The Expressions of NMU, PPBP and GNG4 in Colon Cancer and Their Influences on Prognosis

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Abstract

Objective

This study aims to identify the core genes that influence the prognosis of colon cancer, and analyze their relationships with clinical characteristics.

Methods

The gene expression profiles were downloaded from The Cancer Genome Atlas (TCGA) database. The differentially expressed genes (DEGs) were identified. The top ten core genes were selected by bioinformatics tools, and screened through the Oncomine database. The expressions of core genes in colon cancer cells and tissues were validated by immunohistochemistry, immunoblotting and quantitative real-time polymerase chain reaction.

Results

There were 1665 DEGs that have been identified from the TCGA database. Bioinformatics analysis found that GNGT1, NMU, PPBP, AGT, and GNG4 were differentially expressed in colon cancer tissue. Over-expression of NMU, PPBP, AGT, and GNG4 in colon cancer was associated with shortened survival time ($p<0.05$). In the validation studies, the high expression levels of NMU, PPBP and GNG4 in colon cancer cells and tissues were confirmed as compared to the control groups ($p<0.05$), and were adverse prognostic markers ($p<0.01$). The combination prognostic model of the three core genes predicted the 1, 3, and 5-year survival of colon cancer with the AUC of 0.868, 0.635 and 0.770, respectively.

Conclusions

The high levels of NMU, PPBP, and GNG4 were associated with poor prognosis in colon cancer. The combination prognostic model of these three genes could be a new option.

Introduction

In 2021, colon cancer (CC) is the third leading cause of death[1]. The relevant signaling pathways of CC including Wnt/β-catenin[2], PI3K-Akt/mTOR[3,4], MAPK[5], p53[6] and NF-κB[7] and etc. Molecular markers for predicting the prognosis of colon cancer patients are becoming increasingly important. Bioinformatics is a combination of biological and informatics methods to identify key disease-causing factors, so to facilitate the exploration of new treatment methods and ultimately solve challenging medical problems, such as cancer. There are growing number of researches using the public databases, such as TCGA[8] and Oncomine[9]. Statistical tools, such as R data analysis package, Cytoscape visualization software, provide researchers with more intuitive methods for bioinformatics analysis [10,11]. Gene Ontology (GO)[12] and Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genetic.jp/)[13] is a widely regarded as useful tools for genetic analysis. The Database for Integrated Discovery, Visualization and Annotation

(DAVID, http://david.abcc.ncifcrf.gov/)[14] contains comprehensive biological knowledge and series of analysis tools that can be used to extract genetic biological information. These databases and bioinformatics tools provide a lot of information for tumor research and also contribute to precision medicine.

This study is carried out to to identify the DEGs of human colon cancer and analyze their relationships with the clinical characteristics. We also evaluate the impact of these genes on clinical prognosis.

Materials And Methods

Differential expression genes screening, signaling pathway enrichment and functional enrichment analysis

The study design was demonstrated in the flow diagram (Figure 1). The entire clinical and mRNA data of colon cancer and tumor-adjacent tissues were achieved from the TCGA database for DEG screening. R software was used to convert gene expression into numerical values, average repeated genes, filter low-expressed genes, and draw heat maps and volcano maps. False Discovery Rate (FDR) < 0.05 and Fold Change ($|\log_2 FC|$) > 2 were set as the criteria to screen statistically significant DEGs. The KEGG pathway enrichment and the GO enrichment analysis of the hub genes were executed in the DAVID online tool. The statistical significance was defined with P value beneath 5%.

Protein-protein interaction (PPI) network construction

Import DEGs into STRING[15] online tool (https://string-db.org/) to assemble a network of PPI. The criteria for protein interaction screening were set (confidence 0.9) . The network was reconstructed by degree algorithm of cytohubba in Cytoscape software v3.7.1[16] (http://www.cytoscape.org). This module was applied to obtain the top ten hub genes.

Oncomine database screening and survival analysis

In order to identify the overlap key genes across Oncomine (www.oncomine.org) and TCGA database, the hub genes obtained from the cytohubba module were searched in the Oncomine database for cross selection. Therefore, the genes screened out both in TCGA and Oncomine were obtained.

Cell culture and qRT-PCR

Human normal colonic epithelial cells (NCM460) and human colorectal cancer cells (RKO, SW480, SW620, HCT116 and COLO678) were obtained from Cell Bank of National Collection of Authenticated Cell Cultures (Shanghai, China). All cell lines were cultured in high glucose DMEM containing 10% fetal bovine serum, 1% Penicillin-Streptomycin and maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

EZ-press RNA purification kit was used to extract cellular RNA. The reverse transcription kit was used to synthesize cDNA, and qRT-PCR assays were performed on the LightCycler® 96 thermal cycler. The primer sequences were shown in Supplementary Table S1. The reagents were purchased from the EZBioscience, USA. The work was carried out according to the instructions.

Immunohistochemistry

Tissue microarray sections containing 69 samples of colon cancer and 55 samples of adjacent tissue were purchased from Weiao Biotechnology (Shanghai, China). The clinical characteristics of the patients were shown in Supplementary Table S2. Xylene was used to deparaffinize and the graded ethanol dilutions were used to rehydrated tissue microarray sections. Followed by incubation at 4℃ overnight with rabbit anti-NMU, anti-PPBP, and anti-GNG4 primary antibody, the peroxide sections were then blocked by 3% hydrogen. After incubation with secondary antibody at room temperature, sections were stained by Diaminobenzidine (DAB) and counterstained with hematoxylin. Based on the proportion of positive cells and the integrated staining intensity，the staining scores were evaluated by two pathologists independently. The final score ranged from 0 to 12. The samples with scores of 6-12 were defined as high expression , while the samples with scores of 0–5 were defined as low expression .

Immunoblotting

Total proteins were extracted from the above-mentioned cells with RIPA buffer (Beyotime, China) that contained protease and phosphatase inhibitors (Beyotime, China). Followed by centrifugation of protein lysates , the bicinchoninic acid assay kit (Pierce, USA) was used to determine the concentration of the supernatants.. The final protein lysates were boiled at 100°C for 5 min with 5× loading buffer (Beyotime, China), and separated through 15% SDS polyacrylamide gel (Fdbio science, China). The gel was transferred onto polyvinylidene fluoride (PVDF) membrane (Bio-Rad, USA). The membrane was blocked in Tris-buffered saline containing 0.1% Tween 20 (TBST)，which was added with 5% skimmed milk for 2 h at room temperature and incubated with antibody against NMU (1:1000, DF4238, Affinity Biosciences, USA), PPBP (1:1000, DF6695, Affinity Biosciences, USA), GNG4 (1:1000, DF9560, Affinity Biosciences, USA), and GAPDH (1:1000, cat. No.5174, Cell Signaling Technology, USA) at 4°C overnight, respectively. After washing with TBST, the PVDF membrane was incubated with methanol for 1 minute. The enhanced chemiluminescence (ECL) substrate (Bio-Rad) was used for detecting and GelView 6000Pro (BLT, Guangzhou, China) was used for digitizing immunoblot.. Band densities were semi-quantified by the ImageJ 1.52.

Statistical analysis

Spearman correlation was used to analyze the relationship between different parameters. The overall survival was exhibited by the Kaplan–Meier method. The area under the curve (AUC) and the receiver operating curve (ROC) were applied to assess the accuracy of genes for predicting prognosis. The levels of relative gene expression were calculated using the comparative threshold cycle ($2^{-\Delta\Delta Ct}$) method. Data

were analyzed using the SPSS 26.0, GraphPad Prism and R software. $P < 0.05$ (bilateral) was considered statistically significant.

Results

Identification of DEGs in colon cancer

This study collected mRNA and clinical information of 480 colon cancers and 41 adjacent tissues from TCGA database. A total of 18449 mRNAs were obtained, and 1665 mRNAs were found differentially expressed, of which 911 were up-regulated and 754 were down-regulated (Figure 2A).

We then performed GO and KEGG enrichment analysis on 1665 genes using the DAVID web tools. The GO analysis exhibited that DEGs were primarily involved in seven biological functions (Figure 2B), including nucleosome assembly, coagulation, digestion, cellular protein metabolism, cell adhesion, cell signal transduction and sodium ion transport. The KEGG analysis indicated that DEGs were involved in many biological pathways closely related to cancer progression, such as drug metabolism-cytochrome P450, neuroactive ligand-receptor interactions, alcoholism, chemical carcinogenesis, and metabolism of cytochrome P450 heterologous organisms (Figure 2C). These findings suggested that the DEGs participated extensively in oncogenesis and progression of colon cancer.

Identification of key genes for prognostic evaluation

We put the proteins encoded by DEGs into STRING tool to construct a PPI network that was composed of 1608 nodes and 3084 edges (Supplementary Figure 1A). After the PPI network was optimized using Cytoscape, the degree algorithm in Cytohubba was used to screen the top ten hub genes. The correlation between the hub genes (Figure 2D) and gene rankings (Table 1) were obtained. The top ten hub genes were then imported into the Oncomine database searching for key genes. GNGT1, NMU, PPBP, AGT, and GNG4 were found differentially expressed in colorectal cancer in the Oncomine database, while the expression of GNG13, LPAR1, NMUR2, CASR and PENK was not significant (Figure 2E).

The clinical characteristics of the colon cancer cohorts in the TCGA database were extracted, and survival analysis was performed with the five key genes respectively (Figure 2F). Among these, the increased expression of NMU, PPBP, GNG4, and AGT indicated a poor prognosis ($p<0.05$), while the increased level of GNGT1 did not affect the survival ($P>0.05$) (Supplementary Figure 1B).

Expression of the key genes in colon cancer cells and tissues

Quantitative RT-PCR was used to detect the mRNA expression levels of key genes GNG4, NMU, PPBP and AGT in colon cancer cells and normal colon epithelial cells. As shown in Figure 3A, the expressions of NMU, GNG4, and PPBP mRNA in colon cancer cells were significantly higher than those in normal colon epithelial cells ($p<0.05$), while the expression of AGT mRNA was similar among different groups ($P> 0.05$). Then, the protein expressions of GNG4, PPBP and NMU in colon cancer cells and normal epithelial

cells were examined (Figure 3B). We found that both the mRNA and protein expressions of GNG4, PPBP and NMU were increased in colon cancer cells as compared to normal epithelial cells.

To confirm the expression of the 3 key genes in human colon cancer, we detected the expressions of the GNG4, PPBP and NMU in a tissue microarray including 69 CC and 55 adjacent normal tissues by immunohistochemistry. The results showed that the positive staining of NMU and GNG4 was mainly located in the cytoplasm, while the PPBP was mainly located in the cytoplasm and interstitial of colon cancer cells. The score of each protein was calculated and a scatter plot involving each case was drawn, respectively (Figure 4A-4C). The expression scores of NMU, GNG4 and PPBP in CC and adjacent tissues were 8.246±3.863 vs 1.761±2.013 ($p<0.0001$), 7.667±3.677 vs 2.109±2.034 ($p<0.0001$), and 5.667±5.011 vs 3.364±3.335 ($p<0.0001$). These suggested that NMU, GNG4 and PPBP were overexpressed in CC tissues when compared with adjacent normal tissues.

Prognostic markers of colon cancer

We then explored the correlation between the expressions of NMU, GNG4, PPBP and the clinical characteristics of the colon cancer patients in the tissue microarray cohort (Table 2). The results showed that positive expression of PPBP was associated with lymph node metastasis, distant metastasis and advanced tumor stages ($p<0.05$).

We used Kaplan-Meier method to validate the relevance between the expression of NMU, GNG4, PPBP and the overall survival of 69 colon cancer cases, respectively (Figure 4D). At the significance level of 5%, the results stated that the survival time of patients with high expression of NMU, GNG4 and PPBP was shorter than those with low expression, respectively (NMU HR = 3.3, $p = 0.032$; GNG4 HR = 5.3, $p = 0.0056$; PPBP HR = 12.8, $p < 0.0001$). The results indicated that NMU, GNG4 and PPBP were adverse prognostic markers of colon cancer.

Subsequently, we drew the ROC to evaluate the sensitivity and specificity of NMU, GNG4 and PPBP in predicting the survival of CC, respectively (Figure 4E). The combination of these three key genes predicted the overall survival of colon cancer patients with the AUC of 0.868 (1-year), 0.635 (3-year) and 0.770 (5-year),. respectively. These results suggested that the combination model surpassed the single marker in predicting the prognosis of colon cancer.

Discussion

Although a substantial number of studies have declared that biomarkers are related to colon cancer, only a few markers showed prognostic value[17-21]. Previous studies showed that as a neuropeptide, neuromedin U (NMU) was not only involved in the immune response, energy balance, and gut-brain axis, but also related to poor survival of cancers, especially colorectal cancer[18,22-27].

PPBP was highly expressed in the specimens of lymph node and peritoneal metastasis of gastric cancer, which could be associated to the CXCR2 signaling pathway[28]. Interference with the CXCR2/PPBP

signaling pathway might be a new option for reversing the resistance of CC patients with liver metastases to conversion therapy[29]. In addition, KINOUCHI et al. found that the abnormal expression of PPBP in peripheral blood cells contributed to the diagnosis of renal cell carcinoma (RCC) [30].

Previous studies of GNG4 mainly focused on the nervous system, suggesting that GNG4 was related to cognitive decline and glioblastoma[31,32]. However, recent studies found that GNG4 could be an adverse marker of rectal cancer and gallbladder cancer[33-35]. Angiotensinogen (AGT) deficiency was related to inflammatory bowel disease and the development of cancer[36]. The studies of AGT in CC mainly focused on the field of liver metastasis[36-39]. In the current study, however, we were not able to validate the differential expression of AGT in colon cancer cells.

Although there were some bioinformatics analyses exploring the differentially expressed genes in colon cancer using GEO or TCGA or both database [18-21,40] for diagnostic and/or prognostic purpose, overlapping genes were identified consistent to our study, seldom did they verify their findings with cells and tissue cohorts. Our study made up the deficiency and focused on prognostic value of these markers. Nevertheless, the pathophysiological roles and the mechanisms of these 3 keys genes participating in the oncogenesis and progression of colon cancer remain to be clarified.

In conclusion, our study found that the combination model of NMU, PPBP and GNG4 expression predicted the overall survival of colon cancer patients at 1 and 5 years with high accuracy. They may serve as reliable prognostic biomarkers for colon cancer.

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Tables

Tables 1 and 2 are available in the supplementary files section.

Figures

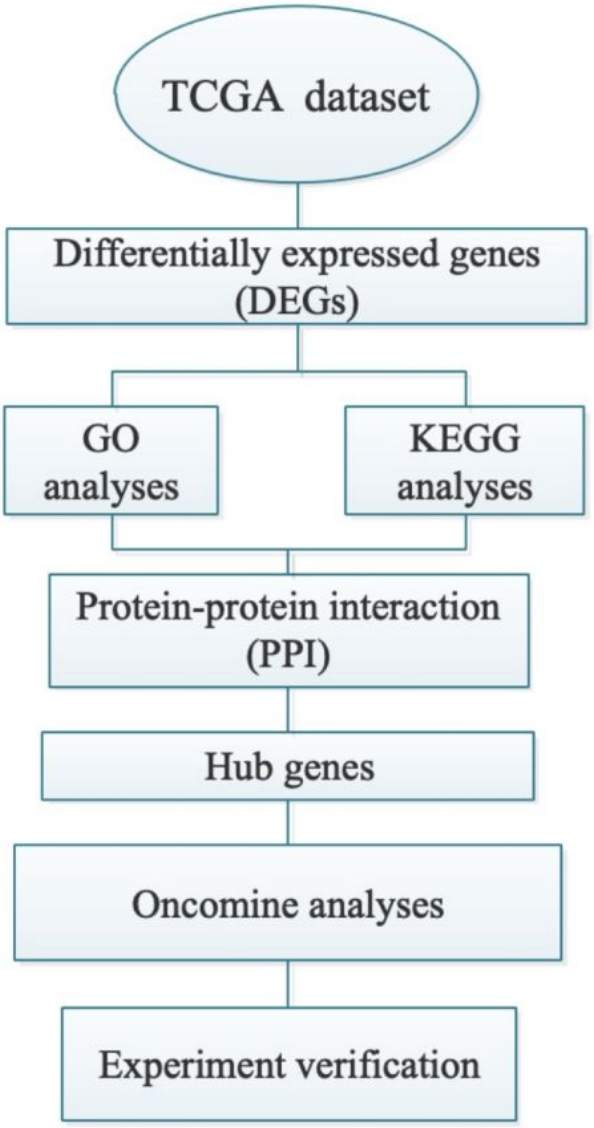

Figure 1

Flow diagram of study design.

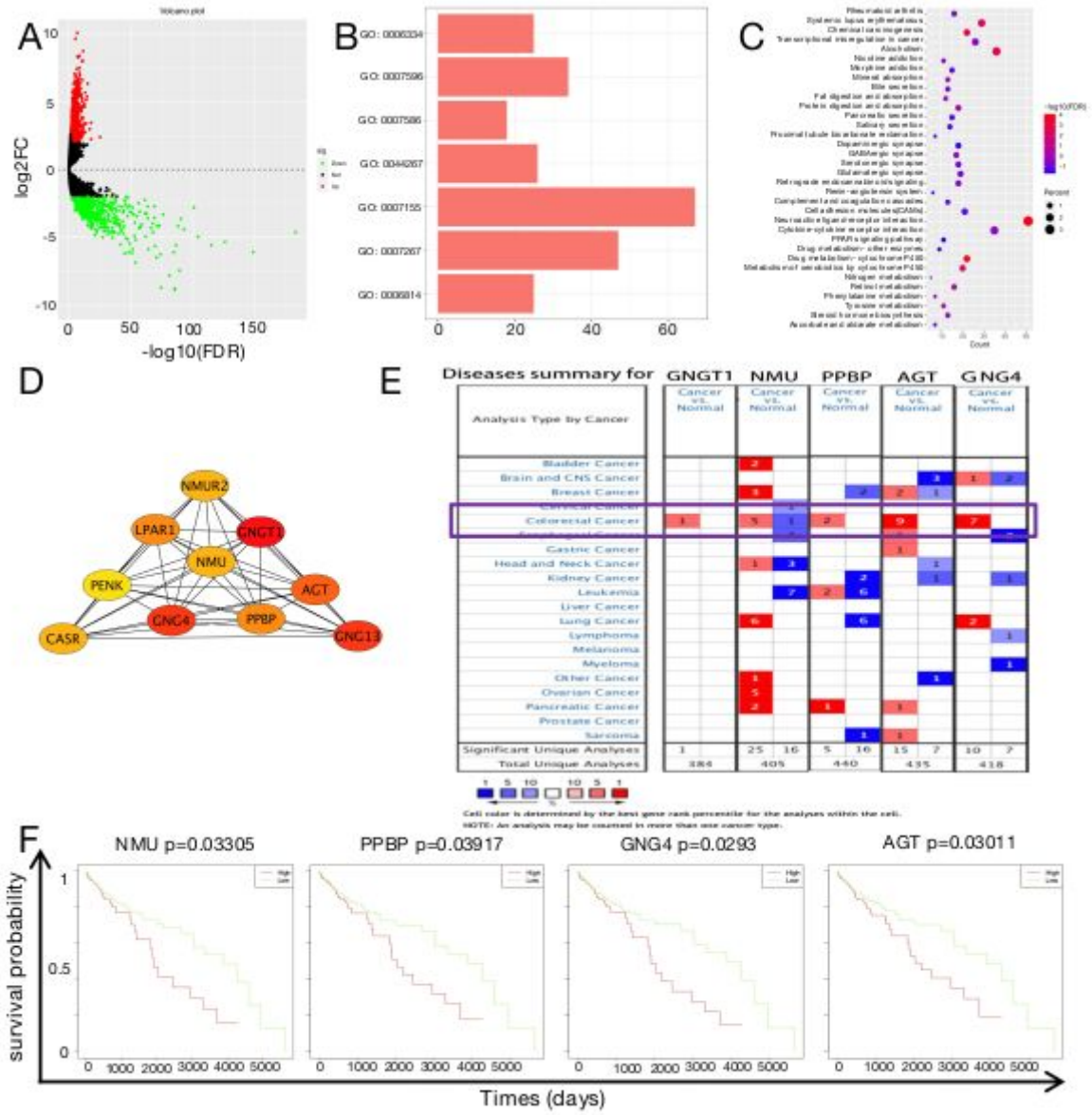

Figure 2

Identification of key genes in colon cancer. A. Volcano map of DEGs. B. GO functional enrichment. C. KEGG pathway enrichment analysis. D. Interrelationship of the top ten genes (Degree algorithm). E. The expression of key genes in Oncomine. F. The overall survival influenced by the key genes (NMU, PPBP, GNG4, and AGT).

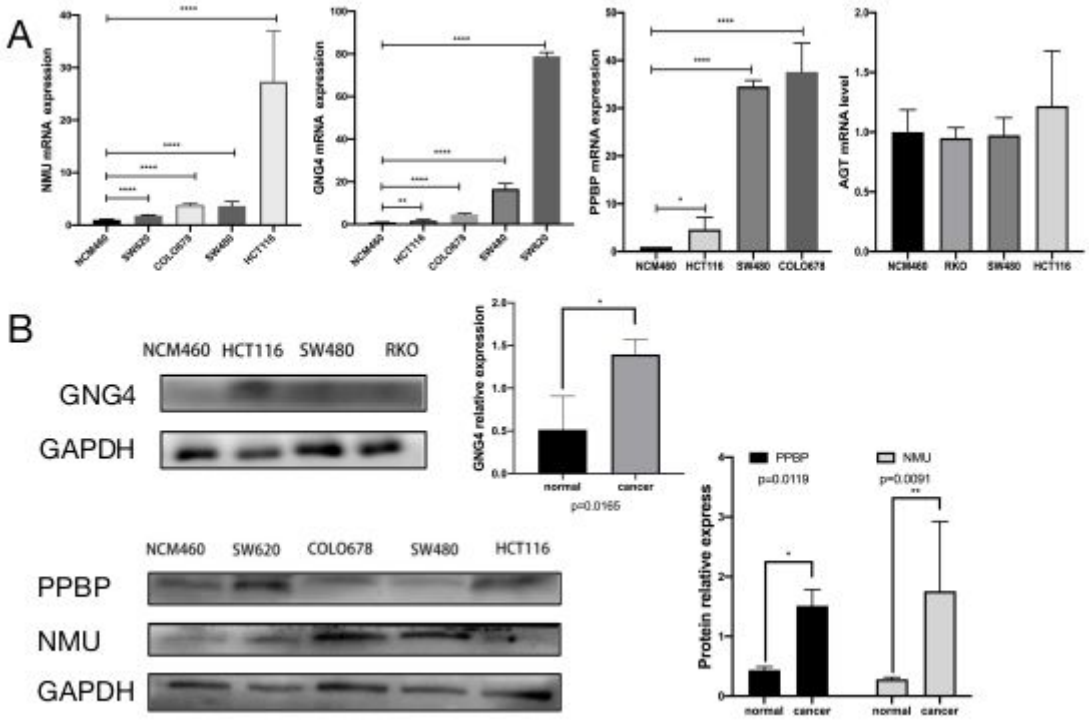

Figure 3

Expression of key genes in colon cancer cells. A. NMU, GNG4 and PPBP mRNA levels were detected by RT-qPCR. B. The expression of NMU, GNG4 and PPBP were detected by immunoblotting.

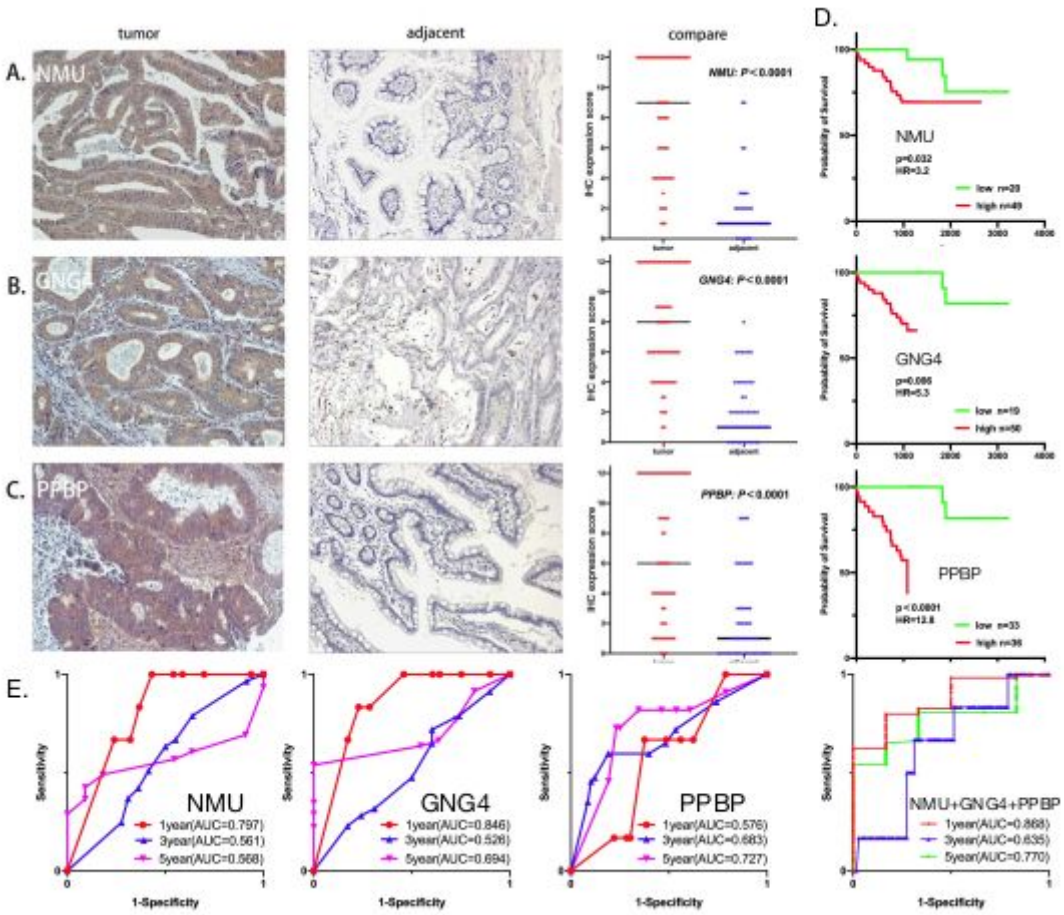

Figure 4

Expression of key genes in colon cancer tissue microarray and their influence on survival. A.NMU, B. GNG4 and C. PPBP were highly expressed in CC tissues. D. The survival curves of the key genes. E. The ROC curves of the key genes and combination model for predicting 1, 3, and 5-year survival.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table1.pdf