Lipoxin A_4通过调节肿瘤相关巨噬细胞对肝癌细胞株HepG2 microRNA表达谱的影响

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摘要 肿瘤与炎症密切相关, 作者以往已证实内源性促炎症缓解介质脂氧素A_4(Lipoxin A_4, LXA_4)能在整体和细胞水平发挥抗肝癌细胞增殖和转移的作用。为进一步探讨LXA_4通过调节肿瘤相关巨噬细胞对肝癌细胞株HepG2 microRNAs(miRNAs)表达谱的影响, 该文首先提取经脂多糖(LPS)或LPS+LXA_4作用24 h的人巨噬细胞株U937培养上清液, 分别称为ACM或LCM, 以模拟肿瘤的炎症微环境, 并用此上清液刺激HepG2细胞, 24 h后提取细胞总RNA, 采用microRNA芯片miRCURY™ LNA Array(V16.0)检测, 计算各样本中的miRNAs标准值及比值。以两组间Hy3荧光标记信号强度的比值≤0.5或≥2为标准判定差异表达miRNA。Real-time PCR检测hsa-miR-623的相对含量以验证基因芯片的结果。结果发现, 与对照组细胞相比, 经过ACM作用24 h的HepG2细胞有35个miRNAs上调、130个miRNAs下调。LCM组与ACM组相比, HepG2细胞有185个miRNAs上调、71个miRNAs下调。 Real-time PCR检测的结果证实, hsa-miR-623的变化与基因芯片趋势一致。综上所述, LXA_4能通过肿瘤相关巨噬细胞而间接发挥其调节HepG2细胞miRNAs表达谱的作用。

关键词 肝癌; 微小RNA; 脂氧素

Effect of Lipoxin A_4 on MicroRNAs Expression Profile in HepG2 Cells through Modulating the Tumor Associated Macrophages

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Abstract On the basis that the pathologic process of tumor is closely related to uncontrolled inflammation, we have proved that Lipoxin A_4 (LXA_4) could inhibit the proliferation and metastasis of HCC cells both *in vitro* and *in vivo*. This study further investigated the indirect effect of Lipoxin A_4 (LXA_4) on the microRNA (miRNA) expression profile in HepG2 cells through modulating the tumor-associated macrophages. Conditioned cell culture media from LPS-stimulated and LPS/LXA_4 co-stimulated U937 cells, as ACM and LCM respectively, were collected.

收稿日期: 2013-10-10 接受日期: 2014-01-20

国家自然科学基金(批准号: 81272313)和中央高校基本科研业务费华中科技大学(批准号2012QN32、2013-313)资助的课题

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Received: October 10, 2013 Accepted: January 20, 2014

This work was supported by the National Natural Science Foundation of China (Grant No.81272313) and the Fundamental Research Funds for the Central Universities, HUST (Grant No.2012QN32, 2013-313)

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网络出版时间: 2014-05-05 10:02 URL: http://www.cnki.net/kcms/doi/10.11844/cjcb.2014.05.0324.html

HepG2 cells were separated into control group, ACM group and LCM group, which were cultured in normal culture media, ACM and LCM for 24 hours, respectively. miRNAs were extracted and hybridized to miR-CURY™ LNA Array (V16.0). It was considered to be up- or down- regulated when the miRNAs fluorescent intensity ration between two groups was over 2 or less than 0.5. Validation of microarray results was carried out by Real-time PCR of hsa-miR-623. Compared with control group, ACM treatment for 24 h up-regulated 35 miRNAs and down-regulated 130 miRNAs in HepG2 cells, while LCM group had 185 miRNAs higher and 71 miRNAs lower than ACM group. Hsa-miR-623 showed similar variation with microarray. Our study indicated that LXA_4 could indirectly regulate the miRNAs expression profile in HepG2 cells through modulating the tumor-associated macrophages.

Key words hepatic carcinoma; microRNA; Lipoxin

肿瘤是目前严重影响人类生命和健康的疾病之一。经过多年的研究, 虽然在原癌基因、抑癌基因以及肿瘤相关的信号转导通路等方面都取得了较多令人兴奋的成果, 但肿瘤发生、发展的确切机制仍有许多未解之谜[1]。microRNAs(miRNAs)是一类新近发现的非编码小分子RNA, 包含21~25个核苷酸, 是哺乳动物基因组中最为丰富的调节基因之一。迄今, miRNA的基因序列数据库miRBase(http://microrna.sanger.ac.uk/)已收录了约1 900种人类miRNA[2]。近年来的研究显示其在肿瘤的发生、发展过程中发挥着重要的调节作用[3]。

肿瘤相关巨噬细胞(tumor-associated macrophages, TAMs)处于炎症和肿瘤交叉路口这一特殊位置, 参与了肿瘤的发生、转移、基质重构、血管新生等几乎各个环节[4]。本课题组以往证实, 内源性促炎症缓解介质脂氧素A_4(Lipoxin A_4, LXA_4)能抑制巨噬细胞的炎症反应[5-6], 亦能在整体和细胞水平发挥抗肝癌细胞增殖和转移的作用[7-8]。在此基础上, 我们还证实, 经过LPS活化的巨噬细胞其上清液作用于HepG2肝癌细胞株后, 对后者的增殖和迁移均有促进作用, 而LXA_4对上述作用具有明显的抑制[7]。那么, LXA_4的这一作用与HepG2细胞的miRNAs表达有何关联, 目前还未见报道。本文采用miRNA芯片技术, 初步研究了LXA_4通过调节TAM对肝癌细胞株HepG2 miRNAs表达谱的影响。

1 材料与方法

1.1 细胞与试剂

人肝癌细胞株HepG2和人单核细胞株U937购自中科院上海细胞库。1640培养基和胎牛血清(FBS)为Gibco公司产品; LXA_4购自Cayman公司; 脂多糖(lipopolysaccharide, LPS)购自Sigma公司; Trizol购自Invitrogen公司; miRNA RT-PCR相关试剂购自QIAGEN公司; miRCURY™ Hy3™/Hy5™ Power labeling kit和miRCURY™ LNA Array kit购自Exiqon公司; 0.45 μm滤器为Fisher Scientific公司产品。

1.2 试验方法

1.2.1 细胞培养 细胞在含有10%胎牛血清、2%谷氨酰胺、100 U/mL链霉素和100 U/mL青霉素的1640完全培养基中, 置于37 °C、5% CO_2培养箱内常规培养, 细胞贴壁达到80%~90%时进行传代。

1.2.2 巨噬细胞上清液的制备和收集 经常规培养、无血清化过夜后, U937巨噬细胞分别于100 ng/mL LPS或100 ng/mL LPS+100 nmol/L LXA_4中培养24 h。弃培养基并用PBS洗涤2次、无血清培养基中继续培养4 h以清除残余刺激物。最后, 细胞在完全培养基中生长24 h后收集上清液, 0.45 μm滤器过滤。经LPS刺激的巨噬细胞上清液称为ACM(activated macrophage-conditioned media), 经LPS+LXA_4作用的巨噬细胞上清液称为LCM(Lipoxin conditioned media)。

1.2.3 实验分组 按本课题组建立的条件[7], HepG2细胞分为空白对照组、ACM和LCM组, 分别采用完全培养基、50% ACM+50%完全培养基、50% LCM+50%完全培养基作用24 h。

1.2.4 细胞总RNA的提取和鉴定 参照Trizol说明书, 采用一步法收集细胞总RNA。紫外分光光度计测定RNA的浓度和纯度。取1 μg总RNA进行1.5%甲醛变性凝胶电泳, 分析RNA质量。

1.2.5 芯片分析差异表达的miRNA 应用丹麦Exiqon公司生产的microRNA芯片miRCURY™ LNA Array(V16.0)进行检测, 由上海康成生物工程有限公司代理检测和分析。该芯片检测了miRBase(版本16.0)收录的1 891个属于人、小鼠或大鼠的miRNA以及66个Exiqon公司独家的microRNA。分别取

1 μg上述各组细胞样本的总RNA, 采用miRCURY™ Hy3™/Hy5™ Power labeling kit进行荧光标记、芯片杂交。其后进行图像扫描, 所得数据通过原始值减去背景值进行修正, 并用中值标准化, 分别计算各样本中的miRNAs标准值及比值。以两组间Hy3荧光标记信号强度的比值≤0.5或≥2为标准判定差异表达miRNAs。

1.2.6 定量PCR验证差异表达的miRNAs 为进一步验证芯片结果, 选取在芯片实验中发现的几组细胞之间含量有明显差异的hsa-miR-623进行实时荧光PCR验证。按照操作指南, 首先以细胞总RNA为模板, 用miScript II RT kit逆转录生成cDNA; 进一步采用hsa-miR-623的特异引物Hs_miR-623_1 miScript Primer Assay和通用引物The miScript Universal Primer, 利用miScript SYBR Green PCT kit进行实时荧光PCR。内参为U6, 其特异性引物采用Hs_RNU6-2_11 miScript Primer Assay。反应条件为: 95 °C预变性15 min; 94 °C 15 s, 55 °C 30 s, 70 °C 30 s, 40个循环; 反应结束后得到各组目的miRNA和内参的*Ct*值。以*ΔΔCt*法计算hsa-miR-623的相对表达量。

2 结果

2.1 细胞总RNA质量检测

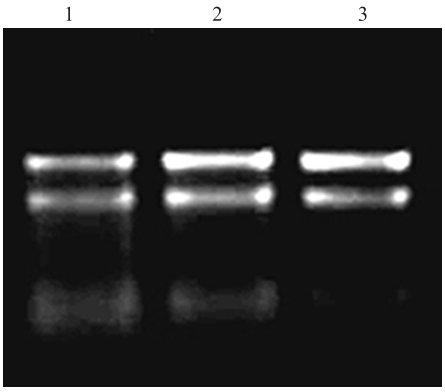
从各组细胞中提取的RNA浓度分别为897.17, 926.88, 849.43 mg/L。$D_{260/280}$值均在2.03~2.04之间。其18S和28S条带清晰, 总RNA纯度达到实验要求且无明显降解(图1)。实验结果提示, 来自3组细胞的总RNA质量较好, 能满足后续miRNA芯片实验的需要。

2.2 miRNA芯片检测结果

来自3组细胞的总RNA经过Hy3荧光标记后, 同miRNA芯片进行杂交。结果显示, 与空白组细胞相比, 经过ACM作用24 h的HepG2细胞有35个miRNA上调、130个miRNA下调。LCM组与ACM组相比, HepG2细胞有185个miRNA上调、71个miRNA下调(图2)。表1列出部分相对含量变化明显的miRNAs, 并总结了其相应的功能。

2.3 hsa-miR-623 Real-time PCR结果

为了进一步验证基因芯片的结果, 我们随机选取了在miRNA芯片中具有差异表达的hsa-miR-623进行RT-PCR检测, 数据表明其变化趋势与基因芯片相同: ACM作用后的HepG2细胞hsa-miR-623表达下调, 相反, LCM组的表达较ACM增高(表2)。

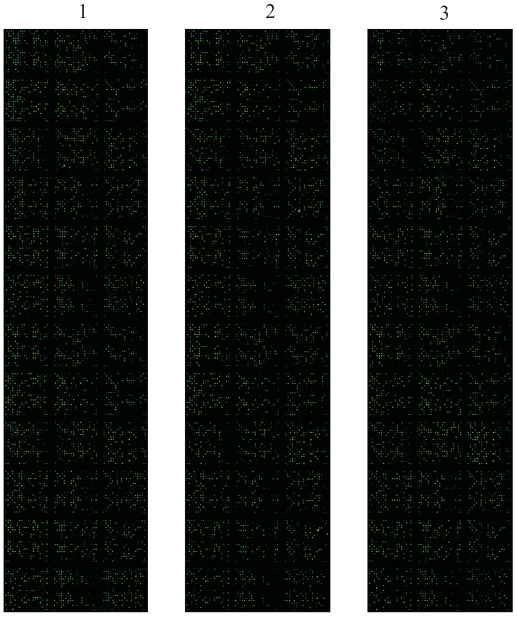


1: 对照组; 2: ACM组; 3: LCM组。

1: control group; 2: ACM group; 3: LCM group.

图1 三组细胞的RNA甲醛变性琼脂糖凝胶电泳图

Fig.1 RNA agarose gel electrophoresis of HepG2 cells



1: 对照组; 2: ACM组; 3: LCM组。细胞分别采用完全培养基、50% ACM+50%完全培养基、50% LCM+50%完全培养基作用24 h, 然后进行miRNA芯片检测。

1: control group; 2: ACM group; 3: LCM group. Before analyzed with miRNA microarray, cells were cultured in completed medium, 50% ACM+50% complete medium and 50% LCM+50% complete medium for 24 h, respectively.

图2 各组HepG2细胞的miRNA芯片扫描图

Fig.2 miRNA microarray maps of HepG2 cells

3 讨论

自1863年最初观察到肿瘤组织周围有炎性细胞浸润[9], 其后大量证据提示肿瘤与炎症息息相关, 肿瘤相关炎症甚至被称为肿瘤的另一半[10]。LX(Lipoxin)是花生四烯酸经过脂加氧酶(lipoxygenase, LOX)代谢途径的产物, 已被证实为体内最重要的抗炎及促炎症消退脂质, 具有“炎症刹车信号”

之称[11]。

我们以往针对移植H22肝癌细胞的小鼠研究证实, LXA_4及其类似物不仅能减缓移植部位肿瘤的生长, 还显著抑制了肿瘤的肺转移[8], 其机制可能与LXA_4抑制肿瘤内部血管新生[8]、减弱肝癌细胞的侵袭能力有关[12]。我们进一步发现, LXA_4处理后的移植肝癌小鼠的癌组织里, TAMs数量减少, 且由弥漫分布于肿瘤内部转向肿瘤周围[8]; 在细胞水平, LXA_4能通过巨噬细胞间接抑制肝癌细胞迁移和增殖[7]。

TAMs处于炎症和肿瘤交叉路口这一特殊位置[4], 为肿瘤微环境的重要组成部分, 也是目前肿瘤治疗策略的研究热点。为深入探讨TAMs在LXA_4抑制肝癌细胞中的作用, 本实验中我们将miRNAs作为研究的靶点。miRNA是一个在进化上高度保守的小分子RNA家族, 主要通过结合于靶mRNA的3′末端非翻译区介导蛋白翻译阻抑, 或者通过诱导靶mRNA的3′端去腺苷酸化或5′端去帽而介导靶mRNA的快速降解, 对靶基因的表达进行调节。其在调节肿瘤细胞增殖、分化、凋亡等方面扮演了重要的角色[3]。

以往对miRNAs的研究多集中于比较其在肿瘤组织与正常组织的差异, 本文采用已发表的方法[7], 以LPS活化巨噬细胞并收集其培养上清液以模拟肿瘤的炎症微环境, 进而用此ACM刺激HepG2细胞以期探讨肿瘤微环境对肝癌细胞miRNA的影响。诚然, 肿瘤微环境中的细胞种类很多, 除了TAM还包括其他如血管内皮细胞、白细胞、纤维母细胞甚至是脂肪细胞等; 同时, 肿瘤的微环境也并非单由炎症环境构成, 其他如缺氧等微环境也影响了肿瘤的发展。因此, 本文所采用的LPS刺激模型只能帮助我们从一个侧面研究LXA_4的作用。结果发现LPS能通过活化巨噬细胞而改变肝癌细胞的miRNAs表达谱(表1): 如能上调hsa-miR-181a, 后者被发现在慢性淋巴细胞白血病细胞中高表达[13]; 上调hsa-miR-125, 后者能抑制抑癌基因*p53*从而促进肿瘤生长[14]; 上调miR-30a, 后者与肿瘤对细胞外基质的黏附有关; 上调促进EMT的miR-141和miR-216。

同时, 为了探讨LXA_4通过肿瘤微环境的间接作用, 我们也收集了LXA_4和LPS共同作用的巨噬细胞上清液刺激HepG2细胞, 数据显示, LXA_4亦能通过对活化的巨噬细胞发挥作用, 间接改变肝癌细胞的miRNAs。一项涉及83例肝细胞肝癌的临床调查显示, 肝癌组织的miR-34a明显低于正常组织, 且TNM III期和IV期的患者低于I期和II期患者, 同时, 发生转移的患者低于未发生转移的患者; 在细胞水平还证实, 转染了miR-34a的肝癌细胞其增殖减缓、转移和浸袭能力减弱[15]。本文中, 芯片结果显示, ACM刺激组miR-34a仅为对照组细胞的45%, 而LXA_4处理过的巨噬细胞上清液明显增加了HepG2细胞的miR-34a的表达。这有可能是LXA_4抑制肝癌的原因之一。除此之外, 结果还发现, LCM能下调其他促癌的miRNAs如miR-27a, 上调抑癌的miRNAs如miR-22等。实体瘤的发展需要血管新生, 本试验中LCM能上调某些抑制血管新生的miRNA, 如miR-320和miR-20b。另外, miRNA芯片结果还表明, 多种涉及到EMT的miRNAs如miR-30a、miR-203也受到LCM

表1 各组HepG2细胞部分miRNAs变化情况

Table 1 Changes of miRNAs in HepG2 cells with different treatments

	miRNAs miRNAs	倍数 Ratio	功能 Function
ACM group *vs* control group	hsa-miR-216b	192.60	Down-regulated the expression of CKIIα in colon cancer[16]
	hsa-miR-181a	12.93	Highly expressed in chronic CLL cells[13] and gastric cancer tissue[17]; In malignant glioma cells, has-miR-181a was down-regulated by radiation treatment[18]
	hsa-miRPlus-G1267-5p	9.84	Patent product of Exiqon
	hsa-miR-30a	8.91	Down-regulated in colon cancer. miR-30a suppresses cell migration and invasion through downregulation of PIK3CD in colorectal carcinoma[19]
	hsa-miR-623	0.05	miR-623 was up-regulated in transient depletion of TIA-proteins in HeLa cells[20]
	hsa-miR-507	0.03	Differently expressed between decidualized and undecidualized endometrial stromal cells[21]
	hsa-miR-1180	0.03	Expressed in human sarcomas[22]
	hsa-miR-3130-5p	0.02	Not reported
	hsa-miR-2114*	0.01	Not reported

(续表1)

	hsa-miR-1224-3p	30.62	The combination of miRs-135b/15b/1224-3p detected bladder cancer with a high sensitivity (94.1%)[23]
	hsa-miR-1909*	22.1	Significantly upregulated in TRG1 patients[24]
	hsa-miR-623	18.52	miR-623 was up-regulated in transient depletion of TIA-proteins in HeLa cells[20]
	hsa-miR-1180	17.43	Expressed in human sarcomas[22]
	hsa-miR-204	16.12	Regulates carcinogenesis in malignant peripheral nerve sheath tumor[25]
	hsa-miR-191*	9.27	Up-regulated in breast cancer[26] and hepatocellular carcinoma[27];
	hsa-miR-3667-5p	0.08	Not reported
LCM group *vs* ACM group	hsa-miR-27b	0.08	Up-regulated in breast cancer[28]
	hsa-miR-30a	0.07	Down-regulated in colon cancer. miR-30a suppresses cell migration and invasion through downregulation of PIK3CD in colorectal carcinoma[19]
	hsa-miR-19b	0.06	Highly expressed in basal cell carcinoma[29]
	hsa-miR-181a	0.03	Highly expressed in chronic CLL cells[13] and gastric cancer tissue[17]; In malignant glioma cells, has-miR-181a was down-regulated by radiation treatment[18]
	hsa-miR-378c	0.03	miR-378c was expressed after treated with X ray for 8 hours in TK6 cells[30]
	hsa-miR-27a	0.02	Significantly higher in breast cancer with lymph node metastasis[31], increase angiogenesis in tumor[32]
	hsa-miR-216b	0.00	Down-regulated the expression of CKIIα in colon cancer[16]

表2 各组HepG2细胞hsa-miR-623表达情况(*n*=8)

Table 2 Expressions of hsa-miR-623 in HepG2 cells with different treatments (*n*=8)

	对照组 Con group	ACM组 ACM group	LCM组 LCM group	ACM/Con ACM *vs* Con	LCM/Con LCM *vs* Con	LCM/ACM LCM *vs* ACM
Hsa-miR-623 *Ct*	22.34±0.14	24.01±0.12	21.43±0.23			
U6 *Ct*	34.45±0.25	33.89±0.23	34.01±0.42			
ΔCt	−12.11±0.29	−9.88±0.26	−12.58±0.94			
ΔΔCt				2.23±0.26	0.47±0.94	−2.7±0.94
$2^{-\Delta\Delta Ct}$				0.21	1.39	6.50
Relative value of Has-miR-623				0.21 (0.18~0.26)	0.72 (0.38~1.39)	6.50 (3.39~12.47)

Hsa-miR-623的检测采用Real-time PCR法, 计算方法采用*ΔΔCt*法。每组细胞重复4次, 每次做2个平行孔。

Expression of Hsa-miR-623 was measure by Real-time PCR and calculated with *ΔΔCt* method. The experiment was repeated for four times with 2 wells for each group every time.

的调节; 而对于能抑制肿瘤干性的miR-183、miR-148b, LCM明显增加了其含量。

总之, 本文首次报道了促炎症缓解的介质LXA_4通过调节TAM而间接影响肝癌细胞的miRNAs表达谱, 这不仅进一步阐明了LXA_4抗肝癌的作用机制, 更为肝癌的治疗提供了新的思路, 但具体的作用信号通路还有待进一步研究。

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