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·基础研究·

miR-204过表达抑制成视网膜细胞瘤细胞的增殖与侵袭及其可能的机制

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[摘 要] **目的**:观察miR-204对成视网膜细胞瘤(retinoblastoma,RB)细胞增殖与侵袭的影响,探讨其可能的调控机制。**方法**:应用实时荧光定量PCR(qRT-PCR)检测RB细胞系Y79、SO-RB50、HXO-Rb44和人正常视网膜色素上皮细胞系hTERT RPE-1中miR-204的表达水平。将Y79细胞系分成阴性对照组和miR-204组,分别应用脂质体转染法转染NC-mimics和miR-204 mimics,CCK-8增殖实验检测miR-204表达对Y79细胞增殖的影响,细胞划痕实验和Transwell小室法检测miR-204对Y79细胞迁移和侵袭的影响,应用生物学信息法预测miR-204的可能作用靶基因,应用qRT-PCR和Western blotting检测miR-204对靶基因高迁移率族蛋白A2(high mobility group AT-hook 2,*HMGA2*)mRNA和蛋白表达的影响。**结果**:miR-204在RB细胞系Y79、SO-RB50、HXO-Rb44中的表达较人正常视网膜色素上皮细胞系hTERT RPE-1明显降低(P<0.01)。转染miR-204 mimics后,Y79细胞中miR-204表达明显升高(P<0.01)、细胞增殖能力明显下降(P<0.01)、迁移及侵袭能力明显降低(P<0.01),miR-204的靶基因*HMGA2* mRNA和蛋白的表达明显下降(P<0.01)。**结论**:miR-204在RB细胞系中低表达,过表达miR-204能够抑制RB细胞的增殖、迁移及侵袭能力,其机制可能与下调*HMGA2*基因的表达有关。

[关键词] 成视网膜细胞瘤;miR-204;增殖;迁移;侵袭;高迁移率族蛋白A2
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Over-expression of miR-204 suppresses proliferation and invasion of retinoblastoma cells and its possible mechanism

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[Abstract] Objective: To observe the effects of miR-204 on the proliferation and invasion of retinoblastoma (RB) cells and to explore the potential regulatory mechanism. **Methods:** The expression level of miR-204 in RB cell lines (Y79, SO-RB50, and HXO-Rb44) as well as in normal human retinal pigment epithelial cell line hTERT RPE-1 was detected using qRT-PCR. The Y79 cells were divided into two groups (negative control group and miR-204 group) by respectively transfecting Y79 cells with NC-mimics and miR-204 mimics using liposome transfection method. The effects of miR-204 on Y79 cell proliferation was detected with CCK-8 assay; while the effect of miR-204 on migration and invasion of Y79 cells were determined by cell scratch assay and Transwell assay, respectively. Besides, the potential target gene of miR-204 was predicted by bioinformatics; and the influence of miR-204 on the expression of high mobility group AT-hook 2 gene (HMGA2) at both mRNA and protein levels was detected using qRT-PCR and Western blotting, respectively. **Results:** miR-204 expression in RB cell lines Y79, SO-RB50 and HXO-Rb44 was remarkably lower than that in normal human retinal pigment epithelial cell line hTERT RPE-1 (P<0.01). miR-204 expression in Y79 cells was markedly up-regulated after transfection with miR-204 mimics (P<0.01) along with significantly reduced cell proliferation, migration and invasion capacities (all P<0.01), and mRNA and protein expressions of HMGA2 were also outstandingly reduced (P<0.01). **Conclusion:** miR-204 is lowly expressed in RB cell lines; in addition, miR-204 over-expression can suppress RB cell proliferation, migration and invasion, the mechanism of which might be related to down-regulation of the expression of *HMGA2*.

[Key words] retinoblastoma;miR-204; proliferation; migration;invasion; high mobility group AT-hook 2 gene (*HMGA2*)
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成视网膜细胞瘤（retinoblastoma，RB）是起源于视网膜上皮层的恶性肿瘤，是最常见的婴幼儿眼内恶性肿瘤，恶性程度高、预后差，如不能及时治疗往往会危及患儿生命[1]。至今RB的致病及进展机制尚不清楚，手术和放化疗等传统的治疗方式效果欠佳。microRNA（miRNA）是一类由21~24个核苷酸组成的高度保守的非编码单链RNA，通过与靶基因不完全或完全互补结合来调控靶基因的表达，在机体基因调控网络中发挥着重要的作用[2-3]。近年来发现miRNA作为癌基因或抑癌基因在恶性肿瘤发生进展中发挥重要作用[4]。在RB中也发现了许多与RB密切相关的miRNA异常表达[5]。miR-204是miRNA家族重要成员之一，其定位于人类第9号染色体，在人视网膜色素上皮细胞中表达极为丰富[6]，参与视网膜色素上皮细胞的分化[7]。近期研究[8]显示，miR-204在包括RB在内许多恶性肿瘤中表达异常，参与肿瘤细胞的增殖、迁移及侵袭等生物学行为，但作用机制尚不明确。本研究观察miR-204在RB细胞系中的表达变化以及其对RB细胞增殖、迁移和侵袭能力的影响，并探讨其可能的作用机制。

1　材料与方法

1.1　主要试剂

miR-204 mimics、阴性对照NC-mimics购自上海吉玛制药公司，TRIzol试剂、Lipofectamine™2000购自美国Invitrogen公司，RNA反转录试剂盒、qRT-PCR试剂盒购自大连宝生生物公司，miR-204、*U6*与高迁移率族蛋白A2（high mobility group AT-hook 2，*HMGA2*）、*GAPDH*引物购自广东锐博生物公司，Transwell小室购自美国Corning公司，基质胶购自美国BD公司，HMGA2和GAPDH一抗购自美国CST公司，HRP标记的二抗购自美国Santa Cruz公司。

1.2　细胞系及其培养

人正常视网膜色素上皮细胞系hTERT RPE-1和人RB细胞系Y79、SO-RB50、HXO-Rb44均购自美国ATCC。Y79、SO-RB50和HXO-Rb44细胞在RPMI 1640培养基(含10%胎牛血清、100 U/ml青霉素和100 μg/ml链霉素)中培养，人正常视网膜色素上皮细胞hTERT RPE-1在含10%胎牛血清和0.01 mg/ml潮霉素B的DMEM/F12培养基中培养，均置于37℃、5% CO_2的饱和湿度恒温箱中常规培养，细胞用0.05% EDTA消化传代，取生长状态良好的对数生长期细胞进行后续实验。

1.3　miR-204 mimics转染Y79细胞

取对数生长期Y79细胞，接种于6孔细胞培养板，常规调节下培养，细胞汇合度达60%～70%时进行细胞转染操作。Y79细胞分为两组，NC-mimics组(对照组)和miR-204 mimics组（实验组），严格按Lipofectamine™2000说明书操作分别将NC-mimics和miR-204 mimics转染至Y79细胞，转染后24 h，qRT-PCR检测miR-204的表达，确定转染效率后再进行后续细胞实验。

1.4　qRT-PCR法检测Y79细胞中miR-204和*HMGA2*基因的表达水平

按照TRIzol试剂说明书提取Y79细胞总RNA，鉴定纯度和含量后，反转录合成cDNA。cDNA合成参照反转录试剂盒说明书操作。以cDNA为模板，按照qRT-PCR试剂盒说明书配置反应体系进行PCR反应。miR-204基因PCR反应条件：94 ℃ 2 min；40个循环（94 ℃ 20 s、55 ℃ 30 s）；*HMGA2*基因PCR反应条件：94 ℃ 2 min；40个循环（94 ℃ 30 s、58 ℃ 30 s、72 ℃ 30 s）。miR-204以*U6*作为内参基因，*HMGA2*以*GAPDH*作为内参基因，相对表达水平采用$2^{-\triangle\triangle Ct}$法计算。实验重复3次。

1.5　CCK-8法检测miR-204过表达对Y79细胞增殖的影响

具体操作参照CCK-8试剂盒说明书进行。收集转染后的各组细胞按5×10^3/孔的密度分别种于96孔板上，每组设3个复孔，每24 h加入10 μl CCK-8反应液37 ℃继续孵育4 h，在Spectra Max 190酶标仪上测定波长450 nm处测定各孔不同时点的光密度（*D*）值，绘制增殖曲线。

1.6　细胞划痕实验检测miR-204过表达对Y79细胞迁移的影响

取转染后24 h的各组细胞，接种培养于6孔板中，孔板底部预先画好标记线，待细胞长到完全融合后，用10 μl的灭菌枪头垂直培养孔底面中央用力划直线沿直线划痕，PBS冲洗2次后继续培养，划痕后即刻和24 h后于显微镜下观察并测量划痕宽度，划痕愈合率（%）=[(0 h时刻划痕宽度−24 h时刻划痕宽度)/0 h时刻划痕宽度]×100%。实验重复3次。

1.7　Transwell小室法检测miR-204过表达对Y79细胞侵袭的影响

基质胶均匀涂抹Transwell小室上室，过夜成膜。收集转染后24 h的各种Y79细胞以无血清培养基重悬制备单细胞悬液，取200 μl含2×10^5个细胞的单细胞悬液加入Transwell小室上室，下室加入500 μl含10%胎牛血清的DMEM培养基，继续培养24 h后取出小室，棉签轻轻擦弃上室内的细胞，4%多聚甲醛固定，染色，风干，显微镜下观察，并随机取5个高倍视野计算穿膜细胞数。实验重复3次。

1.8　Western blotting检测miR-204过表达对Y79细

胞中HMGA2表达的影响

取对数生长期各组Y79细胞，常规提取细胞总蛋白，以BCA法定量蛋白浓度。每个样本取200 μl样品，蛋白经10% SDS-PAGE分离后电转移至PVDF膜，5 %封闭液4 ℃封闭4 h，TBS缓冲液漂洗3遍，加入HMGA2和GAPDH一抗，4 ℃孵育过夜，TBS缓冲液漂洗3遍，加入HRP标记的二抗，室温孵育2 h，ECL显影液显影，Quality One软件分析条带灰度，以HMGA2与GAPDH灰度的比值表示相对表达量。实验重复3次。

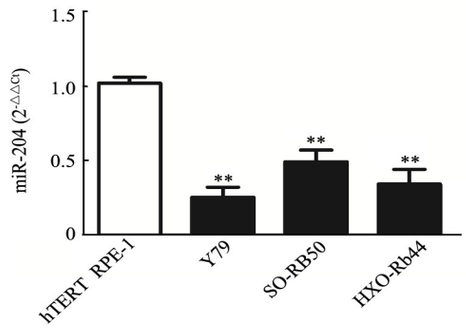
1.9 统计学处理

采用SPSS 20.0软件进行数据分析，计量资料以$\bar{x}\pm s$表示，差异比较采用t检验或单因素方差分析。以$P<0.05$或$P<0.01$表示差异有统计学意义。

2 结 果

2.1 miR-204在RB细胞中低表达

qRT-PCR结果（图1）显示，miR-204在RB细胞系Y79、SO-RB50、HXO-Rb44中的表达较人正常视网膜色素上皮细胞系hTERT RPE-1明显降低（均$P<0.01$）。



**$P<0.01$ *vs* hTERT RPE-1 cell

图1 miR-204在RB细胞和人正常视网膜色素上皮细胞中的表达

Fig.1 Expression of miR-204 in RB cells and normal human retinal pigment epithelial cells hTERT RPE-1

2.2 转染后各组Y79细胞miR-204的表达

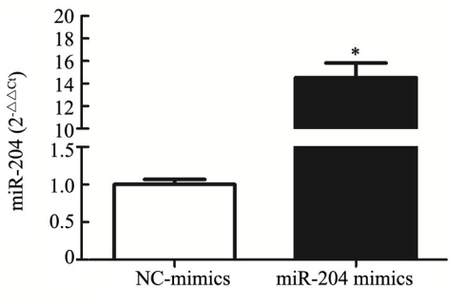
qRT-PCR检测结果（图2）显示，miR-204 mimics组细胞miR-204的表达明显高于NC-mimics组（14.50±1.45 *vs* 1.01±0.06，$P<0.01$），表明miR-204 mimics转染效率较高。

2.3 miR-204过表达抑制Y79细胞的增殖活性

CCK-8增殖实验结果（图3）显示，miR-204 mimics组细胞转染后48、72和96 h时增殖活性明显低于NC-mimics组（$P<0.01$）。

2.4 miR-204过表达抑制Y79细胞的迁移能力

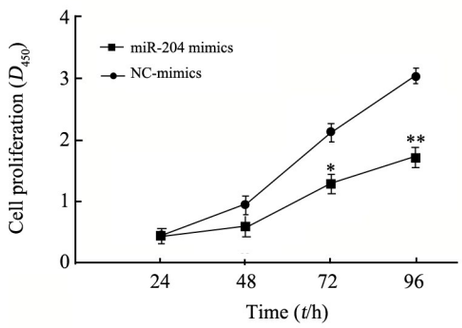
划痕实验结果（图4）显示，与NC-mimics组比较，miR-204 mimics组细胞的划痕愈合率明显下降[（31.3±3.8）% *vs* （60.7±4.9）%，$P<0.01$]，提示miR-204 mimics组细胞的迁移能力明显降低。



*$P<0.01$ *vs* NC-mimics group

图2 转染miR-204 mimics后Y79细胞中miR-204高水平表达

Fig.2 High level of miR-204 expression was detected in Y79 cells after transfection with miR-204 mimics



*$P<0.05$，**$P<0.01$ *vs* NC-mimics group

图3 miR-204过表达抑制Y79细胞的增殖

Fig.3 Over-expression of miR-204 inhibits the proliferation of Y79 cells

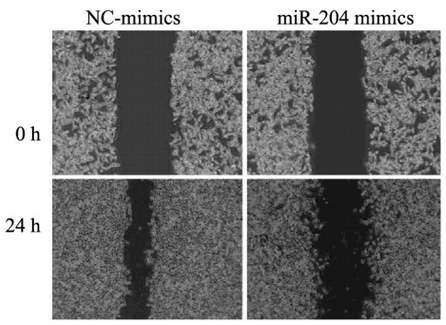
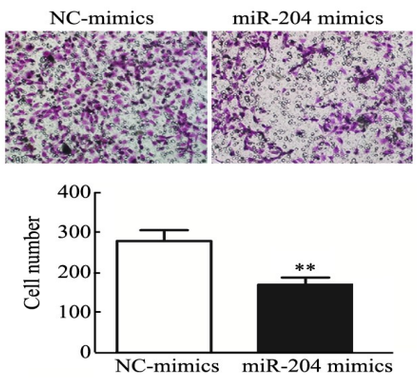


图4 miR-204过表达抑制Y79细胞的迁移(×10)

Fig.4 Over-expression of miR-204 inhibits the migration of Y79 cells (×10)

2.5 miR-204过表达抑制Y79细胞的侵袭能力

Transwell侵袭实验检测结果（图5）显示，miR-204 mimics组穿膜细胞数明显低于NC-mimics组（$P<0.01$），提示miR-204 mimics组细胞的侵袭能力明显下降。



$^{**}P<0.01$ *vs* NC-mimics group

图5 miR-204过表达抑制Y79细胞的侵袭(×200)

Fig. 5 Over-expression of miR-204 inhibits the invasion of Y79 cells (×200)

2.6 *HMGA2*是miR-204的靶基因

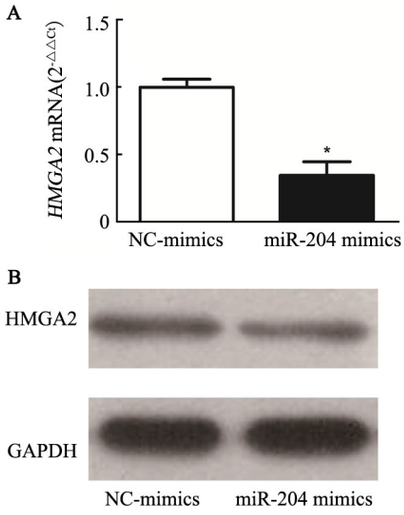
应用生物学信息法预测miR-204可能靶基因，查阅miRanda（http://www.microrna.org）及相关文献[9-11]，预测*HMGA2*可能是miR-204的作用靶基因,miR-204与HMGA2的3'UTR区存在种子序列互补的结合位点，见图6。

2.7 miR-204过表达抑制Y79细胞的*HMGA2* mRNA和蛋白的表达

qRT-PCR检测结果（图7A）显示，miR-204 mimics组细胞*HMGA2* mRNA的表达明显低于NC-mimics组（0.35±0.10 *vs* 1.00±0.06,$P<0.01$）。Western blotting检测结果（图7B）显示，miR-204 mimics组细胞HMGA2蛋白的表达明显低于NC-mimics组（0.18±0.06 *vs* 0.64±0.10，$P<0.01$）。

图6 miR-204和HMGA2的3'UTR区的结合序列

Fig.6 The binding sequence of the 3 'UTR region of miR-204 and HMGA2



$^{*}P<0.05$ *vs* NC-mimics group

图7 miR-204过表达抑制Y79细胞的*HMGA2* mRNA（A）和蛋白（B）的表达

Fig.7 Over-expression of miR-204 inhibits mRNA (A) and protein (B) expression of HMGA2 in Y79 cells

3 讨　论

目前已发现上百种miRNA在人类视网膜、角膜、晶状体等眼部组织中表达，通过对下游靶基因的调控，在维持眼部组织的正常功能方面发挥着重要的作用。目前研究[4,12]证实，机体组织器官中miRNA表达水平的改变与包括肿瘤、炎症等在内的众多疾病的发生进展密切相关。

RB是儿童最常见的眼内恶性肿瘤，许多miRNA在RB的发生发展中发挥着重要的作用。miRNA通过对其靶基因的调控参与肿瘤细胞的侵袭、迁移、增殖以及肿瘤的发生发展。miR-138-5p[13]、miR-200c[14]、miR-320[15]等在RB中表达下调，被认为是类似于抑癌基因在RB的发生发展中发挥重要作用，而miR-498[16]、miR-655[17]、miR-106b[18]等在RB中表达上调，被认为是类似于癌基因发挥作用。miR-204定位于人类第9号染色体，在人视网膜色素上皮细胞表达极为丰富[6]。近期研究显示，miR-204在子宫颈癌[19]、成胶质细胞瘤[20]、肝细胞癌[21]、胃癌[22]等众多恶性肿瘤中表达下调，起抑癌基因的作用。但目前有关miR-204在RB中的研究较少，并且其作用机制尚不清楚。本研究通过qRT-PCR检测RB细胞系Y79、SO-RB50、HXO-Rb44和人正常视网膜色素上皮细胞系hTERT RPE-1中miR-204的表达水平发现，miR-204在RB细胞系中表达明显降低，在miR-204在其他恶性肿瘤中的研究一致，提示miR-204在RB发生发展中可能也起着抑癌基因的作用。

细胞无限增殖、侵袭与转移是恶性肿瘤的基本生物学特性，在其他众多恶性肿瘤的研究中均发现miR-204能够抑制肿瘤细胞的增殖、迁移及侵袭等恶性生物学行为[19-22]。本研究发现，上调Y79细胞中miR-204的表达能够明显抑制Y79细胞的增殖、迁移和侵袭能力，进一步证实miR-204作为抑癌基因能够抑制RB的进展。

miR-204抑制肿瘤细胞增殖、侵袭及迁移能力的下游分子通路尚不完全明确。为了研究miR-204调控RB细胞的下游靶基因，本研究应用生物学信息法预测发现*HMGA2*可能是miR-204的作用靶基因,因为miR-204与*HMGA2*的3’UTR区存在种子序列互补的结合位点。近期WU等[23]研究发现，miR-204通过靶向调控*HMGA2*抑制甲状腺癌细胞的增殖。在口腔癌[24]和结肠癌[25]中也证实，miR-204可以通过靶向*HGMA2*调控肿瘤细胞的恶性生物学行为。本研究发现，上调Y79细胞中miR-204表达后，Y79细胞*HMGA2* mRNA和蛋白的表达水平明显下降，提示miR-204在RB中的作用对*HMGA2*基因的靶向调控有关，上调miR-204的表达能够明显抑制*HMGA2*基因的表达。HMGA2是一种非组蛋白染色体蛋白，是高迁移率族蛋白超家族成员之一,在胚胎组织中高表达，在正常的成熟组织中几乎检测不到[26]。*HMGA2*可以上调细胞周期蛋白的表达，加速G2/M期转化促使肿瘤发生发展[27]，已公认为作为一种癌基因参与恶性肿瘤的发生发展，能够促进肿瘤细胞的增殖、侵袭和迁移能力[26-31]。较多的研究也已经证实*HMGA2*在RB中表达明显增高，抑制*HMGA2*的表达能够明显抑制RB细胞的增殖、侵袭和迁移能力[32-34]。

综上所述，miR-204在RB细胞系中低表达，miR-204能够抑制RB细胞的增殖、迁移及侵袭能力，其机制可能与下调*HMGA2*基因的表达有关，为进一步探索miR-204在RB进展过程中的作用机制提供了实验基础。

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