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

Abstract

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.

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 cell 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 through altering the ratio of Bax/Bcl2l2 and inducing the intrinsic apoptosis pathway.
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 (A570) of each group was calculated by the formula: IR = (X - Y)/X × 100%. X is the A570 of the untreated group and Y is the A570 of each group of cells with cisplatin treatment. The concentration at which each drug produced 50% inhibition of growth (IC50) 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^-ΔΔCT 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

<table>
<thead>
<tr>
<th>Usage</th>
<th>Primer name</th>
<th>Sequence (5’-3’)</th>
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<td>Reverse primer</td>
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A 608 bp fragment of the human wild-type Bcl2l2 coding sequence was amplified by PCR using Bcl2l2-PS and Bcl2l2-PA from the cDNA of HeLa cells and cloned into the pcDNA3HA plasmid (constructed by our laboratory), which allows the expression of a hemagglutinin (HA) tag at the C-terminal of the protein of interest using the EcoRI and Xhol sites. The resulting plasmid was designated as pcDNA3HA/Bcl2l2. All of the constructs were confirmed by DNA sequencing. The primers were provided in Table 1.

2.6. Western blot analysis

Total cellular extracts were extracted using RIPA buffer. Proteins were separated by 12% SDS–PAGE, and proteins of interest were detected using the appropriate antibodies. Rabbit anti-human GAPDH, tubulin, HA, caspase-8, caspase-9 and caspase-3 antibodies were from Saierbio (Tianjin, China). Rabbit anti-human Bcl2l2 and Bax primary antibodies were obtained from Abcam (Cambridge, UK) and Santa Cruz Biotechnology (CA, USA), respectively.

2.7. Target prediction and EGFP reporter assays

Based on bioinformatic prediction (PicTar, miRanda and Target-Scan), Bcl2l2 was selected from anti-apoptotic subset as candidate target of miR-214. The 3’UTR segments of Bcl2l2 containing putative binding sites for miR-214 were obtained by PCR and inserted into pcDNA3/EGFP (constructed previously [9]). The wild-type reporter construct pcDNA3/EGFP-Bcl2l2-UTR and the mutant reporter construct pcDNA3/EGFP-Bcl2l2-UTRmut, in which the site of perfect complementarity to miR-214 was mutated (CTGCTG → CACGAG) using site-directed mutagenesis PCR, were used for miRNA functional analysis. Wild-type and mutant insertions were confirmed by DNA sequencing. All primer information is available in Table 1.

For EGFP reporter experiments, HeLa cells were co-transfected with the wild-type or mutant 3’UTR EGFP construct and the miR-214 expression vector pcDNA3/miR-214 (constructed previously [6]) or pcDNA3 negative control vector. The vector pDsRed2-N1 (Clontech, USA) expressing RFP was used for normalization. EGFP and RFP levels were measured at 48 h after transfection as previously described [9]. Each experiment was repeated at least three times.

2.8. Treatment of cells with 5-aza-2′-deoxycytidine and Trichostatin A

HeLa cells were seeded into 60-mm dishes on day 0 and exposed to the DNA methyltransferase inhibitor 5-aza-CdR (5-aza-deoxycytidine; Sigma–Aldrich; 5 μM) from day 1 to day 4. Meanwhile, the histone deacetylase inhibitor TSA (trichostatin A; Sigma–Aldrich; 0.1 μM) was added to the cells on day 3. Cells treated with 5-aza-CdR, TSA, or both were harvested on day 4. The mock-treated group received a comparable treatment with ethanol. QRT-PCR was used to examine the expression of primiR-214, miR-214 and Bcl2l2. Known target genes of miR-214, including Plexin-B1, MEK3 and JNK1, were used as positive controls. All the primers used are shown in Table 1.

2.9. Statistical analysis

Statistical significance between groups was assessed using ANOVA or Student’s t-test. The data are presented as means ± S.D. Pearson’s correlation was employed to analyze the relationship between two factors. P < 0.05 was considered to be statistically significant.

3. Results

3.1. Over-expression of miR-214 reduces cell survival and enhances cisplatin-induced cytotoxicity in HeLa cells

In order to investigate the role of miR-214 in survival and sensitivity to cisplatin, cell survival assays and cytotoxicity assays were performed using HeLa/miR-214 and HeLa/Neg-Ctrl cells. We found that the viability of HeLa/miR-214 was significantly lower than that of HeLa/Neg-Ctrl cells in all serum conditions (Fig. 1A), and HeLa/miR-214 cells required only half as much cisplatin to achieve the same level of cell death as the HeLa/Neg-Ctrl cells at 48 h (Fig. 1B).

3.2. Bcl2l2 is a direct target of miR-214

To explore the mechanisms through which miR-214 inhibits cell survival and increases cellular sensitivity to cisplatin, we searched for potential targets of miR-214 using bioinformatic algorithms. One predicted miR-214-binding site (2690–2711 nt) was found within the 3’UTR of Bcl2l2 mRNA (Fig. 2A). Then we constructed EGFP reporter plasmids carrying the wild-type or mutant 3’UTR sequence (shown in Fig. 2A) and performed the EGFP reporter assays. As shown in Fig. 2B, the normalized intensity of fluorescence was significantly reduced in the pcDNA3/miR-214- and wild-type EGFP reporter plasmid-transfected group as compared to the
control group. In contrast, there was no significant difference between the pcDNA/miR-214 and the pcDNA3 group when the mutant EGFP reporter plasmid was transfected (Fig. 2B). These results demonstrate that miR-214 can bind directly to the Bcl2l2 3'UTR to repress gene expression.

We went onto measure the endogenous expression of Bcl2l2 in HeLa cells with various levels of miR-214. As shown in Fig. 2C and D, Bcl2l2 mRNA and protein levels were both inhibited in HeLa/miR-214 cells, indicating miR-214 can negatively regulate the expression of Bcl2l2 at the post-transcriptional level. Consistent with previous studies [6,10], we also observed an obvious decrease in the expression of miR-214 and a significant increase in the expression of Bcl2l2 mRNA in cervical cancerous tissues compared to adjacent normal cervical tissues (Fig. 2E and F) which was inversely correlated ($R = -0.826$) (Fig. 2G).

3.3. Knockdown of Bcl2l2 in HeLa cells leads to decreased cell survival and increased cisplatin-induced cytotoxicity

We also transfected a shRNA expression vector targeted against Bcl2l2 (sh-Bcl2l2) into HeLa cells after the validity was confirmed (Fig. 3A) and tested cell survival and sensitivity to cisplatin. As shown in Fig. 3B and C, knockdown of Bcl2l2 in HeLa cells obviously decreased cellular viability in various conditions and increased the sensitivity to cisplatin by ~8-fold. The respective $IC_{50}$ doses for cisplatin were 0.32 $\mu g/ml$ (HeLa cells transfected with sh-Bcl2l2) and 2.4 $\mu g/ml$ (HeLa cells transfected with sh-Neg-Ctrl).

3.4. Restoration of Bcl2l2 expression in HeLa/miR-214 cells reverses the changes in cell survival and resistance to cisplatin induced by miR-214

If the effect of excess miR-214 is specific, co-expression of Bcl2l2 protein should be able to suppress the miR-214 over-expression phenotype. The plasmid expressing Bcl2l2 pcDNA3HA/Bcl2l2, which significantly increased the protein expression of Bcl2l2 (Fig. 4A) was transfected into HeLa/miR-214 cells followed by cell survival and cytotoxicity assays. As shown in Fig. 4B and C, pcDNA3HA/Bcl2l2 significantly increases cell viability under all serum conditions and cisplatin-resistance in HeLa/miR-214 cells compared to the pcDNA3HA-transfected group. To achieve the same level of cell death, HeLa/miR-214 cells with high levels of Bcl2l2 ($IC_{50}$, 13.59 $\mu g/ml$) required 4.85 times the concentration of cisplatin compared to control cells ($IC_{50}$, 2.80 $\mu g/ml$).
3.5. Ectopic expression of miR-214 significantly reduces cell survival and resistance to cisplatin in another cervical cancer C-33A cells, which was counteracted by re-expression of Bcl2l2.

We went on investigating whether miR-214 exert the similar function on cell survival and cisplatin-induced cytotoxicity through targeting Bcl2l2. As expected, in C-33A cells, ectopic expression of miR-214 significantly inhibited Bcl2l2 expression (Fig. 5A), reduced cell survival (Fig. 5B) and induced sensitivity to cisplatin (Fig. 5C). And pcDNA3HA/Bcl2l2 was also able to restore the decreased cell viability and cisplatin-resistance in C-33A cells (Fig. 5B and C).

3.6. Over-expression of miR-214 promotes apoptosis via caspase-8 and -9 activation

We further investigated whether miR-214 could alter the sensitivity of HeLa cells to apoptosis upon serum deprivation using TUNEL assays. As shown in Fig. 6A, HeLa/Neg-Ctrl cells showed almost no green fluorescence regardless of the amount of serum, whereas HeLa/miR-214 cells showed weak or strong fluorescence when incubated with 10% FBS or 0% FBS (Fig. 6A). In addition, enhanced expression of Bax (21 KDa), caspase-9 (47 KDa), caspase-3 (33 KDa), caspase-8 (57 KDa) was found in HeLa/miR-214 cells compared with HeLa/Neg-Ctrl cells, in parallel with the elevated
levels of activated forms of caspase-9 (25 KDa), caspase-3 (16 KDa) and caspase-8 (18 KDa), and the strongest expressions of these apoptosis-related proteins were occurred in HeLa/miR-214 cells under conditions of serum deprivation (Fig. 6B). Moreover, restoration of Bcl2l2 in HeLa cells transfected with pcDNA3/miR-214 abolished the increased expression of all of the above apoptosis-related protein except caspase-8 (the expression of active caspase-8 was not detected, Fig. 6C). Together, these data demonstrate that ectopic expression of miR-214 induces apoptosis through activation of caspase-9 and caspase-8 in HeLa cells upon serum deprivation, which was partly related with Bcl2l2 inhibition.

3.7. DNA demethylation and histone acetylation decrease pri-miR-214 expression, whereas increase miR-214 expression in HeLa cells

It is well known that epigenetic mechanisms, including DNA methylation and histone acetylation, regulate miRNA expression [11–12]. Here we found that the expression of pri-miR-214 was down-regulated, whereas the mature miR-214 was up-regulated upon treatment with 5-aza-CdR and/or TSA (Fig. 7A and B). Furthermore, the most pronounced inhibition of pri-miR-214 and the most significant increase of miR-214 are both present in HeLa cells treated with 5-aza-CdR and TSA simultaneously (Fig. 7A and B). In addition, the expression of Bcl2l2 and other known direct miR-214 target genes, including MEK3, JNK1 and Plexin-B1 [5–6], decreased significantly in HeLa cells treated with 5-aza-CdR and/or TSA compared with mock treated controls (Fig. 7C).

4. Discussion

MiR-214 has been found to be aberrantly expressed in human tumors and to play various roles in cancer development. Previous studies have shown that miR-214 is up-regulated and functions as an oncogene in human ovarian cancer and pancreatic cancer, in which miR-214 can promote cell survival and resistance to cisplatin or gemcitabine [13–14]. However, our previous reports have showed that miR-214 is down-regulated in cervical cancer tissues and suppresses cell proliferation, migration and invasion in cervi-
cal cancer cells by targeting MEK3, JNK1, Plexin-B1 and GALNT7, thus functioning as a tumor suppressor [5–7]. Here, we showed that miR-214 can reduce cell survival, promote apoptosis and induce sensitivity to cisplatin in HeLa and C-33A cells, supporting the tumor suppressor role of miR-214 in cervical cancer.

Identification and characterization of the targets of altered miRNAs may help elucidate the molecular mechanisms involved in carcinogenesis. Bcl2l2, also known as Bcl-w, which is a member of the Bcl-2 protein family, has been reported to promote cell survival [15], reduce apoptosis in response to apoptotic stimuli [16], and contribute to chemoresistance [17]. It has also been found to be over-expressed in non-small cell lung cancer [18], gastric and colon cancers [19–20] and result in their carcinogenesis. In the present study, we found that Bcl2l2 was up-regulated in cervical cancer tissues and promoted cell survival and resistance to cisplatin, which confirmed the previous reports. Furthermore, we showed that miR-214 reduced the mRNA and protein expression of Bcl2l2 at post-transcriptional level through directly binding the 3'UTR of Bcl2l2. The inverse correlation between miR-214 levels and Bcl2l2 mRNA levels in cervical cancer tissues supported this conclusion. Moreover, we showed that enforced expression of Bcl2l2 counteracted the effects of miR-214 on cell survival and resistance to cisplatin in HeLa cells. Together, these data suggest that Bcl2l2 is indeed a direct target gene of miR-214.

We went onto detail the apoptosis-related mechanisms involved in miR-214’s action. Apoptosis induced by serum starvation is generally accepted to be caspase dependent. Caspase-3 activity is essential for apoptosis after serum withdrawal [21], but whether apoptosis occurs via the extrinsic pathway (characterized by caspase-8 activation) or the intrinsic pathway (characterized by caspase-9 activation) remains controversial. Here we found that ectopic expression of miR-214 in HeLa cells increased the expressions of Bax, caspase-9, caspase-8 and caspase-3, which were partly reversed by over-expression of Bcl2l2. These data indicate that not only the extrinsic pathway but also the intrinsic pathway is involved in the apoptosis induced by enforced expression of miR-214. Moreover, the increased expression of Bax and decreased expression of Bcl2l2 observed in response to high levels of miR-214 may contribute to a pronounced increase in the Bax/Bcl2l2 ratio and the increase in apoptosis. Together, our data provide the evidence that miR-214 may induce apoptotic signals at least in part through regulation of the amount of Bcl2l2. However, how miR-214 increases the expression of Bax is still unclear.

Moreover, we showed epigenetic regulation by DNA methylation and histone deacetylation may be responsible for the down-regulation of miR-214 observed in HeLa cells. But the inconsistence between the pri-miR-214 and miR-214 expression level upon epigenetic regulation is hard to explain. Fortunately, the enhanced expression of mature miR-214 via 5-aza-CdR and TSA treatment was also verified in another report by Yamane et al., which support our results [22]. Other than that, they found the down-regulation of miR-214 in squamous cell carcinoma cell line most likely caused by hypermethylation of other promoter regions rather than the miR-214 promoter, which may be one reason for the inhibited
transcription of miR-214 upon 5-aza-Cdr treatment [22]. In addition, Twist-1, the important transcriptional activator of miR-214 [23], has been reported to be remarkably suppressed by seven reactivated miRNAs with hypermethylation in their upstream CpG islands in human ductal carcinomas [24] and high level of miR-214 in intrahepatic cholangiocarcinoma [25]. Thus, the down-regulation of Twist-1 upon demethylation or enhanced miR-214 may lead to the transcriptional inhibition of pri-miR-214. Moreover, histone deacetylase 1 has been shown to enhance miRNA processing via deacetylation of DGCR8 [26]. Therefore, the regulation of pri-miR-214 and miR-214 are too sophisticated to elucidate simply by one or two reasons. The detail mechanism of miR-214 transcription and processing in HeLa cells deserves further investigation.

In summary, we here report that miR-214 expression significantly reduces cell survival and enhances cisplatin-induced cytotoxicity, increasing HeLa cell apoptosis (especially under conditions of serum depletion and cytotoxic agent treatment). Because decreases in apoptosis are critical in cancer development and constitute a major barrier to effective treatment, miR-214 may serve as a novel therapeutic target in cervical cancer.

Acknowledgments

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References


