miR-3174 Contributes to Apoptosis and Autophagic Cell Death Defects in Gastric Cancer Cells by Targeting ARHGAP10

Bowen Li,1,4 Lu Wang,1,4 Zheng Li,1,4 Weizhi Wang,1,4 Xiaofei Zhi,2,4 Xiaoxu Huang,1 Qiang Zhang,1 Zheng Chen,3 Xuan Zhang,1 Zhongyuan He,1 Jianghao Xu,1 Lu Zhang,1 Hao Xu,1 Diancai Zhang,1 and Zekuan Xu1

1Department of General Surgery, The First Affiliated Hospital of Nanjing Medical University, Nanjing 210029, Jiangsu, China; 2Department of General Surgery, The Affiliated Hospital of Nantong University, Nantong 226001, Jiangsu, China; 3Department of Surgery, Vanderbilt University Medical Center, Nashville, TN 37232, USA

Gastric cancer (GC) is a major health problem worldwide because of its high morbidity and mortality. Considering the well-established roles of miRNA in the regulation of GC carcinogenesis and progression, we screened differentially expressed microRNAs (miRNAs) by using The Cancer Genome Atlas (TCGA) and the GEO databases. We found that miR-3174 was the most significantly differentially expressed miRNA in GC. Ectopic miR-3174 expression was also detected in clinical GC patient samples and cell lines and associated with poor patient prognosis. Apoptosis and autophagic cell death are two types of programmed cell death, whereas both are deficient in gastric cancer. Our functional analyses demonstrated that miR-3174 inhibited mitochondria-dependent apoptosis and autophagic cell death in GC. Moreover, high expression of miR-3174 also resulted in Cisplatin resistance in GC cells. Using bioinformatics analyses combined with in vitro and in vivo experiments, we determined that miR-3174 directly targets ARHGAP10. Notably, ARHGAP10 promotes mitochondria-dependent apoptosis by enhancing p53 expression, which was followed by Bax trans-activation and caspase cleavage. ARHGAP10 also facilitated autophagic cell death by suppressing mammalian target of rapamycin complex 1 (mTOC1) activity. Our results reveal a potential miRNA-based clinical therapeutic target that may also serve as a predictive marker for GC.

INTRODUCTION

Gastric cancer (GC) is a major health problem worldwide because of its high morbidity and mortality. Considering the well-established roles of miRNA in the regulation of GC carcinogenesis and progression, we screened differentially expressed microRNAs (miRNAs) by using The Cancer Genome Atlas (TCGA) and the GEO databases. We found that miR-3174 was the most significantly differentially expressed miRNA in GC. Ectopic miR-3174 expression was also detected in clinical GC patient samples and cell lines and associated with poor patient prognosis. Apoptosis and autophagic cell death are two types of programmed cell death, whereas both are deficient in gastric cancer. Our functional analyses demonstrated that miR-3174 inhibited mitochondria-dependent apoptosis and autophagic cell death in GC. Moreover, high expression of miR-3174 also resulted in Cisplatin resistance in GC cells. Using bioinformatics analyses combined with in vitro and in vivo experiments, we determined that miR-3174 directly targets ARHGAP10. Notably, ARHGAP10 promotes mitochondria-dependent apoptosis by enhancing p53 expression, which was followed by Bax trans-activation and caspase cleavage. ARHGAP10 also facilitated autophagic cell death by suppressing mammalian target of rapamycin complex 1 (mTOC1) activity. Our results reveal a potential miRNA-based clinical therapeutic target that may also serve as a predictive marker for GC.

Apoptosis and autophagy, two methods for programmed self-destruction of cells, have long been topics of investigation by the research community. Apoptosis, also known as type I cell death, is one of the best-described types of programmed cell death (PCD), and it activates cellular death signaling cascades such as mitochondria-dependent and death receptor-mediated signaling, which are followed by nuclear chromatin condensation and nuclear fragmentation. In contrast, autophagy is an intracellular self-catabolic degradation process. Under normal conditions, autophagosomes (APs) package and transport bulk cytoplasm, damaged proteins, and organelles to lysosomes to be digested, recycled, and converted to energy, thereby ultimately supporting cancer cell survival or proliferation. However, excessive autophagy can also induce autophagic cell death (ACD), which is also known as type II cell death, another type of PCD. In early ACD, in contrast to the classic morphological changes caused by apoptosis, large numbers of organelles are digested, but cytoskeletal elements are preserved, and this whole process is caspase independent. Typically, stimuli can induce either apoptosis or ACD; however, both modes of death can be simultaneously induced in response to the same stimulation. These characteristics might be attributed to the variable thresholds of both processes. Recently, several genes and pathways have been reported to be involved in the link between apoptosis and ACD, thus indicating that these processes do not occur through isolated pathways. However, cancer cells can develop multiple mechanisms that promote malignant processes, such as continuous proliferation and avoidance of death. Both types of PCD are defective in cancer, and there is currently a lack of suitable therapeutic methods that can simultaneously target both ACD and...
apoptosis. Thus, there is an urgent need to discover therapeutic targets that can reestablish PCD in cancer cells.

MicroRNAs (miRNAs) play significant roles in many types of diseases and regulate the expression of oncogenes and tumor suppressors as well as entire signaling pathways involved in tumorigenesis and cancer progression. miRNAs can also potentially serve as clinical therapeutic targets and predictive markers for GC. Using bioinformatics analysis, we discovered a miRNA, miR-3174, that was significantly upregulated in GC tissues and cell lines. Because the role of miR-3174 has seldom been reported previously, we focused on uncovering the biological function of this miRNA in GC.

ARHGAP10, a member of the Rho GTPase-activating protein family (RhoGAP), catalyzes the GTP of Rho GTPases to GDP, such as RhoA and CDC42, and subsequently inhibits the activities of Rho GTPase as well as its downstream signaling cascade. In addition to a RhoGAP domain, ARHGAP10 also possesses a SRC homology 3 (SH3) domain and a Pleckstrin homology (PH) domain. This configuration allows ARHGAP10 to interact with many other protein species and to regulate multiple cellular processes, such as cytoskeleton and cell junction formation, cardiac mechanical stress, and vesicular trafficking of Golgi bodies. Luo et al. have found that ARHGAP10 plays a tumor-suppressor role in ovarian cancer by inhibiting the activity of CDC42. Azzato et al. have described single-nucleotide polymorphisms of ARHGAP10 in breast cancer. Collectively, these results indicate an association between ARHGAP10 and cancer; however, the mechanism underlying this relationship is unknown.

In the present study, we provide the evidence that miR-3174 expression is significantly upregulated in GC. Further experiments confirmed that miR-3174 upregulation simultaneously inhibits ACD and apoptosis in GC cells by downregulating the expression of ARHGAP10. Upregulation of miR-3174 expression also contributes to cisplatin resistance in GC.

RESULTS

miR-3174 Is Upregulated in Gastric Cancer and Is Associated with Poor Prognosis

To identify candidate miRNAs with a role in GC, we examined changes in miRNA expression (fold change ≥ 2) among 446 GC tissues compared to 45 normal tissues from The Cancer Genome Atlas (TCGA) database and combined these results with the GEO datasets (GEO78091, fold change ≥ 2). Five miRNAs were upregulated; three were downregulated in both TCGA and the GEO libraries. RT-PCR was used to further validate the expression of these eight miRNAs (means and standard deviations of each group of miRNA expression profile were displayed in Table S1). Our results showed that the expression of miR-3174, miR-17-5p, and miR-19a-3p was increased (Figures 2A, 2E, and 2G), whereas there was no...
significant difference in miR-93-5p or miR-20a-5p expression among 50 GC tissues compared with paired adjacent normal tissues (Figures 2C and 2H). The most significant change in expression was observed for miR-3174, thus prompting us to further examine the role of this miRNA in GC malignancy. High expression of miR-3174 was confirmed in an additional 100 paired GC tissues (Figures 2I and 2J). miR-3174 reads with transcripts per million (Tpm) correction were significantly higher in 446 GC tissues compared with 45 healthy tissues in TCGA (Figure 2K). In addition, miR-3174 expression was also increased in several GC cell lines compared with the normal stomach mucosa cell line GES-1 (Figure 2L). Using the median expression value of miR-148a-3p as a cutoff point, the cohort was dichotomized into miR-148a-3p high and low groups (means or variance values of two groups were shown in Table S1); univariate analysis of clinicopathologic features showed that the relative expression of miR-3174 was correlated with tumor size, tumor node metastasis (TNM) stage, and T stage of GC patients (Table S2); furthermore, high expression of miR-3174 contributed to poor prognosis and significantly lower five-year overall survival (Figure 2M). These data indicate that miR-3174 is probably an onco-miRNA in GC.

miR-3174 Inhibits Mitochondria-Dependent Apoptosis in GC Cells

Flow cytometry assays revealed that upregulation of miR-3174 prominently decreased the apoptotic rate in MKN45 cells, whereas repression of miR-3174 improved the apoptotic rate in BGC823 cells. In addition, the pro-apoptotic effect of miR-3174 inhibition was recovered by Z-VAD-FMK, a pan-caspase inhibitor, in BGC823 cells, and the anti-apoptotic effect of miR-3174 reconstitution was similar to the effect of Z-VAD-FMK in MKN45 cells (Figures 3A and 3B). These results were further verified by caspase-3 activity detection kit and cell viability assay (Figures 3C and 3D). To confirm that the inhibition of apoptosis caused by miR-3174 was mitochondria dependent, we measured mitochondrial membrane potential (ΔΨm, MMP) by using JC-1 probes. As expected, miR-3174 reconstitution increased, whereas miR-3174 downregulation decreased the ΔΨm compared with that in controls (Figures 3E and 3F). Besides, levels of cleaved caspase-3 and Bax, both of which play important roles in mitochondria-dependent apoptosis, were decreased in MKN45 cells with miR-3174 reconstitution but increased in BGC823 cells with miR-3174 suppression, whereas Bcl-2 was unchanged regardless of altering miR-3174 expression (Figure 3G, upper panel). Furthermore, another protein that participates in mitochondria-dependent apoptosis pathway, cytochrome c, released from mitochondria to cytosol in miR-3174-suppressed cells but accumulated in mitochondria in miR-3174-overexpressed cells (Figure 3G, lower panel). Thus, we conclude that miR-3174 inhibits mitochondria-dependent apoptosis in GC.

miR-3174 Reinforces the CDDP Resistance in GC Cells

cis-diamine dichloroplatinum/cisplatin (CDDP)-based chemotherapy is the first-line regimen for advanced and metastatic GC. On the basis of the relationship between apoptosis or autophagy with chemo-sensitivity in cancer, we speculated that miR-3174 might also decrease the cytotoxicity of CDDP in GC. To test this hypothesis, MKN45 and BGC823 cells resistant to CDDP (referred to as MKN45CDDP and BGC823CDDP cells, respectively) were established. The CDDP-resistant features of these two cell lines were validated through cell viability assays and subsequent IC50 evaluation (Figures S1B–S1D). miR-3174 expression was significantly
upregulated in MKN45 and BGC823 CDDP-resistant cells compared with the corresponding drug-sensitive parental cells (Figure 5A). miR-3174 reconstitution decreased cell viability with various concentrations of CDDP treatment and elevated the IC_{50} in MKN45 and BGC823 cells (Figures 5B, 5D, 5F, and 5H), whereas downregulation of miR-3174 displayed the reverse effects in MKN45CDDP and BGC823CDDP cells (Figures 5C, 5E, 5G, and 5I). Colony formation assay verified that miR-3174-reconstituted CDDP-sensitive cells showed stronger long-term viability than control cells (Figures 5J, S2B, and S2C, left panels); the opposite results were acquired in miR-3174-depressed CDDP-resistant cells (Figures 5K, S2B, and S2C, right panels). These data indicate that miR-3174 facilitates CDDP resistance in GC cells.

ARHGAP10 Is a Direct Downstream Target Gene of miR-3174

To identify miR-3174 target genes, we screened TCGA for genes that were significantly downregulated in GC (fold change ≥ 2) (Figure 6A) and combined these results with predictive target information regarding miR-3174 obtained from TargetScan (http://www.targetscan.org/). Using this strategy, 345 genes were selected. Subsequently, we identified 466 genes that may increase CDDP sensitivity in 28 GC cell lines based on the Genomics of Drug Sensitivity in Cancer (GDSC) database (Figure 6B; Table S3; p < 0.05) and combined these results with the previous 345 genes. Ultimately, 21 genes were identified that meet the following criteria: (1) are downregulated in 375 GC tissues compared with 32 normal gastric tissues on TCGA (Figure 6C), (2) exhibit CDDP sensitization effect according to GDSC, and (3) are the potential targets of miR-3174 according to TargetScan (Figure 6D). Negative correlations between miR-3174 expression and these 21 potential targets were subsequently verified in GC and normal tissues by the statistics acquired from TCGA (Figures 7A and 7B), whereas no significant changes in ARHGAP10 and CLMP expression were observed (Figures 7C and 7D). Furthermore, CLMP and ARHGAP expression levels showed a clear negative correlation with miR-3174 expression in the GC tissues (Figures 7E and 7F), whereas no clear relationship was observed between ZNF471 or LDOC1 expression and miR-3174 expression (Figures 7G and 7H). Moreover, the augmentation or inhibition of miR-3174 expression respectively downregulated or upregulated the expression of CLMP and ARHGAP10, but not ZNF471 and LDOC1, at both the mRNA and protein level (Figures 7I and 7J). Luciferase reporter assays further revealed that the bioluminescence intensities in miR-3174-reconstituted BGC823 and MKN45 cells decreased after transfection with the wild-type 3' UTRs of ARHGAP10 and CLMP. However, there were no significant differences after transfected wild-type 3' UTRs of ZNF471 or LDOC1 and mutant-type of 3' UTRs of these four genes in cells with or without miR-3174 reconstitution (Figures 7K and 7L). Constructs specific to these four genes were then transfected into GC cells (Figure S2D), and cell viability was measured with CCK-8 assays. Besides, only interference sequence of ARHGAP10 or LDOC1 treatment could increase the viability of MKN45 cells (Figure 7M). Collectively, we further focus on ARHGAP10, a potential tumor suppressor in GC and direct downstream target of miR-3174.

ARHGAP10 Restores the Effects of miR-3174 on Apoptosis and ACD as well as CDDP Cytotoxicity in GC Cells

JC-1 staining revealed that ARHGAP10 overexpression decreased MMP levels compared with that in the control group, and, in MKN45 cells, reduced the MMP level that was upregulated by miR-3174 reconstitution (Figures 8A, left panel, and S3A). In addition, ARHGAP10 repression not only increased MMP level but also restored the downregulation of MMP caused by miR-3174 inhibition in BGC823 cells (Figures 8A, right panel, and S3B). Flow cytometry assay showed that upregulation of ARHGAP10 expression promoted apoptosis in MKN45 cells and reverted the apoptotic inhibition effect of miR-3174 (Figures 8B and S3C). However, miR-3174 downregulation showed reverse findings in BGC823 cells (Figures 8C and S4D). GFP-mRFP-LC3 detection and LC-3 turnover assay demonstrated that ARHGAP10 could antagonize effect of miR-3174 on autophagy in GC cells (Figures 8D–8G, S3E, and S3F). mTOR signaling is one of the most common pathways that regulates autophagy, thus prompting us to examine this pathway through western blotting. The results showed that ARHGAP10 overexpression decreased intracellular levels of p-p70s6k, a result indicative of mTORC1 activity, although there were no significant differences in total mTORC1 or p-AKT. This finding suggested that ARHGAP10 reconstitution promoted autophagy by inhibiting mTORC1 activity. In addition, ARHGAP10 overexpression increased BECN1, Bax, and cleaved caspase-3 levels and decreased p62 and pan-caspase-3 (inactivated form) levels in MKN45 cells, results consistent with our previous findings (Figure 8H). p53 is an upstream mediator of Bax, and as expected, increased p53 levels were detected after ARHGAP10 reconstitution.
Figure 4. miR-3174 Suppresses Cellular Autophagy and Inhibits Autophagic Cell Death in GC Cells

(A) Cells infected with lentivirus particles for expression of GFP-mRFP-LC3 were plated into a 35-mm confocal culture dish, and cellular puncta were observed using confocal microscopy (63x objective magnification; scale bar, 20 μm) after 48 hr. The areas enclosed in white squares were further amplified. (B) Yellow and red puncta were counted as mentioned in the Materials and Methods. (C) Transmission electron microscopy (TEM) detection of autophagic microstructures in cells. The green arrows refer to cellular autophagosome that has a double layer structure or autolysosome generated by fusion of autophagosome with lysosome. The areas enclosed within green squares were

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that functions through inhibition of ARHGAP10 expression in these tissues were clearly negatively correlated with ARHGAP10-positive cells in these tissues (Figure 9J). In addition, the percentages of ARHGAP10 in tumor tissues compared with para-carcinoma tissues (Figure 9I). The results demonstrated significantly negatively correlated with the IC50 value of CDDP in all tested cell lines (Figure 8L, left panel). Both the effects of ARHGAP10 on CDDP sensitivity and antagonizing CDDP resistance induced by miR-3174 were further determined in CDDP-resistant or -sensitive cells. (Figures 8K and 8L, right panel). Collectively, these data show that miR-3174 inhibits mTORC1-mediated ACD and p53-regulated apoptosis as well as CDDP cytotoxicity through post-transcriptional suppression of ARHGAP10.

miR-3174 Regulates ARHGAP10 Expression Level to Moderate GC Progression In Vivo

To investigate the roles of miR-3174 and ARHGAP10 in vivo, we generated xenografts in nude mice (Figures 9A and 9D). Xenograft tumors formed from miR-3174-overexpressing MKN45 cells showed significantly faster growth (Figure 9B), whereas miR-3174-suppressed BGC823 xenografts were smaller in volume than those generated from control cells (Figure 9C). Furthermore, miR-3174 expression was negatively correlated with AHRGAP10 mRNA expression in each group of xenografts (Figures S4A and S4B). Additionally, over-expression of ARHGAP10 in miR-3174-reconstituted MKN45 cells restrained the acceleration of tumor growth caused by miR-3174 (Figure 9E), and suppression of ARHGAP10 in miR-3174-downregulated BGC823 cells displayed reverse effects (Figure 9F) (mRNA levels of ARHGAP10 were shown in Figure S4C). Immunohistochemistry (IHC) staining of xenografts revealed the negative relationship between protein levels of ARHGAP10 and miR-3174 expression (Figure 9G). The IHC scores for ARHGAP10 in these xenografts were also calculated and showed similar results (Figure 9H). The protein levels of ARHGAP10 were further measured in 50 paired GC tissues of ARHGAP10-positive cells in these tissues were clearly negatively correlated with the expression of miR-3174 (Figure 9K). Collectively, these findings illustrate that miR-3174 is a tumor suppressor in GC that functions through inhibition of ARHGAP10 expression in vivo.

DISCUSSION

A single miRNA can influence multiple biological processes by simultaneously regulating several cellular signaling pathways, thus indicating the vast clinical potential of miRNA-based therapy. Using bioinformatics analysis, we identified eight differentially expressed miRNAs in GC. Through further validation in 150 paired GC clinical tissue samples, we determined that miR-3174 is significantly upregulated in GC and correlates with significantly decreased five-year survival. Stark et al. first detected miR-3174 as an essential miRNA expressed in melanoma; however, the precise role of this miRNA was not previously reported, thus prompting us to investigate its function in GC. We specifically explored the effect of miR-3174 expression on the malignant progression of GC. However, it should be noted that miR-3174 may influence other cellular process or biological functions in GC beyond what is reported here, and additional studies into the function of this molecule should be performed.

Apoptosis (type I cell death), ACD (type II cell death), and necrosis (type III cell death) are all forms of PCD. It has been reported that ACD acts as a compensatory mechanism when apoptosis is inhibited. However, cancer cells show severe defects in both type I and II cell death, and the mechanisms underlying these defects remain unclear. Herein, we determined that miR-3174 simultaneously inhibits apoptosis and ACD in GC and has a role in preventing the death of GC cells. In addition, we also demonstrated that specific targets of miR-3174 can reverse these effects.

Because of its GTPase-activating proteins (GAP) domain, ARHGAP10 shows potent Rho GTPase inhibition activity, in agreement with previous findings that ARHGAP10 promotes tumorigenesis in ovarian cancer by suppressing the activity of CDC42. Additionally, ARHGAP10 also possesses an SH3 domain, and a close relationship between this domain and cancer progression has been reported. Thus, beyond its effects in suppressing CDC42, we hypothesized that ARHGAP10 may regulate the expression of other genes and consequently prevent cancer. Interestingly, we found that ARHGAP10 promotes both cell apoptosis and ACD in GC. The ability of mTORC1, a nutrient/energy/redox sensor, to repress autophagy by phosphorylating autophagy protein-13 (Atg13) has been well established. Moreover, the AKT-mTOR signaling cascade is one of the best-defined cascades that are differentially expressed during GC tumorigenesis and progression. In accordance with these relationships, we discovered that ARHGAP10 decreased the activity of mTORC1, but not AKT, and this decreased activity was accompanied by massive autophagy and ACD.
In addition, we found that ARHGAP10 exerts mitochondria-dependent promotion of apoptosis in GC cells, which is potentially regulated by miR-3174. Wild-type of p53, one of the best-known tumor-suppressors, trans-activates several pro-apoptotic factors, such as Bax, thus inducing mitochondria-dependent apoptosis. Besides, p53 translocates to the outer mitochondrial membrane, where it regulates additional downstream targets such as BCL-2, thereby promoting apoptosis. As expected, elevated expression of miR-3174 in CDDP-resistant MKN45 and BGC823 cells and their parental CDDP-sensitive cells was detected with RT-PCR. Cell viability of MKN45 (B) or BGC823 (D) cells with or without miR-3174 reconstitution and (G) MKN45CDDP or (E) BGC823CDDP cells with or without miR-3174 inhibition was measured with CCK-8 assays after exposure of cells to different concentrations of CDDP for 48 hr. Graph represents mean ± SEM; *p < 0.05, **p < 0.01, ***p < 0.001.
both p53 and Bax was observed after ARHGAP10 overexpression. These data illustrate that ARHGAP10 accelerates mitochondria-dependent apoptosis by enhancing the expression of p53 and subsequent Bax trans-activation in GC. Our results were consistent with those of another study reporting that DLC1, another member of the RhoGAP, promotes mitochondria-dependent apoptosis in nasopharyngeal carcinoma.42 Despite these findings, the details of the functional role of ARHGAP10 in cancer remain unclear. Because of its complex structure, we suspect that ARHGAP10 may also be involved in other regulatory mechanisms in GC.

Because of the effect of ARHGAP10 on CDDP sensitivity in GC elucidated here, we examined the relationship between ARHGAP10 expression and the IC50 values of all anti-cancer drugs in 28 different GC cell lines by using data from the GDSC database.43 In addition to CDDP, we identified three anti-cancer drugs, Crizotinib, AG-014699, and QL-VIII-58, whose IC50 values were also significantly negatively correlated with ARHGAP10 expression in GC cells. In contrast, the IC50 values of TAE684, BMS-536924, BMS-509744, and A-443654 had a positive correlation with expression of ARHGAP10, thus suggesting that ARHGAP10 may contribute to GC cells developing resistance to these drugs. Collectively, these findings suggest that ARHGAP10 may be a predictive marker for GC treatment.

In conclusion, by performing bioinformatics analysis of TCGA as well as the GEO database and combining the results with the data obtained from GC tissue and cell experiments, we identified miR-3174, which showed significantly increased expression in GC cells and was correlated with poor prognosis. The oncogenic role of miR-3174 in GC is driven by the simultaneous suppression of p53/Bax-mediated mitochondria-dependent apoptosis and mTORC1-regulated ACD as well as attenuation of CDDP cytotoxicity. Further experiments including data from TCGA, TargetScan, and the GDSC database demonstrated that miR-3174 functions by directly targeting ARHGAP10 (Figure 10). Collectively, these results may partially explain why GC cells show a death-resistant phenotype and further indicate that the miR-3174-ARHGAP10 axis may be an effective therapeutic target and predictive marker for GC.

MATTERIAlS AND METHODS

Tissue Samples and Cell Culture
In total, 150 GC tissue samples were obtained from patients with stage I–III disease who underwent R0 resection at The First Affiliated Hospital of Nanjing Medical University. The specimen collection process followed HIPAA guidelines. Five-year survival analysis and univariate analysis of clinicopathological characteristics were performed in 140 patients who had the complete follow-up information and were divided into two groups by the median relative expression of miR-3174 (0.129758).

All GC cell lines were purchased from Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (ISBS, CAS) and were cultured in RPMI 1640 (Wisen Biocenter, 3500-000-CL, China) supplemented with 10% fetal bovine serum (FBS) (086150023, Wisent Biocenter, China).

To generate CDDP-resistant cell lines, MKN45 and BGC823 cells were cultured with gradually increasing doses of CDDP. The dose at which the cells became insensitive to CDDP was identified, and the cultures were maintained at that dose for another week. Then the cells were stimulated by the addition of 0.5 μM increments of CDDP until the concentration reached 10 μM. The cells were then maintained in culture with this concentration.

Reagents and Antibodies
Cisplatin (1134357), CQ (C6628), 3-MA (M9281), Wortmannin (W1628), and Z-VAD-FMK (V116) were purchased from Sigma Aldrich (USA). The primary antibody anti-ARHGAP10 was purchased from Proteintech (55139-1-AP, China), and the antibodies anti-Bax (5023), anti-phospho-p70s6k (9234), anti-p53 (2527), anti-mTOR (2983), anti-phospho-Akt (4060), anti-caspase-3 (9665), anti-cleaved caspase-3 (9664), anti-BECN1 (3495), anti-SQSTM1/p62 (8025), anti-LC3A/B (12741), and anti-β-actin (3700) were obtained from Cell Signaling Technology (Danvers, PA, USA).

Lentivirus, siRNA, Plasmids and Small Hairpin RNA Transfection
MKN45 cells, which have the highest expression of miR-3174 in six GC cell lines compared with GES-1 were transfected with lentivirus containing miR-3174, which stably expressed miR-3174 (obtained from GenePharma, Shanghai, China). BGC823 cells, which have the relative lowest miR-3174 expression, were infected with lentiviruses containing miR-3174 interference sequence (GenePharma, Shanghai, China). miR-3174 expression after lentivirus transfection in BGC823 and MKN45 cells were validated by RT-PCR (Figure S1A). MKN45CDDP or BGC823CDDP cells, which have the higher expression of miR-3174 compared to their parental sensitive cells, were infected with miR-3174 inhibition lentivirus, and

Figure 6. Bioinformatics Analysis to Identify Potential Downstream Targets of miR-3174
(A) Volcano plot for the mRNA matrix generated on the basis of TCGA statistics from 407 patients. (B) Left panel, candidate genes potentially involved in CDDP sensitivity and resistance were screened according to the GDSC database. Right panel, 36 genes whose expression was negatively coregulated with the IC50 values for CDDP in 28 different GC cell lines are listed. (C) mRNA expression heatmap showing 21 genes whose expression was not only downregulated in 375 GC tissues compared with 32 normal gastric tissues on the basis of TCGA database but also significantly negatively correlated with the IC50 values for CDDP in 28 different GC cell lines. In addition, these genes were also identified as potential targets of miR-3174 based on the TargetScan. The symbols for the 21 genes are shown in the black square. (D) Schematic diagram detailing the exploration of miR-3174 downstream targets. (E) Correlations between the expression levels of miR-3174 and these 21 genes were calculated with Pearson correlation analysis in 404 patients from TCGA database, and the gene name, R value, and p value are shown. The first four genes, ZNF471, LODC1, CLMP, and ARHGAP10, with p < 0.01 were selected for further validation (red font). (F–I) A negative correlation was found between miR-3174 and ZNF471 (F), LODC1 (G), CLMP (H), and ARHGAP10 (I) according to TCGA statistics is displayed. Graph represents mean ± SEM; *p < 0.05, **p < 0.01, and ***p < 0.001.
the interference effectiveness were also validated by RT-PCR (Figure S2A). For miR-3174 overexpression lentivirus, mature miR-3174 sequence (5′-UAGUAGAUUUAGAGAGCAGAGCC-3′) and interference sequence of miR-3174 (5′-GGCUUCUGCAUUUCUACCUA CUA-3′) were cloned into LV2-pGLV-U6-puro vector (GenePharma, Shanghai, China). We chose U6 (belongs to pol III) as the promoter of LV2 vector, which has been showed that is high yield and commonly adapted for miRNA synthesis.43 For lentivirus transfection, 2 × 10^5 BGC823 or MKN45 cells were plated into 6-well plates. After 24 hr, cells were washed with PBS three times and then cultured with 2 mL medium with a certain amount of miR-3174 overexpression or inhibition lentivirus (MOI, 100 for BGC823; 20 for MKN45) and Polybrene (8 μg/mL). Stable pools were obtained in the presence of 2 μg/mL puromycin for 2 weeks. ARHGAP10-overexpression and -repression plasmids were purchased from GenePharma (shARHGAP10-857: 5′-GGGTCAAACTATTTGACATGT-3′; shARHGAP10-1603: 5′-GGAGTGTGTATTTGACACACT-3′). Plasmids were transfected into cells with Lipofectamine 2000 (1575386, Invitrogen, USA) according to the manufacturer’s instructions, and all transfected cells were subjected to puromycin selection. ARHGAP10 expression was verified by western blotting (shARHGAP10#1 with a better inhibitory effect was used for subsequent experiments) (Figure S2E).

CCK-8 and Apoptosis Assays
For Cell Counting Kit-8 assays, approximately 1,000 GC cells were seeded per well into 96-well plates. After 48 hr of incubation, the cells were cultured with 10% CCK-8 (Dojindo, Kumamoto, Japan) reagent for another 2 hr, and the absorbance was detected using a microplate reader.

To assay cell viability, 5,000 cells were plated per well into 96-well plates. After 24 hr of incubation, the culture medium was changed, different concentrations of cisplatin (MKN45CDPP, 0 μM, 1 μM, 2 μM, 4 μM, 6 μM, 8 μM, 10 μM, 12 μM, 14 μM, 16 μM; BGC823CDPP, 0 μM, 1 μM, 2 μM, 4 μM, 6 μM, 8 μM, 12 μM, 16 μM, 20 μM, 24 μM; MKN45 and BGC823, 0 μM, 0.5 μM, 1 μM, 1.5 μM, 2 μM, 2.5 μM, 3 μM, 3.5 μM, 4 μM, 4.5 μM) were added, and the cells were cultured for another 48 hr. Cell viability was quantified using a CCK-8 kit.

For flow cytometry, GC cells were plated into 6-well plates (2 × 10^5 cells/well) and placed into an incubator for 48 hr. The cells were further incubated with Annexin V (3 μL) and propidium iodide (3 μL) for 15 min and examined using a flow cytometer (Galilio, Beckman, USA).

Figure 7. ARHGAP10 Is a Direct Downstream Target of miR-3174 and Acts in a Tumor-Suppressor Role in GC
(A–D) The mRNA expression of CLMP (A), ARHGAP10 (B), ZNF471 (C), and LDOC1 (D) and in 100 paired GC and adjacent normal tissues were detected using RT-PCR. (E–H) Correlations between miR-3174 and these four genes (E, CLMP; F, ARHGAP10; G, ZNF471; H, LDOC1) were measured with Pearson correlation analysis. (I) The mRNA expression of these four genes in MKN45 cells with or without miR-3174 reconstitution and in BGC823 cells with or without miR-3174 inhibition was also determined with RT-PCR. (J) The protein levels of ZNF471, LDOC1, CLMP, and ARHGAP10 were verified by western blotting. (K and L) Dual-luciferase reporter assays with wild-type or mutant-type 3′ UTRs of these four genes were performed in BGC823 (K) and MKN45 (L) cells with or without reconstitution of miR-3174, and fluorescence was quantified and converted into histograms. (M) Cell viability was detected with a CCK-8 kit in MKN45 cells with or without suppression of ZNF471, LDOC1, CLMP, and ARHGAP10. β-actin was used as an internal control. Graph represents mean ± SEM; *p < 0.05, **p < 0.01, and ***p < 0.001.
TEM
In brief, cells were fixed with 2.5% glutaraldehyde overnight at 4°C after being treated with 1% OsO4 and then were dehydrated with ethanol and embedded in Epon. The embedded materials were then sectioned; the sections were further stained with 0.3% lead citrate and imaged with an electron microscope (JEOL, Tokyo, Japan, 2,500 × or 8,800 × magnification).

Dual-Luciferase Reporter Assay
3′ UTRs containing either wild-type or mutant miR-3174 response elements from ZNF371, LDOC1, CLMP, and ARHGAP10 were cloned into a pMIR-REPORT plasmid (H306, Ohio Technology, Shanghai, China). The transfection process followed the manufacturer’s instructions. Firefly and Renilla luciferase activity was assessed with a Dual-Luciferase Reporter System Kit (E1910, Promega, USA).

Xenograft Model of GC and IHC
Five-week-old female BALB/c nude mice were obtained from the Laboratory Animal Centre of Nanjing Medical University and raised under pathogen-free conditions. In brief, 2 × 106 GC cells in 100 μL of PBS were subcutaneously injected into the mice, and the resultant xenograft tumors were measured every 7 days and harvested 4 weeks later.

For IHC assays, sections of GC xenografts and tissue samples from patients were prepared according to a previously described protocol. The sections were then observed with an IHC Imager (DM4000B, Leica, Germany). Cells positively stained with different types of antibodies were detected in an average of five fields per slice, and the following scores used to describe the overall proportion were measured: <10% = 0, 10%–25% = 1, 26%–50% = 2, 51%–75% = 3 and >75% = 4. For analysis of clinicopathological characteristics, the patients were divided into two groups on the basis of the median relative expression of miR-3174: a miR-3174-high group (expression > 0.1297358, n = 70) and a miR-3174-low group (expression < 0.1297358, n = 70).

Bioinformatics Analysis
miRNA expression levels reported for 491 (446 GC patients and 45 healthy subjects) individuals included in TCGA database (https://cancergenome.nih.gov/) were integrated by using Perl, and a matrix describing differentially expressed miRNA (fold change ≥ 2, p value < 0.05) was generated using the edgeR package (Bioconductor software) after Tpm correction and p value adjustment. A matrix of differentially expressed miRNA (fold change ≥ 2, p value < 0.05) was also similarly generated for a cohort of 407 patients (375 GC patients and 32 healthy controls).

To screen for differentially expressed miRNA in the GEO database, the GEO78091 dataset, which contains three GC tissues and paired adjacent normal tissues, was selected and analyzed using R with the limma package (Bioconductor software). Candidate mRNAs were selected with a standard method, and differentially expressed mRNAs were defined as having a fold change ≥ 2 and a p value < 0.05.

To identify potential genes influencing cisplatin sensitivity, we integrated the IC50 statistics of multiple types of anti-cancer drugs in 28 different GC cell lines on the basis of a gene-expression matrix obtained from the GDSC (http://www.cancerrxgene.org) database by using Perl. Correlations between gene expression (number of genes, 17,419) and the IC50 value of CDDP in 28 GC cell lines and correlations between ARHGAP10 expression and the IC50 values of various types of anti-cancer drugs (number of drugs, 265) were assessed using Pearson correlation coefficient analysis in R. A Z test was used to calculate p values. Genes with R < 0 and a p value < 0.05 are shown as green puncta, and those with R > 0 and a p value < 0.05 are shown as red puncta (Figure 6B). Drugs with R < 0 and a p value < 0.01 are shown in green font and as green puncta, and those with R > 0 and a p value < 0.01 are shown as red puncta (Figure 8J).

Statistical Analysis
All experiments were replicated three times, and statistical analysis was performed with SPSS 19.0. All data are shown as the mean ± SEM. Unpaired Student’s t tests were used to analyze the statistical significance of the differences between results. The relative expression of miRNAs or genes in tissue samples was calculated by using a Mann-Whitney U test and/or Wilcoxon signed-rank test. Overall survival rates were evaluated with Kaplan-Meier curves and log-rank tests. A p value < 0.05 was defined as statistically significant.
Figure 9. miR-3174 Displays a Tumor-Suppressing Effect by Inhibiting ARHGAP10 In Vivo
(A) Representative xenografts of sacrificed mice after subcutaneous injection of MKN45 cells with or without miR-3174 reconstitution and of BGC823 cells with or without miR-3174 suppression. (B and C) The volume of each xenograft tumor was measured every 7 days after injection with MKN45 cells with or without miR-3174 overexpression (B) or BGC823 cells with or without miR-3174 inhibition (C). (D) Photograph of xenograft tumors in nude mice after subcutaneous injection of MKN45 miR-3174-reconstituted cells with or without ARHGAP10 overexpression and miR-3174-suppressed BGC823 cells with or without ARHGAP10 inhibition. The length of the photo is equal to that in (A). (E and F) Tumor volume was calculated every seventh day after injection with miR-3174-overexpressed MKN45 cells with or without ARHGAP10 reconstitution (E) or
miR-3174 expression is upregulated in GC cells and leads to cell death defects by directly targeting ARHGAP10, which could not only promote mitochondria-dependent apoptosis by enhancing p53 expression, followed by Bax trans-activation and caspase cleavage, but also facilitate autophagic cell death by suppressing mammalian target of rapamycin complex 1 (mTORC1) activity.

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Supplemental Information

miR-3174 Contributes to Apoptosis and Autophagic Cell Death Defects in Gastric Cancer Cells

by Targeting ARHGAP10

Bowen Li, Lu Wang, Zheng Li, Weizhi Wang, Xiaofei Zhi, Xiaoxu Huang, Qiang Zhang, Zheng Chen, Xuan Zhang, Zhongyuan He, Jianghao Xu, Lu Zhang, Hao Xu, Diancai Zhang, and Zekuan Xu
Figure S1. Lentivirus effectiveness detection and CDDP resistant characteristics of cell lines validation. (a) Expression of miR-3174 was validated after infecting MKN45 cells with lentivirus carrying LV-miR-3174 or LV-miR-NC and BGC823 cells with ANTI-3174 or ANTI-NC. (b,c) CDDP resistance features of MKN45CDDP and BGC823CDDP cells were verified by CCK-8 cell viability assays, and IC50 of each cell line was showed in (d). Graph represents mean ± SEM; *=P<0.05, **=P<0.01, and ***=P<0.001.
Figure S2. Colony numbers quantitation and target genes’ siRNA effectiveness verification. (a) miR-3174 expression was detected after BGC823 or MKN45 cells was infected with lentivirus carrying LV-miR-3174 or LV-miR-NC and BGC823CDDP or MKN45CDDP cells was infected with lentivirus carrying ANTI-3174 or ANTI-NC. (b,c) The colony units in Figure 5j,k were counted, and the data were converted to...
percentages and compared with data for the control groups. (d) mRNA expression of ZNF471, LDOC1, CLMP and ARHGAP10 was determined respectively by RT-PCR after MKN45 cells were treated with their specific RNA interference sequences. (e) Protein levels of ARHGAP10 were detected by Western blot after transfecting MKN45 cells with plasmids overexpressed ARHGAP10 or empty vector plasmids, and BGC823 cells with ARHGAP10 inhibited or control plasmids. Graph represents mean ± SEM; *=P<0.05, **=P<0.01, and ***=P<0.001.
Figure S3. Mitochondria membrane potential level, apoptosis and autophagosome quantitation. (a,b) Fluorescence intensity ratios of red vs green in Figure 8a were calculated in MKN45 and BGC823 cells. (c,d) Apoptotic rates in cells as mentioned in Figure 8b,c were quantitated and transformed to histograms. (e,f) Red and yellow puncta after GFP-mRFP-LC3 transfection and detection as shown in Figure 8d,e were
also quantitated with the same method in Figure 4b. Graph represents mean ± SEM; * = P < 0.05, ** = P < 0.01, and *** = P < 0.001.
Figure S4. miR-3174 and ARHGAP10 expression in xenograft tumors. (a) miR-3174 and (b) ARHGAP10 expression in xenografts as displayed in Figure 9a was determined by RT-PCR. (c) Expression of ARHGAP10 was also measured in xenografts as showed in Figure 9d. Graph represents mean ± SEM; *=P<0.05, **=P<0.01, and ***=P<0.001.
Table S1. Tendency of dispersion of each miRNA profile in GC, and means or variance values of two miR-3174 groups. The means and standard deviations of eight miRNAs’ expression in 50-paired gastric cancer patients, and miR-3174’s expression in 100-paired gastric cancer patients, and the means or variance values of miR-3174’s expression in two groups of survival curve or clinicopathological features analysis cut-off by median expression. SD = standard deviation.
| Clinicopathological features | Number | No. of patients | Pvalue |
|------------------------------|--------|-----------------|--------|
|                              | miR-3174<sub>high</sub> | miR-3174<sub>low</sub> |
| Age(y) ≥ 60                 | 84     | 47              | 37     | 0.084 |
| Age(y) < 60                 | 56     | 23              | 33     |
| Gender Male                 | 105    | 56              | 49     | 0.172 |
| Gender Female               | 35     | 14              | 21     |
| Tumor size ≥ 3.0cm          | 84     | 51              | 33     | 0.002** |
| Tumor size < 3.0cm          | 56     | 19              | 37     |
| Lauren’s classification Intestinal-type | 92 | 50 | 42 | 0.154 |
| Lauren’s classification Diffuse-type | 48 | 20 | 28 |
| Histological grade Well     | 47     | 26              | 21     | 0.371 |
| Histological grade Moderately and Poorly | 93 | 44 | 49 |
| TNM stage I                 | 31     | 10              | 21     | 0.005** |
| TNM stage II                | 38     | 15              | 23     |
| TNM stage III               | 71     | 45              | 26     |
| T stage T1-T2               | 48     | 18              | 30     | 0.033 |
| T stage T3-T4               | 92     | 52              | 40     |
| N stage N0                  | 55     | 29              | 26     | 0.604 |
| N stage N1-N3               | 85     | 41              | 44     |

Table S2. Correlation of relative miR-3174 expression with the clinicopathological characteristics of 140 patients with gastric cancer. * = P < 0.05, ** = P < 0.01, and *** = P < 0.001.
Table S3. Potential genes associated with GC cells CDDP sensitivity or resistance on GDSC. The correlations between gene expressions with IC50 values of CDDP in 28 kinds of GC cells based on GDSC database were measured using Pearson correlation coefficient analysis in R language library followed by Z-test to acquire P-value. Genes meeting the following requirements were showed: P-value < 0.05. “1005” represents Cisplatin (CDDP) on GDSC. (the table was supplied separately as an Excel file: Supplemental Table S3)

Table S4. Candidate drugs whose IC50 in GC cells were related to ARHGAP10 expression in GDSC. The correlations between IC50 values of all types of anti-cancer drugs with ARHGAP10 expressions in 28 kinds of GC cells based on GDSC database were calculated using Pearson correlation coefficient analysis in R language library followed by Z-test to acquire P-value. Genes meeting the following requirements were showed: P-value < 0.05. “1005” represents Cisplatin (CDDP) on GDSC. (the table was supplied separately as an Excel file: Supplemental Table S4)

Abbreviations
GC: Gastric cancer; miRNA: microRNA; mRNA: messenger RNA; CDDP: cis-diamminedichloroplatinum (Cisplatin); ARHGAP10: Rho GTPase activating protein 10; mTOC1: rapamycin complex 1; PCD: Programmed cell death; ACD: autophagic cell death; AP: autophagosome; AL: autolysosome; SH3 domain: SRC hoology 3 domain; PH domain: Pleckstrin homology domain; FBS: fetal bovine serum; BECN1: Beclin 1; SQSTM1: sequestosome 1; RT-PCR: Quantitative real-time PCR; TEM: Transmission electron microscopy; DMSO: dimethyl sulfoxide; PVDF: polyvinylidene difluoride; HRP: horseradish peroxidase; UTR: untranslated region; ZNF471: zine finger 471; LDOC1: leucine zipper down-regulated in cancer 1; CLMP: CXADR like membrane protein; IC50: half maximal inhibitory concentration; CQ: Chloroquine; 3-
MA: 3-methyladenine; WMT: Wortmannin; MMP: mitochondria membrane potential