Cell Death and Survival

The Impact of miRNA-Based Molecular Diagnostics and Treatment of NRF2-Stabilized Tumors

Shinsuke Yamamoto, Jun Inoue, Tatsuyuki Kawano, Ken-ichi Kozaki, Ken Omura, and Johji Inazawa

Abstract

NF-E2-related factor 2 (NRF2) is a master transcriptional regulator that integrates cellular stress responses and is negatively regulated by Kelch-like ECH-associated protein 1 (KEAP1) at the posttranslational level. In human cancers, aberrantly stabilized NRF2, either by mutation of NRF2 or KEAP1, plays a vital role in chemoresistance and tumor cell growth through the transcriptional activation of target genes, suggesting that targeted inhibition of NRF2 is a potential therapy for NRF2-stabilized tumors. MicroRNAs (miRNA) are endogenous small noncoding RNAs that can negatively regulate gene expression by interfering with the translation or stability of target transcripts. Moreover, tumor-suppressor miRNAs have been suggested to be useful for cancer treatment. Here, a reporter-coupled miRNA library screen identified four miRNAs (miR-507, -634, -450a, and -129-5p) that negatively regulate the NRF2-mediated oncogenic pathway by directly targeting NRF2. Importantly, down-regulation of these miRNAs, in addition to the somatic mutation of NRF2 or KEAP1, is associated with stabilized NRF2 and poor prognosis in esophageal squamous cell carcinoma (ESCC). Furthermore, administration of a miR-507 alone or in combination with cisplatin inhibited tumor growth in vivo. Thus, these findings reveal that miRNA-based therapy is effective against NRF2-stabilized ESCC tumors.

Implications: This study determines the potential of miRNA-based molecular diagnostics and therapeutics in NRF2-stabilized tumors. Mol Cancer Res; 12(1); 58–68. ©2013 AACR.

Introduction

NF-E2-related factor 2 (NRF2) is a master transcriptional regulator for cytoprotection against cellular damage from chemotherapy and oxidative stress (1, 2). Under physiologic conditions, NRF2 is ubiquitinated by the cullin 3 (CUL3) Kelch-like ECH-associated protein 1 (KEAP1) ubiquitin E3 ligase complex and is constantly degraded in the proteasome, resulting in a low cellular concentration of the NRF2 protein. Under cellular stress, KEAP1 is inactivated and NRF2 is stabilized in the nucleus, resulting in cell survival through the transcriptional activation of target genes to which NRF2 binds directly to the antioxidative responsive element (ARE) within the promoter in each of the target genes (3, 4). In addition to cellular stress response, it has been recently reported that NRF2 can also contribute to tumor cell growth by modulating metabolism (5). It has been found that gene mutations leading to a gain-of-function for NRF2 or loss-of-function for KEAP1 in various types of human cancers result in NRF2-mediated cancer cell survival and growth due to the constitutive activation of NRF2 (6–9). Interestingly, excess accumulation (as the aggregate) of the p62 protein, a substrate for protein degradation by autophagy, may also stabilize NRF2 by competitively interacting with KEAP1 (10–13). Thus, NRF2 has an oncogenic function in cancer cells, and a high level of NRF2 protein is associated with a poor prognosis (14–16). On the basis of this evidence, the therapeutic inhibition of the NRF2-mediated oncogenic pathway may benefit patients with NRF2-stabilized tumors.

MicroRNAs (miRNA) are endogenous small noncoding RNAs that regulate gene expression by interfering with the translation or stability of target transcripts through binding to the 3’-untranslated region (UTR; refs. 17, 18). Some miRNAs can negatively regulate oncogene(s), and the downregulation of tumor-suppressive miRNAs leads to the activation of oncogenic pathways (19–22). Indeed, we have identified novel tumor-suppressive miRNAs in endometrial cancer and oral squamous cell carcinoma through function-based screening using double-stranded RNAs (dsRNA) mimicking sequences of mature miRNAs.
Importantly, one transcript can be targeted by multiple miRNAs and conversely, one miRNA can target multiple transcripts (26, 27). This suggests that the down-regulation of multiple miRNAs, which target an oncogene, leads to the activation of that oncogenic pathway, and that the therapeutic administration of miRNA that can target multiple genes contributing to one oncogenic pathway may be effective.

In this study, by screening a miRNA library using the ARE-reporter system, we found that four miRNAs, miR-507, -634, -450a, and -129-5p, negatively regulate the NRF2-mediated oncogenic pathway by directly targeting NRF2. Importantly, these miRNAs were frequently down-regulated in esophageal squamous cell carcinoma (ESCC), and the aberrant gene expression of miRNAs in addition to somatic mutations of NRF2 or KEAP1 were closely associated with the increased expression of NRF2 protein and the poor prognosis in ESCC. Furthermore, miR-507 can also directly repress the expression of ME1, a known target of NRF2, and the administration of this miRNA inhibited tumor growth in vivo. Thus, our findings provide information on the potential miRNA-based molecular diagnosis and treatment of NRF2-stabilized tumors.

Materials and Methods

Cell culture and primary tumor samples

HeLa, LK-2, A549, and JHH-5 cells were purchased from the American Type Culture Collection. The cells were cultured in Dulbecco’s Modified Eagle Medium (for HeLa and LK-2 cells), RPMI 1640 medium (for A549 cells), or Williams’ E medium (for JHH-5 cells), RPMI 1640 medium (for A549 cells), or 10-cm dishes. After 24 hours of transfection, the cells (1 × 10⁴ cells per dish) on 10-cm dishes. After 24 hours of transfection, the cells (1 × 10⁴ cells per well) were seeded on 96-well plates, and the next day 20 nmol/L of each of 470 dsRNA from Pre-miR miRNA Precursor Library-Human V3 (Ambion) or Control-miRNA (negative control #1) were obtained from Ambion. Alternatively, the dsRNA mimicking human mature miRNA for miR-507 (C-300847-05) and Control-miRNA (CN-00200-01) from Thermo Scientific Dharmacon were also used. The siRNA for NRF2 (sGEME SMARTpool; www.thermofisher.com). Cell survival was assessed by the crystal violet staining assay. The cells were washed in PBS and fixed with 0.2% crystal violet in 10% formaldehyde in PBS for 3 minutes. Excess crystal violet solution was discarded and after being completely air-dried, the stained cells were lysed with a 2% SDS solution by shaking the plates for 1 hour. Optical density (OD) absorbance was measured at 560 nm using a microplate reader (ARVOmx; PerkinElmer), and the percentage 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.

Screening system using ARE-luciferase reporter

The luciferase reporter plasmid was generated by inserting the human NQO-1 promoter region (5'-TCAGCGCCCTCAATCGCAGTCACAGTGACTCAGAATC-3') containing the anti-oxidative element (ARE) into the MluI/XhoI site (Supplementary Table S4) within the pGL3-vector (Promega; ref. 29). First, the reporter plasmids were transiently transfected into HeLa cells (1 × 10⁴ cells per dish) on 10-cm dishes. After 24 hours of transfection, the cells (1 × 10⁴ cells per well) were seeded on 96-well plates, and the next day 20 nmol/L of each of 470 dsRNA from Pre-miR miRNA Precursor Library-Human V3 (Ambion) or Control-miRNA was transfected. After 2 days, Firefly luciferase activity was measured with a microplate reader (ARVOmx; PerkinElmer) using the Bright-Glo Luciferase Assay System (Promega). At the same time, living cells were enumerated by the crystal violet staining assay. ARE activity was calculated by the luciferase activity per living cell.

Transfection of miRNAs and siRNAs

A total of 20 nmol/L of miRNA or siRNA was transfected individually into cells using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s instructions. The dsRNA mimicking human mature miRNA for miR-507 (PM10509), miR-129-5p (PM10195), miR-450a (PM11192), and miR-634 (PM11538), and Control-miRNA (negative control #1) were obtained from Ambion. Alternatively, the dsRNA mimicking human mature miRNA for miR-507 (C-300847-05) and Control-miRNA (CN-00200-01) from Thermo Scientific Dharmacon were also used. The siRNA for NRF2 (sGEME SMARTpool; www.thermofisher.com). Cell survival was assessed by the crystal violet staining assay. The cells were washed in PBS and fixed with 0.2% crystal violet in 10% formaldehyde in PBS for 3 minutes. Excess crystal violet solution was discarded and after being completely air-dried, the stained cells were lysed with a 2% SDS solution by shaking the plates for 1 hour. Optical density (OD) absorbance was measured at 560 nm using a microplate reader (ARVOmx; PerkinElmer), and the percentage 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.

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M-003755-02-0005) and KEAP1 (siGENOME SMARTpool; M-012453-00-0005) were obtained from Thermo Scientific Dharmacon.

Conventional luciferase assay

Luciferase reporter plasmids were made by inserting the 3′-UTR of NRF2, ME1, or NQO1 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 (Invitrogen). Luciferase reporter plasmids and pTK plasmids as the internal control were cotransfected in HeLa cells, and the next day the dsRNA mimicking human mature miRNA or Control-miRNA was transfected. After 2 days, Firefly and Renilla luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega), and relative luciferase activity was calculated by normalizing the Firefly luciferase reading with its corresponding internal Renilla luciferase control.

Western blotting

Whole cell lysates were subjected to SDS-PAGE, and proteins were transferred to polyvinylidene difluoride (PVDF) membranes (GE Healthcare). After blocking with TBS containing 0.05% Tween-20 and 5% nonfat dry milk for 1 hour, the membrane was reacted with an antibody, overnight. The dilutions for primary antibodies were, rabbit anti-ME1 (1/1,000), and mouse anti-KEAP1 (1/1,000), rabbit anti-NRF2 (1/1,000), and mouse anti-β-actin (1/5,000). The membrane was washed and exposed to horse-radish peroxidase (HRP)–conjugated anti-mouse or rabbit immunoglobulin G (IgG) antibodies (both at 1/5,000) for 2 hours. The bound antibodies were visualized with HRP conjugated anti-mouse or rabbit immunoglobulin G (IgG) antibodies (both at 1/5,000) for 2 hours. The bound antibodies were visualized with HRP staining solution or with an ECL Western detection kit according to the manufacturer’s instructions (Cell Signaling Technology).

Quantitative reverse transcription PCR

Total RNA was isolated using TRIzol reagent (Invitrogen) according to standard procedures. Single-stranded cDNA generated from the total RNA was amplified with a primer set specific for each gene. The quantitative RT–PCR (qRT-PCR) was performed using the KAPA SYBR system (Kapa Biosystems) and an ABI PRISM 7500 sequence detection System (Applied Biosystems) according to the manufacturer’s instructions. Gene expression values are given as ratios (differences between the Ct values) between the genes of interest and an internal reference (glycerinaldehyde-3-phosphate dehydrogenase, GAPDH) or U6, and subsequently normalized with the value in the control cells (relative expression level). Information on the primers and TaqMan probes used is provided in Supplementary Table S2 and S3.

For miRNAs, qRT-PCR was performed using an ABI Prism 7500 Fast Real-time PCR System (Applied Biosystems), Taqman Universal PCR Master Mix (Applied Biosystems), Taqman Reverse Transcription kit (Applied Biosystems), and Taqman MicroRNA Assays (Applied Biosystems), according to the manufacturer’s instructions. Expression levels of miRNA genes were based on the amount of the target message relative to that of the RNU6B transcript as a control to normalize the initial input of total RNA.

Immunohistochemistry

Tumor samples were fixed with 10% formaldehyde in PBS, embedded in paraffin, sectioned into 4-μm-thick slices, and subjected to immunohistochemical staining of NRF2 or ME1 with the avidin–biotin–peroxidase method as described previously (30). Sections from paraffin-embedded tumor samples were deparaffinized with xylene and rehydrated in ethanol. After the retrieval of antigens by boiling in 10 mmol/L citrate buffer (pH 6.0), the sections were treated with 0.3% hydrogen peroxide in methanol to inactivate the endogenous peroxidase. Then, the sections were incubated with an antibody against NRF2 (dilution; 1/1,000) or ME1 (1/500) at 4°C overnight. The bound antibody was visualized using diaminobenzidine as a chromogen (VECTASTAIN Elite ABC kit; Vector Laboratories), and the sections were lightly counterstained with hematoxylin.

Mutation analysis of NRF2 and KEAP1

The genomic regions containing exon 2 of NRF2 or all coding regions of KEAP1 were amplified by PCR using KOD-plus (Toyobo). PCR products were purified using ExoSAP-IT (GE healthcare) and analyzed by sequencing. Information on primers is provided in Supplementary Table S2.

Expression array analysis and pathway analysis using the ingenuity pathway analysis

For the gene expression array analysis, the Agilent 4 × 44 K gene expression array was used according to the manufacturer’s instructions (Agilent Technologies). Each gene array experiment was performed in duplicate and the data were analyzed in GeneSpring (Agilent Technologies).

The expression data and data from the TargetScan program were subjected to an ingenuity pathway analysis (IPA; Ingenuity Systems).

In vivo tumor growth assay and administration of miRNAs

Seven-week-old female BALB/c nude mice were purchased from the Oriental Yeast Co. Ltd and maintained under specific pathogen-free conditions. A total of 1.0 × 10⁷ cells in 200 μL of PBS were subcutaneously injected into the flank of mice. A mixture of 1 nmol of dsRNA (Ambion) and 200 μL of AteroGene (Koken) was administered into the spaces between the tumor and skin. Mice were treated with CDDP by intraperitoneal administration at a dose of 5 mg/kg body weight. At 35 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 the Student t test. Clinicopathologic variables pertaining to the corresponding patients were analyzed for statistical significance by a \( \chi^2 \) or Fisher exact test. For the analysis of survival, Kaplan–Meier curves were constructed for groups based on univariate predictors, and differences between groups were tested by log-rank test. Results of in vivo experiments and the cell survival assay in A549 cells were analyzed for statistical significance using a two-way ANOVA. A calculated P value of <0.05 was considered statistically significant.

Results

Screening of miRNAs negatively regulating the transcriptional activity of NRF2

To identify miRNA negatively regulating the transcriptional activity of NRF2, we screened a miRNA library using the luciferase reporter system, which enabled the measurement of luciferase gene expression driven through the ARE (29). With this reporter system, we confirmed the ARE activity to be actually changed in a manner dependent on the level of NRF2 protein in HeLa cells (Supplementary Fig. S1). For the screening of 470 dsRNAs, we sequentially transfected the reporter plasmid together with each miRNA into HeLa cells, and then calculated the ARE activity in the transfected cells as the luciferase activity per living cell, as measured by the crystal violet staining assay (Fig. 1A). Eight candidate miRNAs demonstrated a relative ratio of ARE activity compared with Control-miRNA–transfected cells of less than 0.5 (Fig. 1B and Supplementary Table S1). We focused on the top four miRNAs (miR-507, -634, -450a, or -129-5p), and Control-miRNA was transfected. After 48 hours of transfection with miRNAs, Firefly or Renilla luciferase activity was measured. The ARE activity relative to that in Control-miRNA–transfected cells is indicated on the vertical axis. Bar, SD. D, expression analysis of NQO1 mRNA in miRNA–transfected cells. HeLa cells were transfected with miR-507, -634, -450a, or -129-5p, or Control-miRNA. After 48 hours of transfection, the mRNA level of the NQO1 gene was measured by qRT-PCR. The expression of the GAPDH gene was used as the internal control. The expression level relative to that in Control-miRNA–transfected cells is indicated on the vertical axis. Bar, SD.
Figure 2. Identification of NRF2 as a direct target of four candidate miRNAs. A, expression analysis of NRF2 in the miRNA-transfected cells. HeLa cells were transfected with miR-507, miR-634, miR-450a, or miR-129-5p, or Control-miRNA. After 48 hours of transfection, the cell lysate was subjected to SDS-PAGE and immunoreacted with the indicated antibodies (top). The mRNA level of the NRF2 gene was measured by qRT-PCR (bottom). The expression of the GAPDH gene was used as the internal control. (Continued on the following page.)
were transfected with SD. D, effect of the transfection of miRNAs on cell survival under cellular stress caused by cisplatin (CDDP) treatment (left) or oxidative stress (right). HeLa cells protein but also signiﬁcantly reduced compared with that for the empty and R1 vectors in cells transfected with each miRNA, compared with Control-miRNA–transfected cells (Fig. 2A). The downregulation of NRF2 by overexpression of those miRNAs was also observed in NRF2-stabilized cancer cell lines, LK2 (non–small cell lung cancer, NSCLC) having a NRF2 mutation, A549 (NSCLC) having a KEAP1 mutation, and JHH-5 (hepatocellular carcinoma) having p62 aggregation (Supplementary Fig. S3; refs. 3, 10, 15). Therefore, to examine whether each miRNA could directly bind to the 3′-UTR of NRF2, we next performed luciferase assays using reporter plasmid vectors having each of two fragments of the 3′-UTR, region-1 (R1) and region-2 (R2), or five different mutants of R2, respectively (Fig. 2B). The luciferase activity for the R2 vector was signiﬁcantly reduced compared with that for the empty and R1 vectors in cells transfected with each miRNA, and completely restored with vectors having mutations within seed sequences of R2 (Fig. 2C). Furthermore, transfection of each miRNA not only inhibited the expression of NRF2 protein but also signiﬁcantly increased sensitivity to treatment with cisplatin (CDDP) or exposure to H2O2, compared with Control-miRNA–transfected cells (Fig. 2D). These results suggest that all four candidate miRNAs can functionally target NRF2 by directly binding to its 3′-UTR, probably inhibiting NRF2-mediated cancer cell survival despite elevated cellular stress.

Identification of NRF2 as a functional target of the candidate miRNAs

To identify the functional target for the four candidate miRNAs, we ﬁrst explored whether genes known to be associated with the NRF2 pathway exist among the targets predicted by the TargetScan program (http://www.targetscan.org) using the IPA (Ingenuity Systems Pathway Analysis; http://www.ingenuity.com). By this in silico analysis, we unexpectedly found that all four candidate miRNAs might functionally target NRF2 itself, because the seed sequences for three miRNAs, though not miR-450a, were mapped within the 3′-UTR of NRF2 (2 sites for miR-507, 1 site for miR-634, and 2 sites for miR-129-5p). In addition, the seed sequence for miR-450a was highly homologous to that for miR-507 (6 of 7 seed sequences). Indeed, we showed that the level of NRF2 protein was remarkably decreased in cells transfected with each miRNA, compared with Control-miRNA–transfected cells (Fig. 2A). The downregulation of NRF2 by overexpression of those miRNAs was also observed in NRF2-stabilized cancer cell lines, LK2 (non–small cell lung cancer, NSCLC) having a NRF2 mutation, A549 (NSCLC) having a KEAP1 mutation, and JHH-5 (hepatocellular carcinoma) having p62 aggregation (Supplementary Fig. S3; refs. 3, 10, 15). Therefore, to examine whether each miRNA could directly bind to the 3′-UTR of NRF2, we next performed luciferase assays using reporter plasmid vectors having each of two fragments of the 3′-UTR, region-1 (R1) and region-2 (R2), or five different mutants of R2, respectively (Fig. 2B). The luciferase activity for the R2 vector was signiﬁcantly reduced compared with that for the empty and R1 vectors in cells transfected with each miRNA, and completely restored with vectors having mutations within seed sequences of R2 (Fig. 2C). Furthermore, transfection of each miRNA not only inhibited the expression of NRF2 protein but also signiﬁcantly increased sensitivity to treatment with cisplatin (CDDP) or exposure to H2O2, compared with Control-miRNA–transfected cells (Fig. 2D). These results suggest that all four candidate miRNAs can functionally target NRF2 by directly binding to its 3′-UTR.

Clinical signiﬁcance of the downregulation of the four identiﬁed miRNAs to ESCC

We examined the level of each miRNA by qRT-PCR analysis in 30 paired samples from patients with ESCC. In this analysis, a more than 50% reduction in expression in primary tumor tissue compared with the corresponding noncancerous tissue was observed in nine cases (30.0%) for miR-507, 12 cases (40.0%) for miR-634, two cases (6.7%) for miR-450a, and 18 cases (60.0%) for miR-129-5p (Fig. 3A and Supplementary Fig. S4). In addition, mutation analysis led us to ﬁnd a missense mutation of NRF2 in three cases (10%) and KEAP1 in two cases (6.7%; Fig. 3A and Supplementary Fig. S5). On the basis of a positive “aberration score” for the mutation of NRF2 or KEAP1 and/or the downregulation of each miRNA expression, we assigned 30 cases to two groups; 14 ESCC cases with 2 to 4 aberrations to a “high score group” and 16 ESCC cases with 0 to 1 aberration to a “low score group” (Fig. 3A). Importantly, the “high score group” was signiﬁcantly correlated with distant metastasis (pM categories, P = 0.0394, Table 1) and closely associated with a worse overall survival as shown by the Kaplan–Meier survival estimates (log-rank test; P = 0.004; Fig. 3B). Immunohistochemical analysis of ESCC samples, which were selected randomly, demonstrated that levels of NRF2 protein were markedly higher in the “high score group” than “low score group” (Fig. 3C). These ﬁndings suggest that the downregulation of expression for these miRNAs in addition to the mutation of NRF2 or KEAP1 also contributes to the NRF2 stabilization in ESCC tumors. Furthermore, an increase in the “aberration score” may be helpful to clarify NRF2-stabilized tumors and to evaluate the poor prognosis of ESCC.

In vivo tumor-suppressive effects by administration of miR-507

Because one miRNA can target multiple genes contributing to one oncogenic pathway, the miRNA may be effective against tumors with this oncogenic pathway. Here, we focused on miR-507 among the four identiﬁed miRNAs and examined whether it can also functionally target genes regulated transcriptionally by NRF2 using expression array
and pathway analyses. In these analyses, we found eight transcriptional target genes of NRF2 to be repressed by transfection of miR-507, and among them, ME1 was also confirmed to be a direct target for miR-507 through binding to the 3'-UTR of NRF2 (Supplementary Fig. S6). These results suggest that miR-507 can inhibit the NRF2-mediated oncogenic pathway by directly targeting both NRF2 and its target gene(s). Indeed, the effect for increased sensitivity to treatment with CDDP by miR-507 was canceled by exogenous overexpression of NRF2 protein, suggesting that its effect was dependent on the miR-507-mediated inhibition of NRF2 (Supplementary Fig. S6). Furthermore, we confirmed the increased sensitivity to treatment with CDDP by miR-507 was confirmed by using different dsRNA (Supplementary Fig. S6). Taken together, these findings suggest that miR-507 can robustly inhibit the NRF2 pathway.

Next, we examined the tumor-suppressive effect in vivo of dsRNA mimicking miR-507 or Control-miRNA into the subcutaneous space surrounding tumors formed from A549 cells. Combined treatment with carboplatin and NRF2-specific siRNA was reported to be effective in inhibiting the growth of tumors formed from A549 cells by Singh and colleagues (31), and this cell line was assigned to the "high score group" with the KEAP1 mutation and the downregulation of three miRNAs, miR-507, miR-634, and miR-129-5p, except for miR-450a (Supplementary Fig. S7A). Furthermore, we showed that the exogenous overexpression of miR-507 mildly inhibited cell growth and increased sensitivity to cellular stress by CDDP in vitro (Supplementary Fig. S7B and S7C). Control-miRNA or miR-507 was administered around the tumors subcutaneously formed from A549 cells weekly a total for four times (7, 14, 21, and 28 days after the injection of A549 cells; Fig. 4A). Furthermore, mice were treated with PBS or CDDP by intraperitoneal administration the next day a total of three times (8, 15, and 22 days after the injection of A549 cells). Tumor

Figure 3. Association between clinical significance and downregulation of miRNAs in primary tumor samples from ESCC. A, summary of results for the expression analysis of four miRNAs (miR-507, -634, -450a, and -129-5p) and the mutation analysis of the NRF2 or KEAP1 gene in 30 primary tumor samples. Filled squares indicate the presence of a mutation for the NRF2 or KEAP1 gene or the downregulation of miRNAs in each case. The number of aberrations in each case was represented as the aberrant score (0, 1, 2, 3, or 4), and 30 cases were assigned into two groups based on the "aberration score": a "high score group" (n = 14; "aberration score" of 2, 3, or 4), and a "low score group" (n = 16; "aberration score" of 0 or 1). B, Kaplan-Meier curves for overall survival rates of 30 patients with ESCC according to the aberrant score. The "high score group" was significantly associated with a worse survival rate (P = 0.004, log-rank test). C, representative images for immunostaining of the NRF2 protein in primary ESCC tumors from the "high score group" (Case-1, -4, and -7; top) and "low score group" (Case-21, -25, and -26; bottom). Scale bar, 100 μm.
found that four miRNAs, miR-507, -634, -450a, and -129-5p, that directly target the NRF2 gene can repress the NRF2-mediated oncogenic pathway. Although recent studies have demonstrated that miR-28 (32) and miR-144 (33) can directly target NRF2, these miRNAs were not highly ranked as candidates that negatively regulate ARE activity in our analysis. NRF2 is constitutively stabilized in various types of human cancers by several mechanisms, including gain-of-function mutations of NRF2, loss-of-function mutations of KEAP1, and the functional inactivation of KEAP1 through p62 aggregation. In addition, here, we showed that four novel miRNAs are aberrantly downregulated in some ESCC cases. Importantly, the aberrant downregulation of one or more miRNAs accompanied by the mutation of NRF2 or KEAP1 was closely associated with NRF2 stabilization in ESCC tumors and the poor prognosis of patients with ESCC. Thus, the downregulation of these miRNAs may help to increase the basal level of NRF2 expression and synergistically stabilize the NRF2 protein with or without genetic aberrations of NRF2 or KEAP1 in ESCC (Fig. 5). Because the inhibition of NRF2 activity is clearly a rational approach to the treatment of NRF2-stabilized tumors, examination of the expression status of the four miRNAs directly targeting NRF2 in this study, in addition to the genetic status of NRF2 and KEAP1, in ESCC, may facilitate the screening of NRF2-stabilized tumors.

Previous studies, including ours (24), have demonstrated that one miRNA can inhibit the expression of multiple targets by directly binding to the 3′-UTR of each gene, suggesting that one miRNA concurrently targets several genes related to one signaling pathway. Actually, miR-16 is known to act as a negative regulator of tumor development and metastasis by directly targeting multiple genes, CCND1, CDK4, CDK6, and MYC (35). Thus, it has been generally considered that the reexpression and/or administration of tumor-suppressor miRNA(s), which can repress one oncogenic pathway, may be effective against tumors with this pathway (19). In this study, we showed that the administration of miR-507 was effective in inhibiting the growth of tumors formed from A549 cells in nude mice through probably targeting NRF2 as well as ME1, a known transcriptional target of NRF2. Although our in vitro analysis revealed that the transfection of miR-507 led to an increase in the sensitivity of cell-growth suppression with cisplatin (CDDP) in A549 cells, we could not show a synergistic suppressive effect in vivo on combined treatment with miR-507 and CDDP in subcutaneous tumors formed from A549 cells in nude mice. In addition to cellular stress in response to chemotherapies, NRF2 also promotes tumor cell growth by modulating metabolism (5). Indeed, we showed the tumor growth to be inhibited by miR-507 both in vitro and in vivo. Thus, the growth inhibition by miR-507 may be attributed to changes in intracellular metabolism through repression of NRF2 and its target genes.

The inhibitory regulation of the NRF2-mediated oncogenic pathway may be a promising strategy for anticancer
Figure 4. Tumor-suppressive effect of miR-507 in vivo. A, the experimental schedule for the combined treatment with miR-507 and CDDP. Tumors were formed by subcutaneous injection of A549 cells in nude mice. Control-miRNA or miR-507 was administered around tumors subcutaneously formed from A549 cells a total of four times (7, 14, 21, and 28 days after the injection of A549 cells). In addition, mice were treated with PBS or CDDP by intraperitoneal administration the next day a total of three times (8, 15, and 22 days after the injection of A549 cells). At 35 days after the injection of A549 cells, mice were sacrificed and the tumors were resected. B, representative images of tumor-bearing nude mice (left) and the resected tumors (right) at 35 days after the injection of A549 cells. C, weight of the resected tumors. Mice were sacrificed at 35 days after the injection of A549 cells, and the weight of each resected tumor was measured. The graph indicates the average ± SD from the results in 4 PBS-treated mice or 3 CDDP-treated mice. Significant differences were analyzed by two-way ANOVA: *P = 0.0486 between Control-miRNA and miR-507; P = 0.5716 between PBS and CDDP. The interaction (miR-507 × CDDP) was P = 0.8757, FmiR-507 = 170.667, FcDDP = 13.4444, FmiR-507 x cDDP = 0.026061. D, expression analysis of miR-507 in the resected tumors. The mRNA expression level of miR-507 was measured by qRT-PCR. The expression of RNU6B was used as the internal control. The expression level relative to that in the Control-miRNA-administered tumor in PBS-treated mice is indicated on the vertical axis. Bar, SD. E, representative images for immunostaining of NRF2 and ME1 in the tumors resected. Scale bar, 100 μm.
MicroRNAs and NRF2-Stabilized Cancer

NRF2-mediated oncogenic pathway at the basal level. MicroRNA expression can negatively regulate the NRF2 activity (38, 39). In particular, drugs such as sulforaphane and curcumin that activate NRF2 are now undergoing clinical trials for cancer chemoprevention (39). These findings suggest the importance of choosing a therapeutic approach in a cellular context-dependent manner according to NRF2 status (38, 39). Furthermore, accurate delivery of the agents to the target tumors is necessary to increase therapeutic potential and reduce possible side effects of miRNA-based therapy (19). When these problems are overcome, diagnosis and treatment using the four miRNAs identified in this study will be useful. Thus, our findings provide a novel approach to the miRNA-based molecular diagnosis and treatment of NRF2-stabilized tumors.

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

Figure 5. Schematic models for the association between NRF2 stabilization and miRNA expression. A, the expression of the four miRNAs, miR-507, -634, -450a, and -129-5p, negatively regulates the stability of NRF2 by directly targeting NRF2. In addition, miR-507 can also directly repress the expression of transcriptional target genes of NRF2. B, in cancer cells, the downregulation of expression for these miRNAs may lead to NRF2’s stabilization, and in cancer cells with a mutation of the NRF2 or KEAP1 gene or aggregation of p62 synergistically contribute to its stabilization. Thus, these miRNAs may negatively regulate the NRF2-mediated oncogenic pathway at the basal level.

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Shinsuke Yamamoto, Jun Inoue, Tatsuyuki Kawano, et al.


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