STEMedicine

RESEARCH ARTICLE

Oleuropein inhibits pancreatic cancer through miR-190b-5p induction

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Abstract

***Background*:** Pancreatic cancer has the highest mortality rate of all major cancers. Till now, surgical resection is considered the only potentially curative therapy. The aim of this study was to study oleuropein (Ole) as an alternative treatment for pancreatic cancer.

***Methods*:** First, we determined the tumor-inhibitory effect of Ole in pancreatic cancer cell lines and the KPC mouse model. Second, we employed microarray and real-time polymerase chain reaction to identify microRNAs (miRNA) regulated by Ole in pancreatic cancer. Third, we mapped out the downstream target of the miRNA candidate by luciferase assay and western blotting. Finally, we measured the tissue expression of the miRNA candidate in clinical samples. Both *in vitro* and *in vivo* studies supported Ole treatment suppressed pancreatic cancer.

***Results*:** We profiled miRNAs changes induced by Ole and identified miR-190b-5p as a novel functional player in Ole-mediated pancreatic cancer suppression. Further on, we reported that TCF4 in Wnt signaling was a direct target of miR-190b-5p. The suppression of Wnt signaling by miR-190b-5p was attributed to Ole-induced tumor inhibition in a pancreatic cancer model. Finally, we showed that miR-190b-5p was downregulated in human clinical cancer samples.

***Conclusion*:** In summary, we discovered that Ole carried anti-pancreatic cancer function. MiR-190b-5p induced by Ole was a potential therapeutic target.

Keywords: *oleuropein*; *miR-190b-5p*; *TCF4*; *Wntlβ-catenin*; *pancreatic cancer*

Received: 21 February 2022; Revised: 5 March 2022; Accepted: 6 March 2022; Published: 4 April 2022

Pancreatic cancer is one of the deadliest types of cancers. More than 200,000 people died of pancreatic cancer every year. The annual incidence of pancreatic cancer is about 40,000 cases in the United States. Due to the high fatality of pancreatic cancer, the mortality rate is almost equal to the incident rate. More than 30,000 cases die from the disease in the United States. The survival rate within 5-year among the pancreatic cancer patients is less than 5%. The causes of pancreatic cancer are still largely unknown. Environmental factors may be implicated to the development of pancreatic cancer. The risk of developing pancreatic cancer is about three times as high among smokers as to those non-smokers. High intake of alcohol, coffee, or aspirin may also be considered as risk factors, though the causative links of these are still under study. Family history of the disease is a high-risk factor of pancreatic cancer. The genetic mutations in DNA repair pathway such as BRCA2 could be one of the genetic bases for this causative link (1, 2).

The cancer originates in the ductal epithelium and manifests from premalignant lesions to metastatic tumor. This pathogenesis of pancreatic cancer is largely involved the accumulation of gene mutations including the activation of oncogene KRAS2 and the inactivation of tumor suppressor gene TP53 or CDKN2A. The activating mutations in KRAS2 have been found in 90% of the pancreatic tumors. Similarly, 95% of the tumors carry inactivating mutations in CDKN2A. TP53 shows abnormal expression in 75% of the tumors (3, 4).

[#]These authors contributed equally to this work.

Citation: STEMedicine 2022, 3(2): e125 - http://dx.doi.org/10.37175/stemedicine.v3i2.125

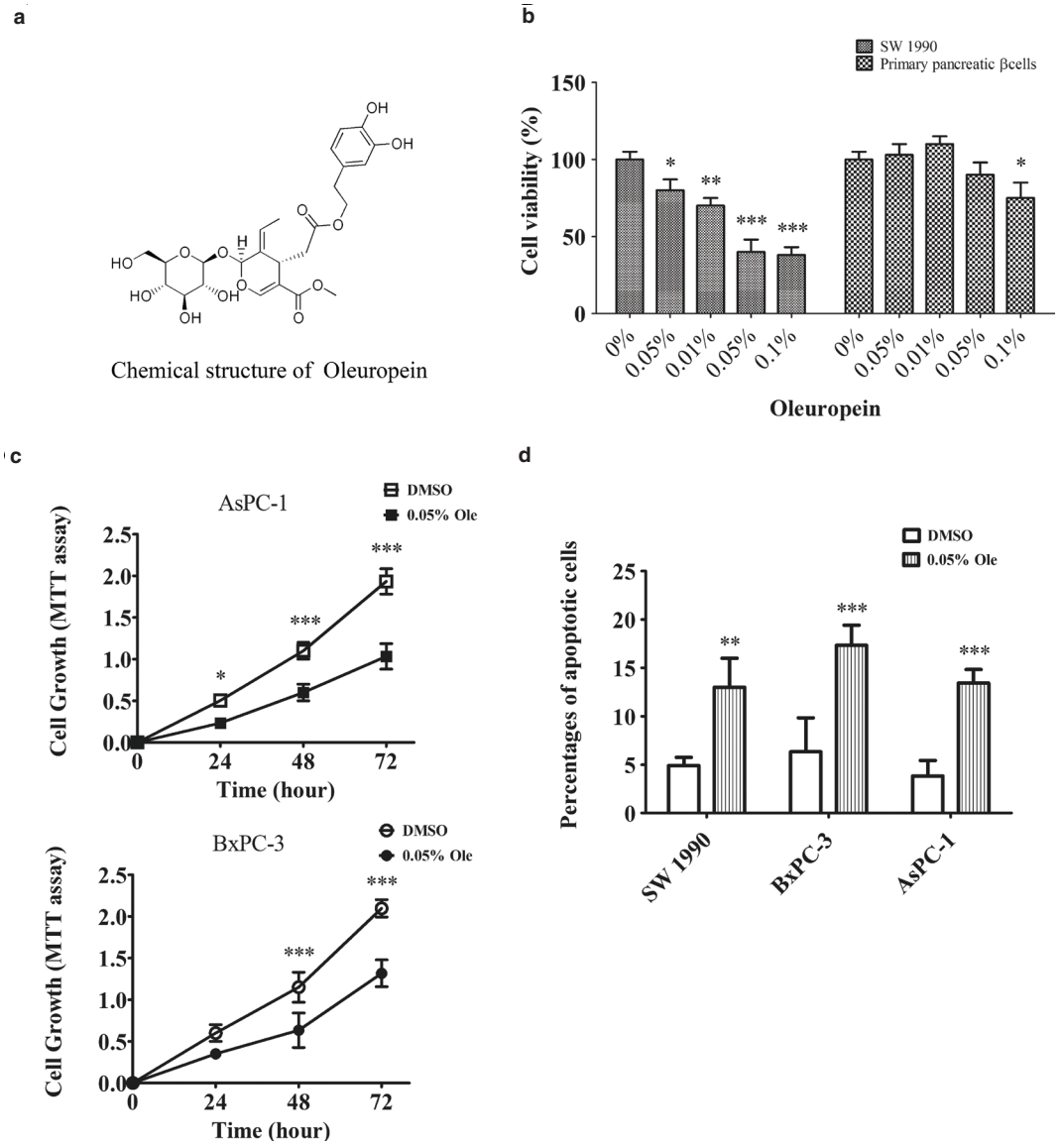

Fig. 1. Ole inhibits pancreatic cancer cells *in vitro.* (a) Schematic diagram of the chemical structure of Ole. (b) Cell viability study of SW 1990 or primary pancreatic β-cells under the treatment of Ole. Cells were treated with different doses of Ole for 48 h. The cell viability was measured by MTT assay. (c) Cell proliferation study of AsPC-1 and BxPC-3 lines under the treatment of Ole. Cells were incubated with either 0.05% Ole or DMSO. The cell growth was assessed by MTT assay every 24 h for 3 days. (d) Cell apoptosis study of pancreatic cancer cell lines under the treatment of Ole. Cells were treated with either 0.05% Ole or DMSO. Forty-eight hours later, the apoptotic cells were counted by Annexin V/PI staining. The data were presented as means ± SD of three independent experiments (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ as compared with control).

Surgery remains to be the most effective treatment for the patients in the early stage of the cancer. Even the tumor is fully resected, the postoperative treatment is critical to reduce the relapse rate and improve the outcome in overall survival. Gemcitabine, a nucleoside analog to induce cell death, is considered one of the standard adjuvant chemotherapies after surgery (5). Identifying new cancer treatment targets or agents will definitely provide better disease management for the cancer patients (6).

Oleuropein (Ole) is one of the major phenolic components found in olives, olive leaves, or olive oil. Its concentration in olives can reach as high as 140 mg/g. Oleuropein is an ester of 2-(3,4-dihydroxyphenyl) ethanol (hydroxytyrosol) belonging to the secoiridoids family (Fig. 1a). Ole has strong physiological implication in health and been explored as healthy supplement in the market. Cumulative studies have revealed that Ole carries biological properties in anti-inflammatory, antioxidant, antimicrobial, antiatherogenic, and anticancer. For instance, Ole

has been shown to inhibit the oxidation of low-density lipoproteins. It can also elicit anti-inflammatory effects by inhibiting 5-lipoxygenase. In addition, Ole can reduce endothelial adhesion molecule expression to confer its antiatherogenic protection (7, 8).

Ole has also been characterized as effective agent in attenuating the initiation, progression, and metastasis of multistage carcinogenesis. Growing evidence from *in vivo* animal studies has suggested the therapeutic potential of Ole in breast cancer, colon cancer, skin cancer, and tongue cancer. *In vitro* mechanistic studies have shown that Ole regulates multiple cellular signaling pathways to suppress tumor growth. For instance, Ole can increase p53 or Bax expression to promote apoptosis in cancer cells. It can also inhibit Akt or mitogen-activated protein kinases cascade to reduce cell proliferation (8, 9).

In our study, we were the first time to determine the antitumor property of Ole in pancreatic cancer with both the *in vitro* cell model and *in vivo* animal model. To investigate the mechanism of Ole-mediated pancreatic cancer suppression, we identified that miR-190b-5p was induced by Ole and validated its functional role in inhibiting pancreatic cancer. To further elucidate its mechanistic role, we identified the downstream target of miR-190b-5p in Wnt/β-catenin. The link between miR-190b-5p and pancreatic cancer was also established at clinical level.

Methods

Reagents and antibodies

Ole (>98%, Cat no. 12247) was obtained from Sigma Aldrich. LiCl (>99.98%, Cat no. 203637) was purchased from Sigma Aldrich. Recombinant Wnt3a (>75%, 5036-WN) was ordered from R&D system. TCF-4 antibody (sc-166699) was from Santa Cruz. Gapdh antibody (ab9485) was purchased from Abcam.

Cell lines

SW 1990, BxPC-3, and AsPC-1 tumor cell lines were obtained from ATCC. They were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and 2 mM L-glutamine (Invitrogen, Waltham, MA, USA) at 5% CO_2/37°C. The cells were passaged every 2–3 days.

Cell viability assay

Cell viability or proliferation rate was measured by the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (ab211091) kit from Abcam. Briefly, cells were seeded in 96-well format with 5,000 cells/well. Twenty-four hours later, cells were treated with different concentrations of Ole dissolved in dimethyl sulfoxide (DMSO). The cell growth rate was assessed every 24-h following the manufacturer's instruction.

Cell apoptosis assay

The cell death rate was quantified using the Dead Cell Apoptosis Kit (V13242, ThermoFisher Scientific, Waltham, MA, USA). Briefly, cells were cultured in 96-well plate with 500 cells/well. Twenty-four hours later, cells were treated with different doses of Ole as indicated in the legend. The cell apoptotic rate was measured 48 h after the treatment. The apoptotic cells were defined as stained positive by both annexin V and propidium iodide as measured in a flow cytometer.

Pancreatic cancer animal model

All animal studies were carried out under the approval of ethical review in Fujian Medical University Union Hospital. KPC (*LSL-Kras*$^{G12D/+}$; *LSL-Trp53*$^{R172H/+}$; *Pdx-1-Cre*) mice spontaneously developed pancreatic cancer lesions as described previously (10). KPC mice were enrolled onto studies when the tumor size was reaching 200 ± 50 mm^3 as measured by 3D ultrasonography. PC (LSL-Trp53R172H/+; Pdx-1-Cre) mice did not develop any pancreatic tumors and were used as healthy controls.

Survival experiment

KPC mice carrying pancreatic tumors of 200 ± 50 mm^3 were randomly assigned to control group (n = 10) receiving no treatment or treatment group (n = 10) receiving 1% Ole in their drinking water, which they consumed ad libitum. The tumor size and mortality endpoint were accessed every 3 days throughout the study. *In vivo* delivery of microRNA (miRNA) was achieved with the aid of nanoparticles-based vector as described previously (11). The mice were received 100 μg of miRNAs complexed with nanovector at 1:4 ratio via tail-vein injection.

MiRNA microarray profiling

Total miRNA from pancreatic cancer cells was purified using the miRNeasy kit (Qiagen, Valencia, CA, USA) and reversed transcribed using the miRCURY LNA RT kit (Qiagen). The miRNA expressional profiling was performed on GeneChip miRNA 2.0 Array according to the standard protocol. The miRNA expressional level was normalized to all miRNAs in the sample. Fold change more than 2.0 with P-value < 0.05 is considered significant.

MiRNA mimic and inhibitor

MiR-190b-5p inhibitor or its mimic as well as their scramble controls was ordered from mirVana Libraries (Thermo Fisher Scientific). The transfection of miR-190b-5p inhibitor or its mimic into cancer cells was achieved with TransIT-X2 Dynamic Delivery System (Mirus Bio) according to the manufacturer's manual.

Realtime-polymerase chain reaction (PCR) analysis

Total RNA was extracted using the Trizol method. The total RNA containing miRNA was reverse transcribed using the miScript II RT kit (Qiagen). The customized primer targeting miR-190b-5p was ordered from Qiagen. The U6 RNA was used as endogenous control. The real-time PCR was carried out on the QuantStudio real-time PCR platform (Applied Biosystems, Waltham, MA, USA) according to the standard protocol.

Luciferase assay

The 3′-UTR with the miR-190b-5p binding site of TCF4 or the corresponding disrupted binding sequence (Fig. 5a) was cloned, respectively, at the downstream of the Firefly luciferase reporter gene in the pMIR-REPORT vector. Cells expressing the TCF4 3'UTR luciferase reporter vector (WT or MUT) and Renilla luciferase as internal control (pRL-SV40, Promega) were further transfected with miR-190b-5p mimics or scramble control. The luciferase activities were measured using the Dual Luciferase Reporter Assay (Promega, Madison, WI, USA) in a plate reader following the manufacturer's instruction.

The Wnt signaling pathway was measured by the TCF/LEF reporter assay kit (#60500, BPS Bioscience). Briefly, cells transfected with miR-190b-5p or scramble control were subjected to the assay measurement according to the user's manual. The activation of Wnt pathway was induced by either WNT3A recombinant protein or LiCl.

Western blotting analysis

Western blotting was carried out according to the standard protocol. Briefly, cells were lysed in the radioimmunoprecipitation buffer. Protein extracts were denatured in the LDS sample buffer (Life Technologies, Pleasanton, CA, USA) and fractionated in 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane for over-night at 4°C. Blotting was performed with the indicated antibodies in 1:1,000 dilution. The signal was captured by the SuperSignal West Pico PLUS chemiluminescent kit.

Clinical pancreatic cancer tissue samples

Clinical evaluation of miR-190b-5p expression in human pancreatic cancer tissues was approved by Fujian Medical University Union Hospital. A total of 10 pancreatic cancer patients were enrolled with an informed consent (Table 1). The tumor tissues and the corresponding adjacent non-tumor tissues were harvested for the study. The tissues were frozen in dry ice and kept in −80°C.

Statistical analysis

The results were presented as means ± standard deviations (SDs). The statistical analyses were performed using Student's *t*-test or one-way analysis of variance (ANOVA) when more than two groups involved. Only *P*-value less than 0.05 was considered significant.

Table 1. The list of clinical characteristics of pancreatic cancer patients (n = 10) enrolled in this study

Clinical characteristics of pancreatic cancer patients		
Parameters	Classes	Number of patients
Total		10
Age	< 50	0
	50–59	2
	60–69	3
	70–79	4
	> 80	1
Gender	Male	6
	Female	4
Tumor stage	I	2
	II	2
	III	4
	IV	2

Results

Ole-inhibited pancreatic cancer cells growth

First, we tested the inhibitory effect of Ole on the viability of SW 1990, a pancreatic cancer line derived from a grad II pancreatic adenocarcinoma. The cancer cells were incubated with different doses of Ole from 0.05 to 0.1% for 2 days. The cell viability was measured using MTT assay. As shown in Fig. 1b, Ole treatment reduced the viability of SW 1990 in a dose-dependent manner. In the condition of 0.05% Ole, there was more than 50% inhibition on cell growth. There was no significant difference between 0.5 and 0.1% treatment, implying the inhibitory effect reached a plateau. In addition, we also tested the cytotoxicity of Ole treatment on primary pancreatic β-cells. As shown in Fig. 1b, only the maximum condition of 0.1% caused a marginal inhibition on normal pancreatic cells. Therefore, we chose 0.05% as testing concentration in our *in vitro* experiments. To confirm the effect of Ole on pancreatic cancer cells, we moved onto test two other caner lines: AsPC-1 and BxPC-3. The cells were treated with 0.05% Ole, and the proliferation rate was measured for 3 days. As shown in Fig. 1c, Ole slowed down cell growth markedly in both lines starting from 24 h post-treatment. Subsequently, we tested the apoptotic effect of Ole on cancer lines. All three cancer lines were treated with 0.05% Ole for 48 h, and the cell death was quantified by Annexin V/PI staining. In control, only about 5% cells were apoptotic in all three types of cancer cells. After treatment, the dead cells increased significantly as shown in Fig. 1d. Taken together, these results strongly suggest that Ole carried inhibitory effect toward pancreatic cancer cells.

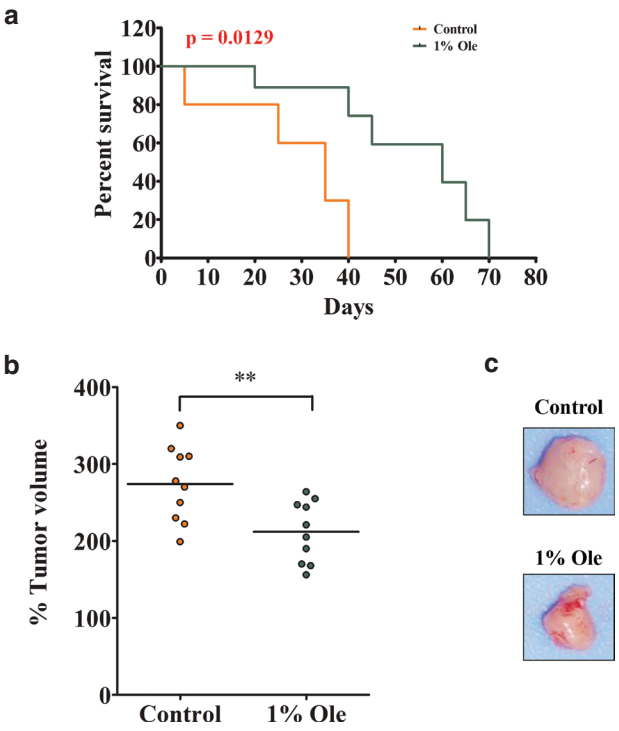

Fig. 2. Ole inhibits pancreatic cancer *in vivo.* (a) Survival study of KPC mice administrated with or without 1% Ole in drinking water. KPC mice were enrolled onto studies when the tumor size was reaching 200 ± 50 mm^3 as measured by 3D ultrasonography. They were randomly assigned to control or treatment group, with 10 subjects in each group. The tumor size and mortality endpoint were accessed every 3 days throughout the study. (b) The tumor sizes of KPC mice in day 10 were compared between control and treatment group as determined by 3D ultrasonography. (c) The representative images of tumor samples harvested from each experimental group (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ as compared with control).

Ole prolonged the survival in pancreatic cancer mouse model

Next, we tested the therapeutic effect of Ole in the pancreatic cancer mouse model. Pancreatic tumors spontaneously developed in KPC (*LSL-Kras*$^{G12D/+}$; *LSL-Trp53*$^{R172H/+}$; *Pdx-1-Cre*) as driven by the mutations of *Kras* and *Trp53*. When the tumors reaching about 200 ± 50 mm^3 as assessed by three-dimensional ultrasonography, the mice started receiving 1% Ole in the drinking water. We then began to measure the survival in a 5-day interval. As shown in Fig. 2a, the median survival time of KPC mice without treatment was about 35 days. They died within 40 days from the onset of the measurement. In comparison, 1% Ole treatment significantly extended the median survival time nearly double to 60 days. With the treatment, the maximum survival period in KPC mice was prolonged to 70 days. The tumor size in KPC mice was also measured in day 10 after the treatment. The increased tumor volume was determined against the baseline. In line with our observation in animal survival improvement, Ole treatment also significantly decreased the tumor growth as shown in Fig. 2b. The representative tumor samples under either control or treatment conditions were shown in Fig. 2c. Our findings in the mouse model confirm the inhibitory effect of Ole in pancreatic cancer.

Ole induced miR-190b-5p in pancreatic cancer cells and mouse model

We hypothesized that miRNAs regulatory circuit might play a role in Ole-mediated pancreatic cancer suppression. We sought to measure the differences in miRNA expression profiles in normal and Ole treatment conditions. SW 1990 was used as a model cell line, and the miRNA expression profiles between the conditions were obtained using the GeneChip miRNA 2.0 Array. As shown in Fig. 3a, there were 16 miRNAs showing most significant changes (more than two folds) after the treatment. Among them, miR-190b-5p showed markedly increase in response to Ole. We confirmed the upregulation of miR-190b-5p in all three cancer cell lines treated with 0.05% Ole (Fig. 3) in real-time PCR. The fold change was between two and three times. Moreover, we also measured the miR-190b-5p expression in pancreatic tumors harvested from KPC mice. As shown in Fig. 3c, the baseline was established in the control group receiving no treatment. In comparison, the treatment group receiving 1% Ole showed notably increased level of miR-190b-5p in the tumor tissues. Taken together, the miR-190b-5p expression was regulated by Ole positively.

MiR-190b-5p induction is required for Ole-mediated pancreatic cancer cells inhibition

To elucidate the role of miR-190b-5p in Ole-mediated tumor suppression, we first validated a miRNA inhibitor against miR-190b-5p in SW 1990 cells (Fig. 4a). About two-third reduction in miR-190b-5p expression was observed when the inhibitor transfected in cells. We then transfected the cancer cells with either miR-190b-5p inhibitor or non-specific control. Forty-eight hours after transfection, the cells were treated with either Ole or DMSO, and their proliferations were monitored through 3 days. As shown in Fig. 4b, Ole treatment significantly slowed down cancer cells growth as expected. Interestingly, knocking-down miR-190b-5p completely abolished its inhibitory effect on cell proliferation. We also studied the role of miR-190b-5p in the apoptotic effect of Ole in cancer cells. The transfected was treated with either Ole or DMSO. The percentages of apoptotic cells were quantified after 48 h. In agreement with the observation on cell proliferation experiment, inhibiting miR-190b-5p totally cleared the apoptotic effect of Ole on SW 1990 cells (Fig. 4b). On the whole, these results indicate Ole-suppressed pancreatic cancer cells through miR-190b-5p pathway.

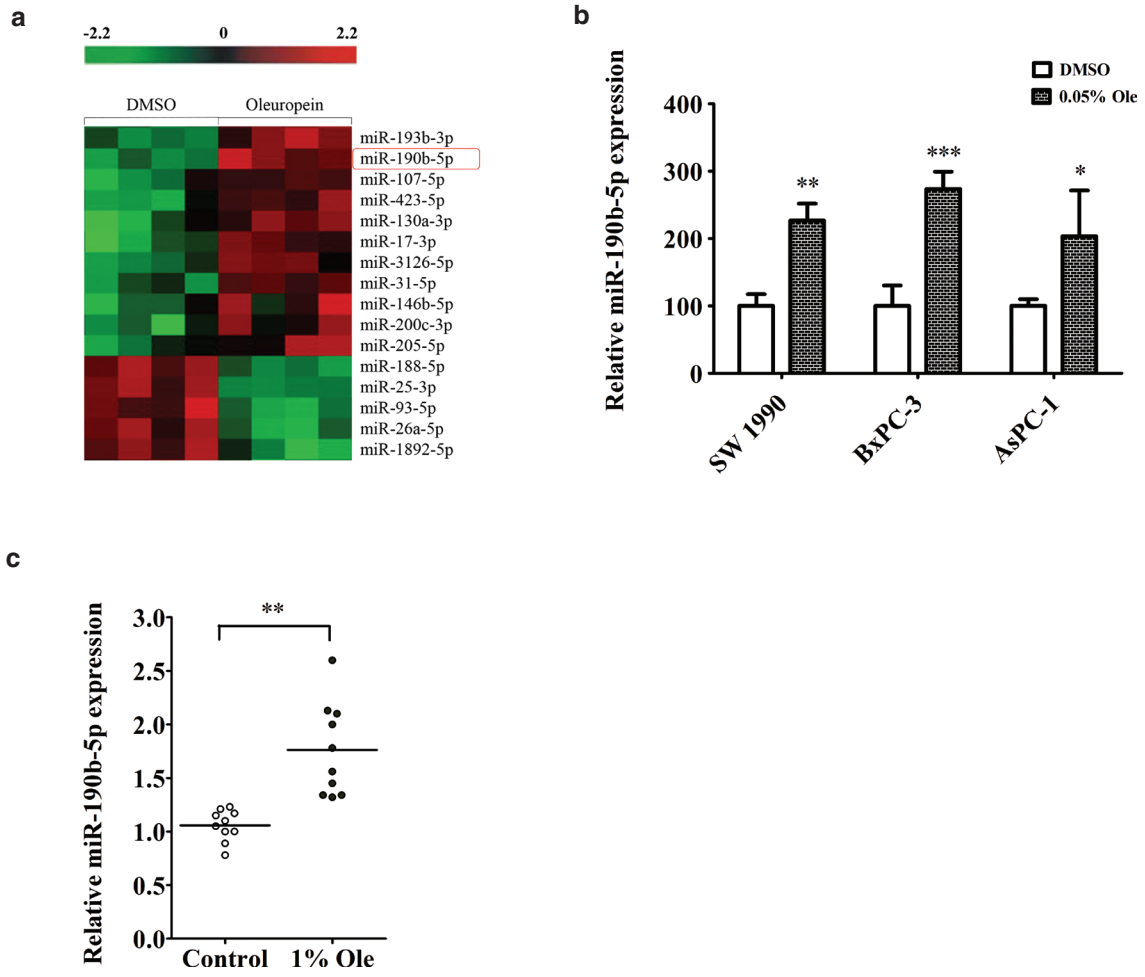

Fig. 3. Ole induces miR-190b-5p *in vitro* and *in vivo*. (a) Hierarchical clustering of selected miRNAs expression regulated by Ole. The SW 1990 (n = 4, each group) was treated with 0.05% Ole or DMSO for 48 h. The miRNAs were harvested and subjected to GeneChip miRNA 2.0 Array study. (b) Realtime PCR analysis of miR-190b-5p induction by Ole. SW 1990, BxPC-3, and AsPC-1 were treated with 0.05% Ole or DMSO for 48 h. The fold induction of miR-190b-5p was determined by real-time PCR. (c) MiR-190b-5p expression levels in pancreatic tumors harvested from KPC mice. Control group received normal drinking water. Treatment group received 1% Ole in drinking water. The data were presented as means ± SD of three independent experiments (*P < 0.05, **P < 0.01, and ***P < 0.001 as compared with control).

MiR-190b-5p-targeted TCF4 in Wnt/β-catenin pathway in pancreatic cancer cells

We used TargetScan to analyze the hypothetical targets of miR-190b-5p (12). Among the top target was TCF4, a key transcriptional factor activated by Wnt/β-catenin. Wnt–β-catenin pathway is one of the most frequently altered pathways in pancreatic cancer (13). The predicted targeting sequence on 3'UTR of TCF4 was shown in Fig. 5a. We employed luciferase gene reporter assay to test TCF4 as downstream target of miR-190b-5p. As shown in Fig. 5a, in WT reporter vector, the luciferase gene was conjugated with 3'UTR of TCF4 containing the miRNA targeting site. In contrast, the MUT vector carried the site mutations on the targeting sequence of 3'UTR of TCF4 that theoretically abolished the binding of miR-190b-5p. We cotransfected WT or MUT reporter vectors with either miR-190b-5p or the scramble miRNA in SW 1990 cells. The luciferase activities were measured in 48 h posttransfection. As shown in Fig. 5b, miR-190b-5p was found to reduce the luciferase activation significantly in the WT system. In contrast, it had no effect on the luciferase activity of the MUT vector, which implied that the binding of miR-190b-5p on the mutated TCF4 3'UTR was interrupted. Subsequently, we validated the direct impact of miR-190b-5p on TCF4 protein expression. Two cancer lines, SW 1990 and BxPC-3, were transfected with miR-190b-5p or scramble control. Seventy-two hours later, the protein expression of TCF4 was markedly downregulated in miR-190b-5p transfected cells (Fig. 5c). Since TCF4 acts as a key player in Wnt/β-catenin signaling, we next asked whether miR-190b-5p carried a functional role in regulating Wnt/β-catenin pathway. We used

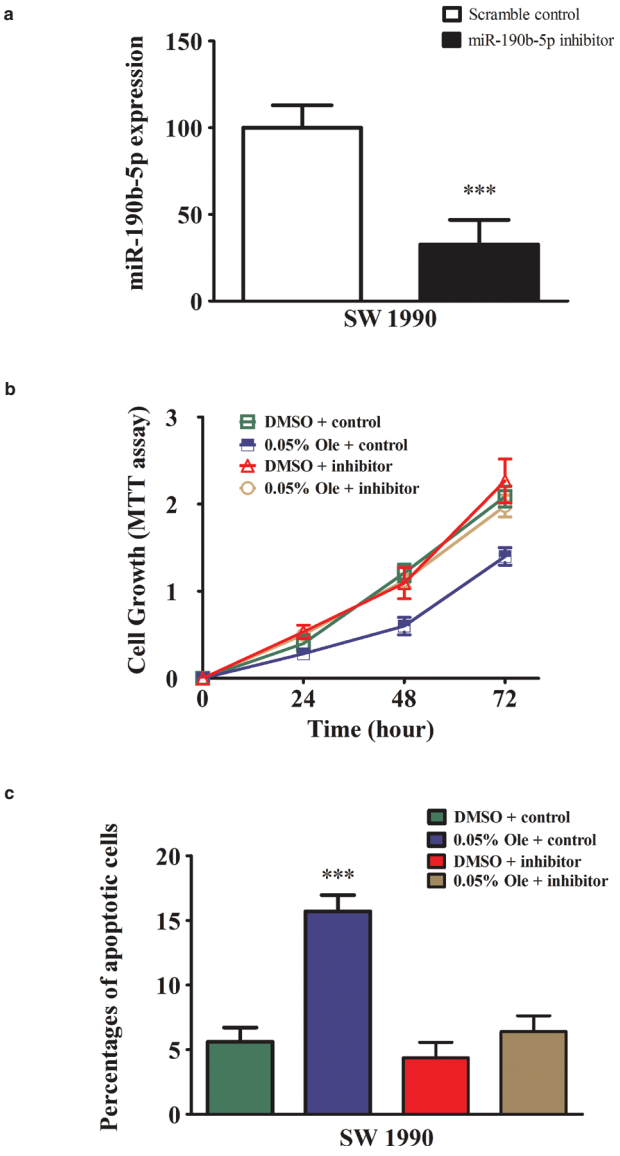

Fig. 4. MiR-190b-5p is required for Ole-mediated pancreatic cancer inhibition. (a) Real-time PCR analysis of miR-190b-5p expression in cells transfected with miRNA inhibitor or scramble control. (b) Cell growth analysis of pancreatic cancer cells treated with DMSO of 0.05% Ole. Forty-eight hours before the treatment, cells were transfected with miR-190b-5p inhibitor or control. Cell proliferation was monitored by MTT assay every 24 h for 3 days. (c) Cell apoptosis analysis of pancreatic cancer cells treated with DMSO of 0.05% Ole. Seventy-two hours before the treatment, cells were transfected with miR-190b-5p inhibitor or control. Apoptotic cells were stained positively with Annexin V/PI. The data were presented as means ± SD of three independent experiments (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ as compared with control).

a TCF/LEF luciferase reporter vector, a Wnt pathway-responsive reporter, to evaluate this hypothesis. In cells transfected with TCF/LEF luciferase reporter gene, the luciferase activities were induced greatly by either LiCl or recombinant Wnt3a, indicating the activation of Wnt signaling (Fig. 5d). Interestingly, the coexpression of miR-190b-5p in cells inhibited the activation of luciferase gene in basal level or in response to LiCl or Wnt3a. Collectively, these results indicate that miR-190b-5p suppressed Wnt/β-catenin signaling by targeting TCF4.

Delivery of miR-190b-5p mimic attenuated pancreatic cancer development in mice

We had showed that miR-190b-5p could mediate Ole-induced tumor suppression in pancreatic cancer through targeting TCF4 pathway. Next, we conducted an *in vivo* study to evaluate the therapeutic function of miR-190b-5p in the KPC pancreatic cancer mouse model. We formulated miR-190b-5p mimic or scramble control in nanoparticle delivery system. One mg/kg of formulated miRNAs was delivered to the mice through tail vein injection following a regimen of 5-day interval. No abnormal phenotypes or weight-loss was associated with miRNAs injection (unpublished results). As shown in Fig. 6a, systematic delivery of miR-190b-5p significantly prolonged the survival time in tumor-bearing mice as compacted to the control group ($P < 0.05$). The median survival time was extended from about 25 days in control group to about 40 days in treatment group. In addition, the average tumor size was also significantly reduced in mice delivered with miR-190b-5p mimic (Fig. 6b). The representative tumor samples treated with either scramble or miR-190b-5p mimic were shown in Fig. 6c. This *in vivo* study strongly suggests that miR-190b-5p may carry therapeutic potential in treating pancreatic cancer.

MiR-190b-5p was downregulated in pancreatic cancer lesions

Finally, to establish the clinical relevance of targeting miR-190b-5p in pancreatic cancer, we proceeded to measure its expressional level in cancer lesions. First, we compared miR-190b-5p expression between tumor-bearing mice (KPC) and non-tumor-bearing parental line (PC). We harvested the pancreatic tissues and quantified miR-190b-5p expression in the tissue samples. As shown in Fig. 7a, miR-190b-5p was found notably decreased in KPC mice. To further confirm the trend, we proceeded to measure miR-190b-5p in human pancreatic cancer patients. The characteristics profile of the patients (n = 10) was summarized in Table 1. The tumor samples represented all the four clinical stages of pancreatic cancers. The expression of miR-190b-5p in between the tumor lesion and the surrounding non-tumor tissue from the same donor was compared. As shown in Fig. 7b, the pancreatic cancer-associated downregulation of miR-190b-5p was recapitulated in human clinical samples. Our new findings strongly imply miR-190b-5p acted as a functional target of Ole in inhibiting pancreatic cancer.

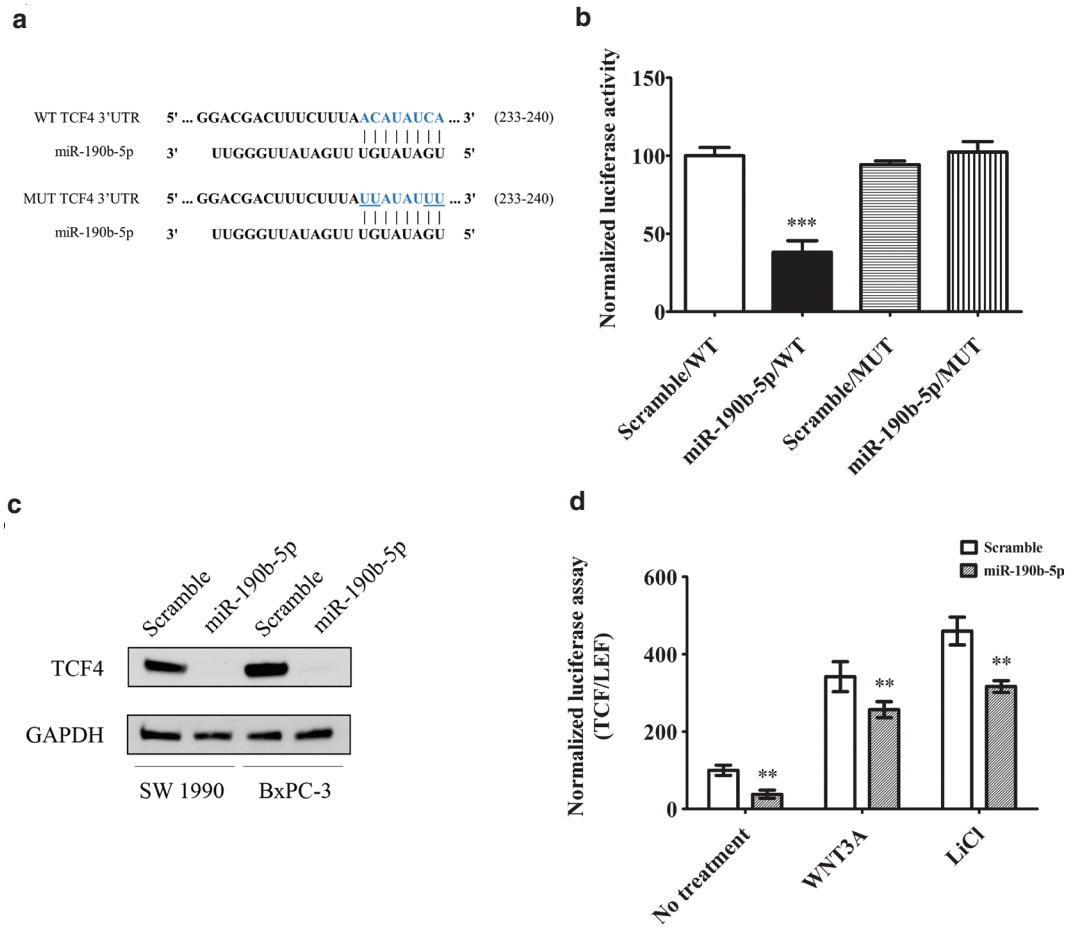

Fig. 5. TCF4 is a direct target of miR-190b-5p. (a) Schematic diagram of a conserved putative miR-190b-5p targeting site in 3'UTR of TCF4 (WT). The site mutations were introduced to miR-190b-5p targeting site of 3'UTR (MUT). (b) Luciferase reporter assay study of the interaction between miR-190b-5p and 3'UTR of TCF4. Pancreatic cancer cells were first transfected with luciferase reporter genes conjugated with either wildtype (WT) or mutant (MUT) 3'UTR of TCF4. Twenty-four hours later, cells were transfected with either miR-190b-5p mimic or scramble control. Transfected cells were cultured for 48 h and then harvested for the measurement of luciferase activities in a plate reader. (c) Western blotting analysis of TCF4 protein expression in cells transfected with miR-190b-5p or scramble control. The cells were transfected with either miR-190b-5p mimic or scramble control for 48 h before harvesting. GAPDH was used as loading control. (d) Luciferase reporter assay study of the regulatory effect of miR-190b-5p in Wnt pathway. Cells were cotransfected with TCF/LEF luciferase reporter genes with either miR-190b-5p mimic or scramble control. Forty-eight hours later, the transfected cells were treated with either WNT3A or LiCl. The activation of Wnt pathway was assessed in a plate reader. The data were presented as means ± SD of three independent experiments (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ as compared with control, in one-way ANOVA).

Discussion

We are in the first time to establish antitumor function of Ole in pancreatic cancer model. In the *in vitro* study, Ole inhibits cancer lines growth and induces cell death specifically. *In vivo* study also supports Ole administration extends survival and reduces tumor volume in the KPC mouse model. We draw the link of Ole-mediated pancreatic cancer suppression to miR-190b-5p expression. Ole induces miR-190b-5p expression in all the cancer lines tested and mouse cancer model. Inhibiting miR-190b-5p abolishes Ole-mediated pancreatic cancer suppression, suggesting miR-190b-5p works downstream of Ole. In contrast, over-expressing miR-190b-5p suppresses pancreatic cancer in both cell and mouse models. Clinical evidence further supports that miR-190b-5p reduction is implicated in pancreatic cancer tumorigenesis. Last but not least, we elucidate the downstream target of miR-190b-5p, TCF4 in Wnt pathway, suggesting targeting Wnt pathway may provide a new avenue for pancreatic cancer treatment development.

Ole is a secoiridoid derived from Olive tree. It has multiple functions in human health, including anti-inflammatory, antimicrobial, antioxidant, neuroprotective, and anticancer. There are cumulative evidence showing the therapeutic potential of Ole in treating different types of cancers. In colorectal cancer, Ole has been shown to

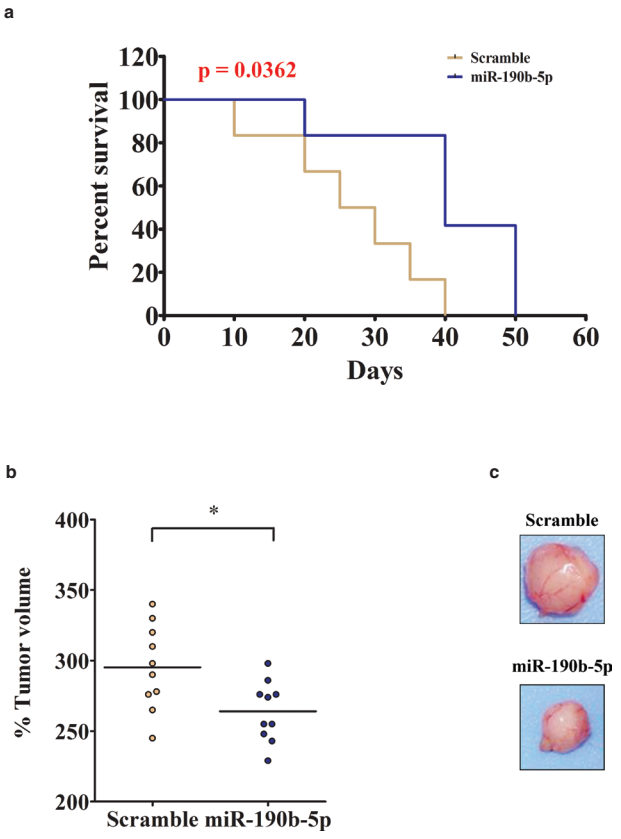

Fig. 6. MiR-190b-5p inhibits pancreatic cancer in mouse model. (a) Survival study of KPC mice administrated with 1 mg/kg of miR-190b-5p or scramble control formulated in nanoparticle-based vectors. KPC mice were enrolled onto studies when the tumor size was reaching 200 ± 50 mm^3 as measured by 3D ultrasonography. They were randomly assigned to receive miR-190b-5p or scramble, with 10 subjects in each group. The tumor size and mortality endpoint were accessed every 3 days throughout the study. (b) The tumor sizes of KPC mice in day 10 were compared between miR-190b-5p or scramble group as determined by 3D ultrasonography. (c) The representative images of tumor samples harvested from each experimental group (*P < 0.05, **P < 0.01, and ***P < 0.001 as compared with control).

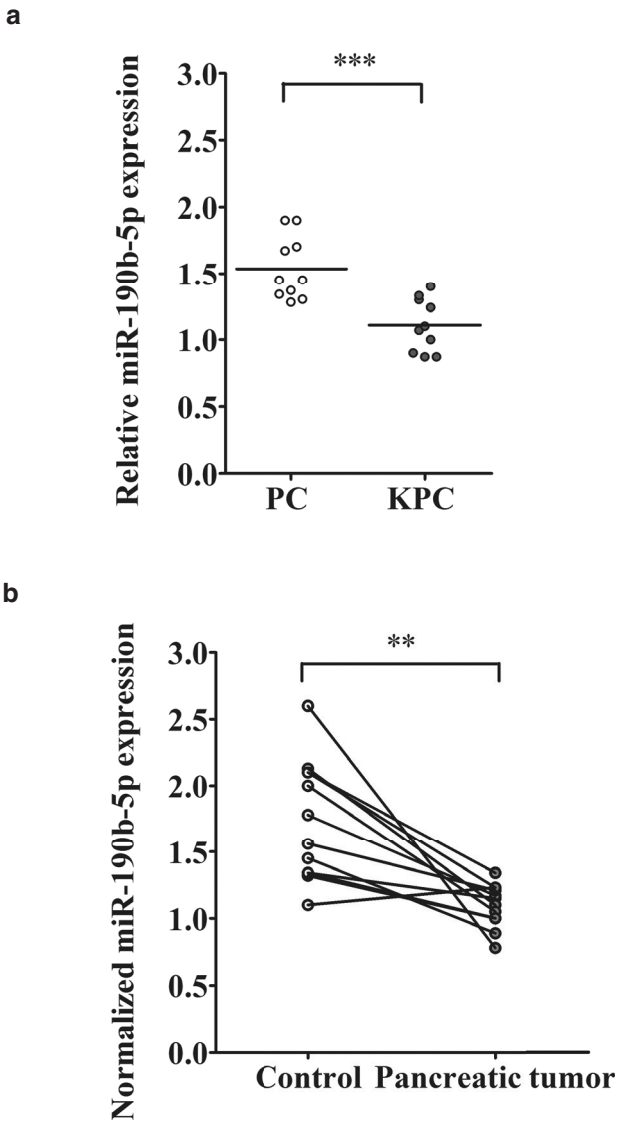

Fig. 7. MiR-190b-5p is downregulated in pancreatic cancer. (a) The comparison of miR-190b-5p expressional levels in pancreatic tissues between PC and KPC mice. The pancreatic tissues were harvested, and the miRNA expression was analyzed by real-time PCR. Ten subjects were studied from each group. (b) The expressional levels of miR-190b-5p in 10 pancreatic cancer tissues and the matched non-tumor tissues were quantified by real-time PCR (**P < 0.01, significantly different from control group in a pair-wise student t-test).

repress the cancer cell proliferation through downregulating HIF-1α and activating p53. But, the exact mechanism is not totally revealed (14). In animal study, Ole has been shown to carry promising protective function against colitis-associated colorectal cancer. Treatment with Ole reduces proinflammatory cytokines production and downregulates Wnt/β-catenin along with other colorectal cancer-related pathways (15). In breast cancer, Ole together with hydroxytyrosol, another polyphenol found in olive oil, inhibits MCF-7 cancer cell proliferation by interfering with ERK1/2 activation (16). Supporting our study, Goldsmith et al. have shown that Ole and hydroxytyrosol carry anticancer potential as demonstrated in three pancreatic cancer lines (BxPC-3, CFPAC-1, and MIA PaCa-2). They have also managed to reveal that the putative mechanism of activation involves c-Jun and c-Fos pathways (17).

In our model, we confirmed the anti-pancreatic cancer effect of Ole in cell lines and mouse model. More importantly, we further dissect out its molecular mechanism in conferring the cytotoxic effect toward cancer cells. In our study, we focus on the role of miRNAs circuit in Ole-mediated tumor suppression. There are cumulative studies revealing the essential function of miRNAs in

pancreatic cancer oncogenesis and suppression. For instance, a recent study (18) has reported that the low level of miR-195 is associated with higher rate of metastasis stage and poor survival in pancreatic cancer patients. The intervention approach confirms the over-expression of miR-195 inhibits the progression of pancreatic cancer in the xenograft mouse model. The authors have also elucidated Doublecortin-like kinase 1 (DCLK1) as a direct downstream target of miR-195 emerging as a novel therapeutic target in pancreatic cancer. In contrast, Wang et al. have reported that miR-182 is over-expressed in human pancreatic cancer specimens and plays an oncogenic function in driving pancreatic cancer cell proliferation and migration (19). β-TrCP2 is identified as downstream target of miR-182 in the same study. The oncogenic effect of miR-182 is reversed by ectopic expression of β-TrCP2. From our microarray-based screening, we have uncovered that the miR-190b-5p is attributed to the therapeutic effect of Ole in these models. The antitumor function of miR-190b has been reported in other forms of cancers. In U2OS osteosarcoma cells, the induction of miR-190b-5p leads to tumor cell proliferative inhibition and apoptosis, suggesting it as a tumor suppressor (20). In this study, the upregulation of miR-190b-5p is associated with the downregulation of Bcl-2 that subsequently leads to Bax and apoptotic executive gene Caspase-3 activation. However, the direct target of miR-190b-5p in this study has not been identified. In gastric cancer cells, miR-190b-5p has been shown to confer the radio sensitivity (21). Consistently, miR-190b-5p induction is linked to the downregulation of Bcl-2, though the direct target has not been revealed. The repression effect on the tumor radiation resistance by Ole has also been observed in the xenograft model of ovarian cancer (22). Our study is the first one to provide a comprehensive picture on miR-190b-5p-mediated tumor suppression in the pancreatic cancer model. First, the ectopic expression miR-190b-5p suppressed cancer progression. Second, miR-190b-5p is downregulated in pancreatic cancer clinical samples and induced by Ole treatment. Third, TCF4 in Wnt/β-catenin pathway is a direct target of miR-190b-5p. All these evidences strongly suggest that miR-190b-5p could be both biomarker and therapeutic target of pancreatic cancer.

However, there are some controversy studies that show miR-190b-5p plays an opposite role in cancer biology. In human hepatocellular carcinoma, the upregulation of miRNA-190b-5p decreases IGF-1 that induces insulin resistance to confer the protection (23). In hormone-dependent breast cancer, miR-190b-5p is the most upregulated miRNA and has been proposed as a biomarker for ER positive breast cancer, though its oncogenic function is yet to be confirmed in this subtype of breast cancer (24). Therefore, the role of miR-190b-5p may be cell-type specific. The downstream target also defines the pathway it regulates and the implication to the tissue physiology. In pancreatic tissue, we have showed miR-190b-5p targeting TCF4 in Wnt/β-catenin pathway. Given the genes in Wnt/β-catenin pathway is also frequently mutated in cancers (25), it will be intriguing to evaluate whether Wnt signaling inhibitors can confer the protection or suppression against pancreatic cancer.

Conclusion

Finally, our study has revealed the potential role of Ole as therapeutic agent to treat pancreatic cancer. As from natural origin, Ole carries little risk potential on human health. To evaluate the efficacy of Ole in human pancreatic will be the next important step to test this hypothesis. Surgery removal of the pancreatic cancer tissue remains the mainstream for the treatment (26). Delivery of Ole may benefit the pancreatic patients for recovery and prevent the relapse after the surgery. We have very promising outcome in testing this hypothesis in mouse model through the gastrointestinal system. However, the digestive system between mouse and human to process Ole may be different. More work needs to be done to evaluate the efficiency and efficacy of delivering Ole through GI tract to treat human pancreatic cancer.

Conflict of interest and funding

The authors have declared that no conflicts of interest exist. The authors have not received any funding or benefits from industry or elsewhere to conduct this study.

Acknowledgements

Not applicable.

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