CDKN1A, CDX2, ERBB2, HRAS, MAP2K1, TERT]
KEGG:05230	Central carbon metabolism in cancer	6.98E-10	9	[AKT1, ERBB2, FGFR1, HIF1A, HRAS, MAP2K1, NTRK3, PDK1, PTEN]
KEGG:05235	PD-L1 expression and PD-1 checkpoint pathway in cancer	2.14E-06	7	[AKT1, HIF1A, HRAS, MAP2K1, NFATC3, PTEN, TLR2]

Table(6)

gene expression difference between normal tissue and bladder tumor

Gene	**statistical significance**
ERBB2	1.56630000003322E-06
TERT	6.08069150587198E-13
HRAS	3.17120000037363E-07
BCL2	2.83109979903884E-10
FGFR1	7.11640000261582E-08
PTEN	8.188700E-04
YES1	1.69970999999158E-05

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Gene	statistical significance
CDX2	3.41909944800989E-10
MYB	1.70774505647842E-12
MAP2K1	5.13659999978877E-07
PTCH1	2.2163E-02
SIAH1	1.744020E-03
VEGFA	2.6162E-02
CCND3	2.29529999999922E-05
CDKN1A	1.634450E-02
IRS1	2.521700E-03
PDK1	1.62470037423645E-12
TJP1	3.46179999999308E-06
TLR2	1.50089995987202E-09
ZEB1	1.916850E-03
TWIST1	2.8403E-02
AR	2.35690000000011E-05
NTRK3	4.024600E-03
ATR	4.024600E-03
TIMP	2.720000E-02