

Overexpression of miR-200c Induces Chemoresistance in Esophageal Cancers Mediated Through Activation of the Akt Signaling Pathway

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Abstract

Purpose: To determine the relationship between resistance to chemotherapy and microRNA (miRNA) expression in esophageal cancer, we focused on miRNAs known to be associated with maintenance of stem cell function.

Experimental Design: Using 98 formalin-fixed, paraffin-embedded samples obtained from patients with esophageal cancer who had received preoperative chemotherapy followed by surgery, we measured expression levels of several miRNAs that are considered to be involved in the regulation of stem cell function (e.g., let-7a, let-7g, miR-21, miR-134, miR-145, miR-155, miR-200c, miR-203, and miR-296) by real-time reverse transcriptase PCR. Then, we examined the relationship between miRNA expression and prognosis or response to chemotherapy. To investigate the mechanism of miRNA-induced chemoresistance, *in vitro* assays were carried out using esophageal cancer cells.

Results: Analyses of the 9 miRNAs expression showed that overexpression of miR-200c ($P = 0.037$), underexpression of miR-145 ($P = 0.023$), and overexpression of miR-21 ($P = 0.048$) correlated significantly with shortened overall duration of survival. In particular, miR-200c expression correlated significantly with response to chemotherapy ($P = 0.009$ for clinical response; $P = 0.007$ for pathologic response). *In vitro* assay showed significantly increased miR-200c expression in cisplatin-resistant cells compared with their parent cells (~1.7-fold). In anti-miR-200c-transfected cells, chemosensitivity to cisplatin and apoptosis after exposure to cisplatin was found to increase as compared with the negative control. Western blotting showed that knockdown of miR-200c expression was associated with increased expression of PPP2R1B, a subunit of protein phosphatase 2A, which resulted in reduced expression of phospho-Akt.

Conclusions: Results of this study emphasized the involvement of miR-200c in resistance to chemotherapy among esophageal cancers and that this effect was mediated through the Akt pathway. *Clin Cancer Res*; 17(9); 3029–38. ©2011 AACR.

Introduction

Esophageal cancer is the eighth most common incident cancer and the sixth most common cause of cancer death (1). Surgery is considered the standard management approach for esophageal cancer, but the prognosis of patients who receive only surgery is poor, with a 5-year survival rate ranging from 15% to 39% (2, 3). To improve

the survival rate of patients with esophageal cancer, multimodal treatment, including chemotherapy plus surgery and chemoradiotherapy plus surgery, has been developed. In fact, some clinical trials have shown that these multimodal therapies prolonged survival of esophageal cancer patients (4, 5). The most commonly used chemotherapeutic regimen in esophageal cancers is cisplatin-based chemotherapy, such as combination chemotherapy of cisplatin and 5-fluorouracil (5-FU). However, the reported response rate to chemotherapy including cisplatin is only 19% to 40% (3, 6), and about half of these patients do not achieve a good response to chemotherapy. Thus, chemoresistance is a major obstacle in the treatment of esophageal cancers. A better understanding of the mechanism of chemoresistance in esophageal cancer is needed to improve prognosis.

MicroRNAs (miRNA) bind to the 3'-untranslated region of their target mRNAs, and such binding leads to translational repression or reduced stability of the mRNA (7).

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Translational Relevance

In this study, we examined the expression of several miRNAs known to regulate stem cell function by using formalin-fixed, paraffin-embedded (FFPE) tissues obtained from patients with esophageal cancer who received preoperative chemotherapy followed by surgery. The results showed that overexpression of miR-200c was closely associated with poor response to preoperative chemotherapy and poor prognosis, and in the *in vitro* study, we found that miR-200c directly targeted PPP2R1B and resulted in activation of Akt signaling. The results suggested that miR-200c is a potentially useful predictor of chemosensitivity in patients with esophageal cancer.

MiRNAs play important roles in various biological processes, such as cell differentiation, cell proliferation, apoptosis, and metabolism. In addition, miRNAs have emerged as central regulators of cancer (8), and their aberrant expression in many tumors indicate that they could function as tumor suppressors or oncogenes.

Recent studies have shown that certain miRNAs (e.g., let-7, miR-134, miR-296, miR-302, miR-367, and miR-470) are involved in the regulation of stem cell function, such as self-renewal, pluripotency, and differentiation (9, 10). In particular, miR-145 directly regulates the reprogramming factors (OCT4, SOX2, and KLF4), inhibits human embryonic stem cell self-renewal, and represses the expression of pluripotency genes (11), whereas miR-203 directly represses the expression of p63, which is an essential regulator of stem cell maintenance in epithelial tissues (12). On the other hand, the "cancer stem cells (CSC)" hypothesis has attracted significant attention. The hypothesis suggests that cancers are maintained in a hierarchical organization of rare, slowly dividing cancer stem cells (or tumor-initiating cells), rapidly dividing amplifying cells, and differentiated tumor cells. There are several similarities between CSCs and normal stem cells with regard to maintenance of self-renewal and pluripotency. Therefore, miRNA may play an important role in the regulation of CSCs and normal stem cells. In fact, a recent study showed accumulation of tumor-initiating cells after initiation of chemotherapy in breast cancer and reduced let-7 expression in these tumor-initiating cells, which helped maintain their undifferentiated status and proliferative potential (13). CSCs are considered responsible for resistance to anticancer treatment, such as chemotherapy and radiotherapy (14).

The main hypothesis of the present study was that miRNAs that regulate stem cell function are involved in resistance to chemotherapy in esophageal cancer. To test this hypothesis, we examined the expression levels of several miRNAs considered to be involved in the maintenance of stem cell function, such as miRNAs from the let-7 family, miR-145, miR-200c, miR-21, miR-296, miR-155, miR-134, and miR-296, and analyzed their association

with the response to chemotherapy and prognosis after chemotherapy followed by surgery in esophageal cancers. Among these miRNAs, the results pointed to the involvement of miR-200c in chemoresistance in esophageal cancers.

Material and Methods

Patients and tissue samples

All tissue samples were obtained from patients who had undergone radical esophagectomy with lymph node dissection for thoracic esophageal cancers between 1999 and 2006 at the Department of Gastroenterological Surgery, Graduate School of Medicine, Osaka University, Japan. Informed consent was obtained from each patient prior to participation in the study. Furthermore, these patients had received chemotherapy before the surgery ($n = 98$). The preoperative chemotherapeutic regimen can be described as follows: Cisplatin was administered at 70 mg/m², adriamycin was administered at 35 mg/m² by rapid intravenous infusion on day 1; and 5-FU at 700 mg/m² administered by continuous intravenous infusion starting on day 1 and until day 7. Two courses of chemotherapy were used, separated by a 4-week interval. All patients were staged pre- and postoperatively according to the criteria of the International Union Against Cancer. The median duration of follow-up was 28.8 months (range: 2.3–96.7 months), and 57 patients (58.2%) died during the follow-up period.

Clinical and histopathologic evaluation of response to chemotherapy

Two weeks after completion of chemotherapy, all patients were re-staged through endoscopy, computed tomographic (CT) scan, and positron emission tomographic (PET) scan to evaluate the clinical response to chemotherapy. The clinical response was categorized according to the following criteria [based on the World Health Organization response criteria for measurable disease (15) and the criteria of the Japanese Society for Esophageal Diseases (16)]: A complete response (CR) was defined as total regression of the disease. A CR of the primary tumor represented disappearance of the tumor on CT scan and/or PET scan and endoscopy. A partial response (PR) was defined as more than 50% reduction in primary tumor size and lymph node metastasis, as confirmed by CT scan. Progressive disease (PD) was defined as more than a 25% increase in the primary tumor or the appearance of new lesions. Cases that did not meet the criteria of PR or PD were defined as no change (NC).

After fixation in 10% buffered formalin, the surgical specimens of primary tumors were cut into 5-mm slices. All sliced tissues were embedded in paraffin, cut into 4- μ m thick sections, and then stained with hematoxylin and eosin. The extent of histopathologic tumor regression was classified into 5 categories. The extent of viable residual carcinoma at the primary site was assessed

semiquantitatively on the basis of the estimated percentage of viable residual carcinoma in relation to total carcinoma area. Briefly, the percentage of viable residual tumor cells within the total cancerous tissue was assessed as follows: grade 3, no viable residual tumor cells; grade 2, less than two-third residual tumor cells; grade 1b, one-third to two-third residual tumor cells; grade 1a, more than two-third residual tumor cells; and grade 0, no significant response to chemotherapy (16).

RNA isolation from FFPE and fresh-frozen samples

Portions of esophageal cancer tissue samples were frozen in liquid nitrogen immediately after surgery and stored at -80°C , and the remaining tissues were routinely fixed in 10% formalin and embedded in paraffin wax. Total RNA was isolated from the FFPE samples using the RecoverAll Total Nucleic Acid Isolation Kit (Ambion) according to the manufacturer's instructions. Briefly, each FFPE tissue block was cut into 20- μm thick pieces, and 4 slices were placed in a centrifuge tube. To liquefy the paraffin, 100% xylene and 100% ethanol were added into each tube. After centrifugation, the precipitated samples were air dried and treated with protease in heat blocks for 3 hours at 50°C . Then, each sample was treated with isolation reagent and filtered. Each filter was treated with DNase and incubated for 30 minutes at room temperature. After washing the filter with washing reagents, it was treated with warmed Elution Solution and centrifuged to pass the mixture through the filter. The eluate contained the isolated RNA.

Total RNA from fresh-frozen samples was isolated by using TRIzol reagent (Invitrogen). Briefly, 100 mg of frozen tissue sample was homogenized in 1.0 mL of TRIzol reagent and incubated for 5 minutes at room temperature. Each sample was treated with 0.2 mL of chloroform and, after incubation at room temperature for 3 minutes, centrifuged at $12,000 \times g$ for 15 minutes at 4°C . Following centrifugation, the colorless supernatant was transferred to a fresh tube, and 0.5 mL of isopropyl alcohol was added before incubation at room temperature for 10 minutes. After centrifugation at $12,000 \times g$ for 10 minutes at 4°C , the supernatant was removed and the pellet was washed with 75% ethanol and then centrifuged at $7,500 \times g$ for 5 minutes at 4°C . The RNA pellet was dried and dissolved in RNase-free water. RNA concentration was quantified using the NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific Inc.).

To evaluate the quality of the RNA extracted from FFPE samples, we compared the expression levels of miRNAs in these samples with the RNAs extracted from fresh-frozen tissue samples. There was no significant difference in the expression of RNU48, which was used as the internal control miRNA, between the fresh-frozen and FFPE tissue samples. As reported previously (17), there was a significant correlation in miRNA expression level between fresh-frozen and FFPE tissue samples (Supplementary Fig. S1). These results validated the use of FFPE samples in our study for examination of miRNA expression.

Quantitative real-time reverse transcriptase PCR

The cDNA was synthesized from 10 ng of total RNA, using the TaqMan miRNA Reverse Transcription Kit and specific stem-loop reverse transcription primers (Applied Biosystems) according to the manufacturer's protocol. Reverse transcription conditions were as follows: 16°C for 30 minutes followed by 40°C for 30 minutes and 85°C for 5 minutes. Real-time PCR reaction was done using TaqMan Universal PCR Master Mix, No AmpErase UNG, and TaqMan miRNA specific PCR-primers (Applied Biosystems). Twenty microliters of the reaction product was incubated in a 96-well optical plate at 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds, and then at 60°C at 1 minute, using ABI PRISM 7900HT (Applied Biosystems). The miRNA expression value was expressed relative to that of RNU48 (control) and analyzed using the $2^{-\Delta\Delta\text{Ct}}$ method (18).

Cell lines and culture conditions

Five established cell lines derived from esophageal squamous cell carcinoma (SCC; i.e., TE-1, -8, -10, -13, and -15) were obtained from the Riken Cell Bank (Tsukuba, Japan). All cell lines were cultured in RPMI 1640 (Life Technologies), containing 10% FBS (Sigma-Aldrich Co.) and 1% penicillin/streptomycin (Life Technologies Inc.), in a humidified atmosphere with 5% CO_2 at 37°C .

Establishment of cisplatin-resistant cell lines

A cisplatin-resistant cell line (TE8-R) was developed through a stepwise increment of cisplatin concentration as follows: The initial concentration of cisplatin was $2 \mu\text{mol/L}$ and, after 3 days, the cells were passed through a cisplatin-free medium. Upon reaching confluence, the cells were treated with a higher concentration of cisplatin (1.5- to 2.0-fold). The dose of cisplatin was gradually escalated with every few passages, until it reached a concentration of $35 \mu\text{mol/L}$ over a period of 2 months. The cisplatin-resistant subline (TE8-R) of cells was 5.8-fold more resistant to cisplatin than the parent cell line (TE8-P). The IC_{50} values for TE8-P and TE8-R were 8.33 and $48.19 \mu\text{mol/L}$, respectively.

Anti-miRNA transfection

Cells were cultured to 60% to 80% confluence and transfected with 5 nmol/L of anti-miR-200c or negative control oligonucleotides (Applied Biosystems), using siPORT NeoFX Transfection Agent (Ambion) in accordance with the manufacturer's protocol. After transfection, cells were cultured for 72 hours and intermediate samples were collected at 12, 24, and 48 hours and analyzed by immunoblotting, reverse transcriptase PCR (RT-PCR), MTT assay, and flow cytometry.

MTT assay

The MTT assay was used to assess IC_{50} (the 50% inhibition concentration) value for cisplatin. Cells were seeded into 96-well plates at a concentration of 5×10^3 per well and incubated overnight under the usual culture

conditions; then, they were exposed to cisplatin at various concentrations ranging from 0 to 400 $\mu\text{mol/L}$ (0, 3.125, 6.25, 12.5, 25, 50, 100, 200, and 400 $\mu\text{mol/L}$) for 6 hours. After treatment, the medium was changed to a cisplatin-free medium. Following 48 hours of incubation, 10 μL of MTT solution was added to each well and the plates were incubated for another 3 hours at 37°C and formazan crystals were dissolved with 100 μL of 0.04N HCl-isopropanol. The absorbance of individual wells was read at 550-nm test wavelength and 655-nm reference wavelength, using a microplate reader (Bio-Rad Laboratories). The IC_{50} value for cisplatin was calculated from the dose-response curve.

Apoptosis assay

Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) stains were used to determine the percentage of cells undergoing apoptosis and necrosis among the cells treated with 30 $\mu\text{mol/L}$ of cisplatin for 4 hours. An apoptosis assay was conducted using the protocol supplied by the manufacturer (BioVision Inc.). Briefly, cells were trypsinized gently and resuspended with 500 μL of $1 \times$ binding buffer and then treated with 5 μL of Annexin V-FITC and 5 μL PI. After incubation for 5 minutes on ice, each sample was analyzed immediately using the FACSCalibur flow cytometer (Becton Dickinson). Approximately 15,000 cells were detected for each sample. Cytogram analysis was done using the Cell Quest software, and unstained cells were classified as "live"; cells stained by Annexin V only were "early apoptotic"; cells stained by both Annexin V and PI were "late apoptotic"; and cells stained by PI only were "dead" cells.

miRNA target prediction

The analysis of miR-200c predicted targets was determined using algorithms called Target Scan (<http://www.targetscan.org/>) and miRBase Targets database (<http://microrna.sanger.ac.uk>).

Immunoblotting

Adherent cells were washed with ice-cold PBS and lysed in radioimmunoprecipitation assay (RIPA) buffer (Thermo Fisher Scientific Inc.) or Sample buffer (Wako Pure Chemical Industries) on ice. Lysates were spun and the supernatant was collected. Equal amounts of cell extracts (15 μg) were fractionated by SDS-PAGE (Bio-Rad Laboratories Inc.) and transferred onto membranes (ImmobilonP). After blockade induced through incubation with milk, the membranes were incubated overnight at 4°C with the primary antibodies and with secondary antibodies for 1 hour at room temperature. The following antibodies were used in this study: anti-actin (dilution 1:1,000; Sigma-Aldrich Co.); anti-PTEN, anti-Apaf1, anti-TFAP2 α , and anti-SOCS6 (for all, dilution 1:200; Santa Cruz Biotechnology); anti-LATS2 (dilution 1:1,000) and anti-A20 (dilution 1:200; Abcam); anti-Akt (dilution 1:200), anti-pAkt (dilution 1:200), anti-PPP2CA (dilution 1:500), and anti-PPP2R1B (dilution 1:500; Cell Signaling Technology).

Immune complexes were detected using the Detection Kit (GE Healthcare).

Statistical analysis

All data are expressed as mean \pm SD. The relationship between miRNA expressions and each clinicopathologic variable was comparatively analyzed by the χ^2 test, the Fisher exact test, or the Mann-Whitney *U* test. Time to recurrence was defined as the time interval between the date of surgery and the date of diagnosis of first recurrence, or last date of follow-up if recurrence was not observed. Overall survival time was censored at the date of the last follow-up if death did not occur. For survival analysis, the Kaplan-Meier method was used to assess survival-time distribution according to miRNA expression level, and the log-rank test was used to examine the differences between groups. A *P* value of less than 0.05 indicated the presence of statistically significant difference between groups. All statistical analyses were carried out with JMP ver.8.0 software (SAS Institute Inc.).

Results

Overexpression of miR-200c was associated with poor response to preoperative chemotherapy and poor prognosis in patients with esophageal cancers

Several miRNAs are known to regulate stem cell function. In this study, we used quantitative RT-PCR (qRT-PCR) to quantify the expression of 9 miRNAs (let-7a, let-7g, miR-21, miR-134, miR-145, miR-155, miR-200c, miR-203, and miR-296) in esophageal cancer tissues harvested during surgery after a course of preoperative chemotherapy. The expression of miR-200c, but not of any other miRNAs, correlated inversely and significantly with the response to chemotherapy (Table 1, Supplementary Table S1). Furthermore, overexpression of miR-200c, underexpression of miR-145, and overexpression of miR-21 correlated significantly with shorter overall survival duration of patients who received preoperative chemotherapy (Fig. 1, Supplementary Fig. S2). On the other hand, the expressions of other miRNAs, including let-7a, let-7g, miR-134, miR-155, miR-203, and miR-296, did not correlate with duration of survival. These findings suggest the involvement of miR-200c overexpression in chemoresistance and poor prognosis.

Table 2 summarizes the relationship between miR-200c expression and other clinicopathologic parameters of patients who received preoperative chemotherapy followed by surgery. The expression of miR-200c correlated significantly with tumor depth ($P < 0.001$), lymphatic invasion ($P = 0.022$), and vessel invasion ($P = 0.002$). In addition, we measured miR-200c expression level in paired tumor and nontumor surgical tissues from 15 patients. The expression level of miR-200c was higher in the tumor tissues than in nontumor tissues in most patients (Fig. 2A), and the mean expression level of miR-200c in tumor tissues was significantly higher than that in nontumor tissues (Fig. 2B).

Table 1. Relationship between miRNA expression and response to preoperative chemotherapy in patients with esophageal cancer

	High expression (n = 49)	Low expression (n = 49)	P
miR-200c			
Clinical response CR/PR/NC/PD	0/17/31/1	1/31/16/1	0.009
Pathologic response 2/1b/1a/0	1/5/29/14	8/14/16/11	0.007
miR-145			
Clinical response CR/PR/NC/PD	1/21/25/2	0/27/22/0	0.290
Pathologic response 2/1b/1a/0	6/9/24/10	3/10/21/15	0.287
miR-21			
Clinical response CR/PR/NC/PD	0/23/24/2	1/25/22/0	0.393
Pathologic response 2/1b/1a/0	4/6/27/12	5/13/17/13	0.371

Inhibition of miR-200c expression increases chemosensitivity to cisplatin and apoptosis in esophageal cancer cells

In the next series of studies, we established the relationship between miR-200c expression and chemoresistance, using esophageal SCC cell lines. We first established the cisplatin-resistant cell line (TE8-R) because the expression level of miR-200c was lower in TE8 than in other esophageal cancer cell lines (Fig. 3A). The IC₅₀ of TE8-R was approximately 5.8-fold higher than that of parent cells (TE8-P; Supplementary Fig. S3). Real-time RT-PCR confirmed a significantly higher miR-200c expression in TE8-R than in their parent cells (~1.7-fold change; Fig. 3B).

To investigate the role of miR-200c in the proliferative activity and chemosensitivity in esophageal cancer, we

transfected TE13 cells, which overexpressed miR-200c (Fig. 2C), with a specific inhibitor of miR-200c (anti-miR-200c) (Supplementary Fig. S4). In the proliferation assay, inhibition of miR-200c expression did not have a significant impact on the proliferative activity (Fig. 3A). Furthermore, the MTT assay showed that inhibition of miR-200c expression with anti-miR-200c reduced the IC₅₀ value for cisplatin (17.84 μmol/L), compared with the negative controls (163.40 μmol/L, Fig. 3B), suggesting the role of miR-200c in mediating sensitivity to cisplatin in esophageal cancer cells.

Furthermore, we examined the effect of miR-200c knockdown on apoptotic cell death. For this purpose, we used flow cytometry to determine the percentages of Annexin V-positive cells among anti-200c-transfected cells

Figure 1. Kaplan–Meier curves of overall survival rates of 98 patients with esophageal cancer who received preoperative chemotherapy followed by surgery, according to miRNA expression score as low expression level (below the median value) and high expression (above the median value). High expression levels of miR-200c (A) and miR-21 (C) and low expression levels of miR-145 (B) correlated significantly with shorter overall survival.

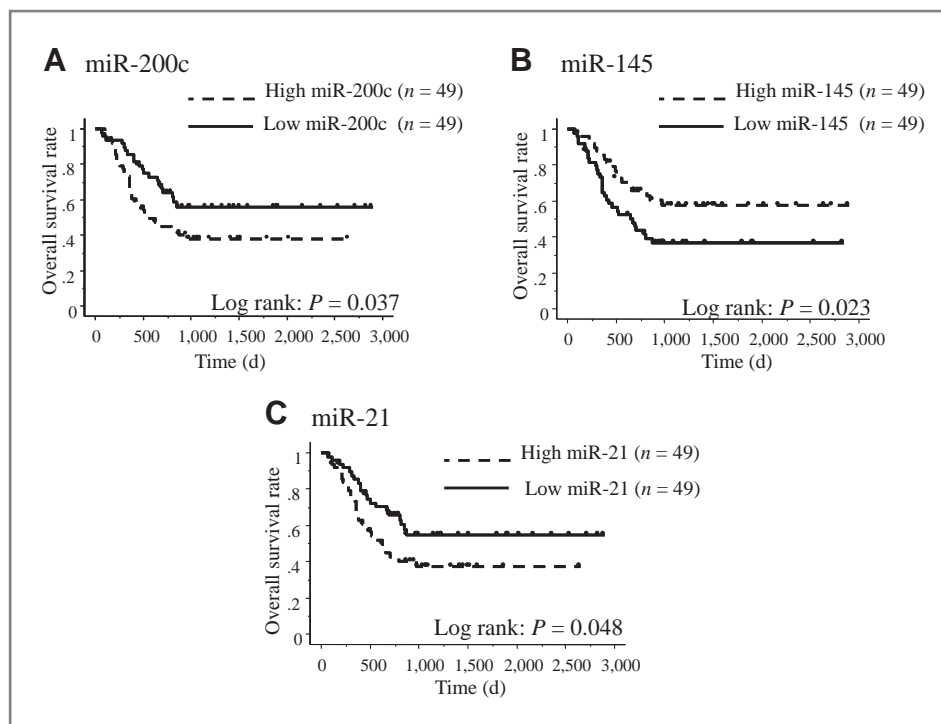


Table 2. Correlation between miR-200c expression and clinicopathologic features of patient who received preoperative chemotherapy followed by surgery

	High expression (n = 49)	Low expression (n = 49)	P
Age (mean ± SD), y	63.2 ± 8.5	60.0 ± 8.6	0.07
Sex			0.774
Male	41	43	
Female	8	6	
Differentiation			0.278
Well differentiated SCC	10	8	
Moderately differentiated SCC	23	24	
Poorly differentiated SCC	14	10	
Other	2	7	
Stage (I/II/III/IV)	1/13/16/19	5/15/12/17	0.262
pT (T1/T2/T3/T4)	2/3/34/10	11/12/20/6	<0.001
pN (N0/N1)	11/38	18/31	0.184
Number of metastatic lymph nodes (mean ± SD)	7.59 ± 18.32	4.08 ± 10.23	0.245
pM (M0/M1)	30/19	32/17	0.712
ly			0.022
ly0	3	12	
ly1-3	46	37	
v			0.002
v0	17	33	
v1-3	32	16	
Recurrence			0.276
Present	30	26	
Absent	19	23	

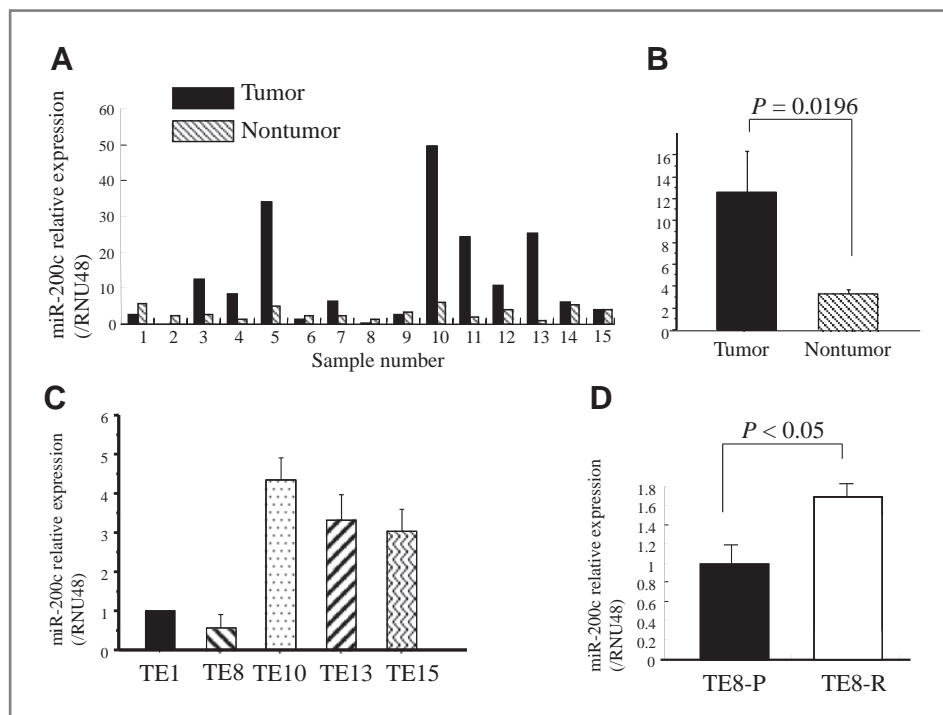


Figure 2. A and B, comparison of miR-200c expression in paired tumor and nontumor tissues of patients with esophageal cancers. The miR-200c expression level was determined by qRT-PCR. A, miR-200c expression in paired tumor and nontumor samples of 15 patients. B, mean expression levels of 15 samples. Data are presented as mean ± SD. C and D, expression level of miR-200c in cell lines determined by qRT-PCR. C, the miR-200c expression level varied in different esophageal carcinoma cell lines. D, comparison of miR-200c expression levels between cisplatin-resistant cells and parental cells. The expression level of miR-200c in the established cisplatin-resistant cells (TE8-R) is significantly higher than that in the parent cells (TE8-P). Data are presented as mean ± SD. All assays were done in triplicate, and values represent the mean of 3 independent experiments.

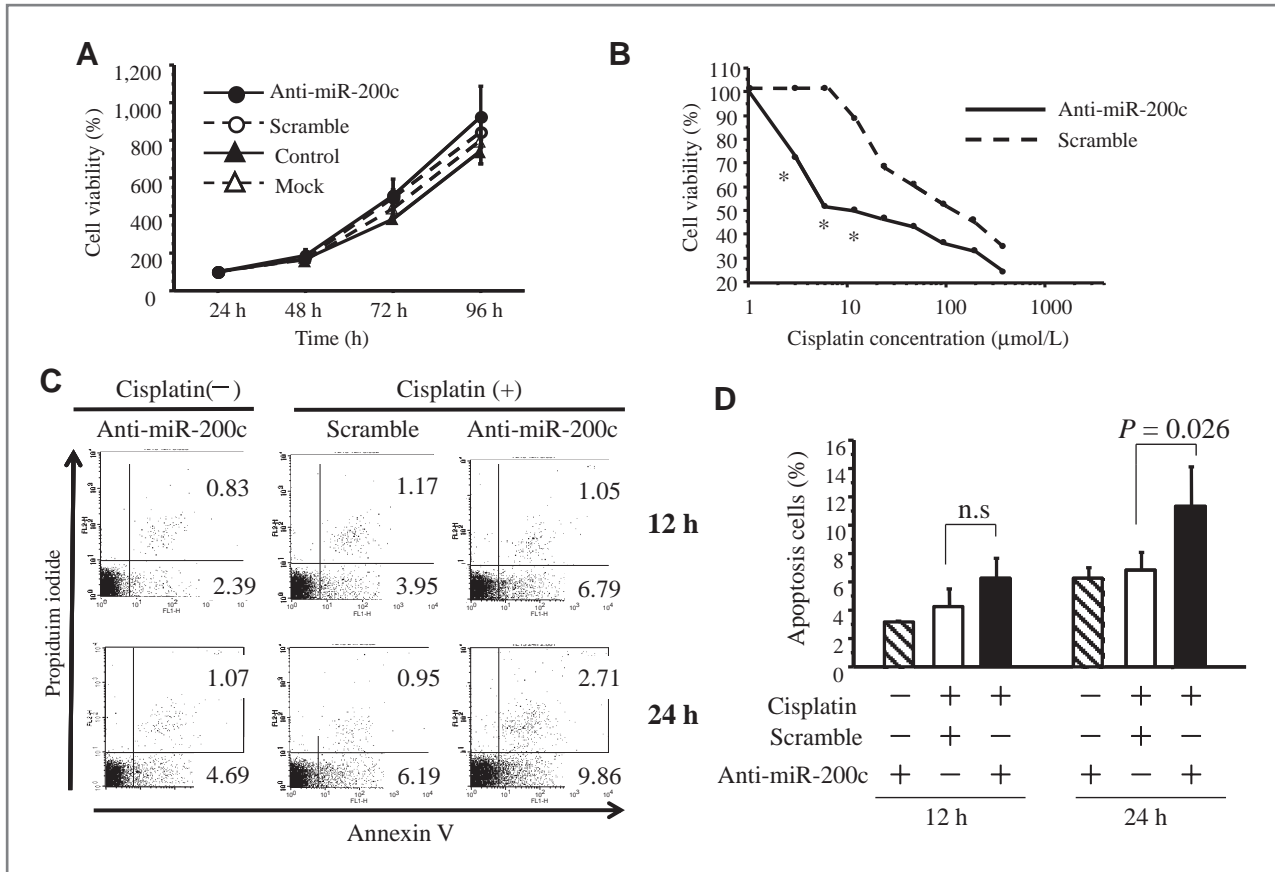


Figure 3. Effect of inhibition of miR-200c expression on cellular behavior in esophageal carcinoma cell line (TE13). A, cells were transfected and, at 24, 48, 72, and 96 hours, their viability was determined by the MTT assay. The viability of control cells, cells transfected with anti-miR-200c, negative control (scramble), and mock-transfected cells was similar. Inhibition of miR-200c expression had no impact on the proliferative activity. B, resistance to cisplatin was determined by the MTT assay 24 hours after transfection of cells. Anti-miR-200c-transfected cells are significantly more sensitive to cisplatin than were negative control cells. *, $P < 0.01$. C, TE13 cells were stained with PI and Annexin V and examined 12 and 24 hours after treatment with anti-miR-200c or scramble. Early and late apoptotic cells are shown in the right quadrant. D, Annexin V-positive cells were quantified by flow cytometry. Data are presented as mean \pm SD. of 3 independent experiments.

and control cells treated with cisplatin. Knockdown of miR-200c expression significantly increased the proportion of apoptotic cells after cisplatin treatment, compared with the negative control ($11.30 \pm 2.76\%$ vs. $6.82 \pm 1.26\%$ at 24 hours, $P = 0.026$, Fig. 3C and D).

Overexpression of miR-200c-induced chemoresistance is mediated through Akt pathway

To explore the downstream mechanism through which miR-200c expression modulates the chemosensitivity of esophageal cancer cells, we searched for potential targets of miR-200c by using the Target Scan (19) and miRBase Targets database (20). Several putative miR-200c targets, including Apaf-1, PTEN, TFAP2 α , and LATS2, were chosen as potential direct targets of miR-200c. We considered these proteins to be candidate targets of miR-200c when their inhibition resulted in decreased apoptosis. First, we employed Western blotting to examine whether exogenous repression of miR-200c expression affected the expression

of these proteins. Knockdown of miR-200c expression had no apparent effect on the expression of Apaf-1, TFAP2, and LATS2. Furthermore, low expression of miR-200c had no effect on PTEN expression (Fig. 4A) but resulted in decreased expression of Akt, which is downstream of PTEN and is known to be involved in chemoresistance (Fig. 4B). To explore the mechanism through which downregulation of miR-200c reduces the expression of Akt, we searched again for potential direct target(s) of miR-200c that is (are) involved in the regulation of Akt signaling by using the Target Scan and miRBase Targets database. Thus, in these experiments, we examined the expression of several potential miR-200c targets, such as PPP2CA, PPP2R1B, SOCS6, and A20, all of which are known to negatively regulate Akt signaling (21–23). Knockdown of miR-200c expression resulted in increased expression of PPP2R1B, a subunit of protein phosphatase 2A (PP2A), which is known to inhibit the phosphorylation of Akt (21), at 48 hours after treatment (Fig. 4B). However, knockdown of miR-200c

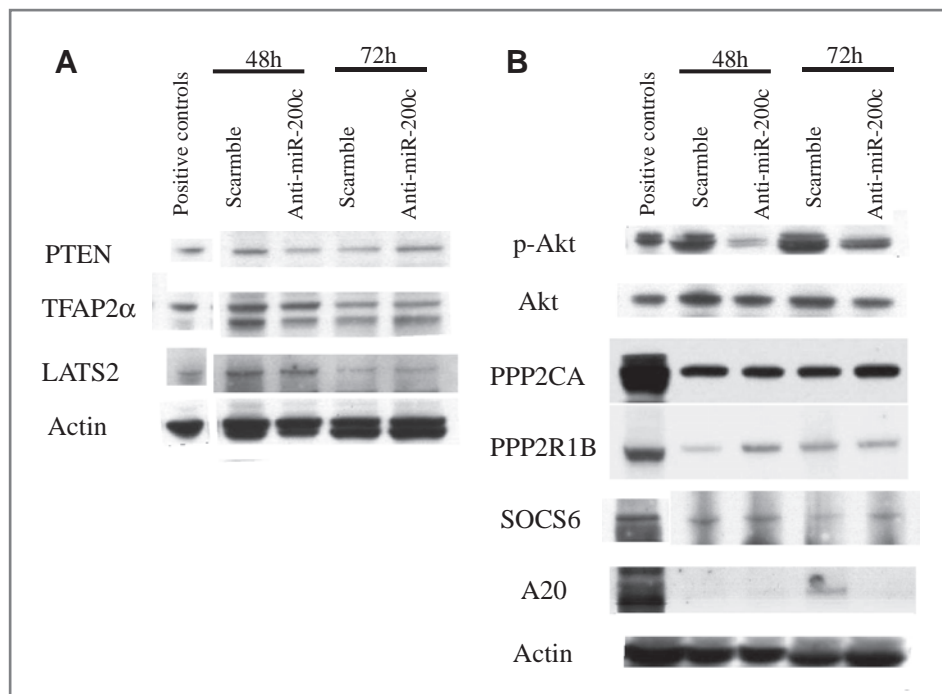


Figure 4. Western blot analysis of SCC cells (TE13). Western blot analysis of differential expression of proteins considered to be candidate targets of miR-200c at 48 and 72 hours after treatment with anti-miR-200c or scramble is shown. The expression level of each protein was normalized to that of β -actin in each sample. Full-length blots are presented in Supplementary Figure 5. A, knockdown of miR-200c expression had no apparent effect on the expression of Apaf-1, TFAP2, and LATS2. B, knockdown of miR-200c expression resulted in decreased expression of p-Akt and increased expression of PPP2R1B 48 hours after treatment. However, knockdown of miR-200c had no apparent effect on the expression of PPP2CA, SOCS6, and A20.

had no apparent effect on the expression of PPP2CA, SOCS6, and A20 (Fig. 4B). Considered together, these results indicate that the involvement of miR-200c expression in chemoresistance occurs by directly targeting PPP2R1B following upregulation of Akt signaling.

Discussion

In the clinical scenario, development of resistance to chemotherapy is one of the major challenges in the treatment of patients with cancer. In this study, we focused on miRNAs that are known to maintain stem cell function as regulators of sensitivity to anticancer therapies. We reported 3 new findings in this study. First, in esophageal cancer, high expression of miR-200c was closely associated with poor response to preoperative chemotherapy. Second, high expression of miR-200c correlated with poor prognosis. Third, the effects of miR-200c on chemosensitivity were mediated through the Akt pathway.

The results showed that overexpression of miR-200c correlated with poor response to cisplatin-based chemotherapy in patients with esophageal cancer. Several miRNAs are known to be associated with chemotherapeutic efficacy. For example, overexpression of miR-214 is reported to induce cisplatin resistance by targeting PTEN in ovarian cancer (24) and high expression of miR-199a is reported to be involved in chemoresistance in cervical carcinoma (25). On the other hand, low expression levels of let-7i, miR-181a, miR-630, miR-7, and miR-345 are associated with chemoresistance in patients with ovarian (26), lung (27), and breast cancers (28). To our knowledge, the relationship between miR-200c expression and che-

mo-resistance in human cancers has not been analyzed previously. In esophageal cancers, a recent study described the relationship between miRNA expression and chemoresistance (29). The study suggested that miR-296 mediated drug resistance, in part, through MDR1 and apoptosis, although the study was limited by the small sample size. In the present study, we showed that miR-200c expression is significantly associated with the response to chemotherapy in a large sample of patients with esophageal cancer.

Furthermore, the results showed that high expression of miR-200c was closely associated with poor prognosis of patients with esophageal cancer. Several studies examined the prognostic value of miR-200c expression in human cancers. The expression of miR-200c was found to be upregulated in cancers of epithelial ovarian (30) and hepatocellular (31) cancer tissues as compared with relative noncancerous tissues. Our results are in agreement with those of previous studies, which showed significant correlation between high expression of miR-200 and poor prognosis of patients with ovarian (32) and colorectal cancers (33). Other studies, however, reported downregulation of miR-200c in bladder cancer (34) and renal clear cell carcinoma (35). Thus, the impact of miR-200c expression on the progression and prognosis of patients with malignancies seems to vary according to human cancer type, histopathologic type (36), and stage of cancer (37).

In addition, the present study showed that the effect of miR-200c on the development of chemoresistance is mediated through the Akt pathway. Akt, a serine/threonine kinase, plays a pivotal role in oncogenesis, and its altered expression is observed in various human cancers. Furthermore, Akt is involved in resistance to chemotherapeutic

agents and to radiotherapy in various cancers (38, 39). In our previous study, we reported an increase in phospho-Akt (p-Akt) expression after chemotherapy and that high expression correlated with poor prognosis in patients with esophageal SCC (40). Recently, several studies have examined the relationship between miRNA expression and Akt-mediated chemoresistance. In breast cancer, the oncosuppressor miR-205, which is downregulated in cancer tissue, directly targeted the HER3 receptor and inhibited the activation of the downstream mediator Akt (41). In pancreatic cancer, overexpression of miR-21 downregulates the expression of PTEN and consecutively upregulates p-Akt, resulting in reduced apoptosis of gemcitabine-treated cells (42). In ovarian cancer, miR-214 is reported to enhance cell survival and cisplatin resistance, primarily through targeting the PTEN/Akt pathway (24). The present study showed, for the first time, that resistance of esophageal cancer to chemotherapy is induced in the presence of high expression of miR-200c through its action on the Akt pathway, and this effect is probably mediated through the downstream target of miR-200c, PPP2R1B, rather than via PTEN.

The PPP2R1B gene encodes the β -isoform of the A subunit of the PP2A, which is one of the major cellular serine/threonine phosphatases and a well-recognized regulator in the control of Akt activity (21). Previous studies showed that increased PP2A activity leads to inhibition of tumor invasiveness and enhancement of chemosensitivity through dephosphorylation of Akt in various cancers (43–45), suggesting that PP2A plays a tumor-suppressive role through downregulation of Akt signaling. Similarly, reduced expression of PPP2R1B is reported in human lung, colorectal (46), and ovarian cancers (47). Recently, Wong and colleagues (48) showed that overexpression of miR-222 enhanced Akt signaling by directly targeting PP2A and this change enhanced the metastatic potential of hepatocellular carcinoma. In this study, we identified PPP2R1B as a potential target of miR-200c, and we propose that PPP2R1B is a probable intermediate that interacts between miR-200c and the Akt pathway in esophageal cancer.

In the present study, we focused on 9 miRNAs that are known to be involved in the regulation of stem cell function (9–13), and, among those miRNAs, we found miR-200c expression to be correlated with chemoresistance and poor prognosis in esophageal cancer. However, it is possible that miRNAs other than those that are involved in stem cell function are also associated with chemoresistance. In fact, we analyzed the miRNA expression profile of a cisplatin-resistant esophageal cancer cell line by using miRNA microarray, and compared the profile with that of the parental cell line. The results showed that the expression of other miRNAs that are unlikely to regulate stem cell function was significantly altered in the chemoresistant cell line compared with the parental cell line (data not shown); however, the expression of several miRNAs that are involved in stem cell function were significantly up- or downregulated in the chemoresistant cell line compared with the parental cell line (miR-200c was 1.7-fold upregulated; miR-296 was 0.37-fold downregulated; miR-145 was 0.52-fold downregulated). Further studies are needed to investigate whether miRNAs, other than those that are involved in stem cell function, are also associated with chemoresistance.

In summary, we examined, in the present study, the role of various miRNAs, which regulate stem cell function, in mediating resistance to chemotherapy in esophageal cancer and we identified miR-200c as the miRNA responsible for chemoresistance in esophageal cancer. Moreover, the results showed that miR-200c-induced resistance is mediated through the Akt pathway. Further studies are needed to confirm the clinical role of miR-200c in the response to chemotherapy in patients with esophageal cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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