MiR-15b促进急性早幼粒细胞白血病细胞分化并抑制增殖

袁桢[1,2] 刘北忠[1,2] 刘冬冬[1,2] 姚娟娟[1] 钟鹏强[1] 刘俊梅[1] 姚仕菲[1]
赵毅[1] 陈敏[1] 刘路[1,2] 李连文[1] 钟梁[2]*
([1]重庆医科大学附属永川医院中心实验室, 重庆 402160;
[2]重庆医科大学, 临床检验诊断学教育部重点实验室, 重庆 400016)

摘要 该文探讨了miR-15b在全反式维甲酸(all-trans retinoic acid, ATRA)治疗急性早幼粒细胞白血病(acute promyelocytic leukemia, APL)过程中发挥的作用。采用实时荧光定量PCR检测miR-15b的表达; 流式细胞术检测细胞的分化情况; CCK-8实验检测细胞增殖; 双荧光素酶报告实验检测miR-15b与CCNE1 3′UTR端的结合能力; Western blot检测下游靶基因*CCNE1*的表达。结果显示, ATRA促进miR-15b的表达; 过表达miR-15b增强了ATRA对APL细胞的分化作用, 而抑制miR-15b表达后则出现相反结果; miR-15b抑制了APL细胞的增殖能力; 双荧光素酶报告实验显示miR-15b与CCNE1的3′UTR端结合; Western blot显示miR-15b可以抑制下游靶基因*CCNE1*的表达。这些结果表明, miR-15b通过抑制CCNE1的表达促进APL细胞分化, 抑制细胞增殖。

关键词 急性早幼粒细胞白血病; miR-15b; 细胞分化; 细胞增殖; CCNE1

MiR-15b Induces Acute Promyelocytic Leukemia Cells Differentiation and Inhibits Proliferation

Yuan Zhen[1,2], Liu Beizhong[1,2], Liu Dongdong[1,2], Yao Juanjuan[1], Zhong Pengqiang[1], Liu Junmei[1], Yao Shifei[1], Zhao Yi[1], Chen Min[1], Liu Lu[1,2], Li Lianwen[1], Zhong Liang[2]*
([1]*Central Laboratory, Yongchuan Hospital Affi liated to Chongqing Medical University, Chongqing 402160, China*;
[2]*Key Laboratory of Laboratory Medical Diagnostics Designated by the Ministry of Education, Chongqing Medical University, Chongqing 400016, China*)

Abstract This study aimed to investigate the effects of miR-15b on cell differentiation and proliferation in acute promyelocytic leukemia cell line NB4 cells. In this study, the expression of miR-15b was detected by qRT-PCR, the expression of myeloid differentiation marker CD11b was detected by flow cytometry and cell proliferation was examined by CCK-8 assay. Luciferase reporter assays and Western blot were used to identify miR-15b regulating its target genes. These results indicted that miR-15b gradually increased upon ATRA treatment in a time dependent manner. MiR-15b mimic treatment significantly increased the percentage of CD11b-positive cells and inhibited cell proliferation. MiR-15b inhibitor significantly decreased the percentage of CD11b-positive cells. Luciferase reporter assays and Western blot demonstrated miR-15b could regulate the level of CCNE1. These re-

收稿日期: 2018-12-06 接受日期: 2019-03-08
国家自然科学基金(批准号: 81772280)资助的课题
*通讯作者。Tel: 023-68485388, E-mail: 530659075@qq.com
Received: December 6, 2018 Accepted: March 8, 2019
This work was supported by the National Natural Science Foundation of China (Grant No.81772280)
*Corresponding author. Tel: +86-23-68485388, E-mail: 530659075@qq.com
网络出版时间: 2019-06-13 18:00:52 URL: http://kns.cnki.net/kcms/detail/31.2035.Q.20190613.1800.024.html

sults suggest that miR-15b inhibits cell proliferation and promotes cell differentiation by inhibiting the expression of CCNE1.

Keywords acute promyelocytic leukemia; miR-15b; cell differentiation; cell proliferation; CCNE1

急性早幼粒细胞白血病(acute promyelocytic leukemia, APL)主要特征是15号和17号染色体易位, 导致PML和RARα基因融合, 从而产生PML-RARα融合蛋白[1-3]。PML-RARα融合蛋白通过调控下游基因的表达导致粒细胞阻滞于早幼粒阶段。临床上, 药理学浓度的全反式维甲酸(all-trans-retinoicacid, ATRA)可以降解PML-RARα融合蛋白, 从而诱导早幼粒细胞分化[4-5]。虽然急性早幼粒细胞白血病的治疗取得了巨大进展, 但ATRA的治疗机制尚未完全阐明。

微小RNA(microRNA, miRNA)是一类19~25个核苷酸构成的非编码小RNA, 主要通过结合下游靶基因mRNA的3'UTR区从而导致靶基因mRNA 降解, 抑制蛋白的翻译[6-7]。既往研究表明, miR-15b在结肠癌[8]、舌癌[9]和胶质母细胞瘤[10]中均发挥了巨大作用。研究同时还指出, 过表达miR-15b能够抑制细胞周期蛋白D2(cyclinD2)的表达, 从而抑制B细胞慢性淋巴瘤细胞的增殖能力[11]。虽然miR-15b在多种肿瘤中可发挥抑癌的作用, 但在APL发生发展中的作用尚未报道。

CCNE1是细胞周期蛋白家族的一员, 在调控细胞周期和激活周期蛋白依赖性激酶中发挥了重要的作用。在细胞分裂过程中, CCNE1通过与细胞依赖性激酶2(cyclin-dpendent kinase 2, cdk2)结合, 从而调控细胞周期[12]。

1 材料与方法

1.1 材料

ATRA购于美国Sigma公司, 溶解于二甲基亚砜(dimethyl sulphoxide, DMSO)中保存于−80 °C。人早幼粒细胞白血病细胞株NB4细胞由重庆医科大学检验诊断学教育部重点实验室保存。RPMI-1640培养基购于Gibco公司。新鲜胎牛血清(fetal bovine serum, FBS)购于美国Gemini公司。MiR-15b mimic和inhibitor购于上海吉玛制药技术有限公司。PE标记CD11b流式抗体购于Biolegend公司。CCK-8试剂购于Abcam公司。兔抗人CCNE1单克隆抗体购于Abcam公司。鼠抗人β-actin单克隆抗体购于上海博士德生物技术有限责任公司。RNA提取试剂、逆转录试剂盒、SYBR Green PCR试剂盒购于日本TaKaRa公司。Lipofectamine购于美国Invitrogen公司。引物均由生工生物工程(上海)股份有限公司合成。

1.2 细胞培养

NB4细胞培养于含10%胎牛血清的RPMI-1640培养基中, 并置于37 °C、5% CO_2的恒温培养箱中进行无菌培养。

1.3 流式细胞术

离心收集已处理的细胞, 并用预冷的磷酸盐缓冲液(phosphate buffered solution, PBS)洗涤3遍。用50 μL PBS重悬细胞, 加入5 μL CD11b-PE荧光抗体冰上避光孵育30 min后再用PBS洗涤3遍, 流式细胞仪检测CD11b的表达。

1.4 CCK-8实验

将转染scramble和miR-15b mimic的NB4细胞铺于96孔板。孵育相应时间后加入CCK-8试剂, 37 °C避光孵育一定时间后于450 nm处测定每孔吸光度值。

1.5 miR-15b模拟物(mimic)或抑制物(inhibitor)转染NB4细胞

取对数生长期NB4细胞接种于6孔板中, 按照lipofectamine说明书步骤分别加入mimic、inhibitor和转染试剂。

1.6 qRT-PCR(quantitative Real-rime polymerase chain reaction)

采用RNAiso Plus提取总RNA, 再使用逆转录试剂盒将总RNA反转录为cDNA。qRT-PCR检测miR-15b, *U6*作为内参, 用$2^{-\Delta\Delta Ct}$方法计算miR-15b的相对表达量。

1.7 Western blot

收集对照组和实验组细胞并提取总蛋白, BCA法测蛋白浓度。取等量蛋白样品上样, 经SDS-聚丙烯酰胺凝胶电泳分离后, 按照蛋白相应分子量进行转膜, 5%脱脂奶粉常温封闭2 h。相应一抗(1:1 000) 4 °C封闭过夜, 用TBS和TBST洗膜后加HRP标记的二抗(1:4 000)室温孵育2 h, 洗膜后用ECL化学发光成像分析。

1.8 双荧光素酶报告实验

利用miRNA数据库TargetScan(www.genes.mit.

edu/targetscan)预测miR-15b的下游靶基因。构建野生型和突变型CCNE1片段, 克隆到载体上, 构建成pGL3-3′UTR wt、pGL3-3′UTR mut质粒。取生长状态良好的HEK293T细胞, 转染质粒48 h后, 根据Promega公司操作说明, 检测荧光素酶活性。。

1.9 统计学分析

实验数据以mean±S.D.形式表示, 数据经GraphPad Prism 5软件进行统计学分析, 多组间均数比较采用单因素方差分析(One-Way ANOVA), $P<0.05$表示有显著差异。

2 结果

2.1 ATRA促进miR-15b的表达

为探讨ATRA对miR-15b表达的影响, 用ATRA分别处理NB4细胞0、24、48、72 h。提取细胞的RNA, qRT-PCR检测miR-15b的表达。结果显示随着处理时间的增加miR-15b表达也不断升高(图1A)。

2.2 miR-15b增强ATRA促进APL细胞的分化

为探讨miR-15b对NB4细胞分化的影响, 取对数生长期的NB4细胞, 分别将miR-15b mimic和scramble转染进NB4细胞, 处理48 h后, qRT-PCR检测转染效率(图2A), 流式细胞术检测CD11b表面抗原的表达(图2C)。然后用ATRA处理转染后的细胞0、24、48 h, 检测细胞CD11b表面抗原的表达(图2D)。将anti-con和anti-15b分别转染进NB4细胞, qRT-PCR检测转染效率(图2B), 在ATRA处理下检测CD11b的表达(图2E)。此结果表明, miR-15b可以促进APL细胞的分化, 并且可以增强ATRA对APL细胞的促分化作用。

2.3 MiR-15b对APL细胞增殖的影响

为探讨miR-15b对NB4细胞增殖能力的影响, 将miR-15b mimic转染进NB4细胞, 并转染scramble作为阴性对照, 分别培养细胞0、24、48、72、96 h。CCK-8检测细胞的增殖能力。结果显示miR-15b抑制了APL细胞的增殖(图3)。

2.4 生物信息学预测miR-15b下游靶基因

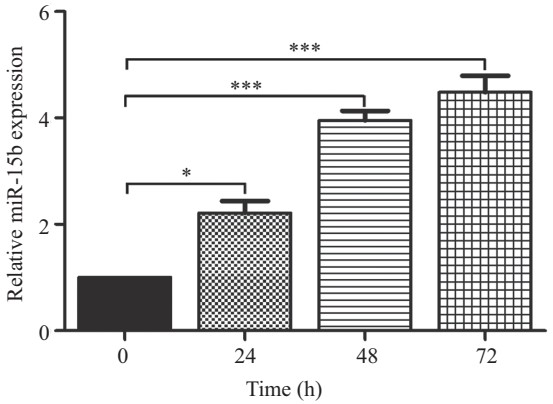
运用生物信息学软件(TargetScan)预测miR-15b与CCNE1的结合位点(图4A), 根据序列构建CCNE1的3′UTR野生型和突变型质粒, 双荧光素酶报告实验结果显示CCNE1是miR-15b的直接靶点(图4B)。

2.5 Western blot检测miR-15b对CCNE1的影响

向NB4细胞中分别转染miR-15b mimic或anti-15b, 并转染scramble和anti-con作为阴性对照, Western blot检测CCNE1的表达。同时在ATRA的处理下, Western blot检测CCNE1的表达。结果表明, miR-15b mimic抑制了CCNE1的表达(图5A), anti-15b促进了CCNE1的表达(图5B), miR-15b mimic可以促进ATRA对CCNE1的抑制作用(图5C), 而anti-15b削弱了ATRA对CCNE1的抑制作用(图5D), 从而说明了在NB4细胞中miR-15b可以抑制CCNE1的表达。

3 讨论

APL的特点是粒细胞异常增殖并阻滞于早幼粒阶段。ATRA可以通过降解PML-RARα融合蛋白, 诱导早幼粒细胞分化从而治疗APL。但其中的机制尚未完全阐明。目前, 研究显示, miRNA可以调节造血细胞增殖分化, 其异常表达与白血病的发生发展密切相关[13]。近年来研究表明, miR-29a和miR-142-3p

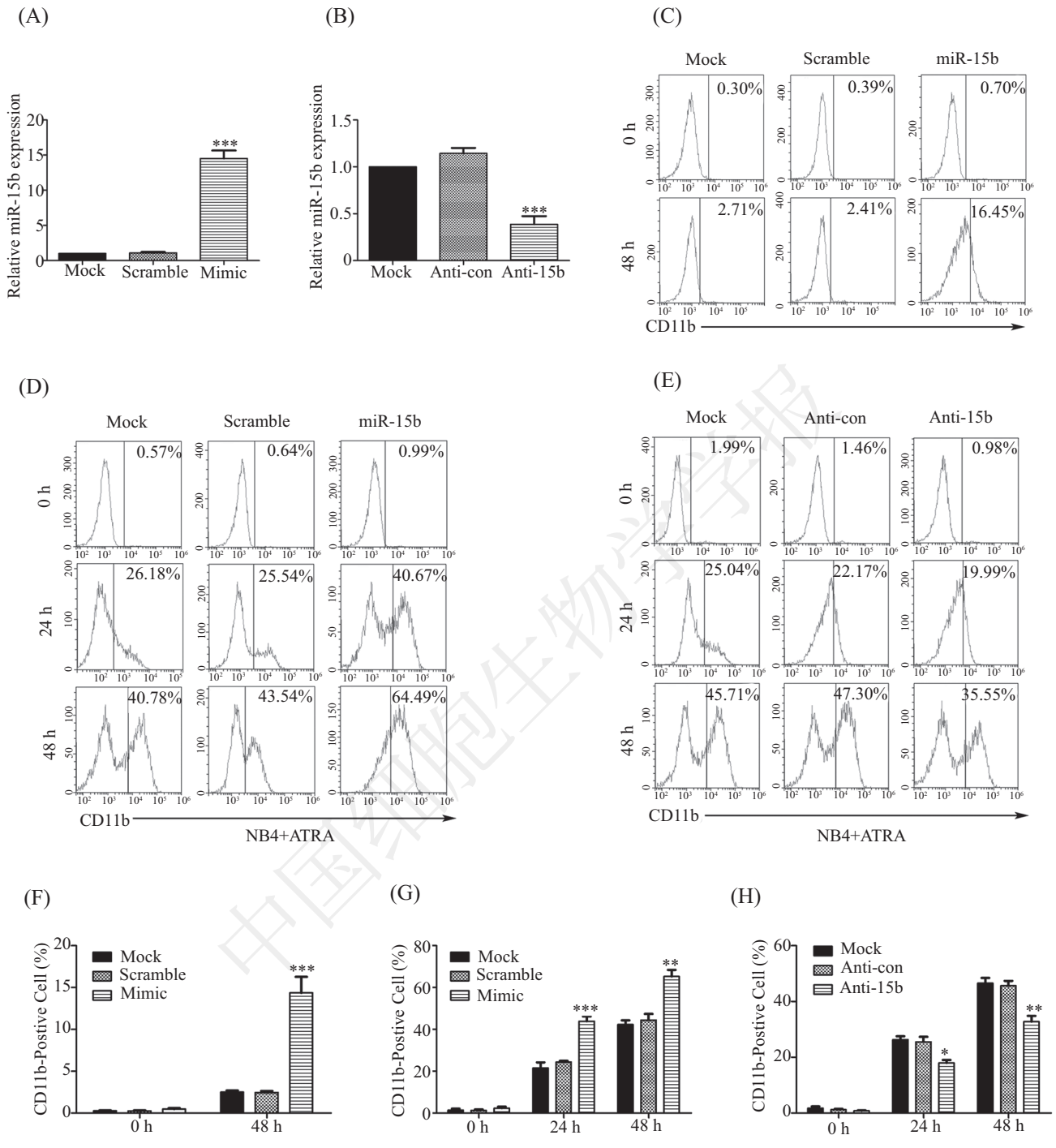


实时荧光定量PCR检测ATRA处理NB4细胞后miR-15b的表达情况。*$P<0.05$, ***$P<0.001$。

The expression of miR-15b in NB4 cells treated with ATRA was determined by qRT-PCR. *$P<0.05$, ***$P<0.001$.

图1 ATRA对miR-15b表达的影响

Fig.1 The expression of miR-15b induced by ATRA

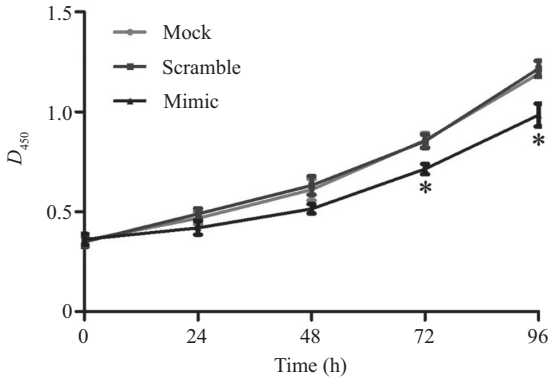


A: qRT-PCR检测过表达miR-15b的效率; B: qRT-PCR检测敲低miR-15b的效率; C: 将miR-15b mimic处理NB4细胞0、48 h后, 流式细胞术检测CD11b表达情况; D: ATRA处理过表达miR-15b的NB4细胞0、24、48 h后, 流式细胞术检测CD11b表达情况; E: ATRA处理抑制miR-15b表达的NB4细胞0、24、48 h, 流式细胞术检测CD11b表达情况; F~H: 统计学分析CD11b在NB4细胞的表达比例。*P<0.05, **P<0.01, ***P<0.001, 与mock组比较。

A: the overexpression of miR-15b was detected by qRT-PCR; B: the knockdown of miR-15b was detected by qRT-PCR; C: NB4 cells were treated with miR-15b mimic for 0h or 48h, then the expression of CD11b was detected by flow cytometry; D: CD11b positive cells were measured by flow cytometry in NB4 cells transfected with scramble or miR-15b mimics under ATRA treatment; E: CD11b positive cells were measured by flow cytometry in NB4 cells transfected with anti-con or anti-15b under ATRA treatment; F-H: The expression rate of CD11b in NB4 cells was analysed by statistics. *P<0.05, **P<0.01, ***P<0.001 compared with mock group.

图2 miR-15b对APL细胞表面分化抗原CD11b表达的影响

Fig.2 Effect of miR-15b on the expression of granulocyte surface differentiation antigen CD11b in APL cells

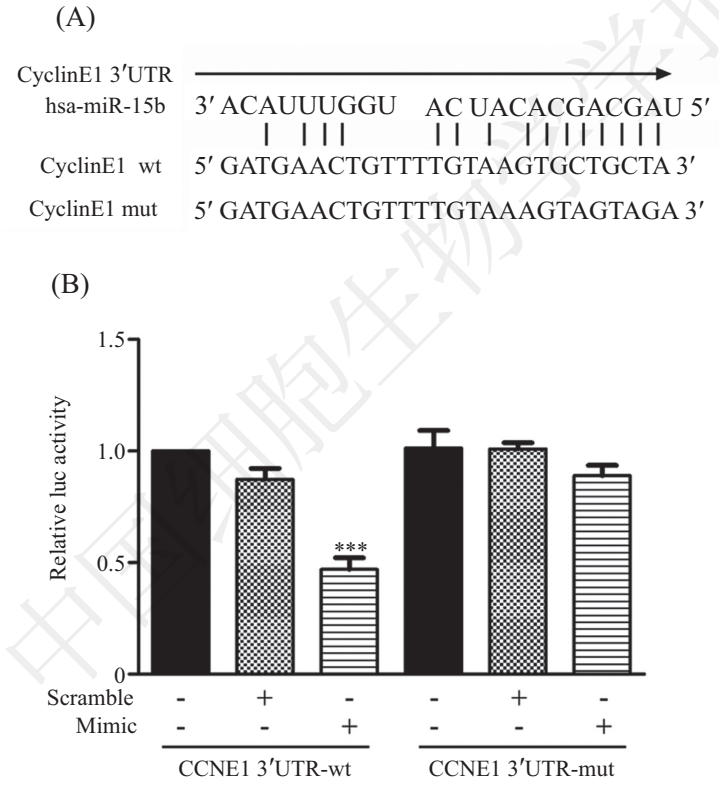


CCK-8检测miR-15b mimic对NB4细胞的增殖情况。*P<0.05, 与mock组比较。

The viability of NB4 cells was detected by CCK-8 after transfecting with scramble or miR-15b mimics. *P<0.05 compared with mock group.

图3 miR-15b对APL细胞增殖的影响

Fig.3 Effect of miR-15b on the proliferation of APL cells



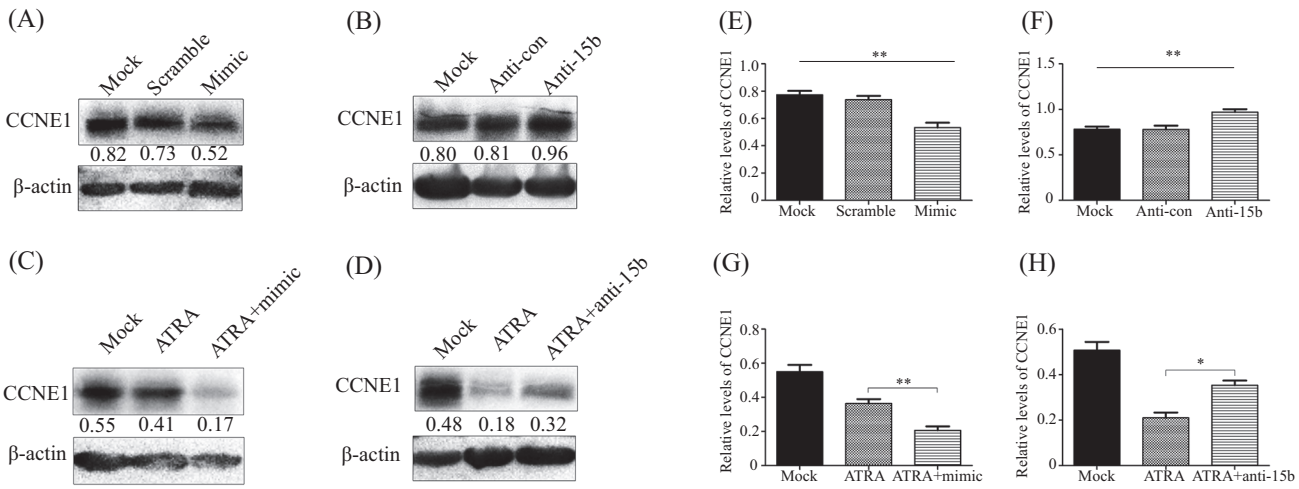
A: CCNE1的3′UTR野生型和突变型序列; B: 双荧光素酶检测miR-15b对CCNE1的调节。***P<0.001, 与对照组比较。

A: wild sites and mutation sites of CCNE1; B: dual luciferase reporter assays verified miR-15b could regulate CCNE1. ***P<0.001 compared with control group.

图4 双荧光素酶报告基因实验验证miR-15b与CCNE1的靶向关系

Fig.4 Dual luciferase reporter assays verify the relationship between miR-15b and CCNE1

可以作为AML的早期诊断指标[14]。De Marchis ML等[15]报道miR-342可以促进粒细胞的分化。近年来研究表明, ATRA可以促进一系列miRNA的表达, 其中包括miR-15b[16-17]。但目前关于miR-15b在APL中的作用尚未有文献报道。因此本研究探讨了miR-15b对APL细胞增殖、分化的作用以及相关的机制。本研究证明, ATRA促进miR-15b的表达, 且呈时间依赖性。MiR-15b可以促进粒细胞表面分化标志物CD11b的表达, 且ATRA和miR-15b mimic联合作用时比单独使用ATRA时CD11b的表达增加。CCK-8实验证明, miR-15b可以抑制APL细胞的增殖。以上实验证明, miR-15b具有诱导APL细胞分化并且抑制



A: 分别用scramble和miR-15b mimic处理NB4细胞48 h, Western blot检测CCNE1蛋白水平; B: 分别用anti-con和anti-15b处理NB4细胞48 h, Western blot检测CCNE1蛋白水平; C: 分别用ATRA或ATRA+miR-15b mimic处理NB4细胞48 h,Western blot检测CCNE1蛋白水平; D: 分别用ATRA或ATRA+anti-15b处理NB4细胞72 h,Western blot检测CCNE1蛋白水平; E~H: 统计学分析CCNE1的相对水平。*P<0.05, **P<0.01。

A: NB4 cells were treated with scramble or miR-15b mimic for 48 h, then the levels of CCNE1 were determined by Western blot; B: NB4 cells were treated with anti-con or anti-15b for 48 h, then the levels of CCNE1 were determined by Western blot; C: NB4 cells were treated with miR-15b mimic and ATRA for 48 h, then the levels of CCNE1 were determined by Western blot; D: NB4 cells were treated with anti-15b and ATRA for 72 h, then the levels of CCNE1 were determined by Western blot; E-H: the relative levels of CCNE1 proteins were analysed by statistics. *P<0.05, **P<0.01.

图5 miR-15b对CCNE1蛋白表达的影响

Fig.5 Effect of miR-15b on expression of CCNE1

细胞增殖的作用。

通过生物信息学预测miR-15b的下游靶基因, 结果显示, *CCNE1*是miR-15b的靶基因, 并且CCNE1的3′UTR端可以与miR-15b紧密结合。有研究表明, CCNE1可以结合CDK2, 从而磷酸化Rb, 磷酸化的Rb是调控细胞周期的关键蛋白[18]。因此, CCNE1可以通过调控细胞周期从而调节细胞的增殖和分化。本文选择CCNE1作为主要研究对象。研究结果, 过表达miR-15b可以降低CCNE1的表达, 敲低miR-15b可以增加CCNE1的表达。同时miR-15b可以促进ATRA对CCNE1的抑制作用。综上, 我们得出, miR-15b可以通过抑制CCNE1的表达促进APL细胞的分化。

综上所述, miR-15b可以通过调节CCNE1的表达促进APL细胞分化, 抑制细胞增殖。本研究进一步表明miR-15b可以作为白血病的新型治疗靶点。

参考文献 (References)

1 Puccetti E, Ruthardt M. Acute promyelocytic leukemia: PML/RARalpha and the leukemic stem cell. Leukemia 2004; 18(7): 1169-75.

2 de Thé H, Lavau C, Marchio A, Chomienne C, Degos L, Dejean A. The PML-RAR alpha fusion mRNA generated by the t(15;17) translocation in acute promyelocytic leukemia encodes a functionally altered RAR. Cell 1991; 66(4): 675-84.

3 Kakizuka A, Miller WH Jr, Umesono K, Warrell RP Jr, Frankel SR, Murty VV, *et al*. Chromosomal translocation t(15;17) in human acute promyelocytic leukemia fuses RAR alpha with a novel putative transcription factor, PML. Cell 1991; 66(4): 663-74.

4 Wang ZY, Chen Z. Acute promyelocytic leukemia: from highly fatal to highly curable. Blood 2008; 111(5): 2505-15.

5 Vitaliano-Prunier A, Halftermeyer J, Ablain J, de Reynies A, Peres L, Le Bras M, *et al*. Clearance of PML/RARA-bound promoters suffice to initiate APL differentiation. Blood 2014; 124(25): 3772-80.

6 Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004; 116: 281-97.

7 Calin GA, Croce CM. MicroRNA signatures in human cancers. Nat Rev Cancer 2006; 6: 857-66.

8 Chen R, Sheng L, Zhang HJ, Ji M, Qian WQ. miR-15b-5p facilitates the tumorigenicity by targeting RECK and predicts tumour recurrence in prostate cancer. J Cell Mol Med 2018; 22(3):1855-63.

9 Sun L, Yao Y, Liu B, Lin Z, Lin L, *et al*. MiR-200b and miR-15b regulate chemotherapy-induced epithelial-mesenchymal transition in human tongue cancer cells by targeting BMI1. Oncogene 2012; 31: 432-45.

10 Wang J, Liu H, Tian L, Wang F, Han L, Zhang W, *et al*. miR-15b inhibits the progression of glioblastoma cells through targeting insulin-like growth factor receptor 1. Horm Cancer 2017; 8: 49-57.

11 Lovat F, Fassan M, Gasparini P, Rizzotto L, Cascione L, *et al*. miR-15b/16-2 deletion promotes B-cell malignancies. Proc Natl

Acad Sci USA 2015; 112: 11636-41.
12 Kanska J, Zakhour M, Taylor-Harding B, Karlan BY, Wiedemeyer WR. Cyclin E as a potential therapeutic target in high grade serous ovarian cancer. Gynecol Oncol 2016; 143(1): 152-8.
13 Manodoro F, Marzec J, Chaplin T, Miraki-Moud F, Moravcsik E, *et al*. Loss of imprinting at the 14q32 domain is associated with microRNA overexpression in acute promyelocytic leukemia. Blood 2014; 123(13): 2066-74.
14 Wang F, Wang XS, Yang GH, Zhai PF, Xiao Z, Xia LY, *et al*. miR-29a and miR-142-3p downregulation and diagnostic implication in human acute myeloid leukemia. Mol Biol Rep 2012; 39(3): 2713-22.
15 De Marchis ML, Ballarino M, Salvatori B, Puzzolo MC, Bozzoni I, Fatica A. A new molecular network comprising PU.1, interferon regulatory factor proteins and miR-342 stimulates ATRA-mediated granulocytic differentiation of acute promyelocytic leukemia cells. Leukemia 2009; 23(5): 856-62.
16 Careccia S, Mainardi S, Pelosi A, Gurtner A, Diverio D, *et al*. A restricted signature of miRNAs distinguishes APL blasts from normal promyelocytes. Oncogene 2009; 28(45): 4034-40.
17 Garzon R, Pichiorri F, Palumbo T, Visentini M, Aqeilan R, *et al*. MicroRNA gene expression during retinoic acid-induced differentiation of human acute promyelocytic leukemia. Oncogene 2007; 26(28): 4148-57.
18 E Hermosilla V, Salgado G, Riffo E, Escobar D, Hepp MI, *et al*. SALL2 represses cyclins D1 and E1 expression and restrains G_1/S cell cycle transition and cancer-related phenotypes. Mol Oncol 2018; 12:1026-46.