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MiR-214 reduces cell survival and enhances cisplatin-induced cytotoxicity via down-regulation of Bcl2l2 in cervical cancer cells

Fang Wang, Min Liu, Xin Li, Hua Tang *

Tianjin Life Science Research Center and Basic Medical School, Tianjin Medical University, Tianjin 300070, China

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A B S T R A C T

MiR-214 has been shown to inhibit cell growth, migration and invasion. Here we demonstrate that ectopic expression of miR-214 reduces cell survival, induces apoptosis and enhances sensitivity to cisplatin through directly inhibiting Bcl2l2 expression in cervical cancer HeLa and C-33A cells. Further analysis reveals that apoptosis induced by miR-214 is correlated with increased expression of Bax, caspase-9, caspase-8 and caspase-3. Moreover, we show that miR-214 is regulated by DNA methylation and histone deacetylation. Taken together, these data suggest that miR-214 might be a candidate target for the development of novel therapeutic strategies to treat cervical cancer.

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1. Introduction

Cervical cancer is a highly prevalent cancer that affects women across the world. Most women with early lesions are cured with surgery or radiation alone. However, in patients with metastatic diseases or those with more advanced lesions who are at significant risk of recurrence, chemotherapy is the only option. The standard regimen in this setting has been systemic cisplatin, but the response rate is very low, mainly because the cancer cells frequently develop resistance to chemotherapy agents. The cellular mechanisms of drug resistance include over-expression of p-glycoproteins that eject anticancer drugs from cells, acquired mutations in drug target cells and defects in cellular apoptosis pathways [1]. Recent reports have shown that microRNAs (miRNAs), which are critical for many important processes such as development, differentiation and even carcinogenesis, can regulate the chemosensitivity of tumor cells [2]. MiRNAs are approximately 22 nt in length and regulate gene expression by binding to the 3′untranslated regions (UTRs) of mRNAs, leading to mRNA degradation or repression of translation. It has been reported that miR-122 sensitizes HCC cancer cells to adriamycin and vincristine by modulating the expression of MDR and inducing cell cycle arrest [3]. Over-expression of miR-15b and miR-16 sensitizes human gastric cancer cells to VCR-induced apoptosis by reducing the levels of Bcl-2 [4]. However, the exact mechanisms underlying drug resistance in cervical cancer cells have not been completely characterized. Therefore, clarifying the mechanisms underlying cancer cell drug resistance in detail and improving chemosensitivity should improve the survival rate in cervical cancer patients.

Our previous studies have demonstrated that miR-214 is frequently down-regulated in cervical cancer tissues and that ectopic expression of miR-214 can inhibit cellular proliferation, growth, migration and invasion in the HeLa cervical cancer cell line [5–7]. Here, we showed that miR-214 significantly reduced cell survival and rendered cell sensitivity to cisplatin through inhibiting the anti-apoptotic protein Bcl2l2 using the stable cell lines HeLa/miR-214 expressing high level of miR-214 and HeLa/Neg-Ctrl as negative control cell line (established in our previous work [6]) and another cervical cancer C-33A cell line. Furthermore, we found that miR-214 can induce apoptosis and enhance the expression of Bax, caspase-9, caspase-3 and caspase-8, indicating that miR-214 induced cell apoptosis partly though altering the ratio of Bax/Bcl2l2 and inducing the intrinsic apoptosis pathway.

Abbreviations: miRNA, microRNA; miR-214, microRNA-214; IC_{50}, inhibit concentration 50; UTR, untranslated region; EGFP, enhanced green fluorescence protein; HA, hemagglutinin; 5-aza-CdR, 5-aza-deoxycytidine; TSA, trichostatin A; Bcl2l2, BCL2-like 2; MEK3, MAPK/ERK kinase 3; JNK1, c-jun N-terminal kinase 1; GAPDH, glyceraldehyde phosphate dehydrogenase; ANOVA, analysis of variance

* Corresponding author. Address: No. 22 Qi-Xiang-Tai Road, Tianjin 300070, China. Fax: +86 22 23542503.

E-mail address: htang2002@yahoo.com (H. Tang).

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2. Materials and methods

2.1. Human tissue specimens and cell culture

Eleven paired human cervical cancer tissues and adjacent normal tissues were obtained from the Tumor Bank Facility of Tianjin Medical University Cancer Institute and Hospital and National Foundation of Cancer Research. Histologically, all the biopsies belonged to squamous cell carcinoma. The collection of human tissue samples was approved and supervised by the Ethics Committee of Tianjin medical university. Cervical cancer HeLa and C-33A cell lines were obtained from the ATCC. HeLa/miR-214 cells and HeLa/Neg-Ctrl cells were established and maintained in our laboratory [6]. These cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 mg/ml streptomycin. All transfections were performed using LipofectAmine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations.

2.2. Cell survival and cytotoxicity assays

Cells were seeded in a 96-well plate (8×10^3 HeLa cells or 1.2×10^4 C-33A cells per well). Twenty-four hours later, the cells medium was replaced with fresh medium containing five different concentrations of FBS (0%, 1%, 3%, 6% and 10%) for cell survival assays or six different concentrations of cisplatin (0, 0.04, 0.2, 1, 5 and 25 μg/ml) for cytotoxicity assays. The cells were then incubated at 37 °C for another 24 or 48 h and cell viability was determined by MTT assays. The absorbance at 570 nm (A_{570}) of each well was read on a spectrophotometer. The inhibition rate (IR) of each group was calculated by the formula: $IR = ((X-Y)/X) * 100\%$. X is the A_{570} of the untreated group and Y is the A_{570} of each group with cisplatin treatment. The concentration at which each drug produced 50% inhibition of growth (IC_{50}) was estimated using the IR. Three independent experiments were performed in triplicate.

2.3. TUNEL assays

HeLa/miR-214 and HeLa/Neg-Ctrl cells (4×10^3 cells/well) were seeded on a 14-well slide (Cel-Line/Erie Scientific Co., NH, USA) in triplicate. Twenty-four hours later, the medium was replaced with fresh medium containing 0% FBS or 10% FBS for another 48 h. Apoptosis was detected using an In situ Cell Death Detection Kit with Fluorescein (Roche Applied Science, IN, USA). DNA was stained with DAPI (Sigma–Aldrich, St Louis, MO, USA). The TUNEL assays were quantified by counting the DAPI and TUNEL-positive cells in five individual fields via fluorescence microscopy respectively. The apoptotic rate was calculated by dividing the average number of TUNEL-positive cells by that of the DAPI-positive cells in one field. The assays were repeated three times.

2.4. RNA extraction and qRT-PCR

Total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. QRT-PCR was performed as previously described to assess the expression level of miR-214, primary transcript of miR-214 (pri-miR-214) and the mRNA levels of Bcl2l2, JNK1, MEK3 and Plexin-B1 using the $2^{-\Delta\Delta CT}$ method [6]. U6 snRNA or β-actin mRNA was used as internal standards to normalize the expression of miR-214 or the expression of pri-miR-214 and other mRNAs, respectively. The specific primer pairs were shown in Table 1.

2.5. Plasmids construction

According to the known functional siRNA sequence [8], the sequences, shBcl2l2-Top and shBcl2l2-Bot, were annealed and inserted into the BamHI and HindIII sites of pSilencer2.1-U6 neo (Ambion, Austin, USA) vector to generate a shRNA expression plasmid against Bcl2l2, pSilencer2.1-U6 neo/Bcl2l2 (sh-Bcl2l2). PSilencer2.1-U6 neo negative control (sh-Neg-Ctrl, Ambion, Austin, USA) was used as a negative control plasmid.

Table 1
Primers used in the construction of plasmids and qRT-PCR.

Usage	Primer name	Sequence (5′–3′)
Construction for Bcl2l2 EGFP reporters	Bcl2l2-UTR-S	5′-GAGTAAGCGGCCGCGGCTGGGCAATTTGCCCTCA-3′
	Bcl2l2-UTR-MA	5′-CCAACTTTTTTTCTCGTGCAAACCCCTTAATG-3′
	Bcl2l2-UTR-MS	5′-CATTAAGGGGTTTGCACGAGAAAAAAAGTTGG-3′
	Bcl2l2-UTR-AS	5′-CAGTCGCTCGAGCTCCTCTTGGCTAAAGGTCC-3′
qRT-PCR for miR-214	miR-214 RT	5′-GTCGTATCCAGTGCAGGGTCCGAGGTGCACTGGATACGACACTGCCTG-3′
	miR-214 Forward	5′-TGCGGACAGCAGGCACAGAC-3′
	Reverse primer	5′-CCAGTGCAGGGTCCGAGGT-3′
qRT-PCR for U6 snRNA	U6 RT	5′-GTCGTATCCAGTGCAGGGTCCGAGGTGCACTGGATACGACAAAATATGG-3′
	U6 Forward	5′-TGCGGGTGCTCGCTTCGGCAGC-3′
	Reverse primer	5′-CCAGTGCAGGGTCCGAGGT-3′
Construction for pSilencer2.1-U6 neo/Bcl2l2	shBcl2l2-Top	5′-GATCCCGCTCGGTCCTGCGATTATTATTCAAGAGATAATAATCGCAGGACCGAGTTTTTTGGAAA-3′
	shBcl2l2-Bot	5′-AGCTTTTCCAAAAAACTCGGTCCTGCGATTATTATCTCTTGAATAATAATCGCAGGACCGAGCGG-3′
Construction for pcDNA3HA/Bcl2l2	Bcl2l2-PS	5′-CAGGAATTCGCCACCATGGCGACCCCAGCCTCGGCCCCAG-3′
	Bcl2l2-PA	5′-GCCCTGCTCGAGGCCTTGCTAGCAAAAAAGGCCCCTAC-3′
qRT-PCR for β-actin	β-Actin-S	5′-CGTGACATTAAGGAGAAGCTG-3′
	β-Actin-AS	5′-CTAGAAGCATTTGCGGTGGAC-3′
qRT-PCR for pri-miR-214	pri-miR-214-S	5′-CATAGGATCCAGATCTGCTGAACTCTGACTACATG-3′
	pri-miR-214-AS	5′-GGGCGGAATTCTATTTCATAGGCACCACTC-3′
qRT-PCR for Plexin-B1	Plexin-B1-S	5′-CGCGGATCCCCTGGTGAAGCCAAGTGAT-3′
	Plexin-B1-AS	5′-CGCGAATTCTGGTGTCCTGGTCGGAGAT-3′
qRT-PCR for MEK3	MEK3-S	5′-CGCGGATCCAAAGAAGCAGACC-3′
	MEK3-AS	5′-CGGAATTCAAAGCCGGGATAGAGG-3′
qRT-PCR for JNK1	JNK1-S	5′-CGCGGATCCAAAGATCCCTGACAAG-3′
	JNK1-AS	5′-CGGAATTCCTAACCGACTCCCCATC-3′

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