Int J Clin Exp Pathol 2020;13(10):2615-2627
www.ijcep.com /ISSN:1936-2625/IJCEP0117403

Original Article

Diagnostic and prognostic values of forkhead box D4 gene in colonic adenocarcinoma

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Received July 2, 2020; Accepted August 31, 2020; Epub October 1, 2020; Published October 15, 2020

Abstract: Previous studies found that Forkhead box D4 (*FOXD4*) overexpressed in human colorectal cancer had the worst prognosis. However, the diagnostic value and further mechanism have not been fully researched. Statistical examinations for *FOXD4* expression colon adenocarcinoma (COAD) patients were obtained from The Cancer Genome Atlas (TCGA). Survival analysis was used to assess its prognostic value. Nomogram model was used for visual prediction of patient survival rate. The online functional enrichment analysis tool was used to evaluate the biological functions and pathways of FOXD4 and its co-expressed genes. Receiver operating characteristic curve analysis suggested that FOXD4 might be a diagnostic biomarker for COAD (P<0.001, area under the curve [AUC]=0.728, 95% confidence interval [CI]=0.669-0.787). Low expression of *FOXD4* was associated with a good clinical outcome (P=0.001, HR=0.517, 95% CI=0.341-0.782). A total of 797 genes were correlated with *FOXD4* and associated with cell proliferation, cell differentiation, nuclear matrix, Rap1 signaling pathway, RNA transport, and VEGF signaling pathway. In conclusion, expression of *FOXD4* may be a diagnostic and prognostic biomarker in COAD.

Keywords: FOXD4, forkhead box D4, biomarker, diagnosis, prognosis, colon adenocarcinoma

Introduction

Colon adenocarcinoma (COAD) is a high incidence gastrointestinal cancer in the world, which is a histological subtype of colorectal cancer (CRC). According to statistics, there were 145,600 estimated new cases and 51,020 estimated death cases of CRC in 2019, USA [1]. The prognosis of colorectal cancer was significantly correlated with tumor stage at the time of diagnosis [2]. It is important to find biomarkers for diagnosis and prediction of prognosis for CRC and COAD. Recently, there were several biomarkers used for the diagnosis, including *SFRP2* [3], *SEPT9* [4], *BCAT1*, *IKZF1* [5], and some other genes. Other genes were used for the prognosis, including *MGMT* [6], *NDRG4*, *BMP3* [7], *HOPXB* [8]. Other genes have emerged in recent years. However, there were still lake of the golden standard biomarker for diagnosis and prognosis in COAD, which was significant for the specific treatment of COAD patients.

Forkhead box D4 (FOXD4) protein was encoded by the corresponding gene *FOXD4*. That gene belonged to the forkhead box (*FOX*) gene family. FOX protein plays an indispensable role in the regulation of cell growth, proliferation, differentiation, and lifespan-related gene expression, as well as for embryonic development [9, 10]. Recent studies about FOXD4 were correlated with non-cancer disease [11, 12], and lack of further study. Previous studies found that FOXD4 in human leukemia cell lines [13], which was up-regulated in CRC and with wore prognosis [14]. Based on those evidences, we assume that FOXD4 could be served as an indicator for COAD diagnosis and clinical outcome prediction.

In this study, based on the TCGA data, we performed the uni- and multi-survival analysis and joint-effects analysis for the expression level of FOXD4. Some clinical information included gender, age and tumor stage. A receiver operating

characteristic (ROC) curve was created for predicting the diagnosis value of FOXD4. A nomogram model for visual prediction of patient survival rate was created. Then we made a bioinformatic analysis which included gene ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), Protein-protein Interaction (PPI) for FOXD4, and related genes in TCGA COAD cohort, to predict the mechanism.

Methods

Data preparation

The dataset of gene expression of current study was obtained from TCGA (https://cancergenome.nih.gov/, accessed in June 1, 2019). The corresponding patients' baseline data and overall survival (OS) information was obtained from University of California, Santa Cruz (UCSC) Xena (https://xena.ucsc.edu/, accessed in June 1, 2019).

The expression level of FOXD4

The online database, GEPIA, was used to assess the FOXD4 mRNA expression between normal colon tissues and tumor tissues. These matched in TCGA para-carcinoma tissues and normal colon tissues of GTEx dataset (http://gepia.cancer-pku.cn, accessed in June 5, 2019) [15]. We also used the TCGA expression data to make violin plots to express the different expression level of FOXD4 gene in paracancerous tissue and colon cancer tissue.

Diagnosis value of FOXD4

Receiver operating characteristics (ROC) curves were used to investigate the diagnostic value of the mRNA expression of *FOXD4* in TCGA cohort. The area under the curve (AUC) >0.5, as well as $P<0.05$, were considered to have reached statistical significance.

Prognostic value investigation of FOXD4

The univariate survival analysis was presented to find out which factor including gender, age, tumor stage, and *FOXD4* expression, may influence the overall survival of COAD. The expression of FOXD4 was divided into 2 groups, low and high expression group, which were according to the median value of *FOXD4* expression. The Kaplan-Meier survival analysis with log-rank test was used to assess the survival rate. In addition, the Cox proportional hazards regression model was used to univariate and multivariate survival analysis. Hazard ratios (HRs) and 95% confidence intervals (CIs) were computed by the Cox proportional hazards regression model. Hierarchical analysis, which the group methods was based on the expression level of *FOXD4* for clinical information, to control the influence of confounding factors on the clinical outcome.

Joint-effects survival analysis was identified to illustrate the relationship of prognosis between the expression level and tumor stages.

Nomogram

A nomogram was constructed to assess the survival rate according to the contribution of each factor which may affect the prognosis of COAD. As for the clinical parameters and prognostic information, we used the tumor stage and FOXD4 to construct the nomogram model. The points for each parameters and total points could be calculate, and year related survival rates could also be calculated.

Identification of FOXD4 co-expressed genes in COAD

The *FOXD4* co-expressed genes were assessed by Pearson correlation coefficient in the COAD tumor tissues of TCGA cohort. Whole genome co-expressed analysis was performed using the RNA-Seq dataset of COAD tumor tissues. The absolute value of correlation coefficient (r)>0.2 was considered as significantly correlated. A gene-gene interaction online tool: GeneMANIA (http://genemania.org/, accessed by June 30, 2019) was used to predict the co-expression relationship of those co-expressed genes and FOXD4 [16].

Function assessment of FOXD4 in COAD

To explore the functionality of FOXD4 and correlated genes in COAD, the online database of the database for the purpose of Annotation, Visualization, and Integrated Discovery (DAVID) v.6.8 (https://david.ncifcrf.gov/tools.jsp, retrieved July 10, 2019) [17, 18], were used together with BiNGO (https://www.psb.ugent.be/cbd/papers/BiNGO/Home, accessed by June 15,

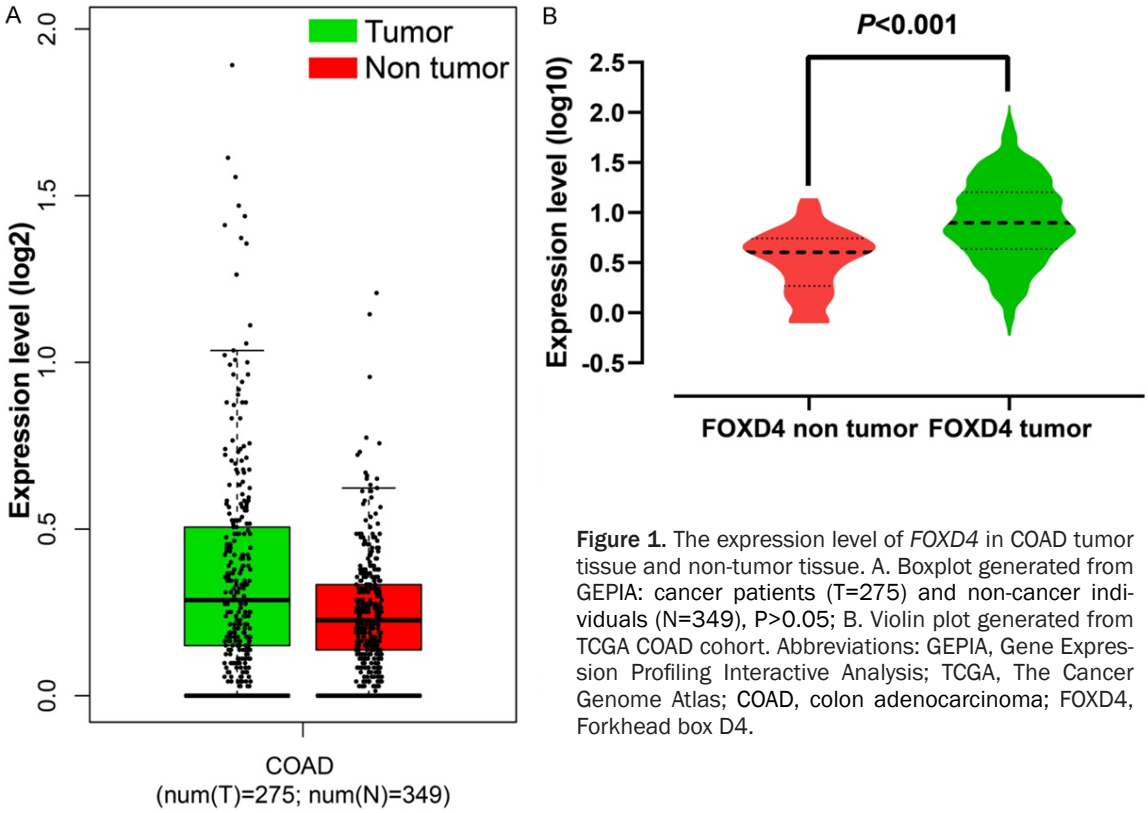

Figure 1. The expression level of *FOXD4* in COAD tumor tissue and non-tumor tissue. A. Boxplot generated from GEPIA: cancer patients (T=275) and non-cancer individuals (N=349), P>0.05; B. Violin plot generated from TCGA COAD cohort. Abbreviations: GEPIA, Gene Expression Profiling Interactive Analysis; TCGA, The Cancer Genome Atlas; COAD, colon adenocarcinoma; FOXD4, Forkhead box D4.

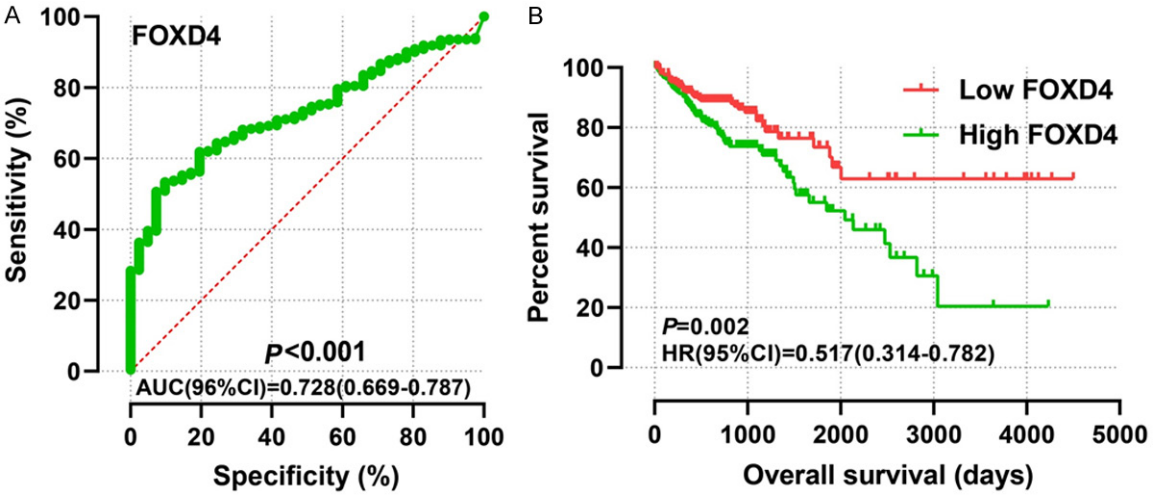

Figure 2. Diagnostic and prognostic analysis results. A. ROC curves of *FOXD4*: cancer patients (T=480) and non-cancer individuals (N=41), the sensitivity is 0.940, and the specificity is 0.976; B. Kaplan-Meier curves of *FOXD4*: median raw count expression value of *FOXD4* is 7.570, and the number of patients in both the high- and the low-FOXD4 expression groups were 219. Abbreviations: ROC, receiver operating characteristic; FOXD4, Forkhead box D4.

2019) [19], to explore the gene ontology (GO) functional examination, including molecular functionality (MF), together with biological process (BP), and cell component (CC), and Kyoto Encyclopedia of Genes and Genomes (KEGG).

The Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database (http://string.embl.de, accessed July 25, 2019) is an online tool that can be used to plot the protein-protein interactions (PPI) network [20]. In the

Table 1. Hierarchical analysis of clinical factors

Variables		FOXD4 low expression				FOXD4 high expression			
		NO. of patients	NO. of events (%)	HR (95% CI)	Log-rank P	NO. of patients	NO. of events (%)	HR (95% CI)	Log-rank P
Gender	Female	100	13 (13.0%)	0.410 (0.214-0.785)	0.007	104	31 (29.8%)	Ref.	N/A
	Male	119	22 (18.5%)	0.632 (0.367-1.091)	0.100	115	32 (27.8%)	Ref.	N/A
Age (years)§	<65	89	12 (13.5%)	0.502 (0.238-1.056)	0.069	79	17 (21.5%)	Ref.	N/A
	≥65	130	23 (17.7%)	0.537 (0.324-0.890)	0.016	138	45 (32.6%)	Ref.	N/A
Tumor stage£	Early stage	105	5 (0.05%)	0.261 (0.100-0.681)	0.006	134	26 (19.4%)	Ref.	N/A
	Advanced stage	109	29 (26.6%)	0.521 (0.314-0.685)	0.012	78	33 (42.3%)	Ref.	N/A

Notes: §Information of age was unavailable in 2 patients; £Information of tumor stage was unavailable in 11 patients. Abbreviations: COAD, colon adenocarcinoma; HR, hazard ratio; CI, confidence interval; N/A, not available; Ref. reference.

Table 2. Joint-effect survival analysis grouping information and results

Group	FOXD4 expression level	Tumor stage£	MST	NO. of patients	NO. of events (%)	HR (95% CI)	Log-rank P
1	Low expression	Early stage	N/A	106	5 (4.7%)	0.076 (0.030-0.193)	<0.001
2	Low expression	Advanced stage	2003	109	29 (26.6%)	0.456 (0.277-0.750)	0.002
3	High expression	Early stage	2532	134	26 (19.4%)	0.306 (0.183-0.511)	<0.001
4	High expression	Advanced stage	1158	78	37 (43.5%)	Ref.	N/A

Notes: £Information of tumor stage was unavailable in 11 patients. Early stage corresponds to TNM stage I and II, and late stage corresponds to TNM stage III and IV. Abbreviations: COAD, colon adenocarcinoma; MST, median survival time; HR, hazard ratio; CI, confidence interval; N/A, not available; Ref. reference.

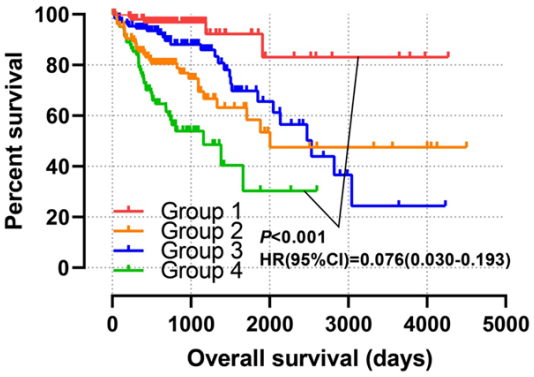

Figure 3. Joint-effects survival analysis curve for the combination of *FOXD4* and tumor stage. Notes: Early stage corresponds to TNM stage I and II, and late stage corresponds to TNM stage III and IV. Abbreviations: FOXD4, Forkhead box D4.

present study, we investigate the relationship of *FOXD4* and its co-expressed genes with a score >0.400 in the STRING parameter setting.

Statistical analyses

We compared the difference in expression levels between tumor and non-tumor tissues using the independent sample t test. Survival analysis was completed using the log-rank test and Cox proportional hazard regression model. Co-expressed gene screening used Pearson correlation coefficient. SPSS v.25.0 software was used to perform the statistical analyses (IBM Corp., Armonk, NY, USA). GraphPad Prism v.7.0 was used for plotting (GraphPad Software, Inc., La Jolla, CA, USA). The nomogram was performed in R v.3.5.1 platform (R Foundation for Statistical Computing, Vienna, Austria). Cytoscape v.3.7.1 was used to construct the gene-gene interaction network plot [21].

Results

Data overview

There were 480 tumor tissues expression dataset of COAD and 41 cases of paracancerous data. After, we deleted the cases without survival data. There were a total of 438 patients who were further included into the survival analysis. The clinical parameters: age, gender, and tumor stage were included.

The expression level of FOXD4 in COAD

In the Gene Expression Profiling Interactive Analysis (GEPIA, http://gepia.cancer-pku.cn/, accessed on June 20, 2019) [15] data base,

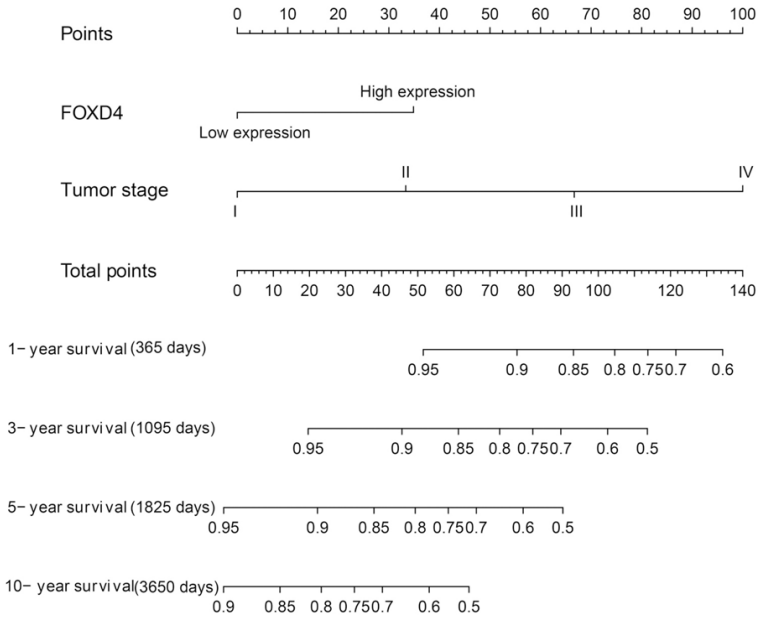

Figure 4. Nomogram. Abbreviations: FOXD4, Forkhead box D4.

the expression level of FODX4 in COAD tumor tissue was higher than non-tumor tissue (**Figure 1A**). We analyzed the expression data form TCGA and found that the expression level in tumor tissue was significantly higher than non-tumor tissue (*P*<0.001, **Figure 1B**).

The diagnosis value of FOXD4

We plotted a ROC curve to assess the power of FOXD4 expression in distinguished COAD tumor and paired colon tissues (**Figure 2A**). The ROC curve analysis suggested that FOXD4 might be a diagnostic biomarker for COAD with a high test efficiency (*P*<0.001, area under the curve [AUC] =0.728, 95% CI=0.669-0.787, **Figure 2A**).

The prognosis value of FOXD4 and clinical information

The univariable prognosis analysis indicated that tumor stage of COAD was significantly related to COAD OS, and early stage were significantly related to a good OS (*P*<0.001, HR=0.323, 95% CI=0.210-0.498, Table S1). The Kaplan-Meier curve of the FOXD4 is shown in **Figure 2B**. Low expression levels of FOXD4 was significantly associated with good prognosis (P=0.001, HR=0.517, 95% CI=0.341-0.782). The tumor stage was included in the multivariate Cox proportional hazards regression model for adjustment, lower expression levels of *FOXD4* significantly associated with good OS (adjusted *P*< 0.001, HR=0.395, 95% CI= 0.255-0.611).

Hierarchical analysis of clinical factors

The hierarchical analysis results is shown in **Table 1**. We found that under the low expression level of FOXD4, patients in female, elder, and tumor stage (both early and advanced stage) groups had favorable prognosis (all *P*< 0.05).

Joint-effect survival analysis results

The joint-effects survival analysis grouping information and results is shown in **Table 2** and **Figure 3**. We found that low expression of *FOXD4* combined with early stage (Group 1) was related to good prognosis (*P*<0.001, HR=0.076, 95% CI=0.030-0.193). High expression of *FOXD4* combined with advanced tumor stage was associated with worse prognosis (**Figure 3**).

Nomogram

The nomogram was assessed based on the expression level of *FOXD4*, tumor stage to assess the 1-, 3-, 5-, and 10-year related survival percentage (**Figure 4**).

Correlated genes at co-expression level of FOXD4

In the TCGA cohort, all correlated genes at co-expression level in COAD with *FOXD4* is shown in **Figure 5**. There were a total of 797 genes which were either negatively or positively correlated with *FOXD4*. The co-expression relationship is shown in **Figure 6**.

Prediction of FOXD4 and correlated gene function

The GO and KEGG enrichment analysis by DAVID results is shown in **Figures 7** and **8**. We found that BP, *FOXD4,* and correlated genes were associated with cell proliferation, cell differentiation, and some other processes (**Figure**

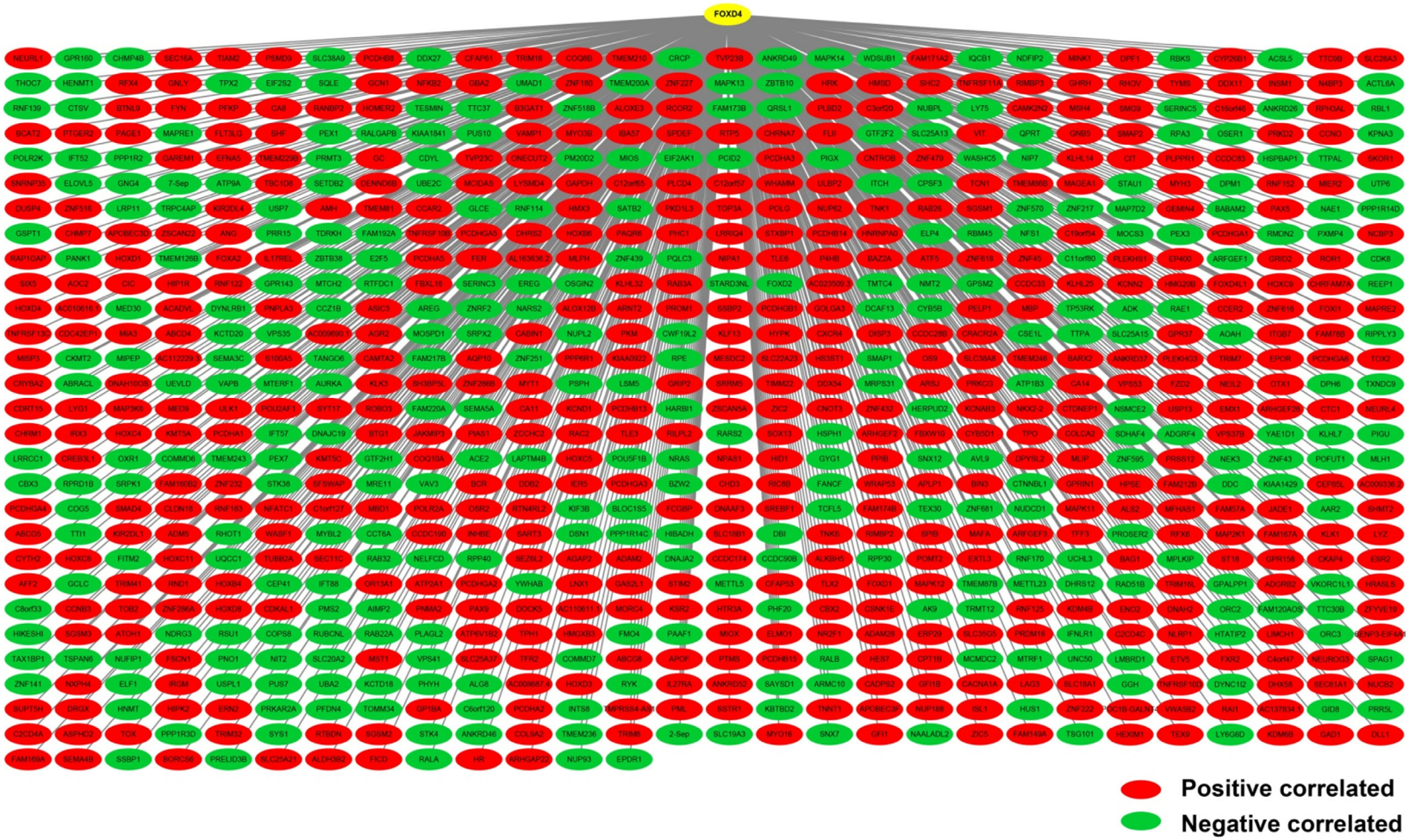

Figure 5. Correlation analysis between FOXD4 and TCGA COAD cohort genes. Abbreviations: FOXD4, Forkhead box D4.

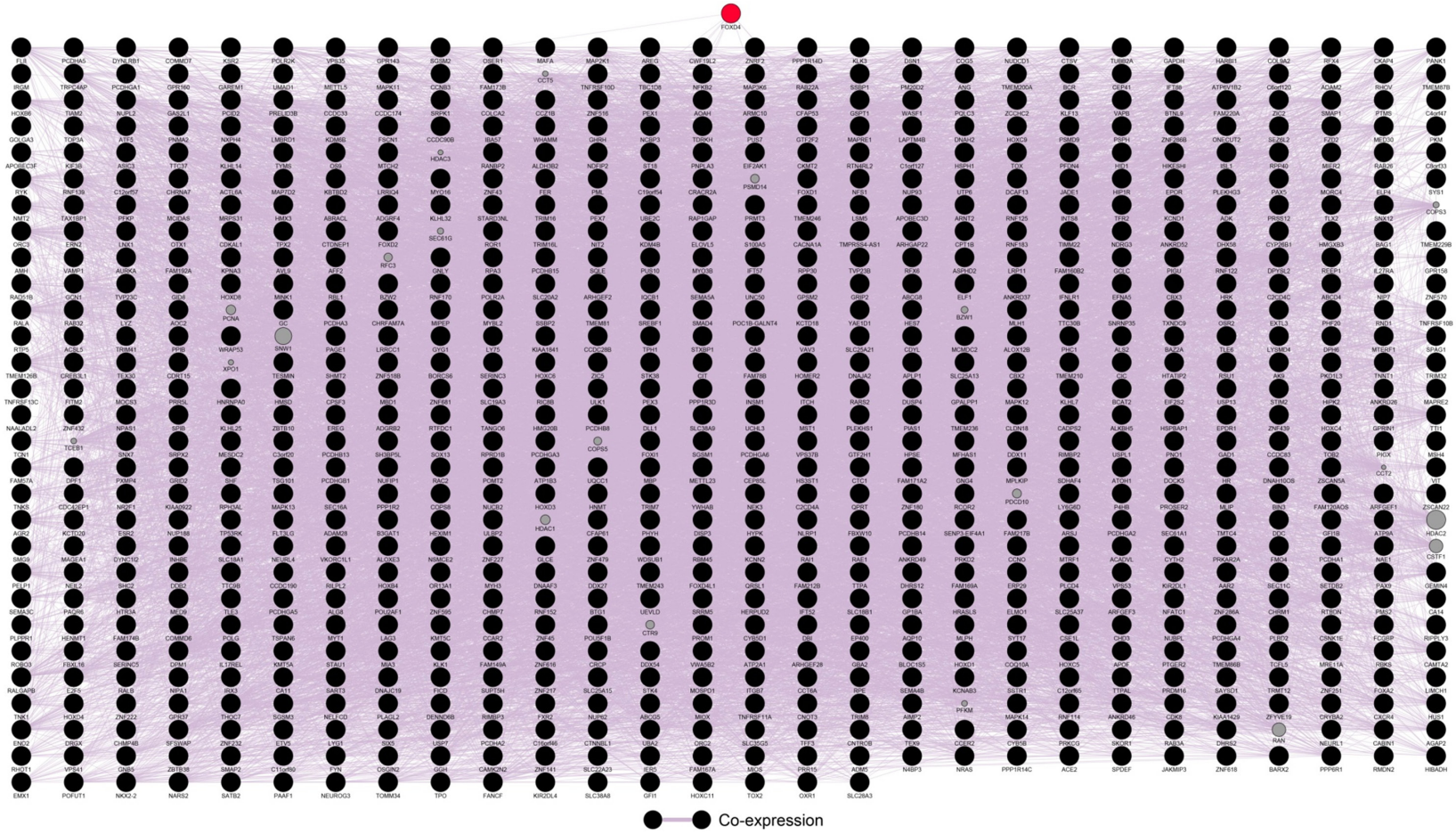

Figure 6. Co-expression network between FOXD4 and correlated genes by GeneMANIA. Abbreviations: FOXD4, Forkhead box D4.

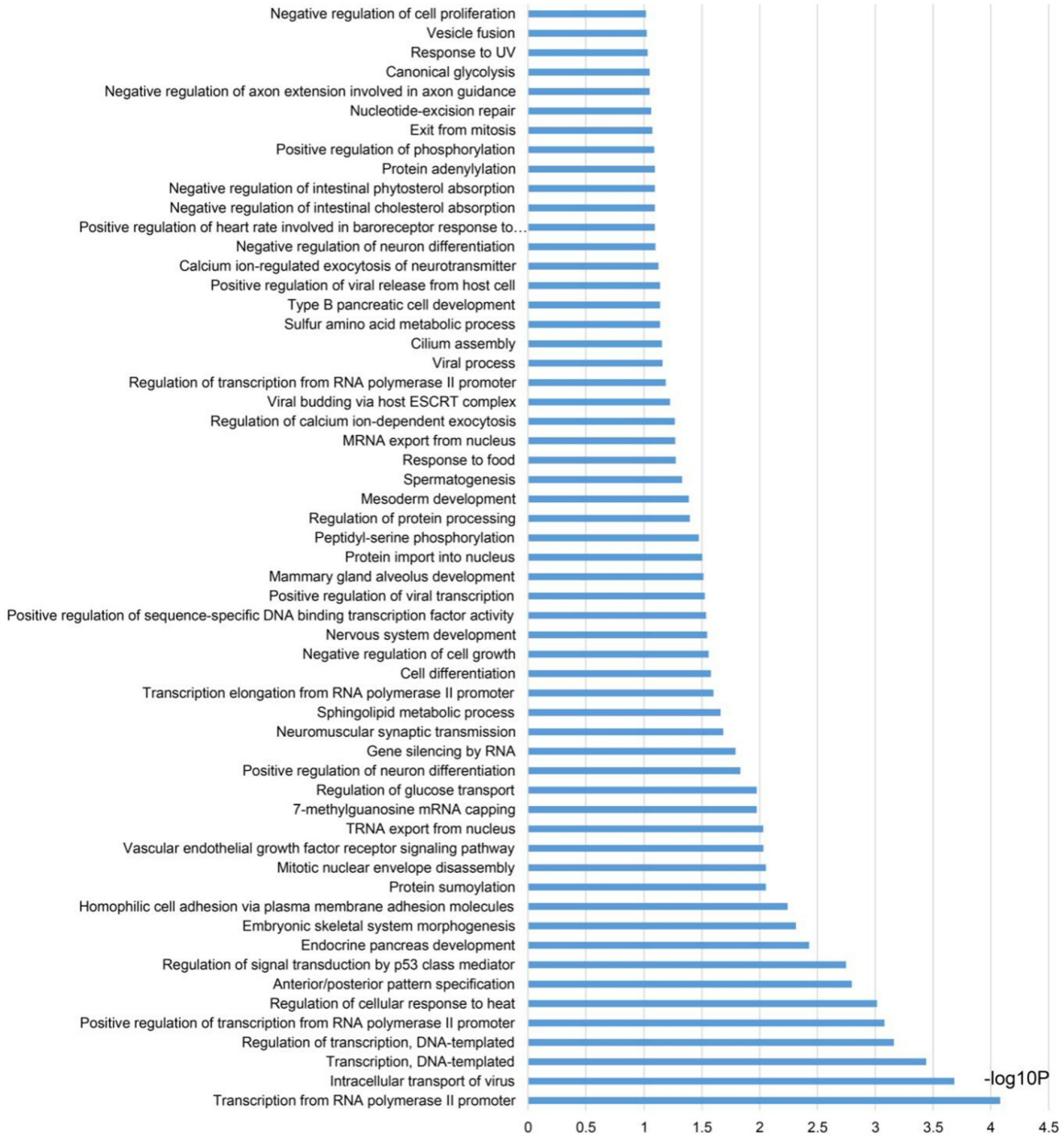

Figure 7. BP enrichment analysis of GO for FOXD4 and correlated genes by DAVID. Abbreviations: BP, biological process; GO, gene ontology; Forkhead box D4; DAVID, Annotation, Visualization, and Integrated Discovery.

6). CC, *FOXD4,* and correlated genes were involved in nuclear matrix, nuclear pore, nuclear envelope, and other cell components (**Figure 8A**). *FOXD4* and correlated genes were involved in Rap1 signaling pathway, RNA transport, and VEGF signaling pathway (**Figure 8B**). In MF, most of those genes were involved in factor and substance binding function (**Figure 8C**). GO results enriched by BiNGO were basically the same as those of DAVID (**Figure 9**).

At protein co-expression level, *FOXD4* was found to correlate with FOXD4L1, PPP2R2A, and PD gene families (**Figure 10**).

Discussion

Forkhead box protein (FOX) is a transcription factor (TF) that plays an indispensable role in development, organ formation, metabolic regulation, and immune system processes [11, 22,

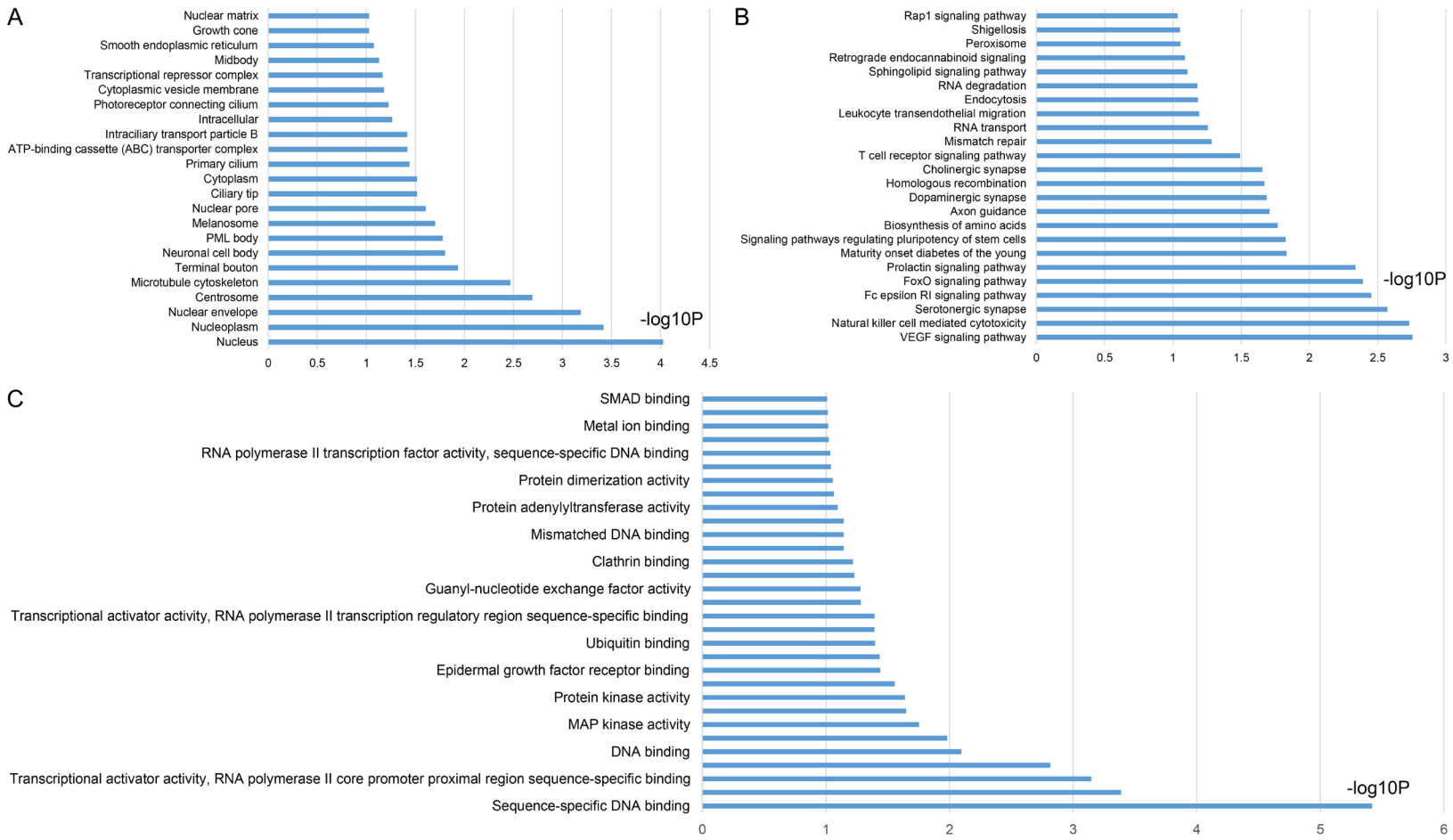

Figure 8. MF, CC of GO enrichment and KEGG enrichment analysis for FOXD4 and correlated genes by DAVID. (A) CC; (B) KEGG; (C) MF. Abbreviations: MF, molecular function; CC, cellular component; GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; Forkhead box D4; DAVID, Annotation, Visualization, and Integrated Discovery.

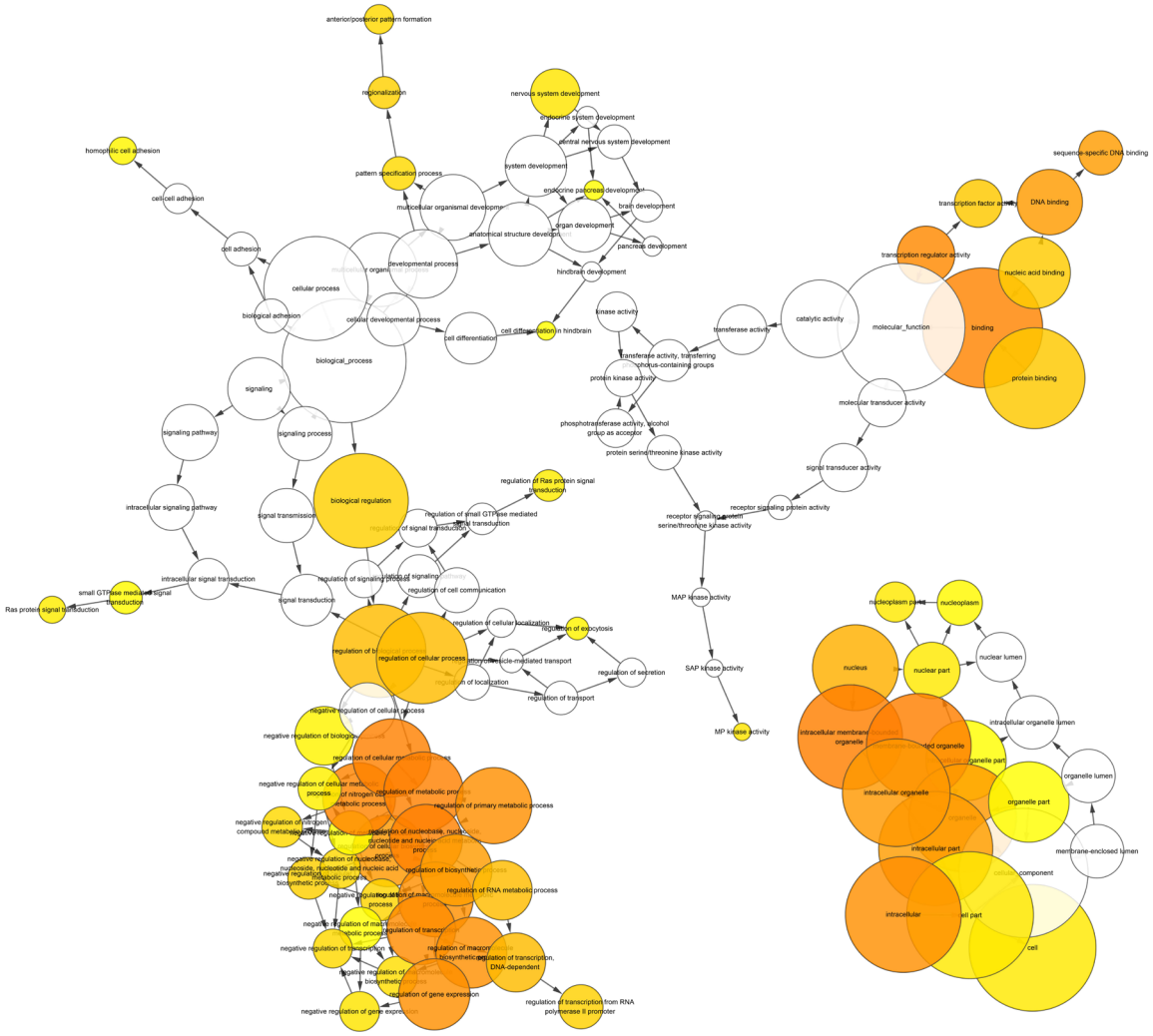

Figure 9. GO analysis for FOXD4 and correlated genes by BiNGO. Abbreviations: GO, gene ontology; Forkhead box D4.

23]. FOX genes are critical in multiple cancers, which could influence the tumor occurrence, development, and outcome [24]. At present, there are many studies on the FOX family genes, but research on *FOXD4* is still lacking.

Mutation of *FOXD4* was found to correlate with some non-cancer diseases, including dilated cardiomyopathy, obsessive-compulsive disorder, and suicidality [11, 12]. In recent years, most of the research on *FOXD4* has been on the role of embryonic overgrowth in the nervous system. It is reported that abnormal expression of *FOXD4* may cause anorectal malformations [25]. FOXD4 was also reported to be involved in establishing neural cell fate and for neuronal differentiation [26-28].

However, there were only a few reports that discussed the relationship between *FOXD4* and cancers. It is reported that *FOXD4* was expressed in human leukemia cell lines [13], which may play a role in leukemogenesis. Previous studies found that the *FOXD4* was up-regulated in colorectal tumor tissues and increased the metastatic ability of colorectal cancer cells [14].

In our study, we also found an up-regulated *FOXD4* in COAD tumor tissue and the patients with low expression of *FOXD4* had a favorable OS. In the clinical information level, tumor stage was an important factor for influence the prognosis of COAD. Survival rate decreases as tumor stage increases. In combination of tumor stage and *FOXD4* expression level by joint-effects survival analysis, patients with low *FOXD4* expression and advanced tumor stage have worse prognosis. In order to integrate all factors affecting the prognosis of COAD to facil-

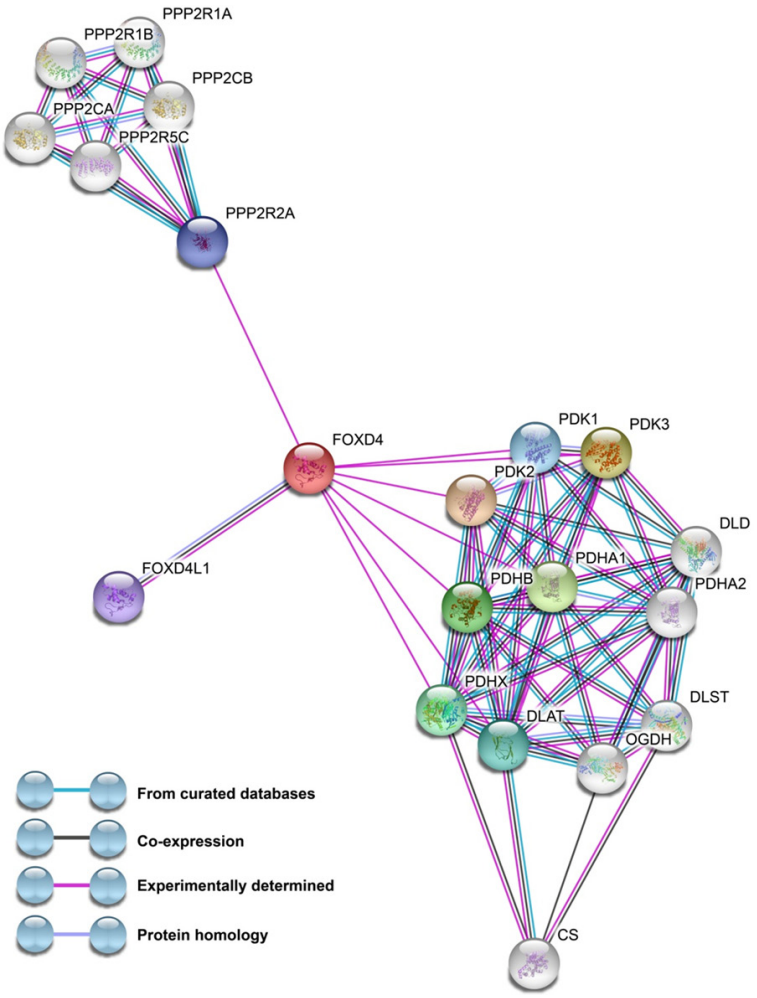

Figure 10. PPI network for FOXD4 by STRING. Abbreviations: PPI, protein-protein interaction; Forkhead box D4; STRING, Search Tool for the Retrieval of Interacting Genes/Proteins.

itate predicting prognosis, we established a nomogram model.

In the nomogram model, we found that tumor stage has a higher score compared with other risk factors, and by this model, every risk factor of an individual COAD patient could be scored. The total points reflected the 1-, 3-, 5-and 10-year survival rate; the higher the total score, the lower the survival rate. This nomogram model was significant for predicting the prognosis of patients with COAD.

The ROC curve showed that the expression level of FOXD4 also had a sensitive diagnosis value in COAD. Those results indicated that *FOXD4* could be used as prudential diagnosis and prognosis biomarker of COAD.

To further explain the value of *FOXD4* in the diagnosis and prognosis of COAD, the bioinformatics analysis was used. We found that KEGG pathway, *FOXD4,* and correlated genes were associated with some cancer related pathways, including VEGF signaling pathway [29], FOXO signaling pathway [30, 31], prolactin signaling pathway [32, 33], and sphingolipid signaling pathway [34]. The GO enrichment analysis results included several biological process for *FOXD4* and related genes, which could be used as a predictor of *FOXD4* and related genes in cancer, and an explanation of the mechanism. In the PPI network, *FOXD4* was found to correlate with *PD* family genes and *PPP2R2A*. It was found that the *PDK1* could act as an intriguing and underexplored target for cancer therapy [35, 36]. Inhibition of *PDHX* in breast cancer could promote cancer progression [37]. *PDHB* acted on cogenic effect as a target of a microRNA [38]. Down regulated *PPP2R2A* in ovarian cancer promoted cell proliferation and inhibited cell apoptosis [39]. These recent evidences have certain guiding significance for the future basic research value of *FOXD4* in COAD. We assume that the function of *FOXD4* in cancer was to influence the correlated genes in co-expression level, to affect the outcome of COAD patients.

This research also has some limitations. First, we only used the TCGA cohort. More cohorts should be included to test and verify those results. Second, in the mechanism prediction section, the results were only based on the bioinformatic analysis. Basic experimental research is needed to verify the result.

Conclusion

In this study, we found that the up-regulated *FOXD4* in COAD tumor tissue and the patients

with low expression of *FOXD4* had a favorable OS, which could be used as a prognostic biomarker. The different expression levels of *FOXD4* in COAD tumor tissue and normal tissue could be used as diagnostic biomarker. We also built a nomogram model, *FOXD4* expression level, age, and tumor stage were included, which could intuitively predict the survival rate of individual COAD patients. Those results require validation in future research.

Acknowledgements

This work was supported in part by National Nature Science Foundation of China (No. 81360200), Guangxi Science Foundation (No. Z2008150). The authors thank the contribution of TCGA (https://cancergenome.nih.gov/) and the UCSC Xena (http://xena.ucsc.edu/) database for sharing the COAD data with open access.

Disclosure of conflict of interest

None.

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Table S1. Demographic and clinical data for 438 COAD patients

Variables	Patients (n=438)	No. of events (%)	MST (days)	HR (95% CI)	Log-rank *P*
Gender					0.545
Male	234	54 (23.1%)	2475	Ref.	
Female	204	44 (22.6%)	N/A	1.131 (0.759-1.686)	
Age (years)					0.114
≥65	168	29 (17.3%)	2475	Ref.	
<65	268	116 (25.4%)	N/A	1.420 (0.919-2.194)	
Missing	2				
Tumor stage					<0.001
Advanced stage	187	62 (33.2%)	1711	Ref.	
Early stage	240	31 (12.9%)	3042	0.323 (0.210-0.498)	
Missing	11				

Abbreviations: COAD, colon adenocarcinoma; MST, median survival time; HR, hazard ratio; CI, confidence interval; N/A, not available.