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LTF 基因在鼻咽癌细胞系中生物学功能的初步研究*

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摘要 目的: 初步探讨野生型 LTF 基因在鼻咽癌细胞系中的生物学功能。**方法:** 野生型 LTF 导入鼻咽癌细胞系, G418 筛选, RT-PCR 和 Western-blotting 分别在 mRNA 和蛋白质水平进行验证, 得到稳定表达 LTF 基因的鼻咽癌细胞系。流式细胞术、平板克隆形成实验和 MTT 法分别检测细胞周期、细胞的克隆形成能力和细胞生长曲线。**结果:** 成功导入 LTF 并稳定表达的鼻咽癌细胞系, G0-G1 期细胞百分比比例明显增加(72.01 % vs 62.31 %), G2-M 期细胞百分比比例减少(6.26 % vs 10.81 %); 克隆形成能力降低(39.5 % vs 59.7 %), 体外瘤细胞增殖能力降低($P < 0.05$)。**结论:** LTF 基因可阻滞细胞周期、抑制鼻咽癌细胞系的增殖能力和克隆形成率, 同时为进一步的体内试验研究奠定基础。

关键词: LTF; 鼻咽癌; 真核表达; 抑瘤基因

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The Preliminary Study of Biological Activity of LTF Gene in NPC Derived Cell Line*

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ABSTRACT Objective: To preliminarily study the biological characteristics of lactotransferrin (LTF) gene in nasopharyngeal carcinoma (NPC) derived cell lines. **Methods:** Wild-typed LTF gene was transfected into NPC derived cell line. After G418 selection and confirmation the expression at mRNA and protein level by RT-PCR and Western blotting analysis respectively, Cell cycle, cell proliferation and colony formation ability were detected by flow cytometry(FCM) analysis, MTT analysis and colony formation assay respectively. **Results:** LTF expression in NPC cell line could block the cell cycle progression in G1 phase, leading to an increase in the number of cells in G1 phase (72.01 % vs 62.31 %) and a decrease in the number of cells in G2/M phase (6.26% vs 10.81%); And they had a much lower colony efficiency (39.5 % vs 59.7 %) and proliferated much more slowly($P < 0.05$). **Conclusions:** Evidence showed that LTF blocked the cell cycle progression in G1 phase and had an inhibitory effect on proliferation and colony formation efficiency of NPC cells. The obtainment paved the way for further study on biological functions of LTF in vivo.

Key words: LTF; NPC; Eukaryotic expression; Tumor inhibitory gene

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前言

鼻咽癌(nasopharyngeal carcinoma, NPC)是一种上皮源性的恶性肿瘤, 好发于我国南方及东南亚^[1]。遗传易感性、Epstein-Barr 病毒(Epstein-Barr virus, EBV)感染及环境因素等与鼻咽癌的发生密切相关。研究发现染色体 3p 的杂合性缺失是鼻咽癌中的早期遗传学变异事件^[2,3]。3p21.3 上存在多个鼻咽癌候选抑瘤基因, 如 DLC1、RASSF1A、SEMA3B、BLU 和 GNAT 等, 都在鼻咽癌中都有不同程度的表达下调或缺失。

LTF (lactotransferrin, LTF)基因定位于 3p21.3。LTF 在胶质细胞瘤、乳腺癌、胃癌和肺癌等恶性肿瘤中均表达下调^[4-6]。提示其表达对肿瘤具有抑制作用。LTF 在慢性鼻咽炎组织中表达正常, 在鼻咽癌组织和多个鼻咽癌细胞株^[7,8]中均表达下调。鼻咽癌系统发生树模型和基因芯片数据提示及 LTF 的多态性分析^[9]

提示, LTF 是鼻咽癌中可能的候选抑瘤基因。为了进一步探讨 LTF 与鼻咽癌的关系, 本研究将野生型 LTF 基因导入鼻咽癌细胞株, 并体外观察其生物学特性的改变。

1 材料和方法

1.1 材料

1.1.1 细胞系 CNE1: 鼻咽高分化鳞状细胞癌细胞系(中国科学院肿瘤医院研究所建系), 细胞呈上皮样、贴壁生长。RPMI1640+10% FBS 的培养基、37 °C、5 % CO₂、饱和湿度条件下培养。

1.1.2 菌株和载体 E.Coli DH5 α 菌株为本校重点实验室保存。pcDNA3.1 (-) 载体 (Invitrogen), pCMV6-XL5-LTF 载体 (Origene)。

1.1.3 主要试剂和试剂盒 LipofectAMINE2000、TRIzol、逆转

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录试剂盒、RPMI1640、OPTI-MEM(Invitrogene);G418(Merck);LA Taq 酶及 *Not*I 内切酶(Takara);MTT(Amresco);T4 DNA Ligase、预染蛋白质分子量标准(Fermentas);BCA Assay Reagent Kit、ECL 化学发光试剂盒(Pierce);Hybond 硝酸纤维素膜(0.45 μ m)(Amersham pharmacia Biotech);Rabbit polyclonal to lactotransferrin(Abcam);Mouse monoclonal to α -Tubulin、monoclonal goat anti-rabbit immunoglobulins/HRP、Goat anti-mouse IgG/HRP(Santa Cruz)。

1.2 方法

1.2.1 构建 LTF 基因真核表达载体 pCMV6-XL5-LTF 载体包含 LTF cDNA 序列(NM_002343.1),测序并 blast 比对进行验证。*Not*I 单酶切并回收 LTF 序列,体外连接 pcDNA3.1(-)载体,转化 DH5 α ,筛选、挑克隆、测序,blast 分析插入方向。

1.2.2 建立稳定转染 LTF 的鼻咽癌细胞系 阳离子脂质体法转染:对数期细胞(80%融合度)洗涤 2 次。配 A 液:50 l OPTI-MEM+2 l lipofectAMINE2000;B 液:50 l OPTI-MEM+0.5 g 质粒。A、B 两液混合,加 0.4 mL OPTI-MEM,混合后均匀滴加细胞表面,培养 6h 后换成完全培养基;36h 后 900 μ g/ml G418 筛选,出现多个抗性克隆,挑克隆,半量 G418 维持并扩大培养,获得稳定的抗性克隆。同时转染空载体作为对照。

1.2.3 制备用于 Western blotting 的蛋白质 取细胞 1 瓶,约 1×10^6 个,PBS 洗 4 次,加入蛋白抽提 lysis buffer (50 mM Tris-Cl (pH8.0),150 mM NaCl,0.02 % 叠氮钠,0.1 % SDS,100 μ g/ml PMSF,1 μ g/ml Aprotinin,1% Nonidet P-40 or Triton X-100,0.5 % Na-deoxycholate),冰上放置 20 min;用刮子刮下并转移至 1.5 mL 离心管。冰上超声裂解细胞 30 s;12000 $\times$ g,4 $^{\circ}$ C 离心 10 min,吸上清,分装,存于 -70 $^{\circ}$ C。BCA 法测定蛋白质浓度。

此外,细胞总 RNA 的提取、RT-PCR 实验,Western blotting 检测实验,流式细胞仪检测细胞周期实验、克隆形成率与 MTT 细胞活性检测实验等具体步骤参照文献进行^[10]。

2 结果

2.1 LTF 真核表达载体测序鉴定

构建 LTF 真核表达载体并小量摇菌测序。Blast 比对结果显示 LTF 序列及连接方向正确(图 1)。获得的重组载体命名为 pcLTF。

2.2 建立稳定转染 pcLTF 的鼻咽癌细胞系

转染 pcLTF 和空载体 pcDNA3.1(-)入 CNE1 后,获得稳定的抗性克隆分别命名为 CNE1.pcLTF 和 CNE1.pcDNA(图 2)。

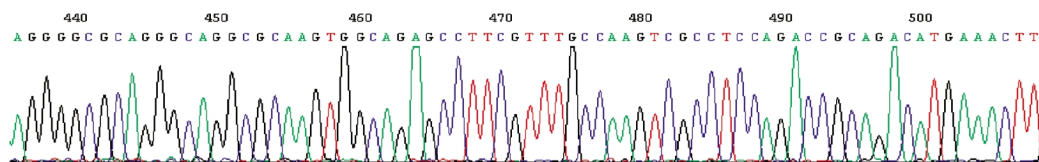


图 1 构建的 LTF 真核表达载体的部分测序图

Fig.1 Sequencing results of recombinant vectors containing LTF gene

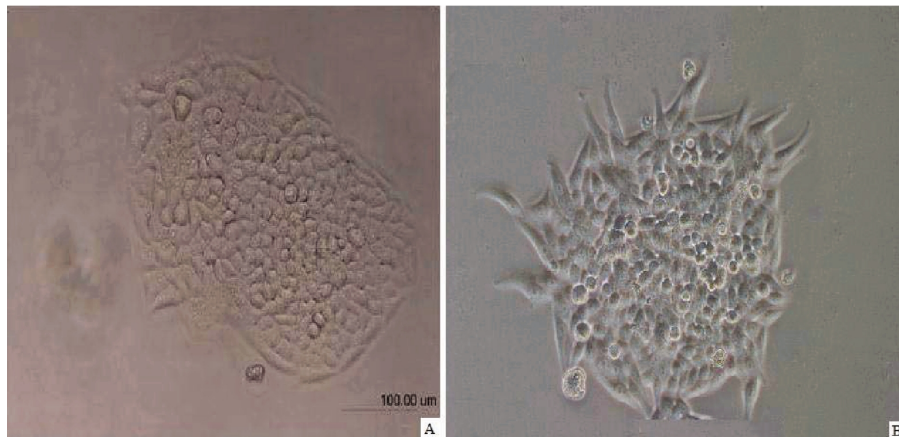


图 2 G418 pcLTF 与 pcDNA3.1(-)分别转染 CNE1 细胞,经筛选得到抗性克隆

A: CNE1.pcLTF 细胞克隆 B: CNE1.pcDNA 细胞克隆 ($\times 40$ 倍)

Fig.2 After pcLTF and pcDNA3.1(-) transfection respectively, several G418-resistant cell clones were obtained on the basis of G418 selection

A: CNE1.pcLTF cell clone; B: CNE1.pcDNA cell clone ($\times 40$)

2.3 检测 CNE1.pcLTF 细胞中 LTF 在 mRNA 和蛋白质水平的表达

LTF 基因的表达情况:在 mRNA 水平上,对照组中不表达,CNE1.pcLTF 细胞的 3 个克隆中均表达(图 3);在蛋白质水平上:两组内对照 α -Tubulin (约 55KD)均表达,在对照组中未检测到 LTF 蛋白的表达,CNE1.pcLTF 细胞中均检测到 LTF 蛋

白(约 80KD)的表达(图 4)。

2.4 检测 LTF 对鼻咽癌细胞系生物学特性的影响

2.4.1 LTF 对鼻咽癌细胞系细胞周期的影响 与转染空白载体的细胞相比,CNE1.pcLTF 细胞 G0-G1 期细胞比例明显增加(72.01% vs 62.31 %),S 期 (21.73 % vs 26.86 %) 和 G2-M 期 (6.26 % vs 10.83 %)细胞比例减少(图 5)。

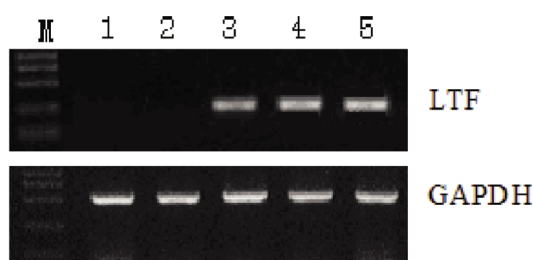


图3 RT-PCR 检测 LTF 基因在 CNE1.pcLTF 细胞中 mRNA 水平的表达 (1.0%琼脂糖凝胶)。M:DL2000;Lane1: CNE1 细胞;Lane2: CNE1.pcDNA 细胞;Lane3-5: CNE1.pcLTF 细胞的 3 个阳性克隆。

Fig.3 LTF expression in CNE1.pcLTF cells at mRNA level detected by RT-PCR. (electrophoresis on a 1.0% agarose gel). (M: DL2000 marker; Lane1:CNE1cells; Lane2:CNE1. pcDNA cells; Lane3-6:CNE1.pcLTF cells.)

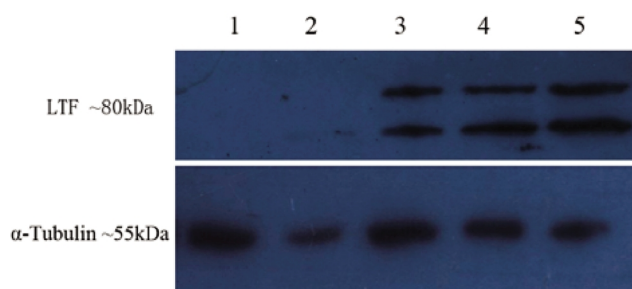


图4 Western blotting 检测 LTF 基因在 CNE1.pcLTF 细胞中蛋白水平表达情况。Lane1: CNE1 细胞;Lane2: CNE1.pcDNA 细胞;Lane3-5: CNE1.pcLTF 细胞。

Fig.4 LTF expression in CNE1.pcLTF cells at protein level detected by Western blotting analysis. (Lane1: CNE1cells; Lane2:CNE1.pcDNA cells; Lane3-5:CNE1.pcLTF cells.)

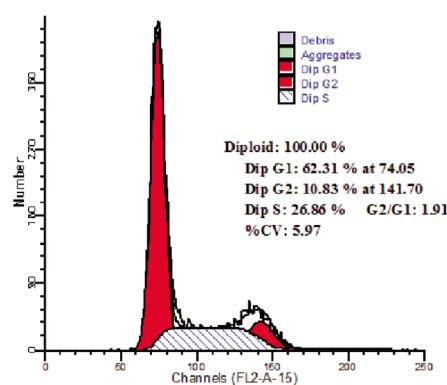
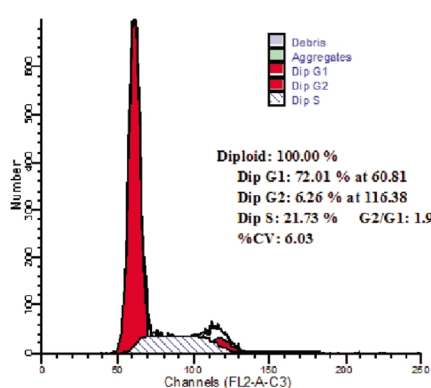


图5 流式细胞术检测 LTF 基因对 CNE1.pcLTF 细胞周期分布的影响 (左: CNE1.pcLTF 细胞;右: CNE1.pcDNA 细胞)

Fig.5 Flow cytometric analysis showed the effect of LTF on the cell cycle of CNE1.pcLTF(left: CNE1.pcLTF cells; right: CNE1.pcDNA cells)

2.4.2 LTF 对鼻咽癌细胞系增殖能力的影响 实验重复 3 次, 4.7%, 明显低于 CNE1.pcDNA 的平板克隆形成率 $59.7\% \pm 3.8\%$ (图 6)。结果显示: CNE1.pcLTF 细胞的平板克隆形成率为 $39.5\% \pm 4.7\%$ 。

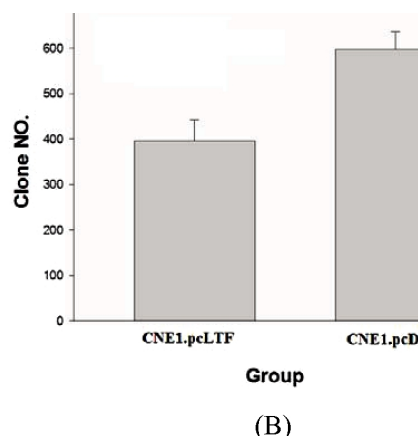
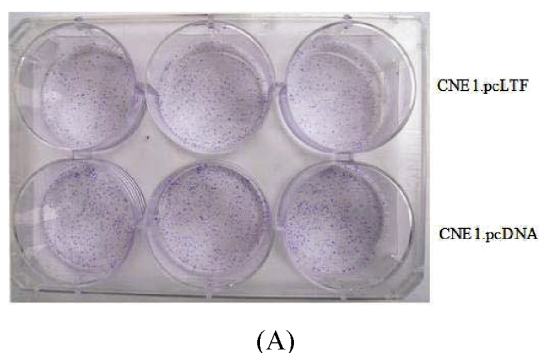


图6 CNE1.pcLTF 和 CNE1.pcDNA 细胞平板克隆形成情况 (CNE1.pcLTF 克隆数: 395 ± 47 ; CNE1.pcDNA 克隆数: 597 ± 38)

Fig.6 Colony formation efficiency of CNE1.pcLTF cells and CNE1.pcDNA cells detected by Colony formation assay. (Colony number: CNE1.pcLTF: 395 ± 47 ; CNE1.pcDNA: 597 ± 38)

2.4.3 LTF 对鼻咽癌细胞系生长曲线的影响 生长曲线结果表明 CNE1.pcDNA 和 CNE1.pcLTF 两组细胞的增殖速度于第 3 天时出现显著性差异并持续至第 7 天 ($P < 0.05$)。

3 讨论

LTF 基因具有调节机体免疫、抗炎症、抗微生物及抗肿瘤

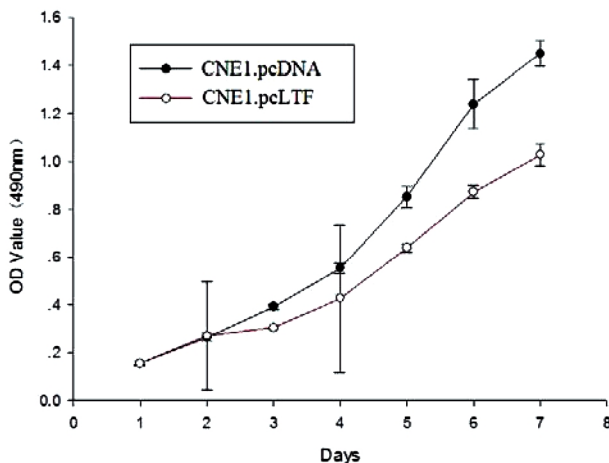


图 7 MTT 法检测稳定转染 LTF 对 CNE1 细胞生长的影响横坐标:时间(天) $n=7$;纵坐标:OD490 值

Fig.7 Effect of LTF expression on of proliferative ability of CNE1 cells assessed by MTT assay. X-bar: Days the cells were cultured Y-bar: The average absorbance values obtained from wells were served as a blank and were subtracted from all other results. ($P<0.05$).

等多种生物学功能,其中的抗肿瘤作用引起了无数肿瘤研究者的广泛关注。LTF 基因表达产物乳铁蛋白是一种分泌性糖蛋白,可表达于乳汁和其它外分泌液中,是非特异性免疫的重要组成部分,对细菌、病毒、真菌等多种微生物具有抵抗作用。人乳铁蛋白有三个糖基化位点,分别是 N-lobe 的 Asn138 和 C-lobe 的 Asn479,Asn624^[11,12]。由于糖基化机会不均等,造成人乳铁蛋白在电泳上主要表现为两条带:主带和副带。本实验中的 Western blotting 结果与之相吻合。鼻咽癌的发生与 EBV 病毒(Epstein-Barr virus)感染有关,Zheng 等^[13]研究表明 LTF 可以通过抑制 EB 病毒的感染,从而通过抗微生物作用来降低鼻咽癌的发生。本研究结果显示,稳定表达野生型 LTF 的鼻咽癌细胞系 CNE1,G0-G1 期细胞比例明显增加,G2-M 期细胞比例减少,细胞周期阻滞于 G0/G1。但其发生的机制尚未完全明确。Son 等^[14]发现 LTF 蛋白可以上调低磷酸化 Rb(retinoblastoma protein,Rb)蛋白的表达,Rb 蛋白通过结合 E2F1 从而持续性抑制 E2F1 相关基因的作用;同时低磷酸化 Rb 通过上调 p21 的表达,抑制 G1-S 期 CDKs(Cyclin-dependent kinases,细胞周期素依赖性激酶)的活性,实现细胞周期阻滞。此外,LTF 蛋白还通过激活 NF- κ B(nuclear factor-kappaB,NF- κ B)途径反式激活肿瘤抑制基因 p53 的表达而实现对细胞周期阻滞^[15]。此外, Δ LTF 蛋白过表达可增强 Skp1(S-phase kinase-associated protein,S 期激酶相关蛋白)表达,参与细胞周期调控^[16]。Zhou 等^[17]采用 LTF 处理鼻咽癌细胞系 5-8F,结果显示细胞周期素 1(CyclinD1)和 Rb 表达下调,p21 和 p27 表达上调。Deng^[17]等在鼻咽癌中研究表明,LTF 基因可以通过两个独立的机制抑制 AKT 信号通路:①抑制 MAPKs(the mitogen-activated protein kinase)/c-Jun 通路,抑制 PDK1(3-phosphoinositide-dependent protein kinase 1);②LTF 和角蛋白 K18(keratin 18)相互作用,阻断 K18-14-3-3 复合物的形成,从而下调 K18 介导的 ALK 的活化。综上所述,LTF 蛋白可通过调控细胞周期的多条通路,诱

导肿瘤细胞周期阻滞,从而抑制包括鼻咽癌在内的多种肿瘤细胞增殖。

LTF 可以通过多种机制发挥抗肿瘤作用:①化学预防作用^[18],它能降低化学致癌物诱导的恶性肿瘤的发生。②通过抗微生物来降低肿瘤的发生。③通过调节免疫反应增强机体的抗肿瘤作用^[19]。④直接抗肿瘤作用,LTF 可直接抑制肿瘤细胞的增殖、促进凋亡、抑制血管形成^[20]和肿瘤转移^[21]。LTF 在鼻咽癌中表达下调,是鼻咽癌发生发展的一个重要原因。Zhang 等^[22]通过对 7 株鼻咽癌细胞系的分析,发现 LTF 基因甲基化率为 100%(7/7),表明启动子区高甲基化可能是鼻咽癌细胞系中 LTF 基因表达失活的重要原因之一。表遗传学因素和遗传学因素共同参与 LTF 在鼻咽癌中表达调控。

本研究进一步证实 LTF 基因是鼻咽癌的一个重要候选抑瘤基因。下一步我们将进行体内实验及其具体作用机制的研究,进一步探讨该区域的 LTF 在 NPC 中的功能,也有利于阐明染色体 3p21.3 区与 NPC 发生发展的确切关系。

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