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LncRNA NEAT1对肝癌细胞增殖、迁移和侵袭的影响及其机制

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[摘 要] **目的：**观察长链非编码RNA NEAT1(long non-coding RNA NEAT1，lncRNA NEAT1)对肝癌细胞增殖、迁移及侵袭的影响，并探讨其机制。**方法：**qRT-PCR用于测定正常肝细胞系L02和肝癌细胞系HepG2，SMMC-7721及Huh7中lncRNA NEAT1表达水平。将HepG2细胞系分为NEAT1-siRNA组、Control-siRNA组和Mock组，NEAT1-siRNA组和Control-siRNA组经Lipofectamine™2000分别转染pcDNA3.1-lncRNA-NEAT1-shRNA及pcDNA3.1-lncRNA-NEAT1-NC，Mock组为阴性对照。CCK-8法、细胞划痕实验和Transwell实验分别用于测定细胞增殖、迁移和侵袭能力，qRT-PCR和Western印迹分别测定miR-121和特异AT序列结合蛋白2(SATB2)的表达。**结果：**LncRNA NEAT1在肝癌细胞系HepG2，SMMC-7721及Huh7中的表达量高于正常肝细胞系L02(P<0.05)。CCK-8示：转染72及96 h后，NEAT1-siRNA组$OD_{450\ nm}$值显著低于Control-siRNA组和Mock组(P<0.05)。NEAT1-siRNA组细胞划痕愈合率低于Control-siRNA组[(30.38±4.19)% vs (54.78±5.83)%，P<0.05]。NEAT1-siRNA组侵袭细胞数少于Control-siRNA组[(107.5±8.1)个 vs (178.1±13.4)个，P<0.05]。NEAT1-siRNA组miR-211相对表达量高于Control-siRNA组(P<0.001)。NEAT1-siRNA组SATB2相对表达量低于Control-siRNA组(P<0.01)。**结论：**LncRNA NEAT1在肝癌细胞系中高表达，沉默lncRNA NEAT1表达可抑制肝癌细胞增殖、迁移和侵袭，其机制可能与调控miR-211/SATB2信号途径有关。

[关键词] 长链非编码RNA NEAT1；肝癌；增殖；迁移；侵袭

Effect of lncRNA NEAT1 on proliferation, migration and invasion in hepatocarcinoma cell line and its mechanism

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Abstract **Objective:** To observe the effect of lncRNA NEAT1 on cell proliferation, invasion and migration in hepatocellular cell lines, and investigate the mechanism of it. **Methods:** qRT-PCR was set out to detect the expression pattern of lncRNA NEAT1 in normal hepatocyte line, L02, and hepatocellular cell lines, HepG2, SMMC-7721 and Huh7. The HepG2 cell line was divided into a NEAT1-siRNA group, a Control-siRNA group and a Mock group, which was transfect with pcDNA3.1-lncRNA-NEAT1-shRNA, pcDNA3.1-lncRNA-

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NEAT1-NC and PBST by Lipofectamine™ 2000. CCK-8 assay, cell wound scratch and transwell assay was used to test the proliferation, migration and invasion ability. The expression level of miR-121 and SATB2 protein was measured by qRT-PCR and Western blot. **Results:** LncRNA NEAT1 expression level was significantly higher in hepatocellular cell lines, HepG2, SMMC-7721 and Huh7, than normal hepatocyte cell line L02 (P<0.05). CCK-8 showed that $OD_{450\ nm}$ of NEAT1-siRNA group was significantly lower than that in the Control-siRNA group and the Mock group after transfect for 72 and 96 h (P<0.05). The scratch healing rate of the NEAT1-siRNA group was significantly lower than that in the Control-siRNA group [(30.38±4.19)% vs (54.78±5.83)%, P<0.05]. The invasive cell number of NEAT1-siRNA group was significantly lower than that in the Control-siRNA group [(107.5±8.1) vs (178.1±13.4), P<0.05]. The expression level of miR-211 in the NEAT1-siRNA group was significantly higher than that in the Control-siRNA group, the expression level of SATB2 protein was significantly lower than that in Control-siRNA group (P<0.01). **Conclusion:** LncRNA NEAT1 is high expressed in hepatocellular cell lines. Silencing expression of lncRNA NEAT1 can inhibit proliferation, migration and invasion of hepatocellular cells, which mechanism may be associated with miR-211/SATB2 cell signaling.

Keywords LncRNA NEAT1; hepatocarcinoma; proliferation; migration; invasion

肝癌是全球第三大常见癌症死亡原因，占所有原发性肝癌的90%[1]。每年新发肝癌患者多达100万人，死亡人数超过75万[1-2]。随着医疗技术不断发展，肝癌治疗取得长足进展，但在防止转移和复发方面治疗仍十分有限[3]。肝癌发生发展机制非常复杂，涉及多种基因和信号通路，因此，深入探索肝癌发生发展机制对开发新的肿瘤治疗手段具有重要的意义。长链非编码RNA(long non-coding RNA，lncRNA)是一类转录长度超过200 nt，具有5'帽子结构和3'聚腺苷酸结构的非编码RNA[2]。研究[4]发现：lncRNA能靶向转录因子、激活子、抑制子等多种目标基因，调控基因的转录和表达。LncRNA广泛参与各种生物学过程，包括细胞增殖、凋亡和侵袭等[5]。LncRNA NEAT1是lncRNA家族成员之一，包括NEAT1_1(3.7 kb)和NEAT1_2(23 kb)两种亚型[6]。目前，已在多种人类肿瘤中发现lncRNA NEAT1表达发生变化，且与肿瘤转移及发生发展密切相关[7-8]。但是，lncRNA NEAT1在肝癌中的研究还很有限。本研究旨在体外研究lncRNA NEAT1对肝癌细胞增殖、迁移和侵袭的影响及其机制。

1 材料与方法

1.1 材料

肝癌细胞系HepG2，SMMC-7721，Huh7及正常人肝细胞系L02均由武汉大学医学院赠予，实验所用的SATB2及GAPDH一抗均购自美国BD公司，二抗购自美国Invitrogen公司，RPMI 1640培养基购自上海经科化学科技有限公司，pcDNA3.1载体均由上海吉玛生物科技有限公司提供。Lipofectamine™2000转染试剂购自美国BD公司。RT-PCR仪购自美国BD公司，HBS-1096B酶标仪购自南京德铁实验设备有限公司，蛋白免疫印迹电泳设备购自美国Bio-Rad公司。

1.2 细胞培养、转染及分组

肝癌细胞系HepG2，SMMC-7721，Huh7及正常人肝细胞系L02均用RPMI 1640培养基培养于37 ℃，5% CO_2培养箱中，于48 h后消化传代。取HepG2细胞系分成3组，NEAT1-siRNA组、Control-siRNA组和Mock组，NEAT1-siRNA组和Control-siRNA组经Lipofectamine™2000分别转染pcDNA3.1-lncRNA-NEAT1-shRNA及pcDNA3.1-lncRNA-NEAT1-NC，Mock组以磷酸盐缓冲液(phosphate buffered solution，PBST)为阴性对照。LncRNA-NEAT1-shRNA序列：正向5'-GUGA-GAAGUUGCUUAGAAACUUUCC-3'，反向5'-GGAAAGUUUCUAAGCAACUUCUCAC-3'；lncRNA-NEAT1-NC序列：正向5'-UACUG-UCUAGUCGCCGUAC-3'，反向5'-GUACGGC-GACUAGACAGUA-3'，转染浓度为300 nmol/孔。

1.3 RNA 提取及 qRT-PCR

用All-in-One miRNA抽提试剂盒和All-in-One miRNA qRT-PCR检测试剂盒提取和分离RNA，

提取RNA后，采用NanoDrop1000分光光度计对RNA的浓度进行测定，7900HT荧光定量PCR系统(美国Applied Biosystems)测定lncRNA NEAT1的表达量。通过阈值分析比较，采用循环数(Ct)法对数据行分析处理。LncRNA NEAT1引物序列：上游5'-GUCUGUGUGGAAGGAGGAATT-3'，下游5'-UUCCUCCUUCCACACAGACTT-3'。GAPDH引物序列：上游5'-CAGCCAGGAGAAATCAAACAG-3'，下游5'-GACTGAGTACCTGAACCGGC-3'，使用$2^{-\Delta\Delta Ct}$方法定量，计算lncRNA NEAT1的相对表达量。

1.4 细胞增殖能力测定

CCK-8法测定NEAT1-siRNA组、Control-siRNA组和Mock组3组细胞增殖能力。将NEAT1-siRNA组、Control-siRNA组和Mock组3组细胞消化成单细胞悬液后，以2×10^3个/孔将3组细胞种植于96孔板上，每个孔按200 μL的体积上样，经0，1，2，3，4 d培养后，每孔加入20 μL CCK-8溶液，继续培养1 h后，在450 nm波长下，用酶标仪测定各孔吸光度值，以时间为横坐标，吸光度值为纵坐标绘制细胞增殖曲线。

1.5 细胞迁移能力测定

1.5.1 细胞划痕实验

将NEAT1-siRNA组和Control-siRNA组两组细胞培养于12孔板中，待细胞长满融合后，用20 μL无菌Tips枪头画直线，在0和48 h显微镜下观察修复情况，划痕愈合率=(划痕后即刻的划痕面积–划痕后48 h的划痕面积)/划痕后即刻的划痕面积×100%。实验在同一情况及条件下重复测量3次。迁移能力与划痕愈合率成正比。

1.5.2 Transwell 实验

将NEAT1-siRNA组、Control-siRNA组两组细胞，每组取3×10^4个细胞后接种于Transwell小室表面，于37 ℃条件下培养24 h后，将小室膜下面的细胞用甲醛固定，并采用0.2%结晶紫溶液染色10 min，显微镜下随机检查10个视野(200×)，计算膜下细胞数，在同一条件下实验重复3次。侵袭细胞数越多表示侵袭能力越强。

1.6 Western 印迹

将NEAT1-siRNA组和Control-siRNA组两组细胞裂解、变性后，上样量为30 μg/孔，浓缩胶条件为50 min 80 V，分离胶条件为100 min 100 V，常规转膜，加入特异AT序列结合蛋白2(special AT-rich sequence binding protein 2，SATB2)一抗，抗体浓度为1:200，于4 ℃孵育过夜，二抗(1:1 000)经37 ℃孵育4 h后，PBST漂洗3次，在ECL发光液下显影，Quantity One 1-D分析目标蛋白灰度值，目标蛋白相对表达量=目标蛋白灰度值/GAPDH灰度值，实验重复3次，取平均值。

1.7 miR-211 表达量测定

转染24 h后，用All-in-One miRNA抽提试剂盒和All-in-One miRNA qRT-PCR检测试剂盒提取和分离NEAT1-siRNA组和Control-siRNA组RNA，反应体系：2×All-in-One™ Q-PCR Mix 10 μL，引物序列(10 μmol/L)1 μL，反转录cDNA 1 μL，加双氧水至20 μL。反应条件：95 ℃预变性10 min，95 ℃变性10 s，60 ℃退火20 s，72 °C延伸30 s，共40周期，miR-211引物序列：上游5'-CGGGTGTCCGCTTCGCAC-3'，下游5'-AGGCAGGTCCGGGT-3'，U6小核引物序列：上游5'-CGCAAGGATGACACG-3'，下游5'-GAGCAGGCTGGAGAA-3'。在ABI 7500实时定量PCR仪中，以U6小核RNA作为内参，使用$2^{-\Delta\Delta Ct}$方法定量，量化miR-211相对表达水平。

1.8 统计学处理

采用SPSS 20.0统计软件进行分析，计量资料以均数±标准差($\bar{x}\pm s$)表示，两组间的比较采用*t*检验，3组比较先用方差分析，有统计学意义时，两两比较再用LSD-*t*检验，$P<0.05$为差异有统计学意义。

2 结果

2.1 LncRNA NETA1 在肝癌细胞系中高表达

qRT-PCR示lncRNA NEAT1在肝癌细胞系HepG2，SMMC-7721，Huh7相对表达量分别为7.47±0.55，4.36±0.27及3.42±0.21，正常人肝细胞系L02的相对表达量为1.0±0.07，lncRNA NEAT1在肝癌细胞系HepG2，SMMC-7721，Huh7，细胞系相对表达量高于正常人肝细胞系L02($P<0.05$，图1)。

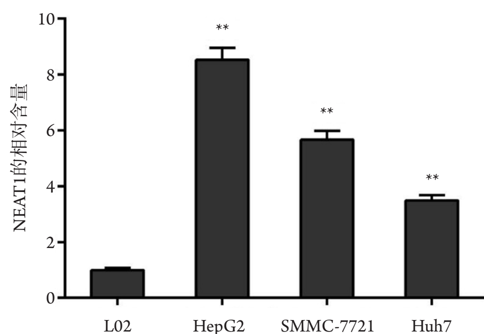

图1 LncRNA NETA1在肝癌细胞系及正常人肝细胞系中的表达

Figure 1 Expression of lncRNA NETA1 in normal hepatocyte line and hepatocellular carcinoma cell lines

与L02相比，$^{**}P$<0.01。

Compared with L02, $^{**}P$<0.01.

2.2 LncRNA NEAT1 沉默对肝细胞增殖的影响

转染HepG2细胞系24 h后，qRT-PCR示NEAT1-siRNA组中lncRNA NEAT1的相对表达量为0.27±0.02，Control-siRNA组为1.05±0.09，Mock组为1.14±0.08，NEAT1-siRNA组lncRNA NEAT1的相对表达量低于Control-siRNA组和Mock组(P<0.01；图2A)，提示沉默表达成功，可行后续实验。

CCK-8法示：转染后0，24，48 h后，NEAT1-siRNA组与Control-siRNA组的吸光度值分别为0.13±0.02 vs 0.12±0.01，0.20±0.02 vs 0.22±0.02，0.30±0.02 vs 0.41±0.04，差异无统计学意义(P>0.05)；转染后72及96 h后，NEAT1-siRNA组与Control-siRNA组的吸光度值分别为0.45±0.04 vs 0.79±0.07(P<0.05)及0.65±0.05 vs 1.17±0.09，差异有统计学意义(P<0.01)。Mock组转染0，24，48，72及96 h后的吸光度值为0.13±0.01，0.23±0.02，0.45±0.04，0.75±0.06及1.21±0.10，与Control-siRNA组比较差异无统计学意义(P>0.05；图2B)。

2.3 LncRNA NEAT1 沉默对迁移与侵袭的影响

细胞划痕实验示：NEAT1-siRNA组细胞划痕愈合率为(30.38±4.19)%，Control-siRNA组为(54.78±5.83)%，NEAT1-siRNA组细胞划痕愈合率低于Control-siRNA组，差异有统计学意义(P<0.05；图3A，3B)。

Transwell细胞侵袭实验示：NEAT1-siRNA组侵袭细胞数为(107.5±8.1)个，Control-siRNA组为(178.1±13.4)个，NEAT1-siRNA组侵袭细胞数少于Control-siRNA组，差异有统计学意义(P<0.05；图3C，3D)。

2.4 LncRNA NEAT1 沉默对 miR-211/SATB2 信号通路的影响

qRT-PCR示：NEAT1-siRNA组miR-211相对表达量为7.32±0.59，Control-siRNA组为1.0±0.03，NEAT1-siRNA组miR-211相对表达量高于Control-siRNA组(P<0.001；图4A)；Western印迹示：NEAT1-siRNA组SATB2相对表达量为0.46±0.04，Control-siRNA组为1.0±0.03，NEAT1-siRNA组SATB2相对表达量低于Control-siRNA组(P<0.01；图4B，4C)。

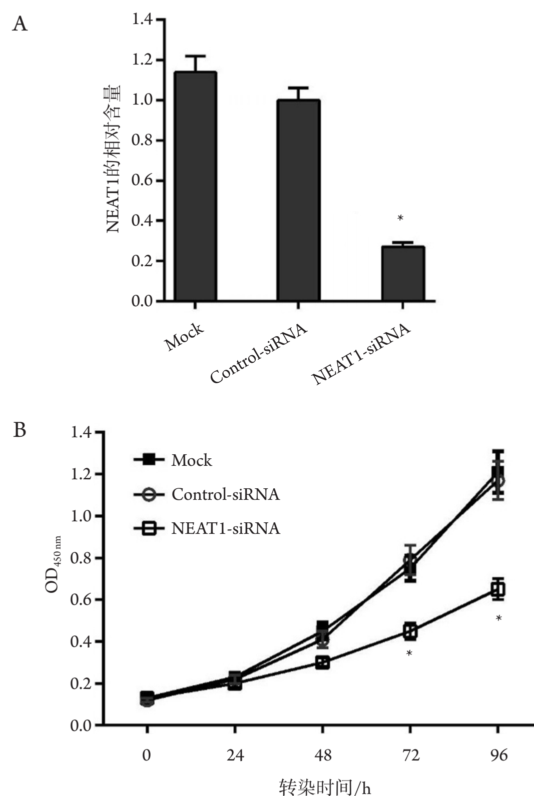

图2 LncRNA NEAT1沉默抑制肝癌细胞增殖

Figure 2 Silencing expression of LncRNA NEAT1 inhibits the proliferation of hepatocellular carcinoma cell

(A) LncRNA NEAT1在3组中的相对表达量比较；(B)3组的细胞增殖曲线比较。3组lncRNA NEAT1表达量及$OD_{450\ nm}$值比较，$^{*}P$<0.05。

(A) Comparison of lncRNA NEAT1 expression among the 3 groups. (B) Comparison of proliferation curve among the 3 groups. Comparison of lncRNA NEAT1 expression and $OD_{450\ nm}$ among the 3 groups, $^{*}P$<0.05.

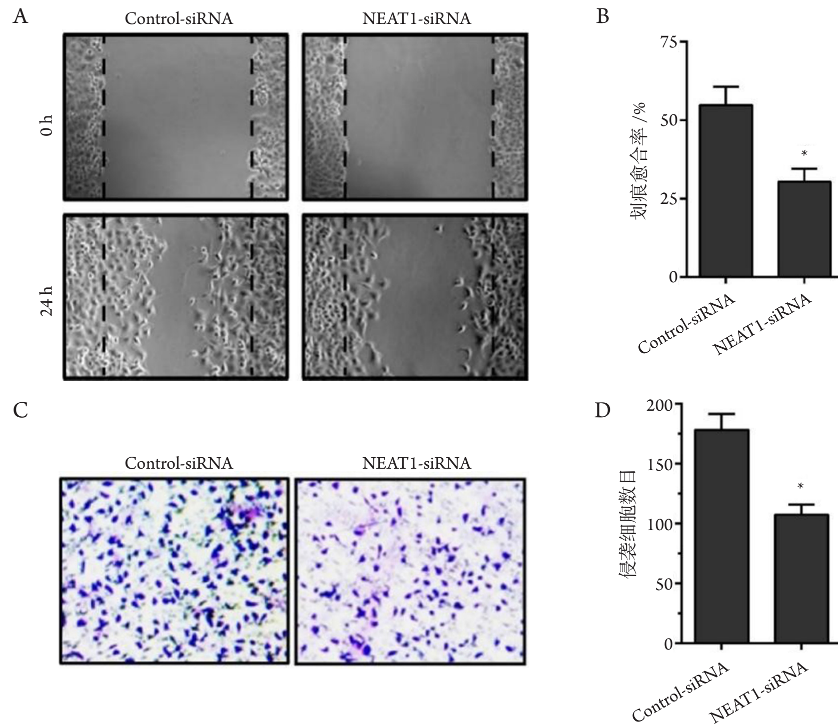

图3 LncRNA NEAT1沉默抑制肝癌细胞迁移和侵袭

Figure 3 Silencing expression of lncRNA NEAT1 inhibit the migration and invasion of hepatocellular carcinoma

(A) NEAT1-siRNA组与Control-siRNA组细胞划痕实验(×200)；(B)NEAT1-siRNA组与Control-siRNA组细胞划痕愈合率比较；(C)NEAT1-siRNA组与Control-siRNA组Transwell实验(结晶紫染色，×200)；(D)NEAT1-siRNA组与Control-siRNA组侵袭细胞数比较。与Control-siRNA组比较，$^*P<0.05$。

(A) Cell wound scratch of the NEAT1-siRNA group and the Control-siRNA group(×200). (B) Comparison of scratch healing rate between the NEAT1-siRNA group and the Control-siRNA group. (C) Transwell assay in the NEAT1-siRNA group and the Control-siRNA group (crystal violet staining, ×200). (D) Comparison of number of invasive cell between the NEAT1-siRNA group and the Control-siRNA group. Compared with the Control-siRNA group, $^*P<0.05$.

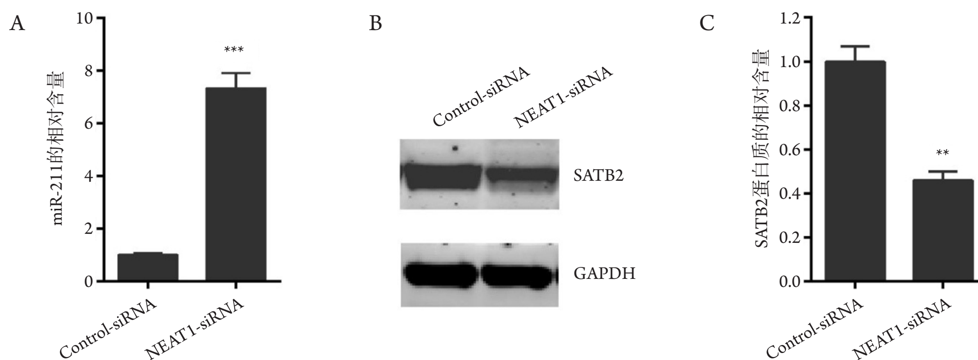

图4 LncRNA NEAT1沉默对miR-211/SATB2信号通路的影响

Figure 4 Effect of silencing expression of lncRNA NEAT1 on miR-211/ SATB2 cell signaling

(A) NEAT1-siRNA组与Control-siRNA组miR-211的相对表达量比较；(B)Western印迹示NEAT1-siRNA组与Control-siRNA组SATB2蛋白的表达；(C)NEAT1-siRNA组与Control-siRNA组SATB2蛋白表达量比较。与Control-siRNA组比较，$^{**}P<0.01$，$^{***}P<0.001$。

(A) Comparison of miR-211 expression between the NEAT1-siRNA group and the Control-siRNA group. (B) Western blot shows the expression of SATB2 protein in the NEAT1-siRNA group and the Control-siRNA group. (C) Comparison of SATB2 protein expression between the NEAT1-siRNA group and the Control-siRNA group. $^{**}P<0.01$, $^{***}P<0.001$ vs the Control-siRNA group.

3 讨论

亚太地区是肝癌高发区，慢性乙型肝炎病毒感染是一个重要的致病因素[9]。据统计，2015年我国肝癌新发病例46.61万，其中男34.37万，女12.23万；死亡病例42.21万，其中男31.06万，女11.15万，是我国最常见的癌症和癌症死亡的主要原因[10]。

LncRNA在人类正常发育和疾病发生发展中发挥重要作用[11]。LncRNA的表达具有组织特异性，通常在多种类型肿瘤中表达失调，部分lncRNA已被证实与肿瘤复发和不良预后有一定关联[12]。LncRNA可通过表观遗传修饰、剪切、RNA降解、翻译后修饰等调控肿瘤抑癌基因或促癌基因，进而参与肿瘤的发生和转移等肿瘤生物学过程[13]。LncRNA NEAT1起初发现于家族性肿瘤综合征多发性分泌腺瘤I型11号染色体[14]。Li等[15]发现非小细胞肺癌组织中lncRNA NEAT1高表达，且与TNM分期、淋巴结转移、远处转移、预后差等临床病理特征有关，沉默该基因后，肿瘤细胞增殖和侵袭明显受抑制。Chai等[16]报道，在卵巢癌肿瘤组织和癌细胞中lncRNA NEAT1高表达，不仅与临床分期和淋巴结转移相关，且高表达后促进肿瘤细胞恶性生物学行为，而敲除后则相反。另有Ke等[17]发现lncRNA NEAT1通过miR-548调控乳腺癌细胞存活。Gao等[18]证实lncRNA NEAT1通过抑制乳腺癌抵抗蛋白抑制白血病患者多药耐药性。

细胞增殖、迁移、侵袭对肿瘤转移和复发起重要作用，本研究通过qRT-PCR发现lncRNA NEAT1在HepG2，SMMC-7721，Huh7细胞系中的表达显著高于正常肝细胞，提示其可能作为促癌基因参与肝癌发生发展过程。LncRNA NEAT-siRNA载体干扰HepG2细胞中lncRNA NEAT1表达后，其增殖、转移、侵袭能力均明显受削弱。已有研究[19]证实：miR-211通过下调SATB2抑制肝细胞癌生长和转移。本研究中，笔者采用qRT-PCR发现NEAT1-siRNA组中miR-211 mRNA及SATB2蛋白表达水平分别是Control-siRNA组的(7.32±0.59)倍和(0.46±0.04)倍，表明lncRNA NEAT1负调控miR-211、正调控SATB2表达。

MiR-211是miRNA家族成员，参与细胞增殖、凋亡、侵袭、分化、血管生成、免疫应答、物质代谢等多种生物学过程[20]。近年来，miRNAs与肿瘤发生发展的密切关系引起人们广泛关注，miR-211作为肿瘤抑制因子参与调节卵巢癌[21]、黑色素瘤[22]、胰腺癌[23]等肿瘤增殖、迁移或侵袭等恶性细胞生物学行为。更重要的是，miR-211被发现在肝癌肿瘤组织中表达较低而在临近正常组织中表达较高，在肝癌HepG2和SMMC7221细胞中过表达miR-211后，肿瘤细胞增殖和侵袭显著受到抑制[19]。此外，荧光素酶报告基因和蛋白检测发现SATB2是miR-211的直接靶基因，miR-211通过负调控SATB2发挥抑癌功能。

SATB2是一种富含AT序列的DNA结合蛋白，编码733个氨基酸，能够特异性结合到核基质附着区，参与转录调控和染色质重塑[24]。Magnusson等[25]采用免疫组织化学分析大量结肠癌患者的SATB2蛋白表达，发现SATB2在结肠癌原发灶和转移灶中表达较高。Patani等[26]发现乳腺癌组织表达SATB2的水平高于正常组织，SATB2随肿瘤分级增加而表达上升，且高表达的患者生存率往往较差。Jiang等[19]发现：肝癌患者SATB2过表达促进HepG2细胞生长，且SATB2能阻碍miR-211介导的肝癌细胞增殖和侵袭。本研究发现lncRNA NEAT1敲除后HepG2细胞中miR-211表达显著上调，SATB2表达下调。基于Jiang等[19]证实miR-211与SATB2 3'UTR存在绑定结合，我们推测lncRNA NEAT1沉默表达抑制肝癌细胞增殖、侵袭、迁移，可能是通过负调控miR-211，进而影响miR-211与SATB2的绑定结合，因此lncRNA NEAT1作为肿瘤促癌因子参与肝癌生长、转移等生物过程。

当然，由于本研究在体外细胞系中进行，尚存在一定的局限，如lncRNA在肝癌组织和正常肝组织中的表达水平及其其对预后的影响尚不得而知，值得更进一步研究，同时也是本研究的后续研究方向。

综上，本研究表明lncRNA NEAT1在肝癌细胞系中高表达，沉默lncRNA NEAT1的表达可抑制肝癌细胞增殖、迁移和侵袭，其机制与调控miR-211/SATB2信号途径有一定关联。该研究对lncRNA NEAT1肿瘤生物学功能所做的基础性研究，将为肝癌发病机理的阐明和新的治疗手段的开发提供一定的理论基础。

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