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Oleanolic acid inhibits the migration and invasion of hepatocellular carcinoma cells by promoting microRNA-122 expression

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MicroRNAs are emerging as important endogenous regulators of gene function and they are playing an important role in the occurrence and development of cancer. They are also regarded as robust biomarkers of cancer diagnosis and prognosis. Hepatocellular carcinoma (HCC) is a common and complex human malignancy with high mortality and morbidity in the world. MicroRNA-122 (miR-122) is a liver-specific microRNA and is closely associated with HCC metastasis, which makes miR-122 a promising target for drug design and development. In this study, we performed a cell-based screening method for discovering miR-122 activators and found that oleanolic acid (OA), a natural pentacyclic triterpene, specifically increased miR-122 expression in a concentration-dependent manner. Two HCC cell lines (HepG2 and Sk-hep-1 cells) were used to evaluate the effect of OA on cell migration and invasion abilities. The results indicated that OA attenuated the migration and invasion abilities of HCC cells by upregulating miR-122 expression. In addition, OA increased the expression of E-cadherin and decreased the expression of β-catenin, N-cadherin and vimentin. After knocking down miR-122 with miR-122 inhibitor, we found that the effect of OA on these epithelial-to-mesenchymal transition (EMT) related molecules was significantly weakened, indicating OA exhibited anti-EMT effect by increasing the expression of miR-122. These finding may help to better understand the molecular mechanism of OA's anti-metastasis activity.

1. Introduction

MicroRNAs (miRNAs) are evolutionarily conserved small non-coding RNAs with a length of 21-23 nucleotides. They are important regulators of gene function by directly binding to the 3'-untranslated region (UTR) of target genes, resulting in gene inactivation by inhibiting mRNA transcription or inducing mRNA degradation (Dai et al. 2020; Lin and Gregory 2015; Wei et al. 2019). MiRNA regulation of gene expression is of great significance in a variety of biological pathways, such as proliferation, migration, invasion and apoptosis (Balatti and Croce 2020; Santoni et al. 2020). Abnormally expressed miRNAs are related to the incidence and development of a variety of diseases (Esquela-Kerscher and Slack 2006; Shenouda and Alahari 2009), and because of their small size and stable secondary structure, miRNAs have now been considered as interesting biomarkers and indicators for disease diagnosis (Ameres and Zamore 2013; Karbasforooshan et al. 2020).

Cell migration and invasion are significant biological processes of tumor metastasis which is the main reason of cancer patient mortality (Hunter et al. 2008). Recently, miRNAs attract more and more attention as potential molecules in anti-metastasis drug targets (Aigner 2011). Hepatocellular carcinoma (HCC) is a common and complex human malignancy. Chromosome instability and epigenetic instability cause HCC complexity. The abnormal expression of coding/non-coding genes is also related to HCC complexity. Among them, non-coding genes include miRNAs (Tsai et al. 2009). In a large family of miRNAs, 20 miRNAs have been recognized as important factors which are related to metastatic HCC. Abnormal expression of these miRNA-related target genes may be involved in the occurrence or recurrence of metastasis (Tsai et al. 2009). MicroRNA-122 (miR-122), as a member of miRNAs associated with HCC metastasis, is a liver-specific miRNA and one of the most abundant miRNA in the liver (Bandiera et al. 2015). MiR-122 is an important tumor suppressor that can induce apoptosis and inhibit metastasis (Coulouarn et al. 2009; Ma et al. 2010). MiR-122 expression is downregulated in HCC and the loss of miR-122 expression is related to HCC metastasis (Coulouarn et al. 2009). Epithelial-to-mesenchymal transition (EMT) is a well-known pathway for tumor metastasis, because the phenotypic changes from epithelial cells to mesenchymal cells makes cancer cells invasive (De Craene and Berx 2013). It has been reported that miR-122 deletion promotes EMT and spontaneous HCC formation (Turato 2014).

Oleanolic acid (OA) is a natural pentacyclic triterpenoid compound and distributed widely in various herbs and vegetables. OA has many bioactivities, including hepatoprotection, anti-inflammatory, anti-virus and anti-tumor activities (Duan et al. 2019; Liu 2005). OA exhibits anti-tumor activity in a variety of cancers by inducing apoptosis and cell cycle arrest or by radiosensitization in radiotherapy (Wang et al. 2019). Although there is a certain understanding of OA inhibiting cancer cell metastasis (Duan et al. 2019; Guo et al. 2013; Wang et al. 2019), the mechanism that OA inhibits the migration and invasion abilities of HCC cells still remains unclear. Here, OA was identified to specifically increase miR-122

expression by a cell-based screening method. In this study, we used several HCC cell lines to investigate whether miR-122 is related to the anti-metastasis activity of OA on tumor cells.

2. Investigations and results

2.1. OA was a miR-122 activator in Huh7 cells

To identify chemical compounds that regulate miR-122 expression, we established a cell-based screening assay with our chemical library containing 1431 compounds and a stable transfected cell line Huh7-luciferase-miR-122 (Tai et al. 2012). Since the miR-122 gene in the Huh7-luciferase-miR-122 plasmid is the complementary sequence of miR-122, the miR-122 in Huh7 cells can be complementary paired with it to affect the expression of luciferase (Fig. 1A). Treatment of the Huh7-luciferase-miR-122 cell line with the nucleotide-based miR-122 mimic or inhibitor suppressed or increased the expression of luciferase signal, respectively (Fig. 2), thereby suggesting that this stable cell line could be used to discover miR-122 regulators. After the hit was confirmed again, OA (Fig. 1B) was identified to potently inhibit the expression of luciferase reporter gene, which meant that OA promoted miR-122 expression (Fig. 1C). The result of OA as an activator of miR-122 is consistent with the previously reported that OA inhibited the proliferation of lung carcinoma cells by inducing miR-122 expression (Zhao et al. 2015). OA inhibited luciferase expression in a concentration-dependent manner and the IC_{50} value of OA for inhibition of luciferase expression was 14.07 μM (Fig. 1D). OA was re-assayed with both pCIneo-luciferase-miR-122 plasmid and pCIneo-luciferase control plasmid (no miR-122 complementary sequence), confirming its activity as miR-122 activator and validating that they did not inhibit luciferase expression in a non-miR-122 specific manner (Fig. 3). The result showed that OA significantly inhibited luciferase expression in Huh7 cells. These results indicated that OA promoted the expression of miR-122.

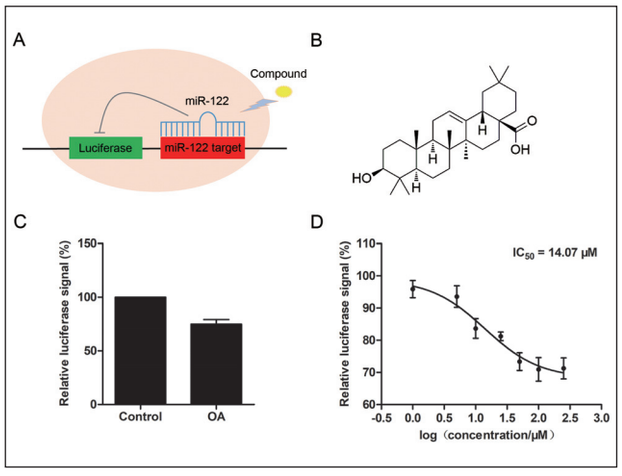

Fig. 1: The effect of OA on miR-122 expression in Huh7-luciferase-miR-122 cells. (A) Assay for miR-122 function via control of luciferase by a 3'miR-122 target sequence. (B) Chemical structure of OA. (C) Changes in the luciferase signal after treatment with OA (10 μM) for 24 h. (D) Luciferase assay dose response curve for OA. The results were shown as mean ± SD of three independent experiments.

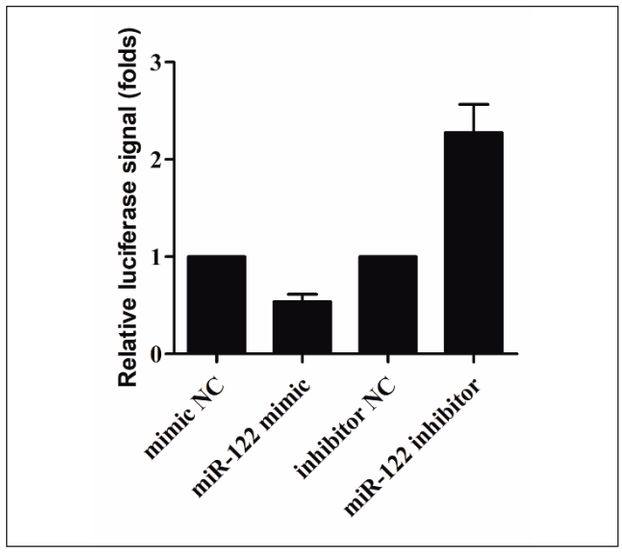

Fig. 2: Huh7-luciferase-miR-122 cells were seeded in 24-well plates and transfected with miR-122 mimic (50 nM) or inhibitor (100 nM) for 48 h. Changes in the luciferase signal were monitored by luciferase assay. The results were shown as mean ± SD of three independent experiments.

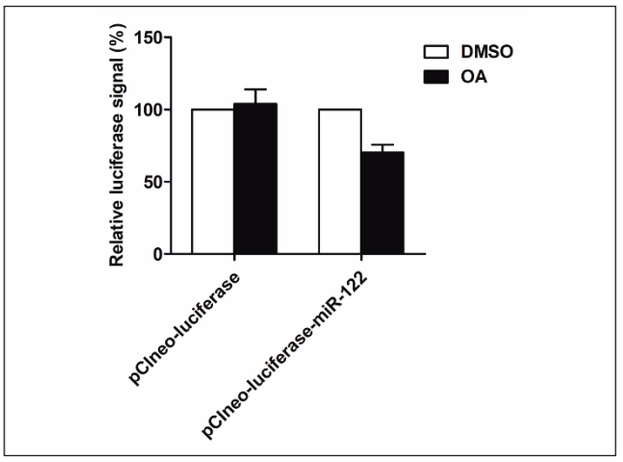

Fig. 3: Huh7 cells growing in 24-well plates transiently transfected with pCIneo-luciferase plasmid or pCIneo-luciferase-miR-122, and then treated with OA (10 μM) for 24 h. Changes in the luciferase signal were monitored by luciferase assay. The results were shown as mean ± SD of three independent experiments.

2.2. OA enhanced the biogenesis of miR-122 in Huh7 cells

To further investigate the mechanisms of OA promoting the expression of miR-122, quantitative RT-PCR was used to measure the effect of OA on the expression of pri-miR-122 and

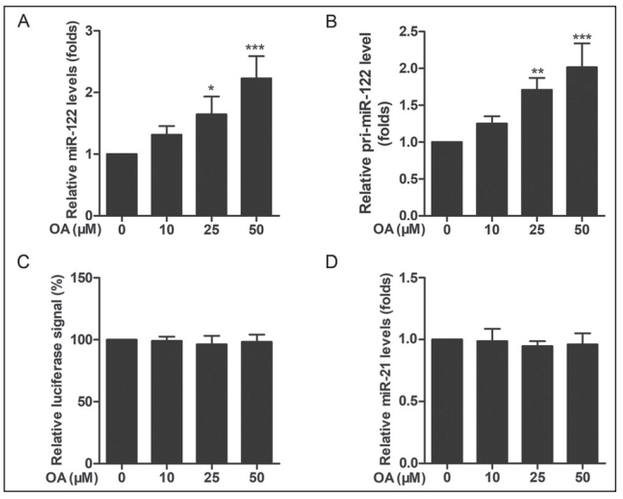

Fig. 4: The effect of OA on the biogenesis of miR-122. RT-PCR quantification of miR-122 (A) and pri-miR-122 (B) in Huh7 cells exposed to OA. (C) Changes in the luciferase signal after treatment with OA for 24 h were monitored in the HeLa-luciferase-miR-21 cell line. (D) RT-PCR quantification of miR-21 in HeLa cells exposed to OA. The results were shown as mean ± SD of three independent experiments. (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$.

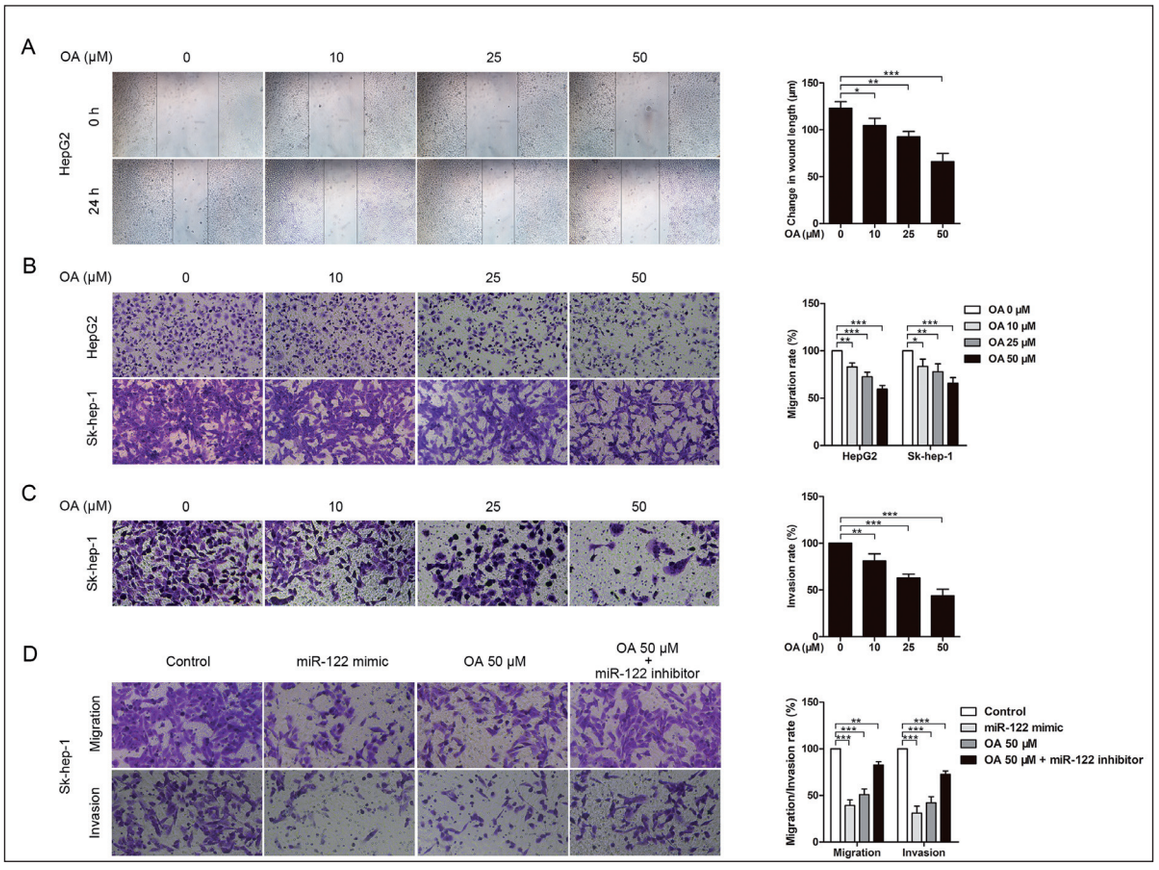

Fig. 5: The effect of OA on the migration and invasion of HCC cells. (A) HepG2 cells were seeded in 6-well plates and grown to full confluence. The migration of HepG2 cells was detected by wound-healing assay after treatment with OA for 24 h. (B) The migration of HepG2 and Sk-hep-1 cells was detected by transwell migration assay after treatment with OA for 24 h. (C) The invasion of Sk-hep-1 cells after was detected by transwell invasion assay after treatment with OA for 24 h. (D) The migration and invasion of Sk-hep-1 cells after transfection with miR-122 mimic or inhibitor, or treatment with OA, were detected by transwell migration and invasion assays, respectively. The results were analyzed with Image J software and shown as mean ± SD of three independent experiments. (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$.

mature miR-122 in Huh7 cells. As shown in Fig. 4A and B, OA significantly enhanced the expression of pri-miR-122 and mature miR-122 in a concentration-dependent manner. The result showed that OA increases the expression of miR-122 by promoting the transcription process of miR-122. To examine the specificity of OA for miR-122, OA was assayed with another stable transfected cell line HeLa-luciferase-miR-21. As shown in Fig. 4C, OA at concentrations of 10-50 μM had no obvious effect on luciferase expression. OA also did not affect the expression of mature miR-21 in HeLa cells by quantitative RT-PCR (Fig. 4D). These results suggested that OA was not a general activator in the miRNA pathway, but showed a certain degree of specificity for miR-122.

2.3. OA inhibited the migration and invasion of HCC cells through enhancing miR-122 expression

Since the downregulation of miR-122 is closely related to HCC metastasis, we selected HepG2 and Sk-hep-1 cells to evaluate the inhibitory effect of OA on cell migration and invasion. As shown in Fig. 5A, we used a wound-healing assay to assess the inhibitory effects of OA on the migration of HepG2 cells. After 24 h corresponding treatment, the gap between the cell layers of the OA-treated group was wider than that of the control group. Quantitative analysis showed that migration was inhibited in a concentration-dependent manner. As shown in Fig. 5B, the results of transwell assay also showed that migration was significantly decreased in HCC cells. Furthermore, we used matrigel-coated transwell chambers to investigate whether or not OA inhibits Sk-hep-1 cell invasion. Compared with the control group, the number of cell invasion through the matrigel-coated filter was reduced in OA-treated group (Fig. 5C). Therefore, OA markedly inhibited Sk-hep-1 cells invasion. To further verify that OA exerts an inhibitory effect on cell migration and invasion by enhancing miR-122 expression, overexpression of miR-122 with miR-122 mimic could inhibit cell migration and invasion, which was consistent with the results of the OA-treated group; Knockdown of miR-122 with miR-122 inhibitor, showed that the inhibitory effect of OA on cell migration and invasion was weakened (Fig. 5D). Taken together, these results suggested that OA exerted an inhibitory effect on cell migration and invasion by promoting miR-122 expression.

2.4. OA exerted anti-EMT effect through enhancing miR-122 expression

Since EMT plays a very important role in cell metastasis, we also evaluated the effects of OA on EMT. EMT associated molecules were firstly detected by western blot assay, the results showed that the overexpression of miR-122 with miR-122 mimic increased the expression of E-cadherin and decreased the expression of β-catenin, N-cadherin and vimentin, which was consistent with the results of the OA-treated group. Knockdown of miR-122 with miR-122 inhibitor showed that the effect of OA on EMT-related molecules was weakened (Fig. 6A). In addition, the same experimental results were found in immunofluorescence assay (Fig. 6B). These data emphasized the important role of miR-122 in the anti-EMT effect. These results suggested that OA suppressed the EMT process of Sk-hep-1 cells by promoting miR-122 expression.

3. Discussion

The incidence of HCC has increased dramatically. HCC is a tumor with poor prognosis and few treatment options (Aleksic et al. 2011). It has been well known that hepatitis B virus (HBV), hepa-

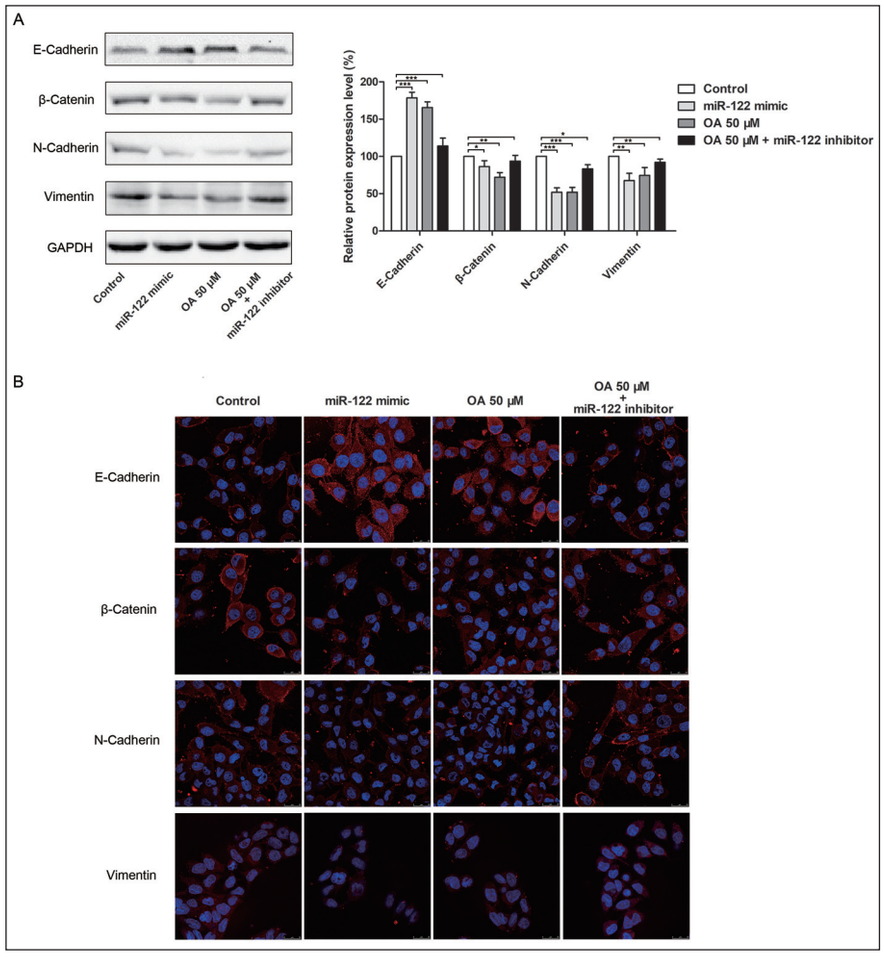

Fig. 6: The effect of OA on EMT in Sk-hep-1 cells. Sk-hep-1 cells were transfected with miR-122 mimic or inhibitor for 24 h and then with or without OA (50 μM) for another 24 h. E-Cadherin, β-Catenin, N-Cadherin and Vimentin were then measured by western blot assay (A) and Immunofluorescence assay (B). The results were shown as mean ± SD of three independent experiments. (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$.

titis C virus (HCV), alcoholic or non-alcoholic steatohepatitis, and aflatoxin intoxication are key factors in the development of HCC (Thorgeirsson et al. 2006). However, tumor metastasis is the main reason of cancer patient mortality (Hunter et al. 2008). Surgery is currently the best treatment for HCC patients. However, only a 10%-15% incidence is suitable for exairesis at diagnosis, resulting in catastrophic outcomes for inoperable HCC (Wang et al. 2013). In addition to surgical treatment, chemoprevention through the use of one or more non-toxic naturally occurring or synthetic agents has become a key approach to control HCC. Since miR-122 is repressed in HCC and the loss of miR-122 is associated with HCC metastasis (Coulouarn et al. 2009), miR-122 raises more attention for its potential as a therapeutic target for anti-tumor and anti-metastasis therapy. In this study, we established a cell-based screening assay by using a stable transfected cell line Huh7-luciferase-miR-122, and wanted to find chemical compounds that act as miR-122 activators. OA, a natural pentacyclic triterpene, was identified to increase the expression of miR-122 from our chemical library. Our results indicated that OA promoted miR-122 transcription. OA had no effect on the expression of another miRNA, miR-21, indicating that OA specifically increased miR-122 expression.

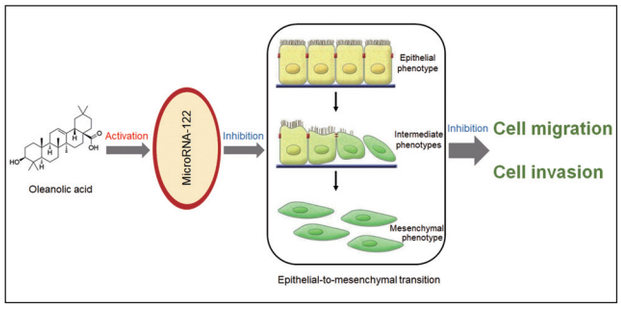

Fig. 7: Overview of the study design.

Although OA has been studied as an activator of miR-122 to exhibit anti-tumor effects (Zhao et al. 2015), the anti-metastasis effect of OA as an activator of miR-122 has not been reported. We found that OA inhibited the migration and invasion of HCC cells by upregulating miR-122 expression. The mechanism of OA inhibiting tumor cell metastasis is diverse. For examples, OA has an inhibitory effect on the proliferation, migration, and invasion of SW579 cells by targeting forkhead transcription factor A (Duan et al. 2019); OA inactivates the MAPK/ERK signaling pathway to inhibit the migration and invasion of malignant glioma cells (Guo et al. 2013); OA and its analogues are identified as inhibitors of breast cancer cell migration and invasion by inhibiting the Brk/Paxillin/Rac1 signaling pathway (Elsayed et al. 2015); OA even inhibits the proliferation and invasion of Kras transformed cells through autophagy (Liu et al. 2014).

As the understanding of how metastasis occurs, it becomes important to explore the unknown molecular mechanism of OA's anti-metastasis effect. EMT, a process of phenotypic changes from epithelial cells to mesenchymal cells, is crucial in HCC progression and metastasis (Nieto et al. 2016). As EMT is considered to be the underlying cause of high cancer mortality, the EMT pathway has important therapeutic significance for reducing the morbidity and mortality of HCC. E-Cadherin, β-catenin, N-cadherin and vimentin are important EMT markers that play an important role in maintaining the phenotype of tumor cells (Ming et al. 2021). This study showed that OA had a significant effect on the expression of these related molecules, increasing the expression of E-cadherin,

and reducing the expression of β-catenin, N-cadherin and vimentin. This result was consistent with the result of miR-122 mimic treatment group. After knocking down miR-122 with miR-122 inhibitor, we found that the effect of OA on these EMT related molecules was significantly weakened, indicating OA exhibited anti-EMT effect by increasing the expression of miR-122.

In conclusion, we identified that OA exerted its anti-metastasis effect on HCC cells through promoting miR-122 expression. Furthermore, targeting miR-122 may be effective therapeutic anti-tumor metastasis strategies.

4. Experimental

4.1. Cell culture and chemicals

HCC cell lines Huh7, HepG2, Sk-hep-1, and human cervical cancer cell line HeLa were obtained from the Cell Bank of Chinese Academy of Science (Shanghai, China). These cells were cultured in Dulbecco's modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and 1% penicillin/streptomycin (Beyotime, Shanghai, China) at 37 °C under 5% CO_2 in a saturated humidified incubator (Thermo Fisher Scientific, Waltham, USA). OA and dimethylsulfoxide (DMSO) were purchased from Solarbio (Beijing, China).

4.2. Transient transfection

MiR-122 mimic, inhibitor and their negative controls (NCs) were obtained from RiboBio (Guangzhou, China). According to different experimental purposes, HCC cells were plated in 24-well plates, 6-well plates or 35mm petri dishes. The siRNAs/lipofectamine 2000 (Invitrogen) complex was formed by incubating in Opti-MEM (Invitrogen) for 20-30 min at room temperature. Then, the complex was transfected into HCC cells for 24 h or 48 h. HCC cells were collected for further analysis after indicated treatment.

4.3. Establishment of Huh7-luciferase-miR-122 and HeLa-luciferase-miR-21 stable cell lines

The pCIneo-luciferase-miR-122 plasmid and pCIneo-luciferase-miR-21 plasmid were constructed by inserting miR-122 and miR-21 genes into the pCIneo-luciferase plasmid between the SalI and NotI restriction sites. The miR-122 and miR-21 genes in the plasmid are the complementary sequences of miR-122 and miR-21, respectively. They were amplified using PCR method. The PCR primers: SalI-miR-122-NotI: 5'-TCGACAGTAGAGCTCTAGTACAAACACCATTGTCACACTCCAGC-3', NotI-miR-122-SalI: 5'-ggccgcTGGAGTGTGACAATGGTGTTTGTACTAGAGCTCTACTg-3', SalI-miR-21-NotI: 5'-TCGACAGTAGAGCTCTAGTAtcaacatcagtctgataagctaGC-3', NotI-miR-21-SalI: 5'-ggccgctagcttatcagactgatgttgaTACTAGAGCTCTACTg-3 (Sangon Biotech, Shanghai, China). Huh7 and HeLa cells were transfected with pCIneo-luciferase-miR-122 plasmid and pCIneo-luciferase-miR-21 plasmid in DMEM medium without antibiotics by lipofectamine 2000 transfection reagent, respectively. The cells after 48 hours of transfection were trypsinized and separated, and then re-plated in DMEM with 10% FBS and 600 μg/mL G418 (Solarbio). The clones with G418 resistance were selected and expanded with DMEM containing 300ug/ml G418 for 4 weeks. The Huh7-luciferase-miR-122 cells and the HeLa-luciferase-miR-21 cells were maintained in DMEM with 300 μg/mL G418.

4.4. Screening and luciferase activity assay

Stable cell line Huh7-luciferase-miR-122 cells were added into 96-well plates (1×10^5 cells/well) and incubated with the test compounds for 24 h. Huh7-luciferase-miR-122 cells were lysed with cell lysis buffer, and the luciferase reporter expression was measured using a Luciferase Reporter Assay System (Promega, Beijing, China) and detected by a chemiluminescence analyzer (Thermo Fisher Scientific).

4.5. Quantitative real-time PCR

Huh7 and HeLa cells were cultrued in 6-well plates and treated with various concentration of OA (0, 10, 25, 50 μM) for 24 h, respectively. RNA was obtained from cells with the TRIzol reagent (Invitrogen). Then, extracted RNA (2 μg) was reverse transcribed into cDNA with M-MLV Reverse Transcriptase (Promega) and oligo dT_{18} primer (Promega). cDNA (2 μL) was subjected to RT-PCR with SYBR Green I (Thermo Fisher Scientific). The primer information was as listed below: miR-122-F: 5'-GGGGTGGAGTGTGACAATG-3'; miR-122-R: 5'-CAGTGCGTGTCGTGGAGT-3'; miR-21-F: 5'-GCCCGCTAGCTTATCAGACTGATG-3'; miR-21-R: 5'-GTGCAGGGTCCGAGGT-3'; pri-miR-122-F: 5'-GCTCTTCCCATTGCTCAAGATG-3'; pri-miR-122-R: 5'-GTATGTAACAACAGCATGTG-3'; U6-F: 5'-GCGCGTCGTGAAGCGTTC-3'; U6-R: 5'-GTGCAGGGTCCGAGGT-3'; GAPDH-F: 5'-TGCACCACCAACTGCTTAGC-3'; GAPDH-R: 5'-GGCATGGACTGTGGTCATGAG-3'. U6 and GAPDH serve as reference genes for mature miRNAs and pri-miRNAs, respectively. The relative expression levels of mature miRNAs and pri-miRNAs were analyzed by using the $2^{-\Delta\Delta Ct}$ method.

4.6. Western blot assay

Sk-hep-1 cells were cultured in 6-well plates and transfected with miR-122 mimic or inhibitor for 24 h. Then, Sk-hep-1 cells were incubated with/without OA for another 24 h. Sk-hep-1 cells were lysed using RIPA buffer and protein concentrations in the supernatant were determined with a BCA protein assay kit (Bestbio, Shanghai, China). The protein was mixed with 5 × loading buffer and boiled for 5 min at 100 °C. The protein (50 μg) was subjected to SDS-PAGE and then transferred onto 0.45 mm Nitrocellulose membrane. Membrane was blocked with 5% bovine serum albumin (BSA) and placed on a shaker at room temperature for 2 h. Then, the proteins were incubated with primary antibodies, including anti- E-Cadherin, β-Catenin, N-Cadherin and Vimentin antibodies (Proteintech, Chicago, USA) (1:1000) in a blocking solution overnight at 4 °C. The proteins were then incubated with the corresponding secondary antibodies (1:5000) for 2 h at room temperature, which was followed by enhanced chemiluminescence detection system (Bio-Rad). The intensity of the blots were quantified using Quantity One software.

4.7. Wound-healing assay

HepG2 cells were plated in 6-well plates. Pipette tips (200 μL) were used to scratch the cells to form wounds. HepG2 cells were incubated with OA (0, 10, 25, 50 μM) for 24 h. The gap of each experimental group was observed and taken photos by an inverted phase contrast microscope at 0 h and 24 h, and the results were analyzed with Image J software.

4.8. Transwell assay

The migration and invasion abilities of HepG2 cells and Sk-hep-1 cells were measured by transwell chamber assay. For migration assay, 4×10^4 cells in 200 μL DMEM medium without serum were added to the upper chamber. 500 μL DMEM medium with 10% FBS was added to the lower chamber and used as the chemoattractant. Then the cells were treated with OA (0, 10, 25, 50 μM) for 24 h. After 24 hours, the medium inside the upper chamber was abandoned. The non-migrated cells were removed by cotton swabs. The cells transferred through the filter membrane were fixed in 4% paraformaldehyde for 20 min and stained with 0.1% crystal violet for 20 min at room temperature. Finally, photographs were taken with a microscope. For invasion assay, extracellular matrixgel (50 μL) (BD, Franklin Lakes, USA) was filled in the upper surface before the cells were added. The remaining steps were the same as previous described. In order to determine whether miR-122 plays an important role in the process of OA inhibiting HCC cell migration and invasion, cells transfected with miR-122 mimic (50 nM) or inhibitor (100 nM) were added to the upper chamber and incubated with/without OA (50 μM) for 24 h. The remaining steps of migration and invasion assays were the same as above.

4.9. Immunofluorescence assay

Sk-hep-1 cells (5×10^4/well) were added to 35 mm dishes with glass bottom. The cells were transfected with/without miR-122 mimic (50 nM) or inhibitors (100 nM) for 24 h, and then treated with or without OA (50 μM) for another 24 h. The cells were washed thrice with 1 × PBS buffer, fixed with 4% paraformaldehyde for 20 min and incubated with 0.1% Triton X-100 for 5 min. Washing thrice with 1 × PBS buffer again, the cells were blocked with 5% BSA for 30 min at room temperature. Then the cells were incubated with primary antibodies E-Cadherin, β-Catenin, N-Cadherin and Vimentin (1:200) overnight at 4 °C. After three times PBS washing, the cells were incubated with the corresponding fluorescently conjugated secondary antibodies (Beyotime) for approximately 30 min at room temperature protecting from light. DAPI (Beyotime) was used to stain the nuclei and pictures were taken by using a laser scanning confocal microscope (Nikon).

4.10. Statistical analysis

GraphPad prism 5.0 software (GraphPad, La Jolla, USA) were applied to data analysis. Results were presented as the mean±SD. Significant differences were assessed by one-way ANOVA and Dunnett's post-hoc test. (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$ were used for statistical significance.

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Conflict of interest: None declared.

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