miR-222 promotes invasion and migration of ovarian carcinoma by targeting PTEN

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Received November 30, 2017; Accepted April 30, 2018

DOI: 10.3892/ol.2018.8743

Abstract. Ovarian carcinoma is the most lethal of the gynecologic malignancies worldwide. Increasing evidence suggests dysfunction of microRNAs (miRNAs) plays an important role in human cancers. The function of miR-222 was detected in ovarian carcinoma to verify the regulation of phosphatase and tensin homolog (PTEN) by miR-222. miR-222 expression in ovarian carcinoma tissues and cell lines were examined using RT-qPCR. Transwell assay was used to detect miR-222 effects on ovarian carcinoma cell migration and invasion. Western blot analysis and luciferase assays were performed to validate PTEN as miR-222 targets. miR-222 expression was upregulated in ovarian carcinoma tissues and three cell lines (A2780, SKOV-3 and OVCAR-3). Ectopic overexpression of miR-222 in ovarian carcinoma cells was sufficient to promote invasion and migration. PTEN acted as a direct target of miR-222. Overexpression of PTEN inhibited human ovarian carcinoma cell migration and invasion. In summary, our findings suggest that miR-222 plays an important role in promoting ovarian carcinoma cell invasion and migration and miR-222/PTEN may be a novel therapeutic target of miRNA-mediated promotion of cell invasion and migration in ovarian carcinoma.

Introduction

Ovarian carcinoma is the most lethal of the gynecologic malignancies in women worldwide (1). Epithelial ovarian carcinoma (EOC), which accounts for 90% (2) of ovarian carcinoma and for 4.2% of all cancer deaths in women globally, is a heterogeneous group of neoplasms (3,4). Due to several factors of insidious nature, manifesting with little or no symptoms until the disease progresses to metastasis, along with a wide diversity of histological subtypes and corresponding clinical behavior, poses significant therapeutic challenges (5). Although significant progress in the diagnosis and treatment including surgery, radiotherapy and chemotherapy have been made in the treatment of ovarian carcinoma, the 5-year survival rate for all stages is below 45% and is decreased to 25% among patients with advanced ovarian carcinoma (6,7). Inability to prolong patient remission is a critical gap in the clinical management of ovarian carcinoma (5). The underlying cause of this problem stems in part from insufficient basic knowledge of the biology and mechanisms supporting ovarian carcinoma carcinogenesis and progression. Therefore, it is crucial to find the underlying molecular mechanisms of ovarian carcinoma and identify the promising therapeutic targets to improve treatment strategies.

MicroRNAs (miRNAs) are a class of small, highly conserved, non-coding RNAs with a length of approximately 21-24 nucleotides that bind to the 3′-untranslated region (3′-UTR) of target messenger RNAs (mRNAs) to induce translational degradation or repression (8). Given the ability of miRNAs to regulate gene expression (9), they unsurprisingly became the critical point in their involvement in cancer. Increasing evidence indicated that miRNAs are frequently dysregulated in cancers (10-12) where they have been shown to contribute to disease pathogenesis, as well as cancer cell death, differentiation, proliferation, metastasis, and apoptosis (13-15). Previous investigations have identified the dysregulated expression of various miRNAs involved in human ovarian carcinoma tumorigenesis and progression, such as cell proliferation, metastasis, migration and DNA methylation (16,17). Among such a large number of miRNAs that have been identified as having significant roles in tumorigenesis, miR-222 was identified as a key miRNA, located on human chromosome Xp11.3 as a single transcript, and showing high sequence identity (18). Functional studies showed that miR-222 reinforced cancer cell biological progression (19), promoted tumor cell proliferation, migration and microtubule formation, and the novel regulatory axis miR-222-3p/GNAI2/AKT is a potential therapeutic target for EOC patients in the future (20). Although the importance of miRNAs in cancer pathological processes have attracted much attention in recent decades, the pathological relevance and significance of the majority miRNAs in ovarian carcinoma remains unclear.
Phosphatase and tensin homolog (PTEN) is well recognized as a tumor suppressor gene and is involved in the regulation of cancer cell biological behavior. PTEN could antagonize PI3 kinase (PI3K) activity by converting phosphatidylinositol (3,4,5)-trisphosphate in the cytoplasm into phosphatidylinositol (4,5)-bisphosphate. The PI3K dysregulation can result in Akt hyper-activation, and promote tumor cell radioresistance, proliferation, migration, and invasion. PTEN has been identified as one direct target of miR-222 (21), we thus reasoned miR-222 may promote the invasion and migration by targeting PTEN of ovarian carcinoma, and be responsible for ovarian carcinoma progression and tumorigenesis.

In order to explore the regulatory mechanism whereby miR-222 was involved in ovarian carcinoma cell migration and invasion, we proved miR-222 could positively regulate PTEN expression and confirmed miR-222 functioned as a vital part in cell migration and invasion of ovarian carcinoma regulated by PTEN. These results may indicate a novel regulatory mechanism for cell migration and invasion in ovarian carcinoma and provide a new avenue for exploring ovarian carcinogenesis.

Materials and methods

**Clinical specimens.** Forty paired tissues and the adjacent normal tissues (ANT) of ovarian carcinoma were collected at the People's Hospital of Rizhao (Rizhao, China), from 2013 to 2015. Patients did not receive radiotherapy or chemotherapy prior to surgery. The Ethics Committee of People's Hospital of Rizhao approved the present study. All samples were obtained with written informed consent from participating patients. All the tissues obtained from biopsy or surgery were frozen at -80°C. The clinical characteristics are shown in Table I.

| Characteristics          | Total cases (n=40) | Low (%) | High (%) | P-value |
|--------------------------|-------------------|---------|----------|---------|
| Age (years)              |                   |         |          |         |
| ≤40                      | 18                | 8 (71.43)| 10 (28.57)| P>0.05  |
| >40                      | 22                | 10 (69.23)| 12 (30.77)|         |
| Tumor size (cm)          |                   |         |          |         |
| <5                       | 18                | 12 (66.67)| 6 (33.33)| P>0.05  |
| ≥5                       | 22                | 6 (72.73)| 16 (27.27)|         |
| Histological grading     |                   |         |          |         |
| 1-2                      | 31                | 13 (90.32)| 18 (9.68)| P<0.01  |
| 3                        | 9                 | 5 (55.56)| 4 (44.44)|         |
| Distant metastasis       |                   |         |          |         |
| Yes                      | 25                | 9 (90.00)| 16 (10.00)| P<0.05  |
| No                       | 15                | 9 (63.33)| 6 (36.76)|         |
| FIGO stage               |                   |         |          |         |
| I-II                     | 29                | 12 (41.38)| 17 (58.62)| P<0.01  |
| III-IV                   | 11                | 6 (76.47)| 5 (23.53)|         |

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from culture cells and cancer tissues using the TRizol reagents (Ambion; Thermo Fisher Scientific, Inc.). PTEN was relative to β-actin and miR-222 was normalized relative to U6 endogenous control using the 2^-∆∆Cq method. The primers used were: miR-222 forward, 5'-AGCTACATCTGGCTACTGG-3', and reverse, 5'-GTATCCAGTGCGAGGGTCC-3'; U6 forward, 5'-CTCGCTTCGGCACTTCT-3' and reverse, 5'-CACGCGATGCCGACGACA-3'; pGL3-PTEN 3'UTR and pGL3-NC were obtained from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). The sequences of miR-222 mimics/inhibitor were: miR-222 mimic, AGCUACAUCUGGCUCUGGGU; and inhibitor, GCCAUGUAGACCCGACCCCA.

**Transwell assay.** Cell Transwell assays were conducted using chambers (Costar, NY) with or without 2 mg/ml Matrigel (Clontech Laboratories, Inc., Mountainview, CA, USA). OVCAR-3 and SKOV-3 cells were transfected with the miR-222 mimic/inhibitor and NC and isolated to make a final concentration at 2×10^5/ml, then placed into the upper chamber.
Lower chambers were filled with medium containing 20% fetal bovine serum as a chemo-attractant. The cells were fixed for 15 min using 1 ml/well 4% paraformaldehyde, stained with Giemsa (JRDUN Biotechnology Co. Ltd., Shanghai, China) for 30 min, and washed three times with 1X phosphate-buffered saline. Finally, stained cells were counted under a microscope (Olympus, Tokyo, Japan).

**Western blot analysis.** Cultured cells were collected and lysed with radioimmune precipitation assay lysis buffer (Beyotime Institute of Biotechnology, Haimen, China). Protein samples were transferred to polyvinyl difluoride membranes (EMD Millipore, Billerica, MA, USA), and the membranes were incubated in primary antibodies rabbit monoclonal anti-PTEN (ab109454; 1:1,000; Abcam, Cambridge, MA, USA) followed by incubation with horseradish peroxidase-coupled secondary antibody goat anti-rabbit IgG-HRP (sc-2004; 1:3,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). β-actin was used as internal control. ImageJ software (NIH, Bethesda, MD, USA) was used to quantify the protein bands.

**Luciferase reporter.** The PTEN mutant (MT) and wild-type (WT) 3’UTR were cloned from human genomic DNA. Cells were seeded on 24-well plates for luciferase assay. miR-222 mimic plus PTEN WT or MT 3’UTR were transfected into OVCAR-3 and SKOV-3. Then, we used Luciferase Reporter Assay (Promega Corporation, Madison, WI, USA) according to the manufacturer’s protocols.

**Statistical analysis.** Statistical results were analyzed by SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA) statistics package. The results are presented as the mean ± standard deviation (SD). The Student’s t-test was used to analyze experimental data. The unpaired two group comparison and multiple comparisons were made with analysis of variance (ANOVA) with Tukey-Kramer post hoc test. Correlation between mRNA and miRNA was estimated using the Spearman’s correlation method. P<0.05 was considered to indicate a statistically significant difference.

**Results**

miR-222 expression is upregulated and inversely correlates with PTEN in ovarian carcinoma. The miR-222 expression levels were detected in 40 matched pairs of ovarian carcinoma tissues and ANT by RT-qPCR. In comparison to the normal cervical tissues, miR-222 expression was significantly higher in the ovarian carcinoma tissues (Fig. 1A; P<0.01). **P<0.01, *P<0.05. PTEN, phosphatase and tensin homolog; ANT, adjacent normal tissues; RT-qPCR, reverse-transcription quantitative PCR; HOSEpiC, human ovary surface epithelial cell line.**
miR-222 promotes invasion and migration of ovarian carcinoma. To reveal the functional role of miR-222 in affecting the migration and invasion of ovarian carcinoma, SKO-3 and OVCAR-3 cell lines were both transfected with the miR-222 mimic/inhibitor/NC. The results showed SKO-3 and OVCAR-3 were transfected with miR-222 mimics both expressed at a relatively high level compared with cell lines transfected corresponding to negative control (Fig. 2A). However, miR-222 expression level of cell lines transfected with miR-222 inhibitor were relatively lower than cells transfected with corresponding negative control (Fig. 2B). Transwell assays without Matrigel were used to examine the miR-222 function on the cell migratory potential, and Transwell assays with Matrigel were used to detect the miR-222 effects on the cell invasive potential. Overexpression of miR-222 significantly increased the migration and invasion capacities in the OVCAR-3 and SKO-3 cells (Fig. 2C). Similarly, Fig. 2D showed re-expression of miR-222 in OVCAR-3 and SKO-3
cells transfected with the miR-222 mimic/inhibitor compared with corresponding NC. In summary, these findings indicated that miR-222 may act as an oncomiR and promotes cell migration and invasion during ovarian carcinoma progression.

PTEN is a target and downregulated by miR-222. Predictive tools (TargetScan Huma http://www.targetscan.org/vert_71/) were used to find target genes of miR-222. The miR-222 putative binding sites were found in the 3'-UTR of PTEN at 1269-1275 bp (Fig. 3A). To determine whether miR-222 can regulate PTEN expression, the WT plasmids pGL-UTR and MT plasmid pGL-mUTR of PTEN 3'-UTR were constructed based on luciferase reporter assay, which were then both transfected into OVCAR-3 with or without miR-222 overexpression. Decreased expression of PTEN was observed after transfecting by miR-222 mimic. However, miR-222 inhibitor increased PTEN expression (Fig. 3B). Furthermore, the PTEN mRNA and protein expression were increased by miR-222 inhibitor, and miR-222 mimic had the opposite effect on PTEN expression (Fig. 3C and D).
Overexpression of PTEN promotes migration and invasion of ovarian carcinoma. Having confirmed that miR-222 acts to reduce PTEN levels in ovarian carcinoma, we then determined whether PTEN is involved in the migration and invasion of ovarian carcinoma. We therefore transfected the control group and PTEN plasmid into the miR-222 cells and NC for Transwell assay. The successful overexpression of PTEN was verified by RT-qPCR (Fig. 4A). We further analyzed the migration and invasion of OVCAR-3 and SKOV-3. We found that an increase of the expression of PTEN could effectively reverse the promotion effect on cell migration and invasion induced by miR-222 overexpression in OVCAR-3 and SKOV-3 (Fig. 4B and C). These findings suggest that PTEN is responsible for the tumorigenic effects in ovarian carcinoma cells, and PTEN may be important in migration and invasion of ovarian carcinoma cells.

Discussion

A number of miRNAs have been proved to be encoded in ovarian carcinoma progression and development through effect on cancer cell proliferation, migration, apoptosis, and invasion (16, 17). For example, microRNA-223-3p promoted cell growth and invasion through targeting SOX11 expression of ovarian carcinoma (22). Xu et al demonstrated that miR-28-5p promotes the progression and development of ovarian carcinoma through inhibition of N4BP1 (23). Thus, identification of cancer-specific miRNAs and their involved targets is pivotal for comprehending their function in tumor migration and invasion, and to provide critical clues for diagnosis and therapy of ovarian carcinoma.

Previous studies have suggested miR-222 was altered in ovarian carcinoma and its functional role was extremely tangled as it could act as promoting target for therapeutic purposes (20). miR-222 was significantly increased in ovarian carcinoma cell lines and tissues, and its expression was positively correlated with PTEN. Further studies revealed that miR-222 promoted ovarian carcinoma cell migration and invasion. The quantity of cell invasion and migration of miR-222 with/without overexpression group showed by Transwell assay was increased compared with negative control group, and the number of cell invasion and migration of miR-222 without overexpression group was decreased. These outcomes suggested that miR-200 might serve as a novel biomarker or therapeutic target for ovarian carcinoma.

PTEN, which is well recognized as a tumor suppressor gene in human cancer, has been found involved in the regulation of cancer cell biological behavior and playing an important part in the progression and development of various kinds of human cancer. PTEN has been reported as a target of miR-222 (21). Shen et al (24) reported that miR-222/PTEN/Akt/FOXO1 axis mediated ADR-resistance and prognosis of breast cancer patients. It was also suggested that miR-222 could decrease the sensitivity of breast cancer cells to adriamycin through PTEN/Akt/p27 kip1 signaling pathway (25). In the present study, we determined PTEN as the target of miR-222 in ovarian carcinoma, a significantly increased expression of PTEN was observed after transfection by miR-222 mimic. However, miR-222 inhibitor decreased PTEN expression. Furthermore, to the best of our knowledge, for the first time, interference of PTEN was shown to promote migration and invasion. These studies together demonstrated that miR-222 might be a powerful anti-ovarian carcinoma candidate.

In conclusion, we revealed that miR-222 could promote ovarian carcinoma cell migration and invasion. PTEN was identified as a functional target of miR-222. The newly identified miR-222/PTEN may provide new insight into pathogenesis and represents a potential therapeutic target for ovarian carcinoma.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors’ contributions

LG and WZ contributed to the conception of the study, YY contributed significantly to data analysis and manuscript preparation, XX performed the data analyses and wrote the manuscript, HL and GZ helped perform the analysis with constructive discussions. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of People’s Hospital of Rizhao (Rizhao, China). All patients signed the informed consent.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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