CXC类趋化因子受体4基因RNA干扰对人肝癌细胞HepG2和SMMC-7721增殖和侵袭的影响

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摘要：目的 利用RNA干扰（RNA interference，siRNA）技术抑制人肝癌细胞HepG2和SMMC-7721中CXC类趋化因子受体4（CXC chemokines receptor 4，CXCR4）基因的表达，探讨其在肝癌细胞体外增殖和侵袭过程中的作用及部分机制。**方法** 设计合成CXCR4特异性siRNA，转染人肝癌细胞HepG2和SMMC-7721，siRNA组与对照组采用脂质体转染法转染CXCR4-siRNA与siRNA-对照，空白组以等剂量生理盐水代替。采用逆转录聚合酶链反应（reverse transcription-polymerase chain reaction，RT-PCR）验证siRNA效果，采用MTT法、流式细胞仪及Transwell小室检测细胞增殖、细胞周期和细胞侵袭情况，采用蛋白质免疫印迹法检测细胞中基质金属蛋白酶-9（matrix metalloproteinase-9，MMP-9）与血管内皮生长因子（vascular endothelial growth factor，VEGF）蛋白的表达。**结果** siRNA组HepG2细胞和SMMC-7721细胞的增殖率分别为0.45 ± 0.12和0.42 ± 0.03，对照组为1.02 ± 0.44和1.07 ± 0.51，空白组分别为1.08 ± 0.44和1.11 ± 0.08，siRNA组细胞增殖率均显著低于对照组和空白组（*P*均＜ 0.05）。siRNA组HepG2细胞和SMMC-7721细胞的S期与G_2/M期比例显著高于对照组和空白组，G_0/G_1期比例显著低于对照组和空白组，差异有统计学意义（*P*均＜ 0.05）。siRNA组HepG2细胞和SMMC-7721细胞的侵袭数分别为（4.55 ± 1.49）个和（2.48 ± 0.48）个，显著低于对照组 [（42.48 ± 3.18）个和（42.00 ± 2.78）个]和空白组 [（38.27 ± 2.47）个和（38.19 ± 3.11）个]，差异有统计学意义（*P*均＜ 0.05）。siRNA组HepG2细胞MMP-9与VEGF蛋白表达量分别为2.09 ± 0.13和0.78 ± 0.12，SMMC-7721细胞MMP-9与VEGF蛋白表达量分别为2.11 ± 0.14和0.81 ± 0.13，均显著低于对照组和空白组（*P*均＜ 0.05）。**结论** RNA干扰CXCR4基因的表达可抑制人肝癌细胞HepG2和SMMC-7721的增殖和侵袭，其可能是通过调控MMP-9及VEGF蛋白的表达来调节肝癌细胞的增殖和侵袭。

关键词：CXC类趋化因子受体4；RNA干扰；肝癌；增殖；侵袭

Effects of CXC chemokines receptor 4 gene RNA interference on the proliferation and invasion of human hepatoma cells HepG2 and SMMC-7721

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Abstract: Objective RNA interference (siRNA) technology was used to inhibit the expression of CXC chemokines receptor 4 (CXCR4) gene in human hepatocellular carcinoma cells HepG2 and SMMC-7721 to explore the role and mechanism of CXCR4 gene in the proliferation and invasion of hepatoma cells *in vitro*. **Methods** CXCR4 specific siRNA were designed and synthesized, and were transfected to the human hepatoma cells HepG2 and SMMC-7721. The siRNA group and the control group were transfected with CXCR4-siRNA and siRNA-control by liposome transfection. The blank group was set with an equal dose of normal saline. The effects of siRNA was verified by reverse transcription-polymerase chain reaction (RT-PCR). Cell proliferation, cell cycle and cell invasion were detected by MTT, flow cytometry, Transwell assay. The matrix metalloproteinase-9 (MMP-9) and vascular endothelial growth factor (VEGF) expression were detected by Western blot. **Results** The cell proliferation rates of HepG2 and SMMC-7721 in siRNA group were 0.45 ± 0.12 and 0.42 ± 0.03, respectively, which were 1.02 ± 0.44 and 1.07 ± 0.51 in control group, respectively, and 1.08 ± 0.44 and 1.11 ± 0.08 in blank group , respectively. The cell proliferation rate in siRNA group were significantly lower than those in control group and blank group (all $P < 0.05$). Compared with control group and blank group, the proportion of S phase and G_2/M phase in siRNA group increased significantly, and

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the proportion of G_0/G_1 phase decreased significantly, the differences were statistically significant (all $P < 0.05$). The number of cell in vasion of HepG2 and SMMC-7721 in siRNA group were 4.55 ± 1.49 and 2.48 ± 0.48, respectively, which were significantly lower than those in control group (42.48 ± 3.18 and 42.00 ± 2.78, respectively)and blank group (38.27 ± 2.47 and 38.19 ± 3.11, respectively), the differences were statistically significant (all $P < 0.05$). The expression of MMP-9 and VEGF in HepG2 in siRNA group were 2.09 ± 0.13 and 0.78 ± 0.12, respectively, and in SMMC-7721 were 2.11 ± 0.14 and 0.81 ± 0.13, respectively, which were significantly lower than those in control group and blank group (all $P < 0.05$). **Conclusions** RNA interference on CXCR4 gene expression can inhibit the proliferation and invasion of human hepatoma cells HepG2 and SMMC-7721, which may regulate the proliferation and invasion of hepatocellular carcinoma by regulating the expression of MMP-9 and VEGF.

Key words: CXC chemokines receptor 4; RNA interference; Liver cancer; Proliferation; Invasion

肝癌发病率居中国恶性肿瘤发病率第2位，每年死亡病例接近60万，肝癌预后差的主要原因是癌细胞增殖快且易侵犯门静脉，导致肝内播散，肝内转移率高[1,2]。肿瘤的增殖和侵袭是一个多因素、多步骤、多阶段综合作用的发展过程，特别是趋化因子及其受体可参与肿瘤细胞的发生、发展与侵袭，已成为肿瘤学的研究热点[3,4]。趋化因子是一群由机体内间质细胞、淋巴细胞和巨噬细胞等多细胞分泌的低分子质量细胞因子，与相应的趋化因子受体结合后可参与多种生理和病理过程，如调节肿瘤的生长和转移等[5,6]。CXC类趋化因子受体4（CXC chemokines receptor 4，CXCR4）是肿瘤细胞中常见的趋化因子受体，CXCR4表达水平与肿瘤局部复发、5年存活率和淋巴结转移密切相关[7]。CXCR4在淋巴瘤、神经胶质细胞瘤、卵巢癌及胰腺癌组织中均高表达[8-10]。相关研究表明，SDF-1/CXCR4生物学轴参与癌细胞播散转移的全部过程，阻断SDF-1/CXCR4信号转导通路可有效防止恶性肿瘤的转移[11-13]，但在肝癌细胞中的应用尚缺乏足够的客观依据。本研究以人肝癌细胞HepG2和SMMC-7721为研究对象，观察CXCR4基因RNA干扰对人肝癌细胞增殖和侵袭的影响，初步探讨SDF-1/CXCR4生物轴在肝癌侵袭机制中的作用，进一步明确其信号转导通路的调控机制。

1 材料与方法

1.1 实验材料 人肝癌细胞株 HepG2 和 SMMC-7721 购自中国科学院典型培养物保藏委员会细胞库，鼠抗人 CXCR4 单克隆抗体和山羊抗小鼠二抗均由美国 Santa Cruz 公司生产，CXCR4、β-actin/GAPDH 引物由上海生工生物工程有限公司合成，逆转录聚合酶链反应（reverse transcription-polymerase chain reaction，RT-PCR）检测试剂盒为日本 TaKaRa 公司生产。

1.2 实验方法

1.2.1 siRNA序列设计与转染 根据CXCR4的靶序列、涉及酶切位点、Loop环与含有siRNA段信息的DNA序列，设计siRNA序列如下：5’-CGCCGATCAGTGTGAGTATATTTCAAGAGAATATCTCACACTGATCGGCGTTTTTGGA-3’。取对数生长期细胞，实验设CXCR4-siRNA组（siRNA组）、siRNA-对照组（对照组）与空白-对照组（空白组）。siRNA组与对照组采用脂质体转染法转染CXCR4-siRNA与siRNA-对照，空白组以等剂量生理盐水代替。转染48 h后收集生长状态良好的目的细胞，进行RT-PCR以检测细胞中目的基因的表达。检测CXCR4的引物序列：下游引物为5’-GCCAACCATGATCTGCTGAAAC-3’，上游引物为5’-GCCAACGTCAGTAGGCAGA-3’。

1.2.2 MTT实验 取对数生长期的HepG2和SMMC-7721细胞，胰酶消化后制成5×10^5个/ml的细胞悬液，使用96孔培养板常规培养，每孔200 μl细胞悬液；培养3 d后每孔加20 μl 5 mg/ml的MTT母液，孵育2 h后弃上清，加入200 μl二甲基亚砜，充分溶解细胞。采用酶标仪检测490 nm的吸光度值，读取细胞增殖率。

1.2.3 流式细胞仪检测 转染细胞培养48 h后，胰酶消化后制成（1～5）$\times 10^6$个/ml的细胞悬液。加入1 ml预冷PBS重悬细胞，1000 r/min离心5 min（离心半径为10 cm），用PBS重悬细胞，逐滴加入1.5 ml预冷的无水乙醇，4 ℃放置过夜固定。取出固定样品，1000 r/min离心5 min（离心半径为10 cm），用PBS重悬细胞，离心收集细胞，用30目滤网过滤。加入100 μl PI工作液，4 ℃避光染色30 min。上流式细胞仪检测，分析PI荧光直方图上细胞各周期的百分率。

1.2.4 Transwell小室实验 取对数生长期细胞，洗涤后加入无血清培养液继续培养48 h，收集上清。放置Transwell小室于室温状态，加入含碳酸氢钠的温培养基，孵育2 h，移除培养基，将上清放置预先铺有基质胶的小室，加入细胞，孵育24 h。取出，拭去膜内层细胞，0.1%结晶紫染色30 min，光学显微镜下计数。

1.2.4 蛋白质免疫印迹法检测 采用蛋白质免疫印迹法检测基质金属蛋白酶-9（matrix metalloproteinase-9，MMP-9）与血管内皮生长因子（vascular endothelial growth factor，VEGF）的相对

表达水平。转染细胞培养至48 h，离心收集细胞并提取蛋白，根据BCA蛋白浓度测定试剂盒操作手册测定各样品蛋白浓度，进行SDS-PAGE，于凝胶成像仪中进行自动曝光，一抗浓度（抗MMP-9抗体：抗VEGF抗体）1∶1000，二抗浓度1∶5000，蛋白条带采用Gelpre32软件检测，以目的蛋白与内参蛋白（β-actin）的比值作为目的蛋白表达水平的参数。

1.3 统计学处理 采用 SPSS 22.00 软件进行统计学分析，细胞增殖率、G_2/M 期比例及 G_0/G_1 期比例等计量资料均符合正态分布，以 $\bar{x} \pm s$ 表示，多组间比较采用方差分析，两两比较采用 LSD-t 检验。以 $P < 0.05$ 为差异有统计学意义。

2 结果

2.1 siRNA 验证结果 RT-PCR 检测结果显示，HepG2 和 SMMC-7721 细胞中 siRNA 组 CXCR4 mRNA 表达水平高于空白组和对照组，见图 1。

2.2 细胞增殖率 MTT 检测结果表明，与空白组和对照组相比，siRNA 组细胞增殖率显著降低（P 均 < 0.05），见表 1。

2.3 细胞周期比例 流式细胞仪检测显示，与空白组和对照组相比，siRNA 组 S 期和 G_2/M 期比例显著增加，G_0/G_1 期比例显著降低，差异有统计学意义（P 均 < 0.05），见表 2。

2.4 细胞侵袭情况 Transwell 小室检测显示，与空

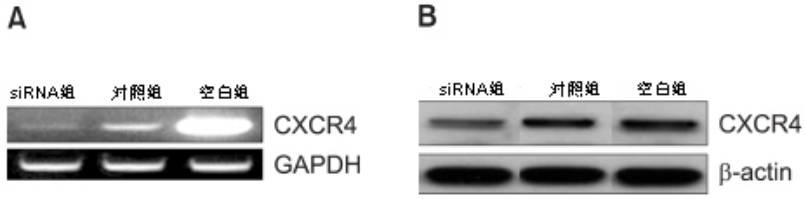

图 1 siRNA 组、对照组及空白组 CXCR4 mRNA 表达的 RT-PCR 检测结果

注：A 为 HepG2 细胞，B 为 SMMC-7721 细胞

表 1 siRNA 组、对照组及空白组细胞增殖率（$\bar{x} \pm s$）

组别	HepG2细胞	SMMC-7721细胞
siRNA组	0.45 ± 0.12	0.42 ± 0.03
对照组	1.02 ± 0.44	1.07 ± 0.51
空白组	1.08 ± 0.44	1.11 ± 0.08
F值	12.482	14.092
P值	＜ 0.001	＜ 0.001
t_1值	6.822	7.142
P_1值	0.013	0.010
t_2值	7.919	7.555
P_2值	0.006	0.008
t_3值	0.194	0.222
P_3值	0.893	0.888

注：siRNA 组为 CXCR4-siRNA，对照组为 siRNA- 对照，空白组为空白 - 对照；t_1、P_1 为 siRNA 组与对照组相比，t_2、P_2 为 siRNA 组与空白组相比，t_3、P_3 为对照组与空白组相比

表 2 siRNA 组、对照组及空白组细胞周期比例（$\bar{x} \pm s$，%）

组别	HepG2细胞			SMMC-7721细胞		
	G_0/G_1期	S期	G_2/M期	G_0/G_1期	S期	G_2/M期
siRNA组	48.29 ± 1.48	42.58 ± 1.49	8.85 ± 1.19	48.10 ± 2.22	42.98 ± 2.58	8.91 ± 1.44
对照组	85.32 ± 1.77	13.21 ± 1.22	0.95 ± 0.66	85.30 ± 2.18	13.22 ± 2.48	0.98 ± 0.45
空白组	87.45 ± 2.44	11.60 ± 2.14	0.94 ± 0.22	87.20 ± 3.78	11.67 ± 3.11	0.96 ± 0.32
F值	9.832	24.583	34.111	9.672	13.955	33.533
P值	＜ 0.001	＜ 0.001	＜ 0.001	＜ 0.001	＜ 0.001	＜ 0.001
t_1值	5.202	12.042	16.925	5.694	8.103	15.694
P_1值	0.029	＜ 0.001	＜ 0.001	0.023	0.005	＜ 0.001
t_2值	5.881	12.747	18.002	5.104	7.822	16.092
P_2值	0.021	＜ 0.001	＜ 0.001	0.030	0.008	＜ 0.001
t_3值	1.032	1.773	0.092	1.332	1.932	0.144
P_3值	0.133	0.102	0.913	0.156	0.100	0.932

注：siRNA 组为 CXCR4-siRNA，对照组为 siRNA- 对照，空白组为空白 - 对照；t_1、P_1 为 siRNA 组与对照组相比，t_2、P_2 为 siRNA 组与空白组相比，t_3、P_3 为对照组与空白组相比

白组和对照组相比，siRNA 组的细胞侵袭数显著减少（P 均 ＜ 0.05），见表 3。

2.5 MMP-9 与 VEGF 的相对表达水平 Western blot 结果显示，siRNA 组 HepG2 细胞和 SMMC-7721 细胞的 MMP-9 与 VEGF 蛋白表达量均低于空白组和对照组（P 均＜ 0.05），见图 2、表 4。

表 3 siRNA 组、对照组及空白组的细胞侵袭数（$\bar{x} \pm s$，个）

组别	HepG2细胞	SMMC-7721细胞
siRNA组	4.55 ± 1.49	2.48 ± 0.48
对照组	42.48 ± 3.18	42.00 ± 2.78
空白组	38.27 ± 2.47	38.19 ± 3.11
F值	13.583	24.583
P值	＜ 0.001	＜ 0.001
t_1值	8.284	12.455
P_1值	0.004	＜ 0.001
t_2值	6.833	12.774
P_2值	0.014	0.000
t_3值	2.014	1.772
P_3值	0.092	0.167

注：siRNA 组为 CXCR4-siRNA，对照组为 siRNA- 对照，空白组为空白 - 对照；t_1、P_1 为 siRNA 组与对照组相比，t_2、P_2 为 siRNA 组与空白组相比，t_3、P_3 为对照组与空白组相比

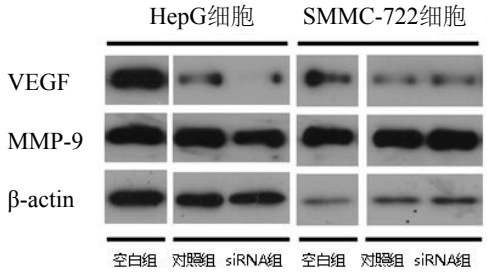

图 2 siRNA 组、对照组及空白组 MMP-9 与 VEGF 的蛋白质免疫印迹图

表 4 siRNA 组、对照组及空白组 MMP-9 与 VEGF 的相对灰度（$\bar{x} \pm s$）

组别	HepG2细胞		SMMC-7721细胞	
	VEGF	MMP-9	VEGF	MMP-9
siRNA组	2.09 ± 0.13	0.78 ± 0.12	2.11 ± 0.14	0.81 ± 0.13
对照组	3.11 ± 0.23	1.00 ± 0.02	3.08 ± 0.31	1.03 ± 0.04
空白组	4.59 ± 0.22	5.09 ± 0.14	4.61 ± 0.23	5.11 ± 0.12
F值	8.144	16.024	7.883	14.002
P值	0.003	＜ 0.001	＜ 0.001	＜ 0.001
t_1值	5.024	8.945	5.633	8.914
P_1值	0.022	0.001	0.014	0.001
t_2值	4.194	4.868	4.753	7.882
P_2值	0.029	0.024	0.028	0.007
t_3值	2.292	1.842	1.492	1.564
P_3值	0.041	0.102	0.123	0.121

注：siRNA 组为 CXCR4-siRNA，对照组为 siRNA- 对照，空白组为空白 - 对照；t_1、P_1 为 siRNA 组与对照组相比，t_2、P_2 为 siRNA 组与空白组相比，t_3、P_3 为对照组与空白组相比

3 讨论

肝癌的侵袭与转移是决定患者预后的关键因素，特别是有远处器官或淋巴结转移的肝癌患者病死率极高[14]。肝癌的增殖与侵袭是多步骤、多因素相互作用的复杂过程，具体机制尚未完全明确。趋化性细胞因子及其受体网络参与肿瘤增殖与侵袭的各个环节[15]，成为当前的研究热点。CXCR4是G-蛋白偶联受体超家族中的CXCR类趋化因子受体，其

与配体SDF-1结合后组成SDF-1/CXCR4生物轴[16]。SDF-1与CXCR4结合后可改变CXCR4结构，从而影响肿瘤细胞的增殖和侵袭等生物学行为。目前研究表明，CXCR4基因在乳腺癌、鼻咽癌、肺癌及肝癌组织中均高表达[17,18]。有研究者发现小分子非肽类CXCR4特异性抑制剂可显著抑制乳腺癌细胞的侵袭转移能力[19]。RNAi是一种成熟、有效的沉默基因方法，其可特异性降低相应靶基因的表达。有研究通过RNAi沉默食管癌细胞中CXCR4的表达，结果表明食管癌细胞的侵袭转移能力显著受到抑制[20]。本研究中siRNA组细胞增殖率显著低于空白组和对照组。最新研究认为当SDF-1与CXCR4结合后，促进了钙离子的活化，细胞内AMP减少，PI3K/Akt信号转导通路被激活，从而提高细胞的生存能力[21]。

CXCR4是目前公认的SDF-1唯一受体，其编码352个氨基酸，具有7个跨膜G蛋白偶联受体。SDF-1/CXCR4生物学轴与多种肿瘤的发生和转移过程有关，CXCR4表达强度可能与肝癌的进展密切相关[22]。还有研究表明，肝癌细胞中CXCR4的表达与肿瘤的发生和发展显著相关[23]。有研究证实，低转移潜能的肝癌细胞系HepG2中可检测到CXCR4表达，SDF-1可能增强Huh7的迁移和浸润能力[24]。表达CXCR4的HepG2细胞在外源性SDF-1趋化诱导作用下，其趋化侵袭能力显著增强，且对SDF-1a呈浓度依赖性[25]。本研究表明，与空白组和对照组相比，siRNA组S期与G_2/M期比例显著升高，G_0/G_1期比例显著降低，提示CXCR4 siRNA转染后HepG2和SMMC-7721细胞在细胞周期中出现了S和G_2期阻滞[26]。

原发性肝癌易侵犯门静脉分支，癌栓经门静脉形成肝内播散。肿瘤细胞的侵袭是一个多级联过程，SDF-1/CXCR4生物学轴对肿瘤的影响是多方面的。SDF-1/CXCR4可增加细胞的定向运动性和趋化性，也能提高肿瘤细胞的存活率及增殖能力[27]。特异性靶向siRNA能够阻断SDF-1/CXCR4生物轴功能，降低恶性肿瘤细胞的体外侵袭转移能力[28]。有研究表明，SDF-1/CXCR4生物轴在CXCR4高表达的肿瘤细胞迁移中发挥重要的驱动和侵袭作用[29]。本研究中siRNA组的细胞侵袭数显著低于空白组和对照组。也有研究表明，表达CXCR4的HepG2细胞具有一定的侵袭能力，在外源性SDF-1趋化诱导作用下，其趋化侵袭能力呈剂量依赖性；而CXCR4 mAb可抑制HepG2细胞的穿膜侵袭能力[30]。表达CXCR4的肿瘤细胞可沿SDF-1浓度梯度转移至SDF-1高浓度的细胞中，通过自分泌或旁分泌机制形成侵袭[31]。

CXCR4参与肿瘤增殖与侵袭的各个关键环节，包括肿瘤微血管淋巴管形成与细胞外基质的降解，而MMP-9和VEGF是调控这些环节的重要因子。本研究表明，与空白组和对照组相比，siRNA组MMP-9与VEGF表达量显著降低（$P < 0.05$）。可推测CXCR4被激活后能够调控MMP-9和VEGF的分泌，从而影响细胞的侵袭[32]。有研究表明，肝癌细胞中CXCR4与VEGF呈显著正相关，沉默CXCR4基因后，肝癌细胞中VEGF的表达水平也随之下调[33]。从机制上分析，CXCR4与SDF-1结合后可激活其磷脂酰肌醇3-激酶路径（PI3K），促进PI3K磷酸化，进而激活AKT，上调VEGF的表达，促进肿瘤侵袭[34]。还有研究表明，VEGF及核转录因子在乳腺癌细胞中可增强CXCR4表达并增强肿瘤的趋化侵袭能力[35]。本研究尚未在动物水平进一步验证CXCR4的功能，在相关机制研究方面也有待进一步完善。

综上，RNA干扰CXCR4基因的表达可抑制人肝癌细胞HepG2和SMMC-7721的增殖与侵袭，其可能通过调控MMP-9及VEGF的表达来调节肝癌细胞的增殖和侵袭。

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