Research Article

MicroRNA-21 antisense oligonucleotide improves the sensitivity of A375 human melanoma cell to Cisplatin: an in vitro study¹

Running title: MicroRNA-21 AS-ODN and Cisplatin resistance of A375

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¹ This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi:[10.1002/jcb.26455](https://doi.org/10.1002/jcb.26455)

ABSTRACT

This study explored Cisplatin resistance effect of microRNA-21 (miR-21) antisense oligonucleotide (AS-ODN) in human melanoma A375 cell. AS-ODN was transfected in melanoma A375 cells and Cisplatin-resistant cell line A375/CDDP, and divided into the AS-ODN, nonsense oligonucleotide (NS-ODN) and normal groups. Cell ultrastructure changes were observed through transmission electron microscope. MiR-21 AS-ODN could be tested cell growth effect in different time periods by trypan blue exclusion. MiR-21 mRNA expression change was detected by quantitative fluorescence PCR. Cell apoptosis, cycle distribution and miR-21 AS-ODN effect on proliferation and Cisplatin sensitivity were tested by flow cytometry, MTT assay, TUNEL, and Clonogenic assay. Cell apoptosis was observed after transfection 24 h with the AS-ODN group, while the NS-ODN and normal group cells had no apoptotic symptoms; Compared with the normal group, the AS-ODN group began to show obvious cell growth inhibition effect after transfection 24 h lasting 72 h (all $P < 0.05$), but the NS-ODN group had no significant difference ($P > 0.05$). miR-21 mRNA expression in the AS-ODN group was obviously decreased with rising apoptosis rate (all $P < 0.05$) and there was no significant difference in the NS-ODN group ($P > 0.05$). MiR-21 AS-ODN could remarkably increase A375 cell and A375/CDDP cell sensitivity to Cisplatin ($P < 0.05$), while A375 cell sensitivity to Cisplatin between the NS-ODN group and the normal group had no difference. MiR-21 AS-ODN decreased IC₅₀ and increased Cisplatin sensitivity for A375 cells and A375/CDDP cells, which would be a new target of melanoma treatment.

KEYWORDS: MicroRNA-21; Antisense oligonucleotide; Melanoma; Cisplatin; Drug resistance; IC₅₀

INTRODUCTION

Melanoma, a malignancy deriving from pigment-producing melanocytes, is the most aggressive form of skin cancer and accounts for the leading cause of skin cancer deaths in the world [Jiao et al., 2015]. Globally, the incidence of malignant melanoma is increasing at a faster pace than any other cancer [Xu

et al., 2012]. Though surgical treatment of early melanoma can yield some 90% cure rates in patients with melanoma, advanced melanoma is notoriously known for its high mortality with intrinsic resistance to chemotherapy treatment, aggressive clinical behavior and tendency to metastasize faster than expected [Villanueva et al., 2010]. Metastatic melanoma has a poor prognosis with stage IV melanoma ranging from 8 to 18 months after diagnosis and median overall survival in the range of 6~10 months with chemotherapy [Chapman et al., 2011, Wolchok et al., 2013]. It has been found out previously that antibodies blocking the inhibitory receptor can release negative immune regulatory pathways, and lead subsequently to durable responses in some patients with metastatic melanoma and improved overall survival of patients [Hamid et al., 2013]. Besides, serum miRNAs are shown to be closely related to the development of melanoma [Fleming et al., 2015]. Therefore, it is of vital importance to focus on genetic and regulatory mechanisms at play in the development and progression of melanoma so as to bring about better prevention, diagnosis, and clinical management [Howell et al., 2010].

MicroRNAs (miRs) are a relatively novel class of endogenous, non-protein coding small RNA molecules that can regulate hundreds of genes through binding to the 3'UTR of target mRNAs [Long et al., 2012]. Mature miRNA is able to regulate gene expression at the post-transcriptional level, binding through partial complementarity for the most part to the 3'UTR of target mRNAs, and resulting concurrently in mRNA degradation and translation inhibition [Iorio and Croce, 2009]. Previous studies have revealed that miRNAs are potential clinically relevant biomarkers of prognosis in metastatic melanoma [Segura et al., 2010, Stark et al., 2015]. Studies have shown that miR-21 takes part in a variety of cancer such as breast cancer and pancreatic ductal adenocarcinoma by regulation of genes [Kadera et al., 2013, Petrovic et al., 2014]. Meanwhile, it has drawn attention how miR-21 plays a role in melanoma, the result of which indicating that up-regulation of miR-21 in melanocytes resulted in increased proliferation and decreased apoptosis [Satzger et al., 2012]. miR-21 has also been shown to enhance melanoma invasiveness [Martin del Campo et al., 2015], and it might be potential biomarker

for malignant melanoma [Jiao et al., 2015]. Moreover, one study has demonstrated that miR-21 inhibition is associated with blockade of melanoma metastasis [Li et al., 2016]. Cao et al. found in their study that Survivin antisense oligodeoxynucleotide (AS-ODN) can inhibit the proliferation of human malignant melanoma cells (HMMC) A375 in a concentration-time dependent manner and it induces G2/M stage block and promotes its apoptosis [Cao et al., 2010]. Li et al illustrated that miR-21 inhibition mediated by antisense could significant hamper human cutaneous melanoma growth, enhance apoptosis, and increase its chemo-or radio-sensitivity [Jiang et al., 2012]. However, no study has yet been reported to find out what an effect miR-21 AS-ODN has on melanoma. This study is hereby initiated to probe its effect on melanoma A375 with miR-21 AS-ODN used to stain HMMC A375 in vitro, in hopes of laying a theoretical foundation for the treatment of malignant melanoma with miR-21 AS-ODN.

MATERIALS AND METHODS

Cells culture and grouping

Human malignant melanoma cell line A375 and Cisplatin-resistant cell line A375/CDDP was both purchased from American Type Culture Collection (ATCC). DMEM/F12 basal medium (GE Healthcare HyClone Cell Culture) was used for cells culture, into which 10% fetal calf serum (Gibco, Inc.), 0.1U/L penicillin and 0.1 µg/L streptomycin (GE Healthcare HyClone Cell Culture) were added. The fetal calf serum was first processed by water bath at 56°C for 30 min for the purpose of inactivation, and then the serum was preserved at -80°C for later experiment. The cell line was incubated in a 5%CO₂ incubator (Thermo Scientific) at 37°C. In the study, the following 3 groups were divided: miR-21 antisense oligonucleotide (AS-ODN) group, nonsense oligonucleotide (NS-ODN) group and normal group, among which the NS-ODN group was negative-control oligonucleotide with no human genome sequence homology. The sequences of oligonucleotide in the AS-ODN group and the NS-ODN group

are shown in Table 1, and the above sequences were synthesized by Shanghai Genepharma gene Co., Ltd. after BLAST alignments in NCBI databases.

Cells transfection

A375 or A375/CDDP cells were cultured at a density of $4 \times 10^4/\text{mL}$ and seeded in a 24-well plate. Once cells growing in adherence to 80%, they were divided into different groups and transfected. In the normal group, serum-free and double antibody-free mediums were only added; whereas in the AS-ODN group and the NS-ODN group, corresponding oligonucleotide encapsulated by liposome lipofectamin 2000 (11668019, Thermo Fisher Scientific, San Jose, CA, USA) (final concentration was 20 $\mu\text{mol/L}$) together with serum-free and double antibody-free mediums were added respectively. The transfected cells were then cultured for 4h without serum, after which 10% fetal calf serum was added to culture the cells continuously in 5% CO_2 incubator at 37°C . In order to measure the efficiency of oligonucleotide transfection in this manner, oligonucleotide labeled by Cy3 (sequence: 5'-CACTTGATGGTGCCTACAC-3', synthesized by Shanghai Genepharma gene Co., Ltd) was also transfected in the same manner as above to make a contract and then observed under fluorescence microscope after 24-h culture to measure the efficiency of cells transfection. Pictures were taken 24 h, 48 h, and 72 h after transfection.

Transmission electron microscope

A375 or A375/CDDP cells were successively gathered 24 h, 48 h and 72 h after transfection and digested with normal trypsin (Gibco, Inc.). Cells were centrifuged for cells collection, washed by phosphate-buffered saline (PBS), fixed with 2.5% glutaraldehyde (Shanghai Junrui Biotechnology. Co., Ltd.) for 2h at 4°C and then with 1% osmium acid (from Shanghai Junrui Biotechnology. Co., Ltd.) for 2 h. After that, the transfected cells were dehydrated with a gradient ethanol, permeated with the mixture of propylene and Epon 812 resin (Hede Entrepreneurship (Beijing) biotechnology Co., Ltd.),

embedded, polymerized and sectioned into ultrathin sections (no more than 100nm thick). After being stained with 3% uranyl acetate-lead citrate (Shanghai Yiweixin Info Technology Co., Ltd.), the transfected cells were placed under a transmission electron microscope (JEM100CX-II, Japan) to observe their ultrastructure.

Trypan blue exclusion

During the transfection culture, the A375 or A375/CDDP cells were, at the 24th h, 48th h and 72th h respectively, digested with 0.25% trypsin and collected, after which, 1 mL cell suspension in each group was fully mixed with 0.4% trypan blue (Cusabio Biotech Co., Ltd.). The numbers of the total cells (named xxx[the group title]-sum), viable cells (named as xxx-alive), and dead cell (named xxx-dead), and calculate the cell viability (named viable cell) in different groups were counted three times by Beckman-Coulter cell counter (Beckman Vi-Cell XR) to obtain the averages. Inhibitory rate of cell growth = (normal-sum – treated-alive or normal-sum – normal-alive) / normal-sum × 100%; viable cells (%) = (total number of viable cells per ml of aliquot / total number of cells per ml of aliquot) × 100.

Quantitative fluorescent real-time polymerase chain reaction (qRT-PCR)

After being transfected for 24 h, 48 h, and 72 h, A375 or A375CDDP cells were digested with trypsin and then collected. The total RNA was extracted with Trizol reagent (Thermo Fisher Scientific Inc.), and the concentration and purity of RNA were measured with Nano Drop2000 (Thermo Fisher Scientific Inc.). PrimeScript®RT reagent Kit was used to conduct reverse transcription, and cRNAs of miR-21 and U6 were synthesized via reverse primer with specific stem-loop structure. The sequence of the reverse transcription primers was as follows: *miR-21*: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCACTGGATACGACTCAACA-3'; *U6*: 5'-GTCGTACCAGTGCAGGGTCCGAGGTATTTCGCACTGGATACGACAAAATA-3'. According to

gene sequence released by GenBank, the PCR primers were designed respectively with the Primer5.0, (Table 2) and the primers were synthesized by Shanghai Genepharma gene Co., Ltd. after being ensured correct through comparison. ABI PRISM 7500 real-time PCR System (ABI) and SYBR Green I fluorescence PCR diagnostic kit (Takara Biotechnology (Dalian) Co., Ltd.) were used in PCR reaction, additionally, U6 served as internal reference and dissolution curves were used to evaluate the reliability of PCR result, thus the CT value (transition of PCR amplification curve) was accordingly obtained. $\Delta Ct = CT(\text{targeted gene}) - CT(\text{internal reference})$, $\Delta\Delta Ct = \Delta Ct(\text{treated group}) - \Delta Ct(\text{normal group})$, and the relative expression of targeted gene was figured out by $2^{-\Delta\Delta Ct}$ [Tuo et al., 2015].

Western blotting

A375 or A375CDDP cells were lysed at 4°C for 30 min with protein lysis buffer (shaken every 10 min), and then centrifuged at 12000 r/min at 4°C for 20 min. After the lipid layer was discarded, the supernatant was collected for detection of protein concentration with the bicinchoninic acid (BCA) protein assay reagent kit (20201ES76, Shanghai Yeasen Biotechnology Co., Ltd., Shanghai, China). The loading buffer with 30 µg for each protein lane was adjusted with deionized water. Sodium dodecyl sulfate (SDS) separation gel (10%) and concentration gel (5%) were prepared. Samples in each group were mixed with corresponding 5XSDS sample loading buffer, boiled at 100°C for 5 min, ice-bathed, centrifuged, and equally added into each lane by micropipette for electrophoretic separation. Subsequently, the protein was transferred to the nitrocellulose membranes, which were fixed with 5% skim milk powder at 4°C overnight. Membrane were added with the following diluted rabbit anti-rat polyclonal antibody (1 : 500): anti-SMAD7 (Abcam, ab216428); anti-GAPDH (Abcam, ab8245) antibodies, incubated overnight, washed with PBS three times at room-temperature (5 min each). Afterward, membranes were incubated at 37°C for 1 h with diluted horseradish peroxidase (HRP)-labeled goat anti-rabbit immunoglobulin G (IgG) (1 :10000, A21020, Abbkine, USA) and washed with PBS three times (5 min each). Membranes reacted with enhanced chemiluminescence

(ECL) solution (ECL808-25, Biomiga, USA) for 1 min. The liquid was discarded, and the membrane covered and observed after X-ray exposure (36209ES01, Shanghai qcbio Science&Technologies co., Ltd., Shanghai, China). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was taken as the internal reference, and the relative expression of protein was expressed as the ratio between the gray-scale values of the target band and that of the internal reference band. The experiment was conducted three times on each group.

Flow cytometry

The A375 or A375CDDP cells that were cultured for 24 h, 48 h and 72 h respectively after transfection were digested with trypsin, collected by centrifugation, washed by cold PBS and resuspended to be 1×10^6 /mL cell suspension with buffer (PBS with Calcium). The cell suspension (100 μ L) was put into tube at room temperature, and then 5 μ L Annexin V/FITC labeled by fluorescence and 10 μ L propidium iodide (PI) with the concentrations of 20 μ g/mL were added and fully mixed with the cell suspension. After culture at room temperature without light for 15 min, 400 μ L staining buffer was added, which was immediately followed by detection and analysis by flow cytometry. During the process, 10^4 cells were taken each time and statistically analyzed by Cell Quest software. Annexin V-positive cells were identified as apoptosis, whereas PI-positive and Annexin V-negative cells, as necrosis. Apoptosis rate = (Annexin V⁺PI⁺ cells number + Annexin V⁺PI⁻ cells number) / $10^4 \times 100\%$. As to detection of cell cycle, 100 μ L cell suspension was put into a tube, and 1 mL PI/TritonX-100 staining buffer (with 0.2 mg RNase A, 20 μ g PI, 0.1% TritonX-100) was then added and fully mixed with the cell suspension. After culture at 4°C for 30min, the cell suspension was put onto flow cytometry (Attune NxT, Thermo Fisher, Carlsbad, CA, USA) to analyze and detect the cell cycle.

Methylthiotetrazole (MTT) assay

A375 or A375/CDDP cells were transferred into 96-well plate at a density of 5×10^3 /mL, and once

cells growing in adherence to 80%, they were divided into different groups and transfected. In the normal, serum-free and double antibody-free mediums were only added; whereas in the AS-ODN group and the NS-ODN group, serum-free and antibody-free mediums of corresponding AS-ODN and NS-ODN encapsulated by liposome were added. The transfected cells were then cultured without serum for 4h, after which the medium without serum was replaced by 10% fetal calf serum one. Cisplatin (Jinan Qilu Pharmaceutical Co., Ltd.) of the final concentrations being 1 µg/mL, 2 µg/mL, 4 µg/mL, 8 µg/mL, 16 µg/mL, 32 µg/mL and 64 µg/mL was added in order into A375/CDDP cells; Cisplatin (Jinan Qilu Pharmaceutical Co., Ltd., Qilu, Shandong, China) of the final concentrations being 0.2 µg/mL, 0.4 µg/mL, 0.8 µg/mL, 1.6 µg/mL, 3.2 µg/mL, 6.4 µg/mL and 12.8 µg/mL (Jinan Qilu Pharmaceutical Co., Ltd., Qilu, Shandong, China) was added in order into A375 cells. The cell line was continuously cultured in 5%CO₂ incubator at 37°C. During the culture, at the 24th h, 48th h and 72th h , 120 h respectively, 20 µL freshly prepared 5 mg/mL MTT solution (Sigma-Aldrich Co. Ltd.) was added into each well, followed by culture at 37°C for 4 h. The medium was then removed and the 150 µL dimethyl sulfoxide (DMSO) (Sigma-Aldrich Co. Ltd.) was added. In each group, 4 repeated wells were set, and 490 nm optical density (OD) was detected by microplate reader (GloMax, Promega, Beijing, China) three times. The IC₅₀ (the concentration of AM necessary to produce 50% inhibition of cell growth) was calculated from the following nonlinear equation of the survival fraction curve: $Y = aX^3 + bX^2 + cX + d$ (Y is the surviving fraction when there is a 50% inhibition of cell growth; X is dose of cisplatin induces 50% inhibition; a, b, c and d are constant values) [El Habbash et al., 2017].

TdT-mediated dUTP-biotin nick end-labeling (TUNEL) assay

A375 or A375/CDDP cells were seeded into a 24-well plate at a density of 4×10^4 cells/mL. As cells growing in adherence to 80%, cells were transfected and assigned in into the normal, AS-ODN, NS-ODN, Cisplatin + AS-ODN, and Cisplatin groups. The normal group was added with serum-free and double antibody-free mediums; the AS-ODN and NS-ODN groups was respectively added with

serum-free and double antibody-free mediums supplemented liposome-encapsulated AS-ODN or NS-ODN; the Cisplatin + AS-ODN group was added with Cisplatin (0.45 µg/mL [72h IC₅₀, Jinan Qilu Pharmaceutical Co., Ltd., Qilu, Shandong, China] for A375 cells; 2.19 µg/mL [72h IC₅₀, Jinan Qilu Pharmaceutical Co., Ltd., Qilu, Shandong, China] for A375/CDDP cell) and serum-free and double antibody-free mediums supplemented with liposome-encapsulated AS-ODN; the Cisplatin group was added with equal dose of Cisplatin as the Cisplatin + AS-ODN group. Cells were cultured for 4 h in serum-free blocking medium, and later cultured in 10% FBS medium supplemented with Cisplatin in a 5% CO₂ incubator at 37°C for 72 h. Afterward, cells were fixed with 4% paraformaldehyde for 10 min, cleaned with 0.1% Triton X-100 for 5 min, and cultured for 1 h with TUNEL reactant (In Situ Cell Death Assay Reagent Kit, MultiSciences, Hangzhou, Jiangsu). Subsequently, cells were labelled with 4',6-diamidino-2-phenylindole (DAPI) for 5 min, observed under fluorescence microscopy (OLYMPUS). TUNEL positive cells were considered as apoptotic cells. Each sample had three replicates and the experiment was conducted three times.

Clonogenic assay

A375 or A375/CDDP cells were seeded into a 24-well plate at a density of 2×10^4 cells/mL. As cells growing in adherence to 80%, cells were transfected and assigned in into the normal, AS-ODN, NS-ODN, Cisplatin + AS-ODN, and Cisplatin groups. The normal group was added with serum-free and double antibody-free mediums; the AS-ODN and NS-ODN groups was respectively added with serum-free and double antibody-free mediums supplemented liposome-encapsulated AS-ODN or NS-ODN; the Cisplatin + AS-ODN group was added with Cisplatin (0.45 µg/mL [72 h IC₅₀, Jinan Qilu Pharmaceutical Co., Ltd., Qilu, Shandong, China] for A375 cells; 2.19 µg/mL [72 h IC₅₀, Jinan Qilu Pharmaceutical Co., Ltd., Qilu, Shandong, China] for A375/CDDP cell) and serum-free and double antibody-free mediums supplemented with liposome-encapsulated AS-ODN; the Cisplatin group was added with equal dose of Cisplatin as the Cisplatin + AS-ODN group. Cells were cultured for 4 h in

serum-free blocking medium, and later cultured in 10% FBS medium supplemented with Cisplatin in a 5% CO₂ incubator at 37°C for 72 h. Cells were then digested, spread on a 10 cm culture dish at a density of about 10 cells/cm². Two weeks later, clones were stained with crystal violet and then counted.

Statistical methods

SPSS 21.0 software was employed for data statistical analysis, in which measurement data was shown by mean value $\pm$ standard deviation, comparison between two groups of measurement data accorded with normal distribution was tested by *t*-test, while comparison among groups was tested by One-Way Analysis of Variance (ANOVA); Categorical data was expressed by percentages and tested by chi-square test; $P < 0.05$ meant differences were statistically significant.

RESULTS

miR-21 AS-ODN decreased the relative expression of miR-21

After transfection of 48 h, the results of qRT-PCR (Fig. 1) of the A375 and A375/CDDP cells in each group showed that compared with the normal group, relative expression of miR-21 in the AS-ODN group was significantly lower ($P < 0.05$), while miR-21 expression in the NS-ODN group reduced but no statistical significance ($P > 0.05$) 24h, 48h and 72 after transfection; forty-eight h after transfection, the expression of SMAD7, a basic target gene of miR-21, was higher in the AS-ODN and NS-ODN groups than in the normal group. The above results indicated that miR-21 AS-ODN downregulated the expression of miR-21.

The AS-ODN group showed evident morphological changes after and before transfection

Inverted microscope was used to observe the entire phenomenon of A375 and A375/CDDP cells. In the normal group, the difference was not significant before and after cell transfection, which had many flat

polygons, round nuclei in the middle of the cell with one or more of kernels, and the cells were arranged closely and connected with each other with epithelioid cell morphology; Cells of the AS-ODN group turned round in transfection miR-21 AS-ODN, and when cytoplasmic processes were small, vacuoles appeared in cells. Cell membrane boundary was fuzzy, visible cell fragmentation differed itself from the cell morphology before transfection, and the cell state changed drastically over time; Cell morphology and growth state of the NS-ODN group were similar to the normal group (Fig. 2).

Cells in the AS-ODN group showed apoptosis conditions

The ultrastructure of A375 and A375/CDDP cells was observed by transmission electron microscope 24 h, 48 h, and 72 h after transfection. In the AS-ODN group, a lot of cells had early and middle stage apoptosis symptom, reduced cell bodies, and concentrated nuclear chromatin and tokaryolemma, which was shrunk into chunks with Kernel discretization and partial mitochondria edema. Moreover, the degranulation phenomenon was obvious and a large number of vacuoles were appeared in cytoplasm in which cells showed apoptosis conditions and the cell morphology changed drastically over time; On the contrary, cells were plump in the normal and NS-ODN groups, which presented round or oval shape with irregular and big nucleus and obvious nucleolus, rich chromatin in the nucleus and heterochromatin margination. There were no obvious morphological characteristics of apoptosis in the normal and NS-ODN groups (Fig. 3).

Cells in the AS-ODN group showed obvious cell growth inhibition

Trypan blue dye exclusion assayed inhibition of AS-ODN to A375 and A375/CDDP cell growth. It is illustrated that cell growth of the normal and NS-ODN groups was basically unaffected; 24 h after transfection, cells in the AS-ODN group showed obvious cell growth inhibition (lasted 72 h) compared with the normal and NS-ODN groups and marked decreased of viable cell (%) compared with the

NS-ODN group (Fig. 4). Cell counting with trypan blue is shown in Table 3, and inhibition rate of cell growth in each group is shown in Figure 4.

MiR-21 AS-ODN improved cell apoptosis

After transfection for 24 h, 48 h, and 72 h, apoptosis rate of A375 and A375/CDDP cells were detected by flow cytometry. The cell apoptosis rate in the NS-ODN group had no statistically difference in comparison with that in the normal group; On the contrary, apoptosis rate in the AS-ODN group was obviously high than that in the normal and NS-ODN groups, and increased over time (all $P < 0.05$) (Fig. 5). It is suggested from the above data that downregulation of miR-21 induced cell apoptosis in both A375 and A375/CDDP cells.

miR-21 AS-ODN could significantly increase the sensitivity of A375/CDDP cells to Cisplatin

After treated by different concentrations of Cisplatin, A375/CDDP cell growth appeared different degrees of inhibition. In comparison with the normal group, inhibitory effect of Cisplatin on cells growth in the NS-ODN group was not obvious, which was obvious in the AS-ODN group and had statistical difference (all $P < 0.05$) (Fig. 6). After transfecting 24 h, 48 h, 72 h, changes of the cell Cisplatin IC₅₀ value were calculated and analyzed. Compared with the normal group, cell Cisplatin IC₅₀ in the NS-ODN group had no obvious change, while Cisplatin IC₅₀ in each time of Cisplatin IC₅₀ group point decreased significantly ($P < 0.05$) (Table 4). These results indicated that miR-21 AS-ODN could significantly increase the sensitivity of A375/CDDP cells to Cisplatin.

miR-21 AS-ODN decreased the resistance of A375 and A375/CDDP cells to Cisplatin

Seventy-two h after A375 and A375/CDDP cells were transfected and/or treated with Cisplatin, the result of TUNEL assay (Fig. 7) demonstrated that compared with the normal and NS-ODN groups, the AS-ODN and Cisplatin groups had increased apoptotic rate ($P < 0.05$); the Cisplatin + AS-ODN group

showed reduced viable cells and elevated apoptotic rate ($P < 0.05$). It is, therefore, suggested that AS-ODN decreased the resistance of A375 and A375/CDDP cells to Cisplatin.

miR-21 AS-ODN increased the sensitivity of A375 and A375/CDDP cells to Cisplatin

Seventy-two h after A375 and A375/CDDP cells were transfected and/or treated with Cisplatin, cells were spread on a 10 cm culture dish at a low density. The counting result two weeks later (Fig. 8) showed that compared with the normal and NS-ODN groups, the AS-ODN and Cisplatin groups exhibited half the number of monoclonal colonies; the Cisplatin + AS-ODN group had declined number and smaller size of monoclonal colonies. Thus, AS-ODN was shown to increase the sensitivity of A375 and A375/CDDP cells to Cisplatin.

DISCUSSION

The morbidity of melanoma has increased at a rapid rate [Gabriel and Skitzki, 2015], and it has the highest mortality among all types of skin cancer [Yang and Qian, 2014]. A study demonstrated that over 50% of miRNA genes are located in fragile sites or in cancer-associated genomic regions, suggesting that miRNAs may play an essential role in the pathogenesis of a range of human cancers [Zhang et al., 2007]. Besides, chemotherapy drug resistance is a major problem facing current cancer treatment [Holohan et al., 2013]. Therefore, in the present study, miR-21 was processed by antisense oligonucleotide in order to discuss its effects on Cisplatin-resistant melanoma cell line A375.

Firstly, we found in our study that in melanoma after treatment with miR-21 AS-ODN, miR-21 was downregulated, and cell morphology changed evidently, presenting apoptosis condition. It has been reported miR-21 is significantly higher in melanoma samples, compared with normal skin specimens [Jiao et al., 2015], and its inhibition is associated with the suppression of melanoma metastasis [Li et al., 2016]. MiR-21 expression has been shown to be associated with human umbilical

vein endothelial cell morphological change [Liu et al., 2016]. Apoptosis induces deep changes in the cell ultrastructural organization, including difference in nuclear/cytoplasmic ratio and cell diameter, and cell debris [Zamai et al., 1993]. Hence, AS-ODN-mediated miR-21 downregulation led to drastically changed morphology indicating apoptosis.

In the study, more apoptosis in early-middle stage, higher cells apoptosis rate and higher inhibitory rate of cell growth were found in cell line A375 transfected with miR-21 AS-ODN than in normal group and NS-ODN group. Previous studies show that increased levels of miR-21 expression would post-transcriptionally down-regulates programmed cell death 4 (*PDCD4*) [Asangani et al., 2008], which further regulates factors involved in cell cycle, tumor progression and differentiation [Lankat-Buttgereit and Goke, 2009]. In the present study, it is shown that the decreased miR-21 inhibited the proliferation of A375, which is confirmed by a previous study, in which suppression of miR-21 percentage of apoptotic breast cancer cells [Mei et al., 2010]. Additionally, Increased expression of miR-21 has been revealed to enhance the invasive potential of MM cell lines [Martin del Campo et al., 2015]; AS-ODN reverse regulated miR-21 has been shown to inhibit migration and induce apoptosis in leukemic K563 cells and human colon carcinoma cells [Hu et al., 2010, Tao et al., 2015], reduce apoptosis of glioblastoma cells [Song et al., 2015], which is in accordance with the result of the present study.

Cisplatin as one of the most common anticancer drugs, kills cancer cells by damaging their DNA [Kang et al., 2010], however, the development of Cisplatin-resistance represents various serious clinical problems [Marzano et al., 2007]. Previous studies have already confirmed the positive role of miR-21 in inducing Cisplatin resistance in gastric cancer and ovarian cancer [Yang et al., 2013, Pink et al., 2015]; suppression of miR-21 is associated with promoted Cisplatin-induced apoptosis in tongue squamous cell carcinoma, increased Cisplatin sensitivity in A549 cells, and improved sensitivity to Cisplatin in oral squamous cell carcinoma [Ren et al., 2014, Zhang et al., 2013, Zhou et al., 2014]. Additionally, miR-21 has also been demonstrated to contribute to drug resistance of glioblastoma cells

[Papagiannakopoulos et al., 2008, Li et al., 2009]; plays a crucial role in resistance to chemotherapeutic drugs in renal cancer and in cell line K562/daunorubicin (DNR) [Gaudelot et al., 2017, Bai et al., 2011]. Moreover, one study revealed that sensitivity of cancer cells the anticancer agents that aberrant miR-21 expression can reduce includes tamoxifen, docetaxel, gemcitabine, and 5-fluoro-Uracil [Pan et al., 2010]. From the above all, miR-21 represents a promising target for therapeutic manipulation to enhance chemotherapeutic agents in treating cancers.

However, the dose dependence of the effect is focusing further experiment to relate to such a dose dependence. A definite answer may be provided in the near future. In conclusion, it was confirmed in the study that miR-21 AS-ODN can increase the sensitivity of melanoma cell line A375 to Cisplatin, laying a theoretical foundation for miR-21 AS-ODN in treating cancers clinically.

ACKNOWLEDGMENTS

We would like to acknowledge the helpful comments on this paper received from our reviewers.

COMPETING INTERESTS

None.

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Legends

Fig. 1. Comparisons of miR-21 expression in the A375 and A375/CDDP cells in the normal, AS-ODN, and NS-ODN groups

Notes: A, expression of miR-21 in A375 cells in each groups 24 h, 48 h, and 72 h after transfection; B, expression of miR-21 in A375 cells in each groups 24 h, 48 h, and 72 h after transfection; C, protein expression of SMAD7 (a major target of miR-21) in the A375 and A375/CDDP cells in each group; *, $P < 0.05$ compared with the NS-ODN and the normal groups; AS-ODN, antisense oligonucleotide; NS-ODN, nonsense oligonucleotide; The experiment had three replicates and was repeated three times.

Fig. 2. Comparisons of morphological changes of the A375 and A375/CDDP cells before and after transfection among the normal, AS-ODN, and NS-ODN groups

Notes: A, Brightfield of the cell morphology 0 h after transfection; B, Brightfield of the cell morphology 24 h after transfection; C, Brightfield of the cell morphology 48 h after transfection; D, Brightfield of the cell morphology 72 h after transfection; AS-ODN, antisense oligonucleotide; NS-ODN, nonsense oligonucleotide; The experiment had three replicates and was repeated three times.

Fig. 3. Comparisons of ultrastructural changes of the A375 and A375/CDDP cells after transfection of 24h in the normal, AS-ODN, and NS-ODN groups

A, transmission electron microscopy of cell morphology 0 h after transfection; B, transmission electron microscopy of cell morphology 24 h after transfection; C, transmission electron microscopy of cell morphology 48 h after transfection; D, transmission electron microscopy of cell morphology 72 h after transfection; AS-ODN, antisense oligonucleotide; NS-ODN, nonsense oligonucleotide; The experiment had three replicates and was repeated three times.

Fig. 4. Comparisons of inhibition rate of cell growth in the normal, AS-ODN, and NS-ODN groups

A, inhibition rate of A375 cell growth 24 h, 48 h, and 72 h after transfection; B, inhibition rate of A375/CDDP cell growth 24 h, 48 h, and 72 h after transfection; AS-ODN, antisense oligonucleotide; NS-ODN, nonsense oligonucleotide; *, $P < 0.05$ compared with the NS-ODN and normal groups.

Fig. 5. FACS result by Annexin/PI in the normal, AS-ODN, and NS-ODN groups

Notes: A, FACS result of the A375 and A375/CDDP cells in each group 24 h, 48 h, and 72 h after transfection; B, diagram of the FACS data in A; AS-ODN, antisense oligonucleotide; NS-ODN, nonsense oligonucleotide; FACS, flow cytometry; PI, propidium iodide; *, $P < 0.05$ compared with the NS-ODN and normal groups.

Fig. 6. Comparisons of cell survival rate (%) in the normal, AS-ODN, and NS-ODN groups 24 h, 48 h, 72 h, and 120 h after been treated with different concentrations of Cisplatin

Notes: A, A375 cell survival rate in each group 24 h, 48 h, 72 h, and 120 h after been treated with different concentrations of Cisplatin; B, A375/CDDP cell survival rate in each group 24 h, 48 h, 72 h, and 120 h after been treated with different concentrations of Cisplatin; AS-ODN, antisense oligonucleotide; NS-ODN, nonsense oligonucleotide; *, $P < 0.05$ compared with the NS-ODN and normal groups; The experiment had three replicates and was repeated three times.

Fig 7. Cell apoptosis by TUNEL assay in the normal, NS-ODN, AS-ODN, Cisplatin, and Cisplatin + AS-ODN groups

Notes: A375 and A375/CDDP cell apoptosis 72 h after transfection and treatment with Cisplatin; B, Positive rate testes by TUNEL assay; TUNEL, TdT-mediated dUTP-biotin nick end-labeling; AS-ODN, antisense oligonucleotide; NS-ODN, nonsense oligonucleotide; *, $P < 0.05$ compared with the NS-ODN group; #, $P < 0.05$ compared with the AS-ODN group; &, $P < 0.05$ compared with the Cisplatin group; The experiment had three replicates and was repeated three times.

Fig 8. Clonogenic assay result in the in the normal, NS-ODN, AS-ODN, Cisplatin, and Cisplatin + AS-ODN groups

Notes: A, the staining result of the A375 and A375/CDDP cells by crystal violet in each group; B, number of colonies in A; AS-ODN, antisense oligonucleotide; NS-ODN, nonsense oligonucleotide; *, $P < 0.05$ compared with the NS-ODN group; #, $P < 0.05$ compared with the AS-ODN group; &, $P < 0.05$ compared with the Cisplatin group; The experiment had three replicates and was repeated three times.

Table 1. Sequence of oligonucleotide segment

Group	Sequence
AS-ODN (MicroRNA-21 anti-sense oligonucleotide)	5'-TCAACATCAGTCTGATAAGCTA-3'
NS-ODN (Negative-control oligonucleotide)	5'-CATTAATGTCTGGACAACCTCAAT-3'

Notes: ASODN, antisense oligonucleotide; NS-ODN, non-sense oligonucleotide.

Table 2. Primer sequence of quantitative fluorescent PCR

Gene	Forward	Reverse
miR-21	5'-GTGCAGGGTCCGAGGT-3'	5'-GCCGCTAGCTTATCAGACTGAGT-3'
U6	5'-CTCGCTTCGGCAGCACA-3'	5'-AACGCTTCACGAATTTGCGT-3'

Note: PCR, polymerase chain reaction.

Table 3. Cell counting in the normal, AS-ODN, and NS-ODN groups (4 × 100)

		0 h	24 h	48 h	72 h
Normal	A375 -alive	0.993 ± 0.011	2.810 ± 0.025	4.644 ± 0.535	10.409 ± 0.090
	A375 -dead	0.007 ± 0.003	0.257 ± 0.005	0.568 ± 0.122	0.584 ± 0.008
	A375 -sum	1.000 ± 0.009	3.067 ± 0.029	5.212 ± 0.658	10.993 ± 0.082
	viable cells(%)	0.993 ± 0.003	0.916 ± 0.001	0.892 ± 0.009	0.947 ± 0.001
	A375/CDDP -alive	0.997 ± 0.011	2.880 ± 0.021	5.558 ± 0.047	10.617 ± 0.081
	A375/CDDP -dead	0.003 ± 0.002	0.259 ± 0.008	0.407 ± 0.006	0.565 ± 0.007
	A375/CDDP -sum	1.000 ± 0.013	3.139 ± 0.026	5.965 ± 0.053	11.182 ± 0.087
	viable cells(%)	0.997 ± 0.002	0.917 ± 0.002	0.932 ± 0.000	0.949 ± 0.000
AS-ODN	A375 -alive	0.992 ± 0.009	1.890 ± 0.015	2.741 ± 0.023	4.083 ± 0.040
	A375 -dead	0.008 ± 0.001	0.536 ± 0.003	0.968 ± 0.010	1.768 ± 0.015
	A375 -sum	1.000 ± 0.010	2.426 ± 0.013	3.710 ± 0.026	5.851 ± 0.037
	viable cells(%)	0.992 ± 0.001	0.779 ± 0.002	0.739 ± 0.002	0.698 ± 0.003
	A375/CDDP -alive	0.992 ± 0.010	1.930 ± 0.016	2.837 ± 0.024	4.085 ± 0.038
	A375/CDDP -dead	0.008 ± 0.002	0.477 ± 0.003	0.941 ± 0.011	1.524 ± 0.013
	A375/CDDP -sum	1.000 ± 0.010	2.407 ± 0.016	3.778 ± 0.017	5.609 ± 0.040
	viable cells(%)	0.992 ± 0.002	0.802 ± 0.002	0.751 ± 0.004	0.728 ± 0.002
NS-ODN	A375 -alive	0.990 ± 0.011	2.377 ± 0.598	5.203 ± 0.048	9.708 ± 0.554
	A375 -dead	0.010 ± 0.001	0.337 ± 0.056	0.460 ± 0.002	0.684 ± 0.057
	A375 -sum	1.000 ± 0.012	2.714 ± 0.027	5.663 ± 0.049	10.392 ± 0.497
	viable cells(%)	0.990 ± 0.001	0.869 ± 0.053	0.919 ± 0.000	0.934 ± 0.009
	A375/CDDP -alive	0.994 ± 0.008	2.247 ± 0.184	3.921 ± 1.148	9.808 ± 0.666
	A375/CDDP -dead	0.006 ± 0.001	0.274 ± 0.002	0.410 ± 0.006	0.643 ± 0.051
	A375/CDDP -sum	1.000 ± 0.008	2.521 ± 0.182	4.331 ± 1.154	10.451 ± 0.699
	viable cells(%)	0.994 ± 0.001	0.910 ± 0.000	0.901 ± 0.022	0.938 ± 0.004

Notes: xxx-alive, viable cell number; xxx-dead, dead cell number; xxx-sum, total cell number; viable cell (%), cell survival rate; AS-ODN; antisense oligonucleotide; NS-ODN, nonsense oligonucleotide.

Table 4. Comparisons of Cisplatin IC₅₀ value in each group after transfection of 24 h, 48 h, 72 h (ug/mL)

	group	24 h	48 h	72 h	120 h
A375	normal	7.89 ± 1.17	1.68 ± 0.12	0.45 ± 0.06	0.36 ± 0.06
	AS-ODN	4.15 ± 0.38*	1.01 ± 0.20*	0.31 ± 0.11*	0.22 ± 0.11*
	NS-ODN	7.36 ± 0.93	1.63 ± 0.15	0.44 ± 0.03	0.36 ± 0.04
A375/CDDP	normal	36.03 ± 3.23	22.07 ± 2.35	6.51 ± 0.72	3.85 ± 0.53
	AS-ODN	14.29 ± 1.58*	8.05 ± 1.06*	2.19 ± 0.58*	1.01 ± 0.66
	NS-ODN	35.87 ± 3.17	21.64 ± 2.39	6.13 ± 0.59	3.43 ± 0.39

Note: *, $P < 0.05$ in comparison of the NS-ODN and blank control groups.

Figure 1 .

Figure 2 .

Figure 3 .

Figure 4 .

Figure 5 .

Figure 6 .

Figure 7 .

Figure 8 .

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Title:

MicroRNA-21 antisense oligonucleotide improves the sensitivity of A375 human melanoma cell to Cisplatin: An in vitro study (Retracted article. See vol. 122, 2021)

Date:

2018-04-01

Citation:

Zhang, H. -L., Si, L. -B., Zeng, A., Long, F., Qi, Z., Zhao, R. & Bai, M. (2018). MicroRNA-21 antisense oligonucleotide improves the sensitivity of A375 human melanoma cell to Cisplatin: An in vitro study (Retracted article. See vol. 122, 2021). JOURNAL OF CELLULAR BIOCHEMISTRY, 119 (4), pp.3129-3141. <https://doi.org/10.1002/jcb.26455>.

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