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MiR-223-3p通过靶向*SORBS1*增加结直肠癌细胞对5-氟尿嘧啶的耐药性

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[摘 要] 目的：5-氟尿嘧啶(5-fluorouracil，5-FU)是结直肠癌(colorectal cancer，CRC)的一线治疗药物，CRC细胞对5-FU的耐药是导致化学治疗(以下简称“化疗”)失败的主要原因，然而耐药机制仍不明确。本研究旨在探究参与CRC细胞对5-FU耐药的抑癌基因，并寻找对该基因存在调控作用的微RNA(microRNA，miRNA)。**方法：**在高通量基因表达(Gene Expression Omnibus，GEO)数据库中下载CRC数据集GSE28702和GSE69657，分别分析2个数据集中对FOLFOX化疗方案应答组和无应答组患者的差异表达基因并确定目标基因；采用在线生物信息学数据库TargetScan、miRwalk和miRDB预测靶向目标基因含山梨醇和SH3结构域蛋白1(sorbin and SH3 domain containing 1，*SORBS1*)的miRNA。采用瞬时转染技术在CRC细胞系HCT116、SW620中分别转染siSORBS1、HA-SORBS1、miR-223-3p mimic、anti-miR-223-3p及其相应的阴性对照(siNC、HA、miR-NC、anti-miR-NC)，在HCT116细胞中共转染miR-NC和HA、miR-223-3p mimic和HA、miR-223-3p mimic和HA-SORBS1、anti-miR-NC和siNC、anti-miR-223-3p和siNC、anti-miR-223-3p和siSORBS1。采用实时反转录PCR(real-time reverse transcription PCR，real-time RT-PCR)和/或蛋白质印迹法检测细胞中*SORBS1*和*miR-223-3p*的表达水平。转染后用不同浓度的5-FU处理细胞。采用四甲基偶氮唑盐(methyl thiazolyl tetrazolium，MTT)法检测细胞活力，双荧光素酶报告分析验证miR-223-3p与SORBS1的靶向关系。**结果：**GSE69657数据集和GSE28702数据集应答组分别有409和528个高表达基因，共有22个高表达交集基因。在22个高表达交集基因中，与CRC化疗敏感性有关的抑癌基因有3个，选择*SORBS1*作为目标基因进一步探索。3个在线生物信息学数据库预测的靶向*SORBS1*的miRNA为miR-223-3p。用5-FU(25 μmol/L)处理HCT116、SW620细胞12~36 h后，2种细胞中*miR-223-3p*的表达水平均显著下调(均$P<0.05$)。转染siSORBS1或miR-223-3p mimic后，HCT116、SW620细胞中SORBS1表达水平均下调，细胞活力均增加(均$P<0.05$)；转染HA-SORBS1或anti-miR-223-3p后，HCT116、SW620细胞中SORBS1表达水平均上调，细胞活力均下降(均$P<0.05$)。双荧光素酶报告分析结果显示：共转染*SORBS1* 3'-非翻译区(untranslated region，UTR)野生型质粒和miR-223-3p mimic的细胞荧光素酶活性显著低于共转染*SORBS1* 3'-UTR野生型质粒和miR-NC的细胞($P<0.05$)。与共转染miR-223-3p mimic和HA的细胞比较，共转染miR-223-3p mimic和HA-SORBS1的细胞活力明显降低($P<0.01$)；与共转染anti-miR-223-3p和siNC组比较，共转染anti-miR-223-3p和siSORBS1组细胞活力明显增加($P<0.05$)。**结论：**MiR-223-3p通过靶向*SORBS1*基因增加CRC细胞对5-FU的耐药性，miR-223-3p有望成为临床治疗CRC的新靶点。

[关键词] 结直肠癌；含山梨醇和SH3结构域蛋白1；微RNA-223-3p；5-氟尿嘧啶；化学治疗；耐药

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MiR-223-3p increases resistance of colorectal cancer cells to 5-fluorouracil via targeting *SORBS1*

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ABSTRACT

Objective: 5-Fluorouracil (5-FU) is the first-line drug for treating colorectal cancer (CRC), and the resistance of tumor cells to 5-FU is the main cause of chemotherapeutic failure. However, the resistant mechanism is still unclear. This study aims to explore the tumor suppressor genes involved in 5-FU resistance in CRC, and to find the microRNA (miRNA) that regulates these genes.

Methods: CRC data sets GSE28702 and GSE69657 were downloaded from Gene Expression Omnibus (GEO) database, and gene expression profiles of patients in the FOLFOX chemotherapeutic response group and the non-response group were analyzed, and differential expression genes were identified between the 2 groups. Target gene was then selected. Online bioinformatics databases TargetScan, miRwalk, and miRDB were used to predict miRNA targeting the interested gene sorbin and SH3 domain containing 1 (*SORBS1*). siSORBS1, HA-SORBS1, miR-223-3p mimic, anti-miR-223-3p, and their corresponding negative controls (siNC, HA, miR-NC, and anti-miR-NC) were transfected into CRC cell lines of HCT116 and SW620 by transient transfection technique, respectively. Co-transfection was done with miRNA and plasmid (miR-NC+HA, miR-223-3p mimic+HA, or miR-223-3p mimic+HA-SORBS1) or anti-miRNA and siRNA (anti-miR-NC+siNC, anti-miR-223-3p+siNC, or anti-miR-223-3p+siSORBS1) in HCT116 cells. Real-time reverse transcription PCR (real-time RT-PCR) and/or Western blotting were used to detect the expression levels of *SORBS1* and *miR-223-3p* in cells. After transfection, the cells were treated with different concentrations of 5-FU, and the cell viability was detected by methyl thiazolyl tetrazolium (MTT) method. The targeting relationship between miR-223-3p and SORBS1 was comfirmed by dual luciferase reporter gene assay.

Results: There were 409 and 528 highly expressed genes in the FOLFOX chemotherapeutic response group of GSE69657 and GSE28702, respectively. There were 22 overlapping genes in the response group, among which exist 3 tumor suppressor genes might be involved in chemosensitivity in CRC, and *SORBS1* was selected as the target gene for further study. Three online bioinformatics databases predicted miRNAs targeting *SORBS1* and obtained an intersection molecule miR-223-3p. After treatment with 5-FU (25 μmol/L) for 12－36 h, the levels of *miR-223-3p* in HCT116 and SW620 cells were significantly down-regulated (all $P<0.05$). After transfection with siSORBS1 or miR-223-3p mimic, the expression levels of SORBS1 in HCT116 and SW620 cells were down-regulated, and the cell viability was increased (all $P<0.05$). After transfection with HA-SORBS1 or anti-miR-223-3p, the expression levels of SORBS1 in HCT116 and SW620 cells were up-regulated, and the cell viability was decreased (all $P<0.05$). The result of dual

luciferase reporter gene assay showed that the luciferase activity of cells co-transfected with *SORBS1* 3'-UTR wild plasmid and miR-223-3p mimic was significantly lower than that of the 3'-UTR wild plasmid and miR-NC cells (P<0.05). Compared with co-transfection with miR-223-3p mimic and HA, the cell viability of cells co-transfected with miR-223-3p mimic and HA-SORBS1 was decreased significantly (P<0.01). Compared with the co-transfected anti-miR-223-3p and siNC, the cell viability of the co-transfected anti-miR-223-3p and siSORBS1 was significantly increased (P<0.05).

Conclusion: MiR-223-3p increases 5-FU resistance in CRC cells by targeting *SORBS1*, and miR-223-3p is expected to become a new target for clinical treatment of CRC.

KEY WORDS colorectal cancer; sorbin and SH3 domain containing 1; microRNA-223-3p; 5-fluorouracil; chemotherapy; drug resistance

结直肠癌(colorectal cancer，CRC)是人类最常见的消化道恶性肿瘤之一。近年来，随着饮食结构、生活方式的改变以及人口老龄化，CRC的发病率和病死率呈逐年增长趋势。根据全球癌症统计[1]，CRC的发病率和病死率分别排在第3位和第2位，严重危害着人类的健康。手术是早期CRC的主要治疗手段，患者5年生存率可达到90%以上。但是CRC在疾病早期通常无明显的临床症状，确诊时大多已处于中晚期，已经错过最佳的手术时机。对于中晚期CRC的治疗，以化学治疗(以下简称“化疗”)为主的综合治疗占据着重要地位[2]。尽管以化疗为主的综合治疗已取得一定的进展，但患者的5年生存率仍然不高。5-氟尿嘧啶(5-fluorouracil，5-FU)是目前治疗CRC的主要药物，它是一种人工合成的尿嘧啶类似物，在体内通过生成5-氟脱氧尿苷酸，抑制(脱氧)胸苷酸合成酶的活性，进而阻断生长旺盛的肿瘤DNA的生物合成，最终影响细胞的增殖[3]，发挥抗肿瘤的作用。以5-FU为基础的FOLFOX方案(亚叶酸钙和5-FU联用的基础上加用不同剂量的奥沙利铂)是临床常用的CRC化疗方案，然而CRC存在较大异质性，导致FOLFOX方案对于不同患者的治疗效果差异较大，对5-FU的耐受是导致CRC化疗失败和不良预后的重要原因[3]。研究CRC化疗耐药相关机制，以提高患者对化疗药物的敏感性具有重要意义。研究发现：在5-FU耐药的CRC细胞中，存在腺瘤性息肉病(adenomatous polyposis coli，*APC*)[4]、p53肿瘤蛋白(tumor protein p53，*TP53*)[5]和鼠类肉瘤滤过性毒菌致癌同源体B1(v-raf murine sarcoma viral oncogene homolog B1，*BRAF*)[6]等关键基因的突变。基于临床样本的转录组数据，通过生物信息学的手段探索CRC化疗耐药相关基因将为解决CRC化疗耐药提供理论依据。

含山梨醇和SH3结构域蛋白1(sorbin and SH3 domain containing 1，*SORBS1*)编码含有山梨糖和SH3结构域的蛋白，也被称为CAP/ponsin蛋白，参与调节细胞黏附、细胞骨架的形成、胰岛素信号通路和肿瘤的转移[7-10]。在多种恶性肿瘤中，SORBS1的表达下调[11-12]。在乳腺癌患者中，SORBS1的低水平表达与预后不良及肿瘤细胞侵袭能力增强显著相关，SORBS1通过上调乳腺癌细胞中p53蛋白的水平抑制上皮-间充质转化(epithelial-mesenchymal transition，EMT)过程，增强癌细胞对顺铂的敏感性[13]。SORBS1发挥抑制肿瘤的作用，但SORBS1在以5-FU为基础的化疗方案治疗CRC中发挥的作用尚不明确。已有研究[14]表明部分抑癌基因会在化疗药物处理后显著上调，进而促进该化疗药物诱导的细胞死亡，如强力霉素处理会导致肿瘤细胞中的p63肿瘤蛋白(tumor protein p63，*TP63*)水平显著上升，进而促进细胞死亡，而抑制p63则能够减少强力霉素导致的细胞死亡，使细胞获得对强力霉素的耐受力。链霉素、顺铂、依托泊苷等化疗药物处理均能诱导p73的表达[15-16]，敲减p73肿瘤蛋白(tumor protein p73，*TP73*)基因的表达则能够显著减少多种化疗药物引起的细胞死亡[16]。因此，笔者推测：CRC细胞经5-FU处理后，可能会影响某个关键抑癌基因的表达，该基因所翻译的蛋白质参与5-FU诱导的细胞死亡。

微RNA(microRNA，miRNA)是一类长度为18~22个核苷酸的单链非编码RNA，通过与靶基因mRNA的3'-非翻译区(untranslated region，UTR)互补配对，抑制靶基因的翻译或促进靶基因mRNA降解，发挥转录后调节作用。研究[17]表明miRNA的表达失调与肿瘤发生、发展、转移和化疗耐药显著相关。

根据miRNA在肿瘤中发挥的生物学功能，可分为肿瘤抑制性miRNA和致癌性miRNA。设计靶向肿瘤抑制性miRNA的miRNA mimic增强其抑癌功能或靶向致癌性miRNA的anti-miRNA削弱其促癌功能在临床前开发和临床研究中显示出广泛的应用前景[18-19]。

MiR-223-3p在包括CRC在内的多种恶性肿瘤中显著高表达，促进恶性肿瘤的发生和发展[20-23]。miR-223-3p通过靶向叉头框蛋白O3a(forkhead box O3a，*FOXO3a*)基因抑制阿霉素诱导的自噬，增强肝癌的化疗耐药[24]；在非小细胞肺癌中，miR-223-3p通过靶向含F-框和WD重复域蛋白7(F-box and WD repeat domain containing protein 7，*FBXW7*)调节自噬过程和对化疗药物顺铂的耐药[25]；在胃癌中，miR-223-3p通过靶向FBXW7调节细胞周期，从而促进胃癌细胞对顺铂的耐药[26]。由此可见，miR-223-3p可通过不同的分子机制调节多种恶性肿瘤对化疗的耐药性，但miR-223-3p对CRC细胞5-FU耐药的作用机制尚不清楚。本研究分析miR-223-3p与*SORBS1*基因的靶向关系，探究miR-223-3p、SORBS1对CRC细胞5-FU耐药的影响，旨在为研究CRC化疗耐药潜在的药物靶点提供依据。

1 材料与方法

1.1 细胞株和主要试剂

人CRC细胞株HCT116和SW620、人肾上皮细胞系HEK293T购自美国菌种保藏中心(American Type Culture Collection，ATCC)。胎牛血清、青霉素和链霉素均为以色列BI公司产品。DMEM购自兰州市民海生物工程有限公司。转染试剂Lipofectamine 2000购自美国Invitrogen公司。siRNA购自上海吉玛制药技术有限公司。MiR-223-3p mimic及anti-miR-223-3p购自广州市锐博生物科技有限公司。PCR引物购自湖南擎科生物技术有限公司。SORBS1抗体和β-actin抗体购自武汉三鹰生物技术有限公司。质粒购于武汉淼灵生物科技有限公司。TRIzol试剂购自北京全式金生物技术股份有限公司。cDNA合成试剂盒购自上海近岸科技有限公司。Real-time PCR SYBR Green Master Mix和双荧光素酶报告基因检测试剂盒购自翌圣生物科技(上海)股份有限公司。四甲基偶氮唑盐(methyl thiazolyl tetrazolium，MTT)购自北京酷来搏科技有限公司。增强型化学发光(enhanced chemiluminescence，ECL)试剂购自亚科因(武汉)生物技术有限公司。*Bam*H I 内切酶购自纽英伦生物技术(北京)有限公司。

1.2 GEO数据获取

在高通量基因表达(Gene Expression Omnibus，GEO)数据库(https://www.ncbi.nlm.nih.gov/geo/)中，以“colorectal cancer”“chemotherapy”“drug resistance”为检索词进行检索，并对检索到的数据集按以下条件进行筛选：1)样本量大小合适，为30~100；2)CRC患者均采用FOLFOX化疗方案；3)包含患者对FOLFOX方案化疗的应答情况。

根据对化疗的应答情况，将筛选得到的各数据集中的患者分别分为应答组和无应答组。采用在线工具GEO2R对应答组和无应答组进行差异表达基因的筛选，筛选条件设定为$P<0.05$且$|\log_2[$差异倍数(fold change，FC)$]|>0.6$。应答组中高表达的基因可能与化疗敏感性相关，进一步对各数据集中应答组高表达的基因取交集。

1.3 目标基因的确定

用5-FU(25 μmol/L)处理HCT116细胞36 h后，采用实时反转录PCR(real-time reverse transcription PCR，real-time RT-PCR)检测细胞中高表达交集基因中具有抑癌功能的基因的mRNA水平，确定*SORBS1*为本研究的目标基因。并进一步用5-FU(25 μmol/L)处理HCT116细胞、SW620细胞0、9、18、36 h后，分别采用real-time RT-PCR和蛋白质印迹法检测细胞中*SORBS1*的mRNA和蛋白质的表达水平。

1.4 预测靶向目标基因的miRNA

采用3个在线生物信息学数据库Targetscan(https://www.targetscan.org/vert_80/)、miRwalk(http://mirwalk.umm.uni-heidelberg.de/)和miRDB(https://www.mirdb.org/)对靶向目标基因*SORBS1*的miRNA分子进行预测，3个数据库预测的交集miRNA为miR-223-3p。进一步用5-FU(25 μmol/L)处理HCT116细胞、SW620细胞0、12、24、36 h后，采用real-time RT-PCR检测细胞中*miR-223-3p*的mRNA表达水平。

1.5 细胞转染

细胞采用含10%胎牛血清、青霉素和链霉素各100 U/mL的DMEM在37 ℃、5% CO_2条件下培养。取对数生长期的HCT116细胞、SW620细胞接种于12孔板，在细胞单个贴壁且融合度为30%~40%时，采用瞬时转染技术，按Lipofectamine 2000说明书步骤进行转染。

为探索SORBS1与CRC细胞5-FU耐药的关系，

设siSORBS1组和siNC组，每孔加入siSORBS1或siRNA 4 μL；设带有HA标签的过表达SORBS1的质粒(HA-SORBS1)组和阴性对照质粒(HA)组，每孔加入HA-SORBS1或HA质粒500 ng。siNC序列为5'-UUCUUCGAACGUGUCACGUTT-3'，siSORBS1序列为5'-GCAGAGCCCAAGAGCAUUUTTAAAUGC-UCUUGGGCUCUGCTT-3'。

为探索miR-223-3p与CRC细胞5-FU耐药的关系，设miR-223-3p mimic组和miR-NC组，每孔加入miR-223-3p mimic或miR-NC 3 μL；设anti-miR-223-3p组和anti-miR-NC组，每孔加入anti-miR-223-3p或anti-miR-NC 6 μL。

为进一步探索miR-223-3p是否通过靶向SORBS1介导CRC细胞对5-FU的耐受，设miR-NC+HA组、miR-223-3p mimic+HA组、miR-223-3p mimic+HA-SORBS1组和anti-miR-NC+siNC组、anti-miR-223-3p+siNC组、anti-miR-223-3p+siSORBS1组。

转染后收集一部分细胞，采用real-time RT-PCR和蛋白质印迹法检测*SORBS1*的mRNA和蛋白质的表达水平；另一部分细胞用5-FU处理72 h后行MTT法检测细胞活力。

1.6 Real-time RT-PCR

使用TRIzol提取各组细胞总RNA。严格按照cDNA合成试剂盒说明书操作将RNA反转录成cDNA，使用real-time PCR SYBR Green Master Mix试剂盒扩增*SORBS1*基因。反应条件：95 ℃预变性5 min后，95 ℃变性10 s，58 ℃退火30 s，72 ℃延伸20 s，40个循环。用荧光定量PCR仪实时检测荧光值变化，$2^{-\Delta\Delta Ct}$法计算*SORBS1*基因的相对表达量。*β-actin*为内对照基因。Real-time RT-PCR引物序列见表1。

表1 实时反转录PCR引物序列

Table 1 Sequences of real-time RT-PCR primers

引物名称		序列
SORBS1	正向	5'-ATTCCCAAGCCTTTCCATCAG-3'
	反向	3'-TTTTGCTGTTCTCGATTGTGTTG-5'
β-actin	正向	5'-GATTCCTATGTGGGCGACGA-3'
	反向	3'-AGGTCTCAAACATGATCTGGGT-5'
U6	正向	5'-CTCGCTTCGGCAGCACA-3'
	反向	3'-AACGCTTCACGAATTTGCGT-5'

SORBS1：含山梨醇和SH3结构域蛋白1。

1.7 蛋白质印迹法

收集各组细胞，加入蛋白质裂解液提取细胞总蛋白质，行SDS聚丙烯酰胺凝胶电泳(SDS polyacrylamide gel electrophoresis，SDS-PAGE)后，在450 mA下转膜90 min。用TBST缓冲液配置的含5%脱脂奶粉封闭液封闭1 h，按照抗体说明书加入特异性一抗，于4 ℃下孵育过夜，用TBST缓冲液洗膜3次，加入辣根过氧化物酶标记的二抗，在室温下孵育2 h，用TBST缓冲液洗膜后使用ECL试剂检测蛋白质的表达水平。

1.8 构建野生型和突变型重组真核表达载体

使用TargetScan预测miR-223-3p与*SORBS1* 3'-UTR靶向结合的序列。通过PrimerPremier 5.0系统进行引物设计，用PCR扩增包含miR-223-3p结合位点的上下游约150 bp的*SORBS1* 3'-UTR，琼脂糖凝胶电泳回收PCR产物；采用*Bam*H I 单酶切psiCHECK2载体，琼脂糖凝胶电泳回收、纯化酶切产物；将纯化过的PCR产物和酶切载体进行酶联反应；对酶联产物进行转化，次日挑单克隆、摇菌、提取重组载体并进行质粒测序。以测序正确的野生型psiCHECK2-*SORBS1* 3'-UTR(WT-*SORBS1*)质粒为模板，根据结合位点设计突变引物，构建突变型psiCHECK2-*SORBS1* 3'-UTR(MUT-*SORBS1*)质粒。

1.9 双荧光素酶报告分析

接种HEK293T细胞于24孔板中，贴壁后将WT-*SORBS1*和MUT-*SORBS1*分别与miR-NC或miR-223-3p mimic共同转染；培养48 h后，用细胞裂解液在冰上裂解细胞6 min，在4 ℃以12 000 r/m离心2 min；取上清，按照双荧光素酶报告基因检测试剂盒说明书推荐用量加入对应的荧光素酶反应液，分别测定

萤火虫荧光素酶和海肾荧光素酶的活性，计算相对的荧光强度。

1.10 MTT法

各组细胞接种于96孔板，每组设3个复孔，同时设仅加培养基的空白对照。细胞贴壁后加入5-FU(终浓度分别为0、1.48、4.44、13.30、40.00、120.00 μmol/L)。放入37 ℃、5% CO_2的培养箱中培养72 h，按照说明书推荐用量加入MTT，继续培养4 h后，加入MTT溶解液溶解过夜，在室温下震荡15 min。用酶标仪检测570 nm波长处每孔的吸光度值。

1.11 统计学处理

采用GraphPad Prism 9软件进行统计学分析，计量资料以均数±标准差($\bar{x}\pm s$)表示。2组间比较采用配对t检验，同组内不同时间点采用单因素方差分析，不同组别同一药物浓度之间的两两比较采用Dunnett t检验。P<0.05为差异具有统计学意义。

2 结　果

2.1 CRC耐药差异表达基因

GEO数据库中符合要求的数据集为GSE69657和GSE28702。GSE69657数据集应答组高表达基因有409个，GSE28702数据集应答组高表达基因有528个，对2个数据集中应答组患者肿瘤组织中高表达的基因取交集，得到22个交集基因(表2)。

2.2 5-FU上调SORBS1的表达

22个交集基因中包含3个具有抑癌功能的基因(*SORBS1*、*SORBS2*和*CES1*)。通过对GSE28702和GSE69657数据集中FOLFOX方案应答组患者体内高表达的22个交集基因进行基因本体(Gene Dntology，GO)富集分析，结果显示：SORBS1的功能显著富集在细胞-细胞黏附连接、黏着斑、细胞基质黏附、黏附连接组织方面(附图1，https://doi.org/10.11817/j.issn.1672-7347.2023.220345F1)。

Real-time RT-PCR结果显示：用25 μmol/L处理HCT116细胞36 h后，*SORBS1* mRNA的表达提升至基线水平5倍以上(P<0.001，图1)。提示SORBS1可能与5-FU诱导的细胞死亡有关。因此，笔者提出假说：5-FU能够上调SORBS1的表达进而诱导CRC细胞死亡，故最终选择*SORBS1*作为目标基因进行后续研究。

进一步用5-FU(25 μmol/L)处理HCT116细胞、SW620细胞不同时间后，2种细胞中*SORBS1*的mRNA(均P<0.05，图2A)和蛋白质(图2B)的表达水平均显著上调。这提示SORBS1可能与CRC细胞对5-FU的敏感性有关。

表2　FOLFOX应答组高表达交集基因

Table 2　Highly expressed intersection genes in the FOLFOX response group

Gene symbol	Gene title
CHURC1	Churchill domain containing 1
EPHA3	EPH receptor A3
SORBS1	Sorbin and SH3 domain containing 1
MSR1	Macrophage scavenger receptor 1
RDX	Radixin
LYVE1	Lymphatic vessel endothelial hyaluronan receptor 1
ADSSL1	Adenylosuccinate synthase like 1
KCTD15	Potassium channel tetramerization domain containing 15
SORBS2	Sorbin and SH3 domain containing 2
CES1	Carboxylesterase 1
STMN2	Stathmin 2
CXCL12	C-X-C motif chemokine ligand 12
CLU	Clusterin
AGTR1	Angiotensin II receptor type 1
FAM229B	Family with sequence similarity 229 member B
AKAP12	A-kinase anchoring protein 12
ALDH1A1	Aldehyde dehydrogenase 1 family member A1
PDE1A	Phosphodiesterase 1A
LMO3	LIM domain only 3
ECHDC3	Enoyl-Coa hydratase domain containing 3
ADH1B	Alcohol dehydrogenase 1B (Class I), beta polypeptide
CCBE1	Collagen and calcium binding EGF domains 1

2.3 敲低*SORBS1*基因的表达增加CRC细胞对5-FU的耐受性

与siNC组比较，siSORBS1组*SORBS1*的mRNA和蛋白质的表达水平均显著下调(均P<0.05，图3A、3B)，细胞活力明显增加(P<0.05，图3C)。提示敲低*SORBS1*基因增加CRC细胞对5-FU的耐受性。

2.4 过表达*SORBS1*基因增加CRC细胞对5-FU的敏感性

蛋白质印迹结果显示：HA-SORBS1重组蛋白在HCT116细胞、SW620细胞中均成功过表达(图4A)，同时在过表达SORBS1后E-钙黏蛋白表达上调，而

N-钙黏蛋白表达下调(附图2，https://doi.org/10.11817/j.issn.1672-7347.2023.220345F2F3)。MTT结果显示：与HA组比较，HA-SORBS1组细胞活力明显降低(P<0.05，图4B)。提示过表达*SORBS1*基因增加CRC细胞对5-FU的敏感性。

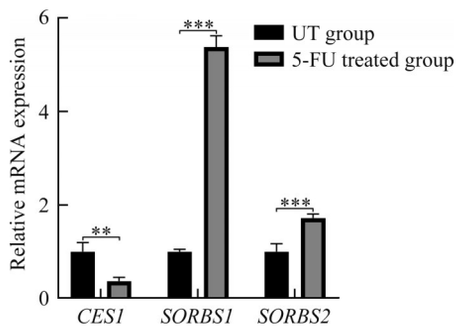

图1 HCT116细胞经5-FU(25 μmol/L)处理36 h后，采用实时反转录PCR检测3种抑癌基因mRNA表达水平

Figure 1 After 25 μmol/L 5-FU treatment for 36 h, the mRNA levels of 3 tumor suppressor genes in HCT116 cells were determined by real-time RT-PCR

Data are represented as the means±standard deviation, n=3. **P<0.01, ***P<0.001. UT: Untreated; 5-FU: 5-Fluorouracil; CES1: Carboxylesterase 1; SORBS1: Sorbin and SH3 domain containing 1; SORBS2: Sorbin and SH3 domain containing 2.

2.5 靶向*SORBS1* miRNA的预测与验证

3个在线生物信息学数据库预测的靶向*SORBS1*基因的交集分子为miR-223-3p(图5A)。在GEO数据库寻找CRC组织mRNA和miRNA测序数据，找到符合要求的数据集GSE41655和GSE41657。匹配临床信息后发现：在53个CRC临床样品中，SORBS1与miR-223-3p的表达量呈负相关(P=0.004，r=−0.388 8；附图3，https://doi.org/10.11817/j.issn.1672-7347.2023.220345F2F3)。

与miR-NC组比较，miR-223-3p mimic组*SORBS1*的mRNA表达水平显著下调(均P<0.05，图5B)，蛋白质表达水平亦明显下调(图5C)。这初步证明miR-223-3p对SORBS1具有负调控作用。与anti-miR-NC组比较，anti-miR-223-3p组SORBS1的蛋白质表达水平显著上调(图5D)。这进一步证明miR-223-3p对SORBS1具有特异性的调控作用。

TargetScan预测的miR-223-3p与*SORBS1*基因3'-UTR靶向结合序列见图5E。双荧光素酶报告分析结果显示：与WT-*SORBS1*+miR-NC组相比，WT-*SORBS1*+miR-223-3p mimic组的荧光素酶活性显著下降(P<0.001)；而Mut-*SORBS1*+miR-NC组与Mut-*SORBS1*+miR-223-3p mimic组的荧光素酶活性差异无统计学意义(P>0.05，图5F)。这提示miR-223-3p与*SORBS1* 3'-UTR互补结合进而在转录后水平调控SORBS1的表达。

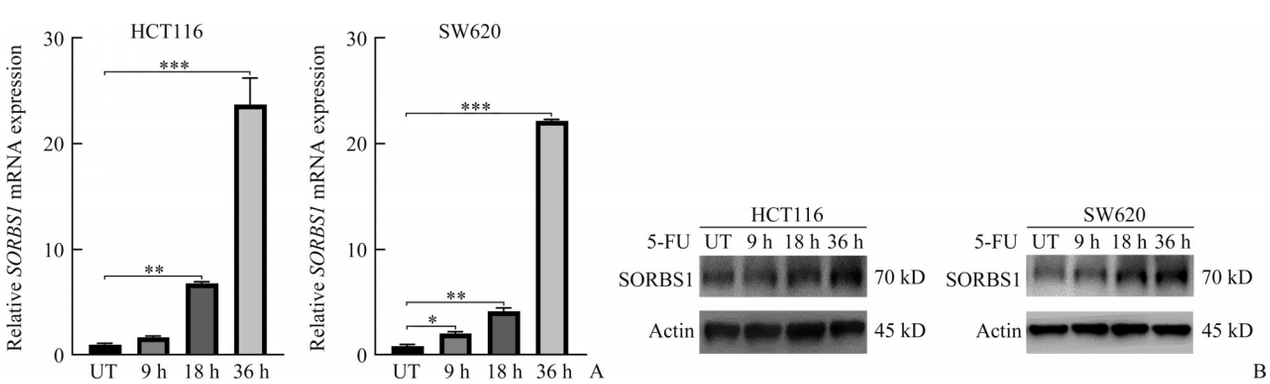

图2 5-FU处理9、18、36 h后，HCT116细胞和SW620细胞中*SORBS1* mRNA和蛋白质表达水平上调

Figure 2 After 5-FU treatment for 9, 18, and 36 hours, the expression levels of *SORBS1* mRNA and protein in HCT116 and SW620 cells were increased

A: mRNA expression was determined by real-time RT-PCR. Data are represented as the means±standard deviation, n=3. *P<0.05, **P<0.01, ***P<0.001. B: Protein expression was determined by Western blotting. UT: Untreated; 5-FU: 5-Fluorouracil; SORBS1: Sorbin and SH3 domain containing 1.

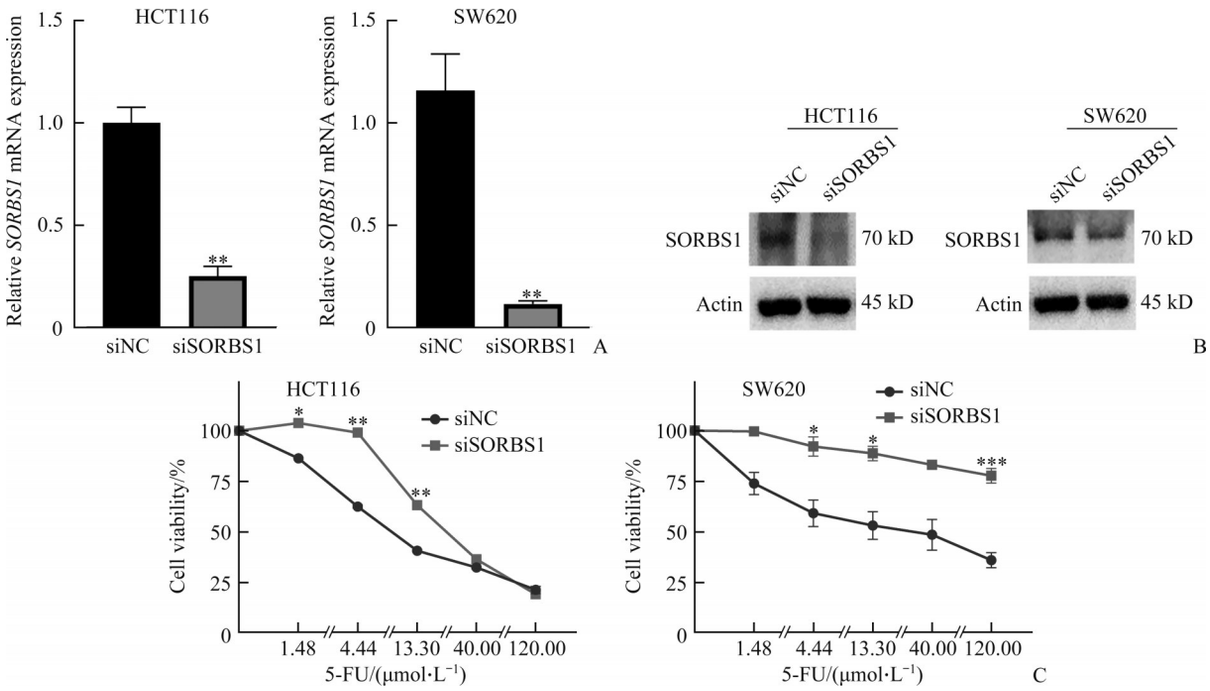

图3　敲低*SORBS1*基因的表达增加CRC细胞对5-FU的耐受性

Figure 3　Knockdown of *SORBS1* gene increases the resistance of CRC cells to 5-FU

A and B: HCT116 cells and SW620 cells were transfected with siNC or siSORBS1. mRNA expression was determined by real-time RT-PCR (A) and protein expression was determined by Western blotting (B). C: HCT116 cells and SW620 cells were transfected with siNC or siSORBS1, and then treated with different concentrations of 5-FU for 72 h. Cell viability was determined by MTT assay. Data are represented as the means±standard deviation, n=3. *P<0.05, **P<0.01, ***P<0.001 vs the siNC group. NC: Negative control; 5-FU: 5-Fluorouracil; SORBS1: Sorbin and SH3 domain containing 1; MTT: Methyl thiazolyl tetrazolium; CRC: Colorectal cancer.

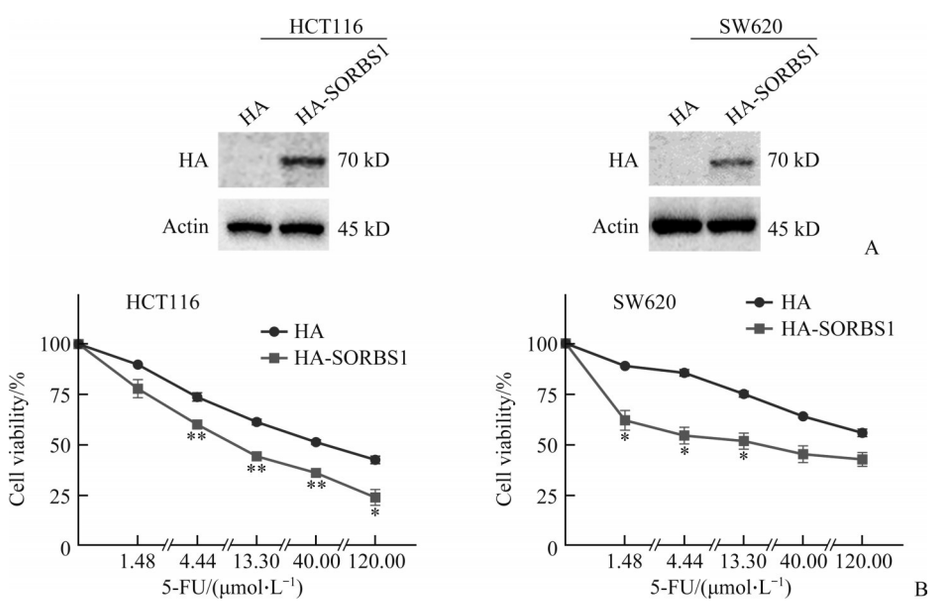

图4　过表达*SORBS1*基因增加CRC细胞对5-FU的敏感性

Figure 4　Overexpression of *SORBS1* gene sensitizes CRC cells to 5-FU

A: HCT116 and SW620 cells were transfected with HA or HA-SORBS1. Protein expression was determined by Western blotting. B: HCT116 and SW620 cells were transfected with HA or HA-SORBS1, and then treated with different concentrations of 5-FU for 72 h. Cell viability was determined by MTT assay. Data are represented as the means±standard deviation, n=3. *P<0.05, **P<0.01 vs the HA group. 5-FU: 5-Fluorouracil; SORBS1: Sorbin and SH3 domain containing 1; MTT: Methyl thiazolyl tetrazolium; CRC: Colorectal cancer.

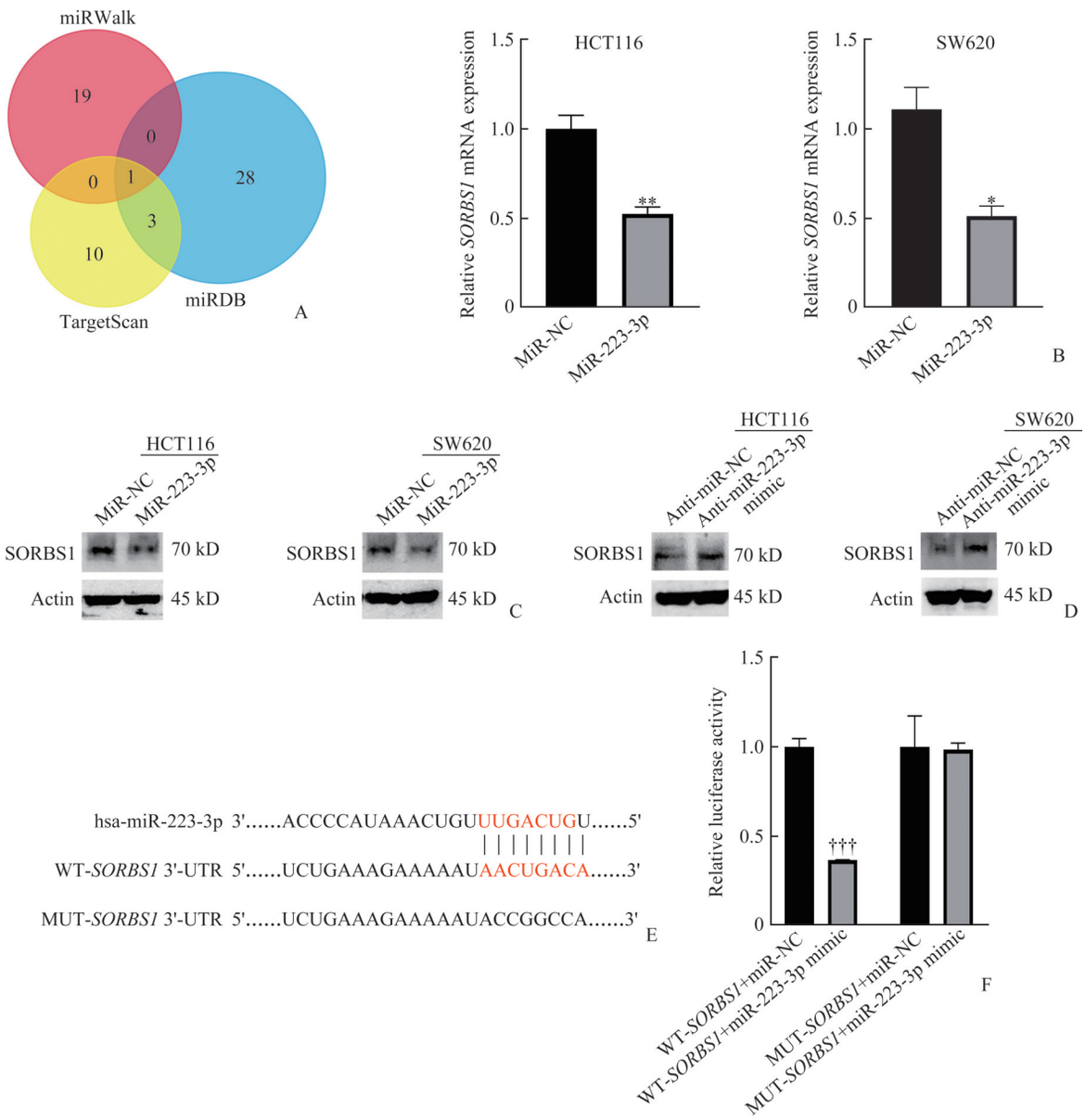

图5 MiR-223-3p靶向调控*SORBS1*基因

Figure 5 MiR-223-3p targets *SORBS1* gene

A: Online databases predict miRNAs targeting *SORBS1*. B and C: HCT116 and SW620 cells were transfected with miR-223-3p mimic or miR-NC. The expression levels of *SORBS1* mRNA (B) and protein (C) were determined by real-time RT-PCR and Western blotting, respectively. D: HCT116 and SW620 cells were transfected with anti-miR-223-3p or anti-miR-NC. The SORBS1 protein expression was determined by Western blotting. E: TargetScan predicts the targeted binding sequence of miR-223-3p and *SORBS1*. F: HEK293T cells were co-transfected with WT-*SORBS1* and miR-223-3p mimic/miR-NC or Mut-*SORBS1* and miR-223-3p mimic/miR-NC. The targeting relationship between miR-223-3p and *SORBS1* was verified by double luciferase reporter assay. Data are represented as the means±standard deviation, n=3. *P<0.05, **P<0.01 vs the miR-NC group; †††P<0.001 vs the WT-*SORBS1*+miR-NC group. SORBS1: Sorbin and SH3 domain containing 1; WT: Wide type; MUT: Mutant; NC: Negative control; UTR: Untranslated region.

2.6 MiR-223-3p水平与CRC 5-FU耐药相关

用5-FU(25 μmol/L)处理HCT116细胞、SW620细胞不同时间后，2种细胞中*miR-223-3p*的表达水平均显著下调(均P<0.05，图6A)。MTT结果显示：与miR-NC组比较，miR-223-3p mimic组细胞活力明显增加(P<0.05，图6B)。与anti-miR-NC组比较，anti-miR-223-3p组细胞活力明显下降(P<0.05，图6C)。提示miR-223-3p与CRC细胞对5-FU耐药相关。

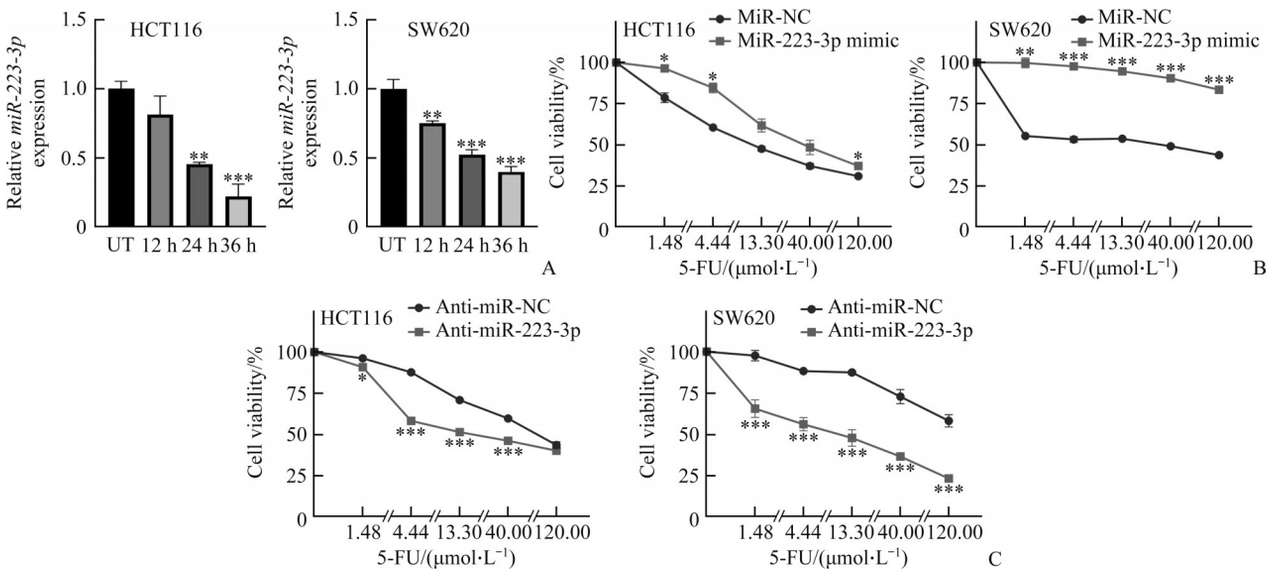

图6　MiR-223-3p与CRC细胞对5-FU耐药有关

Figure 6　MiR-223-3p is associated with 5-FU resistance in CRC cells

A: Real-time RT-PCR results showed that the *miR-223-3p* expression levels in HCT116 and SW620 cells were decreased after 25 μmol/L 5-FU treatment for 12, 24, and 36 h. B: HCT116 and SW620 cells were transfected with miR-223-3p mimic or miR-NC, and then treated with different concentrations of 5-FU for 72 h. Cell viability was determined by MTT assay. C: HCT116 and SW620 cells were transfected with anti-miR-223-3p or anti-miR-NC, and then treated with different concentrations of 5-FU for 72 h. Cell viability was determined by MTT assay. Data are represented as the means±standard deviation, n=3. *P<0.05, **P<0.01, ***P<0.001 vs the UT group or the miR-NC/anti-miR-NC group. 5-FU: 5-Fluorouracil; SORBS1: Sorbin and SH3 domain containing 1; MTT: Methyl thiazolyl tetrazolium; NC: Negative control; CRC: Colorectal cancer; UT: Untreated.

2.7 MiR-223-3p靶向SORBS1增加CRC细胞对5-FU的耐受

与miR-223-3p mimic+HA组比较，miR-223-3p mimic+HA-SORBS1组细胞活力明显降低(P<0.01，图7A)。这说明过表达*SORBS1*基因可以恢复因miR-223-3p表达上调导致的CRC细胞对5-FU敏感性下降。与anti-miR-223-3p+siNC组比较，anti-miR-223-3p+siSORBS1组细胞活力明显增加(P<0.05，图7B)。这说明敲低*SORBS1*基因的表达可以恢复因miR-223-3p表达下调导致的CRC细胞对5-FU敏感性增加。

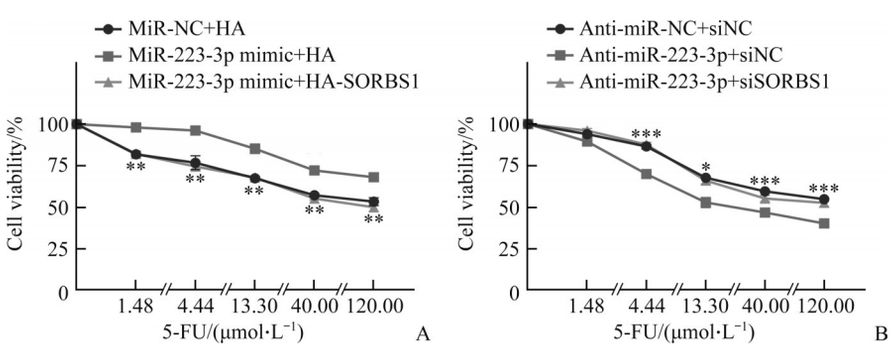

图7　MiR-223-3p靶向*SORBS1*增加CRC细胞对5-FU的耐受

Figure 7　MiR-223-3p via targeting *SORBS1* increases CRC cells resistance to 5-FU

A: HCT116 cells were co-transfected with miR-NC and HA, miR-223-3p mimic and HA, or miR-223-3p mimic and HA-SORBS1, and then treated with different concentrations of 5-FU for 72 h. Cell viability was determined by MTT assay. B: HCT116 cells were co-transfected with anti-miR-NC and siNC, anti-miR-223-3p and siNC, or anti-miR-223-3p and siSORBS1, and then treated with different concentrations of 5-FU for 72 h. Cell viability was determined by MTT assay. Data are represented as the means±standard deviation, n=3. *P<0.05, **P<0.01, ***P<0.001 vs the miR-223-3p mimic+HA group or the anti-miR-223-3p+siNC group. 5-FU: 5-Fluorouracil; SORBS1: Sorbin and SH3 domain containing 1; MTT: Methyl thiazolyl tetrazolium; NC: Negative control; CRC: Colorectal cancer.

3 讨 论

CRC对5-FU化疗耐药是影响患者治疗效果，导致其生存率低的重要原因之一。随着miRNAs在癌症中研究的深入，miRNAs成为减轻癌症化疗耐药的新的生物标志物和临床治疗靶点。MiRNAs在CRC中的异常表达可影响CRC耐药相关靶基因的表达，进而影响CRC细胞对化疗药物的敏感性。

本研究通过生物信息学手段筛选与CRC化疗敏感性相关的抑癌基因。已有研究报道*CLU*基因[27-28]和*CXCL12*基因[29-32]与结直肠癌化疗耐药相关，提示本研究中筛选到的抑癌基因具有较高的可信度。本研究进一步证实*SORBS1*基因与CRC对5-FU的敏感性密切相关：5-FU处理后，CRC细胞内*SORBS1*的mRNA和蛋白质表达水平均上调；敲低*SORBS1*基因的表达后，CRC细胞对5-FU的耐受性增加；过表达*SORBS1*基因后，CRC细胞对5-FU的敏感性增加。提示SORBS1可作为预测CRC细胞对5-FU化疗敏感性的生物标志物。

近年来，miRNAs分子在肿瘤化疗耐药中发挥的作用逐渐被揭示，本研究筛选靶向*SORBS1*基因的miRNA分子，通过降低靶向*SORBS1* miRNA的表达来实现上调*SORBS1*，从而增加患者对5-FU化疗的敏感性，以期为解决CRC化疗耐药提供新思路。

通过在线生物信息学数据库(Targetscan、miRwalk、miRDB)预测到miR-223-3p与*SORBS1*具有靶向关系。MiR-223-3p在多种癌症中高表达，发挥促癌因子的功能[24-26,33]，与SORBS1在癌症中低表达及肿瘤抑制功能相反。在本研究中：在CRC细胞中转染miR-223-3p mimic和anti-miR-223-3p后，*SORBS1*的表达水平相应地下调和上调，这说明miR-223-3p与*SORBS1*可能存在靶向关系。进一步利用双荧光素酶报告分析证实miR-223-3p与SORBS1的3'-UTR中预测到的位点结合，从而发挥转录后调节作用。转染miR-223-3p mimic后，CRC细胞对5-FU的耐受性增加；而转染anti-miR-223-3p后，CRC细胞对5-FU的敏感性增加；转染anti-miR-223-3p的同时敲低*SORBS1*基因的表达，CRC细胞重新获得对5-FU的耐药性；转染miR-223-3p mimic的同时过表达*SORBS1*基因，CRC细胞重新获得对5-FU的敏感性。这些结果进一步说明miR-223-3p通过靶向*SORBS1*基因参与调控CRC细胞对5-FU的耐受性。

SORBS1的功能显著富集在细胞-细胞黏附连接、粘着斑、细胞基质黏附、黏附连接组织方面，这些功能与细胞EMT过程密切相关[34-35]，EMT发生的标志是上皮标志物E-钙黏蛋白在上皮细胞中的表达下调，而间质标志物N-钙黏蛋白在上皮细胞中异位表达[36]。研究[37-38]表明EMT与CRC对5-FU耐药密切相关，这提示SORBS1可能通过参与EMT过程介导CRC对5-FU的敏感性。E-钙黏蛋白和N-钙黏蛋白常用作检测EMT的标志物。本研究通过在CRC细胞系HCT116和SW620中过表达SORBS1，以模拟细胞在经过5-FU处理后SORBS1表达上调，蛋白质印迹法结果显示E-钙黏蛋白在过表达SORBS1后表达上调，同时N-钙黏蛋白表达下调，这说明SORBS1表达量的增加足以影响CRC细胞的EMT进程，并可能因此改变其对5-FU的耐药性。笔者将在后续研究中进一步探索SORBS1介导5-FU耐药的分子机制。

综上，miR-223-3p通过靶向*SORBS1*基因增加CRC细胞对5-FU的耐药性，miR-223-3p有望作为新的临床治疗靶点来改善CRC患者对5-FU的耐药。

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