The role of miR-488-3p on suppressing colorectal cancer by Codonopsis Bulleyana Forest ex Diels in mice

Lily Liu (liulily0518@163.com)
Southwest Forestry University
Yunpeng Luan
Southwest Forestry University

Research

Keywords: cbFeD, CRC, miR-488-3p, PTEN, cell apoptosis

Posted Date: November 22nd, 2022

DOI: https://doi.org/10.21203/rs.3.rs-2243523/v1

Abstract

(Background) Colorectal cancer (CRC) is a frequently occurring cancer. We aimed to evaluate the roles and possible mechanism of microRNAs (miRs) and *Codonopis bulleynana* Forest ex Diels (*cb*FeD) in the development of mice CRC.

(Methods) The differentially expressed miRNAs (DEMs) in CRC mice and their response to *cb*FeD were analyzed using global miRNA expression profiling, and then the role of *cb*FeD on CRC was through regulating the expression of miRNA in mice was identified in MC38 cell lines that was incubated with *cb*FeD and over-expressed with miRNA mimics.

(Results) Results showed that 54 DEMs were found in CRC mice, and they enriched in the processes of cancer development and apoptosis. Among them, miR-488-3p was significantly increased and decreased following exposure to *cb*FeD. In *cb*FeD incubated MC38 cells, the expression of miR-488-3p was significantly decreased, and its target gene PTEN (phosphate and tension homology deleted on chromsome ten) was increased markedly. After over-expressing miR-488-3p mimics in MC38 cells, PTEN was decreased by 20% while a 3-fold increased when exposed to cbFeD for 48h. And it was also found that *cb*FeD could significantly suppress cell proliferation activity and promote apoptosis in MC38 cells, and it could play the above effects following over-expressed miR-488-3p mimics.

(Conclusions) Overall, our results here suggested that there may be a negative correlation between miR-488-3p and PTEN, and targeting PTEN by miR-488-3p may be a mechanism by which *cb*FeD suppresseed the CRC in mice.

Introduction

CRC is one of the most commonly malignant tumor and result in a very high mortality[1]. The current treatments for this malignancy mainly include chemotherapy, radiotherapy and surgery, all of which are aim to suppress cancer cell proliferation and induce apoptosis, while they are not always successful and associated with a high risk of complications. Since cell apoptosis of tumor is a complex process, understanding the key mechanisms and molecules involved in it to develop effective therapeutics for treating CRC. MiRs are small non-coding RNAs that play a crucial role in gene expression through post-transcriptionally regulating the in animals [2]. Especially, numerous studies have found that many annotated miRs were one of the profoundly notable molecules in apoptosis, cell proliferation and metastasis in variety of tumors including colon tumor as the critical mediators of cancer gene silencing[3, 4][5, 6]. A better understanding of the molecular mechanism of miRs in CRC may lead to new ideas for diagnosis and therapies.

*cb*FeD is an obligate shade perennial native of Yunnan province in China. And because of a wide range of pharmacological effects, including its primary anticancer function, it has been one of the most popular medicinal herbs used in Yunnan province. *Cb*FeD is a potential agent in CRC [7], but only limited studies

on it have been carried out. To further understand the anti-cancer mechanism of *cb*FeD, we carry out this study, and explore the regulation of *Cb*FeD on miR in CRC.

Material And Methods

Ethics statement

All procedures for animal handling were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Yunnan University of Chinese Medicine (Permit Number: R-062021G049).

Preparation of cbFeD aqueous extract

The dry *cbFeD* was purchased from Yunnan International Pty, Ltd. (Yunnan, China). 1 kg of dry *cbFeD* was soaked in cold water for 30 min, decocting twice with 1:6 w/v distilled water for 1 h. Filtration was then performed to the appropriate concentrations, the first decoction comprised in 1:10 w/v distilled water for 90 min and second comprised 1:8 w/v distilled water for 60 min. A final quantity of 450 g dried powder was obtained by spray drying at room temperature, which was then sealed and stored in the dark at 4˚C. The *cbFeD* powder was dissolved in normal saline for gavage. The drug containing serum solutions were collected from mice following exposure to the following treatments (once per day for 1 week): Treatment with normal saline by gastrogavage (n = 8; normal control group); 5 g/kg of *cbFeD* by gastrogavage (n = 8; *cb*FeD treat group). The mice were euthanized at day 30, and the colon tissue samples were obtained and stored at 80˚C.

Establishment of CRC mice model and immunohistochemistry (HE)

The Kunming mice (average age is 8 weeks, average live weight is 22 g) were provided by Kunming Medical University, and housed in consistent and standard environmental conditions with laboratory. Based on our criteria, experimental samples were made up of three groups of mice, including CRC group induced by DMH (Dimethylhydrazine, TCI Company, Japan) (CaD) and the control group (C). We set out to compare miRs changes among three groups with a high variation in CRC development. The chosen mice were slaughtered according to guidelines for the ethical use and treatment of animals in experiments in China. Colon tissue were separated and stored in liquid nitrogen until analyzed. The total RNA from colon was extracted by using TRIzol. The quality of miRs was assessed by the 2100 Bioanalyzer (Agilent, USA).

The CRC mice tissues were embedded in paraffin and HE staining was carried out on the 5-μm-thick sections. The sections were dewaxed with xylene for 5 min and repeated for 3 times. Then rinse with anhydrous ethanol for 5 min and repeat for 3 times. Then, 95%, 80%, 75% ethanol and distilled water were used for gradient elution for 2min each time. The sections were stained with hematoxylin drops for 5min, and rinsed with tap water for 2min. After 30s of differentiation with 1% hydrochloric acid ethanol, the water was soaked in antiblue treatment for 15min. The sections were stained with Kay red dye drops for

2min, and rinsed with tap water for 3-5s, dehydration were carried with 70% ethanol, 80% ethanol, 95% ethanol, 100% ethanol (2 times), xylene (3 times) for 1min. Finally, adding neutral resin for seal.

Small RNA library construction and sequencing

After extraction of the total RNA, the RNA molecules with a size range of 18-30nt were enriched by PAGE. Small RNA libraries were then constructed using a NEBNest multiplex small RNA library prep set (NEB E7300, Illumina) according to the manufacturer's protocol. The small RNA libraries were then sequenced on an Illumina HiSeq TM 2500 by Gene Denovo Biotechnology Co. (Guangzhou, China).

Alignment and identification of miRs

Reads obtained from the sequencing machines included dirty reads containing adapters or low quality bases which would affect the following assembly and analysis. Thus, to get clean tags, raw reads were further filtered. After filtering, all of the clean tags were aligned with small RNAs in GeneBank database and Rfam database (11.0) to identify and remove other RNA. All of the clean tags were also aligned with reference genome and then searched against miRBase database (Release 21) to identify known mouse miR. Total miRNA consists of exist miRNA, known miRNA and novel miRNA, based on their expression in each sample, the miRNA expression level was calculated and normalized to transcripts per million.

Bioinformatic analysis of miRs data and target generation

Based on the sequences of the exist miRs, known miRs and novel miRs, the candidate target genes were predicted using three softwares RNAhybrid (v2.1.2) + svm_light (v6.01), Miranda (v3.3a) and TargetScan (Version:7.0). Gene Ontology classification, enrichment and KEGG analysis was performed base on the genes.

Cell culture, miR transfection and cbFeD incubating

The MC38 cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS (cat. no. 10099158; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 μg/ml of streptomycin and 100 U/ml of penicillin in a humidified atmosphere of 5% CO_2 at 37˚C. The logarithmically growing cells were transfected with miR-488-3p mimics and its corresponding control RNA (mimics NC). The sequence of miR-488-3p mimics: 5'-UUGAAAGGCUGUUUCUUGGUC-3'. For transfection, 1.5×10^5 cells were cultured in 6 well plates 1 day for 24 h. And then, the MC38 cells were transfected with of miR-488-3p mimics or mimics NC (10 nmol/L) using INTERFER in siRNA Transfection Reagent (Polyplus Transfections, Illkirch, France). For *cb*FeD incubating, 1.5×10^5 cells were cultured in 6 well plates 1 day for 24 h. And then, the MC38 cells were incubated with 20μg/mL *cb*FeD for 48h. According protocol, cells were harvested after 48h, and total RNA was extracted using TRIzol regent (Invitrogen, CA) and determined by Nanodrop 2000.

Quantitative real-time PCR analysis

Total RNA was isolated from MC38 cells and converted in cDNA using AMV reverse transcriptase (Promega Corp, WI), and then transcript copies were calculated relative to those of the housekeeping gene

GAPDH by qPCR that was performed using TaqMan MicroRNA Assays with the 7300 PCR Assay System (30 cycles, denaturation at 94℃ for 30 s, annealing at 60℃ for 30 s, extension at 72℃ for 30 s with final extension at 72℃ for 10 min). The primers were designed with Primer 5.0 and synthesized by BGI (Beijing Genomics Institute) Co., Ltd. The primer sequences are: PTEN Forward 5'-TTGTTAGCCTCTTCATGTGTGC-3', Reverse 5'- TGGTAGCCAAACGGAACTTCA-3';GAPDH Forward 5'-GAGTCAACGGATTTGGTCGT-3', Reverse 5'-TTGATTTTGGAGGGATCTCG-3'. The relative expression of genes was calculated using the $2^{-\Delta\Delta Ct}$ method and the standard deviation was calculated based on three biological replicates.

Western blot analysis and antibodies

The cells were harvested using protein extraction solution (Intron Biotechnology, Sungnam, Korea), and incubated for 30 min at 4˚C. Following removal of cell debris, the supernatants were collected by centrifuging at 13,000 x g for 15 min at 4˚C and the protein concentrations were determined using a Bio-Rad protein assay reagent, according to the manufacturer's protocol. PTEN (1:500; cat. no. ab137337) level was detected on western blot. 30 μg proteins were separated by 10% SDS-PAGE at 110V of 2hrs. Gel was transferred to PVDF membranes (BioRad, USA). Ponceau-S stain was used to evaluate the transfer efficiency. The strips were blocked with 5% nonfat dry milk powder in 0.1% TBST for 1 hr at RT and then incubated with primary antibodies in 2% BSA/TBST overnight at 4°C or 2 h at room temperature. The membranes were washed with 0.1% TBST buffer for 1 hr followed by probing with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (1:1000 dilution in PBS) (Sigma-Aldrich, USA). The membranes were then washed 0.1% TBST for 1 hr and bound antibody was detected by enhanced chemi-luminescence detection reagents (GE Biosciences, USA) according to manufacturer's instructions. Relative expression was calculated and normalized to the values of β-actin (Santa Cruz, Texas, USA) control.

Cell counting kit assay, flow cytometry apoptosis analysis

Cell counting kit-8 (CCK-8; Dojindo, Tokyo, Japan) was applied to assess cell growth. After 48 hours of transfection, 1×10^3 cells were incubated in each well of the 96-well plates. Then, each well was added with 10 microliters of CCK-8 solution mixed with a non-serum medium at days 1, 2, 3, and 4. After another incubation of 2 hours, the absorbance was examined at 450 nm using a microplate reader.

For analyzing cell apoptosis, cells were seeded in 6-well plates with the destiny of about $1\times l0^4$ each well. After 48 hours of transfection and *cb*FeD incubating, cells were collected, washed 3 times using cold PBS, and re-suspended with binding buffer at a concentration of 1×10^6/mL. Next, the cells were stained using fluorescein isothiocyanate (BioVision, San Francisco, CA) and PE (Beyotime). The signal was captured with the use of a FACS Calibur flow cytometer (BD Biosciences) and subjected to the analysis on FACS Diva software (BD Biosciences).

Statistical analysis

Each treatment was performed in triplicate. All data were compared via analysis of variance and multiple testing using the R-package (R v3.02). Differences were declared significant at $P < 0.05$ and $P < 0.01$.

Results

Establishing CRC mice model

Mice were injected with DMH for 16 consecutive weeks, the red, swollen and unclerated anus were observed. And anal prolapses, fecal glycodiles, colon mucosal folds increased significantly, colon wall tumors and vegetable pattern changes were observed. The HE staining verified that there were cancer cells in mice colon tissues (Fig. 1), which proved that the CRC mice model were established successfully.

RNA sequencing of mice CRC tiusses

A total of 64,559,891 raw reads and 62,661,998 high-quality reads were generated from the control and the CRC mice. We used ACGT101-miR v4.2 to analyze the sequencing data. By removing various un-mappable sequencing reads from the raw sequence reads, 9,707,851 sequences ranging from 18 to 30 nucleotides were obtained after adaptor trimming, accounting for 90.22% of all small RNA (sRNA) sequences. A summary of the data is showed in Table 1. Alignment with miRBase (Release 21) indicated that miRs were highly enriched in all libraries. To assess the efficiency of high-throughput sequencing for sRNA detection, all sequence reads were annotated and classified by alignment with Rfam databases.

Table 1
Statistics of miRNA-seq data of control and CRC mice.

Sample	Total reads	High quality reads	3' adapter null	Insert null	5' adapter contaminants	Clean reads
C1	10425792	10067753	20707	3834	2868	9418676
C2	10480376	10157238	18116	3146	2810	9469913
C3	10514116	10182231	19933	4048	3830	9356959
CaD1	10453386	10181603	22709	8134	5766	9389440
CaD2	11894883	11548547	25257	5654	3331	10938438
CaD3	10791338	10524626	24100	8510	5089	9673680
Sum	64559891	62661998	130822	33326	23694	58247106
Average	10759982	10443666	21803.67	5554.33	3949	9707851

A total of 54 distinct DEMs were identified in C group and CaD group mice (Fig. 2). 15 miRs were significantly up-regulated (≥ 1.2-fold) and 39 miRs were significantly down-regulated (≤ 0.83-fold). The target genes of DEMs were predicted, GO and pathways enrichment analysis were carried according to their biological processes (BPs), cellular components (CCs), molecular functions (MFs) and KEGG

database. As show in Fig. 3, the top 5 GO terms for BPs were enriched in cell differentiation (GO:0030154), developmental process (GO:0032502), cellular developmental process (GO:0048869), cell communication (GO:0007154), cell proliferation (GO:0008283). These biological processes were involved in the cellular development and proliferation. The top 5 GO terms for CCs were cell (GO:0005623), cell part (GO:0044464), intracellular (GO:0005622), cell periphery (GO:0071944), membrane-bounded organelle (GO:0043227). These cellular components were response to the membrane system, may related to cell division. The top 5 GO terms for MFs were mainly enriched in bingding (GO:0005488), DNA binding (GO:0003677), protein binding (GO:0005515), kinase activity (GO:00016301), transferase activity (GO:0016740). The taget genes of DEMs were mainly enriched in the pathways of Axon guidance, Wnt, Rap1, ErbB, TNF and regulation of autophagy. These pathways play important roles in tumor occurrence, progression and metastasis.

cbFeD could decrease miR-488-3p and promote target gene PTEN in MC38 cells

We found miR-488-3p expression was significantly increased ($P < 0.05$) in CRC mice model, and studies shown miR-488-3p always abnormally expressed in CRC [8]. Luan demonstrated *cb*FeD had exerted preventive effect on the chemically induced CRC mice model [9]. These indicated that *cb*FeD may regulate miR-488-3p to excert an anticancer effect in mice CRC. So we focused on the relationship between miR-488-3p and *cb*FeD, and incubated MC38 cells with 20μg/mL *cb*FeD for 48h. As shown in Fig. 4A, *cb*FeD could decrease the expression of miR-488-3p by 54% in MC38 cells.

To understand the mechanism of *cb*FeD regulating miR-488-3p, we assessed the potential target gene of miR-488-3p, and found *PTEN* was predicted to be the target gene (Fig. 4B). We examined the PTEN expression in *cb*FeD incubated MC38 cells and found the mRNA of it was increased by 7.2-fold (Fig. 4C). These findings indicated there may be a negative correlation between miR-488-3p and PTEN.

Further, to verify if *cb*FeD mediated suppression of *PTEN* is dependent on miR-488-3p, we over-expressed miR-488-3p mimics in MC38 cells, and then incubated cells with 20 μg/mL *cb*FeD for 48h. As shown in Fig. 4D, PTEN was increased 3-fold by *cb*FeD, and decreased by 20% after over-expressing miR-488-3p mimics, while a 3-fold increase was found in miR-488-3p mimics over-expressed MC38 cells when exposed to *cb*FeD for 48h. All together, these results are consistent with the hypothesis that the promotion of PTEN by *cb*FeD is dependent on miR-488-3p.

cbFeD could promote apoptosis of CRC cells

We detected the effects of miR-488-3p and *cb*FeD on cell proliferation activity and apoptosis degree in MC38 cells. Compared with the control cells, we found about 10%, 30%, and 25% reduction of cell proliferation activity and 7.26%, 27.79% and 29.25% apoptosis degree in MC38 cells after treating with *cb*FeD, miR-488-3p mimics + cbFeD, and miR-488-3p mimics, respectively (Fig. 4E and F). Our results showed *cb*FeD could significantly suppress cell proliferation activity and promote apoptosis in MC38 cells, and it could play the above effects following over-expressed miR-488-3p mimics. PTEN is the key

player in suppress cell growth and survival in cancer cells, *cb*FeD could increase the PTEN level through miR-488-3p to play key roles in cancer proliferation and apoptosis. Overall, our results here show miR-488-3p and PTEN is at crossroads in the ability of *cb*FeD to exert anti-proliferation activities in mice CRC.

Discussion

A large number of studies have demonstrated miRs extensively deregulated in human cancers, highlighting their roles in cancer growth and development. In our study, we established the CRC mice model and profiled a total of 54 distinct miRs that enriched in the processes of cancer development and apoptosis. The target genes of DEMs were mainly involved in pathways of Axon guidance, Wnt, Rap1, ErbB, TNF, and regulation of autophagy. Wnt signaling pathways play a critical role in regulating homeostasis and self-renewal of tissues, particularly, control cell proliferation and migration[10, 11], is a major contributor to CRC carcinogenesis [12]. Rap1 has high sequence similarity to Ras and could promote invasion and cell migration and metastasis in various cancer types [13, 14]. The ErbB pathway plays pivotal roles in cell-cell communication and could regulate cancer development [15, 16]. TNF is an inflammatory cytokine frequently present in the tumor microenvironment [17]. TNF-α has biphasic effects, by which it could promote tumor angiogenesis and accelerates tumor invasion and metastasis with long-term administration of low doses, while it could also induces tumor cell apoptosis at high doses [18]. The DEMs found in our study are mainly enriched in these pathways, indicates that they could regulate mice CRC development through mediating cell communication, migration, metastasis and apoptosis.

In found DEMs, we detected miR-488-3p was significantly increased in the CRC mice model. This indicated miR-488-3p may play an important role in mice CRC. And it was worth noting that miR-488-3p could be decreased by 54% after *cb*FeD incubating in MC38 cells. This result clearly shown that *cb*FeD may play an antitumor role through regulating miR-488-3p. MiR-488-3p has been reported as a key factor regulates cell proliferation, migration and apoptosis [19]. Studies have shown that miR-488-3p is abnormally expressed in several cancers, such as colorectal [8, 20], glioma [21] and other cancers [22]. All of these data indicating that miR-488-3p is a functional miRNA in tumor development, our findings also shown that increased miR-488-3p related with mice CRC, but the mechanism was not clearly.

To validate the accurate role of miR-488-3p in mice CRC, the target genes of miR-488-3p were predicted and identified. Gene analysis indicated that PTEN was a target of miR-488-3p, which negatively regulated PTEN expression through binding to 3 -UTR site. PTEN is first discovered as a tumor suppressor with growth and survival regulatory functions, could directly oppose the activation of the oncogenic PI3K/AKT/mTOR signalling network [23]. Loss of function of the PTEN tumor suppressor is one of the most common events observed in many types of cancer [24–26], and more prevalent in later stage CRC. Rychahou and Nassif et al. have demonstrated that PTEN expression is lost in 66–70% of CRCs and in approximately 83% of metastatic CRCs [27, 28]. PTEN could be regulated by RNA-RNA interaction, enhanced levels of many miRs are correlated with a concomitant reduction of PTEN [29]. In this study, we found that PTEN was decreased in mice CRC with a concomitant rise of miR-488-3p, consistent with

previous studies. These may indicated that the negative regulatory relationship between miR-488-3p and PTEN in mice CRC.

Major components present in the *cb*FeD are organic acid and its derivatives, flavone, amino acid derivatives etc [30]. Luan and Li have shown that *cb*FeD processed anti-inflammtory and anti-cancer properties [9, 30]. In particularly, a high dose of *cb*FeD could improve long-term constipation-induced intestinal cancer by changing intestinal microbiota in mice [9]. In this study, we found *cb*FeD-treated MC38 cells resulted in a reduction of miR-488-3p and elevated expression of PTEN, and inhibiting cell proliferation and promoted cell apoptosis. After over-expressed miR-488-3p mimics, *cb*FeD can further exert the above effects, indicating some of components present in the *cb*FeD, could have the potential to inhibit miR-488-3p and enhance PTEN expression to plays its antitumor roles in mice CRC, however this needs further study to be confirmed. Therefore a vigorous isolation process of active components of *cb*FeD is underway and future studies regarding this are in the line.

Conclusions

All together, our data suggest that miR-488-3p may be a key miRNA in mice CRC through regulating PTEN, and given the increasing understanding that *cb*FeD and/or its components have potent antitumor activity through inhibiting miR-488-3p and enhancing PTEN expression to suppress cancer cells proliferation and promote cancer cell apoptosis. Our study provides evidence of a possibility that *cb*FeD alone, or in concert with and/or miR-488-3p mimics, may be an important new therapy in the chemoprevention and/or treatment of CRC in human.

Abbreviations

Colorectal cancer (CRC); microRNAs (miRs); *Codonopis bulleynana* Forest ex Diels (*cb*FeD); differentially expressed miRNAs (DEMs); phosphate and tension homology deleted on chromsome ten (PTEN); IACUC (Institutional Animal Care and Use Committee)

Declarations

Ethical Approval and Consent to participate

All procedures for animal handling were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Yunnan University of Chinese Medicine (Permit Number: R-062021G049).

Consent for publication

The Authors confirms that the work described has not been published before, and the publication has been approved by all co-author.

Availability of supporting data

The data used to support the findings of this study are available from the corresponding author upon request.

Competing interests

We declare that we have no conflict of interest.

Funding

This work was financially supported by the Basic Research of Science and Technology Department of Yunnan Province (2019FD06) the Basic Research of Education Edpartment of Yunnan Province (2018JS333), the Scientific Research Foundation of Southwest Forestry Unversity (112119), the National Natural Science Foundations of China (31902152).

Authors' contributions

Lily Liu and Yunpeng Luan contributed to the conception of the study.

Lily Liu and Yunpeng Luan contributed to analysis and manuscript preparation.

Lily Liu performed the data analyses and wrote the manuscript.

Acknowledgements

This work was financially supported by the Basic Research of Science and Technology Department of Yunnan Province (2019FD06) the Basic Research of Education Edpartment of Yunnan Province (2018JS333), the Scientific Research Foundation of Southwest Forestry Unversity (112119), the National Natural Science Foundations of China (31902152).

Authors' information

The first anthor: Prof. Lily Liu, College of Life Sciences, Southwest Forestry University, No.300 Bailong Temple Street, Kunming, Yunnan 650224, China; Phone/Fax: +86 871 63864788, Email: liulily0518@163.com

Corresponding author: Prof. Yunpeng Luan, Key Laboratory for Forest Resources Conservation and Utilization in the Southwest Mountains of China, Ministry of Education, Southwest Forestry University, No.300 Bailong Temple Street, Kunming, Yunnan 650224, China; Phone/Fax: +86 871 63864788, Email: 1760690966@qq.com

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Figures

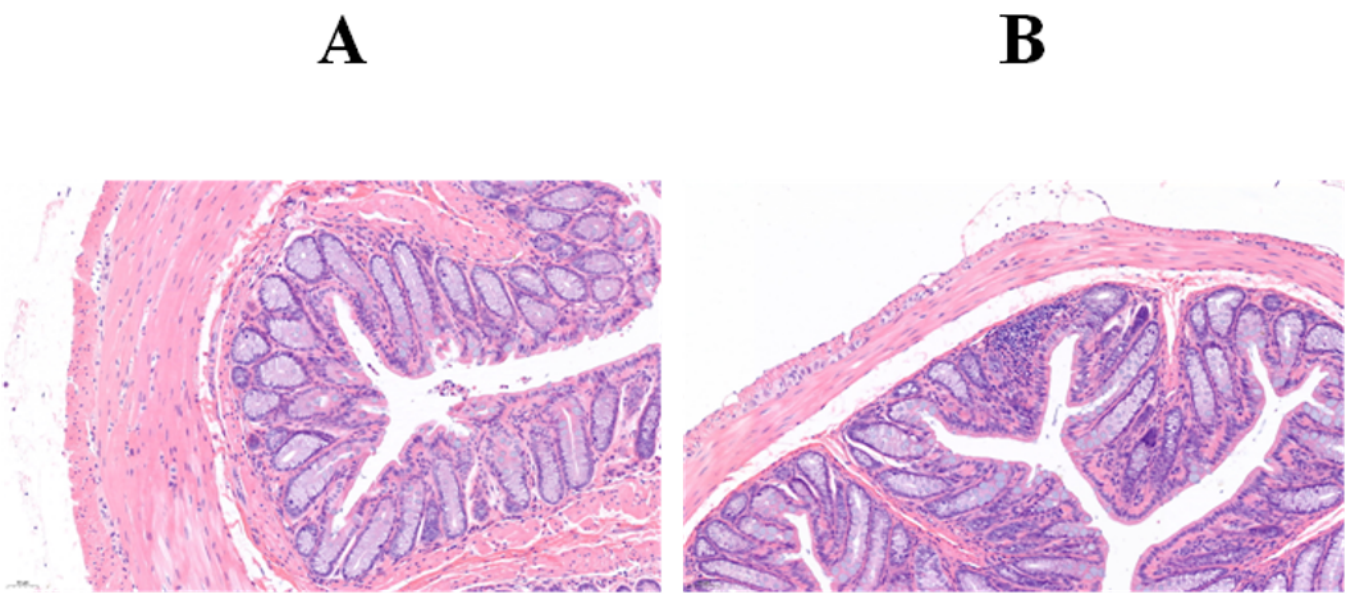


Figure 1

Morphological analysis of colon tissues (40×)

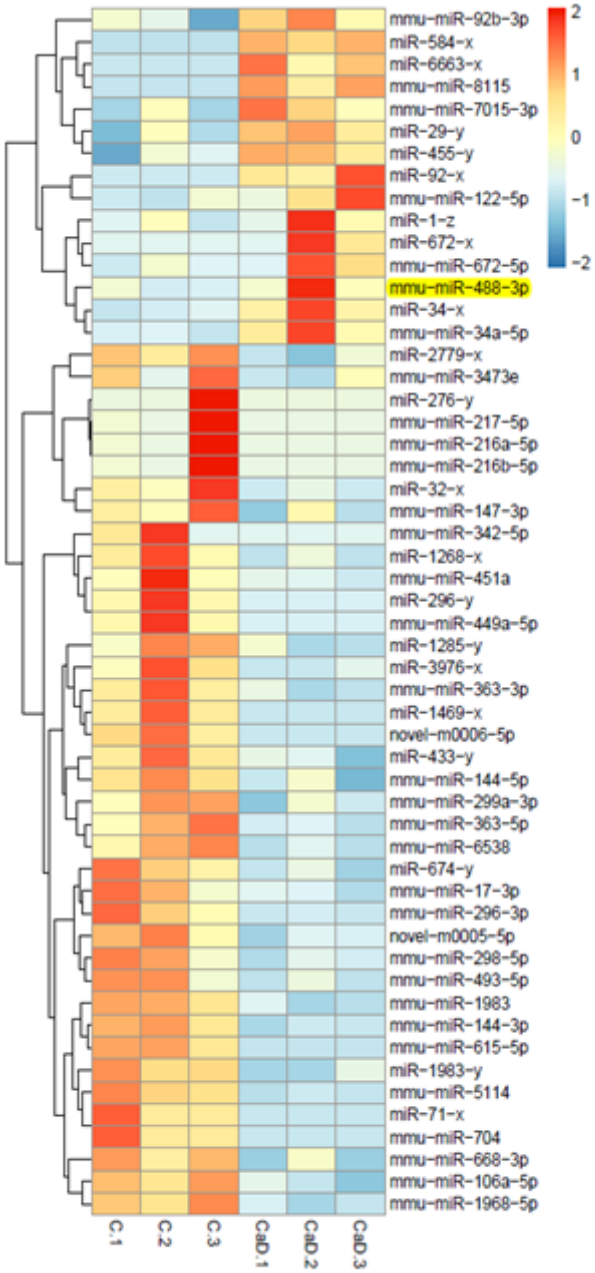
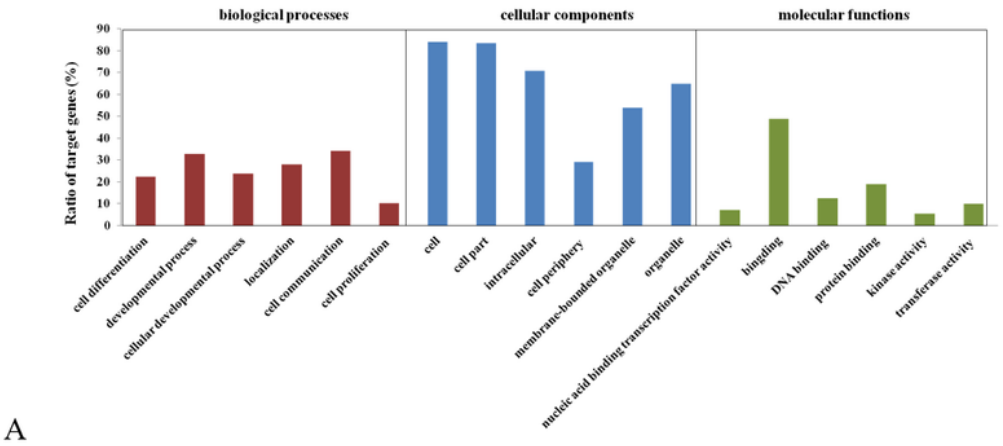
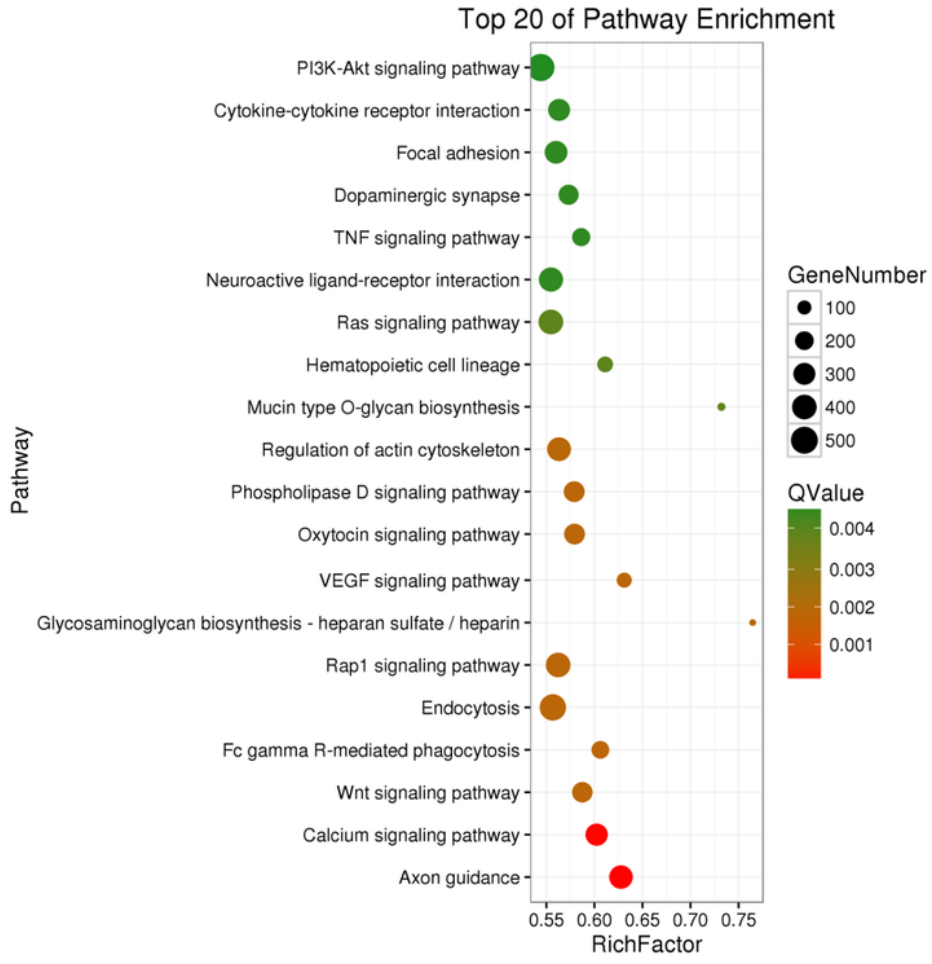


Figure 2

Visual analysis of DEMs in CRC mice model



A



B

Figure 3

GO analysis and KEGG analysis of DEMs

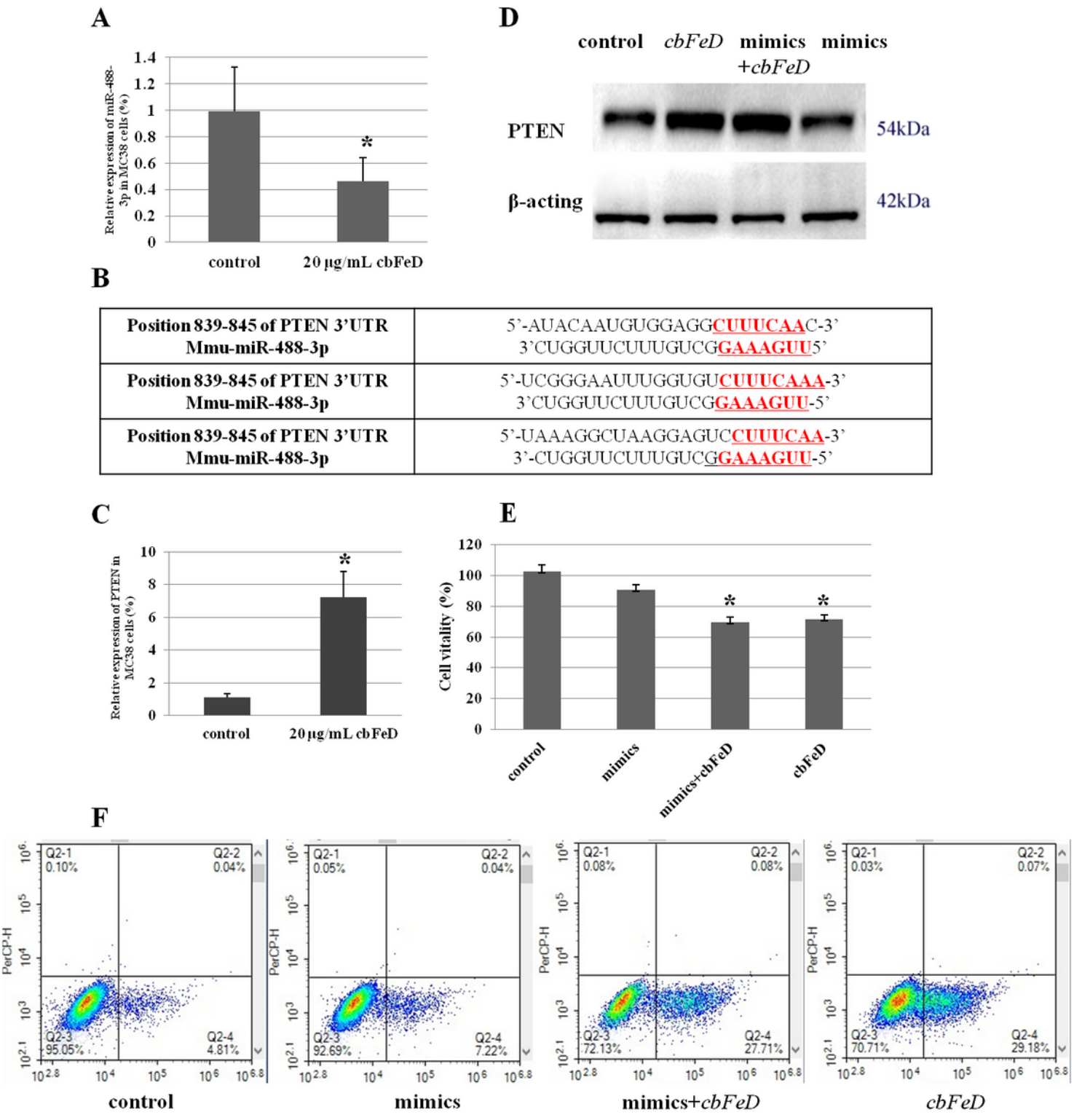


Figure 4

Analysis in MC38 cells after treating with *cb*FeD and miR-488-3p mimics.

A: The relative expression of miR-488-3p in MC38 cells incubated with 20μg/mL *cb*FeD; B: The matching situation of PTEN 3'-UTR and miR-488-3p; C: The relative mRNA expression of *PTEN* in MC38 cells incubated with 20μg/mL *cb*FeD; D: The relative expression of PTEN in MC38 cells after treating with

*cb*FeD, miR-488-3p mimics + *cb*FeD, and miR-488-3p mimics, respectively; E and F: The cell vitality and apoptosis level of MC38 cells after treating with *cb*FeD, miR-488-3p mimics + *cb*FeD, and miR-488-3p mimics, respectively.

*: indicates significant difference, P value < 0.05 from the control.