Identification of the Dysregulated Pathways and Key Gene in Prostate Cancer by Transcriptome Analysis and Cell Biology Experiments

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Research Article

Keywords: Prostate cancer (PCa), Weight gene co-expression network analysis (WGCNA), FERMT2, Focal adhesion pathway.

Posted Date: January 4th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1122595/v1

Abstract

Background: Prostate cancer (PCa) is a common cancer in elderly men with the first increasing new cases and the second leading cause of cancer death, but the molecular mechanisms underlying the pathogenesis of prostate cancer remain unclear.

Methods: Here we mainly used Weight gene co-expression network analysis (WGCNA), Kyoto Encyclopedia of Genes and Genomes (KEGG), protein to protein interaction (PPI), gene set enrichment analysis (GSEA) and cell biology experiments to analyze prostate cancer data in GEO and The Cancer Genome Atlas (TCGA) databases and revealed the main dysregulated pathways and key genes in prostate carcinogenesis.

Results: We found that the focal adhesion pathway was the main pathway in PCa. *FERMT2* was shown to be the key gene for prostate tumorigenesis both in GSE6919 and TCGA datasets. By using WGCNA and GSEA analysis, we found that *FERMT2* was related to the focal adhesion pathway and the ECM interaction pathway. Cell biology experiments demonstrated that FERMT2 inhibited tumor cell proliferation and migration.

Conclusion: Our findings revel that downregulation of *FERMT2* and the focal adhesion pathway are the main characteristics of PCa and *FERMT2* might be the potential biomarker or treatment target for PCa.

Trial registration: The study is not a clinical trial.

Introduction

Prostate cancer (PCa) is one of the most common cancers in men in the United States with the first increasing new cases (21% of newly diagnosed cancers) and the second leading cause of cancer death (an estimated 333300 deaths in 2020) [1]. Most early stages of PCa can be cured by prostatectomy or radiation therapy, but a small percent of patients will develop into castration resistant prostate cancer which leads to fatal metastasis [2].

Some studies have revealed a preliminary understanding of the molecular mechanisms about the occurrence and progression of PCa. The recurrent *TMPRSS2-ETS* family fusion was thought to be primarily characteristic of prostate cancer [3]. ETS family genes, such as *ERG*, *ETV1* and *ETV4*, were found to be sustained activation by fused with the 5'-untranslated region of *TMPRSS2* to promote prostate cancer proliferation and invasion [3–5]. In addition to *TMPRSS2*, three other androgen responsive genes *SLC45A3*, *NDRG1* and *HEPPUD1*, were demonstrated to fuse with *ERG* [6–8]. The incidence of ETS fusion was up to 70% in prostate cancer [9], whereas one study found only 5–10% of samples to be fused with ETS [10]. *BRCA2*, which involved in maintenance of genome stability, was characterized as an important prognostic factor for PCa and found to be mutated but with a small percent (1%-2%)[11, 12]. Somatic mutations in *TP53*, *RB1*, *MYC*, *PTEN* and *AR*, have been discovered in

prostate cancer [13]. However, only 15% of patients with poor prognosis have such mutations [14], the molecular mechanism and pathological process of PCa remain unclear.

Prostate-specific antigen (PSA) in serum was found to be upregulated in prostate cancer and taken as a good standard of diagnosis or screening the early-stage prostate cancer, but was controversial because PSA is a prostate tissue specific and not a PCa specific biomarker [15]. A 5 genes model containing *PDGFR-β*, *HOX6C*, *ITPR3*, *Chromogranin A* and *Sialyltransferase I*, was suggested to be positive related to prostate cancer recurrence [16]. Acetylation and demethylation of Histone3 and Histone4 in primary PCa could be used to predict recurrence independent of PSA level [17]. *EZH2*, a member of polycomb repression group complexes, was found to be significantly correlated to prognosis of prostate cancer patients [18]. Although genes listed above have been suggested to be used as the biomarkers for PCa, the specificity and sensitivity of their detection are not sufficient. It is urgent to discover the new biomarkers for PCa.

Weight gene co-expression network analysis (WGCNA) is a method to analyze gene co-expression network [19]. It mainly divides some genes with similar expression into one module and links the module with clinical traits so as to discover the hub genes. Here we mainly used WGCNA and gene score enrichment analysis (GSEA) to reveal the molecular mechanism of PCa pathological process, and we discovered the hub gene *FERMT2* relating to PCa. Cell biology experiments showed that *FERMT2* inhibited the proliferation and migration of prostate cancer cell lines PC3. *FERMT2* might be the potential biomarkers for diagnosing and give the insight for development of the therapeutical approaches for PCa.

Materials & Methods

Data collection and preprocessing

GEO datasets GSE6919 (18 normal, 63 adjacent, 65 tumor) were download by R package GEOquery and converted from FPKM to TPM. Prostate cancer transcriptome data from TCGA (51 normal, 499 tumor) was download in FPKM format, and converted to TPM format. The flow chart of the analysis is shown in Figure 1.

Differentially expressed genes (DEGs) screening

The R package limma was used to screen the DEGs between each group. The DEGs threshold was set as rules P value<0.05 and |log2(foldchange)|>1. Ggplot2 package was used to plot the boxplot and heatmap.

Co-expression networks construction

Weighted correlation network analysis (WGCNA) was used to co-expression analysis. The top 25% of variance genes were used for analysis. K-core was used to determine the central and core of gene in a network. The soft threshold of each analysis was shown in supplementary figures legends. The key

modules in GSE6919 from WGCNA were defined as rules: a. positive correlation to normal and adjacent group, and negative correlation to tumor group; b. negative correlation to normal and adjacent group, and positive correlation to tumor group.

Gene set enrichment analysis (GSEA)

Prostate tissue datasets were generated in R program and then inputted into GSEA 3.0 software. The number of permutations was set at 1000, permutation type was set at phenotype.

Protein-protein interaction (PPI) network construction

Genes in turquoise module were inputted to STRING search tool (https://string-db.org/), the confidence score was set at 0.90.

Cell and culture

PC3 cells were cultured in DMEM (Thermo Fisher, America) containing 10% fetal bovine serum (Life Technology, America) and 1% antibiotics (100 U/ml penicillin and 100 ug/ml streptomycin) (Life Technology, America) at 37 ℃ and 5% CO_2 atmosphere.

Cell growth

PC3 cells transfected with Flag or Flag-FERMT2 plasmids were inoculated into 96 well plates, and 1000 cells were placed in each well. After cells adhered to the wall, CCK8 reagent (DOJINDO, Japan) was added to each pore, then incubated at 37 ℃ for 1h, and the absorbance value at 450 wavelength was measured at 0, 1, 2, 3 days.

Wound healing assay

PC3 cells transfected with Flag or Flag-FERMT2 plasmids were seed on 6 well plates for 90% convergence degree, and marked off a line by 10 ul tips. The scratches were photographed at 0 h and 48 h respectively.

Colony formation

Five hundred PC3 cell transfected with Flag or Flag-FERMT2 plasmids were seed into 6 well plates and cultured at 37 ℃ for 10 days, and dyed with crystal violet.

Western blotting

Total protein was extracted with cold RIPA lysis buffer. 40 ug proteins per lane was fractionated by sodium dodecyl sulfate-poly-acrylamide gel electrophoresis (SDS-PAGE), transferred onto PVDF membrane (Millipore, America). The membrane was blocked with 5% skimmed milk and incubated with the primary antibody (anti-DDDDK tag antibody, 1:1000, ab1162, Abcam, America; anti-GAPDH antibody, 1:1000, ab8245, Abcam, America) at 4 ℃ overnight, followed by second antibody (goat anti-rabbit IgG-HRP, 1:5000, sc-2301, Santa Cruz, America; goat anti-mouse IgG-HRP, 1:10000, G21040, Thermo Scientific,

America) incubation for 1 h at room temperature. The blots were developed with the ECL system (Thermo Scientific, America) and exposed to X-ray film.

Results

Identification of the hub modules in PCa.

Although PCa is a tumor with high occurrence and good prognosis, its molecular mechanism of tumorigenesis is still unclear. To explore the key molecules in the pathogenesis of PCa, WGCNA was used to analysis the GEO dataset GSE6919 (supplementary Figure 1). Fourteen modules were screened and shown in Figure 2A with the trait normal, adjacent and tumor. The turquoise and magenta modules were picked out as the key modules and shown in Cytoscape (Figure 2B and Figure 2C). Obviously, the magenta module was enrichment in ribosome pathway.

The focal adhesion pathway was the key dysregulated pathway in PCa.

To further clarify the molecular mechanism of PCa, genes from turquoise module were analyzed by enrichGO or enrichKEGG function of clusterProfiler packages in R software, respectively[20]. GO analysis results were enriched in the extracellular structure organization (Figure 3A). The focal adhesion pathway was found to be the foremost pathway by KEGG analysis (Figure 3B). In order to investigate the proteins interaction in turquoise module, the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database was applied for revealing the core protein-protein interaction network. The focal adhesion pathway was also found to be the foremost enriched pathway (Figure 3C). Furthermore, GESA results shown that the focal adhesion pathway ranked the top enriched pathway (Figure 3D and supplementary table 1).

FERMT2 was Screened out as the key gene of PCa.

Genes in key module were candidate for screening the key gene. The turquoise module contained 1450 genes. These genes were sorted by its module membership to turqupise module (MMturqupise), and the top 10 genes were listed in supplementary table 2. FERMT2 was found to be most correlated to turquoise module (R = 0.928), also FERMT2 was positive correlated to normal tissue (GS=0.287, P=0.001) and negative correlated to tumor (GS=-0.184, p=0.028). Take the results from Figure 2C together, FERMT2 was considered as the key gene in PCa.

Validation the main disorder pathway and key gene in TCGA PRAD dataset.

The possible main disorder pathway (Focal adhesion pathway) and key gene (FERMT2) was screened from GSE6919 dataset by using WGCNA, we wanted to see if we could get the same results in other datasets. TCGA prostate adenocarcinoma (PRAD) dataset was performed analysis as the same with GSE6919. Eleven modules were obtained from WGCNA analysis. The green module was the most positive relevant module (r=0.29, p=5e-12) and the brown module was the most negative relevant module (r=-0.5, p=7e-36) in tumor (Figure 4A). The genes in green module were enriched in the ribosome pathway. The

brown module was shown in Figure 4B and mainly enrichment in the focal adhesion pathway (supplementary table 3). FERMT2 was also found to be one the hub genes in TCGA PRAD dataset (Figure 4B), which is consistent with the results from GSE6919 dataset.

FERMT2 was downregulated in pan-cancers and related to focal adhesion pathway.

The expression of FERMT2 in GSE6919 and TCGA were plotted by boxplot in Figure 5A and Figure 5B. FERMT2 was found to be downregulated in PCa tumor tissues compared with controls. To overview the profile expression of FERMT2, expression of FERMT2 in the TCGA cancers with tumor and normal samples was shown in Figure 5C. FERMT2 was found to be downregulated in 19 out of 24 cancers indicating that it is a common feature of tumors. To investigate the relationship of the key gene FERMT2 and the main disorder pathway focal adhesion pathway, genes were sorted according its correlation with FERMT2 and analysis by GSEA. The focal adhesion pathway was found to be shown in top 10 pathways (supplementary table 4).

FERMT2 inhibited the proliferation and migration of PC3 cells.

In order to determine the effect of FERMT2 on the proliferation and migration of PCa cells, Flag-FERMT2 plasmid was constructed and transferred into PC3 cells (Figure 6A). Overexpression of FERMT2 inhibited PC3 cell proliferation (Figure 6B) and Clonogenesis (Figure 6C). We also found that FERMT2 can inhibit PC3 cells migration.

Discussion

Prostate cancer (PCa) is the leading cause of morbidity and mortality in the United States [1, 21], but the molecular mechanism of its occurrence is still not clear. Here, by using the bioinformatics tools to analyze PCa transcriptome data from GEO and TCGA datasets, we revealed that the focal adhesion pathway was the main disorder pathway and *FERMT2* were the key gene in prostate tumorigenesis.

Focal adhesion pathway has an important role in tumorigenesis, such as cell adhesion mediated drug resistance (CAMDR) and cell adhesion mediated radioresistance (CAMRR) [22, 23]. Proteins in focal adhesion pathway participated ECM connection and maintained cell's morphology and cytoplasmic signaling, which is vital for cell survival, proliferation and motility[24]. Depletion of β4 integrin, an adhesion molecules for ECM proteins, delayed the development of breast cancer by down regulated erbB2 and STAT3 [25]. Whereas overexpression of α2β1 promoted breast cancer cells differentiated and reduced proliferation [26]. We found that focal adhesion pathway was the main down regulated pathway in PCa by using KEGG, PPI and GSEA analysis, but the definite mechanism need to be further studied.

FERMT2, also named Kindlin-2, was a member of the kindling protein family and played an essential role in activating integrin [27]. FERMT2 localized in focal adhesion is associated to regulate cell-ECM adhesion [28], whereas the function of FERMT2 localized in nucleoplasm remain unclear. *FERMT2* was upregulated and considered as a tumor promoter in cancers such as breast cancer [29–31], pancreatic

ductal adenocarcinoma [32] and malignant mesothelioma [33]. Here we found that *FERMT2* was downregulated in PCa in 2 datasets and related to tumorigenesis, indicated that it could be functioned as a tumor suppressor. Immunohistochemical analysis of FERMT2 in 34 breast cancer tissues showed that about 50% samples lost FERMT2 expression [34]. Reduced or almost absent expressions of *FERMT2* were found in colonic carcinoma cell lines such as HCT116, LoVo, DLD-1 and HT-29 [35]. FERMT2 could downregulate the level of secreted urokinase-type plasminogen activator to suppress mesenchymal cancer cell invasion [36]. It seems that FERMT2 to be either a tumor suppressor or tumor promoter. Some proteins such as FHL1 could shuttle between the cytoplasm and the nucleus to shift from the tumor suppressor to the tumor promoter [37]. We found that FERMT2 was the key gene both in GSE6919 and TCGA PRAD datasets. KEGG, PPI and GSEA analysis showed that the major disordered genes were enriched in the focal adhesion pathway, which is consistent with the function of FERMT2. Considering that FERMT2 is located in the cytoplasm and the nucleus. The function of FERMT2 in nucleus is not clear, FERMT2 might also exert its dual functions through shuttle between the cytoplasm and the nucleus.

The main limitation of the study was that it is bioinformatics analysis and verified by simply cell biology experiments. Some molecular biology experiments may support the result better [38]. Clinical experimental data may be more able to verify the conclusions of this study. In summary, we have screened out the key gene and the dysregulated pathway in the tumorigenesis of PCa, which might provide the great insights for development the treatments and diagnoses of PCa in clinical.

Conclusions

In this work, we found that focal adhesion pathway was the main dysregulated pathways and the gene FERMT2 was the key gene in PCa by analyzing GEO datasets. Then we validated the results through analyzing TCGA PRAD data. Finally FERMT2 was found to inhibit PC3 cell proliferation and migration. These findings may contribute to the diagnosis and treatment of PCa.

Abbreviations

PCa
prostate cancer
WGCNA
Weight gene co-expression network analysis
KEGG
Kyoto Encyclopedia of Genes and Genomes
PPI
protein to protein interaction
GSEA
gene set enrichment analysis
TCGA

The Cancer Genome Atlas
DEGs
Differentially expressed genes
FPKM
Fragments per Kilobase Million
TPM
Transcripts Per Million

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Authors confirmed that this work can be published. The content of this manuscript is original and it has not yet been accepted or published elsewhere.

Availability of data and materials

Relevant data have been presented within the manuscript and additional files.

Competing interests

The authors declare that they have no competing interests.

Funding

The study was not funded by any funds.

Authors' contributions

SWL and GYX conceived and designed the study; SWL, CZ and GJ performed the analysis procedures. SWL, ZY and GYX contributed to writing of the manuscript. All authors reviewed the manuscript.

Acknowledgements

We appreciate the TCGA PCa datasets and GEO dataset (GSE6919) for free using.

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Supplementary Tables

Supplementary Tables 1-4 are not available with this version.

Figures

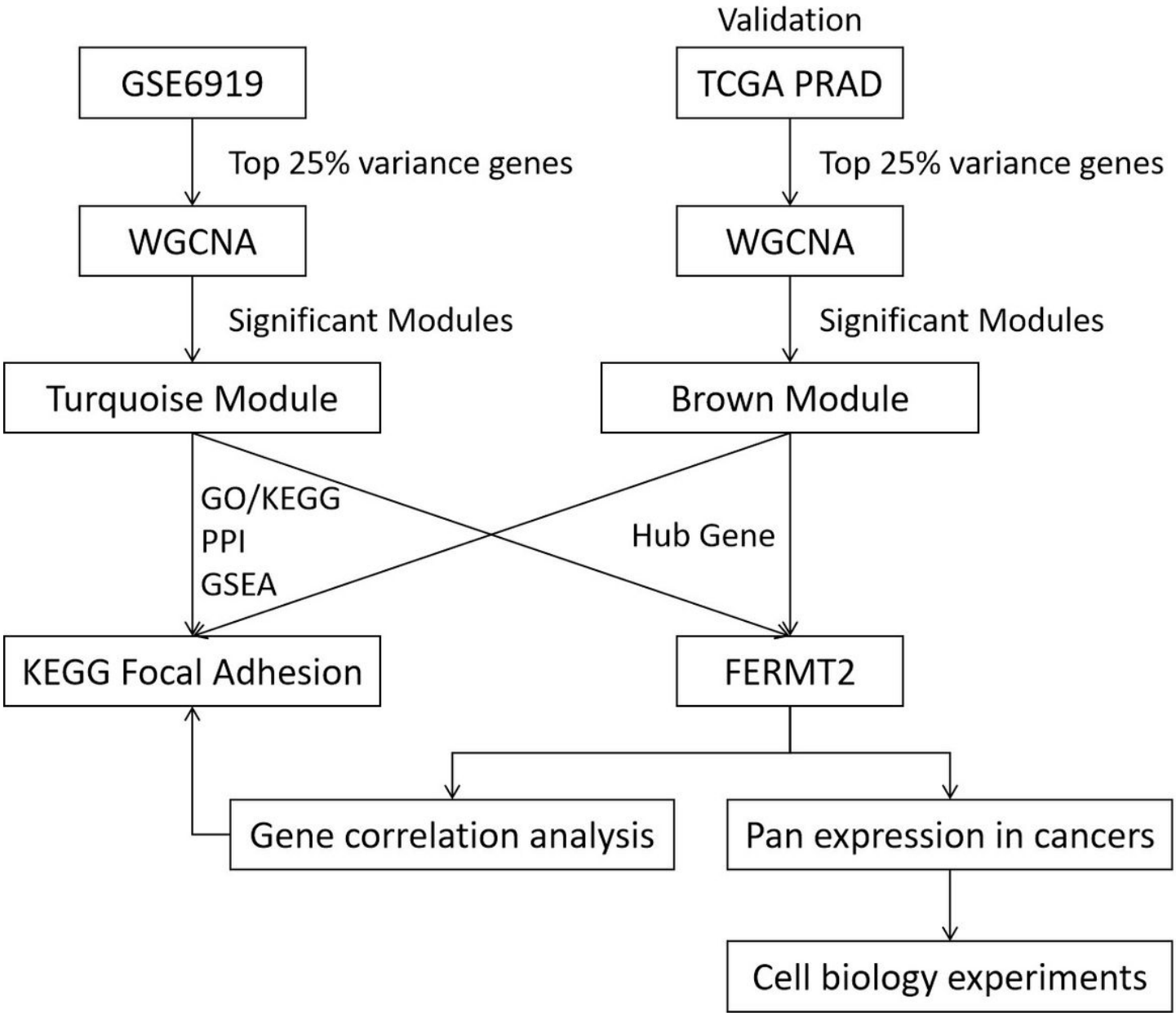

Figure 1

The flow chart of this research.

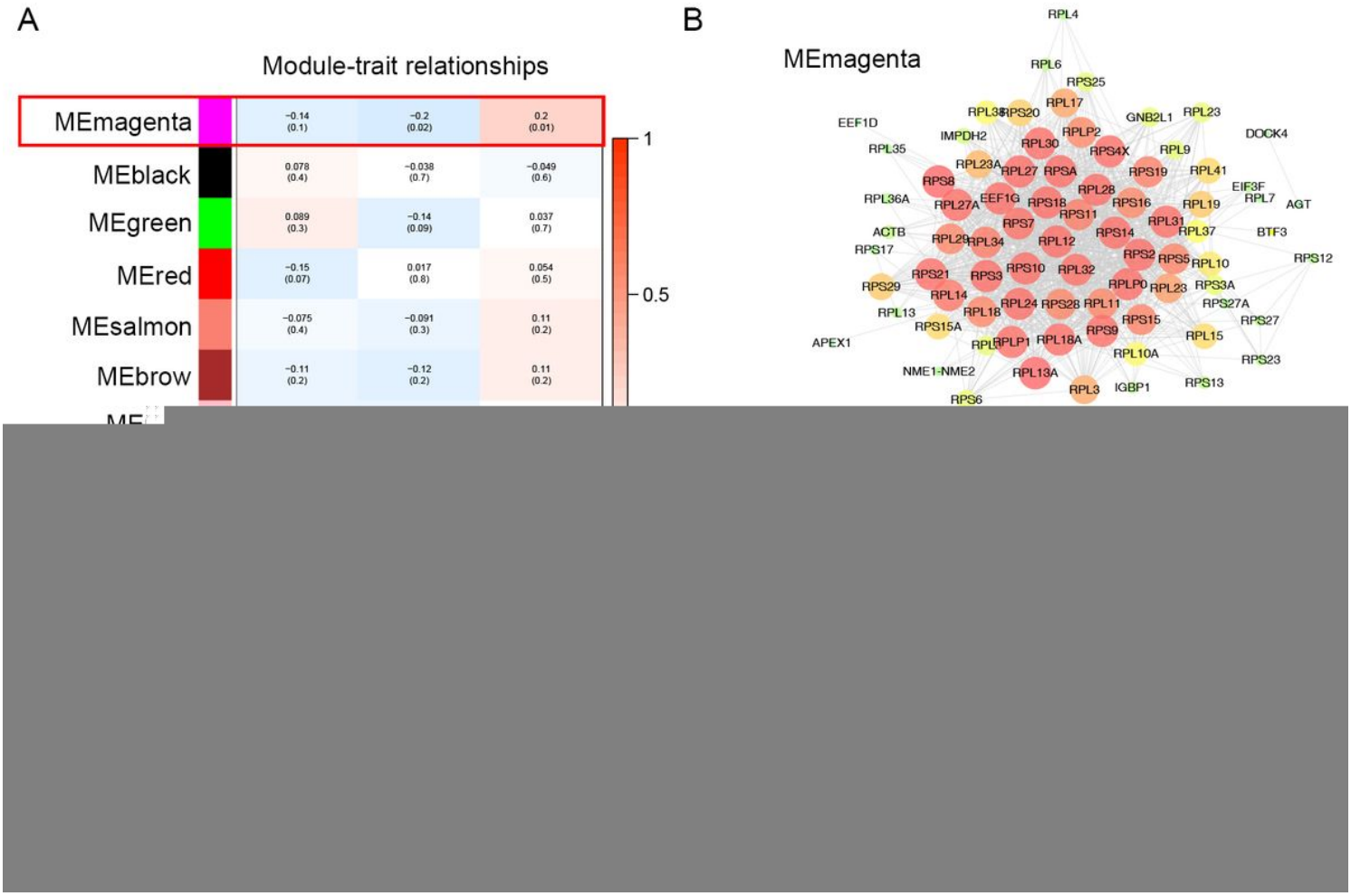

Figure 2

WGCNA analysis of genes in prostate cancer datasets. A. Module-trait relationships in GSE6919. The numbers in each rectangular show the Pearson correlation coefficients and the P values. B and C. The magenta module and the turquoise module from A were constructed by Co-expression networks in Cytoscape respectively. The size and color of the nodes represent the importance of nodes.

Figure 3

Main dysregulated pathway in prostate cancer. A. GO analysis of the turquoise module from Figure2. Genes of turquoise module were analyzed by R package ClusterProfiler，only top 10 GO items were draw. B. KEGG analysis of the genes in turquoise module as A. C. PPI analysis of genes in turquoise, the highest confidence score was set at 0.9. D.GSEA analysis of genes in turquoise module. The most enriched pathway focal adhesion pathway was drown by R package.

Figure 4

WGCNA analysis of genes in TCGA prostate cancers. A. Module-trait relationships in TCGA prostate cancer dataset. B. The brown module from A was shown in Cytoscape as Figure 2.

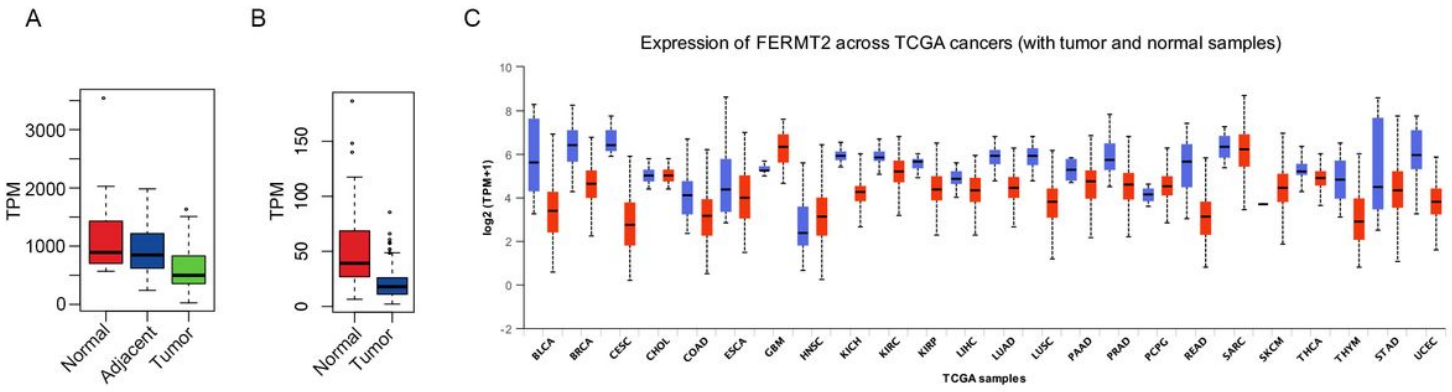

Figure 5

Expression of FERMT2 in cancers. A. Expression of FERMT2 in GSE6919 datasets. TPM: transcripts per million. B. Expression of FERMT2 in TCGA prostate cancers. C. Expression of FERMT2 across TCGA cancers, Red represents tumor samples, blue represents normal samples..

Figure 6

FERMT2 inhibited the proliferation and migration of PC3 cells. A. FERMT2 was tagged with Flag and cloned into PCI-neo vector. The Flag-FERMT2 vector was convery into PC3 cells and Western blot was used to show the Flag-FERMT2 expression. B. PC3 cells were transfected with Control or FERMT2 plasmid, CCK8 was added to cells at different time points and absorbance at 450 nm was measured. This experiment was repeated three times in duplicate and growth curves were plotted with the mean ± SD of absorbance at 450 nm vs. time points. C. Five hundred cells from Flag-FERMT2 transfected PC3 cells were seeded in 6 well plates. Cells were grown for 10 days and cell clones were stained with Coomassie Bright Blue after fixation with ethanol/acetic acid. D. Wound-healing experiments were performed with PC3 cells transfected with Flag-vector or Flag-FERMT2.

Supplementary Files

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- SupplementaryFigure.docx