MicroRNA-1298-3p induces tumor-suppressive effects in human cervical cancer cells via post-transcriptional suppression of ONECUT2

Kun Li1, Min Zhou2 and Yan Zhang1✉

1Department of gynaecology and obstetrics, The Third Affiliated Hospital of Shandong First Medical University (Affiliated Hospital of Shandong Academy of Medical Sciences), Jinan City, Shandong Province, 250031, China; 2Department of gynaecology and obstetrics, Jinan Seventh People’s Hospital, Jinan City, Shandong Province, 250101, China

Recent studies have revealed the negative regulatory role of microRNA-1298-3p (miR-1298-3p) in human carcinogenesis. However, the role of miR-1298-3p is yet to be studied in cervical cancer. The present study showed significant (P=0.03) downregulation of miR-1298-3p in human cervical cancer cell lines. Overexpression of miR-1298-3p significantly (P=0.02) suppressed the proliferation of the cervical cancer cells via induction of apoptosis. The percentage of early and late apoptotic cervical cancer cells increased from 3.33% to 43.37% upon miR-1298-3p overexpression. Additionally, expression of Bax was increased and that of Bcl-2 was decreased in miR-1298-3p overexpressing cervical cancer cells. Overexpression of miR-1298-3p could also suppress migration and invasion of the cervical cancer cells. In vivo study showed that miR-1298-3p overexpression significantly (P=0.02) inhibited the xenografted tumor growth via induction of apoptosis. In silico analysis and subsequent in vitro assays revealed that miR-1298-3p exerts its effects by targeting the expression of ONECUT2. While silencing of ONECUT2 could suppress the proliferation of cervical cancer cells, its overexpression could nullify the tumor-suppressive effects of miR-1298-3p. Collectively, the results revealed the tumor-suppressive role of miR-1298-3p in cervical cancer and thus, indicative of its future therapeutic utility.

Keywords: cervical cancer, microRNA, miR-1298, apoptosis, migration, proliferation.

Received: 22 September, 2021; revised: 17 March, 2022; accepted: 03 April, 2022; available on-line: 12 December, 2022

✉e-mail: yanzhang7006@gmail.com

Acknowledgements of Financial Support: This project was supported by the Shandong Provincial Science and Technology Development Program of Medicine and Health (No. 2017WS269) and the Science and Technology Program of Shandong Academy of Medical Sciences (No. 2017-19)

Abbreviations: AO/EB, acridine orange/ethidium bromide; IHC, immunohistochemical; miRs, Micro-RNAs; NCE, normal cervical epithelial cells; PVDF, polyvinylidene difluoride

INTRODUCTION

Cervical cancer, with its higher incidence, higher mortality and lower survival rates is regarded as one of the lethal malignancies among women, worldwide (Cohen et al., 2019; Arbyn et al., 2020). As per statistical data, more than 0.5 million new cases and 0.25 million deaths are reported annually throughout the globe (Johnson et al., 2019; Sawaya et al., 2019). Although, advancement in screening modalities and awareness regarding health and hygiene in women has led to a slight decline in the mortality rates at the global level, the cervical cancer is still ranked as the second most lethal gynecological cancer (Canfell et al., 2019; Bedell et al., 2020). Cervical cancer is often linked with distant metastasis despite the application of advanced chemo- and radio-therapies (Koh et al., 2019). Researchers are of the view that the pathogenesis of cervical cancer needs to be worked out at the molecular level to formulate better prognostic and curative measures against this deadly cancer. Micro-RNAs (miRs), with the recent findings, have emerged as the molecules of interest to be explored for their specific regulatory role in human cancers (Lacona et al., 2019). MiRs are a heterogenous assemblage of short-length RNA molecules not exceeding in size of 25 nucleotides and regulating the eukaryotic gene expression at the post-transcriptional level (Nahand et al., 2019). MiRs execute their role by binding to the specific regulatory regions in the UTRs of protein-coding genes to cause their silencing, post-transcriptionally (Lacona et al., 2019). The miRs have been shown to exercise a crucial regulatory role in various biological and physiological pathways in eukaryotic cells (Rimsburg et al., 2019). The faulty expression profile of these regulatory RNAs has been witnessed to alter the cellular programming and led to many malignancies including human cancer (Wu et al., 2019). The miRs regulate tumorgenesis of human cancers via their oncogenic or tumor-suppressive roles. The human cervical cancer has also been shown to exhibit expression dysregulation of number of miRs (Miao et al., 2020; Chen et al., 2019; Xu et al., 2019). Previous studies have shown the involvement of miR-1298-3p in the development of glioma (Lu et al., 2018). However, the regulatory role of a tumor-suppressive miR, microRNA-1298 (miR-1298) yet to be studied in cervical cancer. The present study was therefore designed to study the role of miR-1298-3p via modulation of one cut domain family member 2 (ONECUT2) expression.

MATERIALS AND METHODS

Cell cultures and transfection

Four different cervical cancer cell lines (C-33A, CasKi, Hela and SiHa) cells as well as the normal cervical epithelial cells (NCE) were procured from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured using a DMEM medium (Gibco, Carlsbad, CA, USA) containing 10% FBS (Gibco). Humidified CO2 incubator was employed for the mainte-
nance of cell lines at 37°C with 5% CO₂. The miR-1298-3p mimics (overexpression) and the respective microRNA negative control (miR-NC), si-ONECUT2 (knockdown) and the negative control (si-NC) were obtained from RiboBio (Guangzhou, China). The pcDNA3.1 overexpression vector was used for overexpression of ONECUT2 while vector control was used as negative control (NC). Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was used for performing the transfection of CasKi and NCE cells through standard manufacturer protocol.

RNA isolation and RT-qPCR analysis

Total RNA was isolated from cervical cancer cell lines with the help of GenElute mRNA Miniprep Kit (Sigma-Aldrich, St Louis, MO) as per the manufacturer’s guidelines. DNA Reverse Transcription Kit (Applied Biosystems) was used for the reverse transcription of mRNA. The miR reverse transcription was performed using the One Step miRNA cDNA Synthesis Kit (HaiGene, Harbin, China). Using SYBR Select Master Mix (Applied Biosystems), The complementary DNAs (cDNAs) were used for gene expression analyses through RT-qPCR. Human GAPDH and snRNA U6 genes were used as internal controls for studying mRNA and miR expression, respectively. The quantification of relative expression levels was performed with the 2^ΔΔCt method. The primer sequences are given in Table 1.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Direction</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>ONECUT2</td>
<td>Forward</td>
<td>5'-GGATGTTCAAAAAAGGATGTA-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-TCTCTCTCTTTGTGCTG-3'</td>
</tr>
<tr>
<td>miR-1298-3p</td>
<td>Forward</td>
<td>5'-CATTGTTCAAAAAAGGATGTA-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-TCTCTCTCTTTGTGCTG-3'</td>
</tr>
<tr>
<td>U6</td>
<td>Forward</td>
<td>5'-CAGGTCTTTCGCTGAGCCT-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-ATGCTTCAAAAACGCTGTTT-3'</td>
</tr>
<tr>
<td>GADPH</td>
<td>Forward</td>
<td>5'-AAGCGTTGCGCGTTGCTG-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-GCTACACCCGGGAGGAGAAAT-3'</td>
</tr>
</tbody>
</table>

MTT proliferation assay

The stably transfected cancer cells were seeded into 96-well plates at a density of 2x10⁴ cells/well and cultured for varying durations (0, 12, 24, 48 and 96 h). This was followed by the addition of 10 µL 3-(4,5-dimethylthiazol-2-y)-2,5-diphenyltetrazolium bromide (MTT) and the samples were incubated for 3h at 37°C. Afterwards, 100 µL of DMSO was added for the termination of the reaction. Finally, absorbance at 570 nm was recorded for each sample using a spectrophotometer to analyze the cell proliferation.

EdU incorporation assays

For the EdU incorporation assay, the stably transfected CasKi cells were initially cultured for 24 h at 37°C. At this, the cells were incubated with 10 mM EdU for 2 h. The cells were then fixed with 4% paraformaldehyde followed by their permeabilization with 0.5% Triton X-100. The cells were then stained with Apollo fluorescent dye. DAPI was used as a counterstain in the EdU incorporation assay. The cells were lastly examined under a fluorescent microscope (Olympus) for staining analysis.

DAPI and AO/EB staining

Stably transfected CasKi cancer cells were plated into a 96-well plate with 5000 cells per well density. After 24 h incubation at 37°C, the cells were PBS washed and fixed using 4% paraformaldehyde followed by an incubation of 1 h at RT. The cells were then again washed with PBS and treated with 0.1% Triton X-100. Afterwards, the cells were stained with 4',6-diamidino-2-phenylindole (DAPI) (5µg/mL) or acridine orange/ethidium bromide (AO/EB) stain (Solarbio Biotechnology, China) for 15 min. After washing with 200 µL PBS, the cells were finally imaged under a fluorescent microscope.

Annexin V/PI assay

The percentage of apoptotic transfected CasKi cells was studied using flow cytometry. The cells were cultured for 24 h, harvested and thrice washed with PBS followed by staining with Annexin V-FITC (Thermo Fisher Scientific, Waltham, MA) and propidium iodide (PI; Invitrogen, Carlsbad, CA). At last, the FACSCanto II Flow Cytometer (BD Biosciences, San Jose, CA) was used to examine the level of cell apoptosis.

Wound healing assay

The migration of CasKi cancer cells stably transfected with miR-1298-3p mimics or miR-NC was analyzed using a wound-healing assay. Approximately, 2.5x10⁵ cells were added to each well of a 12-well plate. The cells were allowed to grow till 90% confluence and then using a 200 µL micropipette tip, the surface of each well was scratched. The cell surface was washed with PBS and an inverted microscope was used for photographing the scratch-line. Following, the cells were cultured for 24 h at 37°C. The cell surface was again photographed and wound-healing was assessed.

Transwell invasion assay

After being transfected with miR-1298 mimics or miR-NC, the CasKi cells were plated into the upper compartment of a transwell plate at a density of 10⁴ cells per well. The invasion of transfected cells was analyzed using an 8.0 µm Pore Polycarbonate Membrane Insert (Corning, New York, NY, USA) as per the manufacturer’s instructions. The bottom chambers were supplemented with 500 µL of DMEM medium with 10% FBS. The cells were cultured for 24 h at 37°C, fixed with methanol and subsequently stained with 0.5% crystal violet. Finally, the invading cells were counted using five random fields with the help of a light microscope.

Western blotting

For protein expression studies, the cervical cancer cells were lysed with ice-cold RIPA lysis and extraction buffer (Thermo Fisher Scientific). The total amount of proteins in the cell fractions was quantified using the Lowry method (Beyotime Institute of Biotechnology). Exactly, 50 µg of total proteins (per lane) were resolved with 10% SDS-PAGE gels. The PAGE gels were then blotted onto polyvinylidene difluoride (PVDF) membranes (Thermo Fisher Scientific). Next, 5% non-fat milk was used for blocking the membranes at room temperature for 45 min. After being washed with PBS-T, the membranes were incubated with primary antibodies:
anti-Bax (1:1000, Abcam), anti-Bcl-2 (1:1000, Abcam), anti-MMP-2 (1:1000, Abcam), anti-MMP-9 (1:1000, Abcam), anti-ONECUT2 (1:1000, Abcam), and anti-β-actin (1:1000, Abcam) at 4°C overnight. The protein bands were finally detected using chemiluminescence (Thermo Fisher Scientific). Human β-actin protein was used as a loading control.

In vivo tumorigenesis study

Five to six-week-old nude BALB/c mice were procured from the Vital River Laboratory (Beijing, China). The induction of xenograft mice tumors was given by subcutaneously injecting the CasKi cancer cells into the flanks of the animals. As the tumors became apparent, the mice were randomly divided into two groups and intratumoral injections of miR-NC or miR-1298 mimics were given every 3 days. The length and breadth of xenograft tumors were examined every third day to calculate the tumor volume. At the end of the third week, the animals were sacrificed, and the xenograft tumors were resected. Average weight was determined for xenograft tumors from each mouse group.

Immuno-histochemical (IHC) analysis was performed for the xenograft mice tumor sections by immobilizing the latter in paraformaldehyde. This was followed by staining of tumor sections with antibodies against Ki67 and caspase-3 (cleaved) proteins and photography of the sections.

In silico analysis and dual luciferase reporter assay

TargetScan Human (http://www.targetscan.org/vert_72/) online software tool was used for predicting the post-transcriptional target of miR-1298-3p together with specifically identifying binding site in 3’UTR of ONECUT2. The native (WT) and mutant (MUT) binding sites of miR-1298-3p bearing 3’UTR of ONECUT2 were designed and cloned into the pGL3-Control vector with SV40 promoter (#U47296; Promega). The reporter plasmids were co-transfected with miR-1298-3p mimics or miR-NC into CasKi or NCE cells with the help of Lipofectamine 2000 reagent. The transfected cells were assessed for luciferase activity using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) after 48 h of transfection. The luciferase activity was normalized as firefly luciferase by Renilla luciferase activity.

Statistical analysis

Three biological repeats were used for performing all the experimental procedures and the results were presented as the mean ± standard deviation (S.D.). The analyses of the statistical data were performed using GraphPad Prism 7.0 offline software. Student’s t-test and ANOVA followed by Tukey’s test were used for examining the statistical differences. *P<0.05 was taken as the representative of a statistically significant difference.

RESULTS

miR-1298-3p inhibits the proliferation of cervical cancer

To gain insights about the expression of miR-1298-3p in cervical cancer, the miR-1298-3p expression was
miR-1298-3p induces apoptosis in cervical cancer cells

To explore the underlying mechanism of tumor-suppressive effects of miR-1298-3p against cervical cancer cells, DAPI staining was performed. The lesions of cellular damage were also evidenced on DAPI staining (Fig. 2A). Moreover, AO/EB staining also revealed that CasKi cancer cells overexpressing miR-1298-3p showed orange and red fluorescence indicative of apoptosis (Fig. 2B). Flow cytometric analysis revealed that cervical cancer cells overexpressing miR-1298-3p exhibited induction of apoptosis (Fig. 2C). The percentage of early and late apoptotic cervical cancer cells increased from 3.33% to 43.37% upon miR-1298-3p overexpression. Moreover, the western blotting of Bax and Bcl-2 proteins showed that miR-1298-3p overexpression in CasKi cancer cells led to a considerable increase in Bax protein while expression of Bcl-2 was decreased (Fig. 2D). Together, the results are indicative of the induction of apoptosis in cervical cancer cells under miR-1928-3p overexpression.

miR-1298-3p inhibits migration and invasion of cervical cancer cells

To assess if miR-1298-3p regulates the metastatic behavior of cervical cancer cells, the migration and invasion of cervical cancer cells overexpressing miR-1298-3p were studied with reference to the negative control cancer cells, *in vitro* using wound-healing and transwell assays, respectively. The CasKi cancer cells exhibited significantly ($P=0.03$) lower wound closure capacity under miR-1298-3p overexpression suggestive of inhibition of migration (Fig. 3A). Again, the transwell assay showed that CasKi cancer cells overexpressing miR-1298-3p exhibited significant ($P=0.03$) loss of in vitro invasiveness and the relative percentage of invading cells was less than 30% of that of the corresponding negative control cancer cells (Fig. 3B).

miR-1298-3p suppresses xenograft tumor growth, *in vivo*

Results of *in vivo* study revealed that miR-1298-3p negatively regulates the proliferation of cervical cancer. The size of miR-1298 mimics tumors was considerably lower than that of miR-NC tumors (Fig. 4A). As expected, miR-1298 mimics tumor group exhibited higher expression of miR-1298 as compared to miR-NC group (Fig. 4B). Furthermore, western blots showed considerable inhibition of ONECUT2 expression in miR-1298 mimics tumor group as compared to miR-NC group (Fig. 4C). Investigation of tumor volume was made every third day and it was found that the animals exhibited markedly lower ($P<0.05$) tumor volume under miR-1298-3p overexpression (Fig. 4D). Average xenograft tumor weight was also shown to be significantly ($P=0.03$) lower for the mice under miR-1298-3p overexpression (Fig. 4E). The IHC staining indicated that the mice tumors showed significantly ($P<0.05$) lower expression

![Figure 3. miR-1298-3p inhibits the metastasis of cervical cancer cells](image)

![Figure 4. miR-1298-3p inhibits tumor in vivo](image)
miR-1298-3p inhibits the growth of human cervical cancer

Figure 5. miR-1298-3p targets ONECUT2 in cervical cancer
(A) TargetScan analysis showing miR-1298-3p targets ONECUT-2
(B) Dual luciferase assay showing the interaction between miR-NC and miR-1298 mimics in cervical cancer (CaSkI) and normal NCE cells
(C) RT-qPCR showing expression of ONECUT2 in normal and cervical cancer cell lines
(D) western blots showing the expression of ONECUT in miR-NC and miR-1298 transfected cervical cancer cells
(E) western blots showing the expression of ONECUT in si-NC and si-ONECUT2 transfected cervical cancer cells
(F) MTT assay showing cell viability of the si-NC and si-ONECUT2 transfected cervical cancer CaSkI cells
(G) western blots showing the expression of ONECUT in empty vector (NC) and pcDNA-ONECUT2 transfected cervical cancer CaSkI cells
(H) MTT assay showing cell viability of miR-NC, miR-1298 mimics or miR-1298 mimics plus pcDNA-ONECUT2 transfected CasKi cell. The experiments were performed in triplicate and expressed as mean ± S.D. (*P<0.05).

of a proliferative marker, Ki67 and higher Caspase-3 (cleaved) protein levels under miR-1298-3p overexpression (Fig. 4F). The results further suggest that miR-1298-3p experiences a tumor-suppressive role in human cervical cancer.

Tumor suppressive role of miR-1298-3p in cervical cancer is exerted via ONECUT2

In order to find the specific molecular target of miR-1298-3p, in silico analysis was performed which predicted that ONECUT2 acts as the post-transcriptional silencing target of miR-1298-3p for two reasons. Firstly, ONECUT has known the oncogenic role in the development of human cancers (Otsuka et al., 2021) and secondly, ONECUT2 as a target of miR-1298-3p has not been studied before. MiR-1298-3p performs the post-transcriptional silencing of ONECUT2 by specifically interacting with a seeding sequence in its 3′-UTR (Fig. 5A). To validate the online bio-informatics results, a dual luciferase reporter assay was carried out using 3′-UTR of ONECUT2 with native or mutated miR binding site. The luciferase activity was shown to decrease significantly once the CaSkI cancer or NCE cells were co-transfected with miR-1298 mimics and 3′-UTR of ONECUT2 bearing native binding site (Fig. 5B). The decline in luciferase activity was not observed once the mutated miR binding site was used which is indicative of the specific interaction of miR-1298-3p with 3′-UTR of ONECUT2. On the other hand, the expression analysis of ONECUT2 from cervical cancer and normal cell lines revealed that ONECUT2 is significantly up-regulated in cervical cancer cell lines, negatively correlating with the expression pattern of miR-1298-3p (Fig. 5C). Moreover, the CasKi cancer cells overexpressing miR-1298-3p showed considerably lower expression of ONECUT2 protein suggestive of its repression by miR-1298-3p, post-transcriptionally (Fig. 5D). Silencing of ONECUT2 in CasKi cancer cells significantly declined (P = 0.03) cell proliferation, mimicking the anti-proliferative effect of miR-1298-3p overexpression (Fig. 5E and 5F). In contrary,
the tumor-suppressive effects of miR-1298-3p overexpression were reverted by the overexpression of ONECUT2 (Fig. 5G and 5H). Collectively, the results reveal that miR-1298-3p inhibits ONECUT2 expression in cervical cancer to exert its tumor-suppressive effects (Fig. 6).

DISCUSSION

MicroRNA (miR) biology is emerging as one of the growing fields in terms of ever extending horizons of cellular and metabolic activities affected by this diverse class of regulatory molecules (Mollaei et al., 2019). The miRs have gained tremendous research interest in the recent era for their profound regulatory potential to serve as faithful prognostic and therapeutic role in human cancer (Mollaei et al., 2019; Zaheer et al., 2020). Reportedly, a huge number of specific members of a diverse class of eukaryotic RNAs have been deduced to serve either oncogenic function or exercise the tumor restrictive behavior in human cancers (Cao et al., 2020; Chen et al., 2020). MicroRNA-1298-3p (miR-1298-3p) has been implicated for its tumor-suppressive action in a number of human cancers (Du et al., 2021; Ahn et al., 2020). Recently, miR-1298-3p has been shown to inhibit human glioma cell proliferation and invasion via post-transcriptional targeting of Nidogen 1 (Lu et al., 2018). The current study commenced with the exploration of transcript levels of miR-1298-3p in cervical cancer. As miR dysregulation has been elucidated as one of the crucial molecular irregularities reflected by human cancers (Steinbach et al., 2004), thus downregulation of miR-1298-3p was hypothesized to probably have a crucial bearing on the neoplastic behavior of the cervical cancer cells. In this direction, the growth and stability of cervical cancer cells was studied under miR-1298-3p overexpression and interestingly, the cancer cells depicted significantly lower proliferation in vitro and the same was shown to be reflective of the induction of cell apoptosis. Bax and Bel-2 are important marker proteins for apoptosis. Bel-2 is a proto-oncogene that inhibits apoptosis by inactivating Bax pro-apoptotic protein (Korsmeyer et al., 1999). The ratio between anti- and pro-apoptotic proteins enables the maintenance of tissue homeostasis as it affects the sensitivity of cells to inducers of apoptosis (Reed et al., 1999; Reed et al., 1997). Overexpression of Bel-2 has been shown to cause inhibition of apoptosis in several cell types, and this anti-apoptotic function is mainly mediated by its ability to heterodimerize with the apoptosis-inducing Bax protein (Qiu et al., 2018). Herein, we found that miR-1298-3p increases Bax and decreases Bel-2 expression, thereby favoring apoptosis of cervical cancer cells. Previously, miR-1298 has been shown to negatively regulate the metastatic behavior of human cancer cells and in accordance with such reports, miR-1298-3p restrained the migration and invasion of cervical cancer cells (Li et al., 2020; Aalaei-Andabili et al., 2016). The tumor-suppressive role of miR-1298-3p in cervical cancer was also supported by the xenograft tumorigenesis study, in vivo. MiRs implement their molecular task by specifically targeting mRNA transcripts at the post-transcriptional level (Guo et al., 2019). The results revealed ONECUT2 as the functional target of miR-1298-3p in cervical cancer. One cut domain family factor 2 (ONECUT2) belongs to the ONECUT transcription factor family which, as per reports, positively regulates the human cancer progression (Rotinen et al., 2018). The alteration of ONECUT2 silencing by miR-1298-3p resulting in overexpression of ONECUT2 which might be one of the pro-cancerous factors mediating the onset and progression of cervical cancer. Although the present study revealed the role of miR-1298-3p/ONECUT2 axis in cervical cancer, further studies focusing on the identification of other miR-1298-3p in cervical cancer need to be carried out. Moreover, drugs that can modulate the expression of miR-1298-3p need to be identified.

CONCLUSION

Taken together, miR-1298-3p is downregulated in cervical cancer and functions as tumor-suppressive effects. MiR-1298-3p induces apoptosis and negatively regulates the growth, migration and invasion of cervical cancer cells. To exert its regulatory control, miR-1298-3p targets ONECUT2 in cervical cancer, post-transcriptionally. Thus, these findings suggest the therapeutic potential of miR-1298-3p/ONECUT2 molecular axis in cervical cancer.

Declarations

Conflict of interest. All the authors declare that there is no conflict of interest.

REFERENCES


Arshav M, Weiskamm S, Brun I, de Santis E, Serrai M, Ferlay J, Bray F (2020) Estimates of incidence and mortality of cervical can-
miR-1298-3p inhibits the growth of human cervical cancer


