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Identification and analysis of key genes related to efferocytosis in colorectal cancer

Shengliang Zhang[1†], Ying Jiang[1†], Lei Shi[1], Tianning Wei[1], Zhiwen Lai[1], Xuan Feng[1], Shiyuan Li[1] and Detao Tang[1*]

Abstract The impact of efferocytosis-related genes (ERGs) on the diagnosis of colorectal cancer (CRC) remains unclear. In this study, efferocytosis-associated biomarkers for the diagnosis of CRC were identified by integrating data from transcriptome sequencing and public databases. Finally, the expression of biomarkers was validated by real-time quantitative polymerase chain reaction (RT-qPCR). Our study may provide a reference for CRC diagnosis.

Background It has been shown that some efferocytosis related genes (ERGs) are associated with the development of cancer. However, it is still uncertain how ERGs may influence the diagnosis of colorectal cancer (CRC).

Methods In our study, the CRC cohorts were gained from transcriptome sequencing and the gene expression omnibus (GEO) database (GSE71187). Efferocytosis related biomarkers with diagnostic utility for CRC were identified through combining differentially expressed analysis, machine learning algorithms, and receiver operating characteristic (ROC) analysis. Then, infiltration abundance of immune cells between CRC and control was evaluated. The regulatory networks (including mRNA-miRNA-lncRNA and miRNA/transcription factors (TF)-mRNA networks) were created. Finally, the expression of biomarkers was validated via real-time quantitative polymerase chain reaction (RT-qPCR).

Results There were 3 biomarkers *(ELMO3*, *P2RY12*, *and PDK4)* related diagnosis for CRC patients gained. *ELMO3* was highly expressed in CRC group, while *P2RY12* and *PDK4* was lowly expressed. Besides, the infiltrating abundance of 3 immune cells between CRC and control groups was significantly differential, namely activated CD4 memory T cells, macrophages M0, and resting mast cells. We then constructed a mRNA-miRNA-lncRNA network containing 3 mRNAs, 33 miRNAs, and 22 lncRNAs, and a miRNA/TF-mRNA network including 3 mRNAs, 33 miRNAs, and 7 TFs. Additionally, RT-qPCR results revealed that the expression trends of all biomarkers were consistent with the transcriptome sequencing data and GSE71187.

Conclusion Taken together, this study provides three efferocytosis related biomarkers (*ELMO3*, *P2RY12*, *and PDK4*) for diagnosis of CRC, providing a scientific reference for further studies of CRC.

Keywords Colorectal cancer, Efferocytosis, Biomarkers, Diagnosis, Immune

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Introduction

Colorectal cancer (CRC) is the second most common cancer in women and the third most common cancer in men Cancer. Moreover, it is also the fourth leading cause of cancer death, accounting for 9.2% of global deaths [1]. In China, it is estimated that there are over 376,000 new cases and 191,000 deaths annually [2]. The early symptoms of CRC are not obvious. With the development of cancer, bowel habits will change, including bloody stools, diarrhea, alternating diarrhea and constipation, and local abdominal pain [3, 4]. The treatment of CRC includes surgery, chemotherapy, radiotherapy, and targeted therapy [5]. Although screening and treatment strategies have reduced the incidence rate and mortality, about 25% of CRC patients are in advanced stages disease at the time of diagnosis, and about 25% will metastasize [6]. The 5-year survival rate of patients with metastatic CRC is only about 20% [6]. Surgical resection is the main treatment option for CRC. However, there are very few treatment options for metastatic patients. Although chemotherapy is usually recommended, only a few targeted therapies are suitable for cases with specific mutation profiles, and there is an urgent need to develop new molecular targets for CRC [7]. Therefore, there is an urgent need for a non-invasive and efficient method for early diagnosis of CRC patients, in order to provide new ideas for the treatment of CRC.

Efferocytosis refers to the process by which phagocytic cells remove apoptotic cells that have undergone programmed cell death. This clearance process occurs in all tissue development, homeostasis maintenance, and disease processes, and is crucial for organism development, homeostasis tissue renewal and regeneration, as well as the immune system [8]. Abnormal efferocytosis may lead to various diseases. Under normal circumstances, apoptotic cells release seeking and phagocytic signals to stimulate efferocytosis, but with the accumulation of efferocytosis and enhanced inflammation, the reprogramming of macrophages reduces the efferocytosis, and the imbalance of the internal environment creates the conditions for the occurrence of disease, such as autoimmune diseases and cancer. Research has shown that some molecules related to efferocytosis are associated with cancer progression, such as CD47, Axl, MerTK, and TGF- β [9]. In addition, efferocytosis plays an important role in tumor diseases such as breast cancer, prostate cancer and leukemia. For example, in the process of efferocytosis, MERTK and AXL are promoters of tumor cell survival in many hematopoietic malignancies (including acute leukemia, chronic leukemia and multiple myeloma) [10, 11]. In summary, its cleaning function not only can maintain the balance of the body environment, but also confirmed the control of inflammation through immune suppression, the release of mediating factors in the process of complex metabolic state can be found through two steps-find and swallow me, related signals and release degradation products affect the occurrence and development of tumor. However, the relationship between efferocytosis and CRC is not yet clear.

The invasion and recurrence of CRC are the main factors affecting the prognosis, yet, there is still a lack of long-term effective treatment for the metastasis and recurrence of colon cancer.Therefore, looking for targets to inhibit the proliferation, invasion and apoptosis of colon cancer cells has great significance for the diagnosis and treatment of diseases. Studies have shown that cell burial not only promotes resolution of inflammation, also acting as immunosuppressive.It provides an environment for tumor cells to survive and develop, for the last few years, studies on the molecular mechanism of efferocytosis and abroad provide many new ideas and new directions for treating treatment. Targeting apoptotic cell debris, cytokines and surface signals produced during efferocytosis are all expected to be a suitable route for antitumor therapy, besides, there may be some roles of the tumor microenvironment on its development and metastasis, including providing an immune escape environment, regulating the tumor cell proliferation and survival signals, and changing the constituent structure of the extracellular matrix, but the exact mechanism is not yet clear. Overall, the role of immune cells in the different stages of tumor metastasis is highly specific and clearly different, while the specificity and sensitivity of phagocytes as specific immune cells can also vary in the cell burial process of CRC. Further explore the specific mechanism of related genes mediating the regulation of the efferocytosis, thus interfering with the tumor microenvironment and inhibiting the tumor cell proliferation, which is conducive to improving the survival rate of patients.Traditional CRC diagnosis mainly relies on imaging examination, endoscopy, and tissue biopsy. However, these methods have some limitations. Imaging examination may miss early small tumors, endoscopy is invasive, and tissue biopsy may not fully reflect the tumor condition due to material location limitations (https://doi.org/10.3760/cma.j.cn371439-20220520-00108). In the CRC studies, the expression regulation of ERGs is tightly linked to the clinical significance. Abnormal expression of ERGs may lead to changes in the tumor microenvironment, affecting the proliferation, migration, and apoptosis of tumor cells. For example, certain ERGs may be overexpressed in tumor cells and promote the immune escape of tumor cells.(https://doi.org/10.1016/j.tranon.2023.101842) https://doi.org/10.1136/jcp-2022-208350 Therefore, the in-depth study of efferocytosis-related genes (ERGs) in colorectal cancer (CRC) can fill the gap in current diagnostic technology, improve the accuracy of diagnosis, and provide new strategies

and targets for the treatment of CRC. By analyzing the expression patterns and regulatory mechanisms of ERGs, more personalized and precise treatment options for CRC patients.

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In this study, based on self sequencing data and public database datasets on CRC, we identified genes related to efferocytosis in the diagnosis of CRC through bioinformatics methods, providing potential targets for clinical diagnosis and theoretical basis for further understanding of the mechanisms of CRC.

Materials and methods

Sample collection and data sources

In this study, the 10 pairs of samples from CRC patients from The First Affiliated Hospital of Guizhou University of Traditional Chinese Medicine were utilized as the training set, including 10 CRC tumor samples and 10 paracancerous tissue samples. The patients were confirmed by pathology to have CRC. The study was approved by the Ethics Committee of the First Affiliated Hospital of Guizhou University of Traditional Chinese Medicine. All patients had signed an informed consent form.

Additionally, the validation set for the CRC cohort, GSE71187, which includes 157 CRC samples and 32 control samples, was retrieved from the gene expression omnibus (GEO) database [12]. Besides, 71 efferocytosis related genes (ERGs) were gained through published literature [9, 13].

Transcriptome sequencing

Collect tumor tissues from 10 CRC patients and 10 adjacent non-tumor tissues (control) for second-generation transcriptome sequencing. After filtering, comparing, and merging the sequencing raw data, the expression matrix of the sample transcriptome is obtained, which is the count value. Please refer to the attached htseq for details_ Fixed_Result.txt. GSE71187. We downloaded the GSE71187 dataset CRC transcriptome data from NCBI, which comprises 157 CRC samples and 32 control samples. The transcriptome sequencing data expression levels of the samples do not require log2 processing. Please refer to the attachments GSE71187.clinical.tsv.gz and GSE71187.gene for details_ Expression_ Array.tsv.gz.

Firstly, FastQC (version 0.11.9) was utilized for quality assessment of sequencing data. Then, in order to obtain clean data for further analysis, we conducted quality control on the original data and removed any low-quality data. The pre-processed data was compared with the reference genome data (GRGh37) using HISAT2 (version 2.2.1).

The screening of differentially expressed ERGs (DE-ERGs) between CRC and control groups

Based on transcriptome data, the differentially expressed genes (DEGs) between CRC and control groups were identified via "edgeR" R package (version 3.36.0) with $|\log_2 FC| > 1$ and $P<0.05$ [11, 14, 15]. Then, DEGs and ERGs were intersected with DE-ERGs. Subsequently, STRING database (https://string-db.org) (confidence=0.15) was used to create the protein-protein interaction (PPI) network for DE-ERGs. Moreover, enrichment analysis of DE-ERGs was performed via "clusterProfiler" R package (version 4.0.2), based on the Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), and Reactome ($P<0.05$ and count ≥ 1) [16].

Screening of the biomarkers

Two machine learning algorithms, including the least absolute shrinkage and selection operator (LASSO) (via "glmnet" R package (version 4.1-4)) and Boruta (via "Boruta" R package (version 7.0.0)), were utilized to screen the characteristic genes, respectively [17]. The characteristic genes obtained through the two algorithms were intersected to obtain common characteristic genes. Afterwards, the expression levels of common characteristic genes between CRC and control groups were compared of the training set and GSE71187 via Wilcox test ($P<0.05$), and the genes that differed significantly in expression between CRC and control groups and had the same expression trend in separately for the training set and GSE71187 were defined as biomarkers. The diagnostic value of the biomarkers for CRC was assessed using

receiver operating characteristic (ROC) curves in the training set and GSE71187, separately. The correlations of the biomarkers with each other were explored via Spearman algorithm. Additionally, the location of biomarkers on the chromosome was analyzed via "Circos" R package (version 1.2.2) [18].

Creation of a nomogram

The nomogram containing the biomarkers was created to predicted the occurrence of CRC via "rms" R package (version 6.1-0). At the same time, to evaluate the reliability of the nomogram, corresponding calibration curve, decision curve analysis (DCA), and ROC curve were plotted.

Enrichment analysis of the biomarkers

Firstly, the gene-gene interaction network for the biomarkers was created through GeneMANIA (http://genemania.org/search/) database to predicted the function of these genes. Then, to further explore which biological functions or signaling pathways were correlated with biomarkers, the samples in training set were assigned into high and low expressed groups basis on the medium value their expression, and differential analysis was carried out. All genes were ranked according to logFC, and 'c5.go.v7.4.entrez.gmt', 'c2.cp.kegg.v7.4.entrez.gmt' were downloaded as reference gene sets for Gene set enrichment analysis (GSEA) via "clusterProfiler" R package (version 4.0.2) and "org.Hs.eg.db" R package (version 3.13.0) [16]. The threshold values were |NES| > 1, NOM $P<0.05$, and q<0.25. Additionally, to find out which signaling pathways were associated with biomarkers, ingenuity pathway analysis (IPA) was performed.

Immune infiltration analysis

The infiltrating abundance of 22 immune cells of each sample was assessed in CRC and control groups in training set via CIBERSORT algorithm. The differences of infiltrating abundance of 22 immune cells between CRC and control groups were compared by Wilcox Test ($P<0.05$). Therefore, the correlations between biomarkers and immune cells as well as immune checkpoints (TNFSF18, PDCD1LG2, CD40LG, CD28, CD224, ICOS, CD80, BTLA, ADORA2A, HHLA2, TNFSF9, TMIGD2, IDO2, VTCN1, TNFSF25, CD44, CD274, CD86, CD200) were evaluated via Spearman algorithm.

Mutation analysis

The mutation data of CRC obtained from the Cancer Genome Atlas (TCGA) database (Https://portal.gdc.cancer.gov) were processed via "maftools" R package (version 2.8.05) [19]. Mutation rates of three biomarkers in CRC samples were analyzed.

Construction of regulatory network

To further investigate the mechanisms underlying the biomarkers in CRC, we created the mRNA-microRNA (miRNA)-lncRNA network, miRNA/transcription factors (TF)-mRNAs network, and miRNA-single nucleotide polymorphism (SNP)-mRNA network. The miRNAs regulating the biomarkers were predicted via miRWalk database (http://mirwalk.umm.uni-heidelberg.de/) and miRDB database (http://www.mirdb.org). The predicted miRNAs of the two databases were intersected to obtain the common miRNAs that were used to create the mRNA-miRNA-lncRNA network and miRNA/TF-mRNA network. The miRtarBase database (http://mirtarbase.mbc.nctu.edu.tw) was utilized to predict the lncRNAs with regulatory interactions with common miRNAs. The TFs were predicted through TRRUST database (https://link.zhihu.com/?target=http%3 A//www.grnpedia.org/trrust/). Finally, the miRNASNP database (https://bio.tools/mirnasnp-v3#!) was adopt to predicted the single nucleotide polymorphis (SNPs) in the seed regions of the miRNAs, and the SNP locations in the seed region of the miRNAs affected biomarkers were screened. Cytoscape software (version 3.8.2) was utilized to visualize the regulatory networks [20].

Chemotherapy drug sensitivity analysis

We downloaded the RNA-seq expression profile data and DTP NCI-60 compound activity data from CellMiner (http://discover.nci.nih.gov/cellminer/). Correlation between sensitivity of drugs and biomarkers was assessed with |r| > 0.3 and $P<0.05$.

Sample collection

In our study, the 10 patients with CRC at The First Affiliated Hospital of Guizhou University of Traditional Chinese Medicine from From January 6th, 2023 to February 9th, 2023 were recruited. Patients with pathological confirmation of CRC. Ten pairs of CRC tumor tissue and paracancerous tissue samples were gained. This study was approved by Ethics Committee of the First Affiliated Hospital of Guizhou University of Traditional Chinese Medicine. All patients had signed an informed consent form.

Real-time quantitative polymerase chain reaction (RT-qPCR)

The expression of the three biomarkers was further validated via RT-qPCR. The twenty samples' total RNA was extracted using TRIzol (Ambion, Austin, USA) in accordance with the instructions provided by the manufacturer. The SureScript-First-strand-cDNA-synthesis-kit (Servicebio, Wuhan, China) was utilized to perform reverse transcription of total RNA to cDNA the based on the manufacturer's instructions, setting conditions were:

5 min, 50 °C; 15 min, 85 °C, 5 s; 4 °C, hold. RT-qPCR was performed utilizing the 2xUniversal Blue SYBR Green qPCR Master Mix (Servicebio, Wuhan, China). The primer sequences for RT-qPCR were shown in Table S1, the amplification conditions were as follows: pre-denaturation: 95 °C, 1 min; denaturation: 95 °C, 20 s; annealing: 55 °C, 20 s; and extension: 72 °C, 30 s. 40 cycles of the reaction were performed, amplification and lysis curves were made, and Ct values were read. The internal reference gene was GAPDH. The $2^{-\Delta\Delta Ct}$ method was utilized to calculate the expression of biomarkers [21].

Statistical analysis

Graphpad Prism 5 and R software (https://www.r-project.org/) were employed to execute the statistical analysis. Differences were analyzed via the Wilcoxon test. $P<0.05$ represented a significant difference.

Results

Acquisition of DE-ERGs between CRC and control groups

The results of alignment analysis of the transcriptome data demonstrated that the alignment rate of 20 sequenced samples was above 90%, indicating that the sequencing data could be used for subsequent analysis (Table S2). In total, 4504 DEGs between CRC and control groups were screened, containing 2697 up-regulated genes and 1807 down-regulated genes (Fig. 1A-B). These DEGs were intersected with ERGs to obtained 18 DE-ERGs (DNASE1, PDK4, SLC25A10, RAC1, ELMO3, SLC66A1, P2RY12, AXL, SLC12A4, DOCK1, ELMO1, SLC6A6, P2RY6, STAB2, SLC26A6, MERTK, SLC16A2, and ADAM9) (Fig. 1C). Of these proteins, we found from the PPI network that AXL was interacted with multiple proteins, such as PDK4, DOCK1, MERTK, etc. (Fig. 1D) Of the GO results, the DE-ERGs significantly enriched into 294 GO BP items, 16 GO CC items, 60 GO MF items (Table S3), including 'phagocytosis (GO:0006909)', 'guanyl-nucleotide exchange factor complex (GO:0032045)', 'carboxylic acid transmembrane transporter activity (GO:0046943)', etc. (Fig. 1E). Of the KEGG results, there were 11 KEGG pathways markedly enriched (Table S4). For instance, DE-ERGs were involved in 'Bacterial invasion of epithelial cells (hsa05100)', 'Yersinia infection (hsa05135)', 'Shigellosis (hsa05131)', and so on (Fig. 1E). Of the Reactome results, a total of 58 pathways were enriched (Table S5), such as 'Signaling by VEGF (R-HSA-194138)', 'VEGFA-VEGFR2 Pathway (R-HSA-4420097)', 'PTK6 Regulates RHO GTPases, RAS GTPase and MAP kinases (R-HSA-8849471)', and so on (Fig. 1E).

DEGs, differentially expressed genes; CRC, Colorectal cancer; DE-ERGs, differentially expressed efferocytosis related genes (DE-ERGs), PPI, protein-protein interaction.

Acquisition of the biomarkers

For result of LASSO algorithm, there were 8 characteristic genes obtained, namely DNASE1, PDK4, RAC1, ELMO3, P2RY12, AXL, SLC6A6, and STAB2 (Fig. 2A-B). In total, 7 characteristic genes were gained via Boruta algorithm, namely DNASE1, PDK4, RAC1, ELMO3, P2RY12, SLC25A10, and SLC26A6 (Fig. 2C). Subsequently, DNASE1, PDK4, RAC1, ELMO3, and P2RY12 were obtained and considered as common characteristic genes (Fig. 2D). In both training set and GSE71187, ELMO3 was considerably highly expressed in CRC groups, while P2RY12 and PDK4 were considerably lowly expressed in CRC groups (Fig. 2E-F). Therefore, these three genes were defined as biomarkers. Additionally, the AUC of all biomarkers were greater than 0.8 in both training set and GSE71187 (Fig. 2G-H), suggesting that all characteristic genes had diagnostic value for CRC. Moreover, ELMO3 was markedly negatively relevant to P2RY12 (r = -0.53, P=0.016) and PDK4 (r = -0.61, P=0.005), while P2RY12 was significantly positively associated with PDK4 (r=0.91, P=3.24E-08) (Fig. 2I). Afterwards, the location of biomarkers on the chromosome were demonstrated in Fig. 2J, and P2RY12, PDK4 and ELMO3 were located on chromosomes 3, 7 and 16, respectively.

Creation and verification of nomogram

The nomogram containing the biomarkers was created (Fig. 3A). The corresponding calibration curve demonstrated that nomogram passed the calibration degree test (S: $p>0.05$) (Fig. 3B), and the DCA curve suggested the nomogram had a higher net benefit (Fig. 3C), and the AUC of nomogram was 0.96 (Fig. 3D). These results indicated that the nomogram had a high predictive accuracy for the CRC progression.

Enrichment analysis of the biomarkers

To explore the gene-gene interactions of each biomarker, the interaction networks were created (Figure S1). PDK4 and its co-expressed genes (such as PDP2, PDP1, PDPR, etc.) were involved in 'acety1-CoA biosyntheic process', 'acety1-CoA metabolic process', 'acety1-CoA biosyntheic process from pyruvate', 'thioester biosyntheic process', 'acety1 biosyntheic process', etc. (Figure S1A). The 'purinergic nucleotide receptor activity', 'GTPase complex', 'nucleotide receptor activity', 'extrinsic component of cytoplasmic side of plasma membrance', 'G protein-coupled receptor activity', and so on were relevant to P2RY12 and its co-expressed genes (Figure S1B). Additionally, ELMO3 and its co-expressed genes were involved in 'Fc-gamma receptor signaling pathway', 'Rho GTPase binding', 'regulation of epidermal growth factor receptor signaling pathway', 'immune response-regulating cell surface receptor signaling pathway involved in phagocytosis',

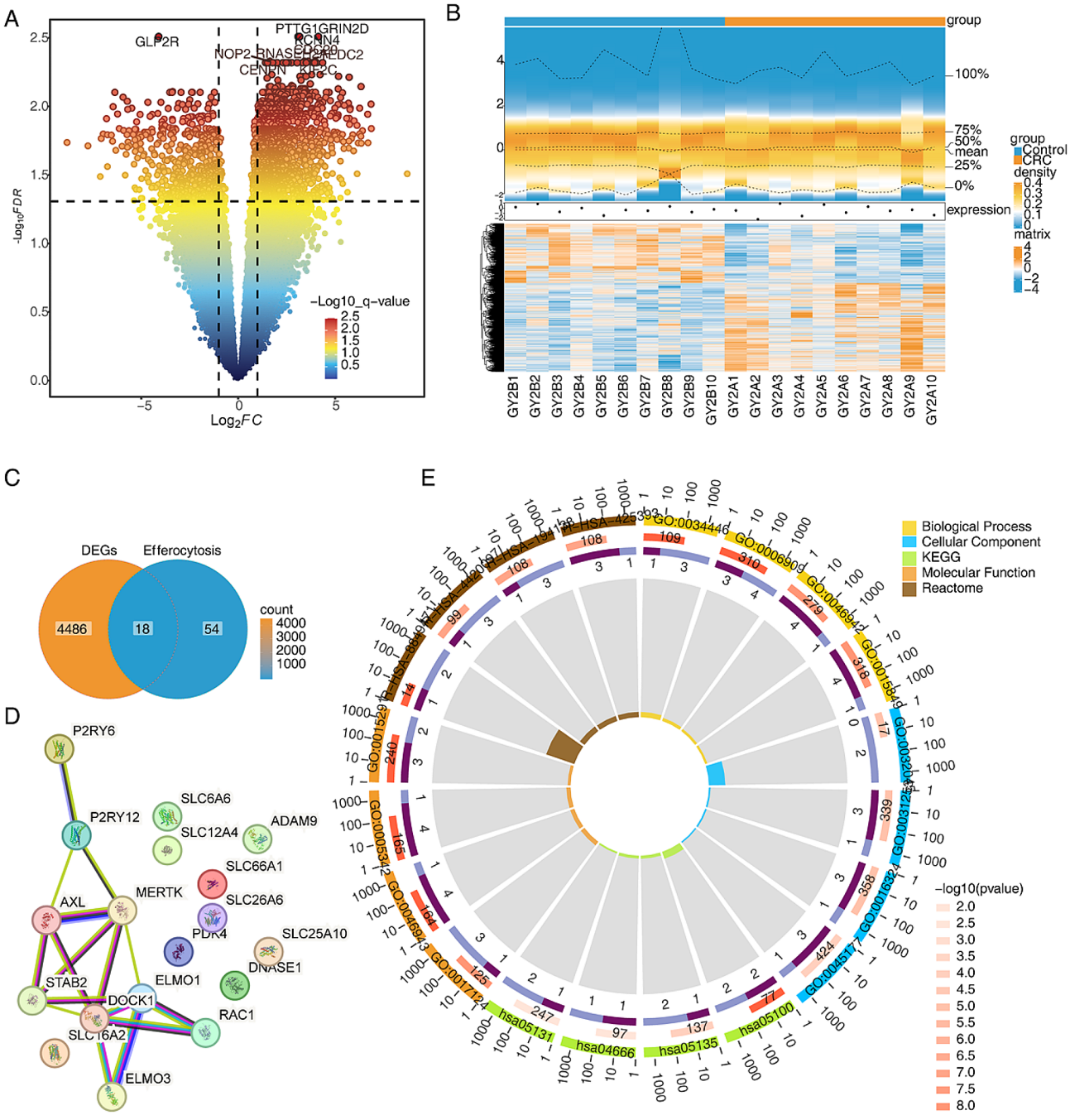

Fig. 1 Identification and function of DEGs. (**A**) Volcano plot and (**B**) Heat map of DEGs between the CRC group and control group. (**C**) Venn diagram, (**D**) PPI network, and (**E**) Enrichment results for DE-ERGs

'Fc receptor mediated stimulatory signaling pathway', etc. (Figure S1C).

GSEA results indicated that, of GO results, all biomarkers were involved in 'collagen containing extracellular matrix' and 'extracellular matrix structural constituent' (Fig. 4A-C). Interestingly, PDK4 and P2RY12 were involved these items in high expressed groups, while ELMO3 was involved these items in low expressed groups (Fig. 4A-C). In addition, PDK4 and P2RY12 were also involved in 'regulation of heart contraction', 'regulation of blood circulation', 'heart process', 'regulation of vasculature development', 'muscle contraction', 'muscle system process', 'muscle organ development' and so on in high expressed group (Fig. 4A-B). For ELMO3, 'immunoglobulin complex', 'immunoglobulin complex circulating', 'structural constituent of ribosome', and so on were enriched in high expressed group (Fig. 4C).

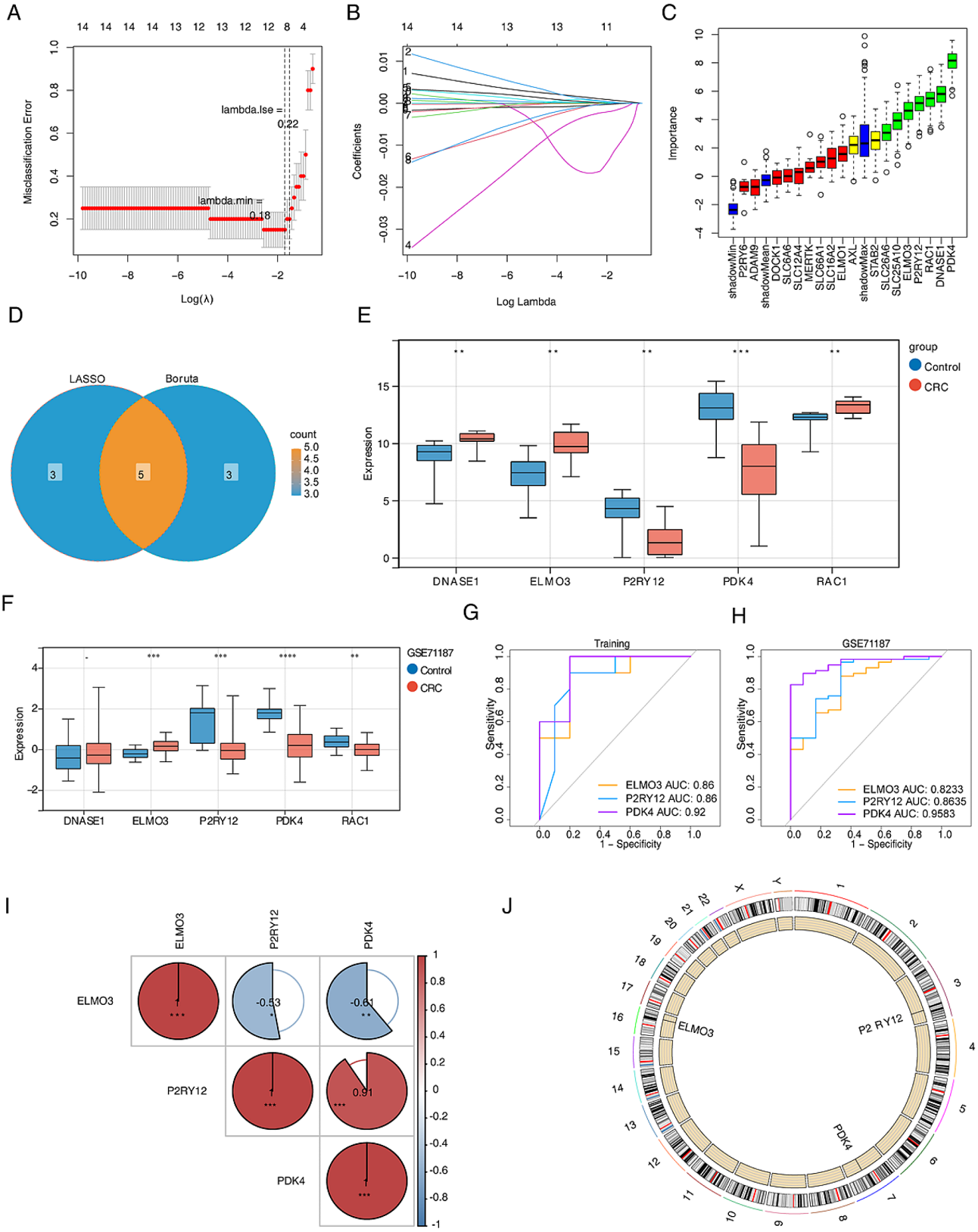

Fig. 2 Screening for biomarkers. (**A, B**) LASSO regression analysis and (**C**) Boruta to screen characteristic genes. (**D**) Venn diagram of common characteristic genes. Expression of candidate key genes in (**E**) training set and (**F**) GSE71187. ROC curves for three biomarkers in (**G**) training set and (**H**) GSE71187. (**I**) Heat map presents biomarker correlations. (**J**) Distribution of biomarkers on chromosomes. **P* < 0.05, ***P* < 0.01, ****P* < 0.001. LASSO, least absolute shrinkage and selection operator; ROC, receiver operating characteristic

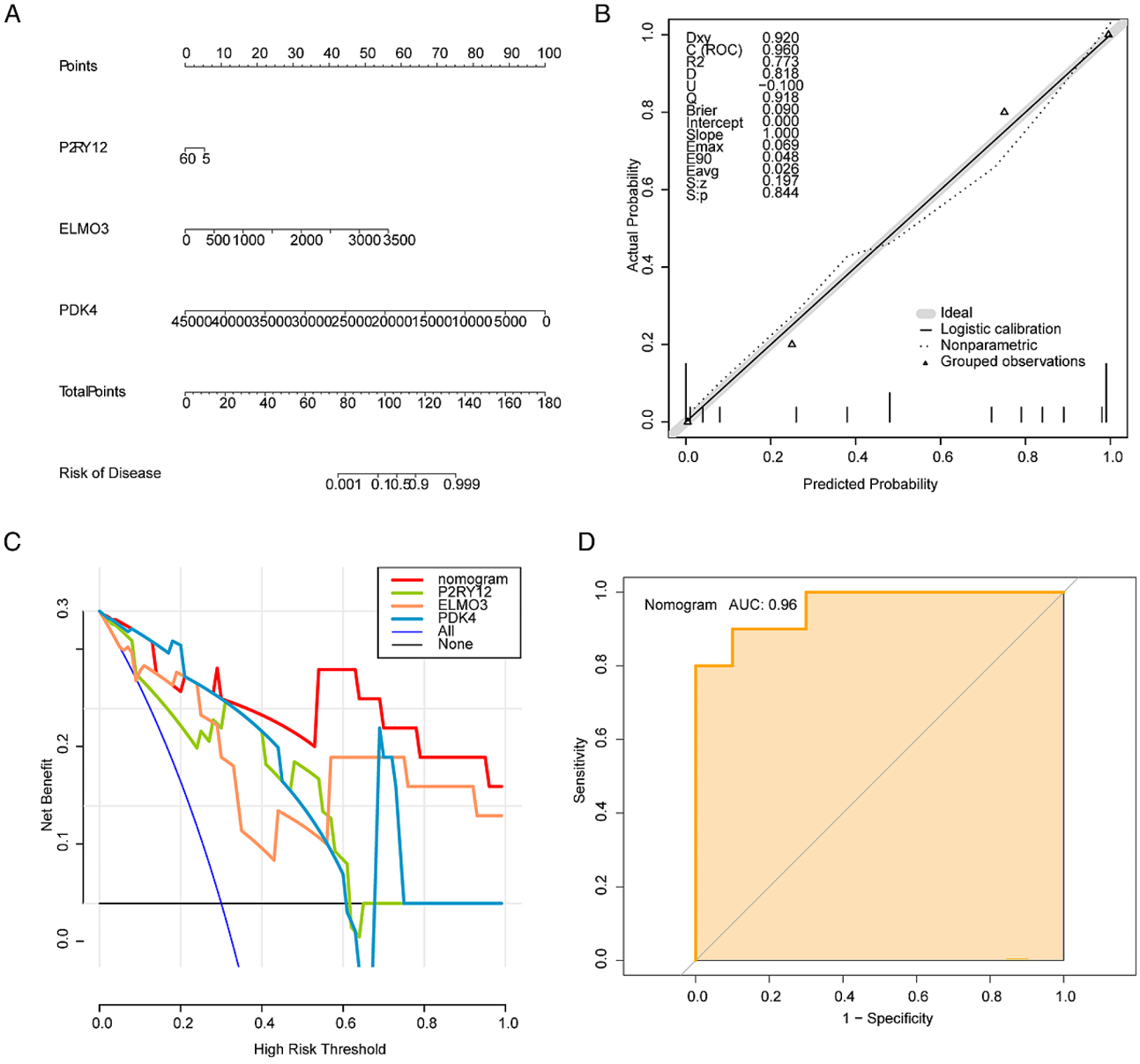

Fig. 3 Creation and verification of Nomogram model. (**A**) Nomogram of biomarkers. (**B**) calibration curve, (**C**) DCA curve, and (**D**) ROC curves for nomogram. DCA, decision curve analysis; ROC, receiver operating characteristic

Of the KEGG results, PDK4 and P2RY12 also showed great functional similarities. In high expressed group, they enriched in 'chemokine signaling pathway', 'cytokine cytokine receptor interaction', 'focal adhesion', 'vascular smooth muscle contraction', 'complement and coagulation cascades', 'calcium signaling pathway', 'MAPK signaling pathway', 'hematopoietic cell lineage', 'TOLL like receptor signaling pathway', etc. (Fig. 4D-E). However, ELMO3 was involved in 'cytokine cytokine receptor interaction' and 'focal adhesion' in low expressed group, and was involved in 'cell cycle', 'oxidative phosphorylation', 'ribosome', and so on in high expressed group (Fig. 4F).

IPA results suggested that the biomarkers played a significant role in 10 pathways (Fig. 4G). The biomarkers were considerably positively correlated with S100 family signaling pathway, while significantly negatively correlated with the remaining pathways, such as 'cAMP-mediated signaling', 'CREB Signaling in Neurons', 'Senescence Pathway', etc.

Immune analysis between CRC and control groups

The infiltrating abundance of immune cells in CRC and control samples of the training set was displayed via column stacking chart (Fig. 5A). After removing the immune cells with infiltration abundance of 0, the infiltrating

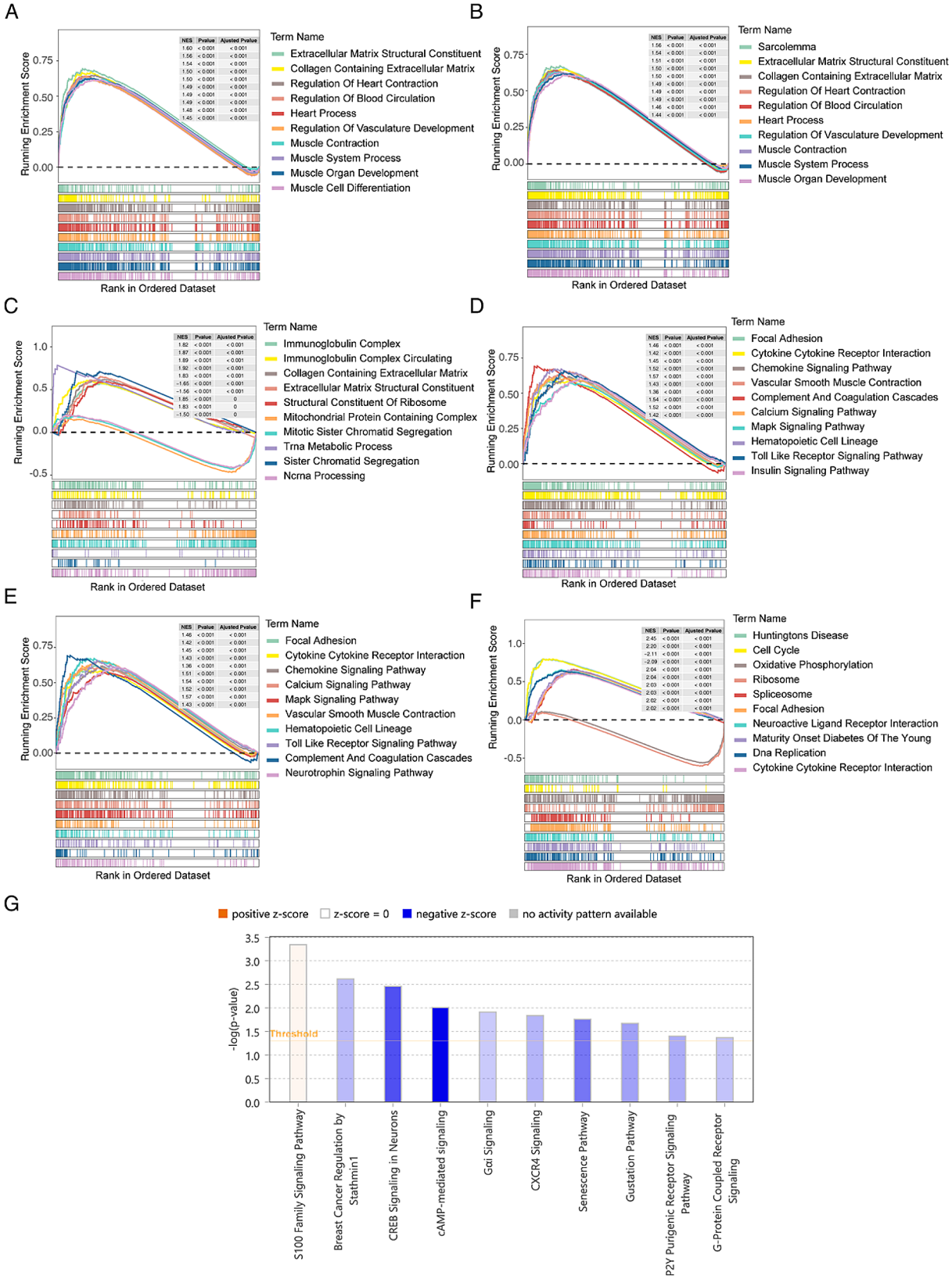

Fig. 4 GSEA and IPA results for biomarkers. GSEA results of (**A**) PDK4, (**B**) P2RY12, and (**C**) ELMO3 in GO. GSEA results of (**D**) PDK4, (**E**) P2RY12, and (**F**) ELMO3 in KEGG. (**G**) IPA results of biomarkers. GSEA, gene set enrichment analysis; IPA, ingenuity pathway analysis; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes

Fig. 5 Immunocorrelation analysis and genetic variation analysis. (**A**) Stacked graph of immune cell percentage. (**B**) Comparison of immune cells between CRA and control group. (**C**) Correlation of biomarkers with immune cells. Correlation of biomarkers with immune checkpoints: (**D**) PDK4, (**E**) P2RY12, and (**F**) ELMO3. (**G**) Mutation frequency of biomarkers in CRC samples. CRC, Colorectal cancer

abundance of 3 immune cells between CRC and control groups was significantly differential, namely activated CD4 memory T cells, macrophages M0, and resting mast cells (Fig. 5B). Moreover, macrophages M0 was considerably negatively relevant to PDK4 (r = -0.57, P=0.008) and P2RY12 (r = -0.45, P=0.045), yet resting mast cells was significantly positively associated with PDK4 (r=0.53, P=0.017) and P2RY12 (r=0.55, P=0.012) (Fig. 5C). Biomarker-immune checkpoint correlation analysis showed that the correlation between PDK4/P2RY12 and immune checkpoints mostly showed an opposite trend to the correlation between ELMO3 and immune checkpoints (Fig. 5D-F). In addition, mutation analysis showed that the three biomarkers had a low mutation rate in CRC samples (Fig. 5G).

The regulation network

Through miRWalk and miRDB databases, a total of 33 common miRNAs were predicted. Through miRTarBase databases, 22 lncRNAs were predicted. Afterwards, the mRNA-miRNA-lncRNA network was created, containing 3 mRNAs, 33 miRNAs, and 22 lncRNAs (Fig. 6A). For instance, the regulatory relationships included MCM3AP-AS1-'hsa-miR-15a-5p'-PDK4, CCDC39-'hsa-miR-19a-5p'-P2RY12, ELFN2-'hsa-miR-663b'-ELMO3 etc. Through TRRUST database, there were 7 TFs predicted for creating of the miRNA/TF-mRNA network (Fig. 6B). We could find that SP1 and CDX2 could regulate ELMO3, and PDK4 might be regulated by PPARA, RELA, NFKB1, PPARD, and E2F1.

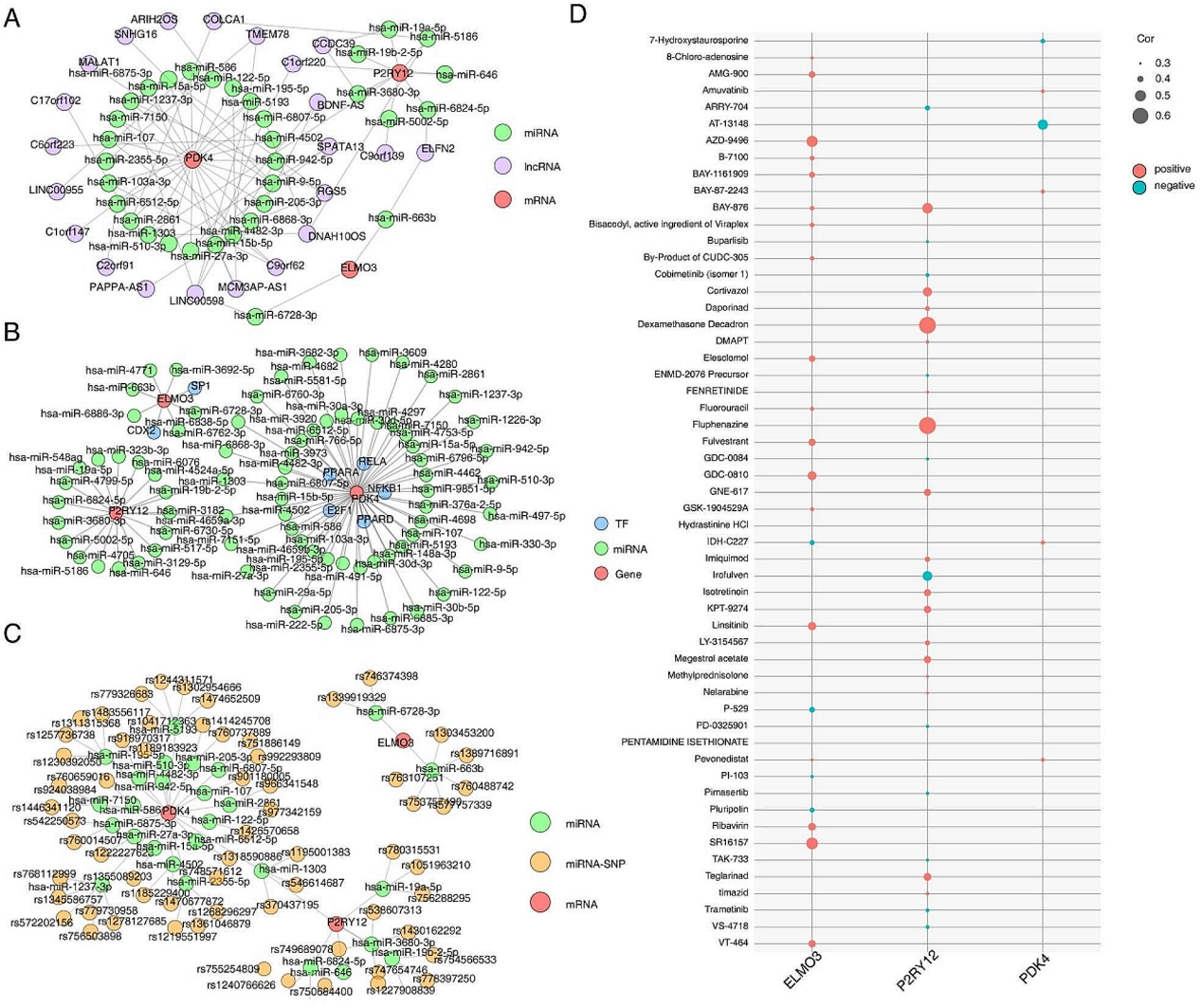

Fig. 6 Molecular regulatory networks and drug sensitization. (**A**) The mRNA-miRNA-lncRNA network for 3 mRNAs (biomarkers), 33 miRNAs, and 22 lncRNAs. (**B**) The miRNA/TF-mRNA network for 3 mRNAs, 33 miRNAs, and 7 TFs. (**C**) The miRNA-SNP-mRNA network for 3 mRNAs, 33 miRNAs, and 63 SNP locations of miRNAs. (**D**) Correlation of biomarker expression with drug sensitization. miRNA, microRNA; TF, transcription factor; SNP, single nucleotide polymorphism

Potential miRNAs targeting the biomarkers were identified via miRNASNP to determine how SNP variants in the miRNA seed region affect the binding of the 3' UTR region of biomarker genes. miRNA-SNP-mRNA network was demonstrated in Fig. 6C. There were 63 SNP locations identified, which might play an important role in binding to biomarkers.

Drug sensitivity analysis, drug prediction, and molecular docking

ELMO3, P2RY12, and PDK4 were significantly correlated with the sensitivity of 22, 28, and 6 drugs, respectively (Fig. 6D). ELMO3 was superlatively positively relevant to SR16157 (r=0.511, P<0.001) (Figure S2). P2RY12 was superlatively positively correlated with Fluphenazine (r=0.617, P<0.001) (Figure S3). PDK4 was superlatively negatively correlated with AT-13,148 (r = -0.480, P<0.001) (Figure S4).

The expression levels of the biomarkers

The RT-qPCR results demonstrated that all biomarkers were differentially expressed between CRC and control groups (Fig. 7A-C). *ELMO3* was highly expressed in the CRC group, while the expression of *P2RY12* and *PDK4* in CRC group showed opposite trends. In conclusion, the results of RT-qPCR matched those of training set and GSE71187, suggesting that all biomarkers have good diagnostic value for CRC.

Discussion

Cancer is basically a genetic disease, and the accumulation of mutations in different genes occurring in a specific group of cells is necessary to trigger tumor development. In contrast, the identification of cancer driver genes and key genes are crucial for personalized treatment, while also well predicting the risk of cancer patients [21, 22]. CRC remains one of the most significant global health burdens" to avoid an absolute statement. Its high incidence rate and mortality have long puzzled the global health organization. Although health screening has reduced the incidence rate and mortality, many CRC patients still have advanced disease at the time of diagnosis. Efferocytosis can be divided into four processes: discovery, recognition, phagocytosis, and digestion and degradation. The phagocytosis of macrophages must be preceded by cell apoptosis in the body. The soluble factors released by apoptotic cells are equivalent to chemoattractants that attract macrophages. The clearance effect during this process can maintain the stability of the internal environment and participate in immunosuppressive and inflammatory responses. Related studies have confirmed that inflammatory factors play an important role in the occurrence and development of tumors [23]. Chronic or excessive inflammation can even induce cancer, which is consistent with the phagocytic effect of macrophages in inhibiting inflammation mechanisms.

By deeply studying the expression pattern of ERGs, specific genes or gene combinations closely related to the occurrence and development of CRC can be selected. These biomarkers can play an important role in the early screening, differential diagnosis and prognosis evaluation of CRC, thus improving the accuracy and timeliness of diagnosis [24]. By regularly testing the expression changes of ERGs in patients, the progression of the disease and the treatment effect can be understood in time, so as to provide a basis for the adjustment of the treatment plan [25]. According to experimental data, all biomarkers have important value in the diagnosis of CRC, with *PDK4*, *ELMO3*, and *P2RY12* showing the most significant differences. Both in vivo cell experiments and in vitro animal experiments showed that miR-9-5p-dependent PDK4 is associated with HCC growth and metastasis, and the promotion of PDK4 expression by inhibiting miR-9-5p expression could inhibit the proliferation, invasion and migration of HCC cells and promote cell apoptosis to inhibit the growth and metastasis of HCC [26]. Related studies confirmed the nature of senescent cells, and revealed the metabolic link between cellular senescence and lactate production, including but not limited to cancer, inhibition of *PDK4* can reduce somatic

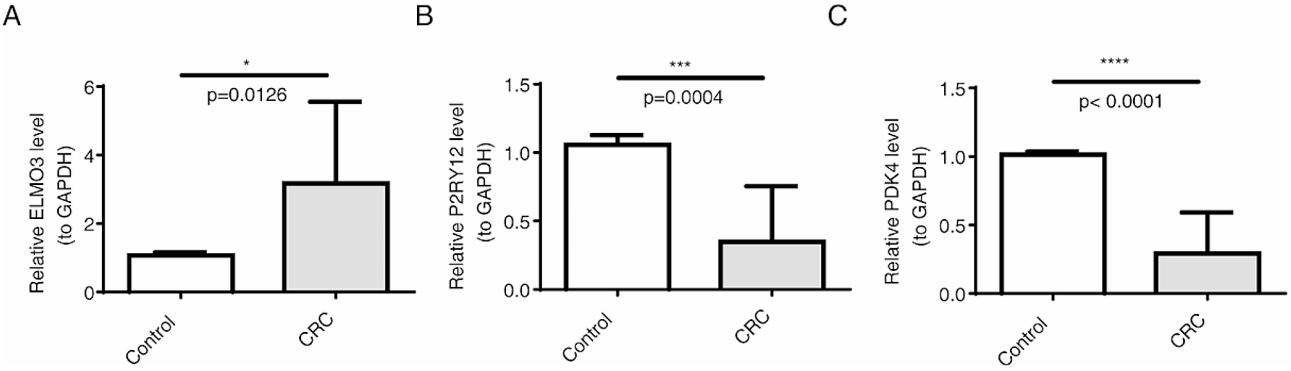

Fig. 7 The expression levels of the biomarkers. (**A**) ELMO3, (**B**) P2RY12, and (**C**) PDK4. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.001

dysfunction, prevent age-related frailty, medium from *PDK4*+stromal cells promotes the malignancy of recipient cancer cells in vitro, while inhibition of *PDK4* leads to tumor regression in vivo [27]. Furthermore, upregulation of the PPARα/*PDK4* pathway can inhibit CSC properties, and pancreatic cancer patients with high *PDK4* gene expression have better prognosis than patients with low expression. This process is related to the induction of cGMP, and the mechanism of action is to inhibit the mitochondrial function of PDACs. The entry point of this study is that the cells produce organelles for energy and metabolism, thus inhibiting the metastasis and proliferation of pancreatic cancer cells [28]. Differently, this paper found that the inhibitory effect of *PDK4* on colon tumor cells does not down-regulate miR-9-5p expression, but acts together with the genes PDP 2, PDP 1 and PDPR on the synthesis, metabolism and thioester biosynthesis of coenzyme A, which act on the synthesis, metabolism, and thioester biosynthesis processes of coenzyme A. The high expression of *ELMO3* in the CRC group may be related to its malignant nature. It is involved in regulating the signaling pathway of epidermal growth factor receptors and phagocytosis, as well as immune response regulation of cell surface receptor signaling pathways, along with related expressed genes such as ADGRB3, ARL4A, DOCK3, etc. It is involved in the growth, invasion, and metastasis of CRC, and is expected to become a new target for the prevention and treatment of CRC [21]. Clinical studies have found that *ELMO3* is mostly applied to the prognostic evaluation criteria of cancer, and it is a protein involved in cell migration and promoting cytoskeletal remodeling [29, 30]. The expression of *ELMO3* is not only CRC, but also widely used in lung cancer, laryngeal cancer and gastric cancer [31]. Knockdown of *ELMO3* by a specific siRNA inhibited the process of cell growth, invasion, and migration in gastric cancer, which was significantly correlated with lymph node metastasis, tumor size, differentiation, and grade of cancer pathological stage, not sex and age [32]. Moreover, *ELMO3* was positively correlated with COX-2 expression, and *ELMO3* inhibited epithelial stromal transformation (EMT), adhesion and metastasis of Lewis lung cancer cells, and the expression of *ELMO3* and epoxygenase-2 (COX-2) was significantly increased in normal tissues compared with adjacent normal tissues [33, 34]. It can be seen that the gene expression role of *ELMO3* in cancer is certain, which is consistent with this experiment. Interestingly, unlike gastric cancer regulating *ELMO3* by special gene chain and inhibiting the external transport process of cancer cells in lung cancer, *ELMO3* expression in this experiment focuses more on the internal cell structure.

The expression of *P2RY12* and *PDK4* were both lowly in the CRC group. Relevant studies [35] have confirmed that miR-15a can directly inhibit colon cancer cells through the target gene *PDK4.* Some scholars found that inhibiting *P2RY12* can directly inhibit the formation of cancer-associated thrombosis and tumor metastasis in pancreatic cancer models, which is similar to the significant correlation between *PDK4* and *P2RY12* and resting mast cells and macrophages M0, and the knockdown of *PDK4* and *P2RY12* could directly inhibit the migration of cancer cells [36]. The expression genes related to *P2RY12* not only exhibit pro-inflammatory macrophage response characteristics, but their expression and localization are also closely related to tumor grading [37, 38]. Therefore, downregulation of *PDK4* and *P2RY12* gene expression can effectively regulate resting mast cells and macrophages M0 to inhibiting the growth and propagation of CRC.

The IPA results indicate that these biomarkers are significantly positively correlated with the S100 family signaling pathway, while in the remaining 9 signaling pathways, such as the "neuronal CREB signaling pathway" and "aging pathway", they are exactly the opposite. *PDK4* and *P2RY12* showed functional similarities in the high expression group, with their signaling pathways acting on the muscle system, blood circulation, body development, and cardiac contraction, while *ELMO3* was concentrated in the expression of cellular structural components such as immunoglobulins. Activated CD4 memory T cells, macrophage M0, and resting mast cells showed significant differences in infiltration abundance during the experiment. Resting mast cells showed a significant positive correlation with *PDK4* and *P2RY12*, while macrophage M0 showed the opposite correlation with *PDK4* and *P2RY12*. The three biomarkers are not only important components of the tumor microenvironment (TME), indicating that they may have a stable effect on tumor occurrence and development, but also exhibit low mutation rates in CRC samples [39]. Numerous studies have shown that activation of S100 family signaling pathway can inhibit invasion and migration of cancer cells in the digestive system, and S100A4 is its direct target. Aberrant activation of Wnt/β -catenin pathway is a key driver of colorectal carcinogenesis [40–42]. Studies by foreign scholars have shown that regulating resting mast cells can effectively weaken anti-tumor immune responses [43, 44]. From these citations, we can know that regulating or inhibiting normal phagocytes can significantly inhibit the migration and proliferation of cancer cells, while reducing the sensitivity of S100 family signaling pathway can directly affect cancer cells, which is consistent with the results of this experiment. Regulation of macrophages and resting mast cells can increase *PDK4* and *P2RY12* expression and thus inhibit CRC tumor cells, through the regulation of the extracellular microenvironment, but not by the cells themselves.

This study found that *ELMO3* can be regulated by SP1 and CDX2 in 22 lncRNA predictions from miRWalk, miRDB, and miRTarBase databases. Previous studies have shown that CDX2 has an unstable predictive role in medullary colon cancer (MCC) [45, 46]. Research has found that IGF1-R antagonists such as linsitinib and aspirin can enhance the expression of CDX2 and PTEN genes, thereby inhibiting the growth of colon cancer stem cell-like cells. *PDK4* may be regulated by PPARA, RELA, NFKB1, PPARD, and E2F1. Related studies have used Fructus Aurantii (CHA) and Angelica sinensis (FRA) to improve the effective mechanism of CRC and predict their potential targets for treating CRC, including PPARA and PPARD [47]. This discovery provides a new basis for studying new compounds in traditional Chinese medicine. In addition, in the study of reducing chemotherapy resistance and sensitive targets of colon cancer with dioscin, it was found that PPARA and other substances involved in regulating cell cycle and apoptosis can reduce their sensitivity and effectively inhibit colon cancer cell proliferation [48]. Network pharmacology studies have found that vitexin and aspirin can inhibit the growth of related cancer cells through the regulation of multiple target proteins such as NFKB1 [49].

VCR combined with CEL may treat colon cancer by inhibiting the activation of PI3K/Akt signaling pathway and the expression of Bcl-2 to achieve improved energy metabolism and inhibit the PI3K signal transduction pathway [50]. In the experiment, *ELMO3*, *P2RY12* and *PDK4* were found to have higher sensitivity to SR16157, fluphenazine and Dexamethasone Decadron and so on. Some studies have found that although 5-FUor fluphenazine treatment for CRC is effective, their drug sensitivity gradually decreases with drug resistance [51, 52]. Fluphenazine is a calmodulin inhibitor that has been found to induce cell cycle arrest, reduce cell proliferation, and cause apoptosis in several cancer cells. This drug is able to target cancer-related proteins and regulate Akt and Wnt signaling pathways through a mechanism likely achieved by disrupting lysosomal function and blocking autophagic flow [53–55]. Combination treatment with cur/mPEG-PCL and erl/mPEG-PCL reduced the expression of α v β 3 integrin in resistant colon cancer cells, thereby increasing *PDK4* gene expression [56]. Unlike the former, the study found that Dexamethasone Decadron mediated PPARA signaling through *PDK4* to effectively reduce colon cancer cell spread and proliferation. The high sensitivity of Fluphenazine to *P2RY12* can effectively inhibit colon cancer cells, but the occurrence of drug resistance has not been involved in this study.

Targeting miR-144-3p-PDK4 axis to regulate glucose metabolism and silencing HCG11 can inhibit colon cancer cell proliferation, migration, invasion, and glucose metabolism. In this study, *PDK4* mainly focused on Amuvatinib (r=0.352, P=0.006) and BAY-87-2243, with significant side effects observed in AT-13,148. Compared with the former, its sensitivity often does not achieve ideal results. Therefore, in clinical practice, *ELMO3* and *P2RY12* related signaling pathways with higher drug sensitivity should be selected, and clinical efficacy will also be relatively improved.Combining Crocin and Sorafenib were highly effective in reducing hepaticβ-catenin overexpression and liver tumor and liver proliferation, withβ-catenin activation in tissue in up to 78% of hepatocellular carcinomas [57]. In other Hcancers, aggravated protein signaling in cancer cells is associated with increased expression levels ofβ-catenin expression [58]. This suggests that the abnormal proliferation of hepatocytes and even carcinogenesis are closely associated with the activation ofβ-catenin causing the downstream target genes to be stimulated to express themselves. At the same time, HCC cells were accompanied by significantly increased levels of CRP, LDH and IL-6, suggesting significant induction of liver injury and inflammation, and P53 is a pro-apoptotic protein playing a critical antitumor role. The combination of CR, SB prevented further damage by inducing cell cycle arrest to either allow DNA repair or by apoptosis to reduce the expression level of p53 after HCC induction [57]. Enhancement of NF-κB activity by multiple pro-inflammatory cytokines is the main mechanism for inducing nephropathy pathogenesis, and Nrf2 can relieve the inflammatory response by blocking the translocation and activation of NF-κB or direct inhibition of pro-inflammatory cytokine gene transcription [59]. The concurrent incremental treatment with DOX effectively attenuated the increase in renal oxidative stress by upregulating renal Nrf2 and SIRT1 expression. Combined with the above results, it can be found that inflammation induction in different tumor cells can directly or indirectly accelerate the proliferation of normal cell function damage and induce tumor cells, and on the other hand, block or inhibit biomarker-mediated signaling pathways or inhibit the response proliferation and metastasis of cells. This is the same as our findings that Fluphenazine can directly block *P2RY12* biotransmission through signal-mediated transmission, and Dexamethasone Decadron reduces or suppresses PPARA signaling through *PDK4* to inhibit colon cancer cell proliferation and promote apoptosis. Due to the particularity of the malignant nature of *ELMO3*, its sensitivity is significantly better than *P2RY12*, *PDK4*, through ADGRB3, ARL4A directly involved in the cell surface receptor signresponse regulation of phagocytosis, which shows that the inhibition of *ELMO3* high expression in CRC effectively block tumor cell growth, invasion and metastasis, its related mechanism is beneficial to develop new ideas for clinical diagnosis and treatment of CRC.Many studies have constructed rat animal models and combined pathological

sections, protein imprinting and DNA cytometry techniques to verify the therapeutic effect of inflammatory markers or drugs on diseases such as hepatocellular carcinoma, liver cirrhosis and renal function. On this basis, we can further use these techniques to validate the effects of biomarkers such as *PDK4*, *ELMO3* and *P2RY12* in the treatment of cancer diseases, so as to achieve precise targeted therapy and predict prognostic efficacy.

In summary, the efferocytosis effect is mediated by its specific biomarkers *PDK4*, *ELMO3*, *P2RY12*, and their related genes. It is not only beneficial for regulating the growth and differentiation of colon cancer cells, but also increases the sensitivity of anti-tumor drugs to achieve precise targeted treatment clinical effects and predict their prognostic efficacy. However, due to the lack of validation of biomarkers and functional experimental exploration in this article. The mechanism of action needs further clinical research.In this study, we did face the problem of a relatively small sample size and possibly some homogeneity, which may affect the stability and reliability of the results. The homogeneity of the sample may also limit the applicability of the study conclusions to a broader population of CRC patients. These findings need to be further validated in larger and more diverse cohorts, ensuring that studies can cover CRC patients of different gender, age, race, socioeconomic status, and disease stage and treatment options. Only then can we more accurately assess the applicability of these findings in different populations of CRC patients and provide more solid evidence support for clinical practice and public health policy. RT-qPCR serves as a highly sensitive, highly specific technique that mainly focuses on changes in mRNA levels, while changes in mRNA levels do not always directly reflect changes in protein levels. This difference may result from a variety of factors, including but not limited to post-transcriptional regulation (e.g., mRNA stability, splice variants), translation efficiency, and protein degradation rate. Therefore, relying on RT-qPCR alone may not fully reveal the true face of biological processes, and a further validation in combination with experiments such as Western Blotting is needed.

By differential expression of three biomarkers, *ELMO3*, *P2RY12* and *PDK4*, in the development of colorectal cancer tumor cells. It not only can expand the clinical diagnosis and treatment thinking of colorectal cancer, but also improve diagnostic specificity and sensitivity. Disrupted development during tumor cell proliferation and development. It is expected to provide treatment ideas for the prognostic problems caused by the continued invasion and recurrence of CRC.

Clinical practice points

This study identified three efferocytosis-related biomarkers (ELMO3, P2RY12, and PDK4) by integrating data from clinical transcriptome sequencing and public databases, and verified their expression by real-time quantitative polymerase chain reaction (RT-qPCR), which provides a scientific reference for further study of colorectal cancer (CRC) in the clinical field. Due to the lack of experimental conditions and funds, we failed to carry out the perfect verification. We plan to verify the efficacy and accuracy of these biomarkers in a larger and more diverse cohort of patients in a future study, through the use of Western Blotting, immunohistochemistry, mass spectrometry to directly observe the expression of proteins or flow cytometry. A series of experimental studies were carried out, using CRISPR-Cas 9 gene editing, RNA interference and chromatin immunoprecipitation and other technologies to explore the specific roles of these biomarkers in the development of diseases.

Ultimately, we expect to be able to apply these biomarkers to clinical practice by designing rigorous clinical trials to evaluate their efficacy in terms of disease diagnosis, prognosis assessment, and treatment monitoring.

Abbreviations

ERGs	Efferocytosis-Related Genes
CRC	Colorectal Cancer
RT-qPCR	Real-Time quantitative Polymerase Chain Reaction
ERGs	Efferocytosis Related Genes
SNP	Single Nucleotide Polymorphism
GEO	The Gene Expression Omnibus
ROC	Receiver Operating Characteristic
TF	Transcription Factors
PPI	The Protein-Protein Interaction
DEGs	The Differentially Expressed Genes
GO	The Gene Ontology
KEGG	Kyoto Encyclopedia of Genes and Genomes
LASSO	The Least Absolute Shrinkage and Selection Operator
GSEA	Gene Set Enrichment Analysis
IPA	Ngenuity Pathway Analysis
TCGA	The Cancer Genome Atlas
VCR	Vincristine
CEL	Celastrol
CR	Crocin
SB	Sorafenib
HCC	Hepatoma Carcinoma Cell

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

Supplementary Material 6

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Author contributions
Zhang Shengliang and Jiang Ying wrote the main manuscript text, Shi Lei, Wei Tianning and Lai Zhiwen are served as project guidance and responsible for data collection of clinical subjects. FengXuan and Li Shiyuan are responsible for the experimental data collation and statistical analysis. Tang Detao is responsible for the publication as the corresponding author.

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Data availability
Data is provided within the manuscript or supplementary information files.

Declarations

Ethics approval and consent to participate
This study followed the guidelines of the Declaration of Helsinki and was approved by the Ethics Committee of the First Affiliated Hospital of Guizhou University of Traditional Chinese Medicine(Approval No. KS2023199). All patients had signed an informed consent form.

Consent for publication
Written informed consent for publication of their clinical details and/or clinical images was obtained from the patient/parent/guardian/ relative of the patient. A copy of the consent form is available for review by the Editor of this journal.

Competing interests
The authors declare no competing interests.

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