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, antiapoptosis, 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, 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. Cancer Res; 75(18); 3890–901. ©2015 AACR.

Introduction

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 biologic processes (1–4). Dysregulated miRNA expression is associated with many human diseases, including cancer (1–4), and downregulation 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; refs. 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 intermembranous 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 caspsases 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 antiapoptosis, 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 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 the ATCC and cultured in Dulbecco’s Modified Eagle Medium containing 10%...
FBS. ESCC cell lines (KYSE850, KYSE170, and KYSE150), which were gifted by Dr. Shimada Y (Toyama University; refs. 14–16), and human lymphoblastoid cell lines (LCL) 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 noncancerous 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, were 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; #2808), were purchased from Cell Signaling Technology; antibodies for β-actin (A5441), LC3B (L7543), and TFAM (SAR1401383) were from Sigma; antibodies for APIP (ab98153), OPA1 (ab42364), and LAMP2 (LAMP2b; ab18529) were from Abcam; and antibodies for NFR2 (sc-13032) and p62 (SQSTM1; sc-28359) were from Santa Cruz Biotechnology. z-VAD-fmk (3188-v) was purchased from Peptide Institute Inc. and added at 100 μmol/L of final concentration simultaneously with transfection of miRNAs, and then cells were evaluated after 48 hours of incubation. Cisplatin (CDDP) was purchased from Sigma. MiRNA or siRNA (for U2OS or HeLa cells) was transfected into cells at indicated concentrations 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, mixtures of 7 siRNAs (in total 70 nmol/L of each 10 nmol/L) or nonspecific control siRNAs (in 70 nmol/L) were transfected into U2OS cells, respectively.

Transfection of miRNAs and siRNAs

The dsRNA mimicking mature human miR-634 (PM11538) and nonspecific control miRNA (negative control #1) were obtained from Life Technologies. The siRNA for NFR2 (M-003755-02), XIAP (M-004098-01), APIP (M-008475-01), BIRC5 (M-003459-03), LAMP2 (M-011715-00), and nonspecific control siRNAs (D-001810-05) were obtained from Thermo Scientific Dharmacon. The siRNA for OPA1 (GHIU1AUGCUGUCGAGCCAGCAG) and TFAM (GUIGUCCCAAAGAACCUUGU) was 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, mixtures of 7 siRNAs (in total 70 nmol/L of each 10 nmol/L) or nonspecific control siRNAs (in 70 nmol/L) 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 560 nm using a microplate reader (ARVOmx; Perkinelmer), and the percent absorbance of every well was determined. The OD absorbance values of cells in every well was determined. The OD absorbance values of cells in every well was determined. The OD absorbance values of cells in every well was determined.

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 blot 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 horse-radish peroxidase-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 cotransfected 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 4 × 44 K (for HeLa cells) or 8 × 60 K (U2OS and KYSE850 cells) array was used according to the manufacturer’s instructions (Agilent Technologies). HeLa, U2OS, and KYSE850 cells were transfected with 20 nmol/L 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. The NCBI GEO accession number is GSE71742.

**Immunofluorescence analysis**

Intracellular mitochondria were stained with 100 nmol/L 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).

**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 nmol/L 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 nmol/L of miR-NC or miR-634. Cell growth rate was assessed with the crystal violet staining assay using a relative ratio compared with day 0. Bar, 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, 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, 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. Cell population analysis was performed using an Accuri Flow Cytometer. The percentage of gated sub-G1 cells is indicated in each histogram.
Induction of Mitochondrial Apoptosis by miR-634

Figure 2.
Identification of miR-634 target genes. A, identification of genes downregulated by miR-634 by expression array. Venn diagram shows the 1,835 genes with downregulated expression of more than 1.2 fold change (Fc) by transfection of miR-634 in three 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 antiapoptosis are significantly enriched within the 626 predicted target genes by IPA. D, Western blot analysis of six 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 bottom panel, cell lysates were prepared under the nonreducing condition (without 2-ME) for detection of LAMP2. E, luciferase assay using reporter plasmids. U2OS cells were cotransfected 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 (top panel in each column). The luciferase activity relative to that of the miR-NC-transfected cells is indicated on the vertical axis (bottom 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, SD.

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, 1/2,000 dilution, and rabbit anti-LC3B, 1/5,000 dilution) for 12 hours at 4°C. Bound antibodies were visualized using Alexa...
Figure 3.
Mitochondrial injury and autophagy impairment induced by miR-634. A, Western blot analysis for miR-634-target genes. Cells were transfected with 20 nmol/L 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 nmol/L of miRNAs or siRNAs and stained with 100 nmol/L 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 nmol/L 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 (top panel). The relative difference in median fluorescence intensity against miR-NC- or negative control-siRNA (si-NC)-transfected cells is shown in the graph (bottom panel). Bar, SD in triplicate experiments. (Continued on the following page.)
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 nmol/L 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 μmol/L of DCFDA for 30 minutes at 37°C with 5% CO₂. Fluorescence intensity in both procedures was 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 μmol/L) 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 (0.5 μmol/L, 1 μmol/L, 2 μmol/L, 4 μmol/L, and 10 μmol/L of CDDP concentration). The cells that could survive at 10 μmol/L of CDDP exposure were defined as the CDDP-resistant cells (KYSE150-R).

Quantitative 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 miR-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 × 10⁶ cells were grown 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 were started. A mixture of 1 nmol dsRNA and 200 μL of AtecloGene (KOKEN) was administered around the tumor (miR-NC to the left flank and miR-634 to the right flank of mice) at days 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 days 7 and 14. At 21 days after the injection of cells, mice were sacrificed and tumors were excised. 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 the Student 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, downregulate 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 nmol/L 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; Fig. 1A and B). This miR-634–induced cell death was not observed in normal cells, such as fibroblast and lymphoblastoid cell lines (LCL; Supplementary Fig. 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 (Fig. 1C); furthermore, these increased levels of cleaved-caspase-3 and -PARP were restored by simultaneous treatment with z-VAD-fmk (Fig. 1D). By FACS analysis, the sub-G₁ cell population, an indicator of apoptotic nuclei, was markedly increased in miR-634–expressing cells compared with 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; Fig. 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 Fig. S2), suggesting that concurrent downregulation 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 downregulated by miR-634, we performed expression array analysis on miR-NC– or miR-634–transfected cells in three cell lines: HeLa, U2OS, and...
KYSE850. As indicated in the Venn diagram in Fig. 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 (Fig. 2B; Supplementary Table S1). Interestingly, in silico analysis using IPA (http://www.ingenuity.com) and GO analysis revealed that several genes associated with mitochondrial biogenesis and antiapoptosis were significantly enriched among the predicted target genes (Fig. 2C; Supplementary Tables 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 antiapoptosis 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 (Fig. 2D). In addition, 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 (Fig. 2D and Supplementary Tables 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 NFE2, a known target gene, was also confirmed to be decreased in miR-634–expressing cells as we previously reported (Fig. 2D; ref. 10).

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 (Fig. 2E) were performed in U2OS cells. The luciferase activity of the WT vectors, except for one of the two seed sequences within the 3′-UTR of XIAP (Region-2), was significantly reduced compared with the empty vector in miR-634–expressing cells, and completely restored with Mut. vector, which had mutations within the seed sequences (Fig. 2E). These results suggest that miR-634 can directly downregulate expression of these 6 novel target genes, in addition to NFE2, 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 Fig. S3), strongly suggesting that concurrent downregulation 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 Fig. 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 Fig. S1).

**Induction of mitochondrial injury by miR-634**

To further evaluate the biologic 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 Fig. 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 (Fig. 3). These results suggest that overexpression of miR-634 triggers the activation of the mitochondrial apoptosis pathway. Furthermore, to estimate the contribution of the downregulation 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 (Fig. 3A). Fragmented mitochondria were observed in OPA1-knockdown cells as previously reported (17), but not in knockdown cells of other genes (Fig. 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 (Fig. 3C and D). These findings suggest that concurrent downregulation 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 (Fig. 3F; refs. 23, 24). Furthermore, these proteins were observed to be frequently colocalized at the same punctate structures in miR-634–expressing cells by immunofluorescence analysis (Fig. 3G). These findings suggest that miR-634 impairs autophagic degradation, possibly due to lysosomal dysfunction via concurrent downregulation 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 nmol/L) of miR-634 and simultaneously treated with CDDP or 5-FU showed drastically decreased cell viability (Fig. 4A). Furthermore, neither transfection with a low concentration (0.2 nmol/L) of miR-634 nor treatment with 2.5 μmol/L of CDDP alone was able to induce cell death, demonstrating combined treatment was necessary for markedly increased cell death (Fig. 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-
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 Fig. S5; ref. 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 Fig. S5). This suggests that upregulation of these miR-634–target genes may contribute to

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 nmol/L 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 nmol/L of miR-NC or miR-634 and were simultaneously incubated in medium with or without CDDP (2.5 μmol/L) for 3 days. Representative phase contrast images are shown in the top panel. The percentage of dead cells is indicated in the graph (bottom panel). C, Western blot 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, the band for cleaved caspase-3 or LC3B form-II. D, attenuation of CDDP resistivity by miR-634. KYSE150 (left) or its CDDP-resistant cells (KYSE150-R, right) were transfected with 0.2 nmol/L 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 with that of miR-NC–transfected cells without CDDP. Bar, SD for triplicate experiments. E, IC50 values based on the cell viability in D were calculated and are indicated in the graph.
acquired resistance to CDDP in KYSE150 cells. Importantly, cell viability assay showed that enforced expression of miR-634 at a low concentration (0.2 nmol/L) attenuated resistance to CDDP (IC50, 11.31 μmol/L in miR-NC-expressing cells, 2.45 μmol/L in miR-634–expressing cells; Fig. 4D and E). 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; Fig. 5A). There were no observations for any adverse events, such as body weight loss (Supplementary Fig. 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 ($P = 0.0081$; Fig. 5B–D). In addition, the enforced expression of miR-634 together with the downregulated expression of OPA1, BIRC5, and NRF2 proteins was confirmed in miR-634–treated tumors (Fig. 5E and F). 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 noncancerous tissue was observed in 25 cases (62.5%; Supplementary Fig. S7); this frequency confirms results from our previous report (10). However, there was no statistical significance in overall survival rate or recurrence-free survival rate between the patients with high or low expression of miR-634 (Supplementary Fig. 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–downregulated 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 downregulation 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 downregulation 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. In addition, concurrent downregulation 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 Fig. 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 physiologic function of miR-634 in environments that 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 has identified several miRNAs that can directly target cytoprotective processes, including the antiapoptosis pathway (XIAP, BIRC3), 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 are 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 nmol/L is not physiologic 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 nmol/L) 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 upregulation 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 noncancerous 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.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: N. Fujiwara, J. Inoue, J. Inazawa
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): N. Fujiwara, J. Inoue, T. Kawano
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Writing, review, and/or revision of the manuscript: N. Fujiwara, J. Inoue, T. Kawano, J. Inazawa
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K.-i. Kozaki
Study supervision: J. Inazawa

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Induction of Mitochondrial Apoptosis by miR-634
miR-634 Activates the Mitochondrial Apoptosis Pathway and Enhances Chemotherapy-Induced Cytotoxicity

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