MicroRNA-221 induces autophagy through suppressing HDAC6 expression and promoting apoptosis in pancreatic cancer

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Abstract. Pancreatic cancer is an aggressive type of cancer with a poor prognosis, short survival rate and high mortality. Therefore, understanding the molecular mechanism underlying the aggressive growth of pancreatic cancer is of importance. An increasing number of studies suggest that numerous microRNAs (miRNAs/miRs) are associated with the tumorigenesis, progression and prognosis of tumors. In a recent study by the present authors, it was revealed that the expression of miR-221 was significantly downregulated in highly aggressive pancreatic cancer cells compared with weakly aggressive pancreatic cancer cells. In addition, miR-221 has been suggested as a novel tumor-associated miRNA, as it is involved in apoptosis, invasion, metastasis and autophagy of tumor cells. However, the function of miR-221 in pancreatic cancer remains yet to be investigated. In the present study, it was revealed that transfection with miR-221 mimic was able to significantly induce apoptosis and autophagy in pancreatic cancer cells compared with the negative control. The protein deacetylase histone deacetylase-6 (HDAC6) has emerged to be an important component in the cellular management of protein aggregates. Studies suggest that HDAC6 serves a function in the clearance of aggresomes, thereby implying a functional association between HDAC6 and autophagy. In the present study, it was revealed that transfection with miR-221 mimic was able to suppress the protein expression of HDAC6 in pancreatic cancer cells compared with the negative control. Immunofluorescence data suggested that the inhibition of HDAC6 was able to induce autophagy in pancreatic cancer cells. Additionally, the results of the present study suggest that the downregulation of miR-221 expression may serve an oncogenic function in the apoptosis and autophagy of pancreatic cancer cells by inducing the expression of HDAC6.

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Introduction

Pancreatic cancer is a malignant tumor of the digestive system identified by an insidious clinical manifestation, rapid progression and a poor prognosis. Furthermore, there are no acceptable screening methods to detect early-stage disease, so the majority of patients present with advanced disease and diagnosis at early stages of the disease is difficult (1-3). Pancreatic cancer has become the fourth leading cause of cancer-associated mortality in the Western world (4,5). Pancreatic cancer is often resistant to known anticancer drugs (6-8). Globally, there has been an increased focus on discovering alternative and more efficient anti-pancreatic cancer agents and gemcitabine sensitizers (8-10). There is a clear requirement to investigate more potential therapeutic agents for the treatment of pancreatic cancer.

MicroRNAs (miRs/miRNAs) are a group of small non-coding RNAs composed of 18-25 nucleotides that regulate protein-coding genes by binding to the 3'-untranslated regions (UTRs) of their target mRNAs (11-15). Since initial discovery by Lee *et al* (16) in 1993, increasing evidence has revealed that miRNAs serve important functions in the generation or development of various tumors, particularly with roles in processes, including cell cycle, apoptosis, migration and invasion (17-21).

Similar studies have also been performed in pancreatic cancer cells (22,23). In a recent study by the present authors (24), miR-221 was found to be differentially expressed between highly and weakly invasive PC cells (PC-1.0 and PC-1) when using miRNA microarray. The PC-1 cell line was established from pancreatic ductal adenocarcinomas induced by N-Nitrosobis (2-oxopropyl) amine in a Syrian golden hamster (25). The PC-1.0 cell line was established from a subcutaneous tumor produced following inoculation

of PC-1 cells. PC-1 cells exhibited a low aggressive potential, whilst PC-1.0 cells exhibited a high aggressive potential (26). These two cell lines also exhibited different growth rates and morphology *in vitro*. PC-1 cells formed island-like cell colonies, whereas PC-1.0 cells primarily grew as single cells (27). The results verified using two hamster pancreatic cancer cell lines (PC-1 and PC-1.0) and two human pancreatic cancer cell lines (Capan-2 and AsPC-1) by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Capan-2 cells exhibited a low aggressive potential, similar to PC-1 cells, whereas the AsPC-1 cell line exhibited a high aggressive potential, similar to PC-1.0 cells. The results revealed that six upregulated microRNAs (miR-34a, miR-193a, miR-221, miR-222, miR-484 and miR-502) exhibited differential expression between PC-1.0/PC-1 and AsPC-1/Capan-2 pancreatic cancer cells. Additionally, Gene Ontology and Kyoto Encyclopedia of Genes and Genomes enrichment analysis revealed that miR-221 were involved in multiple biological functions in tumorigenesis of pancreatic cancer. A number of studies have documented that miR-221 serves an important function in regulating the tumorigenesis and metastasis of glioblastoma, breast and gastric cancer, by affecting processes, including cell cycle, apoptosis, migration and autophagy (13,18). These results suggest that miR-221 may participate in regulating the progression of pancreatic cancer.

Histone deacetylases (HDACs), which are often deregulated in pancreatic cancer and other solid tumor types (28,29), are implicated in the regulation of molecules in growth regulatory and/or apoptotic pathways (28,29). Unique among the HDAC family members, HDAC6 has intrinsic ubiquitin-binding activity and associates with microtubules and the F-actin cytoskeleton (30-33). It has been demonstrated that HDAC6 was able to associate with and deacetylate α-tubulin *in vitro* and *in vivo*. Overexpression of HDAC6 induced the expression of deacetylated α-tubulin and promoted cell motility. Consistent with its effect on cell motility, HDAC6 is predominantly localized in the cytoplasm (34). Notably, malignant mammary epithelial cells exhibit increased HDAC6 cytosolic localization compared with normal cells (35). It has also been reported that HDAC6 also serves a function in the clearance of aggresomes, thereby implying a functional association between HDAC6 and autophagy (36). Giaginis *et al* (36) revealed that HDAC6 may be implicated in pancreatic malignant disease progression, and is considered to have clinical utility with potential use as a therapeutic target. Bae *et al* (37) revealed that c-Jun N-terminal kinase stimulated by hepatocyte growth factor led to the activation of Jun proto-oncogene, AP-1 transcription factor subunit and the transcriptional activation of miR-221, which increased miR-221 expression, thereby serving a critical function in suppressing HDAC6 expression in hepatocellular carcinoma cells. However, there are no studies on miR-221 regulating autophagy in pancreatic cancer via the regulation of HDAC6 expression.

In the present study, the aim was to elucidate the potential function of miR-221 on the apoptosis and autophagy of pancreatic cancer cells. In addition, it was confirmed that miR-221 regulates apoptosis and autophagy in pancreatic cancer cells by regulating HDAC6 expression. This will provide novel treatment methods and strategies for pancreatic cancer.

Materials and methods

Cell culture and cell transfection. PC-1.0 cell line (hamster; sourced from the Kumamoto University Medical School, Kumamoto, Japan) and the AsPC-1 cell line (human; purchased from Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) were cultured in RPMI-1640, supplemented with 10% fetal bovine serum (both Gibco; Thermo Fisher Scientific, Inc.), 1% penicillin-streptomycin (Thermo Fisher Scientific, Inc.) at 37˚C in a humidified atmosphere of 5% CO_2. The growth of cells was observed under an inverted microscope. When the cells had reached 70-80% confluence, they were detached using 0.25% trypsin (Gibco; Thermo Fisher Scientific, Inc.). The medium was changed every other day, and the cells were passaged every 3-4 days. The cells in the exponential phase were selected for further experiments.

The cultured PC-1.0 and AsPC-1 cells were uniformly seeded in 6-well cultured plates at a density of $3x10^5$/ml (2 ml in each well). Subsequent to the cells reaching complete adherence, transiently transfections with miR-221 mimic and mimic negative control (NC; both designed and synthesized by Invitrogen; Thermo Fisher Scientific, Inc.) at a final concentration of 30 nM using 9 μl Lipofectamine® RNAiMAX reagent and 150 μl OPTI-MEM I reduced serum medium (both Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C for 24 h according to the manufacturer's protocol. Untransfected cells were not used as a control. Downregulation of HDAC6 was achieved using 190 nM Apicidin (190 nM; Abcam, Cambridge, UK) at 37˚C for 2 h. The cells that were treated alone with DMSO were used as the NC. The cells were serum-starved overnight prior to transfection. The sequences are as follows: miR-221 mimic, 5'-AGCUACAUUGUCUGC UGGGUUUC-3'; mimic negative control, 5'-UUCUCCGAA CGUGUCACGUTT-3'.

RNA isolation and RT-qPCR. miRNA were extracted using the mirVana™ microRNA isolation kit (Ambion; Thermo Fisher Scientific, Inc.) and the levels of miRNA were determined using the TaqMan® MicroRNA Assay kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. cDNA was amplified using mature microRNA-specific RT primers and the TaqMan® MicroRNA Reverse Transcription kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. RT-qPCR was performed on an ABI 7500 Real-Time PCR system (Thermo Fisher Scientific, Inc.). The amplification reactions were performed in triplicate in a 96-well plate using the following thermocycling conditions: 10 min at 95˚C, followed by 40 cycles of 15 sec at 95˚C and 1 min at 60˚C. The C_q values were calculated using ABI Sequence Detection System software (version 2.1; Thermo Fisher Scientific, Inc.). The relative fold-change of each miRNA was calculated using the comparative C_q ($2^{-\Delta\Delta Cq}$) method (38). The noncoding small nuclear RNA U6 (Thermo Fisher Scientific, Inc.) was used as the endogenous control. The primer sequences were as follows: hsa-miR-221 forward, 5'-GAAACCCAGCAGACAATGTAG CT-3' and reverse, 5'-CTTTGGTGTTTGAGATGTTTGG-3'; and U6 forward, 5'-GCGAATTCTTAAACAGCTCGAATT AAGAATATGTTTCC-3' and reverse, 5'-GCGGATCCGCTA TGGAAGTTTTCTTTATTGATTACTTAATGTG-3'.

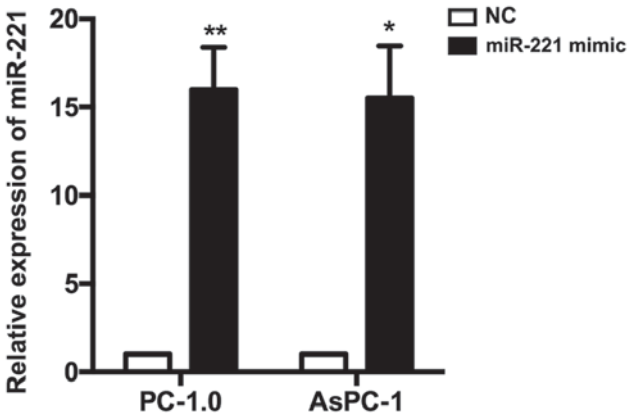

Figure 1. Levels of miR-221 expression following transfection with miR-221 mimic in pancreatic cancer cells. The cells were transfected with miR-221 mimic or mimic NC. The expression levels of miR-221 following transfection were quantified using reverse transcription-quantitative polymerase chain reaction. The results revealed that the expression levels of miR-221 in PC-1.0 and AsPC-1 cells transfected with the miR-221 mimic were significantly increased compared with the NC. U6 was used as an internal control. Data are expressed as the mean ± standard deviation (n=3 in each group). *P<0.05 and **P<0.01 vs. the NC group. miR, microRNA; NC, negative control.

Flow cytometric analysis of apoptosis. Apoptosis was detected using flow cytometric analysis. Following transfection, the cells were washed and re-suspended in 0.5 ml phosphate-buffered saline (PBS; pH 8.0) at a density of $1x10^6$ cells/ml. Then, Annexin V-FITC and propidium iodide dye (Nanjing KeyGEN Biotech Co., Ltd., Nanjing, China) were added to the cells and incubated at room temperature in the dark for 15 min. Apoptosis was detected using FACS LSR II (BD Immunocytometry Systems; BD Biosciences, San Jose, CA, USA) and analyzed using CXP software 2.2 (Beckman Coulter, Inc., Brea, CA, USA).

Western blot analysis. The expression of HDAC6 and LC3 proteins in PC-1.0 and AsPC-1 cells was analyzed. PBS was used as the wash water. The cells were washed three times prior to lysing. The cells were lysed in radioimmunoprecipitation assay buffer containing protease inhibitor cocktail (both Beyotime Institute of Biotechnology, Haimen, China). Total protein was quantified using a Bradford protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Briefly, samples containing equivalent amounts of total protein (20 μg) were run on 7.5-15% SDS-PAGE gels and then transferred onto PVDF membranes (Merck KGaA, Darmstadt, Germany). Blocking solution [PBS + 5% BSA (Sigma-Aldrich; Merck KGaA) + 10% goat serum] was then added at room temperature for 30 min to prevent non-specific binding. Membranes were incubated overnight at 4˚C using primary antibodies. The blots were then incubated with an anti-mouse (cat no. sc-2005) or anti-rabbit (cat no. sc-2004; both Santa Cruz Biotechnology, Inc., Dallas, TX, USA) horseradish peroxidase-conjugated secondary antibody (diluted 1:5,000 in 0.1% Tween-20/PBS) at room temperature for 4 h. The membranes were subsequently visualized using an enhanced chemiluminescence detection system (GE Healthcare, Chicago, IL, USA). Protein bands were quantified using densitometric analysis with ImageJ software (39). The primary antibodies used were as follows: HDAC6 (1:1,000; cat no. ab82667; rabbit polyclonal; Abcam), microtubule associated protein 1 light chain 3 (LC3; 1:500; cat no. sc-398822; mouse polyclonal; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). GAPDH (1:1,000; cat no. sc-25778; rabbit polyclonal; Santa Cruz Biotechnology, Inc.) was used as a protein-loading control.

Immunofluorescence microscopy. Individual sterile coverslips were placed in the wells of a 4-well plate. PC-1.0 and AsPC-1 cells were added and incubated at 37˚C for 24 h. Following treatment with Apicidin at 37˚C for 2 h, the cells were washed using PBS, and then fixed in 3.7% paraformaldehyde for 20 min at room temperature. Subsequently, paraformaldehyde was removed and the cells were washed using PBS. Blocking solution (PBS + 5% BSA + 10% goat serum) was then added at room temperature for 30 min to prevent non-specific binding, and then the cells were incubated with the primary antibodies against HDAC6 (1:200; cat no. ab82667; rabbit polyclonal; Abcam) and LC3 (1:100; cat no. sc-398822; mouse polyclonal; Santa Cruz Biotechnology, Inc.), overnight at 4˚C, on a shaker. The washed slides were incubated for 1 h at room temperature with 1:100 dilutions of Alexa-488 anti-rabbit immunoglobulin IgG (H+L; cat no. ab150077) and Alexa-568 goat anti-mouse IgG (H+L; cat no. ab175473; both Abcam) secondary antibodies. The slides were washed again with PBS, mounted with Vectashield mounting medium (Dako; Agilent Technologies, Inc., Santa Clara, CA, USA), and were observed under a confocal laser scanning microscope (Leica Microsystems GmbH, Wetzlar, Germany).

Statistical analysis. All experiments were performed at least three times in triplicate for each group. The data are presented as the mean ± standard deviation. The differences between groups were calculated with Student's paired t-test using GraphPad Prism software (version 6.0; GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Expression levels of miR-221 following miR-221 mimic transfection. As indicated in in Fig. 1, the levels of miR-221 expression in PC-1.0 and AsPC-1 cells that were transfected with the miR-221 mimic were significantly increased compared with the NC (15.973±2.416 and 15.533±2.936-fold, respectively).

Cell apoptosis is induced by miR-221. Flow cytometric analysis revealed that the apoptotic rate of PC-1.0 and AsPC-1 cells in the miR-221 mimic group were significantly increased compared with the miR-221 NC group following transfection (PC-1.0, P<0.05; AsPC-1, P<0.01; Fig. 2).

Cell autophagy is induced by miR-221. Induction of autophagy was confirmed by western blot analysis of modifications to the endogenous LC3. Data revealed that the protein levels of LC3-II and LC3-I were significantly increased following transfection with the miR-221 mimic group compared with

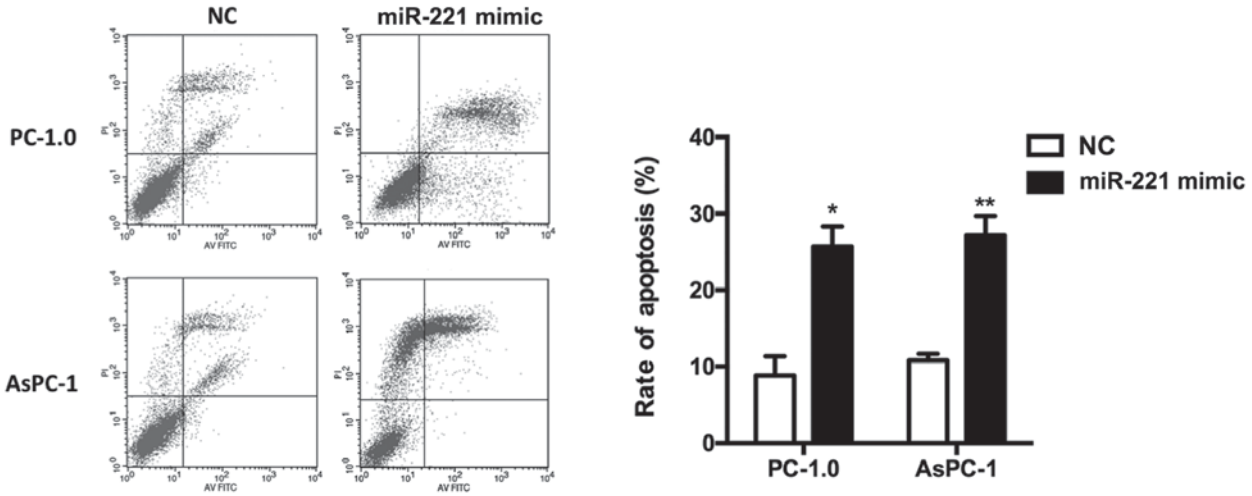

Figure 2. Apoptosis of pancreatic cancers is induced by miR-221. The cells were transfected with the miR-221 mimic or NC mimic. The results indicate that the apoptotic rate of PC-1.0 and AsPC-1 cells following transfection with the miR-221 mimic were significantly. Data are expressed as the mean ± standard deviation (n=3 in each group). *P<0.05 and **P<0.01 vs. the NC group. miR, microRNA; NC, negative control.

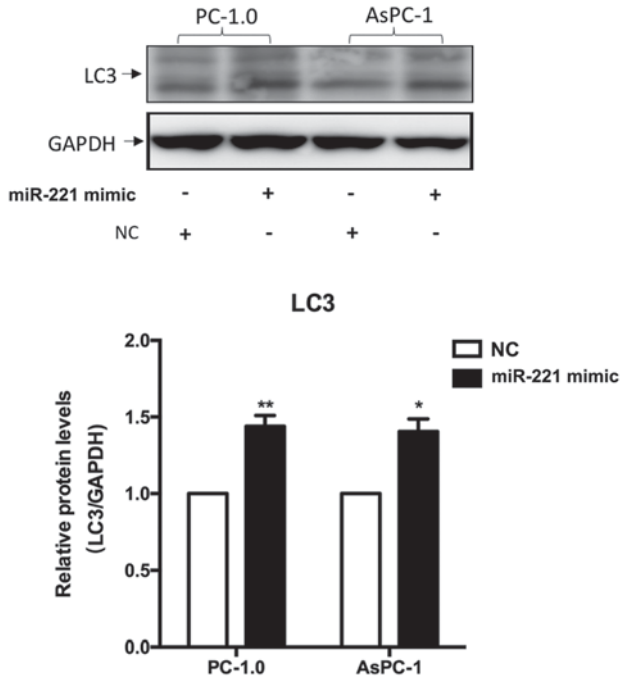

Figure 3. Autophagy of pancreatic cancers is induced by miR-221. The cells were transfected with miR-221 mimic or NC mimic. The levels of LC3 protein were detected using western blot analysis. GAPDH was used as a control. Data are expressed as the mean ± standard deviation (n=3 in each group). *P<0.05 and **P<0.01 vs. the NC group. miR, microRNA; NC, negative control; LC3, microtubule associated protein 1 light chain 3.

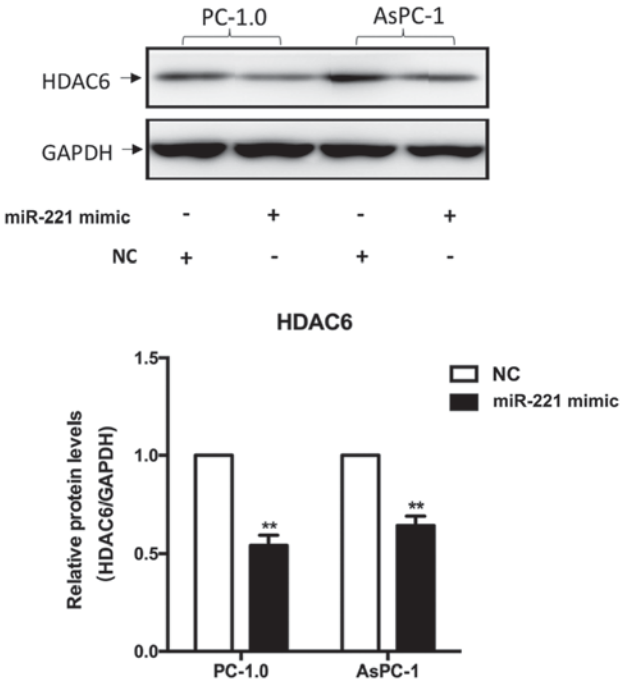

Figure 4. Expression of HDAC6 protein is suppressed by miR-221. The cells were transfected with miR-221 mimic or NC mimic. HDAC6 protein levels were detected using western blot analysis. GAPDH was used as a control. Data are expressed as the mean ± standard deviation (n=3 in each group). **P<0.01 vs. the NC group. miR, microRNA; NC, negative control; HDAC6, histone deacetylase-6.

NC group (PC-1.0, P<0.01; AsPC-1, P<0.05; Fig. 3). These results suggest that miR-221 was able to induce autophagy in pancreatic cancer cells.

HDAC6 expression is inhibited by miR-221. The levels of HDAC6 expression were detected using western blot analysis following transfection. Data revealed that the expression levels of HDAC6 were significantly decreased following transfection compared with the miR-221 NC group (P<0.01; Fig. 4).

Autophagy is induced by the downregulation of HDAC6. To confirm the association between HDAC6 and autophagic activity in pancreatic cancer cells, immunofluorescence was performed on PC-1.0 and AsPC-1 cells using LC3 and HDAC6 antibodies. The immunofluorescence data revealed that LC3 was initially located in the nucleus and cytoplasm (Fig. 5). However, after a 2 h treatment with apicidin, the expression of LC3 was markedly increased in the cytoplasm. Additionally, the results revealed that a decrease in HDAC6 expression was associated with autophagic activity and suggests that HDAC6

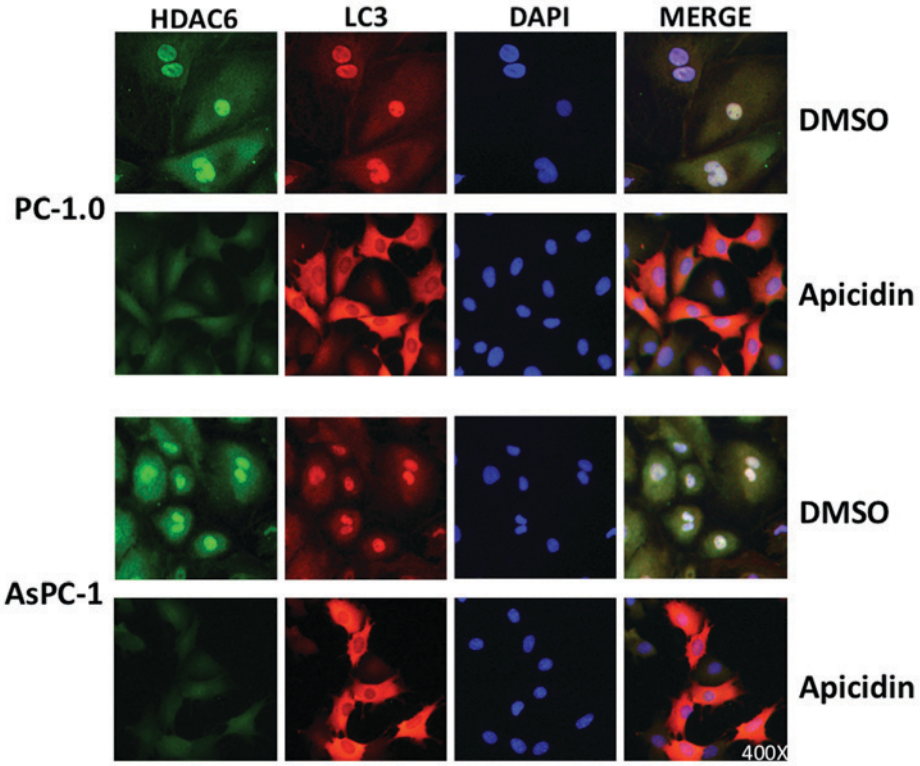

Figure 5. Autophagy is induced by downregulation of histone deacetylase-6. The cells were treated with apicidin or DMSO alone. The cells that were treated with DMSO alone were used as a negative control. LC3 was initially located in the nucleus and the cytoplasm. However, following treatment with apicidin, the expression of LC3 was markedly increased in the cytoplasm. Magnification, x400. HDAC6, histone deacetylase-6; LC3, microtubule associated protein 1 light chain 3.

may serve a key function in modulating autophagy activity in pancreatic cancer cells.

Discussion

Pancreatic cancer is known to be a major cause of cancer mortality globally, with the overall survival time of the majority of patients being <1 year following diagnosis (40,41), and the 5-year survival rate being <5% of all patients (4,5). At time of diagnosis, extensive local invasion and metastasis are observed in patients, thereby precluding curative surgical resection (42). Therefore, understanding the molecular mechanisms underlying high propensity of pancreatic cancer for invasion and metastasis and developing novel therapeutic targets for pancreatic cancer is important.

miRNAs have been revealed to serve important functions in the generation or development of various types of cancer (16-18). The expression and functions of cancer-related miRNAs in pancreatic cancer have been extensively investigated (43,44). A recent study by the present authors indicated that miR-221 was found to be differentially expressed between highly and weakly invasive PC cells and may serve an important role in the regulation of pancreatic cancer (24). This previous data suggests that miR-221 may function as a tumor suppressor in pancreatic cancer. miR-221 is highly expressed in various cancer-derived cells, including pancreatic carcinoma, prostate carcinoma and thyroid carcinoma cells (45-47).

However, the function of miR-221 in pancreatic cancer remains to be determined. In the present study, the carcinogenic function of miR-221 in pancreatic cancer was examined. Flow cytometric analysis revealed that transfection with miR-221 mimic significantly increased the apoptotic rate of PC-1.0 and AsPC-1 cells. Furthermore, western blot analysis also revealed that miR-221 may promote autophagy in pancreatic cancer cells.

HDAC6, a distinct cytoplasmic deacetylase, targets tubulin, heat shock protein 90 and cortactin and therefore may regulate cell adhesion, motility and chaperone function (48). HDAC6 has been demonstrated to be involved in carcinogenic transformation and may modulate the epithelial mesenchymal transition in several types of cancer by regulating a number of critical cellular components. Accumulating evidence indicates that the expression of HDAC6 is associated with oncogenic transformation, anchorage-independent proliferation and tumor aggressiveness (49,50). In the present study, a western blot analysis was performed to reveal that the expression of HDAC6 was suppressed by miR-221 in PC-1.0 and AsPC-1 cells. The immunofluorescence data on PC-1.0 and AsPC-1 cells revealed that LC3 was markedly increased in the cytoplasm following treatment with apicidin, compared with its location in the nucleus and cytoplasm in untreated cells. The results additionally revealed that a decrease in HDAC6 expression may increase autophagic activity and suggested that HDAC6 may serve a key function in modulating autophagic activity in pancreatic cancer cells. The results of the present study indicated that HDAC6 serves as a target of miR-221 and is function-related in pancreatic cancer cells. Taken together with previous studies, it appears that miR-221 may promote autophagy by downregulating HDAC6 expression in pancreatic cancer cells.

In conclusion, miR-221 may significantly induce autophagy by negatively regulating HDAC6 expression and inducing the apoptosis of pancreatic cancer cells. Increasing the levels of miR-221 expression may be a potentially beneficial treatment strategy for patients with pancreatic cancer.

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Availability of data and materials

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

Authors' contributions

YY, YS and XT conceived and designed the study. YY, YS, HW, XM, YW, PL, XL and JZ performed the study. HL, YY, MZ and LZ analyzed the data. YY wrote the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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