jraas

Journal of the Renin-Angiotensin-Aldosterone System
July-September 2016: 1–8

DOI: 10.1177/1470320316663327
jra.sagepub.com
SAGE

miR-205 mediates the inhibition of cervical cancer cell proliferation using olmesartan

Zhang Yue[1,*], Zhang Yun-shan[1,*] and Xue Feng-xia[2]

Abstract

Objective: The renin-angiotensin-aldosterone system has become known as a prerequisite for tumor angiogenesis that is now recognized as a crucial step in the development of tumors, including cervical cancer. The Ang II-AT1R pathway is known to play an important role in tumor angiogenesis. MicroRNAs (miRNAs) are a class of small, regulating RNAs that participate in tumor genesis, differentiation and proliferation. The current study focused on the anti-tumor mechanism of olmesartan, a novel angiotensin II antagonist, on cervical cancer cells.

Materials and methods: qRT-PCR and Western blot were used to demonstrate the effect of olmesartan on miR-205 and VEGF-A expression. miR-205 mimics and VEGF-A shRNA plasmid were separately transfected into HeLa and Siha cells to further validate the function of miR-205 and VEGF-A in cervical cancer cell proliferation.

Results: It was found that olmesartan could upregulate miR-205 and inhibit VEGF-A expression in HeLa and Siha cells. In addition, VEGF-A was proven to be a target gene of miR-205.

Conclusion: This result provides a new idea on the anti-tumor mechanism of olmesartan, which may be used as a novel therapeutic target of cervical cancer.

Keywords

Olmesartan, miR-205, VEGF-A, cervical cancer, proliferation

Date received: 5 February 2016; accepted: 12 May 2016

Introduction

The renin-angiotensin-aldosterone system has become known as a prerequisite for tumors, including cervical cancer.[1] There are convincing data that angiotensin II (Ang II) stimulates cell proliferation in human cancer cell lines[2,3] and induces angiogenesis via the upregulation of vascular endothelial growth factor (VEGF), which is one of the most potent angiogenic factors,[4,5] via angiotensin type 1 (AT1) receptor stimulation. Thus, much attention has been paid to the blockade of the tumoral AT1 receptor as a novel molecular-targeted therapy. Olmesartan is a novel, strong and long-acting AT1 receptor antagonist. It is speculated that olmesartan might have capacity for the inhibition of cell growth and cell proliferation.[6] These studies create the potential mechanism that olmesartan can suppress the angiogenesis and cell proliferation in cervical cancer.

MicroRNAs (miRNAs) are small non-coding RNAs that play a prominent role in tumor genesis, differentiation and proliferation. miRNAs regulate gene expression by binding to 3' untranslated region (UTR). These genes are involved in a variety of biological cell processes. Current studies showed that miRNA acts as an oncogene or tumor suppressor gene in all kinds of cancer. miR-21 is one of the well-understood onco-miRNAs that promotes cancer proliferation, invasion and inhibition of tumor apoptosis by targeting programmed cell death 4 (PDCD4), phosphatase and tensin homolog (PTEN), B-cell CLL/lymphoma 2 (BCL2), tropomyosin 1 (alpha) (TPM1), tumor protein P53 (P53), and the transforming growth factor beta (TGF β)

[1]Tianjin Central Hospital of Gynecology Obstetrics, Tianjin 300052, China
[2]Departments of Gynecology and Obstetrics,Tianjin Medical University General Hospital, Tianjin, China

*These authors contributed equally to this work.

Corresponding author:
Xue Feng-xia, Department of Gynecology and Obstetrics, Tianjin Medical University General Hospital, Tianjin 300052, China.
Email: tianjinxfx@126.com

family.[7–9] miR-10b can also promote tumor invasion of breast cancer.[10] Previous studies have shown that miR-205 is a tumor-suppressing onco-miRNA. In human prostate cancer, miR-205 shows anti-tumor function by targeting protein kinase Cε.[11] Further, miR-205 inhibits breast cancer proliferation and promotes renal cancer cell apoptosis.[12]

VEGF is a member of the platelet-derived growth factor (PDGF)/VEGF group. VEGF-A is a glycosylated mitogen that specifically acts on endothelial cells and has various effects, including angiogenesis and vasculogenesis. Previous studies indicate that VEGF is a positive regulator of tumor growth that promotes tumor migration and invasion, and inhibits tumor apoptosis.[13–15] It can be secreted by tumor cells acting on the endothelial cells of existing blood vessels to promote new blood vessel formation. Overexpression of VEGF has been detected in almost all human cancers investigated, such as glioma, prostate, lung, breast, renal, ovarian, and colorectal cancers.[16–21] Therefore, VEGF is considered a potential therapy for cancer.

In the current study, olmesartan exhibited a high anti-proliferation activity against cervical cancer. It was also found that olmesartan could promote miR-205 expression and inhibit VEGF-A expression. In addition, knocking down miR-205 and overexpression of VEGF-A can modulate the olmesartan sensitivity of cervical cancer. This result provides a new mechanism of olmesartan anti-tumor effect.

Material and methods

Cell culture and olmesartan treatment

HeLa and Siha cells were purchased from the American Type Culture Collection (Rockville, MD, USA) and grown in Roswell Park Memorial Institute (RPMI)-1640 with 10% fetal bovine serum (Gibco). All cultures were maintained at 37°C in a humidified atmosphere containing 5% carbon dioxide. Olmesartan (2.0 mM, Daiichi Sankyo Pharmaceutical) was dissolved in dimethylsulfoxide just before use. The cells were cultured with and without Ang II (0.1 μM) or olmesartan at the time of seeding to examine olmesartan influence on cervical cancer cells.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Quantification of miR-205 expression levels was assessed via qRT-PCR using specific TaqMan® assays according to the instructions of the manufacturer (Applied Biosystems). U6 RNA was used as normalizer.

Proliferation assay

The transfected cells were seeded in 96- or six-well plates and cultured for 48 hours. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to test cell proliferation. For clonogenic assay, cells were plated at a final concentration of 200 cells/ml. Colonies were counted after staining the cells with 0.1% crystal violet within 10 days after plating. Each experiment was conducted in triplicate.

Invasion assay

Cell invasion assay was performed using Transwell cell culture inserts (Corning). Cells (1×10^5) were cultured onto a Matrigel-coated membrane matrix present in the insert of a 24-well culture plate for 24 hours at 37°C. At the end of incubation, the noninvasive cells were removed. Invasive cells were fixed, stained with crystal violet, and photographed under microscope.

DNA construction

miR-205 mimics, control mimics, miR-205 locked nucleic acids (LNA), and control LNA were synthesized by Shanghai Genepharma Co. Ltd. (China). VEGF-A-specific short hairpin RNAs (shRNAs) were generated using oligonucleotide annealing and inserted into pSilencer 2.0 upon BamHI/HindIII restriction. The shRNA sequence was as follows: Forward, 5'-GATCCGCACAGACTCGCGTTGCAAGTTCAAGAGACTTGCAACGCGAGTCTGTGTTTTTTGGAAA-3';Reverse,5'-AGCTTTTCCAAAAAACACAGACTCGCGTTGCAAGTCTCTTGAACTTGCAACGCGAGTCTGTGCG-3'. VEGF-A CDS and full-length VEGF-A with target 3' UTR fragment were obtained from HEK293 cell cDNA library and constructed into pcDNA3.1 plasmid using BamHI/EcoRI restriction. The primers of VEGF-A CDS were as follows:

Forward, 5'-CGCGGATCCACCATGAACTTTCTGCTGTC-3';

Reverse, 5'-CCGGAATTCTCACCGCCTCGGCTTGTCAC-3'.

The primers of full-length VEGF-A with target 3' UTR were as follows:

Forward, 5'-CGCGGATCCACCATGAACTTTCTGCTGTC-3';

Reverse, 5'-CCGGAATTCAGTGCTCTGCGCAGAGTCTC-3'. The oligonucleotide (1 to 180) of VEGF-A 3' UTR was synthesized and inserted into pmirGLO luciferase plasmid using PmeI and XbaI restrictions.

Western blot

Cells were lysed in radioimmunoprecipitation assay lysis buffer containing proteinase inhibitors (Roche). Whole-cell lysates were analyzed using Western blot with anti-VEGF-A

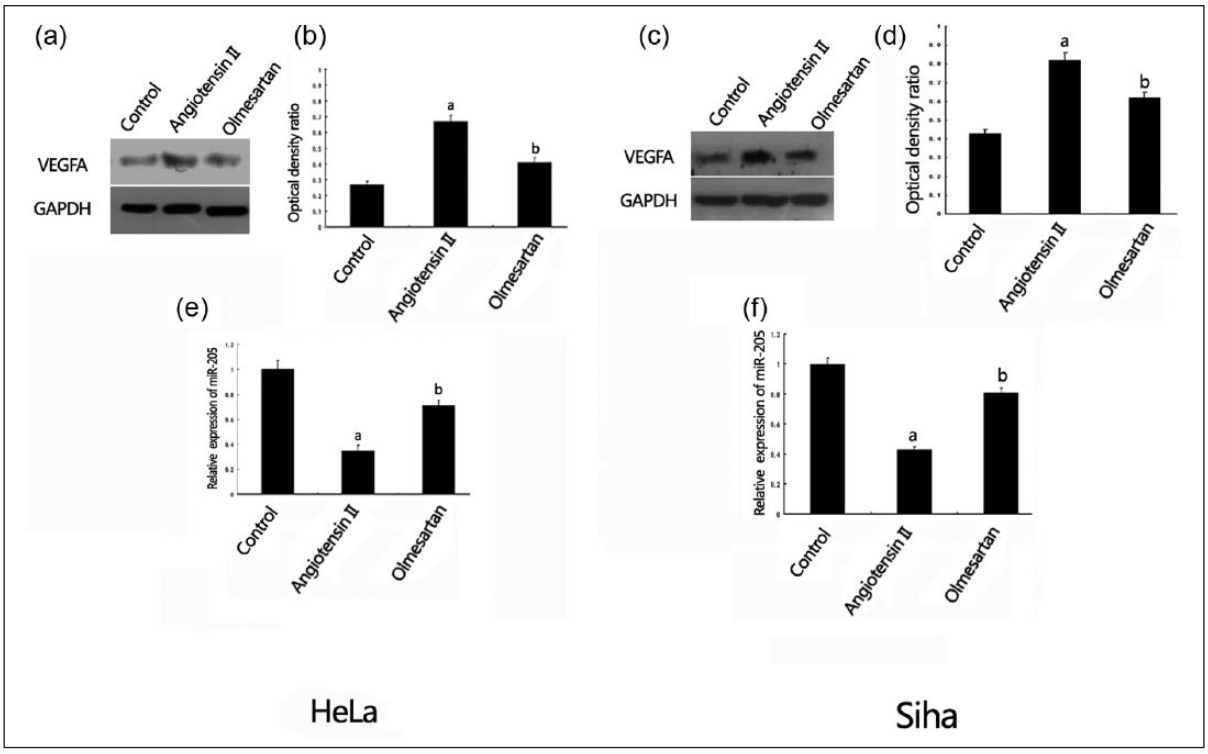

Figure 1. Olmesartan functioned in VEGF-A and miR-205 expression of cervical cancer cells exposed to angiotensin II. (a), (b) Expression changes of protein VEGF-A in HeLa treated with olmesartan (2.0 mM). (c), (d) Expression changes of protein VEGF-A in Siha treated with olmesartan (2.0 mM). (e), (f) Changes of relative expression of miR-205 in HeLa and Siha. $^{a}p < 0.05$ vs control group; $^{b}p < 0.05$ vs angiotensin II group. VEGF-A: vascular endothelial growth factor A; miR-205: microRNA-205.

polyclonal antibody (Abcam) at a ratio of 1:1000 after being subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The expression of glyceraldehyde-3-phosphate dehydrogenase (GADPH) was used as a loading control and was detected using an anti-GADPH polyclonal antibody (Abcam). Enhanced chemiluminescence method was used to determine protein expression.

Statistical analysis

All data in the current study were evaluated using SPSS 16.0 (SPSS Inc, USA). Data were presented as mean ± SEM and were compared by Student *t* test or analysis of variance (ANOVA) followed by a post-Student–Newman–Keuls (SNK) *q* test as appropriate. Differences were considered significant at $p < 0.05$.

Results

Effect of olmesartan on VEGF-A and miR-205 expression exposed to Ang II in cervical cancer cells

HeLa and Siha exposed to Ang II for 24 hours significantly increased cell VEGF-A expression and decreased miR-205 expression compared with control cells ($p < 0.05$). After olmesartan treatment, HeLa and Siha VEGF-A expression was significantly downregulated ($p < 0.05$) and miR-205 was significantly upregulated ($p < 0.05$). See Figure 1.

Olmesartan inhibits cervical cancer cell proliferation and regulates miR-205 and VEGF-A expression

MTT screening analysis of olmesartan demonstrated a strong inhibitory effect on HeLa and Siha cells (Figure 2). HeLa and Siha cells were treated with increasing concentrations of olmesartan. A concentration-dependent inhibition of proliferation was evident at 0.5, 1.0, 1.5, 2.0 and 2.5 mM, as demonstrated using an MTT proliferation assay. MiR-205 and VEGF-A expression levels were identified using miRNA-specific RT-PCR and Western blot analysis to determine the mechanism of olmesartan inhibition of cervical cancer cells. miR-205 was significantly upregulated and VEGF was significantly downregulated after treatment with 0.5–3.0 mM olmesartan.

miR-205 and VEGF-A regulate cervical cancer cell proliferation

miR-205 mimics and VEGF-A shRNA plasmid were separately transfected into HeLa and Siha cells to validate

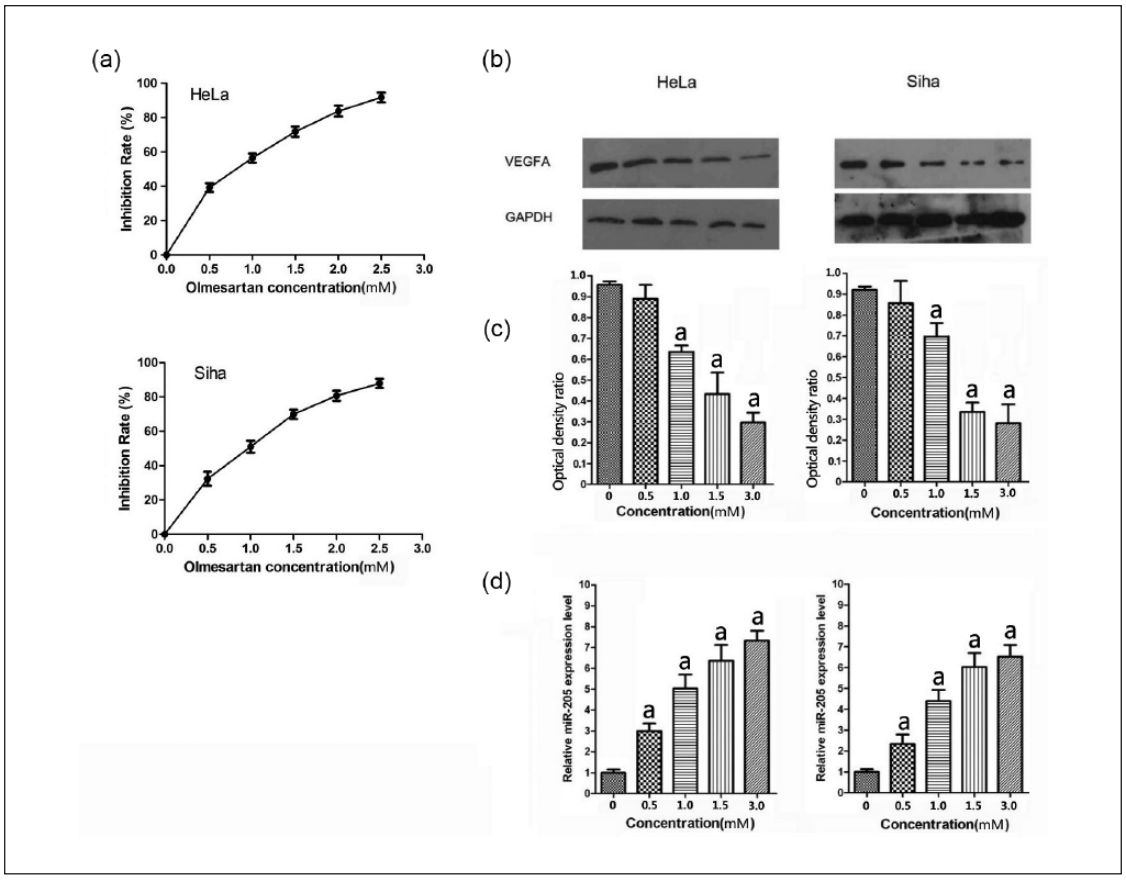

Figure 2. Olmesartan inhibits cervical cancer cell proliferation and regulates miR-205 and VEGF-A expression. (a) MTT assay showed that cell proliferation inhibition was concentration dependent and directly proportional to the olmesartan concentration. (b), (c) Western blot revealed that olmesartan from concentration 1.0–3.0 mM significantly downregulated VEGF-A expression in HeLa and Siha, $p < 0.05$. (d) qRT-PCR revealed that olmesartan from concentration 1.0–3.0 mM significantly upregulated miR-205 expression in HeLa and Siha, $p < 0.05$. $^{a}p < 0.05$ vs concentration 0. VEGF-A: vascular endothelial growth factor A; qRT-PCR: quantitative reverse transcription-polymerase chain reaction.

further the function of miR-205 and VEGF-A in cervical cancer cell proliferation. Colony formation assay and invasion assay were performed to assess the effect on cervical cancer cells. As shown in Figure 3, invasive and colony formation capability was strongly inhibited after miR-205 overexpression or VEGF-A downregulation compared with the control.

miR-205 targets VEGF-A

Bioinformatics database (Targetscan) and luciferase reporter assay were used to examine the relationship between miR-205 and VEGF-A. As shown in Figure 4, VEGF-A expression level and luciferase activity were downregulated, whereas transfected HeLa cells with miR-205 mimics were compared with the control mimics. Further, transfecting VEGF-A CDS plasmid in miR-205-overexpressed HeLa cells has a greater promotion of proliferation than the expression plasmid of VEGF-A with miR-205 targeting the 3' UTR region.

miR-205 LNA and VEGF-A contribute to olmesartan drug resistance

Upregulation of miR-205 was found in olmesartan-treated cervical cancer cells, and inhibition of cell proliferation was mediated by VEGF-A. MiR-205 LNA or VEGF-A expression plasmids were transfected into olmesartan-treated HeLa and Siha cells to assess the effects of miR-205 and VEGFA on olmesartan-dependent cell proliferation inhibition on cervical cancer cells. Upregulation of VEGF-A expression and knock-down of miR-205 expression level can significantly promote the proliferation of olmesartan-treated cervical cancer cells compared with controls (Figure 5).

Discussion

miR-205 is a well-known tumor suppressor in almost all cancers. However, despite the role of miR-205 in cancer[22,23] and the dedication of researchers to the validation of the

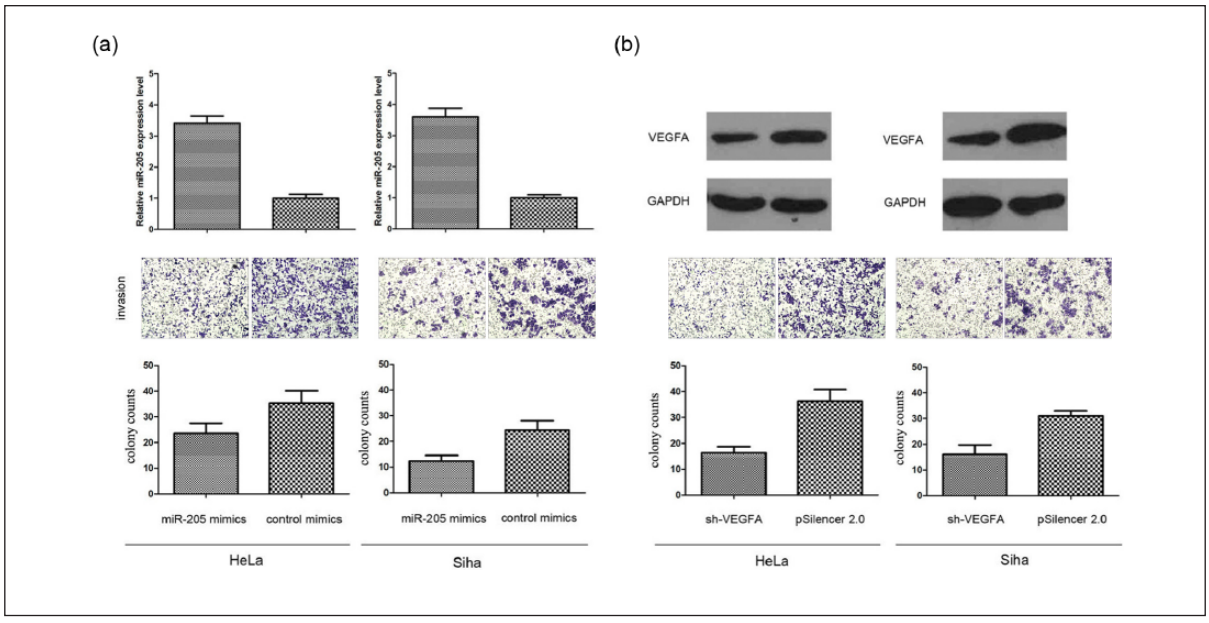

Figure 3. Colony formation assay and invasion assay were performed to analyze the proliferation of miR-205 mimics and VEGF-A shRNA-treated cervical cancer cells. This indicates that both miR-205 mimics and VEGF-A shRNA can inhibit cervical cancer cell proliferation. miR-205: microRNA-205; VEGF-A: vascular endothelial growth factor A; shRNA: short hairpin RNA.

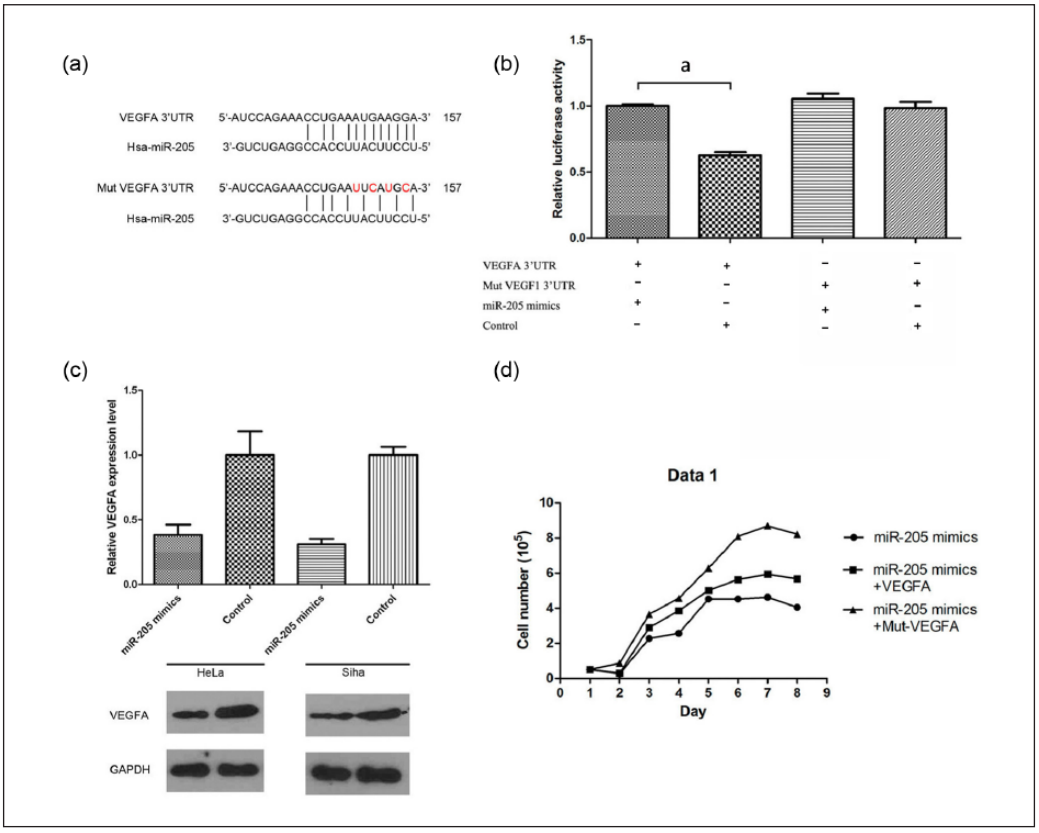

Figure 4. miR-205 targets VEGF-A. (a) A predicted miR-205 target site of the *VEGFA* 3'-UTR. (b) Luciferase reporter assay indicated that the treatment of miR-205 could decrease luciferase activity in the VEGF-A 3'UTR group ($p < 0.05$) but not in the muted VEGF-A 3'UTR group. (c), (d) The growth curve assay showed that VEGF-A could save the cell proliferation of HeLa cells pre-treated with miR-205 mimics. [a]$p < 0.05$ vs control group. miR-205: microRNA-205; VEGF-A: vascular endothelial growth factor A; UTR: untranslated region.

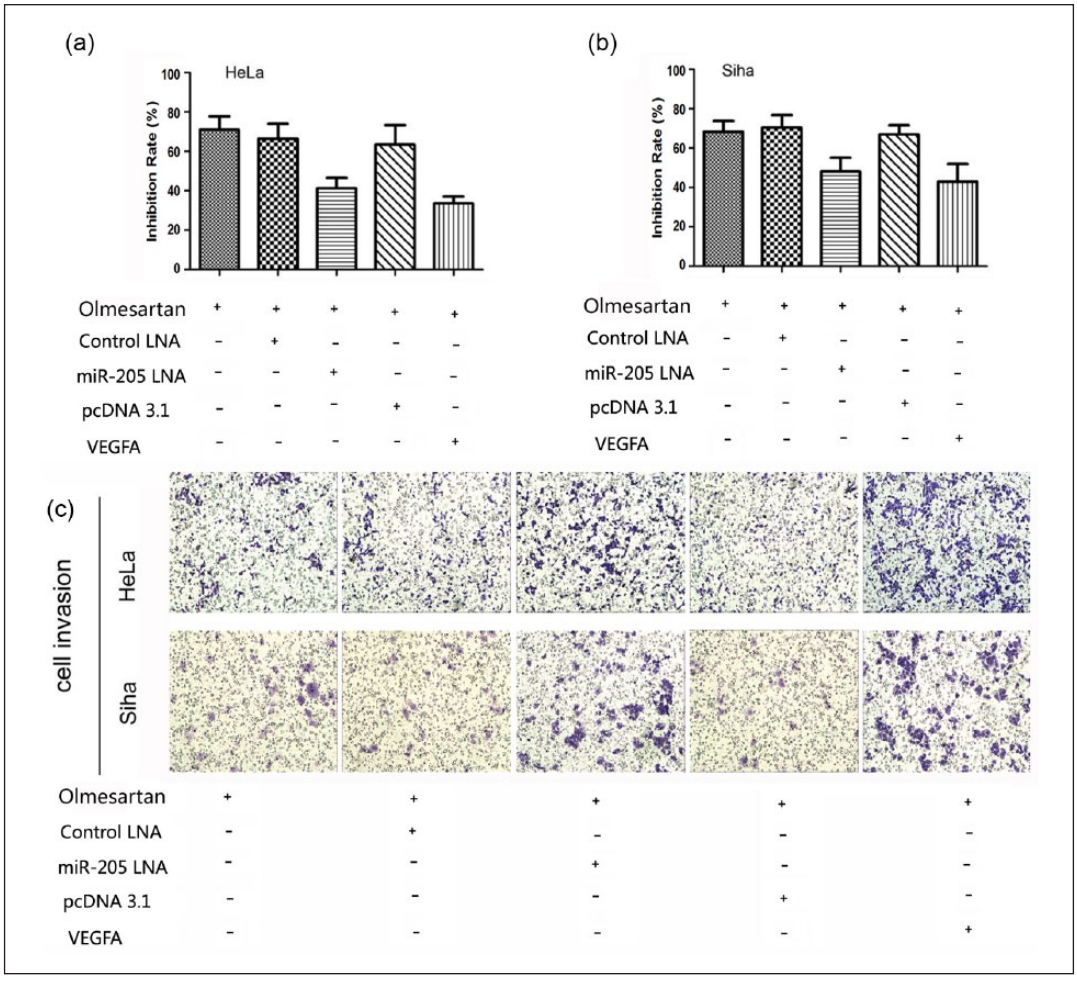

Figure 5. MTT and invasion assay to screen the function of miR-205 and VEGF-A in olmesartan-dependent cervical cancer cell proliferation regulation manner. The MTT and invasion assay revealed that both miR-205 LNA and VEGF-A could rescue the proliferation of cervical cancer cell with olmesartan influence. miR-205: microRNA-205; VEGF-A: vascular endothelial growth factor A; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; LNA: locked nucleic acids.

molecular mechanisms involved in cancer development, the role of miR-205 in cancer drug therapy remains largely unexplored. In the current study, results indicate the dysregulation of miR-205 in cervical cancer cells treated by olmesartan. Genes involved in cancer anti-drug progress include oncogene and anti-tumor genes. Although miRNAs are small non-coding RNAs, they can control tumor genesis, proliferation and differentiation by regulating cancer-related genes. Previous studies have shown that miR-20a contributes to chemotherapeutic resistance in colorectal adenocarcinoma by targeting BCL2 interacting protein 2 (BNIP2).[24] High expression levels of miR-21 contribute to breast cancer drug resistance of Trastuzumab.[25] In the present study, upregulation of miR-205 in cervical cancer cells treated with olmesartan was compared with untreated cells. In addition, VEGF-A as a target gene of miR-205 was validated using luciferase reporter assay. Overexpressions of miR-205 and knockdown of VEGF-A contributed to olmesartan-induced anti-tumor effect on cervical cancer cells. Although early studies by Xie and Ma[26,27] showed that the expression of miR-205 was higher in cervical cancer tissue and cell lines ME-180, C4I and CaSki, while low expression/barely detectable levels were found in HeLa, SW756, SiHa and C33A. This was similar to our results.

Several in vivo and in vitro studies have revealed that renin-angiotensin system (RAS) inhibitors had anti-tumor properties.[28,29] Antiangiogenic drugs (ADs) are one of the key components of frontline therapy in current combination regimens for the treatment of various human cancers. Clinical experiences gained from handling different types of cancers demonstrate that ADs, such as sorafenib, bevacizumab, and sunitinib, in combination with chemotherapy, often produce significant but modest survival benefits.[30,31] These clinical findings have raised several important issues regarding the beneficial mechanisms of antiangiogenic therapy in cancer patients.[32–34] In the current study, olmesartan inhibits tumor cell invasion, and it also has the capability to

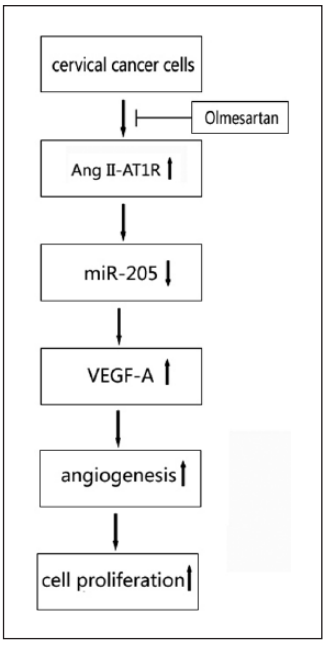

Figure 6. A schematic model of inhibitive effect of olmesartan on cervical cancer cell proliferation. miR-205: microRNA-205; VEGF-A: vascular endothelial growth factor A; LNA: locked nucleic acids.

suppress VEGF-A secretion. Further studies have shown that through miR-205, olmesartan can regulate VEGF-A expression. They also suggest that VEGF-A may be a regulation target of miR-205, which reveals more about the molecular mechanism of olmesartan as an AD. See Figure 6.

The current study suggests that olmesartan inhibits cervical cancer cell proliferation and may serve as a therapeutic option against cervical cancer that might be mediated by upregulating miR-205 and regulate the expression level of VEGF-A. A new mechanism of Ang II receptor blocker (ARB), olmesartan, on anti-tumor therapy was illustrated, which may be useful for further application for human cervical cancer treatment.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) received no financial support for the research, authorship, and/or publication of this article.

References

1. Ino K, Shibata K, Yamamoto E, et al. Role of the renin-angiotensin system in gynecologic cancers. *Curr Cancer Drug Targets* 2011; 11: 405–411.
2. Kikkawa F, Mizuno M, Shibata K, et al. Activation of invasiveness of cervical carcinoma cells by angiotensin II. *Am J Obstet Gynecol* 2004; 190: 1258–1263.
3. Suganuma T, Ino K, Shibata K, et al. Functional expression of the angiotensin II type 1 receptor in human ovarian carcinoma cells and its blockade therapy resulting in suppression of tumor invasion, angiogenesis, and peritoneal dissemination. *Clin Cancer Res* 2005; 11: 2686–2694.
4. Otani A, Takagi H, Suzuma K, et al. Angiotensin II potentiates vascular endothelial growth factor-induced angiogenic activity in retinal microcapillary endothelial cells. *Circ Res* 1998; 82: 619–628.
5. Pupilli C, Lasagni L, Romagnani P, et al. Angiotensin II stimulates the synthesis and secretion of vascular permeability factor/vascular endothelial growth factor in human mesangial cells. *J Am Soc Nephrol* 1999; 10: 245–255.
6. Kurikawa N, Suga M, Kuroda S, et al. An angiotensin II type 1 receptor antagonist, olmesartan medoxomil, improves experimental liver fibrosis by suppression of proliferation and collagen synthesis in activated hepatic stellate cells. *Br J Pharmacol* 2003; 139: 1085–1094.
7. Wickramasinghe NS, Manavalan TT, Dougherty SM, et al. Estradiol downregulates miR-21 expression and increases miR-21 target gene expression in MCF-7 breast cancer cells. *Nucleic Acids Res* 2009; 37: 2584–2595.
8. Papagiannakopoulos T, Shapiro A and Kosik KS. MicroRNA-21 targets a network of key tumor-suppressive pathways in glioblastoma cells. *Cancer Res* 2008; 68: 8164–8172.
9. Zhu S, Si ML, Wu H, et al. MicroRNA-21 targets the tumor suppressor gene tropomyosin 1 (TPM1). *J Biol Chem* 2007; 282: 14328–14336.
10. Ma L, Teruya-Feldstein J and Weinberg RA. Tumour invasion and metastasis initiated by microRNA-10b in breast cancer. *Nature* 2007; 449: 682–688.
11. Gandellini P, Folini M, Longoni N, et al. miR-205 exerts tumor-suppressive functions in human prostate through down-regulation of protein kinase Cepsilon. *Cancer Res* 2009; 69: 2287–2295.
12. Iorio MV, Casalini P, Piovan C, et al. microRNA-205 regulates HER3 in human breast cancer. *Cancer Res* 2009; 69: 2195–2200.
13. Folkman J. Tumor angiogenesis and tissue factor. *Nat Med* 1996; 2: 167–168.
14. Xue Y, Religa P, Cao R, et al. Anti-VEGF agents confer survival advantages to tumor-bearing mice by improving cancer-associated systemic syndrome. *Proc Natl Acad Sci U S A* 2008; 105: 18513–18518.
15. Siironen P, Ristimäki A, Narko K, et al. VEGF-C and COX-2 expression in papillary thyroid cancer. *Endocr Relat Cancer* 2006; 13: 465–473.
16. Ke LD, Shi YX and Yung WK. VEGF(121), VEGF(165) overexpression enhances tumorigenicity in U251 MG but not in NG-1 glioma cells. *Cancer Res* 2002; 62: 1854–1861.
17. Weiss TW, Simak R, Kaun C, et al. Oncostatin M and IL-6 induce u-PA and VEGF in prostate cancer cells and correlate in vivo. *Anticancer Res* 2011; 31: 3273–3278.
18. Dong J, Dai J, Shu Y, et al. Polymorphisms in EGFR and VEGF contribute to non-small-cell lung cancer survival in a Chinese population. *Carcinogenesis* 2010; 31: 1080–1086.

19. Rocca A, Cancello G, Bagnardi V, et al. Perioperative serum VEGF and extracellular domains of EGFR and HER2 in early breast cancer. *Anticancer Res* 2009; 29: 5111–5119.
20. Li L, Wang L, Zhang W, et al. Correlation of serum VEGF levels with clinical stage, therapy efficacy, tumor metastasis and patient survival in ovarian cancer. *Anticancer Res* 2004; 24: 1973–1979.
21. Okuchi Y, Nagayama S, Mori Y, et al. VEGF hypersecretion as a plausible mechanism for pseudo-meigs' syndrome in advanced colorectal cancer. *Jpn J Clin Oncol* 2010; 40: 476–481.
22. Verdoodt B, Neid M, Vogt M, et al. MicroRNA-205, a novel regulator of the anti-apoptotic protein Bcl2, is downregulated in prostate cancer. *Int J Oncol* 2013; 43: 307–314.
23. Li C, Finkelstein D and Sherr CJ. Arf tumor suppressor and miR-205 regulate cell adhesion and formation of extraembryonic endoderm from pluripotent stem cells. *Proc Natl Acad Sci U S A* 2013; 110: E1112–E1121.
24. Chai H, Liu M, Tian R, et al. miR-20a targets BNIP2 and contributes chemotherapeutic resistance in colorectal adenocarcinoma SW480 and SW620 cell lines. *Acta Biochim Biophys Sin (Shanghai)* 2011; 43: 217–225.
25. Gong C, Yao Y, Wang Y, et al. Up-regulation of miR-21 mediates resistance to trastuzumab therapy for breast cancer. *J Biol Chem* 2011; 286: 19127–19137.
26. Xie H, Zhao Y, Caramuta S, et al. miR-205 expression promotes cell proliferation and migration of human cervical cancer cells. *PLoS One* 2012; 7: e46990.
27. Ma Q, Wan G, Wang S, et al. Serum microRNA-205 as a novel biomarker for cervical cancer patients. *Cancer Cell Int* 2014; 14: 81.
28. Arnold SA, Rivera LB, Carbon JG, et al. Losartan slows pancreatic tumor progression and extends survival of SPARC-null mice by abrogating aberrant TGFbeta activation. *PLoS One* 2012; 7: e31384.
29. Funao K, Matsuyama M, Kawahito Y, et al. Telmisartan is a potent target for prevention and treatment in human prostate cancer. *Oncol Rep* 2008; 20: 295–300.
30. Jackson AL, Eisenhauer EL and Herzog TJ. Emerging therapies: Angiogenesis inhibitors for ovarian cancer. *Expert Opin Emerg Drugs* 2015; 20: 331–346.
31. Zhang J, Jiang X, Jiang Y, et al. Recent advances in the development of dual VEGFR and c-Met small molecule inhibitors as anticancer drugs. *Eur J Med Chem* 2016; 27: 495–504.
32. Haas NB, Manola J, Uzzo RG, et al. Adjuvant sunitinib or sorafenib for high-risk, non-metastatic renal-cell carcinoma (ECOG-ACRIN E2805): A double-blind, placebo-controlled, randomised, phase 3 trial. *Lancet* 2016; 387: 2008–2016.
33. González-Vacarezza N, Alonso I, Arroyo G, et al. Predictive biomarkers candidates for patients with metastatic colorectal cancer treated with bevacizumab-containing regimen. *Drug Metabol Pers Ther* 2016; 31: 83–90.
34. Armstrong AJ, Halabi S, Eisen T, et al. Everolimus versus sunitinib for patients with metastatic non-clear cell renal cell carcinoma (ASPEN): A multicentre, open-label, randomised phase 2 trial. *Lancet Oncol* 2016; 17: 378–388.