Research Square

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The value of a group of serum miRNAs in screening prostate cancer

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Abstract

Background

Prostate cancer remains a worldwide public health problem that poses a serious threat to the health of men worldwide. Many studies have found that miRNA in serum has the possible to be a biomarker for cancer. Our study was conducted to investigate the valuation of serum miRNAs in prostate cancer screening.

Methods

We selected 12 miRNAs from past studies associated with prostate cancer. We checked the expression levels of these miRNAs in the serum of 112 prostate cancer patients and 112 healthy controls in a two-stage experiment. We plotted the receiver operating characteristic curve of miRNAs in the validation stage and constructed a four-miRNA panel with the highest diagnostic value using stepwise logistic regression. We also predicted the target genes for these four miRNAs through online databases and performed Gene Ontology functional annotation and pathway analysis.

Results

The results showed that 6 miRNAs (miR-429, miR-10a-5p, miR-183-5p, miR-181a-5p, miR-1231, miR-129-5p) were abnormally expressed in the serum of prostate cancer patients. We used four of these miRNAs including miR-1231, miR-10a-5p, miR-429 and miR-129-5p to construct a combination of miRNAs with high specificity and sensitivity in screening prostate cancer (area under the curve (AUC) = 0.878). Bioinformatic analysis has shown that the genes targeted by these miRNAs can be linked to the development of cancer.

Conclusions

Our study detected and identified a set of miRNAs that serve as screening markers for prostate cancer, which may assist in early diagnosis and treatment of prostate cancer.

Introduction

Prostate cancer(PC) is a common urological disease(1) and the leading cause of cancer deaths associated with men(2). The burden of PC has risen rapidly in Asia recently, and the incidence and prevalence of prostate cancer has risen in almost every country(3). Early stage prostate cancer usually presents only as frequent and painful urination, and the clinical presentation and outcome vary greatly(4). Early diagnosis and monitoring can be effective in managing this disease(5). The following methods are currently used to diagnose prostate cancer: digital rectal examination, determination of

prostate specific antigen (PSA), biopsy, transrectal ultrasound(6). The only test that seems to be available as a screening tool is the serum PSA test. However, PSA lacks specificity as a prostate cancer marker and is also increased in BPH and prostatitis(7). This, in combination with the asymptomatic and slow growth nature of prostate tumors, has caused overdiagnosis or treatment for many men(5). Studies have shown that PSE screening has no positive or substantive effect on the overall survival of patients(8). Hence, finding a biomarker for prostate cancer screening is essential for men's global health.

MicroRNAs (miRNAs) are a kind of small single-stranded endogenous expression-encoding RNAs that govern gene expressing(9). It plays a significant part in various pathophysiological procedures such as cell development, proliferation, differentiation, apoptosis, senescence, and cell recognition(10). Dysregulation of miRNA expression is also linked with the biology of diseases such as cancer(11). MiRNAs have frequently been identified as specific, sensitive, and stable, suggesting that they may be possible specific biomarkers for PC(12). At the same time, miRNA in serum can be stably present and can be obtained by non-invasive methods(13), requiring only simple techniques such as qRT-PCR to detect its expression level(14). Much research has demonstrated the potential of miRNAs to be a screening biomarker for prostate cancer(15, 16).

In our study, we detected some miRNAs related to prostate cancer. Throughout the training and validation phases, the expression levels of different miRNAs in the serum of PC patients and healthy controls (HC) were measured by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) to screen for miRNAs with diagnostic value. We selected miRNAs with high diagnostic valuation to make up a diagnostic panel. Furthermore, we used these miRNAs for target gene prediction and bioinformatics analysis.

Materials And Methods

Participants and Ethics Statement

From January 2018 to September 2020, with the approbation of the Ethics Committee of Peking University Shenzhen Hospital (2019-043), we recruited 224 partakes at Peking University Shenzhen Hospital, including 112 patients with PC and 112 HCs. All participants understood and voluntarily subscribed to the informed authorization form, and the serum sampling procedure complied with the relevant rules published by the Panel. PC patients were initially diagnosed and had not received any treatment prior to taking the serum samples. Healthy controls were free of relevant underlying diseases and history of cancer.

Study design

First, we selected 12 miRNAs in the paper that are associated with PC and are generally expressed aberrant in prostate tissue. During the training phase, we measured the expression levels of these 12 miRNAs in the serum of 28 PC patients and 28 HCs. The miRNAs aberrantly expressed in the sera of PC patients were put into the validation set for further testing. In the validation phase, we increased the

sample size, examined the expression levels of these miRNAs in the serum, calculated their p-values, drew the receiver operating characteristic curve (ROC), and calculated the area under the curve (AUC). Ultimately, a miRNA panel with high specificity and sensitivity was constructed by stepwise logistic regression.

Obtaining serum specimens from patients and healthy controls

The study collected serum samples from 224 men between Jan 2018 and Sept 2020, including 112 patients with pathologically confirmed prostate cancer after surgical resection and 112 healthy check-up men. 10 ml serum sample was taken from each partaker and centrifuged at 3000g for 10 min at 4ºC for 2h. Serum samples were stored at -80°C and frozen for RNA extraction. The clinical characteristics of the participants are shown in Table 1.

Table 1
Clinical and demographic information of 224 participants

	Training stage (n = 56)		**Validation stage (n = 188)**	
	Cases (%)	Controls (%)	Cases (%)	Controls (%)
Total number	28	28	84	84
Age (Mean ± SD)	68.0 ± 7.5	68.2 ± 8.0	68.6 ± 8.1	68.3 ± 8.3
Tumor stage				
<=T2	17 (56.7)		51 (60.7)	
>=T2	13(43.3)		33 (39.3)	
Lymph node metastasis				
Positive	3 (10.7)		8(9.5	
Negative	25(89.3)		76(90.5)	
Bone metastasis				
Positive	2 (7.14)		7(8.33)	
Negative	26 (92.9)		77 (91.7)	
Gleason grade				
Well differentiation	0		0	
Moderate differentiation	18 (64.3)		58 (69.0)	
Poor differentiation	10 (35.7)		26 (30.1)	

RNA extraction and RT-qPCR

We added 2ul cel-miR-54-5p (10 nm/L, RiboBio, China) to each sample, which used to standardize the variability in the extraction process as an internal reference. Subsequently, total RNA from samples were extracted by TRIzol LS isolation kit (Thermo Fisher Scientific, Waltham, MA, United States) and RNA concentration and purity was gauged by NanoDrop 2000c spectrophotometer (Thermo Scientific, United States). RT-qPCR was served to determine the expression level of these miRNAs. The miRNAs were amplified by the reverse transcription-specific primers of the Bulge-Loop miRNA qRT-PCR primer set (RiboBio, Guangzhou, China) in reverse transcription. Then, by the qPCR with the Taqman probe, the expression level of miRNAs was detected on the LightCycler480 Real-Time PCR system (Roche Diagnostics, Mannheim, Germany). The qRT-PCR was performed with 40 cycles following at 95°C for 20s, 95°C for 10s, 60°C for 20s, and 70°C for 10s.

Statistical analyses

We used $2^{-\Delta\Delta Cq}$ method the calculation of miRNA expression levels(17).Categorical variables between groups were reported in the form of numbers and percentages. Continuous variables were presented as the average value ± standard deviation. Kruskal-Wally's rank test was used to compare several distinct phases. Different expression levels of candidate miRNAs in PC and HC samples were anatomized by using Students' T-test or Mann–Whitney test. The diagnostic miRNA panel was designed with the aid of multiple logistic regression analysis. It was estimated that the ROC curve and AUC value were the diagnostic value for miRNAs. P-value < 0.05 was set as statistically significant. SPSS software (SPSS 26.0 Inc, Chicago, IL) GraphPad Prism 8 (GraphPad Prism 9.4.1.681) and Medcalc (Version 19) were used for the statistical processing.

Bioinformatic analysis

MiRWalk3.0 database was used to identify target genes for candidate miRNAs. Genes predicted by over three candidate miRNAs were chosen as target genes, then placed into the David database(18, 19) for Gene Ontology (GO) functional annotation and pathway analysis, and visualized by using ImageGP(20). Through analysis of the GEPIA database(21), we examined target genes whose expression was significantly different in PC patients compared with NCs, which may be related to tumorigenesis and progression of PC.

Results

Demographic and clinical features of participants

In total, our study examined serum mir expression levels in 112 PC patients and 112 HCs. All PC patients were pathologically diagnosed with prostate cancer after surgical removal of cancerous tissue. Apart from this, they had no other history of tumor disease, and none of them had received treatment prior to taking serum samples. Healthy controls were males of the same age who had no prostate disease and no family history of prostate cancer within the cancer group. Table 1 sets out the populations and clinical

outcomes of 224 participants. The difference in age distribution between PC patients and HCs was not statistically significant, with a p-value > 0.05.

Selecting PC-related miRNAs during the screening phase

In the screening phase, we searched the literature for miRNAs associated with prostate cancer. A total of 12 miRNAs were aberrantly expressed in cancer tissues, including: miR-10a-5p, miR-372, miR-29a-3p, miR-183-5p, miR-1231, miR-199b-3p, miR-489-3p, miR-191, miR-129-5p, miR-486-5p, miR-10a-5p, miR-429.

Testing candidate miRNAs at the training phase

During the training phase, the corresponding miRNAs expression levels were measured in the serum of 30 PC patients and 30 HCs using qRT-PCR. The expression levels of these 12 miRNAs in PC patients and HCs are shown in Fig. 1. At a standard of p-value < 0.05, we found a total of 6 miRNAs including miR-429, miR-10a-5p, miR-183-5p, miR-181a-5p, miR-1231, miR-129-5p that remained abnormally expressed in serum in the cancer group and HCs. We further tested the expression levels of these miRNAs during the validation phase.

Further validation of candidate miRNAs

In the validation phase, we further examined the expression levels of these 6 miRNAs in serum samples from 82 PC patients and 82 HCs. The expression levels and ROC curves of these miRNAs are shown in Fig. 2. The results are as follows: the expression level of miR-129-5p, miR-429, miR-1231 were meaningfully upregulated (p-value < 0.001) and the remaining four miRNAs expression levels were all downregulated. The results of AUC values are as follows: 0.644(95% confidence interval[CI]: Fig. 2B) for miR-10a-5p, 0.636 (95% CI: 0.557– 0.709; Fig. 2D) for miR-129-5p, 0.570(95% CI: 0.491– 0.647; Fig. 2F) for miR-181a-5p, 0.671(95% CI:0.594–0.742; Fig. 2H) for miR-183-5p, 0.714(95% CI: 0.639–0.782; Fig. 2J) for miR-429 and 0.646(95% CI: 0.568–0.719;0.566–0.717; Fig. 2L) for miR-1231. Next, in Table 2, Youden index was used to compute the best cut- off value and specify the best specificity and sensitivity of these five miRNAs for PC diagnostics.

Table 2
Outcomes of receiver operating characteristic curves and Youden index for 6 candidate miRNAs and the panel

	AUC	**P value**	**95% CI**	**Associated criterion**	**Sensitivity (%)**	**Specificity (%)**
miR-1231	0.644	< 0.001	0.566–0.717	≤ 0.827	51.19	78.05
miR-181a-5p	0.570	= 0.116	0.491–0.547	≤ 1.027	70.24	47.56
miR-10a-5p	0.671	< 0.001	0.594–0.742	> 1.212	47.62	82.93
miR-183-5p	0.647	< 0.001	0.569–0.720	> 1.228	42.86	85.37
miR-429	0.714	< 0.001	0.639–0.782	> 1.116	67.86	68.29
miR-129-5p	0.636	= 0.002	0.557–0.709	≤ 0.797	40.48	81.71
four-miRNA panel	0.878	< 0.001	0.843–0.941	> 0.435	83.33	79.27

AUC: area under curve; CI: confidence interval

Building a four-miRNA panel for prostate cancer screening

We found that constructing a group of miRNAs had higher diagnostic value than a single miRNA. Therefore, we constructed a four-miRNA diagnostic panel including miR-1231, miR-10a-5p, miR-429 and miR-129-5p through a stepwise logistic regression model. The last logistic regression model is based on the following formula: [logit p= -2.032+(-2.6×miR-1231) +(2.821×miR-10a-5p) +(-5.576×miR-429) + (-5.104×miR-129-5p)]. The roc of this four miRNAs panel is shown in Fig. 3, with an auc value of 0.878 (95% CI: 0.818–0.924; sensitivity = 83.33%, specificity = 79.27%; **Table 3**)

Bioinformatics analysis of four miRNAs

We performed target gene prediction for these four miRNAs including miR-1231, miR-10a-5p, miR-429 and miR-129-5p in mirWalk 3.0. As shown in Fig. 4A, a total of 210 target genes were predicted by three and more miRNAs. In order to further investigate the potential functions of these target genes, an analysis of GO annotation and KEGG enrichment was carried out in the DAVID database(18, 19). GO functional annotation consisting of biological process (BP), cellular component (CC), and molecular function (MF). Figure 5 shows the first five elements of the GO annotation including vesicle-mediated transport(GO:0016192), positive regulation of BMP signaling pathway(GO:0030513), mesoderm formation(GO:0001707), retinal cone cell development(GO:0046549),and ceramide catabolic process(GO:0046514) in the BP category; anchoring junction(GO:0070161), external side of plasma

membrane(GO:0009897), early endosome(GO:0005769), cytoplasmic vesicle membrane(GO:0030659), mRNA cap binding complex(GO:0005845) in the CC category; RNA binding(GO:0003723), myosin phosphatase activity(GO:0017018), receptor tyrosine kinase binding(GO:0030971), chemorepellent activity(GO:0045499), ceramidase activity(GO:0017040). The enriched KEGG pathway contains pathways in PI3K – Akt signaling pathway and EGFR tyrosine kinase inhibitor resistance. We also put the target genes that were simultaneously predicted by the 4 miRNAs into the GEPIA(22) database to search. At the criterion of p-value < 0.01 and |log2FC| Cutoff > 1, NRG1 was discovered to be differentially expressed in PC and normal tissues in Fig. 4B.

Discussion

Prostate cancer is a considerable public health anxiety around the world and remains one of the leading causes of cancer-related morbidity and mortality(23). Early diagnosis and treatment can effectively improve the prognosis of the disease(5). However, the commonly used biomarker PSA has not yet been shown to be a routine screening tool(24) due to the overdiagnosis associated with its poor specificity(25). Many studies have shown that miRNAs are participate in processes such as cell proliferation, differentiation and cancer development (26).MiRNAs in tissues and body fluids can be applied to be a diagnostic and prognostic marker for cancer due to its often dysregulated expression and stable presence(27). In our study, a panel of four miRNAs with the highest diagnostic value was selected through three stages of screening, training, and validation. This panel has the potential to become a prostate cancer screening marker (AUC = 0.878; 95% CI: 0.818–0.924; sensitivity = 83.33%, specificity = 79.27%).

In our panel, miR-10a-5p has been shown to have diagnostic potential for PC in extracellular vesicles(28, 29). MiR-10a-5p can also influence prostate cancer development by regulating the BDNF / TRKB pathway(30). It has also been shown that high levels of mir in tissues are associated with the prognosis of PC(31). These studies have demonstrated the important role of miR-10a-5p in PC.

In previous studies, mir-429 has been reported to be implicated in epithelial-mesenchymal transition, progression, development, invasion, metastases, apoptosis, and drug resistance in a variety of cancers(32). In prostate cancer, miRNAs can contain cell proliferation by targeting p27Kip1(33). In breast cancer, miRNAs can be seen as a potential diagnostic and prognostic marker(34), and our study demonstrates its value in prostate cancer. Currently, there are fewer studies on miR-1231. Expression of miR-1231 is downregulated in both PC tissues and cell lines, and usually acts as a tumor suppressor(35). However, the pathways through which tumors are affected are currently worthy of continued study.

Another miRNA in the diagnostic panel is miR-129-5p. MiR-129-5p acts as an oncogenic factor that can impede cancer cell growth by targeting etv1 and inhibiting DLX1 expression(36, 37). MiR-129-5p has been shown to promote resistance to doxorubicin in PC-3-DR cells by inhibiting CAMK2N1 expression(38). This gives a new direction for the treatment of PC.

To further explore the function and role of this group of miRNAs, we performed target gene prediction in mirWalk 3.0. The results suggest that these aberrantly regulated miRNA targets are associated with many important cancer-related biological processes and pathways such as PI3K – Akt signaling pathway and EGFR tyrosine kinase inhibitor resistance. In the biological process terms, vesicle – mediated transport and positive regulation of BMP signaling pathway are encompassed in the development of many cancers(39–41). An examination by Chen H et al. demonstrated that PI3K – Akt regulates metastasis and invasion of PC cells(42). Sinomenine inhibits prostate cancer cell proliferation, migration, invasion and promotes apoptosis of cancer cells by regulating miR-23a(43). CUDC-907 inhibits PI3K – Akt and histone deacetylases to suppress PC tumor growth in mice(44). This brings a new strategy for the treatment of PC. Many protein molecules can inhibit the growth and metastasis of prostate cancer by affecting EGFR such as FBXW2 and DUSP22(45, 46).

Under the criterion of p-value < 0.01 and |log2FC| Cutoff > 1 based on the expression level, NRG1 was chosen as the target gene among four miRNAs predicted at the same time by the GEPIA database. NRG1 has been less studied in PC and may be an independent risk factor and associated with the development of PC(47, 48). In triple negative breast cancer (TNBC), NRG1 regulates Fra-1 expression and coordinates cancer cell metastasis via the novel ERK1 / 2-Fbxw7-c-Myc pathway. This makes NGR1 a possible target for the treatment of TNBC(49). In the same way, NRG1 has emerged as a promising target for targeted therapy in non-small cell lung cancer(50).

Although it appears that our constructed miRNAs panel has a high diagnostic value from a statistical point of view. Our experiment still has some shortcomings. For instance, we did not test the PSA levels in the serum of the samples and compare them with miRNAs. Our samples are limited in size and come from only one region. We may have overlooked other valuable miRNAs in the serum. These factors may have brought some influence on the experimental results

Conclusions

Our experiments constructed a 4 miRNAs panel with the ability to become a screening marker for PC. This facilitates early detection and timely treatment of PC and may reduce the overdiagnosis and treatment associated with PSA screening. And targets genes NRG1 may be encompassed in the development and progression of PC and holds promise as a new therapeutic target.

Abbreviations

PC, prostate cancer; PSA, Prostate Specific Antigen; ROC, receiver operating characteristic algorithm; NCs, normal controls; qRT-PCR, Quantitative Reverse Transcription-Polymerase Chain Reaction; GO, Gene Ontology; AUC, area under the ROC curve; FC, fold change; BP, biological process; CC, cellular component; MF, molecular function.

Declarations

The authors declare that there are no conflicts of interest.

Ethics approval and consent to participate

The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The studies involving man participants were reviewed and approved by the Ethics Committee of Peking University Shenzhen Hospital (2019-043).

Acknowledgments

Not applicable.

AUTHOR CONTRIBUTIONS

All authors devoted to the generalization and design of the study. Chen Sun and Xinji Li were responsible for investigating and storing the data and wrote the original manuscript. Rongkang Li, Chong Lu and Zhenyu Wen: Formal analysis. Zhenjian Ge , Yingqi LI and Wenkang Chen collected and visualized serum samples. Suolei Sun, Qingshan Yang and Lingzhi Tao assisted with the writing of the manuscript. Yongqing Lai and Hang Li dedicated to review, editing and oversight work. All authors read and approved the final manuscript.

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DATA AVAILABILITY STATEMENT

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Figures

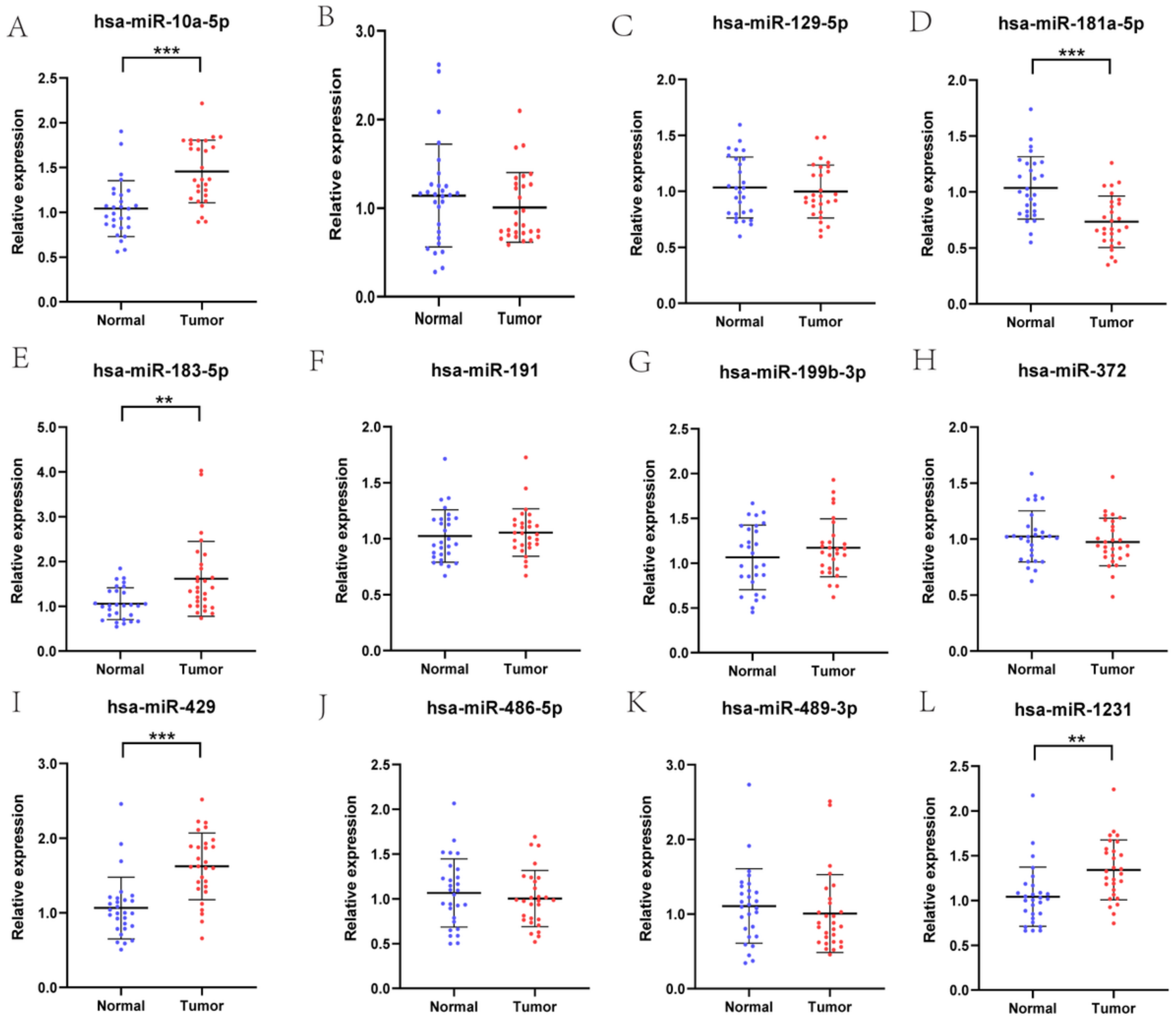


Figure 1

During the training phase, the relative expression levels of 12 miRNAs in serum from 28 PC patients and 28 healthy controls. * represents p<0.05, **represents p<0.01, *** represents p<0.001.

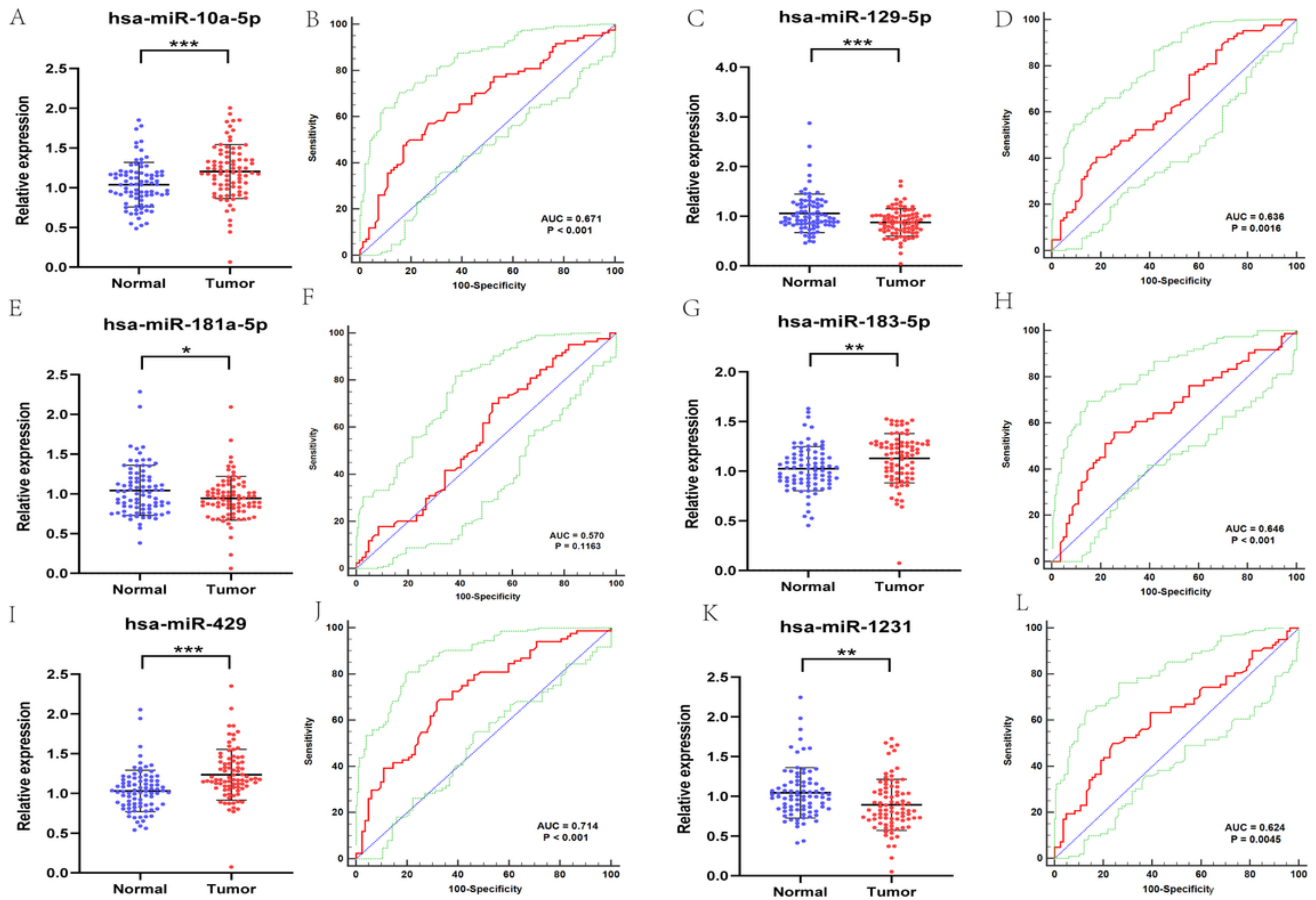


Figure 2

In the validation stage, the relative expression levels of 6 miRNAs in serum of 112 PC patients and healthy controlsand their corresponding receiver operating characteristic curve (ROC). The relative expression levels of (C) miR-129-5p, (E) miR-181a-5p, and(I) miR-1231 in the serum of PC patients were significantly down-regulated while (A) miR-10a-5p, (G)miR-183-5p and (I)miR-429 was up-regulated.

* Represents p<0.05, ** represents p<0.01, ***represents p<0. 001.The red curve represents the receiver-operating characteristic (ROC) curve. The green curve represents 95% ROC confidence interval. The blue line represents diagonal.

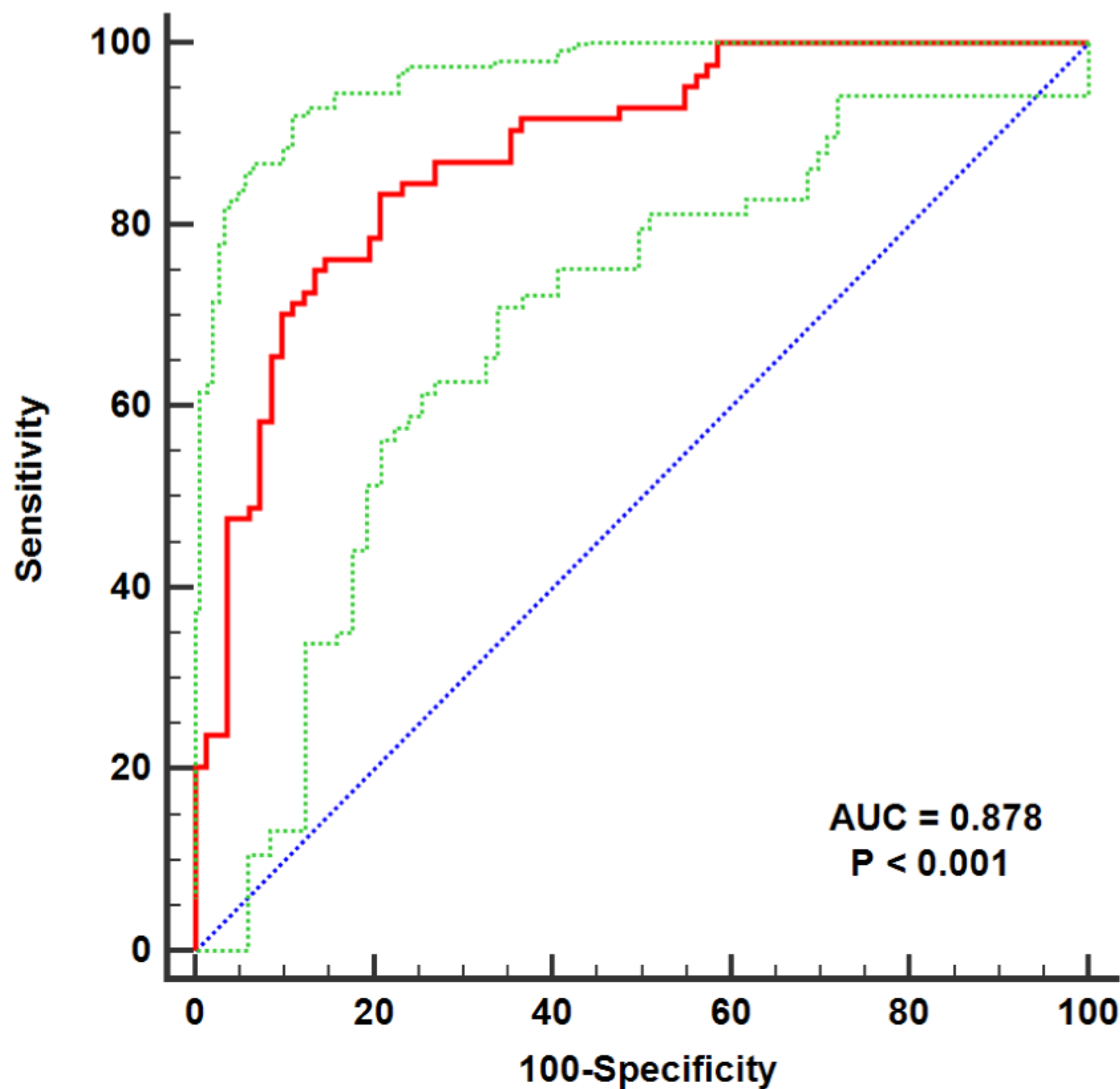
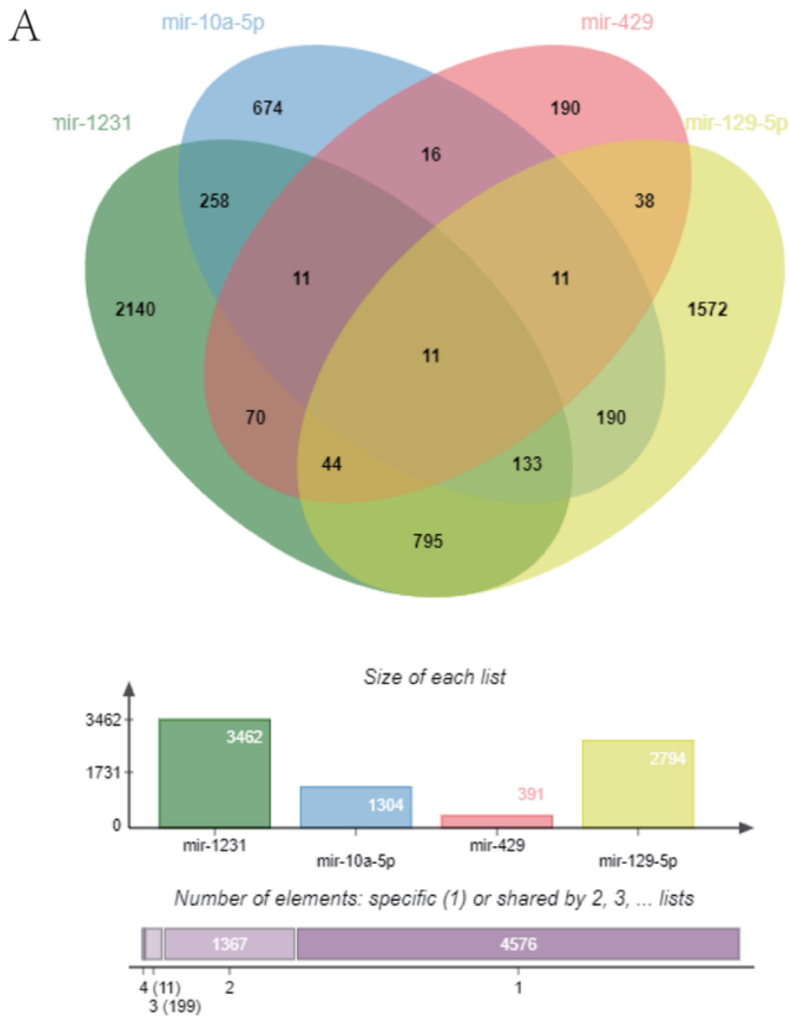


Figure 3

ROC curve analyses of the four-miRNA panel. The AUC of the panel was 0.878(AUC=0.878; 95% CI: 0.843–0.941; sensitivity = 83.33%, specificity = 79.27%). The red curve represents the receiver operating characteristic (ROC) curve. The green curve represents 95% ROC confidence interval. The blue line represents diagonal.



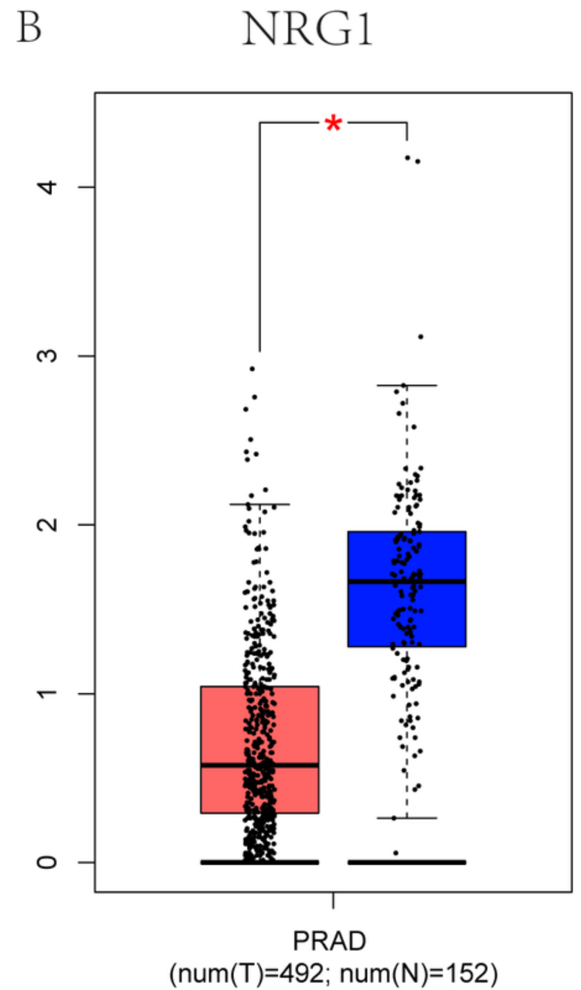


Figure 4

Target genes of the four-miRNA panel predicted from miRWalk 3.0(A). Under the criterion of p-value < 0.01 and |log2FC| Cutoff > 1 based on the expression level, expression levels of NRG1 were significantly dysregulated in 492 PC patients and 152 normal controls.

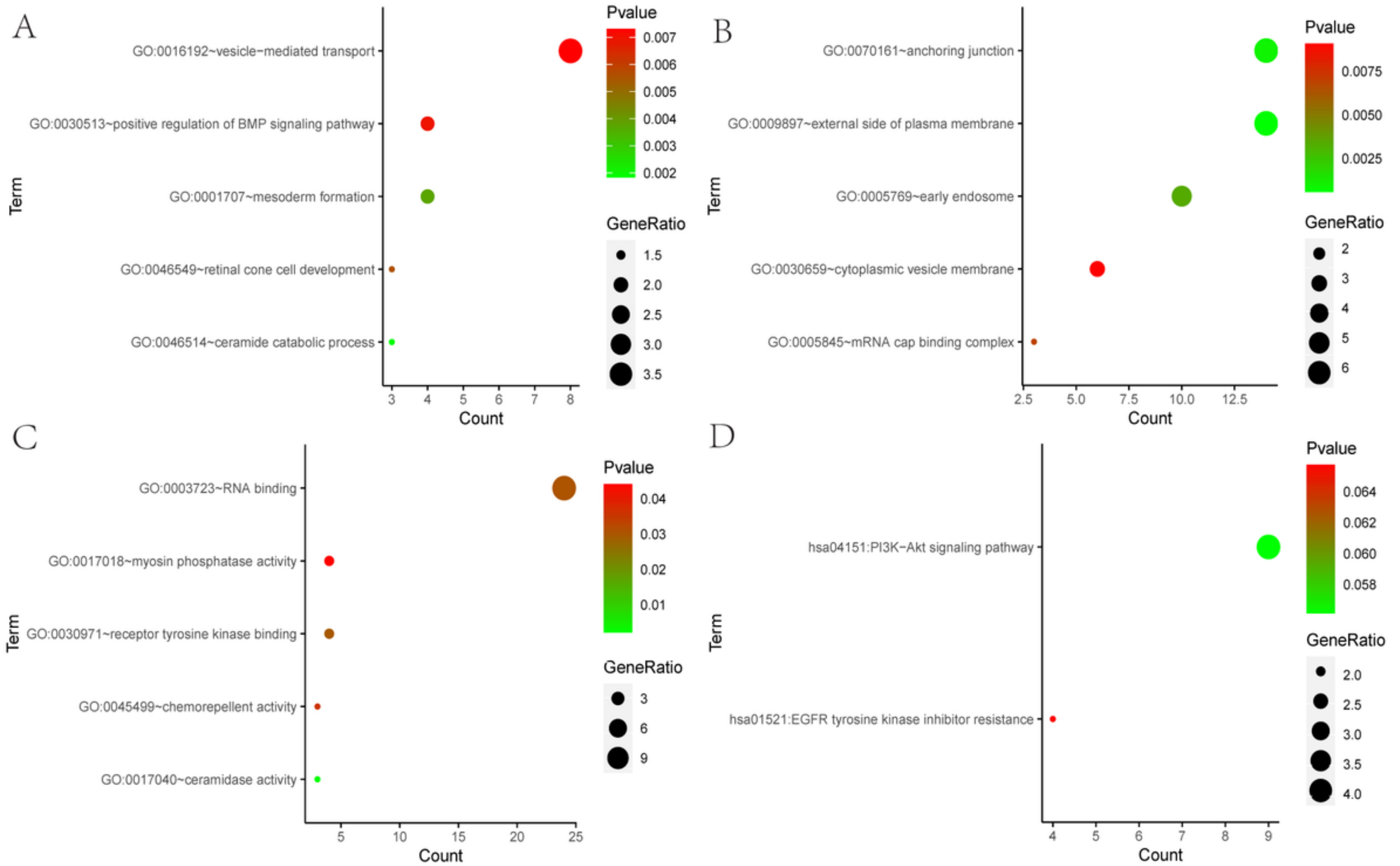


Figure 5

Gene ontology functional annotation KEGG pathway enrichment analysis for the targeted genes of miR-1231, miR-129-5p, miR-10a-5p and miR-429. (A)BP, (B)CC and (C)MF, (D)KEGG pathway analysis. BP: Biological process; CC: Cellular component; GO: Gene ontology; KEGG: Kyoto encyclopedia of genes and genomes; MF: Molecular function.