MiR-634 activates the mitochondrial apoptosis pathway and enhances chemotherapy-induced cytotoxicity

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Abstract

Some tumor suppressing miRNAs target multiple oncogenes concurrently and therefore may be useful as cancer therapeutic agents. Further, such miRNAs may be useful to address chemotherapeutic resistance in cancer, which remains a primary clinical challenge in need of solutions. Thus, cytoprotective processes upregulated in cancer cells that are resistant to chemotherapy are a logical target for investigation. Here we report that overexpression of miR-634 activates the mitochondrial apoptotic pathway by direct concurrent targeting of genes associated with mitochondrial homeostasis, anti-apoptosis, antioxidant ability and autophagy. In particular, we show how enforced expression of miR-634 enhanced chemotherapy-induced cytotoxicity in a model of esophageal squamous cell carcinoma (ESCC), where resistance to chemotherapy remains clinically problematic. Our findings illustrate how reversing miR-634-mediated cytoprotective processes may offer a broadly useful approach to improving cancer therapy.
Introduction

MicroRNAs (miRNAs) are endogenous small non-coding RNAs that regulate gene expression by interfering with the translation or stability of target transcripts via binding to the 3’-untranslated region (UTR), and function as a “fine-tuner” of numerous biological processes (1-4). Dysregulated miRNA expression is associated with many human diseases, including cancer (1-4), and down-regulation of several tumor-suppressive miRNAs has been shown to be associated with tumor progression, including cell proliferation, invasion/metastasis, and chemoresistance. We have previously identified novel tumor-suppressive miRNAs in endometrial cancer, oral squamous cell carcinoma, and esophageal squamous cell carcinoma (ESCC) (5-10). Importantly, some tumor-suppressive miRNAs were found to simultaneously target multiple cancer-promoting genes, and may be useful as a therapeutic agent for cancer therapy.

Mitochondria-mediated apoptosis, an intrinsic pathway of apoptosis, is usually initiated by loss of mitochondrial membrane potential, and proceeds via release of cytochrome c and reactive oxygen species (ROS) from the intermembraneous space of the mitochondria to the cytosol (11). In the cytosol, cytochrome c forms a complex with APAF1 and procaspase-9, and in this complex, known as the apoptosome, an initiator caspase-9 is activated, resulting in cleavage and activation of executioner caspases such as caspase-3 (11). Although cancer treatment by chemotherapy and γ-irradiation kills tumor cells primarily by inducing apoptosis, tumor cells can acquire resistance to apoptosis. Resistance to chemotherapy is a persistent problem during cancer treatment, and consequently, establishing a novel strategy to overcome such resistance is required for the improvement of prognosis in cancer patients (11). Cancer cells are thought to
upregulate cytoprotective processes for their survival, including anti-apoptosis, antioxidant scavenging, and/or autophagic mechanisms; therefore, targeting these processes may be effective in overcoming chemoresistance (11-13).

In the present study, we report that miR-634 induces activation of the mitochondrial apoptotic pathway and may play a supporting role with chemotherapy. Overexpression of miR-634 activated the mitochondrial apoptotic pathway by directly and concurrently targeting multiple genes associated with mitochondrial biogenesis and the cytoprotective processes against apoptosis in various cancer cells. Furthermore, the enforced expression of miR-634 markedly enhanced chemotherapy-induced cytotoxicity in esophageal squamous cell carcinoma (ESCC) cells in vitro and in vivo. Thus, our findings suggest that concurrent miR-634-mediated modulation of cytoprotective mechanisms may be a therapeutically useful tool for cancer therapy.
Materials and Methods

Cell culture and primary tumor samples

HeLa, U2OS, and WI38 cells were obtained from ATCC and cultured in Dulbecco’s Modified Eagle Medium containing 10% fetal bovine serum (FBS). ESCC cell lines (KYSE850, KYSE170, and KYSE150), which were gifted by Dr. Shimada Y (Toyama University) (14-16), and human lymphoblastoid cell lines (LCLs) were cultured in RPMI1640 medium containing 10% FBS. All cell lines were maintained at 37˚C with 5% CO2.

A total of 40 primary ESCC tumor samples and the corresponding non-cancerous esophageal mucosa, obtained from patients treated at the Tokyo Medical and Dental University Hospital from November 2007 to October 2012, frozen immediately in liquid nitrogen, and stored at -80°C until total RNA was extracted, was utilized in this study. The collection and analysis of patient samples were approved by the Tokyo Medical and Dental University Institutional Review Board (approval #2010-5-2), and written consent was obtained from all patients.

Antibodies and reagents

For western blotting and immunofluorescence analysis, antibodies for caspase-9 (#9502), cleaved caspase-9 (#9501), caspase-3 (#9662), cleaved caspase-3 (#9661), cleaved PARP (#9541), XIAP (#2042), and BIRC5 (survivin; #2809), were purchased from Cell Signaling Technology, antibodies for β-actin (A5441), LC3B (L7543), and TFAM (SAB1401383) were from Sigma, antibodies for APIP (ab98153), OPA1 (ab42364), and LAMP2 (LAMP2b; ab18529) were from Abcam, and antibodies for NRF2 (sc-13032) and p62 (SQSTM1; sc-28359) were from Santa Cruz Biotechnology. z-VAD-fmk (3188-v) was purchased from Peptide Institute Inc.
added at 100μM of final concentration simultaneously with transfection of miRNAs, and then cells were evaluated after 48 hours of incubation. Cisplatin (CDDP) was purchased from WAKO, and 5-fluorouracil (5-FU) was from Sigma, and they were used for treatment of cultured cells at indicated concentrations.

Transfection of microRNAs (miRNAs) and small interfering RNAs (siRNAs)

The dsRNA mimicking mature human miR-634 (PM11538) and nonspecific control miRNA (negative control #1) were obtained from Life Technologies. The siRNA for NRF2 (M-003755-02), XIAP (M-004098-01), APIP (M-003459-03), LAMP2 (M-011715-00), and non-specific control siRNAs (D-001810-05) were obtained from Thermo Scientific Dharmacon. The siRNA for OPA1 (GUUAUCAGUCAGCCAGG) and TFAM (GUUGUCCAAAGAAACCUGU) were synthesized by Sigma. MiRNA or siRNA (for U2OS or HeLa cells) was transfected individually into cells at indicated concentrations using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s instructions. For concurrent inhibition of 7 target genes, a mixtures of 7 siRNAs (in total 70 nM of each 10 nM) or non-specific control siRNAs (in 70 nM) were transfected into U2OS cells, respectively.

Cell survival and cell death assay

Cell survival was assessed by the crystal violet (CV) staining assay. Cells were washed in PBS and fixed with 0.1% CV in 10% formaldehyde in PBS for 10 minutes. After excess CV solution was discarded, stained cells were completely air-dried, and then lysed with a 2% SDS solution with shaking for 2 hours. Optical density (OD) absorbance was measured at 560nm using a
microplate reader (ARVOmx; Perkinelmer), and the percent absorbance of every well was
determined. The OD absorbance values of cells in control wells were arbitrarily set at 100% to
determine the percentage of viable cells. Dead cell was counted by trypan blue staining using
the TC20™ Automated Cell Counter (BIO-RAD), and the percentage of dead cells per total cells
was calculated.

Determination of the sub-G1 cell population by FACS analysis

Both attached cells and cells floating in the medium were collected, washed in PBS, and fixed
with 70% cold ethanol overnight at -20˚C. Fixed cells were washed in PBS again, and incubated
in PBS containing RNase (250 µg/ml) for 30 minutes at 37˚C. Cells were then stained with
propidium iodide (Life technologies), and cell population analysis was performed using the
Accuri® Flow Cytometer.

Western blotting analysis

Whole cell lysates were subjected to SDS-PAGE, and proteins were transferred to PVDF
membranes (GE Healthcare). After blocking with TBS containing 0.05% Tween-20 and 5%
non-fat dry milk for 1 hour, the membrane was reacted with an antibody overnight. The dilutions
for primary antibodies were: 1/5,000 for LC3B and β-actin, 1/2,000 for p62, and 1/1,000 for the
other antibodies. The membrane was washed and exposed to horseradish peroxidase
(HRP)-conjugated anti-mouse or rabbit IgG antibodies (both at 1/4,000) for 2 hours. The bound
antibodies were visualized in LAS3000 (FUJIFILM) using a Pierce ECL Western detection kit
according to the manufacturer’s instructions (Thermo Scientific).
Detection of Cytochrome c in the isolated mitochondrial fraction was performed using the Cytochrome c Releasing Apoptosis Assay Kit (Abcam; ab65311) according to the manufacturer's instructions. Mouse monoclonal anti-cytochrome c antibody (Abcam; ab65311) was used at a dilution of 1/500, and mouse monoclonal anti-VDAC1 antibody (Santa Cruz Biotechnology; sc-58649) was used at a dilution of 1/1,000 as a control for the mitochondrial fraction.

**Conventional luciferase assay**

Luciferase reporter plasmids were constructed by inserting the 3′-untranslated region (UTR) of XIAP, BIRC5, APIP, OPA1, TFAM, or LAMP2 downstream of the luciferase gene within the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega). All site-specific mutations were generated using the GeneTailor site-directed mutagenesis system (Life Technologies). Luciferase reporter plasmids and a pTK plasmid as an internal control were co-transfected in U2OS cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions, and after 5 hours, 20 nmol/L of miRNA (miR-NC or miR-634) was also transfected. At 36 hours after transfection of miRNAs, Firefly and Renilla luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega), and relative luciferase activity was calculated by normalizing Firefly luciferase by its corresponding internal 

**Renilla luciferase control.**

**Gene expression array analysis**

For gene expression analysis, the Agilent 4x44K (for HeLa cells) or 8x60K (U2OS and KYSE850
cells) array was used according to the manufacturer’s instructions (Agilent Technologies). HeLa, U2OS and KYSE850 cells were transfected with 20nM of miRNA (miR-NC or miR-634), and RNA was extracted at 48 hours after transfection. Each experiment was performed in duplicate and the data were analyzed in GeneSpring software (Agilent Technologies). After selection, candidate genes were subjected to ingenuity pathway analysis (IPA; Ingenuity Systems) or gene ontology (GO) analysis to determine the enrichment among predicted candidate genes of miR-634 targets. Statistical significance of GO analysis was evaluated according to the hypergeometric distribution.

Immunofluorescence analysis

Intracellular mitochondria were stained with 100 nM of MitoTracker® Red CMX ROS (Life Technologies) for 30 minutes at 37˚C. After fixation with 10% trichloroacetic acid (TCA), images were obtained by confocal fluorescence microscopy (Nikon).

For immunostaining of p62 and LC3B, cells were fixed with 10% TCA, permeabilized with 0.2% Triton X-100, and blocked with PBS containing 0.01% Triton X-100 and 1% BSA for 1 hour, then incubated with primary antibodies (mouse anti-p62, 1/2000 dilution, and rabbit anti-LC3B, 1/5000 dilution) for 12 hours at 4˚C. Bound antibodies were visualized using Alexa Fluor594 anti-mouse IgG antibody or Alexa Fluor488 anti-rabbit IgG antibody (both 1/2000 dilution, Life Technologies), and coverslips were mounted in VECTASHILD containing DAPI (Vector). Images were obtained by confocal fluorescence microscopy (Nikon).

Detection of mitochondrial membrane potential and cellular ROS by FACS analysis
Mitochondrial membrane potential and intracellular ROS production were measured using the TMRE (tetramethylrhodamine, ethyl ester) Mitochondrial Membrane Potential Assay Kit (ab113852) and DCFDA Cellular ROS Detection Assay Kit (ab113851), respectively (both from Abcam). For the TMRE assay, cells were incubated with 200 nM of TMRE for 30 minutes at 37˚C with 5% CO₂, and then suspended in PBS with 0.2% FBS. For the ROS detection assay, cells were harvested and incubated with 20 µM of DCFDA for 30 minutes at 37˚C with 5% CO₂. Fluorescence intensity in both procedures were measured using an Accuri® Flow Cytometer. Median fluorescence intensity was calculated using Flowjo software.

**Generation of CDDP-resistant cells**

Cisplatin-resistant cells from KYSE150, an ESCC cell line, were generated by long-term cultivation in gradually increasing CDDP concentrations. The cells were initially exposed to CDDP at a low concentration (0.5 µM) for 3 days, cultured in CDDP-free medium to confluence, and then exposed to CDDP at a higher concentration; this cycle was repeated 5 times with gradually increasing concentrations (0.5µM, 1µM, 2µM, 4µM, and 10µM of CDDP concentration). The cells that could survive at 10µM of CDDP exposure was defined as the CDDP-resistant cells (KYSE150-R).

**Quantitative Reverse Transcription (RT)-PCR**

Quantitative real-time RT–PCR (qRT-PCR) was performed using an ABI PRISM 7500 Fast Real time PCR System, Taqman Universal PCR Master Mix, Taqman Reverse Transcription kit, and Taqman MicroRNA Assays (Applied Biosystems), according to the manufacturer’s instructions.
Gene expression values are given as ratios (differences between the Ct values) between 
mig-634 and an internal reference, RNU6B.

In vivo tumor growth and administration of miRNAs

Five-week-old female BALB/c nude mice were purchased from Charles River Laboratories, 
Japan. A total of 5.0 x 10^6 cells in 200 μL of PBS were subcutaneously injected into the flanks of 
the mice. After the tumor formation at day 7, the administration of miRNAs and the treatment with 
CDDP was started. A mixture of 1 nmol dsRNA and 200 μL AteloGene (KOKEN) was 
administered around the tumor (miR-NC to the left flank and miR-634 to the right flank of mice) at 
day 7, 9, 11, 14 and 16. Mice were also intraperitoneally administered with PBS (PBS group; 
n=6) or CDDP at a dose of 5 mg/kg body weight (CDDP group, n=8) at day 7 and 14. At 21 days 
after the injection of cells, mice were sacrificed and tumors were enucleated. All experimental 
protocols conducted on the mice were approved by the Tokyo Medical and Dental University 
Animal Care and Use Committee.

Statistical analysis

Differences between subgroups were tested by Student’s t-test. To compare Kaplan-Meier plots 
in two groups, two-sided log-rank tests were used. A P value of < 0.05 was considered 
statistically significant.
Results

Induction of apoptotic cell death by overexpression of miR-634

We have previously reported that four miRNAs, miR-507, miR-634, miR-450a, and miR-129-5p, down-regulate the expression of NRF2 (NFE2L2; nuclear factor, erythroid 2-like 2), a transcription factor involved in the ROS scavenging pathway, by directly targeting its 3′-UTR (10). Interestingly, further examination revealed that overexpression by transfection of 2 or 20 nM of miR-634, and not the other miRNAs, drastically induced cell death in multiple human cancer cell lines, including HeLa (cervical cancer cell line), U2OS (osteosarcoma cell line), and KYSE850 (esophageal cancer cell line) (Figure 1A, and 1B). This miR-634-induced cell death was not observed in normal cells such as fibroblast and lymphoblastoid cell lines (LCLs) (Supplementary Figure S1). In western blotting analysis, expression of the cleaved form of caspase-3, caspase-9, and -PARP was markedly increased in miR-634-expressing cells (Figure 1C); furthermore these increased levels of cleaved-caspase-3 and -PARP were restored by simultaneous treatment with z-VAD-fmk (Figure 1D). By FACS analysis, the sub-G1 cell population, an indicator of apoptotic nuclei, was markedly increased in miR-634-expressing cells compared to miR-NC-expressing cells, and these increases were abrogated by simultaneous treatment with z-VAD-fmk (29.5% to 8.1% in HeLa cells, 27.2% to 4.3% in U2OS cells, and 21.6% to 4.7% in KYSE850 cells) (Figure 1E). These findings suggest that overexpression of miR-634 may drastically induce apoptotic cell death in a caspase-dependent manner in human cancer cells. On the other hand, siRNA-mediated inhibition of NRF2, a known miR-634-target gene, did not induce cell death, but slightly inhibited cell growth (Supplementary Figure S2), suggesting that concurrent down-regulation of additional target genes may be necessary for the
induction of miR-634-induced apoptotic cell death.

Identification of miR-634-target genes

To identify genes that were down-regulated by miR-634, we performed expression array analysis on miR-NC- or miR-634-transfected cells in 3 cell lines: HeLa, U2OS, and KYSE850. As indicated in the Venn diagram in Figure 2A, the expression level of 1,835 genes was decreased by more than a 1.2-fold change in miR-634-expressing cells, compared with miR-NC-expressing cells. By using the TargetScan program (http://www.targetscan.org), among these genes, 626 were predicted to be direct targets of miR-634, due to the presence of miR-634 seed sequences in the 3′-UTRs (Figure 2B, Supplementary Table S1). Interestingly, in silico analysis using IPA (Ingenuity Systems Pathway Analysis, Redwood City, CA) (http://www.ingenuity.com) and GO analysis revealed that several genes associated with mitochondrial biogenesis and anti-apoptosis were significantly enriched among the predicted target genes (Figure 2C, Supplementary Table S2 and S3). The expression level of two mitochondrial genes, OPA1 (optic atrophy 1) and TFAM (transcription factor A, mitochondrial), which are known to play a critical role in mitochondrial homeostasis through mitochondrial fusion and maintenance of mitochondrial DNA (17, 18), and three anti-apoptosis genes, XIAP (X-linked inhibitor of apoptosis, E3 ubiquitin protein ligase), BIRC5 (baculoviral IAP repeat containing 5), and APIP (APAF1 interacting protein), which are known to inhibit the caspase activation cascade (19-21), were markedly decreased in miR-634-expressing cells compared with miR-NC-expressing cells (Figure 2D). Additionally, five lysosome-associated genes (LAMP2; lysosomal-associated membrane protein 2, ATP6V1B2, ATP6V1F, ATP6V0B, and BLOC1S2) were included in the
predicted target genes of miR-634, one of which, LAMP2, showed decreased expression in
miR-634-expressing cells (Figure 2D and Supplementary Table S2 and S3). Thus, we focused
on 6 genes, XIAP, BIRC5, APIP, OPA1, TFAM, and LAMP2, as likely miR-634-target gene
candidates. The expression level of NRF2, a known target gene, was also confirmed to be
decreased in miR-634-expressing cells as we previously reported (10) (Figure 2D).

To examine whether miR-634 can directly bind to each 3’-UTR of these genes,
luciferase assays using reporter plasmid vectors having wild type (WT) or mutant (Mut.) seed
sequences within the fragments of each 3’-UTR (Figure 2E) were performed in U2OS cells. The
luciferase activity of the WT vectors, except for one of the 2 seed sequences within the 3’-UTR of
XIAP (Region-2), was significantly reduced compared to the empty vector in miR-634-expressing
cells, and completely restored with Mut. Vector, which had mutations within the seed sequences
(Figure 2E). These results suggest that miR-634 can directly down-regulate expression of these
6 novel target genes, in addition to NRF2, by binding to the 3’-UTRs. Moreover, we showed that
transfection with a mixture of siRNAs for 7 target genes resulted in remarkable growth inhibition
and increase of cleaved PARP level (Supplementary Figure S3), strongly suggesting that
concurrent down-regulation of these target genes may be critical for the induction of
miR-634-mediated apoptotic cell death.

Furthermore, nevertheless there was no significant difference for endogenous
expression level of miR-634 between cancer cells (Hela, U2OS, and KYSE850) and normal cells
(WI-38 and three LCLs), miR-634-target proteins, especially BIRC5 and NRF2, were highly
expressed in cancer cell lines compared with normal cells (Supplementary Figure S4). This
may be possible explanation for mechanism that the expression of miR-634 was more effective
in cancer cells than in normal cells (Supplementary Figure S1).

Induction of mitochondrial injury by miR-634

To further evaluate the biological features of miR-634-mediated apoptotic cell death, change in mitochondrial status was first evaluated due to multiple miR-634-target genes’ involvement in mitochondrial functions (Supplementary Table S2 and Figure 2). When miR-634 was expressed in U2OS cells, mitochondrial injury was increased in miR-634-expressing cells, indicated by fragmented morphology, decrease in mitochondrial membrane potential, increase in ROS production, and release of cytochrome c from the mitochondria to the cytosol (Figure 3). These results suggest that overexpression of miR-634 triggers the activation of the mitochondrial apoptosis pathway. Furthermore, to estimate the contribution of the down-regulation of miR-634-target genes on mitochondrial injury, we examined mitochondrial status in siRNA-mediated knockdown cells for each gene. Effective knockdown of each gene was confirmed by western blotting (Figure 3A). Fragmented mitochondria were observed in OPA1-knockdown cells as previously reported (17), but not in knockdown cells of other genes (Figure 3B and data not shown). Furthermore, we found that mitochondrial membrane potential was decreased in TFAM- and LAMP2-knockdown cells, and ROS production was increased in TFAM-, LAMP2-, and NRF2-knockdown cells (Figure 3C and 3D). These findings suggest that concurrent down-regulation of multiple miR-634-target genes triggers mitochondrial injury in miR-634-mediated apoptotic cell death. On the other hand, it is known that autophagy partakes in the removal of damaged mitochondria via lysosomal degradation, called mitophagy, contributing to mitochondrial quality control (22). Levels of form-II LC3B, an indicator of
autophagosomes, and p62 protein, a substrate of autophagic degradation, were increased in miR-634-expressing cells (23, 24) (Figure 3F). Furthermore, these proteins were observed to be frequently co-localized at the same punctate structures in miR-634-expressing cells by immunofluorescence analysis (Figure 3G). These findings suggest that miR-634 impairs autophagic degradation, possibly due to lysosomal dysfunction via concurrent down-regulation of lysosome-related genes, including LAMP2, resulting in the accumulation of damaged mitochondria. Taken together, these findings imply miR-634 triggers the activation of the mitochondrial apoptosis pathway by targeting genes involved in mitochondrial homeostasis.

Augmentation of chemotherapy-induced cytotoxicity by miR-634

Because the cytotoxicity of cancer drugs such as cisplatin (CDDP) and 5-FU is closely associated with the mitochondrial apoptosis pathway (11, 25, 26), we speculated that combined treatment with enforced expression of miR-634 may be able to effectively enhance chemotherapy-induced cytotoxicity. To validate this idea, we used KYSE170 cells, an ESCC cell line, because CDDP and 5-FU are the most utilized drugs for chemotherapy of this type of tumor (27). KYSE170 cells transfected with a high concentration (20 nM) of miR-634 and simultaneously treated with CDDP or 5-FU showed drastically decreased cell viability (Figure 4A). Furthermore, neither transfection with a low concentration (0.2 nM) of miR-634 nor treatment with 2.5 µM of CDDP alone were able to induce cell death, demonstrating combined treatment was necessary for markedly increased cell death (Figure 4B). This combined effect of both miR-634 and CDDP was also observed in other ESCC cell lines (data not shown). In western blotting, levels of cleaved caspase-3 and PARP were increased in cells with combined
treatment, compared with transfection of miR-634 or treatment by CDDP alone, and the expression levels of miR-634-target genes were decreased in miR-634-expressing cells (Figure 4C). These findings suggest that enforced expression of miR-634 may effectively enhance chemotherapy-induced cytotoxicity in ESCC cells, even with a low concentration of miR-634.

Next, to examine whether the enforced expression of miR-634 can intervene in ESCC resistance to CDDP, CDDP-resistant cells were generated from KYSE150 cells, an ESCC cell line, which is known to be relatively sensitive to CDDP (Supplementary Figure S5) (28). Interestingly, we showed that the expression level of miR-634-target genes except for OPA1, especially NRF2, was increased in CDDP-resistant KYSE150 cells (KYSE150-R) compared with parental cells, but there was no significant change for expression level of endogenous miR-634 between both cells (Supplementary Figure S5). This suggests that up-regulation of these miR-634-target genes may contribute to acquired resistance to CDDP in KYSE150 cells. Importantly, cell viability assay showed that enforced expression of miR-634 at a low concentration (0.2 nM) attenuated resistance to CDDP (IC₅₀ 11.31 µM in miR-NC-expressing cells, 2.45 µM in miR-634-expressing cells) (Figure 4D and 4E). These results suggest that enforced expression of miR-634 can lessen ESCC acquired resistance to CDDP.

Therapeutic potential of miR-634

The therapeutic effect of miR-634 was investigated in vivo by administration of dsRNA mimicking miR-634 into the subcutaneous space surrounding tumors formed from KYSE170 cells. MiR-NC or miR-634 was administered around the KYSE170-derived tumors subcutaneously a total of five times (7, 9, 11, 14, and 16 days after the injection of cells), in addition to treatment with PBS or
CDDP twice administered intraperitoneally (7 and 14 days after the injection of cells) (Figure 5A).

There were no observations for any adverse events such as body weight loss (Supplementary Figure S6) or local damage around skin area in which miRNAs were administrated. As a result, tumor weights at 21 days after injection of cells were significantly reduced in mice receiving combined treatment of CDDP and miR-634, compared with mice receiving combined treatment of CDDP and miR-NC ($p = 0.0081$) (Figure 5B, 5C, and 5D). In addition, the enforced expression of miR-634 together with the down-regulated expression of OPA1, BIRC5, and NRF2 proteins were confirmed in miR-634-treated tumors (Figure 5E and 5F). Finally, expression analysis of miR-634 by qRT-PCR was performed with 40 paired samples from patients with ESCC. MiR-634 expression reduction of more than 50% in primary tumor tissue compared with corresponding non-cancerous tissue was observed in twenty-five cases (62.5%) (Supplementary Figure S7); this frequency confirms results from our previous report (10), however there was no statistically significance in overall survival rate or recurrence free survival rate between the patients with high or low expression of miR-634 (Supplementary Figure S7).

Taken together, these findings suggest that concurrent modulation of miR-634-target genes may be a therapeutically effective adjunctive treatment with chemotherapy for ESCC tumors, and may benefit patients with miR-634-down regulated ESCC tumors.
Discussion

In this study, a number of mitochondria-associated genes were identified as direct targets of miR-634. Notably, OPA1 and TFAM are known to play a critical role in mitochondrial homeostasis via mitochondrial fusion and maintenance of mitochondrial DNA, respectively. Inhibition of these genes has been suggested to be associated with induction of apoptosis and enhancement of drug-induced cytotoxicity (17, 18, 29-32). Hence, concurrent down-regulation of these mitochondria-associated genes by miR-634 may induce mitochondrial injury or initiate mitochondrial apoptosis. On the other hand, it is known that damaged mitochondria are immediately removed via an autophagy-mediated degradation system in lysosomes (22). In the present study, we observed that overexpression of miR-634 impaired autophagic degradation possibly due to lysosomal dysfunction via down-regulation of lysosome-associated genes, including LAMP2, a well-known lysosomal membrane protein (33), suggesting that accumulation of damaged mitochondria may contribute to the initiation of mitochondrial apoptosis. Additionally, concurrent down-regulation of apoptosis inhibitors, including APIP, XIAP, and BIRC5, together with the ROS scavenger NRF2, may coordinately promote the mitochondrial apoptosis pathway.

In previous studies, it has been suggested that miR-634 strongly inhibits cell growth in nasopharyngeal carcinoma cells and prostate cancer cells; however, direct target genes contributing to this growth inhibition had not been identified (34, 35). As summarized in Figure 6A, our findings in this study suggest that overexpression of miR-634 may trigger the activation of the mitochondrial apoptotic pathway by directly and concurrently targeting multiple genes associated with mitochondrial biogenesis and the cytoprotective processes against apoptosis in human cancer cells. However, the physiological function of miR-634 in environments which...
induce apoptosis in humans remains unknown, requiring further investigation.

Chemotherapy is one of the standard methods of treatment for many cancers, and is often capable of inducing apoptosis in tumors, resulting in a reduction of the tumor bulk; however, many cancer patients experience recurrence and ultimately death due to the acquisition of chemoresistance by tumor cells (11, 36). The ability of tumor cells to acquire chemoresistance is known to be via activation of cytoprotective processes against apoptosis, and therapeutically targeting these processes may be effective in overcoming chemoresistance (11-13). Our group as well as others have identified several miRNAs that can directly target cytoprotective processes, including the anti-apoptosis pathway (XIAP, BIRC5), antioxidant activity (NRF2), and autophagy (ATG4, ATG7), and demonstrated that enforced expression of these miRNAs promotes drug-induced apoptosis in several human cancers (10, 37-43). On the other hand, clinical trials for miRNA therapy of liver and malignant hematopoietic cancers using a synthetic miR-34a mimic is currently ongoing (44, 45). Compared with other strategies, such as siRNA-based therapies, one advantage of miRNA therapy may be its ability to concurrently target multiple genes that are associated within the same network (44, 45). An introduction of miR-634 at high concentration of 20 nM is not physiological levels and may induce some off-target effects. However, in the present study, we indeed showed that the effect of miR-634 at high concentration was slight in normal cells, and importantly the introduction with low concentration (0.2 nM) or the in vivo administration of miR-634 could enhance drug-induced cytotoxicity by concurrently targeting multiple genes associated with the cytoprotective processes, which result in chemoresistance. Since it has been known that some miR-634-target genes were overexpressed in several types of tumor including ESCC tumors and its
up-regulation contribute to chemoresistance, introduction of miR-634 even at low-dose may be therapeutically valuable in cancer cells highly expressing its target genes, although the development of effective delivery system is required for its clinical usage. Thus, miR-634 may be one potential agent in miRNA-based cancer therapy, and a modulator of conventional chemotherapy with small adverse events.

It has been reported that miR-634-target genes that we identified were highly expressed in several tumor cells compared with non-cancerous tissues. In addition, our analysis revealed that the effect of miR-634 was specific in cancer cells, not in normal cells.

ESCC is a cancer with worse prognosis than other cancers, and chemotherapy with or without radiation is used for patients with either unresectable tumors, or with recurrence after radical surgery (46). Despite recent advances in genomic sequencing, effective molecular targeting drugs for ESCC have not yet been established, unlike other types of cancers; therefore conventional chemotherapy, including drugs such as CDDP or 5-FU, remains important for the treatment of ESCC. Development of chemoresistance is a persistent problem in ESCC patients, and establishing a novel strategy to overcome this is needed. Although further studies on delivery, potential off-target effects, and safety are required, our findings suggest that miR-634-mediated modulation of mechanisms involved in chemoresistance may be a reasonable strategy to maximize the outcome of conventional chemotherapy in ESCC.
1 Acknowledgements

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Figure legends

Figure 1. Induction of apoptotic cell death by overexpression of miR-634

A. Phase contrast images of HeLa, U2OS, and KYSE850 cells. Cells were transfected with 20 nM of negative control-miRNA (miR-NC) or miR-634, and images were obtained 2 days after transfection.

B. Cell growth assay of HeLa, U2OS, and KYSE850 cells. Cells were transfected with 2 or 20 nM of miR-NC or miR-634. Cell growth rate was assessed with the crystal violet staining assay using a relative ratio compared to day 0. Bar; standard deviation (SD) for triplicate experiments.

C. Western blotting analysis of HeLa, U2OS, and KYSE850 cells. Cell lysates were subjected to SDS-PAGE and immunoreacted with the indicated antibodies. Arrows indicate the bands for the cleaved form of caspase-3 and -9.

D. Effect of treatment with caspase inhibitor z-VAD-fmk on miR-634-induced apoptotic cell death. Transfected cells were treated with DMSO (as a control) or z-VAD-fmk for 2 days. Cell lysates were subjected to SDS-PAGE and immunoreacted with the indicated antibodies. Arrows indicate the bands for cleaved caspase-3.

E. FACS analysis for the sub-G1 cell population. Cells were treated as described in D., collected, fixed, and stained with propidium iodide (PI). Cell population analysis was performed using an Accuri® Flow Cytometer. The percentage of gated sub-G1 cells is indicated in each histogram.

Figure 2. Identification of miR-634 target genes
A. Identification of genes down-regulated by miR-634 by expression array. Venn diagram shows the 1,835 genes with down-regulated expression of more than 1.2 fold-change (Fc) by transfection of miR-634 in 3 cell lines: HeLa, U2OS, and KYSE850.

B. Prediction of putative miR-634 target genes. Venn diagram shows the 626 predicted candidate targets of miR-634 by the TargetScan program.

C. Graph indicates that genes associated with mitochondrial biogenesis and anti-apoptosis are significantly enriched within the 626 predicted target genes by IPA.

D. Western blotting analysis of 6 candidate target genes and NRF2. Cell lysates were subjected to SDS-PAGE and immunoreacted with the indicated antibodies. A bracket indicates bands for OPA1 variants. In lower panel, cell lysates were prepared under the non-reducing condition (without 2-ME) for detection of LAMP2.

E. Luciferase assay using reporter plasmids. U2OS cells were co-transfected with a reporter plasmid and an internal control vector, and after 5 hours either miR-NC or miR-634 was additionally transfected. After 36 hours, Firefly and Renilla luciferase activity was measured. Seed sequences of miR-634 within the 3’-UTR of each gene and mutant sequences are indicated (upper panel in each column). The luciferase activity relative to that of the miR-NC-transfected cells is indicated on the vertical axis (lower panel in each column). The luciferase activity is weaker for vectors with the WT region than for the empty vector, and is completely restored by inserting a mutation within the seed sequence. Bar; standard deviation (SD).

Figure 3. Mitochondrial injury and autophagy impairment induced by miR-634
A. Western blotting analysis for miR-634-target genes. Cells were transfected with 20 nM of siRNA for each gene. Cell lysates were subjected to SDS-PAGE and immunoreacted with the indicated antibodies.

B. Representative images for mitochondrial staining. U2OS cells were transfected with 20 nM of miRNAs or siRNAs, and stained with 100 nM of MitoTracker® Red CMX ROS for 30 minutes at 37˚C. After fixation, images were obtained by confocal fluorescence microscopy. Nuclei were counterstained with DAPI (blue). Scale bar; 10 µm.

C. Mitochondrial membrane potential assay. Cells were incubated with 200 nM of TMRE for 30 minutes at 37˚C, suspended in PBS with 0.2% FBS, and fluorescence intensity was measured using flow cytometry. Representative histogram is shown (upper panel). The relative difference in median fluorescence intensity against miR-NC- or negative control-siRNA (si-NC)-transfected cells is shown in the graph (lower panel). Bar; standard deviation (SD) in triplicate experiments.

D. Cellular ROS detection assay. Cells were collected, incubated with 20 µM of DCFDA for 30 minutes at 37˚C, and fluorescence intensity was measured using flow cytometry. The relative difference in median fluorescence intensity against miR-NC- or negative control-siRNA (si-NC)-transfected cells is shown in the graph (lower panel). Bar; standard deviation (SD) in triplicate experiments.

E. Western blotting analysis for cytochrome c released into the cytosol. Cytosolic or mitochondrial fractions were isolated, subjected to SDS-PAGE and immunoreacted with the indicated antibodies.

F. Western blotting analysis for p62 and LC3B. Cell lysates were subjected to SDS-PAGE and
immunoreacted with the indicated antibodies. An arrow indicates the band for LC3B form-II, an autophagosome marker.

G. Immunofluorescence analysis of p62 and LC3B. U2OS cells were plated on coverslips and transfected with 20 nM of miR-NC or miR-634. After 36 hours, the cells were fixed, permeabilized, and immunoreacted with the indicated antibodies. LC3B or p62 antibodies were visualized using Alexa Fluor488 anti-mouse IgG antibody (green) or Alexa Fluor594 anti-rabbit IgG antibody (red), respectively. Nuclei were counterstained with DAPI (blue). Bar; 10 µm.

Figure 4. Augmentation of CDDP-induced cytotoxicity by miR-634

A. Cell viability assay for cells with miRNA and CDDP or 5-FU combination treatment. KYSE170 cells were transfected with 20 nM of miR-NC or miR-634, and were simultaneously treated with the indicated concentrations of CDDP or 5-FU for 3 days.

B. Increase in dead cells with miRNA and CDDP combination treatment. KYSE170 cells were transfected with 0.2 nM of miR-NC or miR-634, and were simultaneously incubated in medium with or without CDDP (2.5 µM) for 3 days. Representative phase contrast images are shown in the upper panel. The percentage of dead cells is indicated in the graph (lower panel).

C. Western blotting analysis of cells with miRNA and CDDP combination treatment. Cells were treated as described in B. Cell lysates were subjected to SDS-PAGE and immunoreacted with the indicated antibodies. Arrows indicate the band for cleaved caspase-3 or LC3B form-II.

D. Attenuation of CDDP resistivity by miR-634. KYSE150 (left panel) or its CDDP-resistant cells
KYSE150-R, right panel) were transfected with 0.2 nM of miR-NC or miR-634, and were simultaneously treated with the indicated concentrations of CDDP. Cell survival rate was assessed with the crystal violet staining assay as a relative ratio compared to that of miR-NC-transfected cells without CDDP. Bar; standard deviation (SD) for triplicate experiments.

E. IC50 values based on the cell viability in D were calculated and are indicated in the graph.

Figure 5. Potential of miR-634 as a therapeutic agent

A. The experimental schedule for the miR-634 and CDDP combination treatment. Tumors were formed by subcutaneous injection of KYSE170 cells in nude mice. MiR-NC or miR-634 was administered around the tumors derived from the KYSE170 subcutaneously for a total of 5 times (7, 9, 11, 14, and 16 days after the injection of cells). In addition, mice were treated with PBS or CDDP (5 mg/kg) by intraperitoneal administration twice (7 and 14 days after the injection of cells). At 21 days after the injection of cells, mice were sacrificed and the tumors were evaluated.

B. Representative images of tumor-bearing nude mice at 21 days after the injection of KYSE170 cells. Arrows indicate the tumors.

C. Representative images of resected tumors.

D. Weights of the resected tumors. Tumor weights are shown as a box plot.

E. Expression analysis of miR-634 in the resected tumors. The mRNA expression level of miR-634 was measured by qRT-PCR. Each experiment was performed in duplicate. Expression of RNU6B was used as an internal control. Expression levels relative to those in
the miR-NC-administered tumor are indicated on the vertical axis. Bar; standard deviation (SD).

**F.** Representative images for immunostaining of OPA1, BIRC5, and NRF2 in the resected tumors. Scale bar; 100 μm.

**Figure 6 Schematic models**

**A.** Effective activation of the mitochondrial apoptosis pathway by concurrent down-regulation of miR-634-target genes.

**B.** Contribution of the enforced expression of miR-634 on the reduction of chemoresistance.
Figure 1

A: Cleaved caspase

B: Cleaved PARP

C: Cell growth ratio

D: β-actin

E: FL2-A(x10^6)
Figure 2

A
Down-regulated genes in each 3 cell lines (Fc > 1.2)

HeLa (4078 genes)

U2OS (5664 genes)

KYSE850 (4198 genes)

B
Commonly down-regulated genes (1835 genes)

TargetScan (2465 genes)

C
IPA

Mitochondrial biogenesis

p = 0.00248

Anti-apoptosis

p = 0.0139

D

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Figure 3

A: Western blot analysis of XIAP, BIRC5, APIP, OPA1, TFAM, LAMP2, NRF2, and β-actin using miR-NC or miR-634 siRNA. The relative median fluorescence intensity was compared between miR-NC and miR-634 with statistical significance indicated by p-values.

B: Immunofluorescence images showing the localization of LC3B and p62 in cytosolic and mitochondrial fractions. miR-NC or miR-634 was transfected, and the images were merged with DAPI.

C-D: Flow cytometry analysis for FL1-H and FL2-H channels showing the relative median fluorescence intensity for miR-NC and miR-634 samples with statistical significance indicated by p-values.

E: Western blot analysis of Cyto. c, β-actin, VDAC1, and LC3B in cytosolic and mitochondrial fractions using miR-NC or miR-634 siRNA.
Figure 4

(A) Relative cell viability of KYSE170 cells treated with CDDP at different concentrations with or without miR-634.

(B) KYSE170 cells treated with CDDP (2.5 μM) or no treatment. % of dead cells measured using annexin V and propidium iodide staining.

(C) Western blot analysis of KYSE170 cells treated with CDDP (2.5 μM) or no treatment, showing proteins related to apoptosis and autophagy.

(D) Relative cell viability of KYSE150 and KYSE150-R cells treated with CDDP at different concentrations with or without miR-634.

(E) IC50 of CDDP for KYSE150 and KYSE150-R cells treated with miR-NC, miR-634, miR-634, and no treatment.
Figure 5

A

Subcutaneous injection of KYSE170 cells
Injection of miRNA (10μg) with AteloGene®
Injection of PBS or CDDP (5mg/kg)
Evaluation

B

Injection of PBS or CDDP (5mg/kg)

C

PBS
CDDP

D

Tumor weight (g)

E

Relative miR-634 expression ratio

F

OPA1
BIRC5
NRF2

PBS
CDDP
PBS
CDDP
PBS
CDDP

p=0.0024
p=0.0081

PBS
CDDP
PBS
CDDP

Subcutaneous injection of KYSE170 cells
Injection of miRNA (10μg) with AteloGene®
Injection of PBS or CDDP (5mg/kg)
Evaluation
Figure 6

A

Mitochondria homeostasis (OPA1, TFAM, etc.)

Mitochondrial damage

Cyto. c release

ROS production

Caspase-9

Caspase-3

Apoptosis

miR-634

Autphagic removal (LAMP2, etc.)

Lys

ROS scavenger (NRF2)

APIP

miR-634

XIAP, BIRC5, etc.

B

Chemotherapy

miR-634

Cytoprotective processes for chemoresistance
- Anti-apoptosis
- ROS scavenger
- Mitochondrial quality control by autophagy

Therapeutic effect
MiR-634 activates the mitochondrial apoptosis pathway and enhances chemotherapy-induced cytotoxicity

Naoto Fujiwara, Jun Inoue, Tatsuyuki Kawano, et al.


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