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MicroRNA-221/222 对乳腺癌 MDA-MB-231/DOX 细胞阿霉素耐药性的影响*

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摘要 目的:探讨微小 RNA-221/222(miR-221/222)对乳腺癌 MDA-MB-231/阿霉素(DOX)细胞 DOX 耐药性的影响。**方法:**采用脂质体法转染 miR-221/222 抑制物(miR-221/222 inhibitor)至 MDA-MB-231/DOX 细胞内(Inhibitor 组),同时设立空白对照组和转染无关序列的阴性对照组,采用实时荧光定量 PCR(qRT-PCR)检测 MDA-MB-231 细胞株及 MDA-MB-231/DOX 细胞株的 miR-221/222 表达水平及转染效率;CCK-8 法检测转染 48 h 后 MDA-MB-231/DOX 细胞对 DOX 药物敏感性的变化;流式细胞术(FCM)检测转染 MDA-MB-231/DOX 细胞的细胞凋亡率;蛋白免疫印迹实验(WB)检测转染后 MDA-MB-231/DOX 细胞内促凋亡蛋白 p53 上调凋亡调控因子(PUMA),Bcl2 蛋白修饰因子(BMF)以及细胞周期蛋白激酶抑制因子 p27(p27^{Kip1})的表达情况。**结果:**MDA-MB-231/DOX 细胞中的 miR-221/222 表达水平高于亲本 MDA-MB-231 细胞 ($P<0.05$);MDA-MB-231/DOX 细胞转染 miR-221/222 inhibitor 96 h 后,miR-221/222 的表达水平低于空白对照组和阴性对照组 ($P<0.05$);与空白对照组相比,MDA-MB-231/DOX 细胞转染 miR-221/222 inhibitor 48h 后,DOX 继续处理 48 h 后,细胞的凋亡率明显升高,且细胞内的促凋亡蛋白 PUMA,BMF 以及 p27^{Kip1} 的表达均增加($P<0.05$);DOX 对 inhibitor 组耐药细胞的半数抑制浓度(IC₅₀)显著低于空白对照组细胞及阴性对照组($P<0.05$)。**结论:**miR-221/222 能够增加 MDA-MB-231/DOX 细胞对 DOX 的耐药性,这可能与下调促凋亡蛋白的表达有关;降低 miR-221/222 水平可诱导 MDA-MB-231/DOX 凋亡,并且上调促凋亡蛋白的表达,从而部分逆转 MDA-MB-231/DOX 对 DOX 的耐药性。

关键词:MicroRNA-221/222;阿霉素;耐药性;乳腺癌;MDA-MB-231/DOX

中图分类号:R-33;R737.9 **文献标识码:**A **文章编号:**1673-6273(2020)15-2843-05

Effect of Microrna-221/222 on Adriamycin Resistance of Breast Cancer MDA-MB-231/DOX Cells*

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ABSTRACT Objective: To investigate the effect of microrna-221/222 (miR-221/222) on DOX resistance of breast cancer MDA-MB-231/doxorubicin (DOX) cells. **Methods:** miR-221/222 inhibitor (miR-221/222 inhibitor) was transfected into MDA-MB-231/DOX cells by liposome method, the blank control group and the negative control group were set up meanwhile. The transfection efficiency and expression level of miR-221/222 in MDA-MB-231 cell line and cell line MDA-MB-231/DOX cell line was detected by real time fluorescent quantitative PCR (qRT-PCR); the change of drug sensitivity of MDA-MB-231/DOX cells to dox after 48 hours of transfection was detected by CCK-8 tests; the apoptosis rate of MDA-MB-231/DOX cells was detected by flow cytometry (FCM); the upregulation of pro-apoptotic protein p53 up regulates apoptosis (PUMA), bcl-2 protein modifying factor (BMF) and cyclin kinase inhibitor p27 (p27^{Kip1}) in MDA-MB-231/DOX cells were detected by Western blotting (WB). **Results:** The expression level of miR-221/222 in MDA-MB-231/DOX cells was higher than that in MDA-MB-231 cells ($P<0.05$), the expression level of miR-221/222 in miR-221/222 inhibitor transfected by MDA-MB-231/DOX cells was lower than that in the blank control group and the negative control group ($P<0.05$) after 96 hours of treatment; compared with the blank control group, the expression level of miR-221/222 inhibitor transfected by MDA-MB-231/DOX cells was lower than that in the blank control group ($P<0.05$). The apoptotic rate of the cells increased significantly and the expression of apoptosis promoting protein puma, BMF and p27^{Kip1} in the cells increased ($P<0.05$); the 50% inhibitory concentration (IC₅₀) of drug resistance cells in the inhibitor group was significantly lower than that in the blank control group and the negative control group ($P<0.05$). **Conclusion:** miR-221/222 can increase the resistance of MDA-MB-231/DOX cells to DOX,

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which may be related to down regulating the expression of pro-apoptotic protein, and down regulating the level of miR-221/222 can induce MDA-MB-231/DOX apoptosis and raise the expression of pro-apoptotic protein, which can partially reverse the resistance of MDA-MB-231/DOX to DOX.

Key words: MicroRNA-221/222; Doxorubicin; Drug resistance; Breast cancer; MDA-MB-231 /DOX

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前言

乳腺癌是一种高度异质性恶性肿瘤, 严重威胁着女性的健康, 化疗辅助手术治疗仍是治疗该病的主要方式^[1,2]。目前认为, 化疗耐药情况的发生是乳腺癌患者出现不良预后的重要因素, 因此寻找能够逆转化疗耐药的有关靶标成为当前研究的热点^[3,4]。

微小 RNA (microRNA, miR) 是一类高度保守的长度约 22nt 的内源性非编码小分子单链 RNA, 广泛存在于真核生物细胞中^[5]。近年来大量研究发现, miR 参与转录后基因表达调控, miR 的表达变化与多种恶性肿瘤的发生发展及化疗耐药密切相关^[6-8]。与其他成簇 miR 一样, 人 miR-221 与 miR-222 具有完全同源的核心种子序列(68-72), 定位于 Xp11.3, 呈前排列^[9]。近年来的研究表明, miR-221/222 在乳腺癌的发生及恶性进展中, 发挥着重要作用, 如 Zhao JJ 等^[10] 的研究表明, miR-221/222 能够负调控雌激素受体- α (Estrogen receptor- α , ER- α) 的表达, 从而导致乳腺癌细胞对内分泌治疗药物他莫昔芬产生获得性耐药, 为探讨 miR-221/222 在乳腺癌的化疗耐药中是否发挥作用, 本研究选取乳腺癌 MDA-MB-231/ 阿霉素 (Doxorubicin, DOX) 细胞, 通过改变细胞内 miR- 221/222 表达水平, 观察其在 MDA-MB-231/DOX 细胞的 DOX 耐药中的作用, 报告如下。

1 材料与方法

1.1 细胞培养

乳腺癌细胞株 MDA-MB-231、MDA-MB-231/DOX 在实验室常规培养, 保种, MDA-MB-231/DOX 耐药细胞的耐药指数 (Resistance index, RI)^[11] 为 11.2。MDA-MB-231 细胞培养于 DMEM 培养基 (成分: 10% 的 FBS; 10 $\times$ 10⁴ U/L 的青霉素;

100 mg/L 的链霉素) 中, MDA-MB-231/DOX 细胞培养于 DMEM 培养基 (成分: 1 μ mol/L DOX; 10% 的 FBS; 10 $\times$ 10⁴ U/L 的青霉素; 100 mg/L 的链霉素) 中。有关条件: 37 $^{\circ}$ C 下放于 5% 的 CO₂ 中给予培养, 提取复苏之后的第 4-10 代子细胞实施实验。

1.2 细胞分组和转染

取 4 $\times$ 10⁵ 个对数生长期阶段的实验 MDA-MB-231/DOX 细胞, 将其接种在 6 cm 的培养皿内, 放置 24 h 后, 以脂质体 Lipofectamine[®] 3000 (美国 Invitrogen 公司) 将 miR-221/222 in hibitor (5'-UGGGGUAUUUGACAAACUGACA-3', 上海生工生物工程技术服务有限公司) (inhibitor 组) 及 miR 无关的序列 (设为阴性对照组) 将其转染于 MDA-MB-231/DOX 细胞内, 涉及的步骤遵照说明书进行, 而未转染的 MDA-MB-231/DOX 细胞为空白对照组。分别于 24 h, 48 h, 72 h 及 96 h 后, 收集各组细胞。

1.3 实时荧光定量 PCR (real time quantitative PCR, qRT-PCR)

收集各实验组 3-5 $\times$ 10⁵ 个细胞, 分别加入 1 mL Trizol 试剂 (美国 Invitrogen 公司) 后抽提细胞总 RNA, 遵照试剂盒 (上海谷研实业有限公司) 附带的说明书实施操作。通过紫外分光光度计 (美国 Thermo 公司) 测定出总 RNA 的浓度和纯度 (A₂₆₀/A₂₈₀), 并以逆转录的试剂盒 (美国 Invitrogen 公司) 进行逆转录, 产物置于 -20 $^{\circ}$ C 保存备用。miR-221/222 及内参 U6 的 qRT-PCR 引物序列 (上海生工生物工程技术服务有限公司), 见表 1。定量 PCR 扩增仪 (ABI 7500, 美国 ABI 公司) 检测细胞内 miR-221/222 表达水平。qRT-PCR 扩增体系为 10 μ L, 反应条件: 95 $^{\circ}$ C, 30 s; 95 $^{\circ}$ C, 5s; 60 $^{\circ}$ C, 35s, 实施 40 个循环后通过 2^{- $\Delta\Delta$ CT} 法测定 miR-221/222 的相对表达量, 将实验重复进行 3 次之后, 进行统计分析。

表 1 qRT-PCR 检测 miR-221/222 的引物序列

Table 1 Primer sequence of miR-221 / 222 detected by qRT-PCR

Genes	Primers	Sequence(5'-3')
miR-221/222	RT	GTCGTATCCAGTGCAGGGTCCGAGGTA TTCGCACTGGATACGACACTCACC
	qRT-PCR	
	Forward	CAGCATACATGATTCTTGTGA
	Reverse	CTTTGGTGTTTGAGATGTTTGG
	RT	TGGTGTCGTGGAGTCG
	qRT-PCR	
U6	Forward	CTCGCTTCGGCAGCACA
	Reverse	AACGCTTCACGAATTTGCGT

1.4 药物敏感性实验

转染 48h 后,将各组 MDA-MB-231/DOX 细胞成功接种在 96 孔板内,使每孔接种约 1×10^4 个实验细胞,依次添加不同浓度的 DOX(0,1,2,4,8,16,32 $\mu\text{mol/L}$),并设置 3 个复孔,再培养 48 h。完成后弃去上清,添加 CCK-8 法有关试剂,再培养 4 h,最后记录 450 nm 波长的有关吸光度值。以 SPSS20.0 软件计算 DOX 对各组 MDA-MB-231/DOX 细胞的半数抑制浓度 (50% inhibitory concentration, IC_{50}),并绘图。

1.5 细胞凋亡检测

采用流式细胞术 (Flow Cytometry, FCM),Annexin V-FITC/PI 双染法检测各组 MDA-MB-231/DOX 细胞凋亡率,转染 48h 后,各组 MDA-MB-231/DOX 细胞中分别加入含有 3 $\mu\text{mol/L}$ DOX 的无血清培养液,继续培养 48 h,收集各组细胞,PBS 洗涤 2 次,加入 500 μL Binding Buffer 重悬细胞,随后加入 5 μL Annexin V-FITC 混匀后,加入 5 μL PI,混匀,室温避光孵育 10min,流式细胞仪 (FACSCanto II,美国 BD 公司)检测细胞凋亡率,实验重复 3 次后,进行统计分析。

1.6 蛋白质免疫印迹检测 (Western blotting, WB)

收集约 $4-6 \times 10^5$ 个实验细胞,添加 RIPA 蛋白的裂解液(上海谷研实业有限公司),成功抽提出细胞全蛋白,通过 BCA 蛋白定量试剂盒(美国 Thermo 公司)测定蛋白水平。利用 15%的 SDS-PAGE 胶常规电泳分离出蛋白之后,以湿转法使蛋白转至 PVDF 膜,再用 5%的脱脂奶粉于室温下震荡封闭约 2h,用 TBS-T 洗膜,兔抗人凋亡调控因子 (PUMA) 单克隆抗体 (12450,美国 CST 公司,1:2000 稀释),兔抗人 Bcl2 蛋白修饰因子 (BMF) 多克隆抗体 (5889,美国 CST 公司,1:1000 稀释),兔抗人细胞周期蛋白激酶抑制因子 p27 (p27^{Kip1}) 多克隆抗体

(2552,美国 CST,1:2000 稀释),兔抗人型 GAPDH 单克隆抗体 (2118,美国 CST,1:3000 稀释),在 4°C 下孵育过夜。以 TBS-T 洗膜,而后添加 HRP 所标记的羊抗兔二抗(北京北方同正生物科技发展有限公司,1:10000 稀释),以室温孵育约 1 h,加 ECL 发光液(北京泰新生物科技有限公司)实施化学发光及显影,通过凝胶成像仪 (GelDoc-It,美国 UVP 公司)为蛋白条带实施观察,得到图像后通过 ImageLab 软件实施灰度分析,登记灰度值。实验重复 3 次后,进行统计分析。

1.7 统计分析

通过 SPSS20.0 软件实施资料分析。计量资料经检验均符合正态分布,以 $(\bar{x} \pm s)$ 的形式表示,组间比较采用 t 检验。检验标准设定为 $\alpha=0.05$,以 $P<0.05$ 为差异有统计学意义。

2 结果

2.1 乳腺癌 MDA-MB-231/DOX 细胞中 miR-221/222 表达情况及转染效率

转染前,qRT-PCR 实验结果显示,MDA-MB-231/DOX 细胞中的 miR-221/222 的表达量 (6.34 ± 0.35),显著高于亲本 MDA-MB-231 细胞的 (1.02 ± 0.06) ($P<0.05$),见图 1A,表明 miR-221/222 可能参与并促进了乳腺癌 MDA-MB-231/DOX 细胞对 DOX 的耐药性。采用脂质体转染 miR-221/222 inhibitor 至 MDA-MB-231/DOX 细胞中,转染 24 h 后,实验组的 miR-221/222 水平下降,并且抑制效应可持续至 96 h,miR-221/222 的表达水平分别降低至空白对照组的 (68.92 ± 3.07)%和阴性对照组的 (64.59 ± 2.68)%;且空白对照组与阴性对照组在转染后的各时间点的 miR-221/222 表达水平相比,无统计学意义 ($P>0.05$),见图 1B。

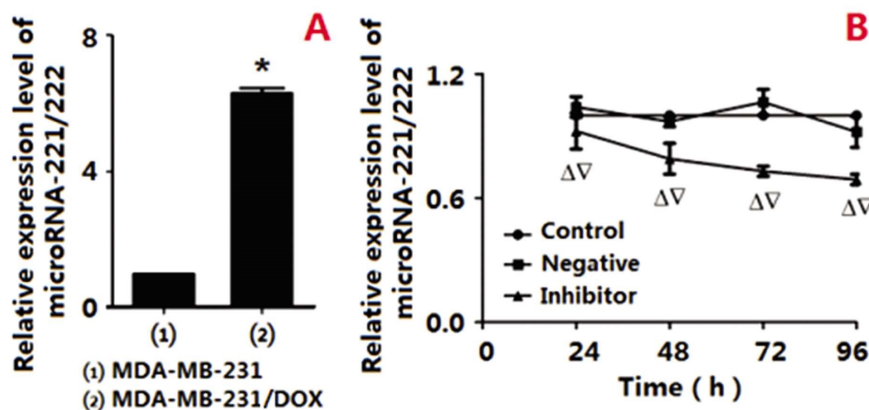


图 1 乳腺癌 MDA-MB-231/DOX 细胞中 miR-221/222 表达情况及转染效率

Fig.1 Expression and transfection efficiency of miR-221 / 222 in MDA-MB-231 / DOX cells of breast cancer

Note: Compared with MDA-MB-231 cells, $*P<0.05$; compared with the blank control group, $P<0.05$; compared with the negative control group, $P<0.05$.

2.2 miR-221/222 inhibitor 转染对 MDA-MB-231/DOX 细胞 DOX 耐药的影响

DOX 对 inhibitor 组耐药细胞的 IC_{50} 值为 (3.11 ± 1.02) $\mu\text{mol/L}$,明显低于空白对照组的 (12.42 ± 1.63) $\mu\text{mol/L}$ 和阴性对照组的 (11.61 ± 1.37) $\mu\text{mol/L}$,差异有统计学意义 ($P<0.05$),空白对照和阴性对照组的 IC_{50} 值无统计学差异 ($P>0.05$),见图 2。

2.3 miR-221/222 inhibitor 转染对 MDA-MB-231/DOX 细胞凋亡

的影响

与空白对照组相比,inhibitor 组耐药细胞在转染 miR-221/222 inhibitor 48 h 后,3 $\mu\text{mol/L}$ DOX 继续处理 48 h,细胞的早期、晚期凋亡率以及总凋亡率均升高,亦高于阴性对照组,差异均有统计学意义 ($P<0.05$);而空白对照组和阴性对照组的早期、晚期凋亡率以及总凋亡率的差异均无统计学差异 ($P>0.05$),见表 2。

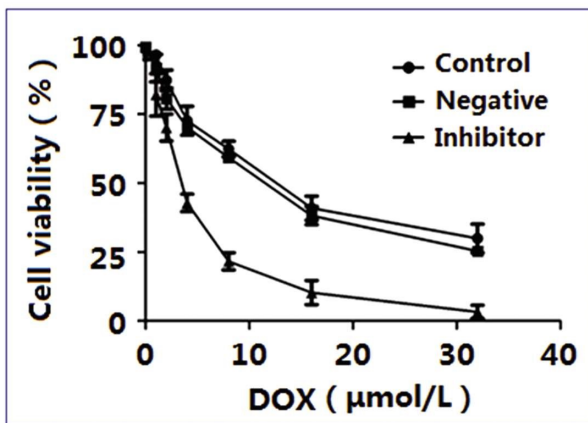


图2 miR-221/222 水平变化对 MDA-MB-231/DOX 细胞对 DOX 药物敏感性的影响

Fig. 2 Effect of miR-221/222 on drug sensitivity of MDA-MB-231/DOX cells to dox

Note: After 48 hours of transfection, DOX (0, 1, 2, 4, 8, 16, 32 μ .mol / L) of different concentrations continued to treat cells for 48 hours, and CCK-8 was used to detect cell viability.

表 2 转染 48h 后, 各组 MDA-MB-231/DOX 细胞的凋亡率(%)

Table 2 Apoptosis ratio of MDA-MB-231/DOX cells in each group after 48 hours of transfection (%)

Groups	Early apoptosis ratio	Late apoptosis ratio	Total apoptosis ratio
Blank control group	2.13±0.25	3.21±0.71	5.61±1.14
Negative control group	1.79±0.17	3.54±0.48	5.46±1.25
Inhibitor group	4.38±1.12 ^Δ ▽	21.93±1.43 ^Δ ▽	26.37± 4.01 ^Δ ▽

Note: Compared with the blank control group, ^Δ P <0.05; compared with the negative control group, ▽ P <0.05.

2.4 miR-221/222 inhibitor 转染对 MDA-MB-231/DOX 细胞中促凋亡蛋白表达的影响

与空白对照组相比, inhibitor 组耐药细胞在转染 miR-221/222 inhibitor 48 h 后, 细胞内的促凋亡蛋白 PUMA, BMF 以及 p27^{Kip1} 的表达均增加(P <0.05); 空白对照组与阴性对照组细胞内的 PUMA, BMF 以及 p27^{Kip1} 蛋白表达水平的差异, 均无统计学意义(P >0.05), 见图 3, 表 3。

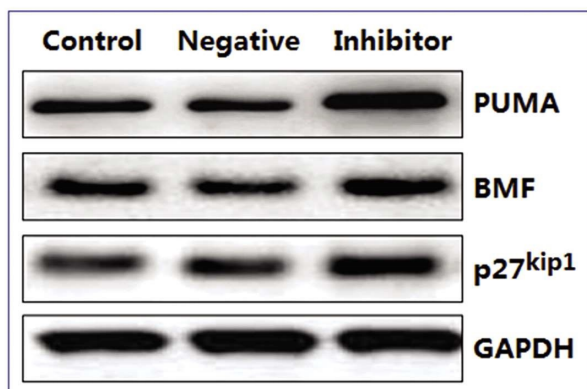


图3 转染 48h 后, MDA-MB-231/DOX 细胞中促凋亡蛋白的表达水平
Fig.3 Expression level of pro-apoptotic protein in MDA-MB-231/DOX cells 48 hours after transfection

Note: 48 hours after transfection, WB detected the expression of puma, BMF and p27^{Kip1} in the cells.

3 讨论

乳腺癌是女性易患的三大癌症之一, 可严重危害生命健康。当前, 化疗是临床上综合治疗方案当中重要的一环, 但在化疗时出现的耐药性往往是引发治疗失败及预后不良, 以及死亡的一个重要因素^[12-14]。有报道表明, 在肿瘤的发生及恶性进展中, 会有大量 miR 发生改变, 提示从 miR 角度探讨肿瘤的恶性进展机制有重要意义^[15-17]。

近年来, miR 与肿瘤耐药之间的关系, 受到越来越多的关注。miR 是通过 Drosha 酶及 Dicer 酶剪切 miR 前体所得, 其可通过和靶基因 mRNA 的 3' 非编码区 (3' Untranslated Region, 3'-UTR) 结合, 在转录水平或者转录后水平, 调节靶基因的表达水平, 进而参与细胞的各项生命活动^[18-20]。据文献报道, miR-221/222 与多种肿瘤的治疗疗效及预后相关, 如前列腺癌^[21]、结直肠癌^[22]及肝癌^[23]。Antoniali G 等^[24]证实, miR-221/222 能够通过靶向 PTEN 信号通路, 调节癌细胞的放疗敏感性。不少国外的研究^[25-27]证明, miR-221/222 能够通过多种途径, 如影

响乳腺癌上皮间质转化、选择性干扰 A20/c-Rel/ 结缔生长因子 (Connective tissue growth factor, CTGF) 信号通路从而影响乳腺癌的发生发展。那么 miR-221/22 在乳腺癌的化疗耐药中, 是否也发挥着诱导和促进作用呢? 本研究探讨了 miR-221/22 在乳腺癌 MDA-MB-231/DOX 细胞 DOX 耐药中的作用。

本研究实验结果发现转染 miR-221/222 inhibitor 后, MDA-MB-231/DOX 细胞的早期, 晚期以及总凋亡率均显著升高, 表明降低 miR-221/222 水平可达到促进细胞凋亡的效果, 提示 miR-221/222 具有原癌基因的作用。此外, 抑制 miR-221/222 水平可提高 MDA-MB-231/DOX 细胞对 DOX 的药物敏感性, 可见抑制 miR-221/222 能够增加乳腺癌 MDA-MB-231/DOX 细胞对 DOX 的药物敏感性, 提示抑制 miR-221/222 有逆转乳腺癌 MDA-MB-231/DOX 细胞对 DOX 耐药的潜能。

多项研究证实^[28-30], miR-221/222 可通过调节靶蛋白的转录表达, 如 PUMA, BMF 以及 p27^{Kip1} 等, 从而在多种恶性肿瘤的发生发展过程中发挥着重要的作用。在本研究中, 我们采用 WB 法检测了 MDA-MB-231/DOX 细胞中, 抑制 miR-221/222 的表达后, 促凋亡蛋白 PUMA, BMF 以及 p27^{Kip1} 的表达水平的变化情况, 实验结果显示, 降低 MDA-MB-231/DOX 细胞内 miR-221/222 水平, PUMA, BMF 以及 p27^{Kip1} 的蛋白表达水平也随之增加, 说明在乳腺癌 MDA-MB-231/DOX 细胞中 miR-221/222 可能参与了促凋亡蛋白 PUMA, BMF 以及 p27^{Kip1}

表 3 转染 48h 后,MDA-MB-231/DOX 细胞中促凋亡蛋白的表达水平

Table 3 Expression level of pro-apoptotic protein in MDA-MB-231/DOX cells 48 hours after transfection

Groups	PUMA	BMF	p27 ^{Kip1}
Blank control group	0.75±0.03	0.59±0.05	0.61±0.04
Negative control group	0.69±0.04	0.52±0.05	0.64±0.06
Inhibitor group	1.13±0.12 ^a ▽	1.19±0.06 ^a ▽	0.96±0.05 ^a ▽

Note: Compared with the blank control group, $P<0.05$; compared with the negative control group, $P<0.05$.

的表达调控。本研究的不足之处在于,并没有针对 miR-221/222 调控促凋亡蛋白 PUMA,BMF 以及 p27^{Kip1} 表达的具体机制作深入研究,接下来,我们将会进一步深入探讨其内在机制,并且探寻 miR-221/222 诱导乳腺癌细胞化疗耐药过程中的新靶蛋白。

综上所述,抑制 miR-221/222 表达可降低 MDA-MB-231/DOX 细胞对 DOX 的耐药性,这可能是通过增加促凋亡蛋白 PUMA,BMF 以及 p27^{Kip1} 表达实现的,提示 miR-221/222 在逆转乳腺癌 DOX 耐药中有一定的应用前景。

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