FMRP通过激活RAS/MAPK信号通路抑制结直肠肿瘤细胞的铁死亡

王 南[1],石 斌[2],马小兰[1],吴伟超[3],曹 佳[4]
宁夏医科大学总医院[1]硕士培养站,[2]急诊科,[4]医学科学研究院外科学研究室,宁夏 银川 750004;[3]银川市第一人民医院耳鼻喉科,宁夏 银川 750004

摘要:目的 探讨脆性X智力障碍蛋白(FMRP)调控结直肠肿瘤(CRC)细胞逃避铁死亡的作用机制。**方法** 使用RT-qPCR和Western blotting方法验证FMRP在CRC细胞中的表达;利用TCGA数据库分析FMRP参与调控CRC进展的生物功能及信号通路;采用慢病毒表达系统和siRNA干扰技术,分别构建FMRP过表达载体(Lv-FMRP)和敲低载体(siFMRP-1、siFMRP-2、siFMRP-3),细胞实验设Control组、NC组、siFMRP-1组、siFMRP-2组、siFMRP-3组、Lv-NC组、Lv-FMRP组;采用CCK8和平板克隆实验检测细胞增殖;采用MDA/ROS/GSH/Fe^{2+}试剂盒检测细胞铁死亡水平;采用JC-1荧光染色法检测线粒体膜电位变化;采用Western blot检测铁死亡相关蛋白及RAS/MAPK信号通路相关蛋白表达;利用裸鼠皮下成瘤实验观察FMRP对肿瘤生长的影响。**结果** 与正常肠粘膜上皮细胞NCM460相比,FMRP在CRC细胞中明显高表达(P<0.05);TCGA数据库联合生信分析显示FMRP与活性氧调控、氧化应激诱导细胞死亡、线粒体呼吸等生物过程相关,与谷胱甘肽代谢通路相关;体外实验结果显示,与Control组相比,FMRP敲低组细胞增殖能力降低,GSH含量降低,MDA和ROS含量升高,Fe^{2+}荧光强度增强(P<0.05),SLC7A11/GPX4蛋白表达降低,线粒体膜电位JC-1荧光强度升高,而FMRP过表达组与上述结果相反;体内实验结果显示,敲低FMRP抑制瘤体生长,抑制SLC7A11表达(P<0.01);进一步检测RAS/MAPK信号通路,发现敲低FMRP导致ERK、MEK、MAPK、RAS蛋白磷酸化水平降低,过表达FMRP上述蛋白磷酸化水平升高(P<0.05)。**结论** FMRP通过激活RAS/MAPK信号通路抑制细胞铁死亡促进CRC恶性进展。

关键词:FMRP;铁死亡;RAS/MAPK信号通路;结直肠肿瘤

High expression of fragile X mental retardation protein inhibits ferroptosis of colorectal tumor cells by activating the RAS/MAPK signaling pathway

WANG Nan[1], SHI Bin[2], MAN Xiaolan[1], WU Weichao[3], CAO Jia[4]
[1]Master's Cultivation Station, [2]Department of Emergency Medicine, [4]Department of Surgical Sciences of Medical Science Research Institute, Ningxia Medical University General Hospital, Yinchuan 750004, China; [3]Department of Otolaryngology, Yinchuan First People's Hospital, Yinchuan 750004, China

Abstract: Objective To investigate the mechanism by which fragile X mental retardation protein (FMRP) regulates ferroptosis evasion in colorectal cancer (CRC) cells. **Methods** We examined FMRP expression levels in CRC cell lines using RT-qPCR and Western blotting and analyzed the biological functions and signaling pathways involved in FMRP-mediated regulation of CRC progression using the TCGA database. A lentiviral FMRP overexpression vector (Lv-FMRP) and 3 knockdown vectors (siFMRP-1, siFMRP-2, and siFMRP-3) were constructed, and their effects on proliferation of HCT116 cells were examined using CCK8 assay and plate clone formation assay; the changes in cell ferroptosis level was determined using MDA/ROS/GSH/Fe^{2+} kits, mitochondrial membrane potential changes were detected using JC-1 fluorescence staining, and the expressions of proteins associated with ferroptosis and the RAS/MAPK signaling pathway were detected using Western blotting. The subcutaneous tumorigenic potential of the transfected cells was evaluated in nude mice. **Results** Compared with normal colonic mucosal epithelial NCM460 cells, the CRC cell lines had significantly higher FMRP expression level. Bioinformatics analysis suggested the involvement of FMRP in regulation of reactive oxygen, oxidative stress-induced cell death, mitochondrial respiration, and glutathione metabolism pathways. In the cell experiments, FMRP knockdown significantly inhibited proliferation of HCT116 cells, lowered cellular GSH content, increased MDA and ROS levels, Fe^{2+} fluorescence intensity, and mitochondrial membrane potential, and decreased SLC7A11/GPX4 protein expressions and the phosphorylation levels of ERK, MEK, MAPK, and RAS proteins; FMRP overexpression resulted in the opposite changes in the cells. In the tumor-bearing nude mice, HCT116 cells with FMRP knockdown showed attenuated tumorigenic potential with lowered xenograft growth rate and reduced SLC7A11 expression in the xenograft. **Conclusion** The high expression of FMRP inhibits ferroptosis in CRC cells and promotes progression of CRC by activating the RAS/MAPK signaling pathway.

Keywords: fragile X mental retardation protein; ferroptosis; RAS/MAPK signaling pathway; colorectal cancer

结直肠肿瘤(CRC)是最常见的消化道恶性肿瘤之一,其发病率和死亡率在我国乃至全球范围内均居前列[1]。CRC发病过程隐匿、病情复杂、缺少有效的诊断标志物。因此探索新的治疗靶点及分子机制,对于CRC临床防治具有重要意义[2]。

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作者简介:王 南,硕士,住院医师,E-mail: 979377808@qq.com
通信作者:曹 佳,博士,副研究员,硕士生导师,E-mail: caojiamed@126.com

脆性X智力障碍蛋白(FMRP),由FMR1基因编码,是一种高度保守的RNA结合蛋白,参与调节RNA可变剪接、mRNA稳定性、蛋白翻译修饰等生物过程[3, 4]。近期研究发现FMRP异常激活促进多种实体瘤的发生发展[5-8],亦有研究报道显示,FMRP在多种人类和小鼠癌症模型中高表达,作为肿瘤微环境与细胞网络交流的重要调节器促使肿瘤发生免疫逃逸[9];结直肠癌中,FMRP调控坏死因子RIPK3激活程序性坏死途径促进肿瘤恶性进展[10];FMRP稳定EGFR m^{6}A甲基化介导CRC增殖转移[11]。以上研究表明FMRP对CRC疾病进展发挥重要的调控作用,具备作为新颖治疗靶点的潜力。铁死亡是新发现的一种铁依赖性程序性细胞死亡,铁积累和脂质过氧化是铁死亡过程启动膜氧化损伤的关键信号[12]。然而FMRP是否通过调控铁死亡途径影响CRC恶性进展,目前尚无相关报道。RAS/MAPK信号通路主要由RAS-RAF-MEK-ERK蛋白激酶组成,通过催化下游蛋白发生磷酸化而激活,促进肿瘤细胞恶性转化[13]。已有研究证实RAS/MAPK信号通路在KRAS突变的CRC中具有关键的调控作用[14, 15]。然而,FMRP能否通过RAS/MAPK信号通路调控CRC细胞的铁死亡,目前尚未见报道。本研究将为完善FMRP调控CRC进展的临床干预决策提供重要的理论基础。

基于此,本课题通过体内外实验验证干预FMRP对CRC细胞增殖及铁死亡的调控作用,检测干预FMRP对RAS/MAPK信号通路核心蛋白磷酸化表达的影响,探讨FMRP调控RAS/MAPK信号通路抑制细胞铁死亡促进CRC发生发展的分子机制,为增敏CRC经铁死亡途径的临床治疗方案提供新思路。

1 材料和方法

1.1 材料

人CRC细胞系HT29、HCT116、SW620、SW480(中科院上海细胞库);DMEM培养基、McCoy'5A培养基、青链霉素混合液、胎牛血清(GIBCO);LipofectamineRNA iMAX助转染试剂(赛默飞);反转录试剂盒及qRT-PCR试剂盒(Takara);蛋白提取试剂盒(南京凯基);BCA试剂盒(Thermo);MDA试剂盒、GSH试剂盒、ROS试剂盒及JC-1试剂盒均(碧云天);Fe^{2+}荧光探针(同仁化学)。

1.2 方法

1.2.1 细胞培养 选择人CRC细胞系HCT116,添加10%胎牛血清、1%双抗McCoy'5A完全培养基,放置37 ℃、5%CO_2培养箱中培养。实验分4组:对照组、NC组、FMRP敲低组(siFMRP)、FMRP过表达组(Lv-FMRP),敲低组利用iMAX助转染试剂转染siRNA,过表达组转染慢病毒载体。将对数生长期细胞HCT116接种于6孔板,待细胞融合度为50%时转染细胞用于后续实验,转染过程严格按照试剂说明书操作。FMRP小干扰载体序列见表1。

表1 siRNA序列

Tab.1 Sequences of the small interfering RNAs

Gene	Sense (5'-3')	Antisense (5'-3')
hFMR1-1	GAGGAUGAUAAAGGGUGAGUUTT	AACUCACCCUUUAUCAUCCUCTT
hFMR1-2	CGAGAUUUCAUGAACAGUUUATT	UAAACUGUUCAUGAAAUCUCGTT
hFMR1-3	GCGUUUGGAGAGAUUACAAAUTT	AUUUGUAAUCUCUCCAAACGCTT

1.2.2 生信分析 从UCSCxena数据库(https://xenabrowser. net/datapages/)获取TCGA直肠癌(READ)、TCGA结肠癌(COAD)数据集,将COAD、READ数据集合并作为CRC数据集,共包含638个肿瘤样本,51个正常样本。从上述数据集中提取FMR1的表达数据,组间使用wilcox计算显著性,比较FMR1的表达情况。将FMR1表达量中位数划分高低表达组,并使用R包"survival"绘制生存曲线。使用R软件包"DESeq2"对数据集以FMR1在Cancer样本中高低表达分组的差异分析,设置差异基因阈值为(|logFC|>1 and P<0.05),筛选差异基因绘制聚类图及火山图,进一步对差异基因采用GO功能和KEGG通路富集分析。最后使用上述差异分析得到的每个基因的logFC值对基因进行排序,使用R软件包对排序后的基因集进行GSEA-GO以及GSEA-KEGG功能富集。

1.2.3 实时荧光定量PCR(RT-qPCR) 收集各组细胞,采用TRizol法提取细胞RNA,按照Takara反转录试剂盒说明书合成cDNA,反应条件为:37 ℃ 15 min,85 ℃ 5 s,4 ℃ ∞。以cDNA为模板配制荧光定量反应液体系20 μL:TB Green 10 μL、上下游引物各1 μL、cDNA 2 μL、ddH_2O 6 μL,经预变性95 ℃ 30 s,PCR反应,95 ℃变性5 s,60℃退火30 s,72 ℃延伸30 s,共经历40次循环。在LightCyder480仪器(Roche Diagnostics)上机操作进行RT-qPCR。人FMR1引物Forward:GGTCAAGGAATGGGTCGAGG,Reverse:AGTTCGTCTCTGTGGTCAGAT;人GAPDH引物Forward:

TGTTGCCATCAATGACCCCTT，Reverse：CTCCAC GACGTACTCAGCG。

1.2.4 Western blot检测 收集各组细胞，选择全蛋白提取试剂盒（KeyGen BioTECH）提取细胞总蛋白，采用BCA（Thermo Fisher Scientific）试剂盒测蛋白浓度。提取的蛋白质经过8%~12%的SDS-PAGE凝胶分离，并转移到PVDF膜，用5%脱脂奶粉封闭2 h，孵育一抗，4 ℃冰箱过夜，1×TBST溶液配制二抗室温孵育1 h，利用化学发光成像仪（ChemiDoc MP）曝光检测并分析。GAPDH作为内参，抗体信息见表2。

表2 抗体信息
Tab.2 Antibody information

Antibody name	Company	Dilution rate
Anti-GAPDH	Cell Signaling technology	1∶1000
Anti-FMRP	Abcam	1∶1000
Anti-HO-1	Abmart	1∶1000
Anti-GPX4	Abmart	1∶1000
Anti-SLC7A11	Abmart	1∶1000
Anti-ERK	Cell signaling technology	1∶1000
Anti-p-ERK	Cell signaling technology	1∶1000
Anti-MAPK	Cell signaling technology	1∶500
Anti-p-MAPK	Cell signaling technology	1∶1000
Anti-RAS	Cell signaling technology	1∶500
Anti-p-RAS	Cell signaling technology	1∶500
Anti-MEK	Cell signaling technology	1∶1000
Anti-p-MRK	Cell signaling technology	1∶1000

1.2.5 细胞活性检测 将各组细胞以5×10³/孔的密度铺至96孔板，每组细胞设5个复孔。37 ℃培养箱分别培养24、48、72 h进行后续实验。更换为含有10% CCK8试剂培养基加入96孔板中继续孵育2 h，酶标仪测450 nm处吸光度。

1.2.6 细胞克隆形成能力检测 将各组细胞以1×10³/孔接种于6孔板中，置37 ℃、5% CO_2培养箱中培养2~3周，2 d/次更换新鲜培养基，定期观察细胞状态。当6孔板中出现肉眼可见的克隆时终止培养，加4%多聚甲醛固定细胞20 min，用1.5 mL 0.2%的晶状体紫染液加入到每个孔中染色10 min。PBS冲洗3次，干燥后拍摄，留存照片。

1.2.7 免疫荧光染色 收集各组细胞接种于提前放入细胞爬片的12孔板中，细胞数为1×10³/孔，待细胞完全贴壁后加入4%多聚甲醛固定，用0.5% Triton-X破膜处理，10%BSA室温封闭，随后按照抗体说明书加入稀释抗体SLC7A11（1∶200），4 ℃冰箱孵育过夜。最后加入FITC标记山羊抗兔IgG（1∶200）和DAPI染色。染色结束后封固，在荧光显微镜下观察并采集图像。

1.2.8 ROS、MDA、GSH、JC-1、Fe^{2+}试剂盒检测 收集各组细胞，严格按照相应试剂盒说明书操作，分别测定细胞ROS、MDA、GSH、JC-1及Fe^{2+}水平。

1.2.9 裸鼠成瘤实验 SPF级BALB/c 6~8周龄裸鼠8只［赛业（苏州）生物科技有限公司］。裸鼠喂养1周使其适应实验室环境，所有动物均在最佳饲养条件下进行。随机将裸鼠分成两笼，4只/组。收集对数生长期Control组、siFMRP-1组的HCT116细胞，向细胞沉淀中加入200 μL冰冷的PBS重悬后放置冰上保持细胞活性。于裸鼠腹股沟区注射细胞悬液，接种细胞量为1×10⁶/只。监测裸鼠状态及皮下移植瘤生长情况，每隔2 d用游标卡尺测量肿瘤体积（mm^3）=1/2最长径（mm）×最短径（mm）²，做好记录。观察结束后，采用颈椎脱臼法处死裸鼠，取皮下移植瘤，称重后用于后续研究。所有动物实验通过相关伦理审核（审批号：TACU23-FY030）。

1.3 统计学分析

采用Graphpad 8统计学软件和SPSS 18.0进行分析，计量资料采用均数±标准差表示，两组数据比较采用独立样本t检验，多组比较采用单因素方差分析，P<0.05为差异有统计学意义。

2 结果

2.1 FMRP在CRC中高表达且与不良预后相关

RT-qPCR和Western blot结果显示：与NCM460细胞相比，FMRP mRNA和蛋白水平在CRC细胞系中均表达上调，选择HCT116作为目的细胞用于后续研究（图1A、B，P<0.05）。TCGA数据库分析结果显示：FMRP在CRC组织中表达升高（图1C，P<0.001），且FMRP高表达CRC患者预后更差（图1D，P<0.05）。

2.2 FMRP与铁死亡信号通路密切相关

将FMR1在TCGA-CRC肿瘤样本表达中位数划分高表达组和低表达组，筛选差异基因绘制聚类图和火山图（图2A、B）；GO富集分析显示，FMRP与活性氧调控、氧化应激诱导细胞死亡、线粒体呼吸等生物学过程密切相关（图2C）；GSEA-GO分析显示，FMRP显著富集ATP能量供应、线粒体转导、氧化磷酸化、铁离子稳态等过程（图2D）；且铁离子稳态中差异基因富集FTH1、SLC25A37、TF、TFR2等铁死亡相关标志物（图2E）；GSEA-KEGG分析显示，FMRP与谷胱甘肽代谢通路相关（图2F）。

2.3 FMRP对细胞增殖表型的影响

Western blotting和RT-qPCR结果显示：与Control组和NC组相比，siFMRP-1、siFMRP-2、siFMRP-3组FMRP蛋白及mRNA表达降低，Lv-FMRP组表达升高

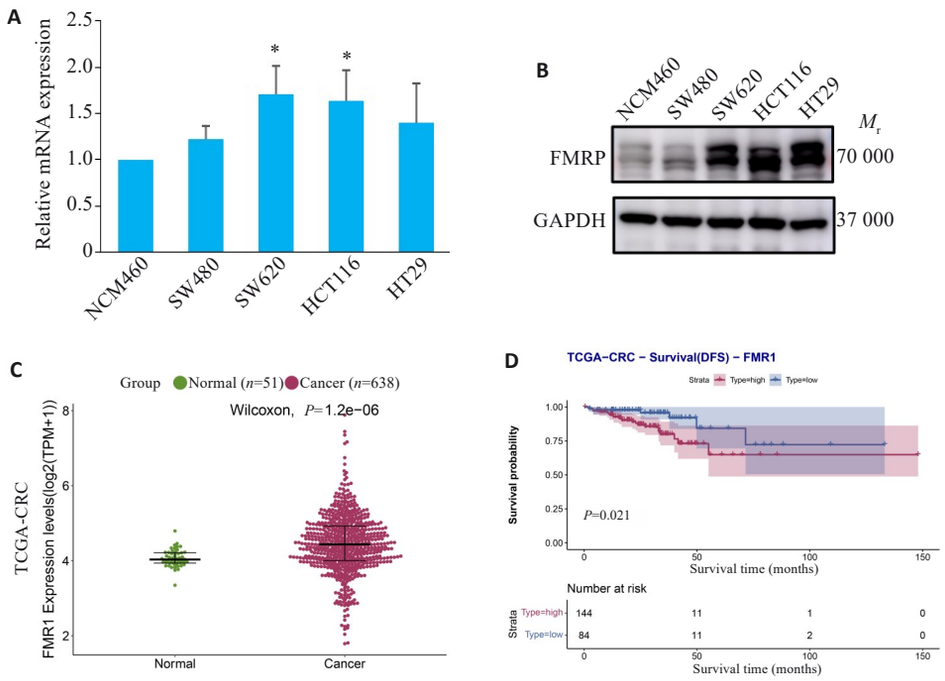

图1 FMRP在CRC中的表达

Fig.1 Expression levels of FMRP in colorectal cancer (CRC) cell lines and tissues. **A**: RT-PCR for detecting the expression of FMRP in different CRC cell lines. **B**: Western blotting for detecting FMRP protein expression in different CRC cell lines. **C**: Expression of FMRP in colorectal cancer tissues and normal tissues. **D**: Relationship between FMRP expression and disease-free survival of CRC patients. *P<0.05 *vs* NCM460 group.

(图3A、B,P<0.01)。CCK8结果提示:干扰组细胞增殖能力较对照组上升缓慢,而过表达组细胞增殖能力明显高于对照组(图3C,P<0.05)。平板克隆结果与CCK8结果一致:干扰组细胞克隆数目较对照组明显减少,而过表达组细胞克隆数目明显高于对照组(图3D,P<0.05)。

2.4 FMRP对细胞铁死亡表型的影响

铁死亡指标检测结果显示:与Control组和NC组相比,siFMRP组GSH含量降低,MDA含量升高,LV-FMRP组结果相反(图5A、B,P<0.05);siFMRP组活性氧ROS较对照组明显增多,而过表达组ROS活性低于对照组(图5C,P<0.05);Fe^{2+}荧光探针染色结果提示:siFMRP组细胞内Fe^{2+}水平较对照组升高,而LV-FMRP组Fe^{2+}水平降低(图5D,P<0.05)。Western blotting结果提示:siFMRP组与对照组相比,SLC7A11、GPX4表达降低,过表达组结果相反(图4E);荧光染色显示,敲低FMRP导致SLC7A11荧光表达减少,线粒体膜电位JC-1荧光增强(图4F、G)。

2.5 敲低FMRP抑制CRC瘤体生长

构建裸鼠皮下成瘤动物模型,结果发现:与对照组相比,干扰组瘤体减小、瘤体质量和体积均降低(图5A~C,P<0.01)。IHC实验结果表明:干扰组FMRP及SLC7A11的表达较对照组均降低(图5D)。

2.6 FMRP调控RAS/MAPK信号通路蛋白磷酸化表达

Western blotting结果显示:与对照组相比,敲低FMRP导致ERK、MEK、MAPK、RAS总蛋白表达不变,磷酸化蛋白表达降低,过表达FMRP上述蛋白磷酸化表达升高(图6,P<0.05)。

3 讨论

FMRP蛋白在脑组织中高度表达,其缺失会引起遗传智力障碍的脆性X综合症[16]。近年来,研究者越来越关注FMRP对恶性肿瘤的调控作用:Pedini等[5]发现FMRP通过调控Wnt信号通路促进胶质母细胞瘤增殖,其过表达与患者预后呈负相关;FMRP通过调控EMT促进三阴性乳腺癌患者肺转移发生[6];FMRP参与调节STAT3 mRNA的定位和翻译,在体内外促进肝癌转移[7, 8];同时,阳慧芝等[17]对FMRP在肿瘤致病机制中的研究进展进行了详细总结,揭示了FMRP在肿瘤细胞增殖、转移、凋亡中的关键作用,为肿瘤治疗提供潜在靶点。本研究发现FMRP在CRC中显著上调,敲低FMRP体内外抑制CRC细胞增殖和生长,与上述研究相符。为了进一步阐明FMRP促进CRC增殖的调控作用,本研究利用TCGA数据库分析发现FMRP与活性氧调控、ATP能量供应、线粒体转导、氧化磷酸化等参与线粒体氧化应激的过程相关;与铁离子稳态、氧化应激诱导细胞死亡、谷胱甘肽代谢等铁死亡途径相关。由此我们推测,FMRP可能通过影响铁死亡途径发挥促进CRC

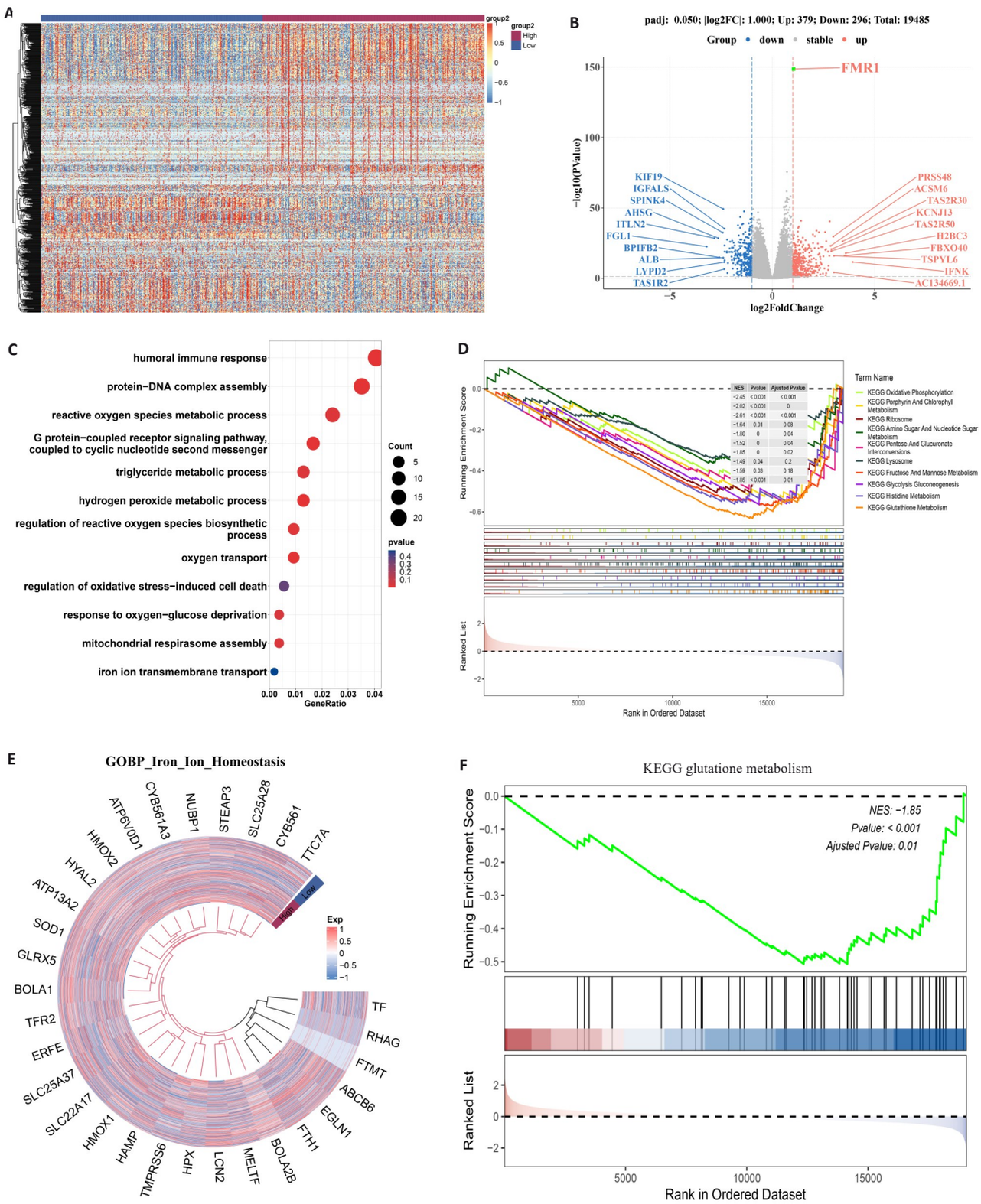

图2 生信分析筛选FMRP富集的生物功能及信号通路

Fig.2 Bioinformatic analysis of biological functions and signaling pathways enriched in FMRP. **A**: Two groups of differential genes. **B**: Up-regulated and down-regulated differential genes. **C**: GO functional enrichment bands. **D**: GSEA-GO enrichment analysis. **E**: Circular enrichment map of differential genes in iron ion homeostasis. **F**: GSEA-KEGG analysis map.

恶性增殖的调控作用。

铁死亡是一种依赖于细胞内铁累积引起毒性脂质过氧化物升高的非调亡细胞死亡形式，其形态学和发生机制均不同于凋亡、自噬和坏死[12]。铁死亡有3个特征：谷胱甘肽生物合成、脂质过氧化和铁代谢，主要调节机制是氧化损伤和抗氧化防御之间的平衡。SystemXc-

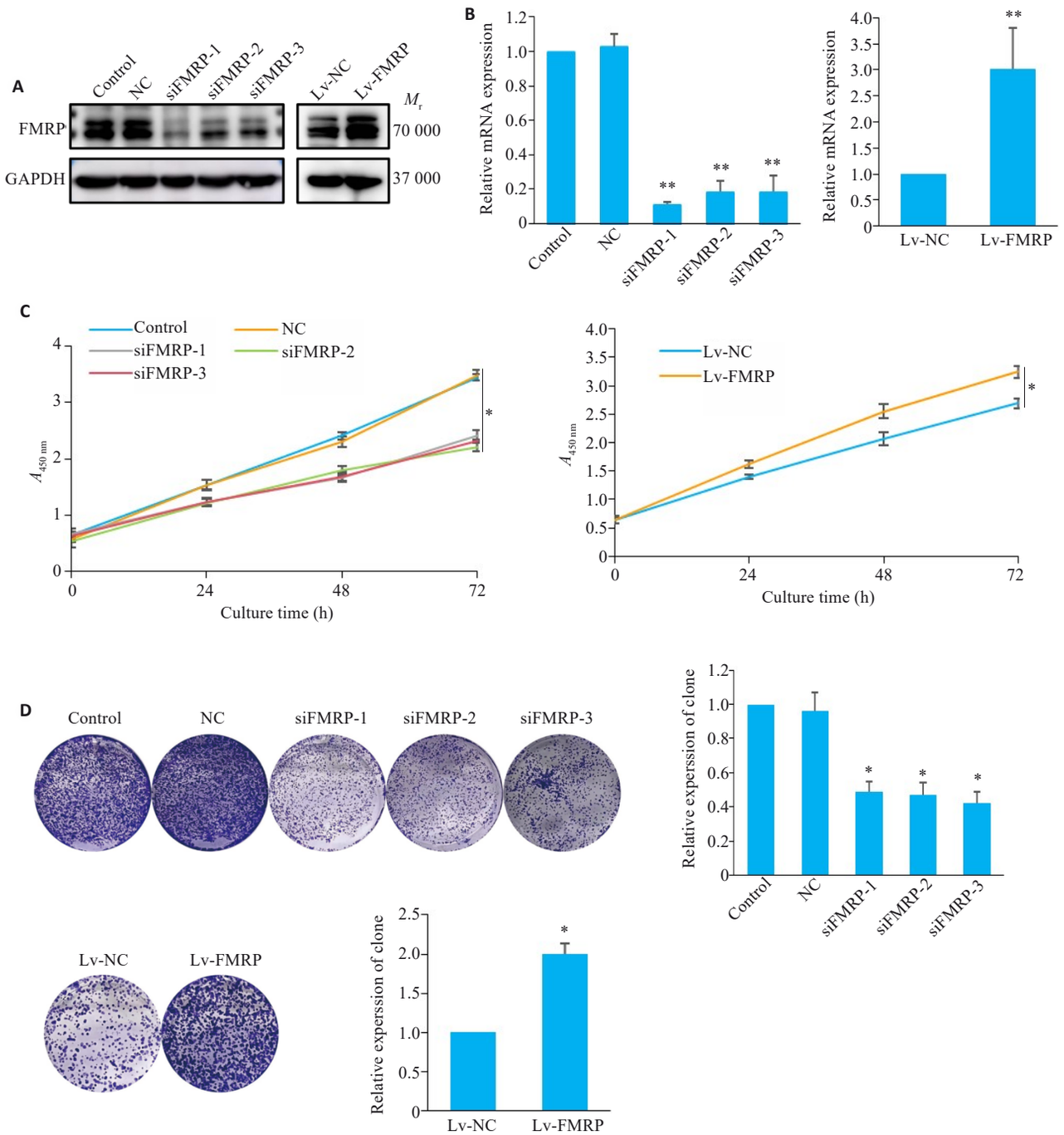

图3 FMRP对细胞增殖能力的影响

Fig. 3 Effect of FMRP knockdown or overexpression on HCT116 cell proliferation. **A**: Western blotting for verifying the efficiency of FMRP knockdown or overexpression. **B**: RT-PCR of FMRP mRNA expression in HCT116 cells. **C**: CCK8 assay of the proliferation of the transfected HCT116 cells. **D**: Plate clone formation assay of cell proliferation. **P*<0.05, ***P*<0.01 *vs* Control or Lv-NC group.

是一种位于细胞膜上的逆向转运蛋白，SLC7A11作为核心成员按一定比例将谷氨酸转运出细胞，将半胱氨酸转运入细胞，并以半胱氨酸为原料合成GSH。GSH是体内重要的抗氧化剂和自由基清除剂。通过抑制SLC7A11的活性，可降低细胞内GSH，从而导致GPX4活性的降低而促进铁死亡[18]。因此，抑制SLC7A11/GSH/GPX4信号轴被认为是目前铁死亡发生的主要作用机制。

既往研究表明[19-22]铁死亡与CRC疾病进展、转移及耐药密切相关，然而FMRP是否通过抑制肿瘤细胞发生铁死亡发挥调控CRC进展的作用，目前尚无报道。本研究发现敲低FMRP促进细胞铁死亡发生，过表达FMRP与之相反，与TCGA生信分析结果一致，表明敲低FMRP通过促进铁死亡途径抑制CRC细胞增殖。

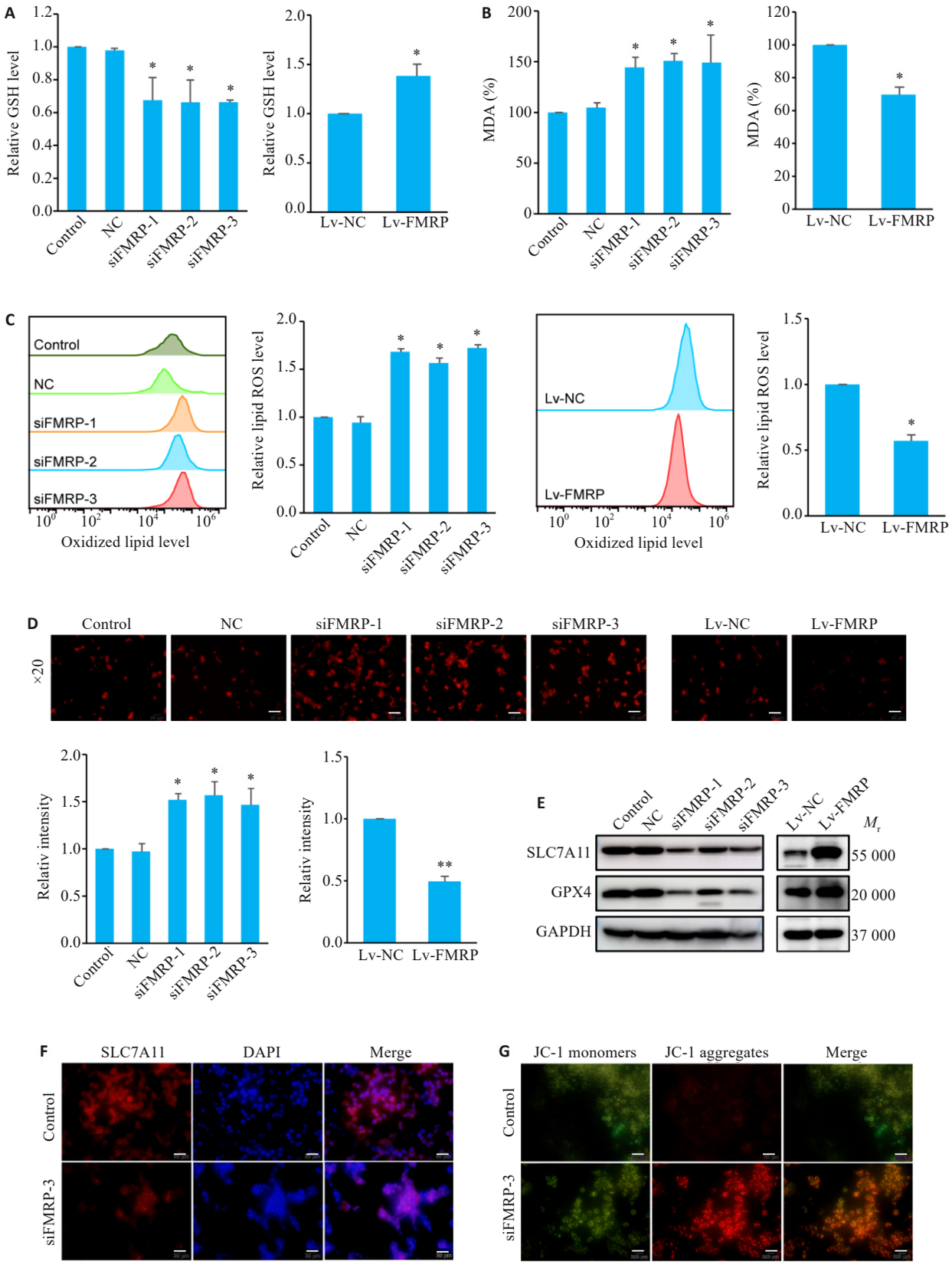

图4 FMRP对细胞铁死亡的影响

Fig. 4 Effect of FMRP knockdown or overexpression on ferroptosis of HCT116 cells. **A**: GSH contents in transfected HCT116 cells. **B**: MDA contents in the transfected cells. **C**: ROS level in the transfected cells. **D**: Fe^{2+} level in the transfected cells (scale bar=50 μm). **E**: Western blots of the ferroptosis markers in the transfected cells. **F**: Immunofluorescence detection of SLC7A11 in HCT116 cells with FMRP knockdown (scale bar=20 μm). **G**: Immunofluorescence detection of mitochondrial membrane potential in HCT116 cells with FMRP knockdown (scale bar=20 μm). **P*<0.05, ***P*<0.01 *vs* Control or Lv-NC group.

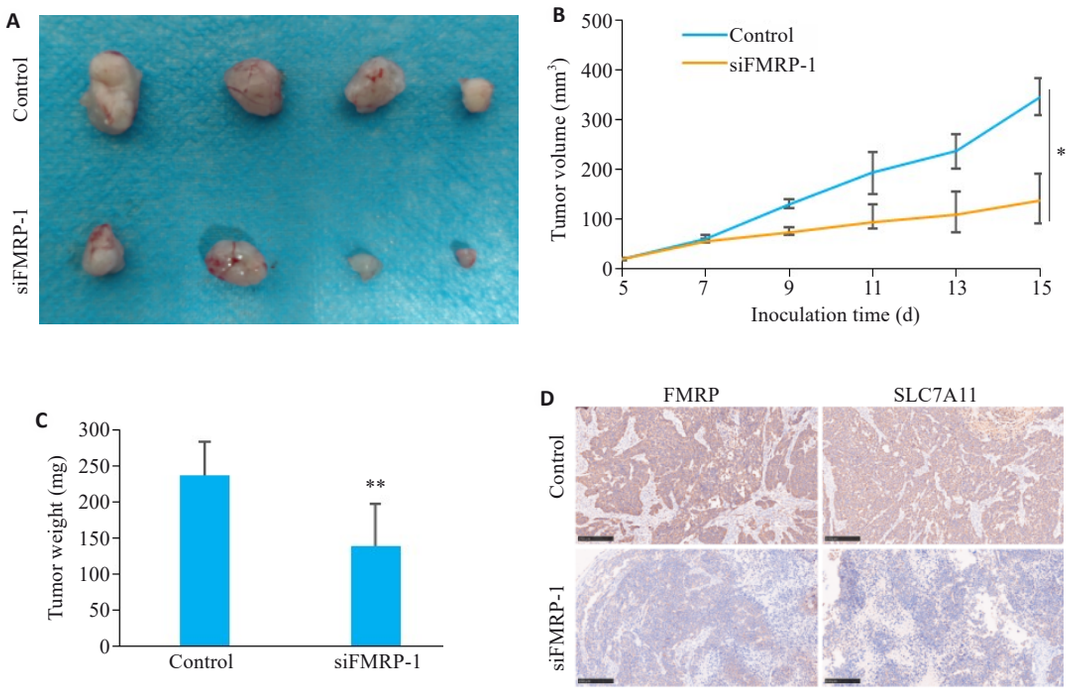

图5 FMRP对裸鼠移植瘤的生长的影响

Fig. 5 Effect of FMRP knockdown on growth of transplanted tumor in nude mice. **A**: The size of subcutaneously transplanted tumor in nude mice. **B**: Changes in tumor weight in nude mice. **C**: Changes in tumor volume in nude mice. **D**: Immunohistochemistry for FMRP and SLC7A11 in the tumor tissue (scale bar=250 μm). **P*<0.05, ***P*<0.01 *vs* Control.

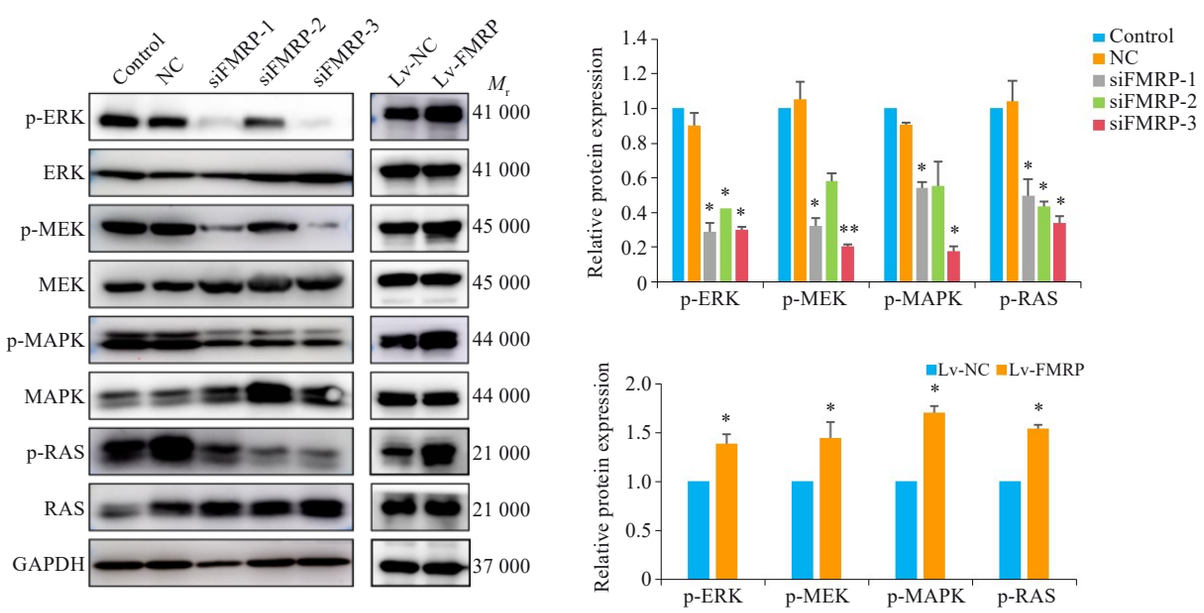

图6 FMRP对RAS/MAPK信号通路的影响

Fig. 6 Effect of FMRP knockdown or overexpression on RAS/MAPK signaling pathway in HCT116 cells detected by Western blotting. **P*<0.05, ***P*<0.01 *vs* Control or Lv-NC group.

CRC发生发展过程复杂，多种信号通路参与调控其恶性进展。目前已报道参与调控细胞铁死亡的信号通路包括AMPK、RAS/MAPK、PI3K/AKT、Wnt/β-catenin通路[23, 24]。其中RAS/MAPK信号通路在KRAS突变的CRC发病机理中扮演重要作用，被认为是人类恶性肿瘤的新型潜在生物标记[25-27]。同时查阅文献发现FMRP协同RAS/MAPK通路在脑肿瘤发生中发挥作用[28]。因此，本研究通过Western blot检测RAS/MAPK信号通路关键蛋白表达，发现敲低FMRP导致RAS/MAPK信号通路关键蛋白ERK、MEK、MAPK、

RAS磷酸化表达降低，过表达FMRP上述蛋白磷酸化水平升高，推测FMRP通过激活RAS/MAPK信号通路诱导CRC细胞发生铁死亡抵抗。

综上所述，本研究证实FMRP通过调控RAS/MAPK信号通路介导铁死亡促进CRC的恶性进展并调控CRC细胞在体内外的增殖。FMRP可能是通过调控铁死亡途径治疗CRC的潜在靶点，对于拓展CRC的临床治疗具有重要的理论和指导意义。

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