基于表观遗传学和代谢组学的野菊花活性部位抗乙肝病毒整体作用分子机制研究

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摘要: 利用表观遗传学和代谢组学理念和方法, 探讨阐明野菊花活性部位 (*Chrysanthemi indici* C, CIC) 抗乙肝病毒 (hepatitis B virus, HBV) 整体作用分子机制。CCK-8和乙肝抗原试剂盒检测CIC对HepG2.2.15细胞增殖和乙型肝炎病毒表面抗原 (hepatitis B surface antigen, HBsAg)、乙型肝炎病毒e抗原 (hepatitis B envelope antigen, HBeAg)、乙型肝炎病毒核酸 (hepatitis B virus-deoxyribonucleic acid, HBV-DNA) 的抑制作用; ELISA法检测CIC对DNA甲基转移酶 (DNA methyltransferases, DNMTs)/去甲基转移酶2 (ten-eleven-translocation-2, TET2) 平衡关系的影响; 利用Illumina 850K甲基化芯片、焦磷酸测序和qPCR技术, 通过GO、KEGG等分析, 确定CIC抗HBV的作用途径和靶点; 80%甲醇提取细胞代谢物, LC-MS等代谢组学方法检测差异代谢物、差异代谢途径及细胞微环境的变化。结果表明, CIC对HepG2.2.15细胞增殖和HBsAg、HBeAg、HBV-DNA有明显的抑制作用, 下调DNA甲基转移酶1 (DNA methyltransferase 1, DNMT1)、DNA甲基转移酶3a (DNA methyltransferase 3a, DNMT3a)、DNA甲基转移酶3b (DNA methyltransferase 3b, DNMT3b), 上调TET2, 恢复DNMTs/TET2平衡; DNA甲基化测序结果表明, CIC抗乙肝病毒的作用靶基因有磷脂酶C-γ2 (phospholipase C gamma 2, PLCG2)、磷脂肌醇3激酶 (phosphoinositide-3-kinase regulatory subunit 3, PIK3R3)、1酰基甘油3磷酸O酰基转移酶2 (1-acylglycerol-3-phosphate *O*-acyltransferase 2, AGPAT2)、5-羟色胺受体2B (5-hydroxytryptamine receptor 2B, HTR2B)、神经生长因子 (nerve growth factor, NGF), 主要涉及脂质代谢、瞬时感受器电位 (transient receptor potential, TRP) 通道炎症介导调节、磷脂酶D信号、糖尿病并发症中晚期糖基化终产物-AGE受体 (advanced glycation end product-receptor for AGE, AGE-RAGE) 信号等通路; 代谢组学研究表明, CIC可以显著影响脂肪酸代谢, 同时对细胞微环境中酚酸、生物碱、脂质类代谢物影响较大。研究结果提示, 野菊花活性部位抗乙肝病毒作用机制可能是通过调节表观遗传表达平衡而调控相关炎症通路、免疫通路、脂代谢等多途径、多靶点的协同作用, 并恢复细胞微环境平衡。

关键词: 野菊花; 活性部位CIC; 抗乙肝病毒; DNA甲基转移酶/去甲基转移酶2; 表观遗传学; 代谢组学

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The overall action molecular mechanism of anti-hepatitis B active extracts in *Flos chrysanthemi indici* based on epigenetics and metabonomics

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Abstract: Using the concepts and methods of epigenetics and metabolomics, to investigate the overall action

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molecular mechanism of *Chrysanthemi indici* C (CIC), the anti-hepatitis B virus (HBV) active extracts from *Flos chrysanthemi indici*. The inhibitory effects of CIC on proliferation and hepatitis B surface antigen (HBsAg), hepatitis B envelope antigen (HBeAg) and HBV-DNA of HepG2.2.15 cells were detected by CCK-8 and antigen kit. The DNA methyltransferases (DNMTs)/ten-eleven-translocation-2 (TET2) equilibrium was detected by ELISA. Illumina 850K methylation chip, pyrosequencing and qPCR were used to determine the action pathway and target of CIC by GO and KEGG analysis. Cell metabolites were extracted with 80% methanol, and the changes of differential metabolites, differential metabolic pathways and cell microenvironment were detected by LC-MS and other metabolomics methods. The results showed that CIC could inhibit the proliferation, HBsAg, HBeAg and HBV-DNA of HepG2.2.15 cells obviously, down-regulate DNA methyltransferase 1 (DNMT1), DNA methyltransferase 3a (DNMT3a) and DNA methyltransferase 3b (DNMT3b), up-regulate TET2, and restore the balance of DNMTs/TET2. The action targets of CIC were phospholipase C gamma 2 (PLCG2), phosphoinositide-3-kinase regulatory subunit 3 (PIK3R3), 1-acylglycerol-3-phosphate *O*-acyltransferase 2 (AGPAT2), 5-hydroxytryptamine receptor 2B (HTR2B), nerve growth factor (NGF), mainly involved in lipid metabolism, inflammation mediated regulation of transient receptor potential (TRP), phospholipase D signaling and advanced glycation end product-receptor for AGE (AGE-RAGE) signaling in diabetic complications pathways. CIC could significantly affect fatty acid metabolism and had great influence on phenolic acid, alkaloid and lipid metabolites in cell microenvironment. These results suggest that the action mechanism of CIC may be the synergistic action of multiple pathways and multiple targets, including related inflammatory pathways, immune pathways and lipid metabolism, through regulating epigenetic expression balance and restoring the balance of cell microenvironment.

Key words: *Flos chrysanthemi indici*; active extract CIC; anti-hepatitis B virus; DNA methyltransferases/ten-eleven-translocation-2; epigenetics; metabolomics

乙型肝炎是由乙型肝炎病毒 (hepatitis B virus, HBV) 引起的, 以肝损伤为主的传染性疾病[1]。乙型肝炎为目前危害大、难以治愈的一种病毒性疾病, 不但严重威胁着人类生命健康, 而且造成较大的社会危害, 但至今国内外对乙肝病毒疾病仍缺乏有效的控制手段和彻底的解决方案[2]。近年来, 随着对乙肝病毒和表观遗传学研究的深入, 认为乙肝病毒侵入并长期寄居在机体肝细胞中, 必将导致乙肝病毒与机体的表观遗传表达改变, 特别宿主和病毒基因的DNA甲基化水平会受到直接影响[3,4]。

表观遗传学是指DNA序列不发生变化, 但基因表达却发生了可遗传的改变。研究表明, 表观遗传学修饰特别是DNA甲基化与HBV感染过程密切相关[5]。当HBV侵入机体后, 病毒基因组和宿主细胞DNA甲基化水平均可能发生改变, 两者甲基化水平的改变可以调节乙肝病毒的复制并影响肝脏的炎症程度[6]。研究表明, DNA甲基化紊乱是众多疾病的共有模式, 针对异常DNA甲基化双向调控的治疗研究日渐成为防治复杂疾病的重要策略, 这与中药调控作用理念也不谋而合[7,8]。同时, 表观遗传学强调遗传因素和环境因素相互作用, 中医药重视内外环境对机体影响的整体观, 表明二者的理念渐趋一致, 可以互相借鉴利用共同发展[9,10], 促进中医药平衡观、整体观科学内涵阐明。

有学者认为, 中医阴阳的生物学根本基础可能就在于DNA高甲基化与低甲基化, 其与DNA甲基化转移酶 (DNMTs, 主要包括DNMT1、DNMT3a和DNMT3b) 和去甲基化转移酶 (特别是TET2) 的平衡调控关系密切[11,12]。从微观层面上看, DNMTs/TET2平衡关系决定了DNA的开 (阳) 与合 (阴), 是细胞生命运动的决策层面, 从而决定了基因转录与表达, 并把遗传和环境结合起来, 多靶点、多途径作用于机体使其恢复微观层面的平衡, 以达到治疗目的。

代谢组学通过对内源性小分子代谢物的整体性、系统性分析, 帮助揭示机体生命活动代谢及机体微环境的状况, 直接反映DNA调控的结果表现, 而细胞微环境又直接影响了DNA的表达[13,14]。

因此, 表观遗传学和代谢组学相结合建立研究模式, 可以相互呼应地阐明药物作用途径和作用靶点对微环境影响及DNA调控作用与细胞微环境间的相互影响, 从而系统阐明中医药基于平衡观和整体观的作用机制[15]。

野菊花 (*Flos chrysanthemi indici*) 来源于菊科植物野菊 (*Chrysanthemum indicum* L.) 的干燥头状花序。性苦、辛、微寒, 归肝、心经, 具有清热解毒, 泻火平肝的功效。现代药理学表明, 野菊花具有抗病毒、抗炎镇痛、抗肿瘤、调节免疫、抗菌、抗病原微生物、保肝等药理学作用[16,17]。本课题组前期研究从野菊花中发现一个具有抗乙肝病毒、肝保护、免疫调节等多途径作用的

活性部位 (CIC), 并系统阐明了CIC的物质基础, CIC主要含黄酮类化合物刺槐素、芹菜素等, 倍半萜类化合物豚草素A和野菊花内酯等。

为在微观层面、分子水平阐明CIC的整体作用机制, 本论文利用HepG2.2.15细胞实验, 系统研究了CIC对乙型肝炎病毒表面抗原 (hepatitis B surface antigen, HBsAg)、乙型肝炎病毒e抗原 (hepatitis B envelope antigen, HBeAg)、乙型肝炎病毒核酸 (HBV-DNA) 的抑制作用和对DNMTs/TET2平衡关系的影响, 利用Illumina 850K甲基化芯片、焦磷酸测序、qPCR技术、LC-MS等代谢组学方法, 通过GO、KEGG等生物信息学分析方法, 确定了CIC抗HBV的作用途径、作用靶点及对细胞微环境的影响, 为在微观层面阐明中医药平衡观、整体观科学内涵探索建立研究模式。

材料与方法

材料 胎牛血清 (Gemini公司); DMEM高糖培养基、胰蛋白酶 (北京索莱宝生物科技有限公司); HBsAg、HBeAg诊断试剂盒、HBV-DNA测定试剂盒 (上海科华生物有限公司); CCK-8试剂盒 (郑州德雅科技有限公司); G418 (北京博奥拓达科技有限公司); DNMTs/TET2检测试剂盒 (上海语纯生物科技有限公司); 细胞/组织基因组DNA提取试剂盒、RNApure高纯总RNA提取试剂盒 (北京百泰克生物技术有限公司); FastKing一步法除基因组cDNA第一链合成预混试剂、SuperReal PreMix Plus (北京天根生化科技有限公司); Real-time PCR反应引物 (上海生工生物工程有限公司); Illumina Infinium Methylation EPIC BeadChip (上海柏豪生物技术有限公司)。

仪器 细胞培养箱、超低温冰箱 (Thermo Fisher Scientific公司); SW-CJ-2D型超净工作台 (苏州名牌之星仪器有限公司); 液氮罐 (新乡市新亚有限公司); 酶标仪 (BioTek公司); BP211D分析天平 (Sartorius公司); 移液枪, 低温高速离心机 (Eppendorf公司); 制冰机 (日本三洋公司); LightCycler 96、LightCycler 8-Tube Strips (Roche公司)。

CIC的制备与质量控制 参照本课题组报道的提取物提取与质量控制方法[18,19]。用10倍量的含水乙醇作溶剂, 超声提取两次, 提取液减压浓缩得到野菊花浸膏, 浸膏经石油醚−乙酸乙酯−乙醇−水梯度萃取和硅胶柱层析, 得到活性部位CIC。主要含有倍半萜 (含量 ≥ 50%) 和黄酮 (含量 ≥ 30%) 两大类化合物, 其中豚草素A含量 ≥ 5.0%, 野菊花内酯含量 ≥ 3.0%, 刺槐素含量 ≥ 0.5%。

CIC母液的配置 用分析天平精确称取30 mg CIC溶于1 mL的DMSO中, 配成30 mg·mL^{-1}母液, 并在超净台中用0.22 μm微孔滤膜过滤。用时用培养基稀释成所需浓度。

细胞培养 HepG2.2.15细胞 [赛百慷 (上海) 生物技术股份有限公司] 采用含10% FBS的DMEM高糖培养基, 在37 ℃、5% CO_2培养箱中培养, 并使用380 μg·mL^{-1} G418严格定期筛选。

CIC对HepG2.2.15细胞增殖的抑制作用 细胞常规培养至对数生长期, 消化、计数后调整细胞密度为每毫升2.8×10^{5}个, 将其接种于96孔培养板中, 每孔100 μL细胞悬液, 于37 ℃、5% CO_2培养箱中培养, 48 h长至单层细胞, 弃去上清液, 换成不同质量浓度 (2.5、5、10、15、20、25、30、35 μg·mL^{-1}) 含CIC的完全培养基, 每孔200 μL, 同时设空白对照组, 每个浓度设4个复孔, 继续生长6天, 每3天更换含药培养基, 于第6天实验结束前4 h, 每孔避光加入5 μL CCK-8试剂, 继续生长4 h, 立即使用酶标仪在450 nm处测定其吸收度 (A) 值, 取平均值为其活力A值, 并计算半数毒性浓度 (TC_{50}) 和有效无毒浓度。

CIC对HepG2.2.15细胞分泌的HBsAg、HBeAg和HBV-DNA的影响 细胞常规培养至对数生长期, 消化、计数后调整细胞密度为每毫升8.4×10^{5}个, 接种于6孔培养板中, 每孔1 mL细胞悬液 (6孔细胞培养板的各孔应提前用1 mL完全培养基润湿), 置于37 ℃、5% CO_2培养箱中培养48 h, 小心弃去上清液, 换为含17.5 μg·mL^{-1} CIC的完全培养基, 同时设空白对照组, 继续生长6天, 每3天更换新鲜的含药培养基。收集第3、6天细胞上清液, 按照诊断试剂盒的要求分别检测HBsAg、HBeAg和HBV-DNA的含量。

CIC对HepG2.2.15细胞内DNA甲基化调控因子DNMTs/TET2的影响 细胞常规培养至对数生长期, 消化、计数后调整细胞密度为每毫升8.4×10^{5}个, 接种于6孔培养板中, 每孔1 mL细胞悬液, 置于37 ℃、5% CO_2培养箱中培养48 h, 小心弃去上清液, 换为含17.5 μg·mL^{-1} CIC的完全培养基, 同时设空白对照组, 继续生长6天, 每3天更换新鲜的含药培养基。收取第6天的细胞, 按照试剂盒说明分别检测DNMTs/TET2的含量。

CIC对HepG2.2.15细胞DNA甲基化水平的影响 按细胞培养的方法, 设空白对照组与CIC组, 收取第6天的细胞, PBS洗涤3遍, 按照细胞/组织基因组DNA提取试剂盒提取DNA, 琼脂糖凝胶电泳检测DNA的完整性。质检合格的DNA样品进行亚硫酸氢盐修饰, 纯化, WGA方式扩增, 芯片杂交, 再通过延伸、染色, 最后利用iScan扫描得出原始数据。采用R软件minfi包

进行预处理, 并利用R软件IMA包筛选样本分组间的全基因组甲基化水平和甲基化位点的差异。

Real-time PCR验证差异甲基化基因的表达水平 按细胞培养的方法, 设空白对照组与CIC组, 收取第6天的细胞, PBS洗涤3遍, 按照RNA pure高纯总RNA提取试剂盒提取RNA, 利用FastKing一步法除基因组cDNA第一链合成预混试剂反转录合成cDNA, 最后用SuperReal PreMix Plus检测RNA的表达水平。各引物序列如表1所示。

Table 1 Primer sequences of real-time PCR reaction. PLCG2: Phospholipase C gamma 2; PIK3R3: Phosphoinositide-3-kinase regulatory subunit 3; AGPAT2: 1-Acylglycerol-3-phosphate *O*-acyltransferase 2; HTR2B: 5-Hydroxytryptamine receptor 2B; NGF: Nerve growth factor

Gene	Name	Sequence
PLCG2	PLCG2-F	GTACCAGGAGAAATGCAAC
	PLCG2-R	CAGAGTGTGAATAGGGCAC
PIK3R3	PIK3R3-F	CAGGAGTAACAAAGCCCATT
	PIK3R3-R	AAGGAAGGCAGAACCACC
AGPAT2	AGPAT2-F	GTCACAGTGCAGGTGCTGGAAG
	AGPAT2-R	TGGAGATGTGGAGGAAGGTGGTC
HTR2B	HTR2B-F	TCTTTTCAACCGCATCCATCA
	HTR2B-R	TGCTGTAGCCCGTGAGTTATA
NGF	NGF-F	AGCTCAGGGTGACAGAGAGAA
	NGF-R	AACCACCAGGGTTTGGAACTG

焦磷酸测序验证差异甲基化位点的甲基化水平 Illumina 850K甲基化芯片检测结果有一定的假阳性率, 为了进一步验证甲基化差异位点, 根据样品表达差异基因的统计学结果, 设空白对照组与CIC组, 利用样本进行焦磷酸测序, 对Illumina 850K甲基化芯片结果进行验证。选取位点信息如表2所示。

利用甲基化转化试剂盒 (EZ DNA Methylation-Gold™ Kit) 将样本进行转化和纯化, 然后将转化后的样品进行PCR扩增, 并利用实时定量焦磷酸序列分析仪进行测序分析。焦磷酸测序扩增引物如表3所示。

CIC对HepG2.2.15细胞代谢微环境的影响 按细胞培养的方法, 设空白对照组与CIC组, 收集第6天的细胞, 用PBS洗3遍, 向6孔板中加入10 mL左右的液氮, 对细胞进行淬灭。将淬灭的细胞样品放置冰上, 向细胞培养皿中加入0.75 mL 80%甲醇 (−20 ℃), 将含有细胞裂解物的混合溶液转移离心管中, 再加入适量的80%甲醇。液氮/室温冻融3次, 低温高速离心机12 000 r·min^{-1}, 4 ℃离心15 min。将上一步得到的样品转入真空浓缩仪进行挥干, 用乙腈−纯水 (v∶v = 75∶25) 复溶, 涡旋5 min, 低温高速离心机12 000 r·min^{-1}, 4 ℃离心15 min, 取上清于进样瓶, 进样量1 μL, 进行LC-MS分析。

统计学分析 所有数据均应用软件SPSS 21.0和GraphPad Prism 7进行分析处理。数据以 ($\bar{x} \pm s$) 表示, 两两比较采用LSD-t检验; 以$P < 0.05$表示结果具有显著性差异。

结果

1 CIC对HepG2.2.15细胞增殖及HBsAg、HBeAg和HBV-DNA的抑制作用

不同质量浓度CIC对HepG2.2.15细胞的增殖有抑制作用, 且CIC对HepG2.2.15细胞的半数毒性浓度 (TC_{50}) 和有效无毒浓度分别为26.27 μg·mL^{-1}和17.5 μg·mL^{-1}; 有效无毒浓度 (17.5 μg·mL^{-1}) 时, CIC在第3天和第6天时对HBsAg、HBeAg和HBV-DNA都有抑制作用, 且第6天的抑制作用强于第3天 (图1)。当CIC对HepG2.2.15细胞增殖的抑制率小于10%时 (有效无毒浓度时), 对HepG2.2.15细胞分泌的乙肝病毒抗原有明显的抑制作用, 说明CIC能够抑制病毒的复制。

Table 2 Difference sites selected by pyrosequencing

Target_ID	Chromosome	UCSC_refgene_name	UCSC_refgene_group	Relation_to_UCSC_CPG_island
cg10064897	1	PIK3R3	5'UTR; TSS200	Island
cg04208291	1	NGF	5'UTR	
cg06255004	9	AGPAT2	Gene body	N_Shore

Table 3 Primers for pyrosequencing

Gene	Name	Sequence
cg10064897	cg10064897-F	AAGGGGAGGTGGTGATGT
	cg10064897-R	CCAACTACCCTCAAACTCTAC
	cg10064897-S	CCCCATACTACCCAC
cg04208291	cg04208291-F	TTTTTTGGTATTTGTGTTTTGATGTGA
	cg04208291-R	CAAATCTTAAAACTAACCCAATACACTAT
	cg04208291-S	TGAATTTTTTGGGAAGG
cg06255004	cg06255004-F	GGTAGTTTAAATAGGGTAGGGAGAAATGTA
	cg06255004-R	AAACTCTTACCTACCCCCTCTC
	cg06255004-S	ATGTAGTAATAGTTTGGGG

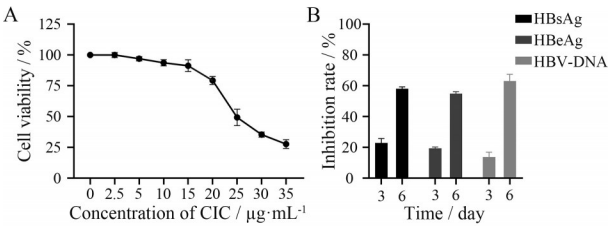

Figure 1　Inhibitory effects of CIC on proliferation, HBsAg, HBeAg and HBV-DNA of HepG2.2.15 cells. A: CIC could inhibit the prolife-ration of HepG2.2.15 cells; B: The inhibition of CIC on HBsAg, HBeAg and HBV-DNA at the effective non-toxic concentration (17.5 μg·mL^{-1}) was tested on days 3 and 6. $n = 3$, $\bar{x} \pm s$. CIC: *Chrysanthemi indici* C; HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen; HBeAg: Hepatitis B envelope antigen

2　CIC对HepG2.2.15细胞内DNA甲基化调控因子DNMTs/TET2的影响

利用ELISA法测定HepG2.2.15细胞中DNMT1、DNMT3a、DNMT3b与TET2的含量。实验结果表明，与对照组相比，CIC组DNMT1、DNMT3a、DNMT3b含量显著降低 (图2A～C，分别 $P < 0.001$, $P < 0.001$, $P < 0.05$)，TET2含量升高 (图2D, $P < 0.05$)。以上结果说明，CIC能够下调甲基化酶DNMTs的表达，同时CIC能够上调去甲基化酶TET2的表达，CIC对DNMTs/TET2平衡具有调控作用。

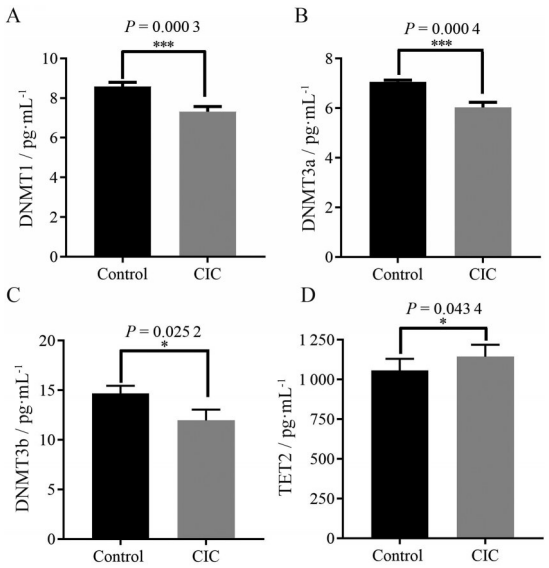

Figure 2　Effects of CIC (17.5 μg·mL^{-1}) on DNA methylated transferase and demethylase levels of HepG2.2.15 cells. A: DNMT1; B: DNMT3a; C: DNMT3b; D: TET2. $n = 3$, $\bar{x} \pm s$. $^{*}P < 0.05$, $^{***}P < 0.001$

3　CIC对HepG2.2.15细胞DNA甲基化水平的影响

3.1　DNA甲基化生物信息学分析

3.1.1　GO功能富集分析　利用Pool.*t*-test方法筛选甲基化差异位点，再将差异位点对应的差异基因映射到Gene Ontology (GO) 数据库的各个条目中，筛选差异显著的GO条目。GO分析结果表明，CIC作用主要影响的生物功能有脂质代谢过程和MAP激酶活性的调节过程 (图3)，与对照组相比，PLCG2、PIK3R3、AGPAT2、HTR2B、NGF、SPRED2基因的表达差异显著。以上结果表明，CIC很可能通过调控相关基因甲基化水平而影响脂质代谢过程和MAP激酶活性的调节过程。

3.1.2　KEGG功能富集分析　将筛选出的甲基化差异位点对应的差异基因进行KEGG富集分析 (图4)，结果表明，CIC作用途径主要有炎症介质调节的TRP通道炎症介导调节、甲状腺激素信号、磷脂酶D信号通路、神经营养信号、糖尿病并发症中AGE-RAGE的信号通路等，作用靶点有PLCG2、PIK3R3、AGPAT2、NGF、PRKCH、HTR2B、DIAPH1、SLCO1C1等。说明CIC通过调控相关靶基因的甲基化水平而影响相关通路。

3.2　Real-time PCR验证差异甲基化基因的表达水平

根据GO分析和KEGG分析确定的主要作用靶标基因有PLCG2、PIK3R3、AGPAT2、NGF和HTR2B，利用Real-time PCR技术对候选靶基因进行mRNA表达水平的测定，结果表明 (图5)，CIC组相比于对照组，NGF、PIK3R3和AGPAT2基因的mRNA表达水平降低且有显著性差异 ($P < 0.01$, $P < 0.05$, $P < 0.05$)；PLCG2 mRNA表达水平升高且有显著性差异 ($P < 0.05$)；HTR2B基因表达水平降低但没有统计学差异，说明CIC作用既能增强相关靶标基因的DNA甲基化修饰，也可以降低一些靶标基因的DNA甲基化水平，此与CIC调控DNMTs/TET2平衡作用一致。

3.3　焦磷酸测序验证候选差异位点的甲基化水平实验结果

焦磷酸测序的差异甲基化位点研究结果表明，NGF的cg04208291位点在CIC组中甲基化水平升高且存在显著差异 ($P < 0.01$)；PIK3R3的cg10064897位点和AGPAT2的cg06255004位点甲基化水平降低 (图6)。该结果与PCR结果一致，NGF基因甲基化水平升高，导致其基因表达降低；PIK3R3甲基化水平降低 ($P > 0.05$)，而PIK3R3基因表达降低；AGPAT2甲基化水平降低 ($P > 0.05$)，其基因表达也降低 ($P > 0.05$)。提示NGF、PIK3R3、AGPAT2基因很可能是CIC抗HBV重要的靶基因。

4　CIC对HepG2.2.15细胞微代谢环境的影响

4.1　代谢数据的分析

采用主成分分析方法 (PCA) 对样本数据进行质控分析 (图7)，红色QC样本检测结果较为密集地聚在一起，说明QC重复性良好，分析系统稳定，所有样本基本均在95%的置信区间内。

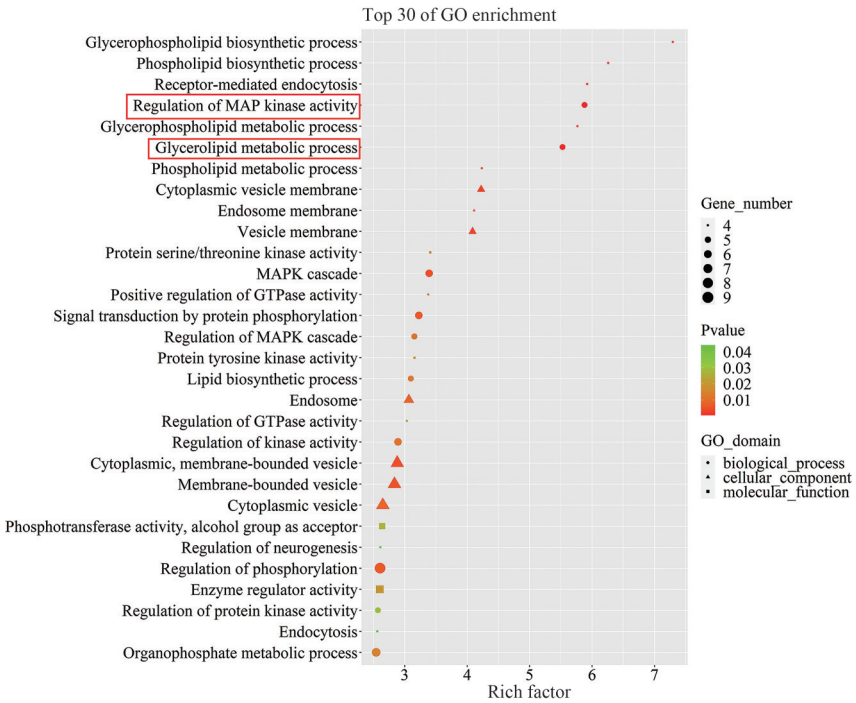

Figure 3 GO enrichment analysis using VIP > 1, $P < 0.05$ as screening conditions, showing the top 30 terms meeting the conditions. The horizontal axis represents rich factor, and different colors represent P value. The larger the rich factor, the smaller the P value (the darker the color), the more significant the enrichment. Differential genes are mainly involved in biological processes including the regulation of MAP kinase activity and glycerol phospholipid metabolic process (biological function)

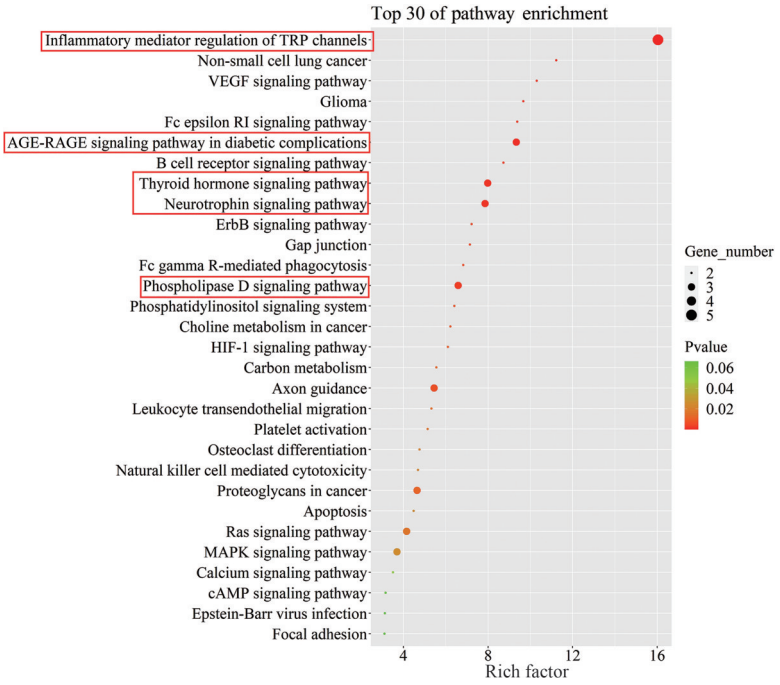

Figure 4 Enrichment analysis of KEGG pathway with VIP > 1, $P < 0.05$ as screening conditions, showing the top 30 pathways meeting the conditions. The horizontal axis represents rich factor, and different colors represent P value. The larger the rich factor, the smaller the P value (the darker the color), the more significant the enrichment. CIC may play an anti-hepatitis B virus role through inflammatory and lipid metabolic pathways

正交偏最小二乘法-判别分析 (orthogonal projections to latent structures-discriminant analysis, OPLS-DA) 可以过滤掉代谢物中与分类变量不相关的正交变量, 对非正交变量和正交变量分别分析, 从而获取更加可

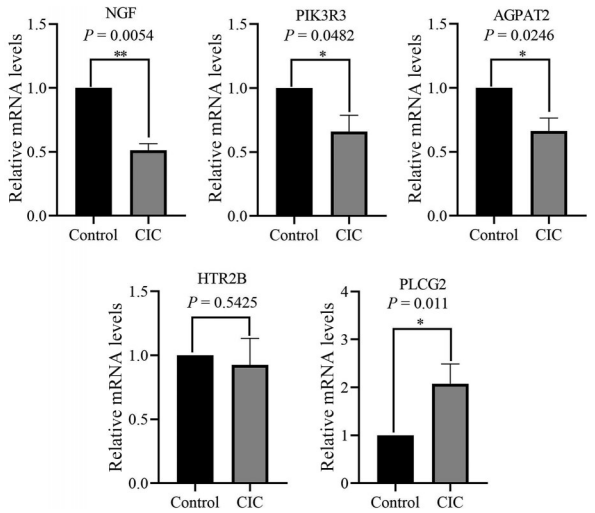

Figure 5　Real-time PCR mRNA expression of differential genes. $^{*}P < 0.05$, $^{**}P < 0.01$

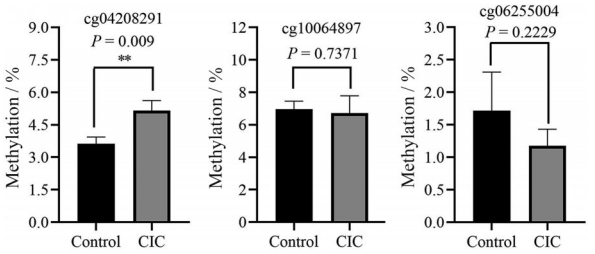

Figure 6　The methylation levels of the candidate sites verified by pyrosequencing. From left to right are cg04208291 of NGF, cg10064897 of PIK3R3 and cg06255004 of AGPAT2. $^{**}P < 0.01$

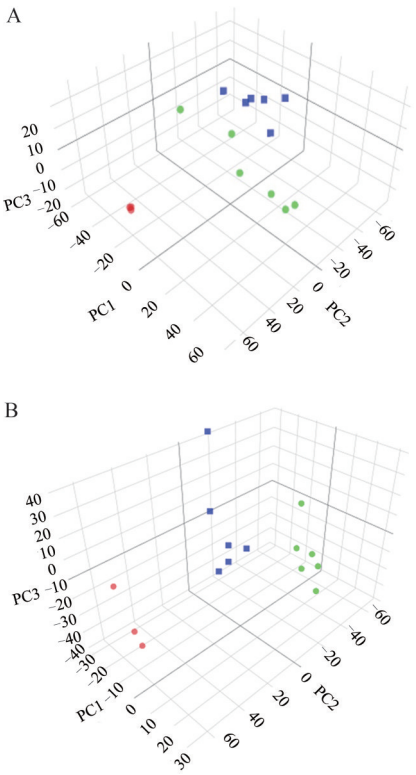

Figure 7　PCA 3D score. A: Negative ion mode; B: Positive ion mode. The red points are QC samples, and good aggregation indicates that the system is stable. The green group is blank, and the blue group is CIC. The closer the distribution points of samples are, the closer the composition and concentration of variables/molecules in the samples are

靠的代谢物的组间差。通过OPLS-DA分析, 表明CIC组与对照组代谢物差异较大, 所有样本基本均在95%的置信区间内 (图8), 说明CIC组与对照组样本质量合格。

4.2　差异代谢物筛选

根据OPLS-DA生成的第1主成分变量重要性值投影值 (VIP) 及 t 检验的 P 值来筛选组间的差异代谢物。对差异代谢物的定量值计算欧式距离矩阵 (Euclidean distance matrix), 以完全连锁方法对差异代谢物进行聚类, 并以热力图进行展示 (图9)。CIC与对照组对比, 差异代谢物有52种, 显著上调的代谢物有31种, 显著下调有21种。利用SPSS 22.0进行变化差异倍数 (fold change, FC) 分析, FC > 4的化合物有黄豆黄素 (glycitein)、叶黄素 (eupatilin)、染料木素 (genistein) 和酚酸类化合物水杨酸异丁酯 (isobutyl salicylate)。差异代谢物的LC-MS图如 (图10) 所示。结果说明, CIC对黄豆黄素、叶黄素、水杨酸异丁酯等代谢物影响较大。在LC-MS分析中, 黄豆黄素的保留时间为8.145 min、相对分子质量为285.067; 叶黄素的保留时间7.963 min、相对分子质量为344.198; 染料木素的保留时间6.643 min、相对分子质量为270.165; 酚酸类化合物水杨酸异丁酯的保留时间和相对分子质量分别为10.858 min和194.121。根据FC值筛选了4种排名靠前与不饱和脂肪酸合成相关的差异代谢物, 分别为 (9*Z*, 12*Z*, 15*Z*)-octadecatrienoic acid (保留时间10.337 min, 相对分子质量278.198)、arachidonate (保留时间12.640 min, 相对分子质量304.220)、(9*Z*)-octadecenoic acid (保留时间14.541 min, 相对分子质量282.198)、dihomo-gamma-linolenate (保留时间13.055 min, 相对分子质量306.220)。

4.3　差异代谢通路分析

基于KEGG数据平台对筛选出的差异代谢物进行代谢通路富集分析 (图11)。正、负离子模式的KEGG通路富集分析发现差异代谢物主要显著富集的代谢途径有2条: 不饱和脂肪酸生物合成、α-亚麻酸的代谢, 表明CIC组作用主要影响了脂质代谢。主要富集到的代谢物有arachidonate、(9*Z*, 12*Z*, 15*Z*)-octadecatrienoic acid和dihomo-gamma-linolenate。

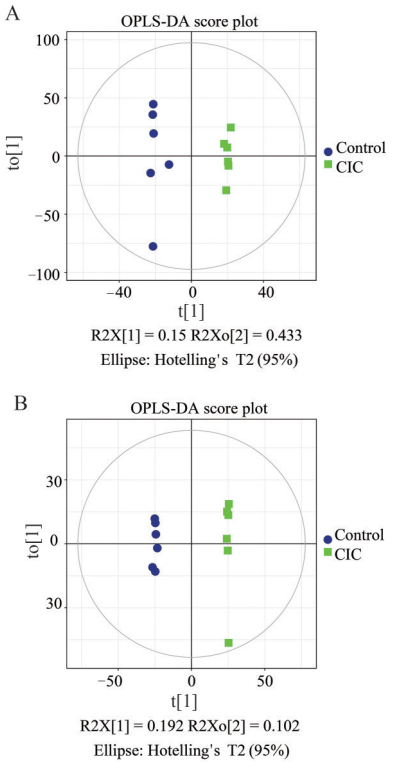

Figure 8 OPLS-DA score graph. A: Negative ion mode; B: Positive ion mode. The horizontal axis represents the predicted principal component score of the first principal component, and the vertical axis represents the variability within the group, which is independent of the grouping problem. Scatter shapes and colors represent different experimental groups, blue for control group and yellow for CIC group

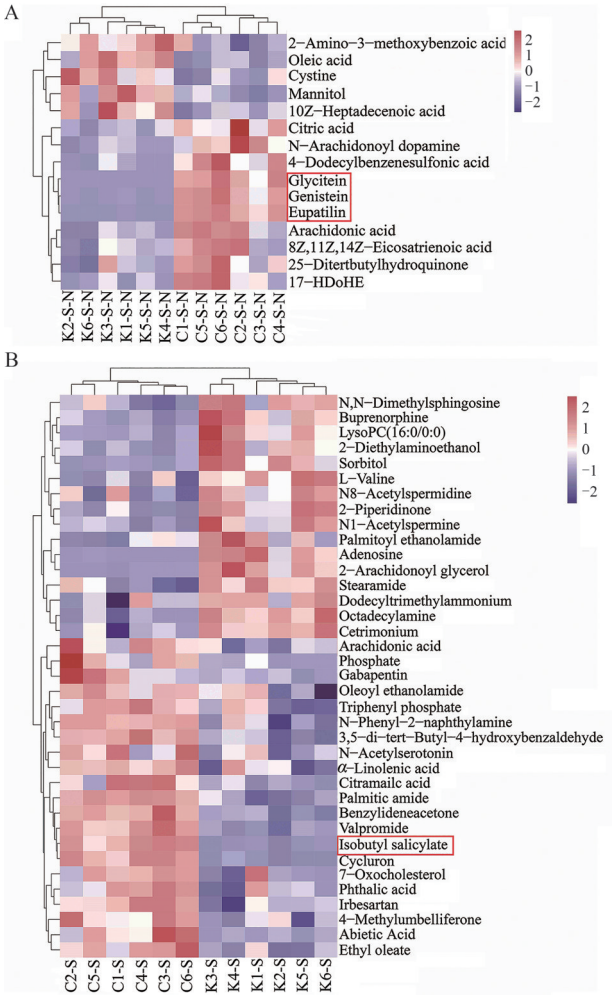

Figure 9 Thermal map of hierarchical cluster analysis of differential metabolites (A: Negative ion mode; B: Positive ion mode) with VIP ≥ 1 and $P < 0.05$. For screening differential metabolites, the greater the VIP value, the greater the contribution to classification. Horizontal coordinate represents different experimental groups, K1-6 are control groups, C1-6 are CIC groups. The ordinate represents the differential metabolites compared in this group. The color blocks at different positions represent the relative expression levels of metabolites at corresponding positions. Red represents up-regulation, blue represents down-regulation, and white represents no difference

4.4 差异代谢通路分类

将KEGG富集得到的差异代谢通路进行分类分析, 发现CIC作用的差异代谢通路主要有6类, 分别为氨基酸代谢、糖类代谢、脂质代谢、维生素及辅酶代谢、核苷酸代谢和生长因子转化, 其中脂质代谢富集到的化合物数量最多, 进一步说明脂质代谢很可能是CIC作用的重要途径。代谢组学研究结果与DNA甲基化测序结果一致。

讨论

研究结果显示, CIC对HepG2.2.15细胞分泌的HBsAg、HBeAg和HBV-DNA均有较好的抑制作用, 表明CIC有良好的体外抗HBV作用。

最新研究表明, HBV持续感染可刺激DNMTs的过表达, 特别是DNMT1、DNMT3a和DNMT3b, 同时, 在肝癌发生中通过激活DNA甲基转移酶诱导启动子高甲基化和肿瘤抑制基因低表达[20-22]。在DNMTs活性下降的情况下, HBV DNA甲基化和去甲基化之间的平衡主要是由TET2调节的[23,24], DNMTs/TET2之间的平衡关系决定了DNA的开与合, 中医药阴阳的生物学根本基础可能就在于DNA高甲基化与低甲基化, 中医药作用的根本也可能主要就是恢复DNMTs/TET2的平衡、恢复表观遗传表达的平衡。本研究结果表明, CIC可以显著降低HepG2.2.15细胞中DNMT1、DNMT3a、DNMT3b的表达, 同时上调TET2的表达, 说明CIC抗HBV作用机制与调控DNMTs/TET2的平衡关系相关。根据中医药理论和表观遗传理论推测, CIC可能通过

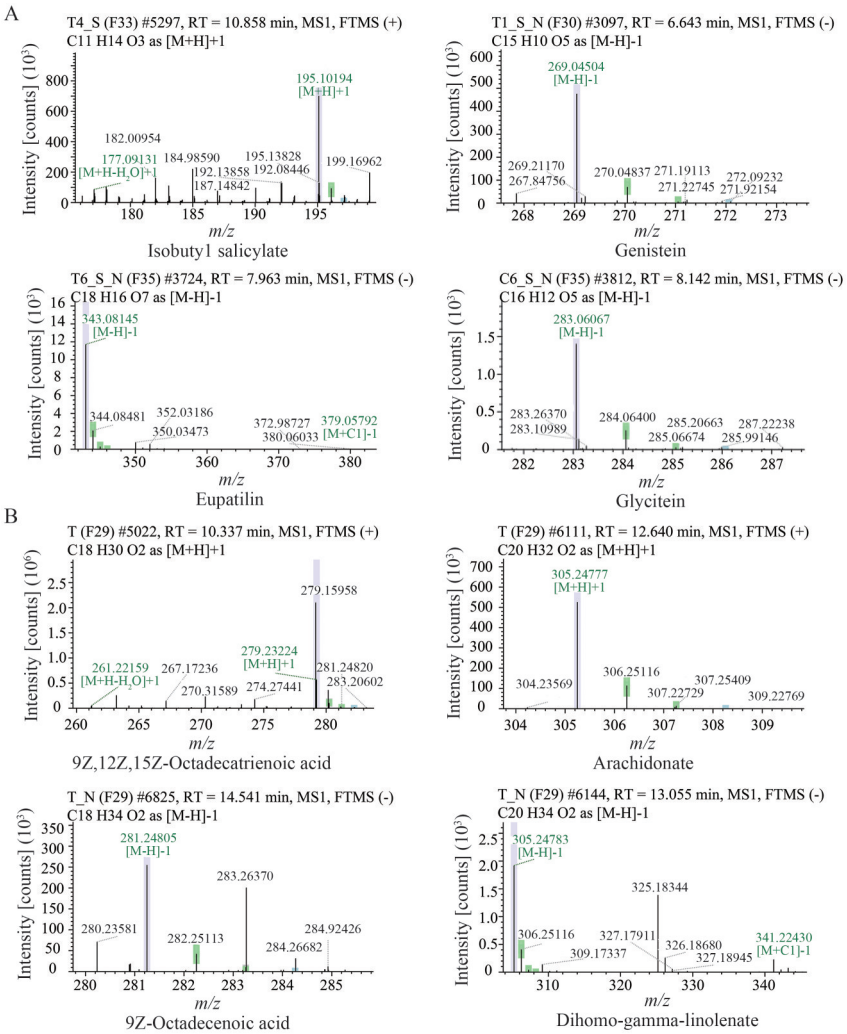

Figure 10 Metabolic ion maps of 8 different metabolites screened in positive and negative ion modes (CIC group *vs* control group). A: FC > 4; B: Unsaturated fatty acid biosynthesis pathway

调节DNMTs/TET2的平衡关系而调控相关作用途径与靶点, 并影响细胞微环境。

为进一步阐明CIC通过调控DNMTs/TET2的平衡作用抗乙肝病毒涉及的具体作用途径和作用靶点, 本研究进行了DNA甲基化测序研究。结果表明, CIC抗HBV的作用途径有脂质代谢、MAP激酶的调节过程、炎症介质调节的TRP通道、B细胞受体信号通路、神经营养因子信号通路。相关研究表明, 这些通路与DNMTs及TET2的调节相关, 也与HBV的发病机制相关[25,26]。而酶动力学研究表明, 肝细胞脂质代谢的重要调节分子25HC3S对DNMTs、DNMT1、DNMT3a和DNMT3b具有特异性抑制作用[27]。也有研究表明, HBV感染与脂肪酸代谢密切相关[28,29]。本论文的表观遗传机制研究和代谢组学研究, 均表明CIC抗HBV作用机制与调节DNMTs/TET2平衡作用相关, 并且与脂质代谢通路密切相关。

对DNA甲基化GO和KEGG分析确定的作用途径和靶点进行作用靶点与作用途径的网络分析 (图12), 进一步确定了CIC作用与DNMTs/TET2相关的重要靶标: PIK3R3 (上调)、PLCG2 (上调)、NGF (上调)、AGPT3 (上调)、HTR2B (下调), 说明CIC作用既能增强相关基因靶标的DNA甲基化修饰, 也可以降低一些相关基因靶标的DNA甲基化水平, 此与CIC调控DNMTs/TET2平衡作用一致。PIK3R3是磷脂酰肌醇3激酶 (PI3K)家族的成员, 有研究显示PIK3R3基因在肝癌、肺癌等多种人类癌症中过表达[30], 肝脏作为脂质代谢的核心脏器, 脂质代谢的紊乱与肝癌的发生也密切相关[31,32], 抑制PIK3R3的表达可调节肝癌细胞的脂质代谢紊乱[33]。PLCG2负责磷脂酰肌醇的水解, 与脂质代谢相关[34]。NGF是一种神经营养蛋白, 有研究显示HBV患者肝组织中NGF mRNA的表达水平显著高于正常肝组织[35,36], DNMT1可以基于mTOR通路抑制NGF表达[37]。AGPAT2

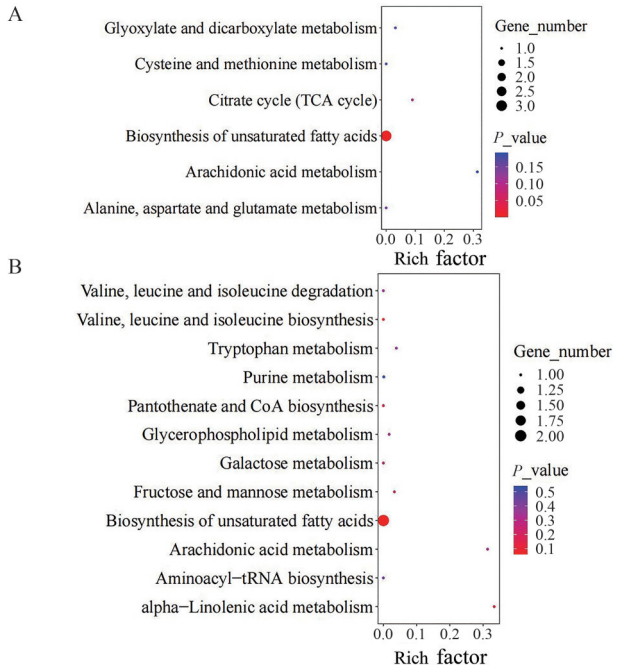

Figure 11 KEGG differential metabolic pathway analysis. A: Negative ion mode; B: Positive ion mode. Ordinate represents different metabolic pathways, abscissa rich factor is for differentially expressed the metabolites of number in the corresponding channel and the channel detection annotation to the ratio of the total number of metabolites. The greater the value, the bigger (or points), the greater the degree of enrichment of the size of the points on the representative enrichment to the corresponding channel number of significant difference of metabolites. The closer the *P* value is to 0 (the redder the color is), the more significant the enrichment is, and the color of the dot represents the *P* value of the metabolic pathway (CIC group *vs* control group)

是一种1-酰基甘油-3-磷酸酰基转移酶, AGPAT2基因的过表达会使脂质代谢发生紊乱, 从而导致疾病的发生[38]。血清素治疗肝癌细胞会诱导自噬, 5-羟色胺诱导细胞脂肪变性, 促进细胞存活, 而抑制自噬可减少5-HTR2B介导的细胞脂肪变性[39]。

代谢组学研究揭示了CIC对细胞微环境的影响。与对照组相比, 在CIC中显著上调的代谢物31种, 包括氨基酸及其衍生物3种、酚酸类5种、黄酮1种、萜类1种、生物碱2种、脂质类6种及糖类及衍生物2种等; 显著下调的代谢物21种, 包括氨基酸及其衍生物3种、核苷酸及其衍生物1种、生物碱6种、脂质类4种及糖类和衍生物2种等。说明CIC对氨基酸及其衍生物、生物碱类、脂质类和糖类及衍生物有不同的调节作用, 从而恢复代谢微环境的平衡。

大量研究表明, 氨基酸代谢、脂质代谢、碳水化合物代谢与花生四烯酸代谢等都与DNMTs和TET2的调控相关[40-49]。作为差异代谢物之一, 染料木素能减少高脂饮食引起的脂质积累, 减轻小鼠体重[50], 染料木素还可以作为表观遗传修饰物, 特别是作为DNMTs抑制剂而被广泛研究[51]。大豆黄素和叶黄素亦为差异代谢物, 研究表明大豆黄素和叶黄素在3T3-L1细胞的脂肪形成过程中显著抑制脂质积累, 可能是通过激活脂肪组织代谢发挥作用[52-54]。本文研究结果提示, 脂质代谢作用途径是CIC抗乙肝病毒的重要作用途径, 并与DNMTs/TET2调控相关。

近年来, HBV调节细胞脂质代谢的研究不断被报道。乙肝抗原的表达与积累能扰乱宿主细胞的脂类代谢过程, HBsAg可上调脂质代谢、促进脂质合成, 提示调节脂质代谢很可能成为抗HBV的潜在有效途径。特别是, 本文DNA甲基化测序和代谢组学研究结果均表明, 脂质代谢作用途径是CIC抗乙肝病毒的重要作用途径。

综上, 本研究利用表观遗传学和代谢组学, 阐明了CIC整体作用机制: 通过调节DNMTs/TET2二者的平

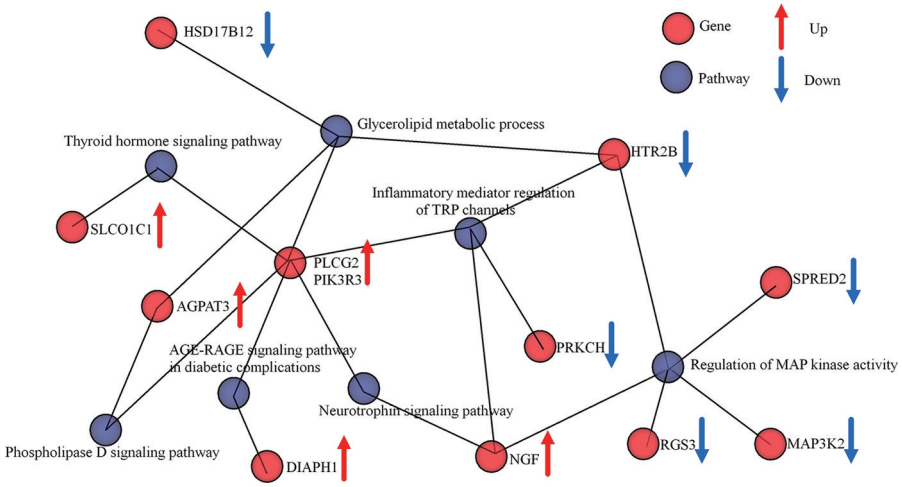

Figure 12 Differential methylation gene-pathway network analysis. Red spheres represent genes, blue spheres represent pathways (CIC group *vs* control group)

衡关系, 调控相关靶标基因PLCG2、PIK3R3、AGPAT2、HTR2B、NGF等甲基化水平变化, 从而调控脂质代谢、相关炎症与免疫等多条通路, 影响细胞微环境, 共同协同作用, 达到了抗乙肝病毒的目的。研究结果也为中医药平衡观、整体观科学内涵阐明建立的新研究模式值得进一步探索和完善。

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利益冲突: 本文不存在任何利益冲突。

References

[1] Su YM, Sun DS. Optimal control of anti-HBV treatment based on combination of traditional Chinese medicine and Western medicine [J]. Biomed Signal Process Control, 2015, 15: 41-48.

[2] Xie X, Lu LG. Advances in antiviral treatments of HBV-induced liver cirrhosis [J]. J Pract Hepatol (实用肝病杂志), 2018, 21: 140-144.

[3] Zhang Dk, Guo SC, Schrodi SJ. Mechanisms of DNA methylation in virus-host interaction in hepatitis B infection: pathogenesis and oncogenetic properties [J]. Int J Mol Sci, 2021, 22: 9858.

[4] Kim GW, Imam H, Khan M, et al. HBV-induced increased N6 methyladenosine modification of PTEN RNA affects innate immunity and contributes to HCC [J]. Hepatology, 2021, 73: 533-547.

[5] Ma L, Yang Y, Zheng XL. Progress in the role of DNA methylation in chronic hepatitis B [J]. Chin Hepatol (肝脏), 2018, 23: 1135-1137.

[6] Ling QX, Chen MQ, Shi GF. Research status of DNA methylation modification in hepatitis B virus persistent infection-associated liver disease [J]. Chin Hepatol (肝脏), 2014, 19: 362-364.

[7] Cui MH, Zheng JY. Research progress in pathogenesis of hepatitis B virus associated hepatocellular carcinoma [J]. China Foreign Med Treat (中外医疗), 2017, 36: 193-195.

[8] Yang CQ, Liu SM. The role of DNA methylation in hepatocellular carcinoma [J]. Chin Bull Life Sci (生命科学), 2021, 33: 646-652.

[9] Zhou QB, Xu FQ, Tong WX, et al. Discussion on the essence of yin and yang in DNA methylation [J]. Chin J Tradit Chin Med Pharm (中华中医药杂志), 2018, 33: 4713-4715.

[10] Li G, Xing XY, Zhang MS, et al. Research progress in epigenetic regulation of myocardial ischemia/reperfusion injury by traditional Chinese medicine [J]. Acta Pharm Sin (药学学报), 2016, 51: 1047-1053.

[11] Liu M, Liu XD, Wang AQ, et al. Research progress of the effect of Chinese medicine on epigenetics [J]. New J Tradit Chin Med (新中医), 2018, 50: 131-134.

[12] Xiang YN, Guo ZM, Zhu PF, et al. Traditional Chinese medicine as a cancer treatment: modern perspectives of ancient but advanced science [J]. Cancer Med, 2019, 8: 1958-1975.

[13] Lyu S, Yang SL, Rao Y, et al. Application of metabolomics and related technologies in research and development field of traditional Chinese medicine [J]. China J Chin Mater Med (中国中药杂志), 2018, 43: 4182-4191.

[14] Perusina Lanfranca M, Thompson JK, Bednar F, et al. Metabolism and epigenetics of pancreatic cancer stem cells [J]. Semin Cancer Biol, 2019, 57: 19-26.

[15] Bi YF, Wang YY, Zhang FP, et al. Research progress on application of epigenetics and metabolomics in traditional Chinese medicine [J]. Chin Tradit Herb Drugs (中草药), 2020, 51: 4837-4845.

[16] Yuan HJ, Lai ZH, Guan YY, et al. Pharmacology study of active constituents from *Chrysanthemum Indicum* [J]. Chin Arch Tradit Chin Med (中华中医药学刊), 2018, 36: 651-653.

[17] Li GD, Chen YY, Wang P, et al. Protection of terpenes and flavonoids from *Chrysanthemi indici flos* on immunological liver injury [J]. Chin Tradit Herb Drugs (中草药), 2013, 44: 3510-3514.

[18] Bi YF, Jia L, Shi SP, et al. New sesquiterpenes from the flowers of *Chrysanthemum indicum* L. [J]. Helv Chim Acta, 2010, 93: 1953-1959.

[19] Fu L, Wang P, Sun YQ, et al. High performance liquid chromatography time of flight electrospray ionization mass spectrometry for quantification of sesquiterpenes in *Chrysanthemi indici Flos* active extract [J]. Pharmacogn Mag, 2015, 44: 740-744.

[20] Vivekanandan P, Daniel HD, Kannangai R, et al. Hepatitis B virus replication induces methylation of both host and viral DNA [J]. J Virol, 2010, 84: 4321-4329.

[21] Xiao T, He GX, Qin WB, et al. Research progress of traditional Chinese medicine in prevention and treatment of atherosclerosis based on DNA methylation regulation [J]. Guid J Tradit Chi Med Pharm (中医药导报), 2021, 27: 110-114.

[22] Rong Z, Zhang JS, Zhu YZ, et al. HBx-induced androgen receptor expression in HBV-associated hepatocarcinoma is independent of the methylation status of its promoter[J]. Histol Histopathol, 2011, 26: 23-25.

[23] Oropeza CE, Tarnow G, Taha TY, et al. Relative DNA methylation and demethylation efficiencies during postnatal liver development regulate hepatitis B virus biosynthesis [J]. J Virol, 2021, 95: e02148-20.

[24] Fan H, Zhang H, Pascuzzi PE, et al. Hepatitis B virus X protein induces EpCAM expression *via* active DNA demethylation directed by RelA in complex with EZH2 and TET2 [J]. Oncogene, 2016, 35: 715-726.

[25] Tsuchiya H, Shiota G. Immune evasion by cancer stem cells [J]. Regen Ther, 2021, 17: 20-33.

[26] Hasegawa T, Adachi R, Iwakata H, et al. ErbB2 signaling epigenetically suppresses microRNA-205 transcription *via* Ras/Raf/MEK/ERK pathway in breast cancer [J]. FEBS Open Bio, 2017, 7: 1154-1165.

[27] Wang YP, Lin WQ, Brown JE, et al. 25-Hydroxycholesterol 3-sulfate is an endogenous ligand of DNA methyltransferases in hepatocytes [J]. J lipid Res, 2021, 62: 100063.

[28] Hu W, Wang XJ, Ding XR, et al. MicroRNA-141 represses HBV replication by targeting PPARA [J]. PLoS One, 2012, 7: e34165.

[29] Xun YH, Zhang YJ, Pan QC, et al. Metformin inhibits hepatitis B virus protein production and replication in human hepatoma cells [J]. J Viral Hepat, 2013, 21: 597-603.

[30] Wang GH, Yang X, Li C, et al. PIK3R3 induces epithelial-to-mesenchymal transition and promotes metastasis in colorectal cancer [J]. Mol Cancer Ther, 2014, 13: 1837-1847.

[31] Liu K, Li XL, Cao YC, et al. MiR-132 inhibits cell proliferation, invasion and migration of hepatocellular carcinoma by targeting PIK3R3 [J]. Int J Oncol, 2015, 47: 1585-1593.

[32] Calvisi DF, Wang CM, Ho C, et al. Increased lipogenesis, induced by AKT-mTORC1-RPS6 signaling, promotes development of human hepatocellular carcinoma [J]. Gastroenterology, 2011, 140: 1071-1083.

[33] Shao SL, Yang X, Xu F. PIK3R3 regulates lipid metabolism and proliferation of hepatocellular carcinoma cells by regulating PPARA [J]. Acta Med Univ Sci Technol Huazhong (华中科技大学学报), 2019, 48: 129-136.

[34] Ichise H, Ichise T, Ohtani O, et al. Phospholipase Cγ2 is necessary for separation of blood and lymphatic vasculature in mice [J]. Development, 2009, 136: 191-195.

[35] Amoras E, Gomes ST, Freitas FB, et al. NGF and P75NTR gene expression is associated with the hepatic fibrosis stage due to viral and non-viral causes [J]. PLoS One, 2015, 10: e0121754.

[36] Pereira L, da Silva Graça Amoras E, da Silva Conde S, et al. NGF (−198C > T, Ala35Val) and p75(NTR) (Ser205Leu) gene mutations are associated with liver function in different histopathological profiles of the patients with chronic viral hepatitis in the Brazilian Amazon [J]. Mol Med, 2020, 26: 12.

[37] Cheng MJ, Lv X, Zhang CH, et al. DNMT1, a novel regulator mediating mTORC1/mTORC2 pathway-induced NGF expression in Schwann cells [J]. Neurochem Res, 2018, 43: 2141-2154.

[38] Gale SE, Frolov A, Han X, et al. A regulatory role for 1-acylglycerol-3-phosphate-*O*-acyltransferase 2 in adipocyte differentiation [J]. J Biol Chem, 2006, 281: 11082-11089.

[39] Niture S, Gyamfi MA, Kedir H, et al. Serotonin induced hepatic steatosis is associated with modulation of autophagy and notch signaling pathway [J]. Cell Commun Signal, 2018, 16: 78.

[40] Akahori H, Guindon S, Yoshizaki S, et al. Molecular evolution of the TET gene family in mammals [J]. Int J Mol Sci, 2015, 16: 28472-28485.

[41] Wu D, Hu D, Chen H, et al. Glucose-regulated phosphorylation of TET2 by AMPK reveals a pathway linking diabetes to cancer [J]. Nature, 2018, 559: 637-641.

[42] Zhang Q, Liu XG, Gao WQ, et al. Differential regulation of the ten-eleven translocation (TET) family of dioxygenases by *O*-linked β-*N*-acetylglucosamine transferase (OGT)[J]. J Biol Chem, 2014, 289: 5986-5996.

[43] Hou YZ, Zhang ZX, Wang Y, et al. 5mC profiling characterized TET2 as an anti-adipogenic demethylase [J]. Gene, 2020, 733: 144265.

[44] Yan CF, Chen JF, Li M, et al. A decrease in hepatic microRNA-9 expression impairs gluconeogenesis by targeting FOXO1 in obese mice [J]. Diabetologia, 2016, 59: 1524-1532.

[45] Zhang X, Zhao S, Yuan QQ, et al. TXNIP, a novel key factor to cause Schwann cell dysfunction in diabetic peripheral neuropathy, under the regulation of PI3K/Akt pathway inhibition-induced DNMT1 and DNMT3a overexpression [J]. Cell Death Dis, 2021, 12: 642.

[46] Zhang H, Li AM, Zhang W, et al. High glucose-induced cytoplasmic translocation of Dnmt3a contributes to CTGF hypo-methylation in mesangial cells [J]. Bioscience Rep, 2016, 36: e00362.

[47] Streck EL, Bussular FP, Wessler LB, et al. Administration of branched-chain amino acids alters epigenetic regulatory enzymes in an animal model of maple syrup urine disease [J]. Metab Brain Dis, 2021, 36: 247-254.

[48] Zhang JB, Luo SJ, Gu ZF, et al. Genome-wide DNA methylation analysis of mantle edge and mantle central from pearl oyster *Pinctada fucata martensii* [J]. Mar Biotechnol, 2020, 22: 380-390.

[49] Fukui T, Soda K, Takao K, et al. Extracellular spermine activates DNA methyltransferase 3A and 3B [J]. Int J Mol Sci, 2019, 20: 1254.

[50] Choi YR, Shim J, Kim MJ. Genistin: a novel potent anti-adipogenic and anti-lipogenic agent [J]. Molecules, 2020, 25: 2042.

[51] Paul B, Li Y, Tollefsbol TO. The effects of combinatorial genistein and sulforaphane in breast tumor inhibition: role in epigenetic regulation [J]. Int J Mol Sci, 2018, 19: 1754.

[52] Kim M, Im S, Cho YK, et al. Anti-obesity effects of soybean embryo extract and enzymatically-modified isoquercitrin [J]. Biomolecules, 2020, 10: 1394.

[53] Kim JS, Lee SG, Min K, et al. Eupatilin inhibits adipogenesis through suppression of PPARγ activity in 3T3-L1 cells [J]. Biomed Pharmacother, 2018, 103: 135-139.

[54] Li F, Tao Y, Qiao Y, et al. Eupatilin inhibits EGF-induced JB6 cell transformation by targeting PI3K [J]. Int J Oncol, 2016, 49: 1148-1154.