

·ARTICLES·

·论著·



DOI: 10.11817/j.issn.1672-7347.2022.210477

神经酰胺合成酶3通过SMAD6基因影响肝细胞癌的侵袭和转移

蔡进中, 刘育齐, 李启洋, 文振超, 李勇, 陈现现

(深圳市人民医院介入科, 深圳 518020)

[摘要] 目的: 由于缺乏早期诊断和有效治疗, 肝细胞癌(hepatocellular carcinoma, HCC)患者预后较差。因此迫切需要更好地了解HCC相关的分子机制, 并确定早期诊断和治疗的有效靶点。本研究旨在探讨神经酰胺合成酶3(ceramide synthase 3, CerS3)在HCC中的表达及其生物学作用。方法: 收集深圳市人民医院159例HCC接受根治性切除术患者的临床标本, 包括HCC组织和邻近非肿瘤组织。采用免疫组织化学染色、蛋白印迹法和real-time PCR检测CerS3在HCC组织和邻近非肿瘤组织中的表达。体外实验中, 将Hep3B细胞分为载体对照组和CerS3载体组, 并分别用含有cDNA对照或CerS3 cDNA的反转录病毒载体转染细胞; 将HCC LM3细胞分为shRNA对照组或CerS3 shRNA组, 并分别用含有shRNA对照或CerS3 shRNA慢病毒载体转染细胞。分别采用MTT、EdU、Transwell和划痕试验测定细胞的增殖、迁移和侵袭, 并进行RNA测序以确定CerS3的下游信号。结果: 与相应的相邻非肿瘤组织相比, HCC组织中CerS3 mRNA和蛋白质水平均升高(均 $P<0.05$)。Cox回归生存模型的单变量和多变量分析显示: 静脉浸润(95% CI: 1.8~9.2, $P<0.01$)、TNM分期(95% CI: 2.3~5.2, $P<0.05$)、组织学分级差(95% CI: 1.4~6.8, $P<0.05$)和CerS3(95% CI: 1.5~3.9, $P<0.05$)与HCC患者总生存率显著相关。此外, 与肿瘤组织中CerS3低表达患者相比, CerS3高表达患者的总生存率显著缩短($P<0.05$)。与载体对照组相比, CerS3载体组Hep3B细胞活力、EdU阳性细胞数、迁移和侵袭细胞数均显著增加(均 $P<0.05$)。与shRNA对照组相比, CerS3 shRNA组HCC LM3细胞活力、EdU阳性细胞数、迁移和侵袭细胞数均显著降低(均 $P<0.05$)。RNA测序将母体抗生物皮肤生长因子同源物6(small mothers against decapentaplegic family member 6, SMAD6)基因鉴定为促进HCC转移的致癌基因。结论: CerS3的过度表达与不良临床特征和不良预后密切相关。CerS3在功能上可通过激活SMAD6基因参与肝癌细胞的增殖、侵袭和转移。

[关键词] 神经酰胺合成酶3; 母体抗生物皮肤生长因子同源物6; 肝细胞癌; 侵袭; 转移

Ceramide synthase 3 affects invasion and metastasis of hepatocellular carcinoma via the SMAD6 gene

CAI Jinzhong, LIU Yuqi, LI Qiyang, WEN Zhenchao, LI Yong, CHEN Xianxian

(Department of Intervention, Shenzhen People's Hospital, Shenzhen 518020, China)

ABSTRACT

Objective: Patients with hepatocellular carcinoma (HCC) have poor prognosis due to lack of early diagnosis and effective treatment. Therefore, there is an urgent need to better

收稿日期(Date of reception): 2021-08-11

第一作者(First author): 蔡进中, Email: caijin6zhong@163.com, ORCID:

通信作者(Corresponding author): 蔡进中, Email: caijin6zhong@163.com, ORCID:

基金项目(Foundation item): 深圳市医学科研基金(B2019045)。This work was supported by the Shenzhen Medical Science Research Fund, China (B2019045).

understand the molecular mechanisms associated with HCC and to identify effective targets for early diagnosis and treatment. This study is to explore the expression and biological role of ceramide synthase 3 (CerS3) in HCC.

Methods: A total of 159 pairs of HCC tissues and adjacent non-tumor tissues were obtained from the patients underwent radical resection in Shenzhen People's Hospital, and the total RNA and proteins from HCC tissues and adjacent non-tumor tissues were obtained. The expression of CerS3 protein and mRNA in HCC was detected by immunohistochemistry, Western blotting and real-time PCR. In vitro experiments, Hep3B cells were divided into a control vector group and a CerS3 vector group, and the cells were transfected with retroviral vector containing control cDNA or CerS3 cDNA, respectively. HCCLM3 cells were divided into a normal control shRNA group and a CerS3 shRNA group, and the cells were transfected with lentiviral vectors containing normal control shRNA or *CerS3* shRNA, respectively. MTT, EdU, Transwell and scratch method were used to detect cell proliferation, migration and invasion. RNA sequencing was performed to determine the downstream signal of CerS3.

Results: Compared with the corresponding adjacent tissues, the mRNA and protein levels of *CerS3* were elevated in the HCC tissues, with significant difference (both $P < 0.05$). The Univariate and multivariate analysis showed that the overall survival rate was significantly correlated with the presence of venous invasion (95% CI 1.8–9.2, $P < 0.01$), TNM stage (95% CI 2.3–5.2, $P < 0.05$), poor histological grade (95% CI 1.4–6.8, $P < 0.05$), and CerS3 (95% CI 1.5–3.9, $P < 0.05$). Furthermore, the high CerS3 expression levels in tumor tissues were significantly associated with shorter overall survival rates compared with the low CerS3 expression ($P < 0.05$). Compared with the vector control group, the Hep3B cell viability, EdU positive cells, and migration and invasion cell numbers in the CerS3 vector group were significantly increased (all $P < 0.05$). Compared with the shRNA normal control group, the HCCLM3 cell viability, EdU positive cells, and numbers of migrating and invasive cells in the CerS3 shRNA group were significantly lower (all $P < 0.05$). The RNA sequencing confirmed that the small mothers against decapentaplegic family member 6 (*SMAD6*) gene as an oncogenic gene could promote the HCC metastasis.

Conclusion: Clinically, the overexpression of CerS3 is closely related to poor clinical features and poor prognosis. Functionally, CerS3 participates in the proliferation, invasion and metastasis of liver cancer cells via activating *SMAD6* gene.

KEY WORDS

ceramide synthase 3; small mothers against decapentaplegic family member 6; hepatocellular carcinoma; invasion; metastasis

肝细胞癌(hepatocellular carcinoma, HCC)是一种常见的消化系统恶性肿瘤,是全球癌症相关死亡的第3大原因^[1]。目前,手术切除、经导管动脉化学药物治疗栓塞、放射治疗和化学药物治疗(以下简称放疗)、射频消融和一些其他靶向方法已用于治疗HCC^[2]。然而,由于缺乏早期诊断和有效治疗,HCC患者预后较差,中位生存期为13~26个月^[3]。因此,迫切需要更好地了解HCC相关的分子机制并确定早期诊断和治疗的有效靶点。代谢异常是肿瘤细胞的主要特征之一^[4]。肿瘤组织通过改变代谢物水平满足

快速生长的生物能量、生物合成和氧化还原需求^[5]。神经酰胺合成酶是真核生物脂质代谢中产生神经酰胺的关键酶^[6],近年来在肿瘤领域受到越来越多的关注。神经酰胺合成酶(ceramide synthase, *CerS*)基因家族包括6个基因(*CerS1-6*),它们编码在不同的染色体上,合成不同链长的脂肪酰辅酶A^[7]。其中,*CerS3*基因主要合成神经酰胺,促进细胞迁移和转移,是细胞凋亡的重要介质^[8]。最近的许多研究^[9-10]表明:*CerS3*基因参与了多种肿瘤的发生和发展,如*CerS3*基因在肺癌和胃癌细胞中高度表达。然而,很

少有研究探讨 *CerS3* 基因在 HCC 发生中的机制作用。母体抗生物皮肤生长因子同源物 6 (small mothers against decapentaplegic family member 6, SMAD6) 是骨形态发生蛋白/SMAD 信号转导的重要反馈抑制调节剂^[11]。发育综合征中骨形态发生蛋白信号的不平衡可以加速疾病的进展, 包括癌症^[12]。在 HCC 中 *SMAD6* 基因的表达上调可促进癌细胞增殖^[11]。然而, *SMAD6* 基因在 HCC 进展中的分子机制尚未完全阐明。本研究分析了 *CerS3* 在 HCC 患者临床标本中的表达情况, 及 *CerS3* 的下调对肝癌细胞转移的影响及相关机制, 旨在为 HCC 转移的治疗提供有力的实验依据。

1 材料与方法

1.1 试剂与仪器

MTT 试剂购自上海 Beyotime 公司; 抗兔 *CerS3* 单克隆抗体、抗兔 *SMAD6* 单克隆抗体、HRP 偶联的山羊抗兔 IgG 购自美国 Cell Signaling Technology 公司; 抗兔 GAPDH 单克隆抗体购自武汉 Proteintech 公司; 10% 胎牛血清、DMEM 培养基购自美国 Gibco 公司; Oligofectamine 转染试剂购自美国 Thermo Fisher Scientific 公司; TRIzol 试剂购自美国 Invitrogen 公司; Fast King RT 试剂盒购自北京天根生化科技有限公司; TB Green Fast qPCR Mix 购自日本 Takara 公司; 蛋白质提取试剂、BCA 蛋白检测试剂盒购自北京 Solarbio 公司; EdU 试剂盒购自美国 Roche 公司; Transwell 室购自美国 BD Biosciences 公司。

ABI 7900HT Fast 型 real-time PCR 系统购自美国 Applied Biosystems 公司; ChemiDoc MP 成像系统购自美国 Bio-Rad 公司; SpectraMax i3x 微孔板读数器购自美国 Molecular Devices 公司; 蔡司荧光显微镜购自德国 Carl Zeiss 公司; DP70 CCD 系统购自日本 Olympus 公司。

1.2 组织标本

收集深圳市人民医院(以下简称本院)收治的 159 例接受 HCC 根治性切除术患者的临床标本, 包括肝癌组织和邻近非肿瘤组织。临床样本储存于 $-80\text{ }^{\circ}\text{C}$ 中, 用于 RNA 和蛋白质的提取。此外, 为了验证 *CerS3* 在肝癌组织中的表达, 从癌症基因组图谱(The Cancer Genome Atlas, TCGA; <https://portal.gdc.gov/>) 下载用于 RNA 测序的基因表达数据。数据集的选择标准如下: 1) 原发性肝细胞癌; 2) 包含癌组织和癌旁匹配组织; 3) HCC 的病因有很多, 包括丙型肝炎病毒和乙型肝炎病毒等病毒感染、酗酒、非酒精性脂肪性肝炎等。研究经本院医学伦理委员会批准(审批号: 2020-05), 所有患者签署知情同意书。

1.3 方法

1.3.1 免疫组织化学分析

HCC 组织和匹配的邻近肝组织标本用多聚甲醛固定并进行石蜡包埋, 切成 $4\text{ }\mu\text{m}$ 厚的切片。将肿瘤切片用二甲苯脱蜡并在不同浓度的乙醇中再水化。用 PBS 洗涤后, 用柠檬酸-盐酸在 $95\sim 100\text{ }^{\circ}\text{C}$ 的温度下进行抗原回收 20 min。将载玻片与含有阻断液的正常马血清孵育。随后, 将切片与抗兔 *CerS3* 单克隆抗体 (1:500) 孵育过夜。次日, 将切片与辣根过氧化物酶偶联的二抗一起孵育 2 h。最后, 用苏木精和二甲苯对载玻片进行复染。

1.3.2 细胞培养和分组

正常肝细胞系 LO2 和 4 种 HCC 细胞系 (Huh7、Hep3B、MHCC97H 和 HCCLM3) 购自中国科学院典型培养物保藏委员会细胞库。将细胞接种在含有 10% FBS 的 DMEM 培养基中, 并在 $37\text{ }^{\circ}\text{C}$ 下含有 5% CO_2 的潮湿培养箱中进行培养。将 Hep3B 细胞分为对照载体组和 *CerS3* 载体组, 并分别用含有对照 cDNA 或 *CerS3* cDNA 的反转录病毒载体转染细胞; 将 HCCLM3 细胞分为 shRNA 对照组或 *CerS3* shRNA 组, 并分别用 shRNA 对照或 *CerS3* shRNA 慢病毒载体转染细胞。此外, 为了考察 *SMAD6* 基因敲低对 *CerS3* 在 HCC 细胞中致癌作用的影响, 将 Hep3B 细胞分为载体 (vector) 对照 + siRNA 对照组、*CerS3* vector + siRNA 对照组和 *CerS3* vector + *SMAD6* siRNA 组, 并分别用含有 cDNA 对照、*CerS3* cDNA 的反转录病毒载体和 *SMAD6* siRNA + 相应的乱序 siRNA 转染细胞。

1.3.3 病毒转导和 siRNA 转染

将 *CerS3* cDNA 克隆到以包装过表达 *CerS3* 的反转录病毒载体。从美国 Sigma Aldrich 公司获得包含 *CerS3*-shRNA 和阴性对照 shRNA (shRNA 对照) 的慢病毒载体。通过将细胞与含有聚凝胺 ($8\text{ }\mu\text{g}/\text{mL}$) 的病毒上清液一起孵育来进行病毒转导。*SMAD6* siRNA 和相应的乱序 siRNA 购自上海 GenePharma 公司。使用 Oligofectamine 转染试剂进行 siRNA 转染。

1.3.4 RNA 提取和 real-time PCR 分析

使用 TRIzol 试剂从组织样品中提取总 RNA, 并采用 FastKing RT 试剂盒制备 cDNA。在 ABI 7900HT Fast 型 real-time PCR 系统中使用 TB Green Fast qPCR Mix 进行 real-time PCR。将反应物在 96 孔板中于 $95\text{ }^{\circ}\text{C}$ 扩增 5 min, 然后 $95\text{ }^{\circ}\text{C}$ 10 s、 $55.7\text{ }^{\circ}\text{C}$ 30 s, 进行 40 个循环。*CerS3*、*GAPDH* 的引物由上海 GenePharma 公司合成。使用 $2^{-\Delta\Delta\text{Ct}}$ 方法计算相对倍数变化 [$\Delta\Delta\text{Ct} = \text{实验组}(\text{Ct}_{\text{目的基因}} - \text{Ct}_{\text{对照基因}}) - \text{对照组}(\text{Ct}_{\text{目的基因}} - \text{Ct}_{\text{对照基因}})$]。*CerS3* 和 *GAPDH* 的正向引物序列分别为 5'-TCGAAGAGTGGTAAGGTTTACA-3' 和 5'-TCAA-GAAGGTGGTGAAGCAGG-3', 反向引物序列分别为 5'-TATATCAAAGGTGTTTAC-3' 和 5'-TCAAAGG-

GTGGAGGAGTGGGT-3'。

1.3.5 蛋白质印迹法

通过蛋白质提取试剂制备来自HCC组织(随机选取第4、7、23、36、47、62号患者)或细胞的蛋白质。采用BCA蛋白质检测试剂盒检测蛋白质浓度。通过SDS-PAGE凝胶分离蛋白质后,使用以下针对指定靶蛋白的一抗进行探测:抗CerS3抗体(1:1 000,条带大小56 kD),抗SMAD6抗体(1:1 000,条带大小48 kD)和抗GAPDH抗体(1:5 000,条带大小37 kD)。洗涤后,转移到PVDF膜,再将PVDF与用HRP偶联的山羊抗兔IgG(1:5 000)在室温下孵育1 h。洗涤后,PVDF膜与ECL底物一起孵育,在印迹上产生化学发光,由ChemiDoc MP成像系统捕获图像并通过Image J软件进行标准化。

1.3.6 细胞增殖分析

采用MTT测定转染后的Hep3B细胞或HCCLM3细胞的活力。将细胞接种在96孔培养板中(1×10^3 个/孔),分别在0、24、48和72 h时,将2 mg/mL MTT溶液添加到细胞中。在培养箱中培养4 h后,加入二甲亚砜以溶解产物。在SpectraMax i3x微孔板读数器上测量450 nm处的吸光度值。使用5-乙炔基-2'-脱氧尿苷(5-ethynyl-2'-deoxyuridine, EdU)试剂盒进行EdU掺入测定以评估细胞的增殖能力。将细胞接种在96孔培养板中(1×10^3 个/孔),培养72 h后,加入100 μ L含50 μ mol/L EdU的培养基中孵育2 h。弃培养液,加入100 μ L 4%多聚甲醛固定细胞15 min,加入100 μ L 0.5% TritonX-100透化细胞30 min。在暗处加入100 μ L $1 \times$ Hoechst33342反应液孵育20 min。冲洗后,使用蔡司荧光显微镜随机选取5个不同视野进行量化。

1.3.7 Transwell 检测

将转染后的Hep3B细胞或HCCLM3细胞重悬于不含FBS的DMEM中,并调整细胞密度至 1×10^6 个/mL。然后,将100 μ L细胞悬浮液加入未包被或用Matrigel基质胶包被的Transwell室的上室。下室充满500 μ L含15% FBS的DMEM,细胞在37 $^{\circ}$ C和5% CO_2 条件下培养24 h。用棉签去除Transwell膜上室细胞,下室细胞用甲醇固定5 min,0.1%结晶紫染色15 min。使用DP70 CCD系统在光学显微镜下选取5个不同视野定量计算通过膜的细胞数,并计算平均值。Transwell小室的孔径为8 μ m。

1.3.8 细胞划痕试验

将Hep3B细胞或HCCLM3细胞接种于6孔板中进行过夜黏附,并通过拖动200 μ L移液管尖端穿过孔中心进行损伤。用磷酸盐缓冲液(pH 7.4)清洗细胞,并用培养基替换。在0和24 h评估划痕愈合或细胞迁移图像并拍照。相对划痕愈合计算为初始划痕面积的百分比。

1.4 统计学处理

数据以均数 \pm 标准差($\bar{x} \pm s$)表示。至少进行3个独立的重复实验用于计算标准差。使用GraphPad Prism 8统计学软件进行数据分析。癌与癌旁的配对样本差异分析用配对 t 检验;不同时间点的细胞活力应用重复测量方差分析。Pearson χ^2 检验用于确定CerS3表达水平与肝癌不同临床病理特征的关系。Student's t 检验用于两组之间比较;单向方差分析用于多组之间的比较;Kaplan-Meier方法用于计算生存曲线。采用Cox回归分析CerS3表达与生存结果之间的相关性,计算风险比(hazard ratio, HR)和95%CI。 $P < 0.05$ 为差异有统计学意义。

2 结果

2.1 CerS3在肝癌组织中高表达与肝癌患者生存率低有关

蛋白质印迹法和real-time PCR检测的结果显示:与相应的癌旁组织相比,HCC组织中CerS3 mRNA和蛋白质水平均升高(分别 $P < 0.01$ 和 $P < 0.001$,图1A、1B)。此外,免疫组织化学分析显示:与癌旁组织相比,HCC组织中CerS3的蛋白质表达上调(图1C)。临床相关性分析显示: CerS3蛋白质高表达与静脉浸润、组织学分级差和晚期肿瘤淋巴结转移(根据TNM分期)显著相关(表1)。Kaplan-Meier分析显示:与CerS3低表达相比,肿瘤组织中CerS3高表达与总生存率显著缩短相关($P < 0.05$,图1D)。与正常组织相比,CerS3在肝癌中的表达较高($P < 0.001$,图1E),并且CerS3高表达HCC患者总生存率显著缩短($P < 0.01$;图1F)。此外,Cox多变量分析显示:静脉浸润、TNM分期、组织学分级和CerS3与患者不良总生存率独立相关(表2)。

2.2 CerS3促进HCC细胞的生长和转移

与永生化正常肝细胞系LO2相比,HCC细胞系中CerS3的表达增加(分别 $P < 0.05$ 和 $P < 0.001$,图2A)。然后,在Hep3B和HCCLM3细胞中分别进行CerS3过表达和敲低以验证CerS3在HCC细胞中的生物学功能(图2B、2C)。结果显示:与vector对照组相比,CerS3 vector组Hep3B细胞活力和EdU阳性细胞均显著增加(均 $P < 0.05$,图2D、2E);与shRNA对照组相比,CerS3 shRNA组HCCLM3细胞活力和EdU阳性细胞均显著降低(均 $P < 0.05$,图2E、2F)。此外,与vector对照组相比,CerS3 vector组Hep3B细胞的迁移和侵袭数目以及划痕愈合显著增加(均 $P < 0.05$);与shRNA对照组相比,CerS3 shRNA组HCCLM3细胞的迁移和侵袭数目以及划痕愈合显著降低($P < 0.05$,图3)。

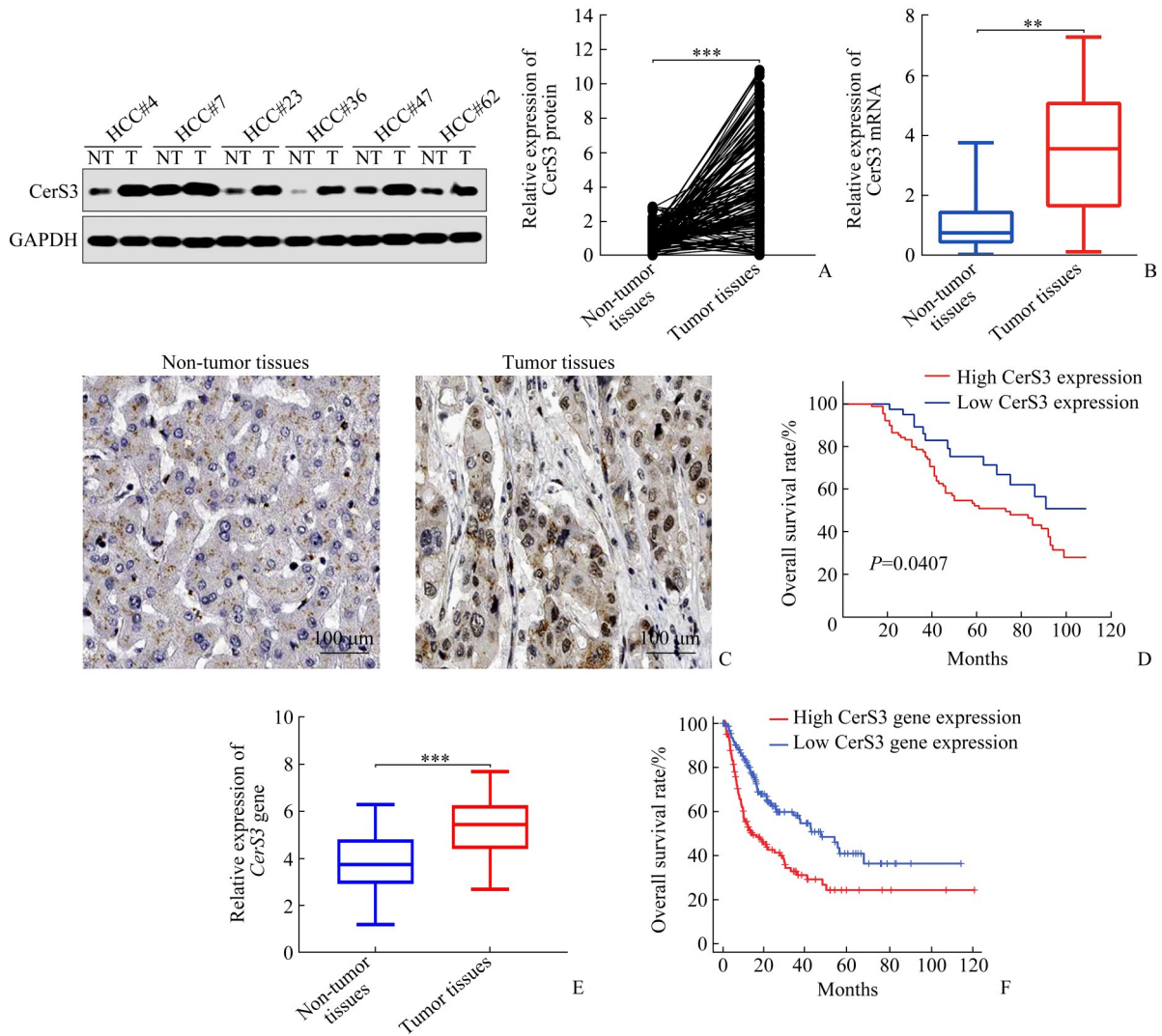


图1 CerS3在肝癌中的相对表达及其临床意义

Figure 1 Relative expression of CerS3 in liver cancer and its clinical significance

A: Expression of CerS3 protein in the hepatocellular carcinoma tissues (HCC#4, 7, 23, 36, 47, and 62 represent the number of hepatocellular carcinoma tissues, respectively) and quantitative analysis by Western blotting. T: Tumor tissues; NT: Non-tumor tissues. *** $P < 0.001$. B: Relative expression of CerS3 mRNA in the hepatocellular carcinoma tissues and non-tumor tissues by real-time PCR. ** $P < 0.01$. C: Immunohistochemical analysis of the expression of CerS3 in the tumor tissues and non-tumor tissues. D: Kaplan-Meier curve analysis of overall survival rate with low and high CerS3 expression in the HCC patients. E: Differential expression analysis of *CerS3* gene in the tumor tissues and non-tumor tissues in the TCGA database. *** $P < 0.001$. F: Correlation of *CerS3* gene expression in TCGA database with overall survival rate in HCC patients.

表1 CerS3表达水平与肝癌患者不同临床病理特征的关系

Table 1 Relationship between the expression level of CerS3 and different clinicopathological features in liver cancer patients

临床病理特征	CerS3 表达/[例(%)]		P
	低表达(n=71)	高表达(n=88)	
性别			0.718
男	59(83.1)	75(85.2)	
女	12(16.9)	13(14.8)	
年龄/岁			0.741
≤50	35(49.3)	41(46.6)	
>50	36(50.7)	47(53.4)	

表 1(续)

临床病理特征	CerS3 表达/[例(%)]		P
	低表达(n=71)	高表达(n=88)	
甲胎蛋白水平/($\mu\text{g}\cdot\text{L}^{-1}$)			0.068
≤ 20	29(40.8)	24(27.3)	
> 20	42(59.2)	64(72.7)	
血清白蛋白水平/($\text{g}\cdot\text{L}^{-1}$)			0.255
< 34	62(87.3)	71(80.7)	
≥ 34	9(12.7)	17(19.3)	
总胆红素水平/($\mu\text{mol}\cdot\text{L}^{-1}$)			0.249
≤ 20.5	66(93.0)	77(87.5)	
> 20.5	5(7.0)	11(12.5)	
肝硬化			0.144
否	25(35.2)	41(46.6)	
是	46(64.8)	47(53.4)	
乙型肝炎病毒感染			0.817
否	8(11.3)	9(10.2)	
是	63(88.7)	79(89.8)	
组织学分级			< 0.001
良好	12(16.9)	3(3.4)	
中等	53(74.6)	54(61.4)	
差	6(8.5)	31(35.2)	
静脉浸润			0.004
否	61(85.9)	57(64.8)	
是	10(14.1)	31(35.2)	
TNM分期			< 0.001
I~II期	45(63.4)	19(21.6)	
III~IV期	26(36.6)	69(78.4)	

TNM: 肿瘤分期系统; CerS3: 神经酰胺合成酶3。

表 2 Cox 回归生存模型的单变量和多变量分析临床病理特征与肝癌患者生存率的关系

Table 2 Univariate and multivariate analysis of the relationship between clinicopathological characteristics and survival in patients with liver cancer by Cox regression survival model

特征	单变量分析			多变量分析		
	HR	95% CI	P	HR	95% CI	P
性别	1.3	0.4~4.2	0.715			
年龄	4.7	0.6~34.8	0.133			
甲胎蛋白水平	0.5	0.2~1.1	0.086	1.4	0.8~2.5	0.281
静脉浸润	4.0	1.8~9.2	0.001	3.3	1.5~7.6	0.004
肝硬化	1.8	0.9~3.7	0.116			
乙型肝炎病毒感染	1.4	0.2~10.2	0.761			
TNM分期	3.4	2.3~5.2	0.002	1.6	1.2~2.6	0.017
血清白蛋白水平	1.4	0.6~2.9	0.426			
总胆红素水平	1.6	0.7~3.3	0.242			
组织学分级	3.1	1.4~6.8	0.005	1.9	1.2~3.1	0.011
CerS3	2.4	1.5~3.9	0.023	1.7	1.1~2.7	0.030

TNM: 肿瘤分期系统; CerS3: 神经酰胺合成酶3。

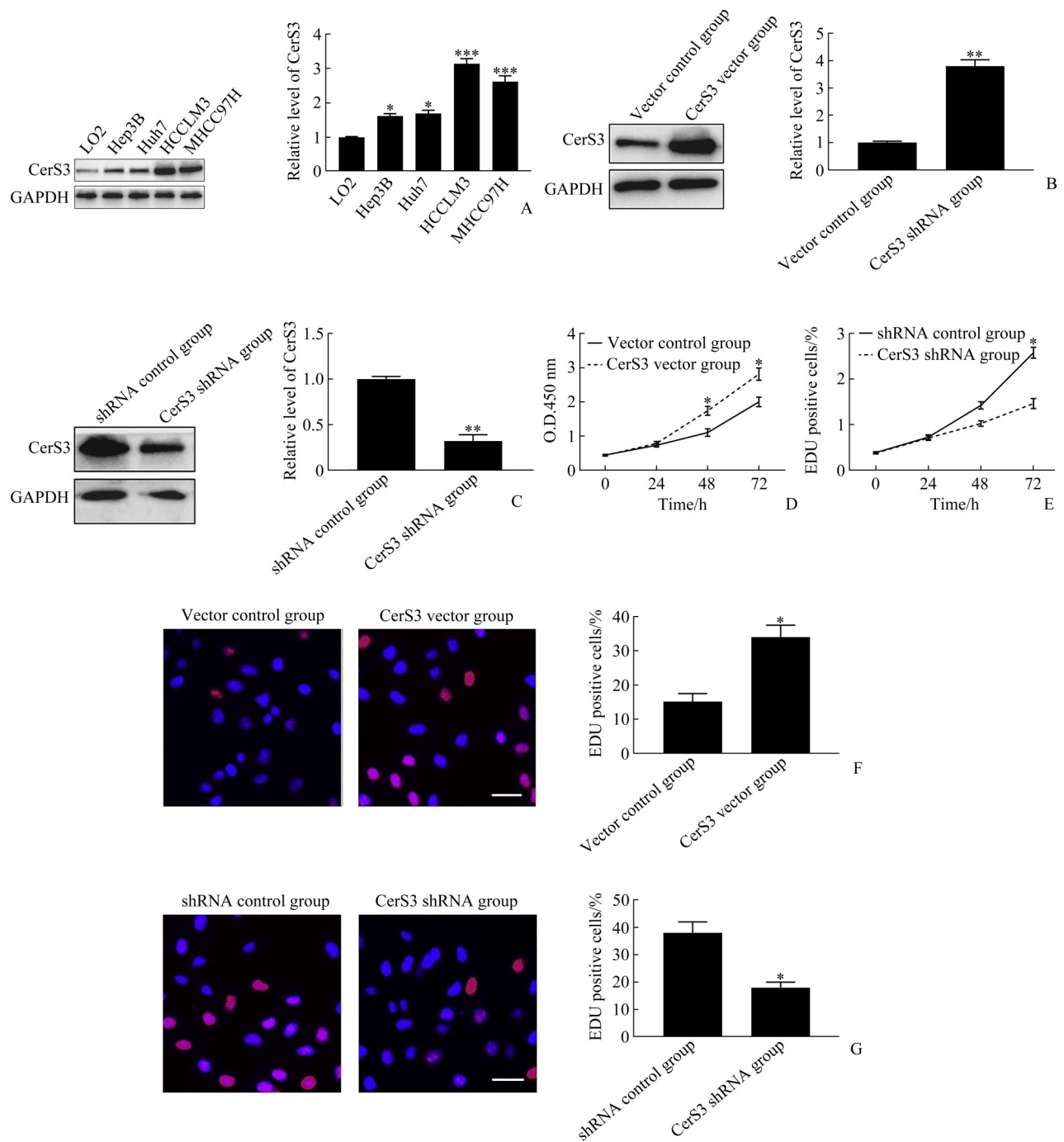


图2 *CerS3* 促进肝癌细胞的增殖

Figure 2 *CerS3* promotes the proliferation of liver cancer cell lines

A: Expression of CerS3 level in the normal liver cell line (LO2) and the 4 kinds of HCC cell lines (Huh7, Hep3B, MHCC97H and HCCLM3) by Western blotting. * $P < 0.05$, *** $P < 0.001$ vs the LO2. B: Detection of CerS3 protein expression in the Hep3B of the vector control group and the CerS3 vector group. *** $P < 0.001$ vs the vector control group. C: Expression of CerS3 in the HCCLM3 of the shRNA control group and the CerS3 shRNA group. ** $P < 0.01$ vs the shRNA control group. D: Changes in cell viability after CerS3 overexpression in Hep3B cells by MTT method in the vector control group and the CerS3 vector group. * $P < 0.05$ vs the vector control group. E: Changes in cell viability after CerS3 knockdown in HCCLM3 cells by MTT method in the shRNA control group and the CerS3 shRNA group. * $P < 0.05$ vs the shRNA control group. F: Changes of cell proliferation after CerS3 overexpression in Hep3B cells by EdU method (scale bar=100 μm) in the vector control group and the CerS3 vector group (scale bar=100 μm). * $P < 0.05$ vs the vector control group. G: Changes of cell proliferation after CerS3 knockdown in HCCLM3 cells by EdU method in the shRNA control group and the CerS3 shRNA group (scale bar=100 μm). * $P < 0.05$ vs the shRNA control group.

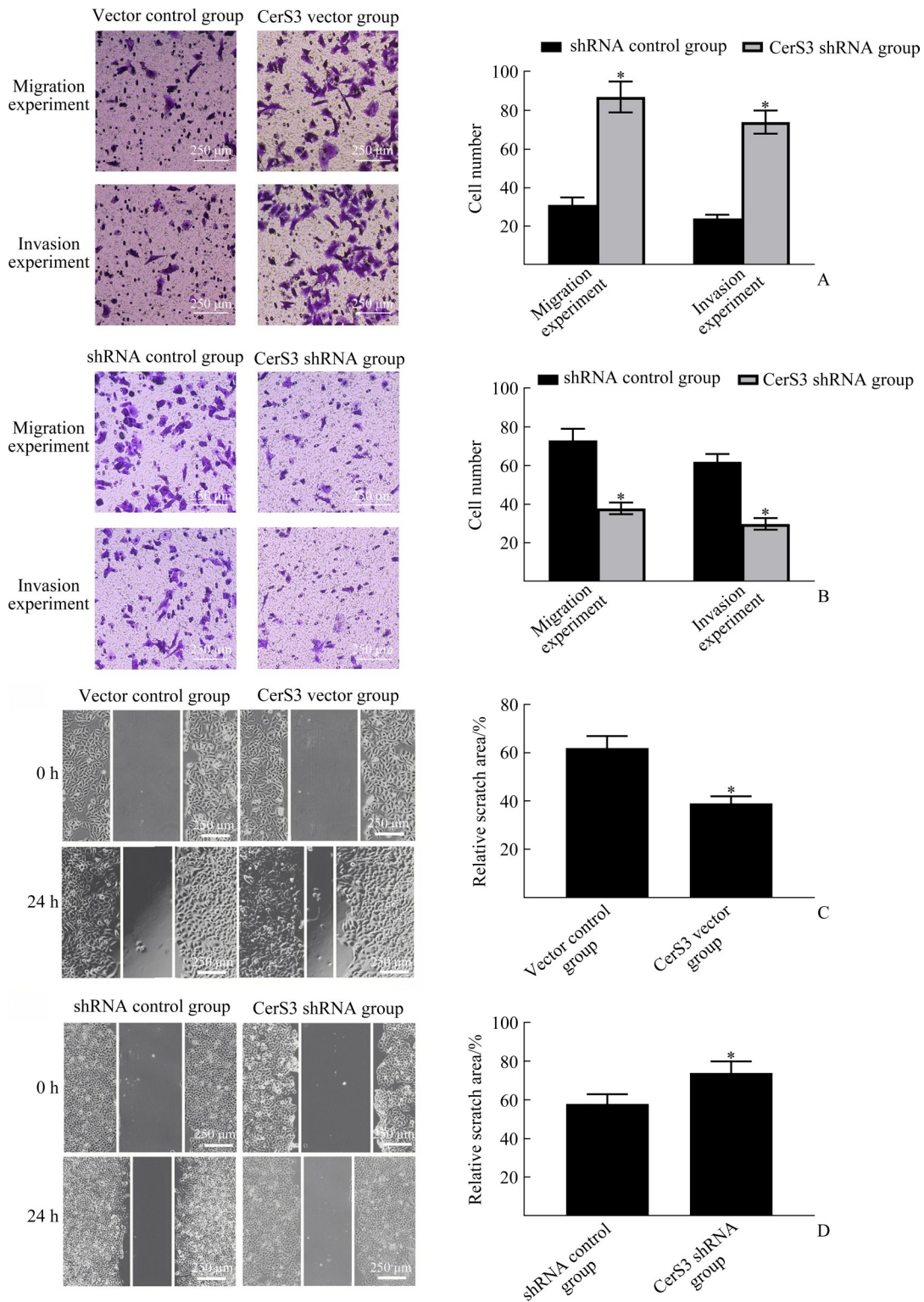


图3 CerS3促进肝癌细胞的细胞迁移和侵袭

Figure 3 CerS3 promotes migration and invasion of liver cancer cell lines

A: Changes of cell migration and invasion after CerS3 overexpression in Hep3B cells in the vector control group and the CerS3 vector group by Transwell method. * $P < 0.05$ vs the vector control group. B: Changes of cell migration and invasion after CerS3 knockdown in HCCLM3 cells in the shRNA control group and the CerS3 shRNA group by Transwell method. * $P < 0.05$ vs the shRNA control group. C: Scratch healing after CerS3 overexpression in Hep3B cells in the vector control group and the CerS3 vector group by scratch assay. * $P < 0.05$ vs the vector control group. D: Scratch healing after CerS3 knockdown in HCCLM3 cells in the shRNA control group and the CerS3 shRNA group by scratch assay. * $P < 0.05$ vs the shRNA control group.

2.3 CerS3介导的HCC细胞转移需要SMAD6

与shRNA对照组相比, CerS3 shRNA组中多种相关基因的表达降低(图4A、4B)。其中, 将SMAD6鉴定为促进HCC转移的致癌基因。与vector对照组相比, CerS3 vector组Hep3B细胞中SMAD6表达显著增加($P<0.001$, 图4C); 与shRNA对照组相比, CerS3 shRNA组HCCLM3细胞中SMAD6表达降低

($P<0.001$, 图4D)。

2.4 敲低SMAD6消除CerS3在HCC细胞株中的致癌功能

与CerS3 vector+siRNA对照组相比, CerS3 vector+SMAD6 siRNA组细胞活力、EdU阳性细胞数、迁移和侵袭细胞数与划痕愈合均显著增加(均 $P<0.05$, 图5)。

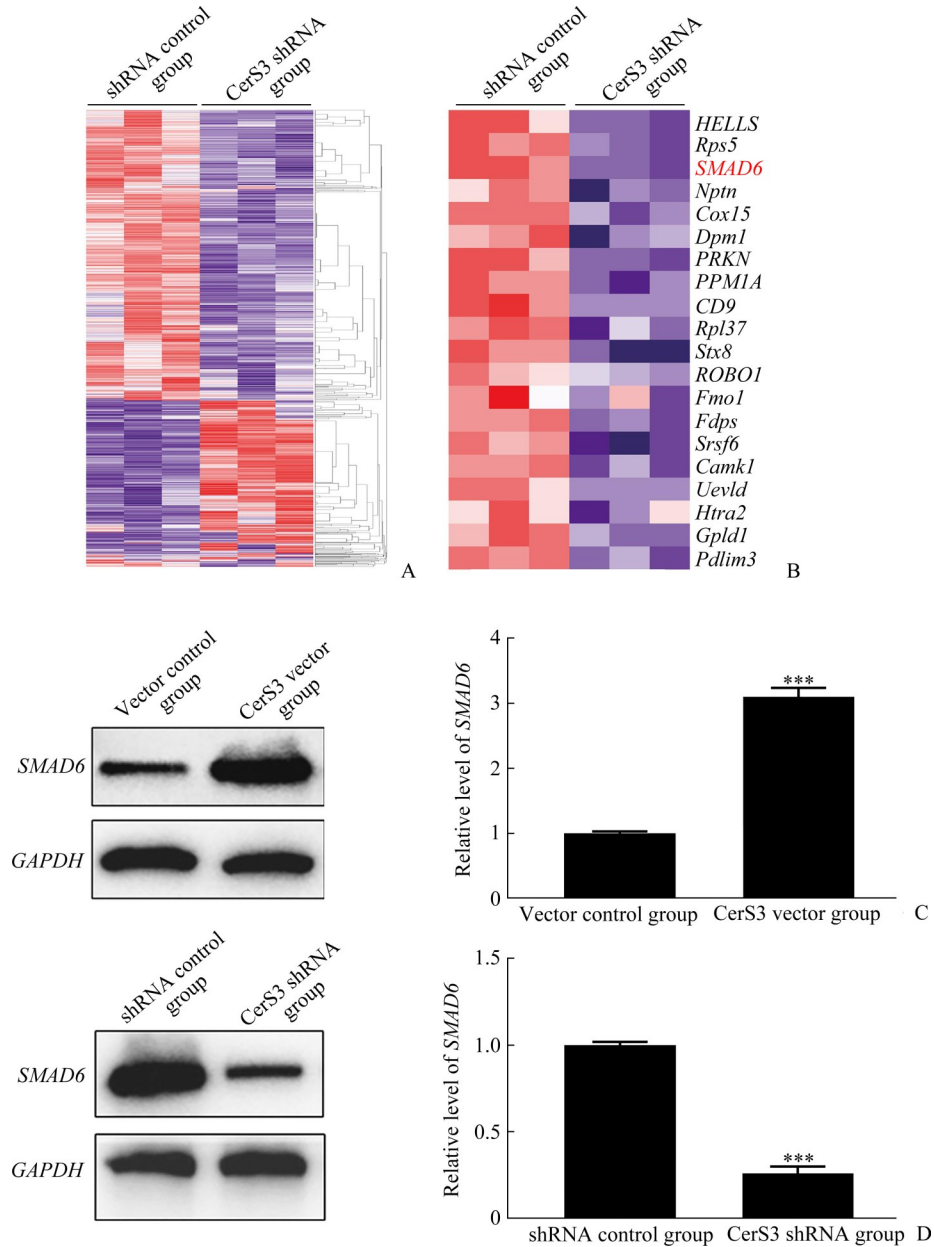


图4 CerS3上调肝癌细胞株中SMAD6表达

Figure 4 CerS3 upregulates SMAD6 expression in liver cancer lines

A: Heat map of mRNA expression abundance in the HCCLM3 cells in the shRNA control group and the CerS3 shRNA group by RNA sequencing. B: Heat map of the top 20 mRNA expression abundance down-regulated genes in the shRNA control group and the CerS3 shRNA group. C: SMAD6 expression in Hep3B cells in the vector control group and the CerS3 vector group. $***P<0.001$ vs the vector control group. D: SMAD6 expression in HCCLM3 cells in the shRNA control group and the CerS3 shRNA group. $***P<0.001$ vs the shRNA control group.

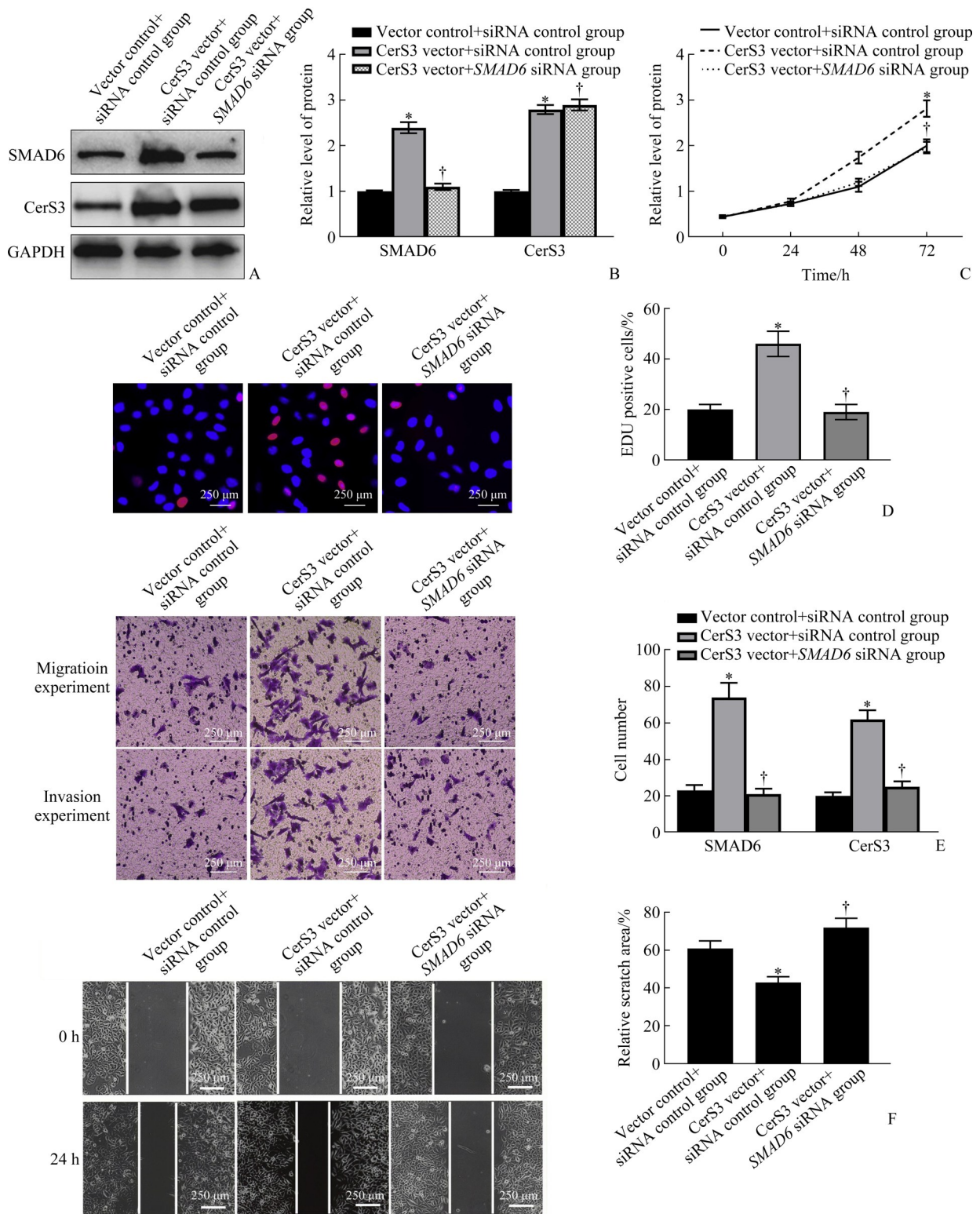


图5 SMAD6敲低消除了CerS3在肝癌细胞株中的致癌作用

Figure 5 SMAD6 knockdown abolished the oncogenic role of CerS3 in liver cancer cell lines

A: Expression of CerS3 and SMAD6 in Hep3B cells in the vector control+siRNA control group, the CerS3 vector + siRNA control group and the CerS3 vector+SMAD6 siRNA group by Western blotting. B: Quantitative analysis of the expression of CerS3 and SMAD6 in Hep3B cells in the vector control+siRNA control group, the CerS3 vector+siRNA control group and the CerS3 vector + SMAD6 siRNA group. C-F: Proliferation, migration and invasion of Hep3B cells by MTT method (C), Edu method (D), Transwell (E), and scratch (F) in the vector control+siRNA control group, the CerS3 vector+siRNA control group and the CerS3 vector + SMAD6 siRNA group. * $P < 0.05$ vs the vector control+siRNA control group; † $P < 0.05$ vs the CerS3 vector+the siRNA control group.

3 讨论

HCC在中国是一种高发病率和病死率的恶性肿瘤。目前,关于HCC发生、发展和转移的分子机制仍不清楚。最近的研究^[13]表明基因表达和信号通路的异常改变是HCC进展的关键。因此,阐明HCC的分子机制对于发现新的生物标志物和治疗策略具有重要意义。CerS的主要功能是合成神经酰胺,神经酰胺是真核细胞膜的重要组成部分^[7]。神经酰胺合成酶在癌症的发病机制中起着重要作用。例如,CerS1和CerS2在乳腺癌中的过度表达可促进肿瘤细胞增殖,并与不良预后相关^[14]。人肝癌组织中CerS4 mRNA和蛋白质水平上调,CerS4促进体外和体内肝癌细胞的增殖^[15]。CerS5已被确定为结直肠癌的肿瘤标志物和预后指标^[16]。研究^[9-10]还发现:CerS3在乳腺癌、非小细胞肺癌、胃癌和头颈癌中高度表达。本研究对CerS3在HCC中的表达和功能进行表征和研究,结果发现CerS3在肝癌组织和细胞系中过度表达。CerS3水平升高与HCC患者的不良临床特征及预后相关;本研究体外实验表明CerS3促进肝癌细胞的增殖、迁移和侵袭。这些结果提示CerS3在HCC中具有致癌作用。

转移是各种肿瘤的重要特征,包括HCC^[17]。研究^[18]进一步使用RNA测序,确定了CerS3对HCC转移的影响可能取决于SMAD6。SMAD6是特异性蛋白家族的成员,参与重要的生物过程,如细胞生长、凋亡和癌变。有证据^[19]表明SMAD6调控的基因表达在各种肿瘤的转移中起关键作用^[19]。高SMAD6蛋白质水平被认为是多种癌症患者的不良预后因素^[20]。研究^[11]发现,SMAD6的上调与HCC的直接肝浸润、静脉浸润和晚期TNM分期显著相关。SMAD6高表达的肝癌患者预后明显较差^[21]。此外,来自体外和体内的研究^[21]显示SMAD6的缺失抑制了肝癌的转移。因此,阐明SMAD6的表达调控机制将有助于了解HCC的转移过程。本研究结果表明:敲除CerS3可降低SMAD6的表达,抑制肝癌细胞的增殖、侵袭和迁移。此外,CerS3对细胞增殖、迁移和侵袭的促进作用可被SMAD6敲低消除。因此,这些数据证实CerS3正调控SMAD6的表达,从而促进HCC的侵袭和转移,表明它是HCC中一个新的SMAD6调节因子。

总之,本研究确定了在肝癌中CerS3的表达上调。临床上,CerS3的过度表达与不良临床特征和不良预后密切相关。在功能上,CerS3通过促进肝癌细胞的增殖、迁移和侵袭,发挥癌基因的作用。本研究还阐明了CerS3增强SMAD6介导的HCC细胞生长和转移,促进了疾病的发展。因此,CerS3是肝癌潜

在的生物标志物和治疗靶点。

作者贡献声明: 蔡进中 实验设计、实施,论文构想;刘育齐、李启洋、文振超 论文撰写,实验研究;李勇、陈现现 参与统计分析。所有作者阅读并同意最终的文本。

利益冲突声明: 作者声称无任何利益冲突。

参考文献

- [1] 周恺乾,孙云帆,杨欣荣,等.肝细胞癌(HCC)肺转移切除术(PME)的预后影响因素分析[J].复旦学报(医学版),2019,46(5): 569-575. <https://doi.org/10.3969/j.issn.1672-8467.2019.05.001>.
ZHOU Kaiqian, SUN Yunfan, YANG Xinrong, et al. Analysis of prognostic factors in hepatocellular carcinoma (HCC) lung metastases (PME) [J]. Fudan University Journal of Medical Sciences, 2019, 46(5): 569-575. <https://doi.org/10.3969/j.issn.1672-8467.2019.05.001>.
- [2] 许智婷,丁红.肝细胞癌(HCC)靶向治疗的研究进展[J].复旦学报(医学版),2019,46(2): 276-280. <https://doi.org/10.3969/j.issn.1672-8467.2019.02.021>.
XU Zhiting, DING Hong. Research progress of targeted therapy for hepatocellular carcinoma (HCC) [J]. Fudan University Journal of Medical Sciences, 2019, 46(2): 276-280. <https://doi.org/10.3969/j.issn.1672-8467.2019.02.021>.
- [3] 王瀚,丛文铭.肝细胞癌组织HepPar-1表达临床意义倾向评分匹配研究[J].中华肿瘤防治杂志,2019,26(1): 31-37. <https://doi.org/10.16073/j.cnki.cjcp.2019.01.006>.
WANG Han, CONG Wenming. Clinical significance of Hep Par-1 in hepatocellular carcinoma based on propensity score matching analysis[J]. Chinese Journal of Cancer Prevention and Treatment, 2019, 26(1): 31-37. <https://doi.org/10.16073/j.cnki.cjcp.2019.01.006>.
- [4] Smith MT, Guyton KZ, Kleinstreuer N, et al. The key characteristics of carcinogens: relationship to the hallmarks of cancer, relevant biomarkers, and assays to measure them[J]. Cancer Epidemiol Biomarkers Prev, 2020, 29(10): 1887-1903. <https://doi.org/10.1158/1055-9965.EPI-19-1346>.
- [5] Sun C, Li T, Song X, et al. Spatially resolved metabolomics to discover tumor-associated metabolic alterations[J]. Proc Natl Acad Sci USA, 2019, 116(1): 52-57. <https://doi.org/10.1073/pnas.1808950116>.
- [6] 阮建佳,杜岩.神经酰胺合酶-神经酰胺通路在青蒿琥酯抑制肝纤维化中的作用[J].中国应用生理学杂志,2020,36(3): 89-92. <https://doi.org/10.12047/j.cjap.5931.2020.061>.
RUAN Jianjia, DU Yan. The role of ceramide synthase-ceramide pathway in the inhibition of liver fibrosis by artesunate [J]. Chinese Journal of Applied Physiology, 2020, 36(3): 89-92. <https://doi.org/10.12047/j.cjap.5931.2020.061>.
- [7] Riley RT, Merrill AH Jr. Ceramide synthase inhibition by

- fumonisin: a perfect storm of perturbed sphingolipid metabolism, signaling, and disease[J]. *J Lipid Res*, 2019, 60(7): 1183-1189. <https://doi.org/10.1194/jlr.S093815>.
- [8] Takeda S, Terazawa S, Shimoda H, et al. β -Sitosterol 3-O-D-glucoside increases ceramide levels in the stratum corneum via the up-regulated expression of ceramide synthase-3 and glucosylceramide synthase in a reconstructed human epidermal keratinization model[J]. *PLoS One*, 2021, 16(3): e0248150. <https://doi.org/10.1371/journal.pone.0248150>.
- [9] Cingolani F, Simbari F, Abad JL, et al. Jaspine B induces nonapoptotic cell death in gastric cancer cells independently of its inhibition of ceramide synthase[J]. *J Lipid Res*, 2017, 58(8): 1500-1513. <https://doi.org/10.1194/jlr.M072611>.
- [10] Brachtendorf S, El-Hindi K, Grösch S. Ceramide synthases in cancer therapy and chemoresistance[J]. *Prog Lipid Res*, 2019, 74: 160-185. <https://doi.org/10.1016/j.plipres.2019.04.002>.
- [11] Chen Z, Lu X, Jia D, et al. Hepatic SMARCA4 predicts HCC recurrence and promotes tumour cell proliferation by regulating SMAD6 expression[J]. *Cell Death Dis*, 2018, 9(2): 59-70. <https://doi.org/10.1038/s41419-017-0090-8>.
- [12] Liu L, Zhang C, Wang J, et al. A high level of lncFGD5-AS1 inhibits epithelial-to-Mesenchymal transition by regulating the miR-196a-5p/SMAD6/BMP axis in gastric cancer[J]. *BMC Cancer*, 2021, 21(1): 453-466. <https://doi.org/10.1186/s12885-021-08192-x>.
- [13] 殷松娜, 张翔, 杜娟, 等. 生物信息学分析鉴定肝细胞癌中的关键基因和信号通路[J]. *基因组学与应用生物学*, 2020, 39(9): 437-444. <https://doi.org/10.13417/j.gab.039.004345>.
YIN Songna, ZHANG Xiang, DU Juan, et al. Identification of key genes and pathways in hepatocellular carcinoma using bioinformatics analysis[J]. *Genomics and Applied Biology*, 2020, 39(9): 437-444. <https://doi.org/10.13417/j.gab.039.004345>.
- [14] Sakharkar MK, Kaur Dhillon S, Chidambaram SB, et al. Gene pair correlation coefficients in sphingolipid metabolic pathway as a potential prognostic biomarker for breast cancer[J]. *Cancers*, 2020, 12(7): 1747. <https://doi.org/10.3390/cancers12071747>.
- [15] Chen J, Li X, Ma D, et al. Ceramide synthase-4 orchestrates the cell proliferation and tumor growth of liver cancer in vitro and in vivo through the nuclear factor- κ B signaling pathway[J]. *Oncol Lett*, 2017, 14(2): 1477-1483. <https://doi.org/10.3892/ol.2017.6365>.
- [16] Markowski AR, Błachnio-Zabielska AU, Guzińska-Ustymowicz K, et al. Ceramides profile identifies patients with more advanced stages of colorectal cancer[J]. *Biomolecules*, 2020, 10(4): 632. <https://doi.org/10.3390/biom10040632>.
- [17] 沈良华, 吴璐华, 张仙丽, 等. PTBP1通过EMT途径促进肝癌细胞的迁移与侵袭[J]. *中国病理生理杂志*, 2019, 35(10): 1819-1825. <https://doi.org/10.3969/j.issn.1000-4718.2019.10.013>.
SHEN Lianghua, WU Luhua, ZHANG Xianli, et al. PTBP1 promotes migration and invasion of liver cancer cells through EMT pathway[J]. *Chinese Journal of Pathophysiology*, 2019, 35(10): 1819-1825. <https://doi.org/10.3969/j.issn.1000-4718.2019.10.013>.
- [18] 马丽萍, 刘小林, 张哲雄, 等. LAMA4通过TGF- β 1/SMAD途径调控肝癌细胞免疫逃逸因子及促进肝癌细胞凋亡[J]. *免疫学杂志*, 2019, 35(4): 300-307. <https://doi.org/10.13431/j.cnki.immunol.j.20190047>.
MA Liping, LIU Xiaolin, ZHANG Zhexiong, et al. LAMA4 regulates immune escape factors and promotes apoptosis of liver cancer cells through TGF- β 1/SMAD pathway [J]. *Journal of Immunology*, 2019, 35(4): 300-307. <https://doi.org/10.13431/j.cnki.immunol.j.20190047>.
- [19] Pan S, Zhou G, Hu W, et al. SMAD-6, -7 and-9 are potential molecular biomarkers for the prognosis in human lung cancer [J]. *Oncol Lett*, 2020, 20(3): 2633-2644. <https://doi.org/10.3892/ol.2020.11851>.
- [20] Wang H, Ji X. SMAD6, positively regulated by the DNMT3OS-miR-134-5p axis, confers promoting effects to cell proliferation, migration and EMT process in retinoblastoma[J]. *Cancer Cell Int*, 2020, 20(1): 23-32. <https://doi.org/10.1186/s12935-020-1103-8>.
- [21] He S, Guo Z, Kang Q, et al. Circular RNA hsa_circ_0000517 modulates hepatocellular carcinoma advancement via the miR-326/SMAD6 axis[J]. *Cancer Cell Int*, 2020, 20(1): 360-372. <https://doi.org/10.1186/s12935-020-01447-w>.

(本文编辑 傅希文)

本文引用: 蔡进中, 刘育齐, 李启洋, 文振超, 李勇, 陈现. 神经酰胺合成酶3通过SMAD6基因影响肝细胞癌的侵袭和转移[J]. *中南大学学报(医学版)*, 2022, 47(5): 588-599. DOI:10.11817/j.issn.1672-7347.2022.210477

Cite this article as: CAI Jinzhong, LIU Yuqi, LI Qiyang, WEN Zhenchao, LI Yong, CHEN Xianxian. Ceramide synthase 3 affects invasion and metastasis of hepatocellular carcinoma via the SMAD6 gene[J]. *Journal of Central South University. Medical Science*, 2022, 47(5): 588-599. DOI:10.11817/j.issn.1672-7347.2022.210477