Molecular and Cellular Pathobiology

Cancer Research

MicroRNA-375 Is Downregulated in Gastric Carcinomas and Regulates Cell Survival by Targeting PDK1 and 14-3-3ζ

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Abstract

We investigated expression profiles of microRNA (miRNA) in gastric carcinomas by use of a miRNA microarray platform covering a total of 470 human miRNAs. We identified 39 differentially expressed miRNAs in gastric carcinoma, of which six were significantly downregulated and the other 33 were upregulated. We found that miRNA-375 (miR-375) was the most downregulated and that its ectopic expression in gastric carcinoma cells markedly reduced cell viability via the caspase-mediated apoptosis pathway. Interestingly, we found that expression of miR-375 inhibited expression of PDK1, which is a direct target of miR-375, followed by suppression of Akt phosphorylation. Further analysis by gene expression microarray revealed that 14-3-3ζ, a potent antiapoptotic gene, was significantly downregulated at both the mRNA and protein levels in cells transfected with miR-375. The activity of a luciferase reporter containing the miR-375 binding sequence at the 3′ untranslated region (UTR) of 14-3-3ζ mRNA was repressed by the ectopic expression of miR-375, suggesting that miR-375 targets the 3′ UTR of 14-3-3ζ. In addition, knockdown of either PDK1 or 14-3-3ζ in gastric carcinoma cells induced caspase activation, which was also observed in miR-375–transfected cells, suggesting that miR-375 may exert its proapoptotic function, at least in part, through the downregulation of PDK1 and 14-3-3ζ. Taken together, we propose that miR-375 is a candidate tumor suppressor miRNA in gastric carcinoma. *Cancer Res; 70(6): 2339–49. ©2010 AACR.*

Introduction

Gastric carcinoma remains one of the most common malignant diseases in spite of a steady decline in its incidence worldwide. Overall, mortality due to gastric cancer has been estimated at 700,000 cases annually (10.4% of all cancer-related deaths), ranking second only to lung cancer, on the basis of the most recent statistics, GLOBOCAN 2002 (1). The clinical outcome of gastric cancer has gradually improved, but the prognosis of patients with advanced disease is still disappointing. Although alterations in a large number of oncogenes and tumor suppressor genes have been reported in gastric carcinomas (2, 3), the molecular mechanisms underlying the development of gastric carcinomas are still poorly understood.

MicroRNAs (miRNA) are non–protein-coding small RNAs in the size range of 19 to 25 nucleotides that are cleaved from 70- to 100-nucleotide hairpin pre-miRNA precursors (4). miRNAs bind to complementary sequences in the 3′ untranslated regions (UTR) of their target mRNAs and induce mRNA degradation or translational repression (5). Via these molecular mechanisms, miRNAs are involved in various biological processes, including cellular differentiation, proliferation, and death (6–9). Furthermore, it has recently been reported that miRNAs are frequently dysregulated in human cancers and that they can act as potent oncogenes and tumor suppressor genes (10–13). The development of microarray platforms for the analysis of miRNA expression has revealed that a number of miRNAs are aberrantly expressed in carcinoma tissues (14–16).

Although there are several reports of aberrant expression of specific miRNAs in gastric carcinoma (17–20), only a few studies have analyzed the global expression pattern of miRNAs (21, 22). Using a miRNA microarray, Petrocca and colleagues recently reported that the miR-106b-25 cluster is upregulated and acts as an oncogenic miRNA in gastric carcinoma (22). However, it is unclear whether the downregulated miRNAs can act as tumor suppressors in gastric carcinoma.

In the present study, we analyzed miRNA expression profiles in advanced gastric carcinomas and identified a set of

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi: 10.1158/0008-5472.CAN-09-2777

differentially expressed miRNAs. One of the most downregulated miRNAs found in this study, miRNA-375 (miR-375), was further evaluated for its function. We found that miR-375 reduced the viability of gastric carcinoma cells and induced apoptosis, suggesting that miR-375 is a candidate tumor suppressor miRNA in gastric carcinoma.

Materials and Methods

Tissues. Gastric carcinoma tissues were surgically resected from 22 patients at Oita University Hospital. Information on the patients is summarized in Supplementary Table S1. Tissue samples were immediately embedded in OCT compound (Sakura Finetech), snap-frozen in liquid nitrogen, and then stored in a deep freezer at −80°C. The use of the tissue samples for all experiments was approved by all the patients and by Oita University Ethics Committee.

Extraction of total RNA and miRNA microarray. Tissue sections were cut from frozen blocks and then stained with cresyl violet (Ambion). Carcinoma cells were selectively collected from the tissue sections by laser-capture microdissection (Arcturus Engineering). Total RNA was extracted from the collected cells with a miRNeasy Mini kit (Qiagen). As a normal control, total RNA was also extracted from tissue sections obtained from five cases of nonneoplastic gastric epithelium (Supplementary Table S1). Extracted total RNA samples were subjected to miRNA microarray analysis essentially as described in our previous study (23). Details of the procedures are provided in Supplementary Data 1.

Analysis of miRNA microarray data. The microarray data were processed and analyzed with GeneSpring GX software version 7.3.1 (Agilent Technologies, Santa Clara) to yield miRNA profiles. Details of the procedures are provided in Supplementary Data 1. The data obtained in the miRNA microarray analysis are available at DDBJ via CIBEX (24), under accession no. CBX80 in a format that conforms to the Minimum Information about a Microarray Gene Experiment guidelines.

Quantitative reverse transcription–PCR. Quantitative reverse transcription–PCR (qRT-PCR) for miRNA and mRNA was performed as described previously (23).

Cell culture and transfection. The gastric carcinoma cell lines NUGC3, AZ521, and MKN74 were purchased from JCRB and maintained in RPMI (NUGC3 and MKN74) and EMEM (AZ521) supplemented with 10% fetal bovine serum (FBS). Cells were transfected with precursor miR-375 (pre375; Ambion), negative control precursor miRNA #2 (preNeg; Ambion), Stealth small interfering RNA (siRNA; Invitrogen) targeting PDK1 (siPDK1), siRNA targeting 14-3-3 (si14-3-3ζ), or Stealth RNAi negative control medium GC Duplex #2 (si-Cont) at a final concentration of 10 nmol/L (NUGC3 and AZ521) or 5 nmol/L (MKN74), using Lipofectamine RNAiMAX (Invitrogen) in accordance with the manufacturer's instructions. The sequences of siPDK1 and si14-3-3ζ are described in Supplementary Table S2.

Treatment of cells with 5-aza-2′-deoxycytidine and Trichostatin A. For treatment with 5-aza-2′-deoxycytidine (5-aza-dC; Sigma-Aldrich), NUGC3 cells (4×10^4) were seeded into 35-mm dishes on day 0 and exposed to 5 μmol/L 5-aza-dC from day 1 to day 4. Trichostatin A (TSA; Sigma-Aldrich) was added into the cells on day 3 at a concentration of 100 nmol/L. The cells treated with either 5-aza-dC, TSA, or both were harvested on day 4.

Gene expression microarray. For gene expression microarray, 300 ng of total RNA extracted from cells transfected with pre375 or preNeg in quadruplicate were subjected to microarray analysis in a similar way to that described in our previous study (25, 26). Details of the procedures are provided in Supplementary Data 1. The data obtained in the analysis of the gene expression microarray are available at DDBJ via CIBEX (24) under accession no. CBX92.

Western blot analysis. At 72 h after transfection with precursor miRNA or siRNA, cells were subjected to Western blot analysis as described previously (27). The primary antibodies used for Western blot analysis were anti-PDK1 antibody (1:1,000; Cell Signaling Technology), anti-phosphorylated Akt antibody (Thr^{308}; 1:1000; Cell Signaling Technology), anti-phosphorylated Akt antibody (Ser^{473}; 1:1,000; Cell Signaling Technology), anti-X-linked inhibitor of apoptosis protein (XIAP) antibody (1:500; R&D Systems), anti-α-tubulin antibody (1:1,000; Cell Signaling Technology), and anti-14-3-3ζ antibody (1:1,500; Santa Cruz Biotechnology).

Proliferation assay. Cells (2×10^4) were transfected with pre375 or preNeg in a 24-well plate for 72 h. They were then treated with trypsin and stained with trypan blue. Viable cells, which excluded trypan blue dye, were counted in quadruplicate with a Countess (Invitrogen). Relative number of viable cells was expressed as percentage of preNeg-transfected cells.

Apoptosis assay. At 72 h after transfection with pre375 or preNeg, cells were evaluated for apoptosis by morphology of nuclei, level of cytoplasmic oligonucleosomal fragment, and activity of caspase-3/caspase-7. For morphologic examination, cells were stained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) and observed by fluorescence microscopy. Cytoplasmic oligonucleosomal fragment was measured with the Cell Death Detection ELISA PLUS kit (Roche Diagnostics) according to the manufacturer's instructions. The activity of caspase-3 and caspase-7 was detected in 96-well format by using the caspase-Glo 3/7 assay kit (Promega) according to the manufacturer's instructions.

Luciferase reporter assay. Double-stranded oligonucleotides corresponding to the wild-type (WT-3′ UTR) or mutant (MUT-3′ UTR) miR-375 binding site in the 14-3-3ζ 3′ UTR were synthesized (Sigma-Aldrich) and ligated between the SpeI and HindIII restriction sites of the reporter plasmid pMIR-Report (Ambion). The oligonucleotides used are described in Supplementary Table S2. At 24 h after transfection with 5 nmol/L of pre375 or preNeg by use of RNAiMAX, a reporter plasmid containing WT-3′ UTR or MUT-3′ UTR and a plasmid expressing *Renilla* luciferase (pRL-CMV, Promega) were cotransfected into MKN74 cells by use of Lipofectamine 2000 (Invitrogen). Firefly luciferase activity derived from pMIR-Report was normalized to *Renilla* luciferase activity from pRL-CMV. Normalized luciferase activity in cells transfected with WT-3′ UTR was

compared with that in cells transfected with MUT-3′ UTR set at 100.

Statistical analysis. The two-sided Student's *t* test was used to analyze differences in experiments with cell lines. Data are reported as mean values ± SD of quadruplicate determinations. *P* values of <0.05 were considered statistically significant.

Results

Identification of miRNAs differentially expressed in gastric carcinoma. We used a miRNA microarray platform covering a total of 470 human miRNAs (Sanger miRBase release 9.1) to investigate expression profiles of miRNAs in gastric carcinomas from 22 individuals, representing 14 of the intestinal type and 8 of the diffuse type, as well as 5 nonneoplastic gastric epithelia (normal control; Supplementary Table S1). Of the 470 miRNAs, 159 whose expression levels were too low in all cases and another 6 that were not registered in miRBase release 13.0 were excluded from the present analysis. Therefore, 305 miRNAs were actually analyzed in this study. Unsupervised hierarchical cluster analysis showed that the five normal control samples were clearly separated from gastric carcinomas (Supplementary Fig. S1), suggesting that the miRNA expression profiles of gastric carcinomas are distinct from those of normal gastric epithelia. Furthermore, no subgroups were identified by unsupervised hierarchical cluster analyses of these 22 gastric carcinomas. By comparing miRNA expression profiles between intestinal-type and diffuse-type gastric carcinomas (ANOVA $P < 0.05$), we could not identify any miRNAs differentially expressed between them, suggesting that, in gastric carcinomas, the miRNA expression profile might not be correlated with histopathologic subtypes.

To identify the miRNAs that are differentially expressed between gastric carcinomas and normal epithelia, we compared the expression levels of miRNAs between them with ANOVA ($P < 0.05$) and found that 17 miRNAs were significantly downregulated and 85 were upregulated in carcinomas (Supplementary Table S3). Among the 17 miRNAs downregulated in carcinoma tissues, we selected 6 miRNAs strongly expressed with raw signal of over 500 in normal tissues (Table 1; Supplementary Table S3). Similarly, among the 85 miRNAs upregulated in carcinoma tissues, we selected 33 miRNAs strongly expressed with raw signal of over 500 in carcinoma tissues (Table 1; Supplementary Table S3).

To validate the microarray data, expressions of miR-17-5p, miR-93, and miR-29c, all of which were differentially expressed in microarray analysis (Table 1), were further analyzed by qRT-PCR. The trends of miRNA expression were all similar to those of the microarray data (Supplementary Fig. S2).

Involvement of DNA methylation and histone deacetylation in downregulation of miR-375. Microarray data revealed that miR-375 was the most downregulated miRNA in gastric carcinomas (Table 1). Further analysis by qRT-PCR revealed that downregulation of miR-375 also occurred in gastric carcinoma cell lines as well as gastric carcinoma tissues (Fig. 1A; Supplementary Fig. S3). Interestingly, we also found that miR-375 expression was decreased in gastric carcinoma *in situ*, suggesting that downregulation of miR-375 occurs in the early phase of gastric tumorigenesis (Supplementary Fig. S4). No relationship between miR-375 expression and lymph node metastasis or histologic subtype was found in the cases analyzed by qRT-PCR (Supplementary Fig. S5). Because genomic loss at 2q35, wherein miR-375 is located, is rare in gastric carcinoma (25, 28), we hypothesized that epigenetic mechanisms, such as DNA methylation or histone deacetylation, may be involved in the downregulation of miR-375 in gastric carcinoma. To test this hypothesis, we treated a gastric carcinoma cell line, NUGC3, with a DNA demethylating agent (5-aza-dC) and/or a histone deacetylase inhibitor (TSA) and then analyzed the expression of miR-375. As shown in Fig. 1B, the expression of miR-375 was significantly increased >6-fold after treatment of NUGC3 cells with either 5-aza-dC or TSA alone. Furthermore, when the cells were treated with both 5-aza-dC and TSA, miR-375 was markedly upregulated by >20-fold, suggesting that DNA methylation and histone deacetylation are involved in the silencing of miR-375.

Exogenous miR-375 reduces the viability of gastric carcinoma cells. To explore the function of miR-375 in gastric carcinoma cells, NUGC3, AZ521, and MKN74 cells, in which miR-375 was downregulated, were transfected with pre375 or preNeg. As shown in Fig. 1C, cells transfected with pre375 for 72 h tended to exhibit a round shape and became detached from the dish, although such changes were rarely observed in cells transfected with preNeg. To determine whether miR-375 affects the viability of gastric carcinoma cells, viable cell numbers were counted at 72 h after transfection by the trypan blue dye exclusion method. The viability of pre375-transfected cells was found to be significantly reduced by 30% to 71% compared with that of preNeg-transfected cells (Fig. 1D).

Exogenous miR-375 induces apoptosis in gastric carcinoma cells. Because the marked morphologic changes observed in cells transfected with pre375 are characteristic of apoptosis (Fig. 1C), we next evaluated nuclear morphology, cytoplasmic oligonucleosomal fragment level, and caspase activity in gastric carcinoma cells transfected with pre375. As shown in Fig. 2A, condensed and fragmented nuclei were apparent in pre375-transfected cells but were rarely detectable in preNeg-transfected cells. Cytoplasmic oligonucleosomal fragment, which is a marker of apoptosis, was markedly induced in pre375-transfected cells (Fig. 2B). Furthermore, caspase-3/caspase-7 activity was highly induced in pre375-transfected cells at 48 h after transfection and was more prominently increased at 72 h (Fig. 2C). These results suggest that ectopic expression of miR-375 induces apoptosis in gastric carcinoma cells.

miR-375 regulates the PDK1/Akt survival pathway in gastric carcinoma cells. It has recently been reported that miR-375 directly targets PDK1 and suppresses its expression in pancreatic β cells (29). The phosphorylated form of Akt, a downstream target of PDK1, has been reported to prevent apoptosis in gastric cancer cells (30). Therefore, we hypothesized that suppression of the PDK1/Akt pathway is involved

in miR-375–induced apoptosis in gastric carcinoma cells. To determine whether miR-375 actually suppresses the PDK1/Akt pathway, we transfected pre375 into gastric cell lines and analyzed the expression of PDK1 and phosphorylation of Akt. As shown in Fig. 2D, ectopic expression of miR-375 caused reduction of PDK1 expression. Furthermore, phosphorylation of Akt on Ser^{473} and Thr^{308} were markedly decreased, whereas the total Akt level was unaffected (Fig. 2D). We also found that expression of XIAP, which is stabilized by the phosphorylated form of Akt (31) and inhibits apoptosis by preventing caspase activation (32), was also decreased in miR-375–transfected cells (Fig. 2D). These results suggest that ectopic expression of miR-375 inhibits the PDK1/Akt survival pathway in gastric carcinoma cells. Next, to analyze whether downregulation of miR-375 can induce Akt phosphorylation, we performed knockdown assays

Table 1. Representative miRNAs differentially expressed between tumor and normal tissues

ID (miRBase 9.1)	Fold change	SD	Raw	Corrected *P*	Mature accession	ID (miRBase 13.0)
(A) Downregulated miRNAs in tumor						
hsa-miR-375	0.15	0.52	809.2	0.0204	MIMAT0000728	hsa-miR-375
hsa-miR-29c	0.16	0.15	1667.4	0.0102	MIMAT0000681	hsa-miR-29c
hsa-miR-148a	0.19	0.43	1176.0	0.0259	MIMAT0000243	hsa-miR-148a
hsa-miR-30a-5p	0.30	0.37	220.0	0.0284	MIMAT0000087	hsa-miR-30a
hsa-miR-30e-5p	0.34	0.28	363.8	0.0147	MIMAT0000692	hsa-miR-30e
hsa-miR-638	0.46	0.34	1480.5	0.0303	MIMAT0003308	hsa-miR-638
(B) Upregulated miRNAs in tumor						
hsa-miR-18a	10.66	7.99	962.8	0.0102	MIMAT0000072	hsa-miR-18a
hsa-miR-106a	9.02	5.21	2212.5	0.0102	MIMAT0000103	hsa-miR-106a
hsa-miR-17-5p	9.01	5.66	1830.2	0.0102	MIMAT0000070	hsa-miR-17
hsa-miR-146a	8.86	18.74	735.5	0.0112	MIMAT0000449	hsa-miR-146a
hsa-miR-93	8.27	5.31	1227.2	0.0102	MIMAT0000093	hsa-miR-93
hsa-miR-19a	7.80	5.18	2188.3	0.0102	MIMAT0000073	hsa-miR-19a
hsa-miR-20a	7.62	4.55	2857.1	0.0102	MIMAT0000075	hsa-miR-20a
hsa-miR-20b	6.84	4.22	798.7	0.0102	MIMAT0001413	hsa-miR-20b
hsa-miR-25	5.57	3.68	1255.5	0.0102	MIMAT0000081	hsa-miR-25
hsa-miR-15b	5.47	2.89	1370.8	0.0102	MIMAT0000417	hsa-miR-15b
hsa-miR-425-5p	5.27	3.47	509.6	0.0102	MIMAT0003393	hsa-miR-425
hsa-miR-92	5.24	3.15	3143.2	0.0102	MIMAT0000092	hsa-miR-92a
hsa-miR-194	5.16	3.24	4713.8	0.0134	MIMAT0000460	hsa-miR-194
hsa-miR-10a	4.77	8.26	607.3	0.0303	MIMAT0000253	hsa-miR-10a
hsa-miR-222	4.70	4.20	936.6	0.0147	MIMAT0000279	hsa-miR-222
hsa-miR-7	4.68	3.87	802.8	0.0102	MIMAT0000252	hsa-miR-7
hsa-miR-106b	4.30	2.86	2382.1	0.0112	MIMAT0000680	hsa-miR-106b
hsa-miR-320	4.20	2.04	1416.7	0.0134	MIMAT0000510	hsa-miR-320a
hsa-miR-21	4.05	2.17	21464.0	0.0102	MIMAT0000076	hsa-miR-21
hsa-miR-34a	4.03	4.00	1422.8	0.0134	MIMAT0000255	hsa-miR-34a
hsa-miR-19b	3.99	2.24	4842.2	0.0102	MIMAT0000074	hsa-miR-19b
hsa-miR-103	3.88	2.10	1857.7	0.0102	MIMAT0000101	hsa-miR-103
hsa-miR-215	3.78	2.10	2395.3	0.0147	MIMAT0000272	hsa-miR-215
hsa-miR-192	3.74	2.04	3743.4	0.0170	MIMAT0000222	hsa-miR-192
hsa-miR-429	3.65	5.92	2019.4	0.0170	MIMAT0001536	hsa-miR-429
hsa-miR-27a	3.32	2.18	1186.0	0.0226	MIMAT0000084	hsa-miR-27a
hsa-miR-223	3.13	4.33	585.6	0.0451	MIMAT0000280	hsa-miR-223
hsa-miR-23a	2.89	2.11	1821.2	0.0226	MIMAT0000078	hsa-miR-23a
hsa-miR-107	2.89	1.72	2475.5	0.0196	MIMAT0000104	hsa-miR-107
hsa-miR-200b	2.57	3.38	5048.1	0.0284	MIMAT0000318	hsa-miR-200b
hsa-miR-24	2.53	2.07	4163.1	0.0344	MIMAT0000080	hsa-miR-24
hsa-miR-15a	2.32	1.03	913.7	0.0170	MIMAT0000068	hsa-miR-15a
hsa-miR-16	1.85	0.77	3698.1	0.0284	MIMAT0000069	hsa-miR-16

NOTE: miRNAs with raw signal over 500 in tumor tissue are shown.

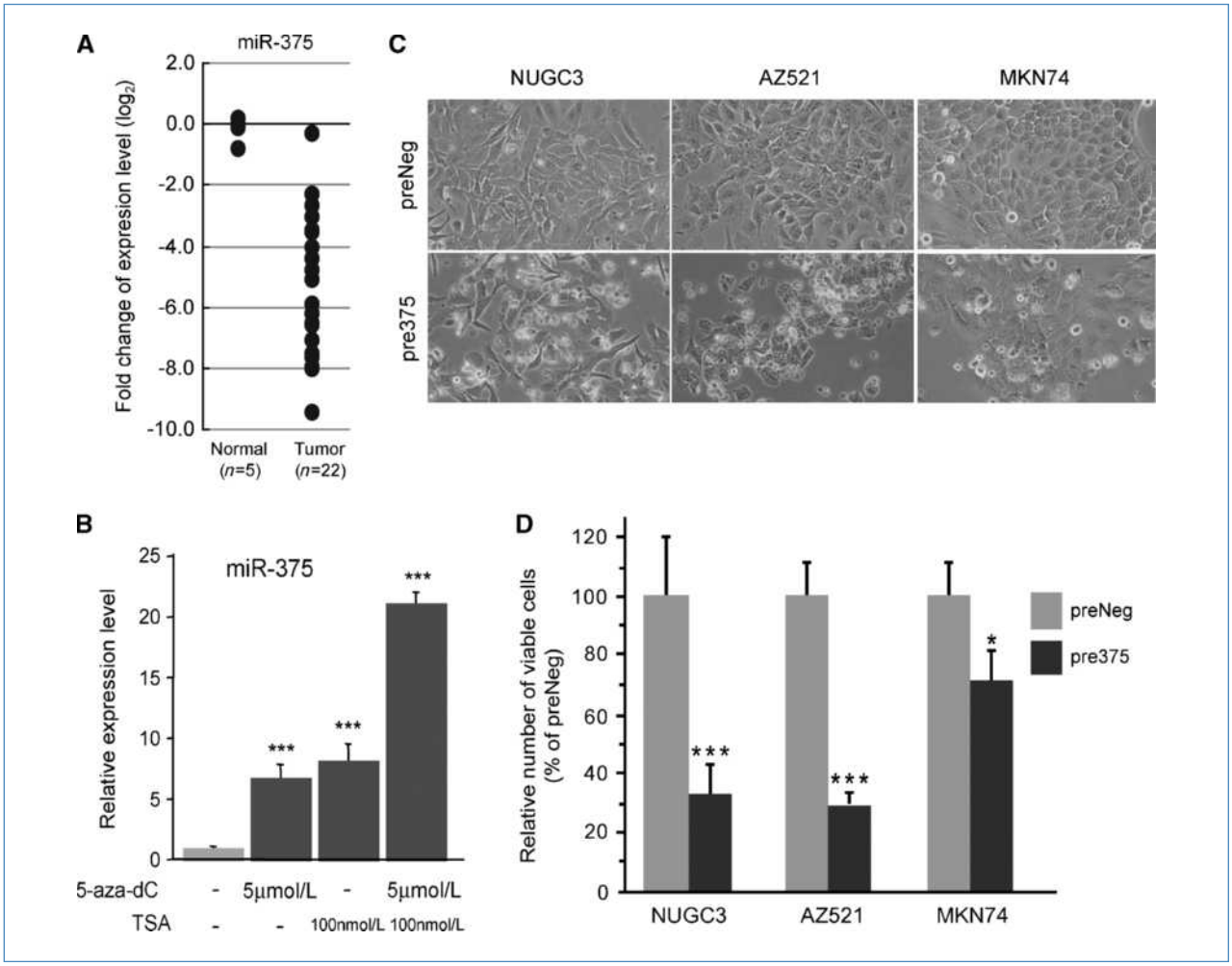

Figure 1. Ectopic expression of miR-375 causes morphologic changes and reduces cell viability. A, quantitative RT-PCR analysis of 27 samples from gastric carcinoma and normal control tissues. Each dot indicates the expression level of an individual case. The *Y* axis displays the expression level (log_2) normalized by the median expression levels of the five normal control tissues. The expression levels of gastric carcinoma (Tumor) were significantly lower than those of normal control (Normal; $P < 0.001$ by Mann-Whitney *U* test). B, induction of miR-375 by treatment of NUGC3 cells with 5-aza-dC or TSA. The expression levels of miR-375 in NUGC3 cells, treated with either 5 μmol/L 5-aza-dC, 100 nmol/L TSA, or both, were determined by qRT-PCR. Columns, means of quadruplicate determinations; bars, SD. C and D, NUGC3, AZ521, and MKN74 cells were transfected with pre375 or preNeg. C, at 72 h after transfection, cells were examined by phase contrast microscopy (original magnification, ×100). D, cell viability was determined by cell count at 72 h after transfection. The numbers of viable cells transfected with preNeg were set at 100%. Columns, means of quadruplicate determinations; bars, SD. Three independent experiments were performed, and representative data are shown. * and *** indicate $P < 0.05$ and $P < 0.001$, respectively.

using an anti-miR miRNA inhibitor targeting miR-375 (anti-miR-375, Ambion) or its control (anti-Cont). Knockdown of endogenous miR-375 in MKN74 cells did not affect the phosphorylation status of Akt (data not shown). This was probably due to the low endogenous level of miR-375 in this cell line. In contrast, knockdown of miR-375 in an islet cell carcinoma cell line, QGP-1, which expresses miR-375 more highly than any of the gastric cancer cell lines analyzed (Supplementary Fig. S3), resulted in induction of Akt phosphorylation (Supplementary Fig. S6), suggesting that downregulation of miR-375 induces Akt phosphorylation in cells that substantially express miR-375.

Expression of apoptosis-related genes is altered by miR-375. To further investigate the mechanism of miR-375–induced apoptosis, we performed expression microarray analysis on NUGC3 and AZ521 cells transfected with pre375 or preNeg for 48 h. After transfection with pre375, 1,399 genes were differentially expressed by >2-fold in NUGC3 cells compared with preNeg-transfected cells, whereas 2,066 genes were differentially expressed in AZ521 cells (Fig. 3A). Comparison of the 1,399 and 2,066 genes revealed that changes in the expression of 522 genes were common to both cell types after transfection with pre375 (Fig. 3A). Among these 522 genes, 400 that had been assigned gene symbols were analyzed further (Fig. 3A; Supplementary Table S4). Of these 400 genes, 19 were found to be apoptosis-related genes in the Gene Ontology (GO) classification (Fig. 3A; Table 2). Interestingly, of the 19 genes extracted, 14-3-3ζ (gene symbol

YWHAZ) and cIAP-2 (gene symbol BIRC3), both of which are potent antiapoptotic genes, were downregulated in pre375-transfected cells (Table 2). Furthermore, BAK1, Bim (gene symbol BCL2L11), and TNFSF12, all of which are proapoptotic genes, were upregulated by pre375 transfection (Table 2), suggesting that dysregulation of these proapoptotic and antiapoptotic genes is involved in miR-375–induced apoptosis. The downregulation of 14-3-3ζ and cIAP-2 and upregulation of Bim by pre375 were further validated by qRT-PCR (Fig. 3B). As shown in Fig. 3C, 14-3-3ζ was also downregulated at the protein level.

miR-375 targets the 3′ UTR of 14-3-3ζ. By using the TargetScan and PicTar algorithms, we found that 14-3-3ζ is a putative target of miR-375. To confirm this possibility, the miR-375 binding sequence present at the 3′ UTR of 14-3-3ζ mRNA (WT-3′ UTR) or its mutant (MUT-3′ UTR) was cloned downstream of the firefly luciferase reporter gene and then cotransfected with preNeg or pre375 into MKN74 cells. When pre375 was cotransfected, the relative luciferase activity of a reporter containing WT-3′ UTR was significantly suppressed by 30% ($P < 0.001$) compared with that of a reporter containing MUT-3′ UTR (Fig. 3D). In contrast, the luciferase activity of the reporter containing WT-3′ UTR was unaffected by simultaneous transfection with preNeg (Fig. 3D). This result was reproducible in the additional two independent experiments [25% and 23% reduction ($P < 0.001$), respectively],

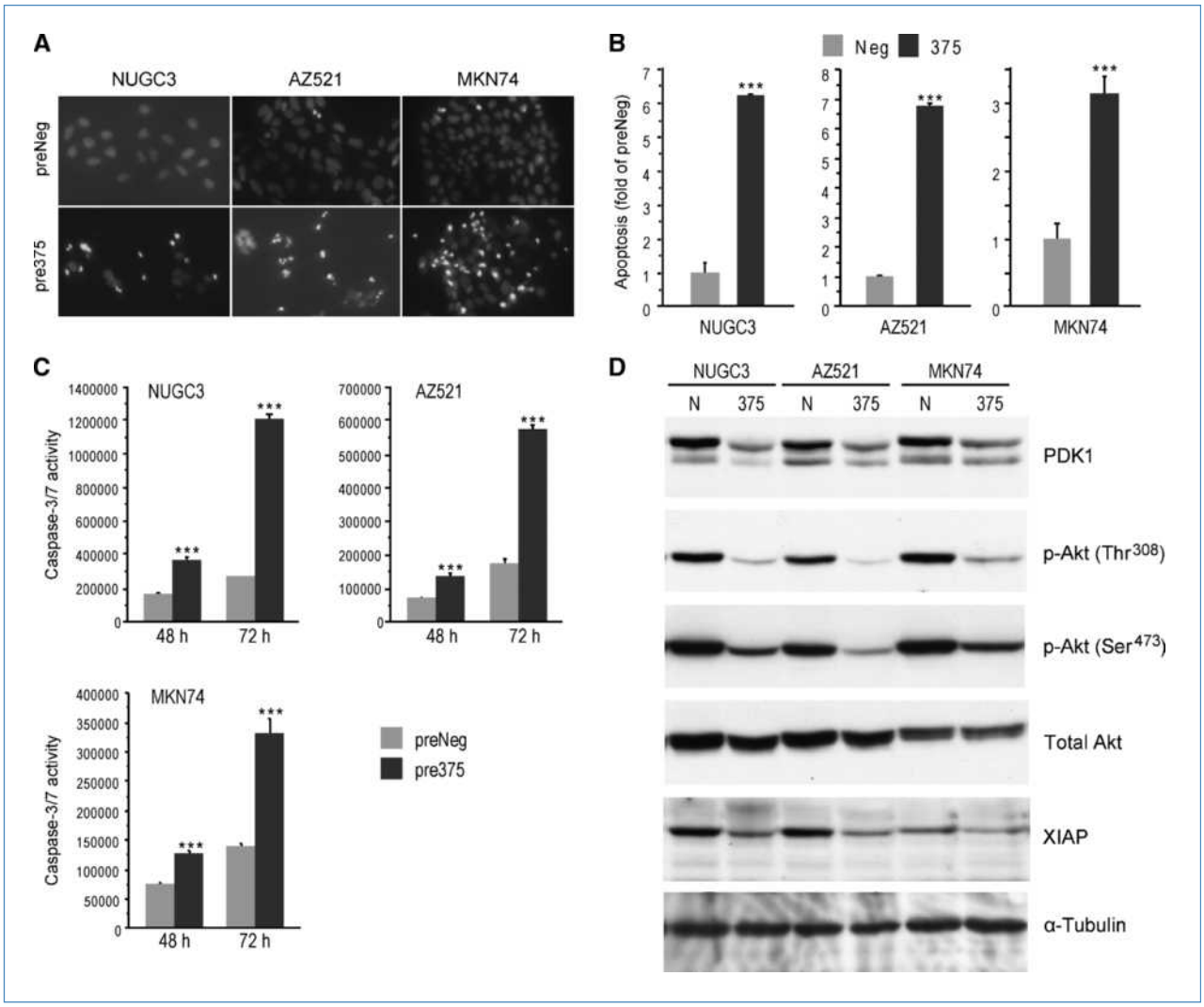

Figure 2. Ectopic expression of miR-375 induces apoptosis in gastric carcinoma cells. NUGC3, AZ521, and MKN74 cells were transfected with pre375 or preNeg. A, at 72 h after transfection, cells were stained with DAPI. B, at 72 h after transfection, cytoplasmic oligonucleosomal fragment was measured by the Cell Death Detection ELISA PLUS assay. C, activity of caspase-3 and caspase-7 was determined at 48 and 72 h after transfection. Columns, means of quadruplicate determinations; bars, SD. Three independent experiments were performed, and representative data are shown. ***, $P < 0.001$ compared with preNeg-transfected cells. D, at 72 h after transfection, cells were subjected to Western blot analysis using antibodies against PDK1, the phosphorylated form of Akt (Thr^{307} and Ser^{473}), total Akt, XIAP, and α-tubulin. α-Tubulin was used as an internal control. Experiments were repeated four times, and a representative result is shown.

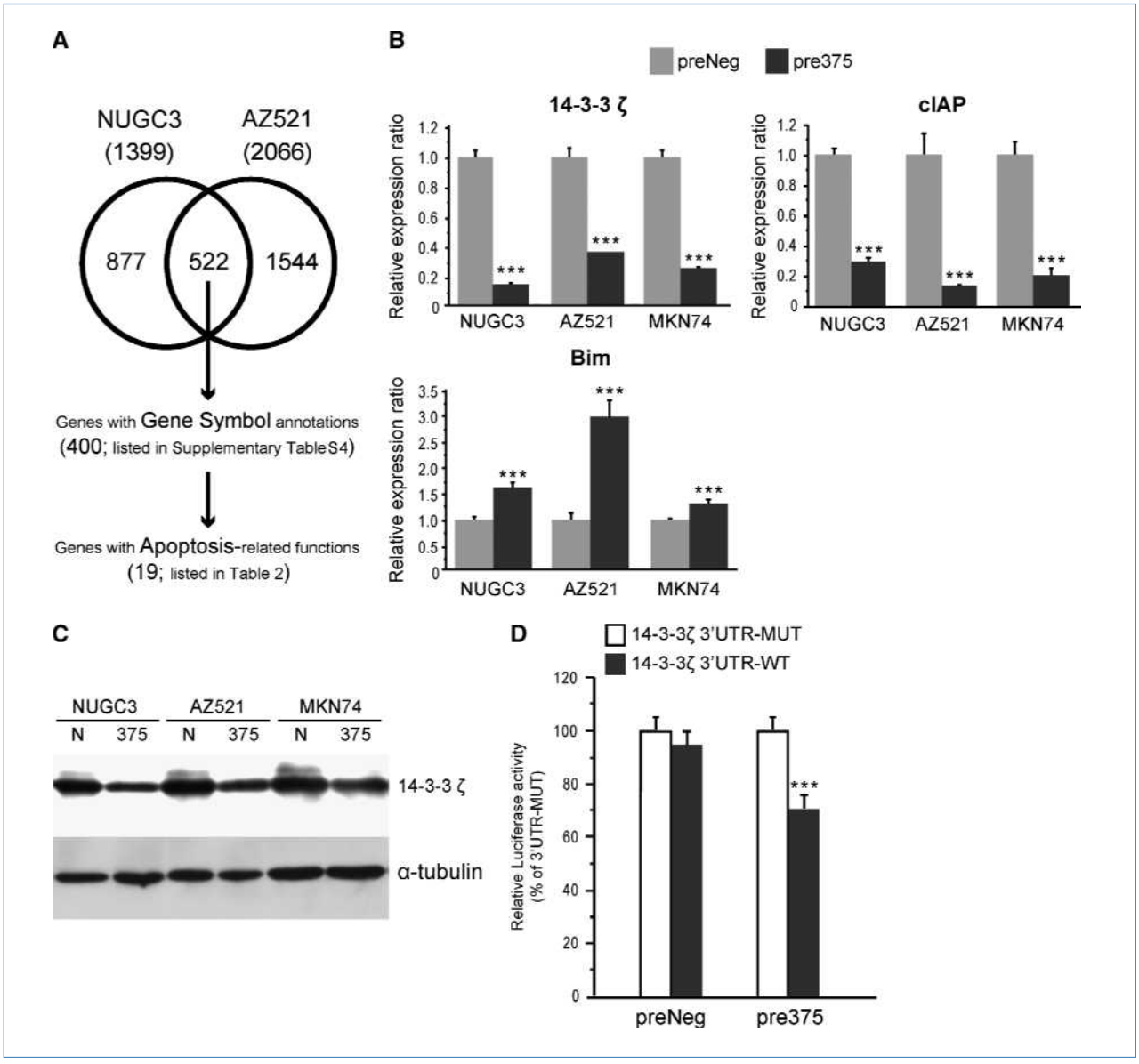

Figure 3. Overexpression of miR-375 changes the expression of apoptosis-related genes. A, gene expression microarray was performed at 48 h after transfection. The Venn diagram shows the relationship between the sets of genes differentially expressed in NUGC3 and AZ521 cells. Expressions of 1,399 and 2,066 genes in NUGC3 and AZ521 cells, respectively, were changed >2-fold by ectopic expression of pre375. Changes in the expression of 522 genes were common to both cell lines. Among these 522 genes, 400 were assigned gene symbols and are listed in Supplementary Table S4. Among the 400 genes, 19 were classified as apoptosis-related genes by Gene Ontology and are listed in Table 2. B, at 48 h after transfection, expressions of 14-3-3ζ, cIAP, and Bim were determined by quantitative RT-PCR and were normalized to expression of KPNA6. Columns, means of quadruplicate determinations; bars, SD. ***, $P < 0.001$ compared with preNeg-transfected cells. C, at 72 h after transfection, expressions of 14-3-3ζ and α-tubulin were analyzed by Western blotting. α-Tubulin was used as an internal control. Experiments were repeated four times, and a representative result is shown. D, interaction of miR-375 with the 3′ UTR of 14-3-3ζ. At 24 h after transfection with 5 nmol/L of pre375 or preNeg, a reporter plasmid containing 14-3-3ζ WT-3′ UTR or MUT-3′ UTR. and a plasmid expressing *Renilla* luciferase (pRL-CMV) were cotransfected into MKN74 cells. Firefly luciferase activity was normalized to *Renilla* luciferase activity. Normalized luciferase activity in cells transfected with WT-3′ UTR was compared with that in cells transfected with MUT-3′ UTR, which was set at 100. Columns, means of quadruplicate determinations; bars, SD. Three independent experiments were performed, and representative data are shown. ***, $P < 0.001$ compared with MUT-3′ UTR.

suggesting that miR-375 targets the miR-375 binding sequence at the 3′ UTR of 14-3-3ζ.

Downregulation of PDK1 and 14-3-3ζ is potentially involved in miR-375–induced apoptosis. To determine whether downregulation of PDK1 and 14-3-3ζ is involved in miR-375–induced apoptosis, we analyzed the effect of knockdown of PDK1 and 14-3-3ζ in NUGC3 cells. As shown in Fig. 4A, transfection of siRNAs that target

PDK1 (siPDK1) and 14-3-3ζ (si14-3-3ζ) into NUGC3 cells led to suppression of the expression of PDK1 and 14-3-3ζ, respectively, at the protein level. Interestingly, both the siPDK1 and si14-3-3ζ transfectants showed marked increases in caspase-3/caspase-7 activity, by 1.9-fold and 3.8-fold, respectively (Fig. 4B). Similar results were also observed when another set of siRNAs targeting PDK1 and 14-3-3ζ was transfected (data not shown). These results suggest that

Table 2. Apoptosis-related genes downregulated or upregulated by miR-375 in both NUGC3 and AZ521 cells

Fold change		Corrected *P*		Gene symbol	Description	Apoptosis-related function in GO biological process
NUGC3	AZ521	NUGC3	AZ521			
Downregulated genes						
0.13	0.23	1.61E-05	6.18E-07	**YWHAZ (14-3-3ζ)**	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, ζ	GO:0006916 (antiapoptosis)
0.24	0.45	7.93E-07	2.30E-06	UNC13B	unc-13 homologue B	GO:0006917 (induction of apoptosis)
0.24	0.41	1.42E-06	8.32E-06	IGFBP3	Insulin-like growth factor binding protein 3	GO:0043065 (positive regulation of apoptosis)
0.25	0.31	1.39E-04	7.43E-07	TGFB2	Transforming growth factor β-2 precursor	GO:0008219 (cell death);GO:0042981 (regulation of apoptosis)
0.27	0.09	5.34E-05	1.74E-07	**BIRC3 (cIAP-2)**	Baculoviral IAP repeat-containing 3	GO:0006916 (antiapoptosis)GO:0042981 (regulation of apoptosis)
0.30	0.40	9.89E-06	5.98E-05	PHLDA1	Pleckstrin homology-like domain, family A, 1	GO:0006915 (apoptosis)
0.33	0.34	3.51E-06	4.51E-07	OPA1	Optic atrophy 1	GO:0006915 (apoptosis)GO:0045768 (positive regulation of antiapoptosis)
0.38	0.46	2.67E-05	5.49E-05	BCL10	B-cell chronic lymphocytic leukemia/lymphoma 10	GO:0007249 (I-κB kinase/NF-κB cascade)GO:0008219 (cell death);GO:0042981 (regulation of apoptosis)
0.40	0.40	8.00E-07	4.28E-07	EBAG9	Estrogen receptor binding site associated, antigen, 9	GO:0006915 (apoptosis)
0.45	0.42	1.07E-05	1.62E-05	CUL5	Cullin 5	GO:0008629 (induction of apoptosis by intracellular signals)
0.48	0.46	1.92E-04	1.13E-05	PRKCA	Protein kinase C, α	GO:0008624 (induction of apoptosis by extracellular signals)
0.50	0.43	0.00129809	2.99E-06	CKAP2	Cytoskeleton-associated protein 2	GO:0006915 (apoptosis)
Upregulated genes						
2.09	3.25	2.58E-04	4.17E-06	IFI6	IFN, α-inducible protein 6	GO:0006916 (antiapoptosis)
2.09	2.55	2.97E-05	3.44E-06	CARD8	Caspase recruitment domain family, member 8	GO:0042981 (regulation of apoptosis)
2.20	2.67	8.40E-05	2.36E-06	**BCL2L11 (Bim)**	BCL2-like 11	GO:0006915 (apoptosis);GO:0006917 (induction of apoptosis)GO:0043065 (positive regulation of apoptosis)
2.25	2.05	2.64E-05	1.25E-04	BAK1	BCL2-antagonist/killer 1	GO:0006917 (induction of apoptosis)GO:0042981 (regulation of apoptosis)
2.29	3.19	2.79E-06	9.32E-06	TNFSF12	Tumor necrosis factor (ligand) superfamily, 12	GO:0006915 (apoptosis);GO:0006917 (induction of apoptosis)
2.70	2.60	1.12E-06	1.67E-04	RRAGC	Ras-related GTP binding C	GO:0006915 (apoptosis)
2.82	2.06	8.72E-06	1.13E-05	LITAF	Lipopolysaccharide-induced TNF factor	GO:0006915 (apoptosis)

NOTE: Genes that were subjected to qRT-PCR are shown in bold.

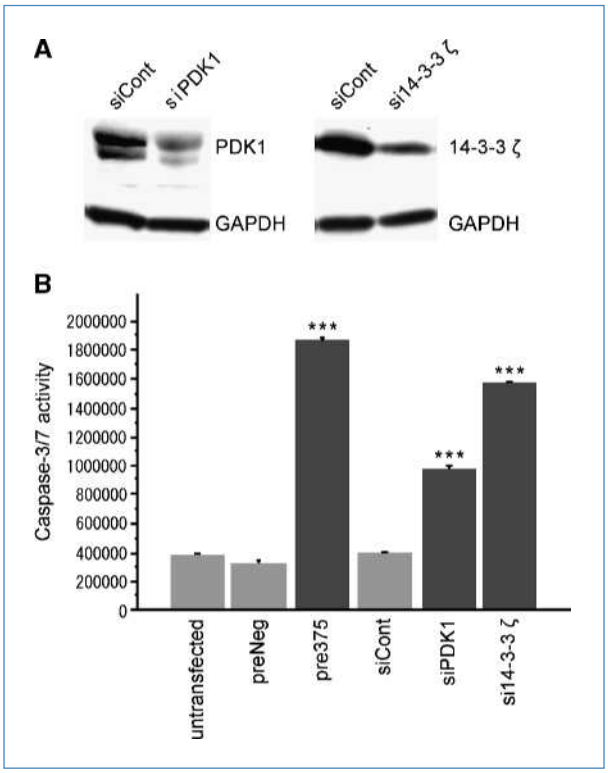

Figure 4. PDK1 and 14-3-3ζ are involved in miR-375–induced apoptosis. A, knockdown of PDK1 and 14-3-3ζ by their respective siRNAs was confirmed by Western blotting. NUGC3 cells were transfected with siPDK1, 14-3-3ζ, or siCont. At 72 h after transfection, cells were subjected to Western blot analysis. B, NUGC3 cells were transfected with pre375, preNeg, siPDK1, si14-3-3ζ, or siCont. At 72 h after transfection, cells were subjected to caspase-Glo 3/7 assay. Columns, means of quadruplicate determinations; bars, SD. Two independent experiments were performed, and representative data are shown. ***, $P < 0.001$. The value for pre375 was compared with that for preNeg, whereas values for siPDK1 and si14-3-3ζ were compared with that for siCont.

downregulation of PDK1 and 14-3-3ζ is involved in miR-375–induced apoptosis.

Discussion

In this study, we analyzed the expression profiles of miRNAs in gastric carcinoma by using a miRNA microarray and found that miR-375 was the most downregulated miRNA. Although miR-375 has been reported to be downregulated in head and neck (33), pancreatic (34), and hepatocellular (35) carcinomas, its function in cancer remains to be determined. In the present study, we showed that reexpression of miR-375 in gastric carcinoma cell lines resulted in induction of apoptosis and reduced cell viability. These results allow us to speculate that downregulation of miR-375 may provide a survival advantage to gastric carcinoma cells. The mechanism responsible for miR-375 downregulation in gastric carcinoma is still unknown. Because loss at 2q35, wherein miR-375 is located, is rarely detected in gastric carcinoma (25, 28), it is unlikely that allelic loss is responsible for the downregulation of miR-375. On the other hand, we found that miR-375 was markedly upregulated when a gastric carcinoma cell line, NUGC3, was treated with both 5-aza-dC and TSA. In addition, computational analysis revealed that miR-375 is located in a CpG island on chromosome 2q35 (National Center for Biotechnology Information). Therefore, it seems possible that epigenetic silencing by, for example, DNA methylation and histone deacetylation may be responsible for the downregulation of miR-375. Further studies will be needed to test this possibility. Taken together, we propose that miR-375 is a candidate tumor suppressor miRNA in gastric carcinoma.

We also found that exogenous miR-375 suppresses the expression of PDK1, resulting in decreased phosphorylation of Akt in gastric carcinoma cells. This is consistent with a recent report showing that miR-375 directly targets PDK1 at the protein level in pancreatic β cells (29). In addition, we found that expression of XIAP, a substrate of Akt, was also decreased by miR-375 overexpression. It has been reported that XIAP, which is phosphorylated and stabilized by Akt, inhibits apoptosis by suppressing caspase activity (32). Indeed, caspase activity was markedly increased by ectopic expression of miR-375. Thus, these results suggest that inhibition of Akt phosphorylation by direct targeting of PDK1 is involved in miR-375–induced apoptosis. Consistent with this speculation, we verified that caspase activity was also increased in siPDK1-transfected cells. Phosphorylated Akt has been reported to promote cell survival in various types of carcinoma cells (36, 37). In gastric carcinoma cells, Akt activation has been reported to promote cell survival against apoptotic stimuli (30, 38). Indeed, Akt activation was reported to correlate with poor prognosis in gastric carcinoma patients (39). Furthermore, we showed that knockdown of endogenous miR-375 increased the phosphorylation of Akt. Therefore, we propose that decreased expression of miR-375 may provide a survival advantage to gastric carcinomas via activation of the PDK1/Akt survival pathway.

In this study, we showed that miR-375 reduces the expression of 14-3-3ζ at both the mRNA and protein levels. The luciferase assay with a reporter containing the miR-375 binding sequence at the 3′ UTR of 14-3-3ζ mRNA suggested that miR-375 directly targets the 3′ UTR of 14-3-3ζ mRNA. However, suppression of the luciferase activity by miR-375 was limited to 23% to 30%, whereas 14-3-3ζ protein was suppressed by at least 50% in miR-375–overexpressed cells. Therefore, we cannot exclude the possibility that other mechanisms may also participate in the reduction of 14-3-3ζ. Further studies will be required to clarify this point. Interestingly, we found that caspase activation was induced in cells transfected with 14-3-3ζ–specific siRNA. Similar results were also obtained in miR-375–transfected cells, suggesting that suppression of 14-3-3ζ is involved in miR-375–induced apoptosis. It has been reported that overexpression of 14-3-3ζ is correlated with poor prognosis in patients with breast or lung carcinomas (40, 41). Fan and colleagues showed that downregulation of 14-3-3ζ sensitizes lung cancer cells to cisplatin-induced cell death (40). Neal and colleagues recently showed that overexpression of 14-3-3ζ enhances anchorage-independent growth and inhibits stress-induced apoptosis in breast

cancer cells (41). Thus, these previous reports suggest that 14-3-3ζ has an antiapoptotic function and promotes survival in carcinoma cells. Together, we can hypothesize that downregulation of miR-375 results in enhanced expression of 14-3-3ζ and provides a survival advantage to gastric carcinoma. Consistent with this hypothesis, we found that a proportion of gastric carcinoma tissues exhibited upregulation of 14-3-3ζ (data not shown).

In contrast, expression microarray analysis revealed that >1,000 genes were upregulated or downregulated by 2-fold or greater in cells transfected with miR-375. Among these genes, several candidate targets for miR-375 were included (data not shown). In addition, the expressions of several apoptotic genes, such as BIRC3 (cIAP-2) and BCL2L11 (Bim), were also affected by exogenous miR-375. Therefore, we cannot exclude the possibility that candidate targets for miR-375 other than PDK1 and 14-3-3ζ might also be involved in miR-375–induced apoptosis. Further study is required to elucidate this possibility.

In our miRNA profiles, 85 miRNAs were significantly upregulated and we selected 33 miRNAs as gastric cancer–related miRNAs on the basis of raw signal intensities. Interestingly, 11 of the 33 miRNAs belonged to the clusters of miR-106b-25 (chr 7), miR-17-92 (chr 13), or miR-106a-92 (chr X). It has already been reported that the miR-106b-25 and miR-17-92 clusters seem to have an oncogenic role (22, 42–44), and the miR-106a-92 cluster shares a high degree of homology with the miR-106b-25 and miR-17-92 clusters (Sanger miRBase, TargetScan). Thus, miRNAs of these three clusters may function in a coordinated manner in gastric cancer tumorigenesis. Of the 17 miRNAs that were significantly downregulated, we selected six miRNAs including miR-375. We noticed that miR-29c was also included among these six miRNAs. Because miR-29c has been reported to play tumor-suppressive roles by targeting DNMT3A and DNMT3B (45), Mcl-1 (46), YY-1 (47), p85α, and CDC42 (48), it is possible that miR-29c is another candidate tumor suppressor for gastric carcinomas. Further studies with the remaining five downregulated miRNAs will be necessary to understand the tumor-suppressive roles of miRNAs in gastric carcinogenesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

Ministry of Education, Science, Sports and Culture of Japan and Grants-in-Aid for Young Scientists (B) 20790286.

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Received 07/27/2009; revised 12/29/2009; accepted 01/09/2010; published OnlineFirst 03/09/2010.

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