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ORIGINAL MANUSCRIPT

miR-27 is associated with chemoresistance in esophageal cancer through transformation of normal fibroblasts to cancer-associated fibroblasts

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Abstract

There is increasing evidence that the expression of microRNA (miRNA) in cancer is associated with chemosensitivity but the mechanism of miRNA-induced chemoresistance has not been fully elucidated. The aim of this study was to examine the role of extracellular miRNA in the response to chemotherapy in esophageal cancer. First, serum expression of miRNAs selected by miRNA array was measured by quantitative reverse transcription-polymerase chain reaction in 68 patients with esophageal cancer who received cisplatin-based chemotherapy to examine the relationship between miRNA expression and response to chemotherapy. The serum expression levels of 18 miRNAs were different between responders and non-responders by miRNA array. Of these, high expression levels of miR-27a/b correlated with poor response to chemotherapy in patients with esophageal cancer. Next, *in vitro* assays were conducted to investigate the mechanism of miRNA-induced chemoresistance. Although transfection of miR-27a/b to cancer cells had no significant impact on chemosensitivity, esophageal cancer cells cultured in supernatant of miR-27a/b-transfected normal fibroblast showed reduced chemosensitivity to cisplatin, compared with cancer cells cultured in supernatant of normal fibroblast. MiR-27a/b-transfected normal fibroblast showed α-smooth muscle actin (α-SMA) expression, a marker of cancer-associated fibroblasts (CAF) and increased production of transforming growth factor-β (TGF-β). Chemosensitivity recovered after administration of neutralizing antibody of TGF-β to the supernatant transfer experiments. Our results indicated that miR-27a/b is involved in resistance to chemotherapy in esophageal cancer, through miR-27a/b-induced transformation of normal fibroblast into CAF.

Introduction

Esophageal cancer is one of the most aggressive and lethal malignancies. Esophagectomy is the standard treatment for resectable esophageal cancers but systemic and local recurrences sometimes occur even after curative resection (1). To improve prognosis, the combination therapies of preoperative chemotherapy followed by surgery and preoperative chemoradiotherapy followed by surgery have been developed and widely implemented. The effects of preoperative chemotherapy differ between patients but the survival outcome is not satisfactory for non-responders (2). Prediction of response to chemotherapy before treatment makes it possible to select those patients who are likely to benefit from the treatment. Clarification of the mechanism of resistance to chemotherapy is important task to improve prognosis.

Recent studies reported that microRNAs (miRNAs) in cancerous tissues or cancer cells are associated with response of

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Abbreviations

CAF	cancer-associated fibroblasts
FBS	fetal bovine serum
HGF	hepatocyte growth factor
IL	Interleukin
miRNAs	microRNAs
NOFs	normal fibroblasts
PARP	poly (ADP ribose) polymerase-1
PDGF-BB	platelet-derived growth factor-BB
qRT-PCR	quantitative reverse transcription-polymerase chain reaction
SMA	smooth muscle actin
TGF	transforming growth factor

chemotherapy (3–6). In our previous studies, we showed that overexpression of miR-200c induces chemoresistance in esophageal cancers mediated through activation of the AKT signaling pathway (3) and that let-7 modulates the chemosensitivity to cisplatin through the regulation of interleukin-6 (IL-6)/STAT3 pathway in esophageal cancer (7). Other studies have demonstrated that miRNAs are consistently detectable in plasma or serum and that miRNAs resist endogenous ribonuclease activity by binding proteins (8,9), or by secretory particles including apoptotic bodies and exosomes (10–12). Thus, blood miRNAs level is considered a potentially useful biomarker for diagnosis and prognosis. However, little information is available on the usefulness miRNA in blood in prediction of response to chemotherapy (13,14).

Cancer tissue microenvironment is currently recognized as an important factor that influences the growth of cancer cells. Cancer-associated fibroblasts (CAF) are a major component of tumor stroma. The role of CAF in tumor growth (15), invasion (16) and tumor metastasis (17) is well-established and its mechanism has been recognized. For example, tumor invasion/metastasis is promoted by remodeling of extracellular matrix and chemokines released from CAF (18). However, whether or how CAF are involved in resistance to chemotherapy is not fully elucidated.

In the present study, we examined whether we could predict the response to chemotherapy in patients with esophageal cancer by measuring miRNA levels in blood. The results showed that high expression of miR-27a/b in blood correlated with poor response to chemotherapy in patients with esophageal cancer. We also examined whether the mechanism of miR27a/b-induced chemoresistance can be through acting on tumor stroma.

Patients and methods

Patients and samples

Between July 2009 and December 2011, 64 patients who underwent neoadjuvant chemotherapy followed by surgery for primary esophageal squamous cell carcinoma at the Department of Gastroenterological Surgery, Graduate School of Medicine, Osaka University were reviewed. These 64 patients (median age 67.5 years, range 45–80 years) and 27 healthy volunteers were included in this study. Esophageal squamous cell carcinoma was confirmed via histology in all patients. Regarding 27 healthy volunteers, they were outpatients who visit our hospital because of benign disease such as hypertension, hyperlipidemia and glaucoma. All patients were staged according to the criteria of the International Union Against Cancer. After obtaining informed consent, 8 ml of peripheral blood were obtained from each patient before chemotherapy for measurement of miRNA level. Immediately after collection, the serum samples were stored at –80°C until use.

The chemotherapeutic regimen was triplet chemotherapy of cisplatin, 5-fluorouracil and adriamycin (19) or cisplatin, 5-fluorouracil, and docetaxel (20). Surgical resection was performed 3–6 weeks after completion of the preoperative therapy. Our standard procedures consisted of subtotal esophagectomy with mediastinal lymphadenectomy via right thoracotomy, upper abdominal lymphadenectomy, reconstruction of a gastric tube and anastomosis in the cervical incision. The study was approved by the appropriate institutional review board and conducted in accordance with the Declaration of Helsinki.

Evaluation of response to chemotherapy

Two weeks after completion of chemotherapy, the clinical response to chemotherapy was assessed in each patient by endoscopy, computed tomography and positron emission tomography, according to the World Health Organization Response Criteria for Measurable Diseases (21). A complete response represented total regression of the tumor, partial response consisted of more than 50% reduction in primary tumor size on computed tomography. Progressive disease was defined as more than 25% increase in the size of the primary tumor or the appearance of new lesion(s). Stable disease represented cases that did not meet the criteria for partial response or progressive disease. For statistical evaluation, patients with complete response and partial response were defined as responders, whereas those with stable disease and progressive disease were defined as non-responders.

Isolation and characterization of fibroblasts

CAFs and normal fibroblasts (NOFs) were isolated according to protocol from previous studies with some modification (22,23). Briefly, CAF were isolated from the cancerous tissue of esophageal cancer, whereas NOF were isolated from the normal part of the esophagus in areas more than 5 cm from the tumor edge. After several washings with sterile phosphate-buffered saline, 2–3 cm^2 piece of cancerous tissue and normal part of the esophagus was minced with scissors. To isolate the CAFs and NOFs, the tissue was digested with DNase (Worthington Biochemical, Lakewood Township, NJ) on an orbital shaker, and collagenase (Roche, Basel, Switzerland) in RPMI 1640 (Life Technologies, NY), 20% fetal bovine serum (FBS) (Sigma–Aldrich Co, St Louis, MO) and 100 U/ml penicillin/100 µg/ml streptomycin at 37°C for 1 h. The tissue was discarded, the cell-containing solution in suspension was centrifuged at 1 500 r.p.m for 5 min, and the pellet was washed twice with 20 ml of RPMI 1640, 20% FBS and 100 U/ml penicillin/100 µg/ml streptomycin. Fibroblasts were plated with RPMI 1640, 20% FBS and 100 U/ml penicillin/100 µg/ml streptomycin. These conditions produced a homogenous population of fibroblasts after 7 days of culture. The fibroblasts were grown in RPMI 1640 with 10% FBS and used for experiments between passages 3 and 10 (24).

Cell lines and culture conditions

The authenticated human esophageal squamous cell line TE10 was obtained from the Riken Bioresource Center Cell Bank (Tsukuba, Japan) and routinely maintained in the laboratory. All cells were cultured in RPMI 1640 medium containing 10% FBS under a humidified atmosphere and 5% CO_2 at 37°C.

RNA extraction

Total RNA was isolated from serum samples using the mirVana PARIS kit (Ambion Austin, TX) according to the instructions supplied by the manufacturer. Briefly, 400 µl of serum were incubated with an equal volume of denaturation solution for 5 min on ice. For normalization of sample-to-sample variation during RNA isolation, 20 fmol of the synthetic *Caenorhabditis elegans* miRNA cel-miR-39 was added to each denatured sample. RNA extraction was performed by the acid-phenol:chloroform method and precipitation was carried out with ethanol and a filter cartridge. The extracted RNA was eluted in 100 µl of preheated Elution Solution and measured on a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Middletown, VA). The RNA samples were immediately stored at –80°C until use.

Total RNA was also isolated from cultured cells using TRIzol reagent (Life Technologies, Gaithersburg, MD) according to the protocol provided by the manufacturer.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) for miRNA expression

Reverse transcription and quantitative PCR (qRT-PCR) were performed using the TaqMan Array Human MicroRNA Assay kit (Applied Biosystems, Foster City, CA) according to the instructions provided by the manufacturer. First, 10 ng of RNA was reverse transcribed and the resulting complementary DNA was amplified using the following specific Taqman MicroRNA assays. Assay IDs were: hsa-miR-16ID: 000391, hsa-miR-19bID: 000396, hsa-miR-25ID: 000403, hsa-miR-27aID: 000408, hsa-miR-27bID: 000409, hsa-miR-107ID: 000443, hsa-miR-223ID: 002295, hsa-miR-373ID: 000561, hsa-miR-378bID: 244177_mat, hsa-miR-449cID: 241086_mat, mmu-miR-451ID: 001141, hsa-miR-490ID: 001037, hsa-miR-519b-3pID: 002384, hsa-miR-520eID: 001119, hsa-miR-598ID: 001988, hsa-miR-648ID: 001601, hsa-miR-875-3pID: 002204, RNU48 ID:001006, cel-miR-39ID: 000200. The PCRs were performed using ABI PRISM 7900HT (Applied Biosystems), as recommended by the manufacturer. Amplification data of cultured cells were normalized to RNU48 expression.

Since there is no established endogenous serum miRNA control for normalization of serum miRNA levels (25), the levels of the target miRNAs were normalized to the level of cel-miR-39 (26). Data were analyzed by the comparative Ct method (2 –ΔCT) (27).

Quantitative real-time polymerase chain reaction

For reverse transcriptase reaction, the Reverse Transcription System (Promega, Tokyo, Japan) was used according to the protocol provided by the manufacturer. qRT-PCRs were conducted with the LightCycler-FastStart DNA Master SYBR Green I kit (Roche Applied Science, IN) with gene-specific oligonucleotide primers listed in Supplementary Table1, available at *Carcinogenesis* Online. Amplifications were performed in triplicate with the LightCycler System (Roche Applied Science), following the procedure provided by the manufacturer. Melting curve analysis was performed to distinguish specific products from non-specific products and primer dimers. The expression was reported relative to the expression of glyceraldehyde-3-phosphate dehydrogenase , which was used as an internal control.

miRNA transfection

TE10 and NOF were transfected with 30 nM pre-miR-27a, pre-miR-27b, anti-miR-27a and anti-miR-27b (Applied Biosystems) using SiPORT NeoFX (Ambion) according to the protocol provided by the manufacturer. Pre-miR negative control oligonucleotides and anti-miR negative control oligonucleotides (Applied Biosystems) were also used as a control.

miRNA array

The purified serum RNAs obtained from five responders and five non-responders were used as samples and assessed as being of high quality by Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) and NanoDrop (NanoDrop Technologies). Next, 500 ng of extracted total RNA was labeled with Hy5 using the miRCURY LNA Array miR labeling kit (Exiqon, Vedbaek, Denmark). The labelled RNAs were hybridized onto 3D-Gene Human miRNA Oligo chips (v.17.0; Toray Industries, Tokyo, Japan). The annotation and oligonucleotide sequences of the probes conformed to the miRBase miRNA data base Release 17 (http://microrna.sanger.ac.uk/sequences/). After stringent washes, fluorescent signals were scanned with the 3D-Gene Scanner (Toray Industries) and analyzed using 3D-Gene Extraction software (Toray Industries). The raw data of each spot were normalized by substitution with a mean intensity of the background signal determined by all blank spots' signal intensities of 95% confidence intervals. Measurements of both duplicate spots with the signal intensities greater than two standard deviations of the background signal intensity were considered to be valid. A relative expression level of a given miRNA was calculated by comparing the signal intensities of the averaged valid spots with their mean value throughout the microarray experiments. The normalized data were globally normalized per array, such that the median of the signal intensity was adjusted to 25.

Immunofluorescence staining

Fibroblasts were grown on glass coverslips. Then, cells were washed twice with phosphate-buffered saline, fixed in 4% paraformaldehyde and processed for immunofluorescence staining.

For immunofluorescence staining, cells were first incubated overnight with the primary antibody (mouse anti-α-SMA; dilution 1:100) at 4°C. After thorough washing, cells were incubated for 1 h with fluorescence-conjugated secondary antibodies (fluorescein isothiocyanate-conjugated goat anti-mouse). Finally, the cells were washed and mounted with mounting medium containing 4′,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA) before imaging by all-in-one-type fluorescence microscopy (BZ-8000; Keyence, Osaka).

Growth inhibition assays with cisplatin therapy and apoptosis assay

After incubation of NOF for 3 days, the supernatant was transferred to a dish of cultured esophageal cancer cell lines. Two days later, esophageal cancer cell lines were exposed to cisplatin for 24 or 72 h. The effects of the supernatant and cisplatin were analyzed by the apoptosis assay and growth inhibition assay, respectively (Figure 3A). Esophageal cancer cells transfected with anti-miR27a and anti-miR-27b were co-cultured with NOF for 24 h, then esophageal cancer cells were exposed to cisplatin for 24 h (Figure 4A). To analyze the effects of hepatocyte growth factor (HGF) and transforming growth factor-β1 (TGF-β1) on apoptosis, we added HGF neutralizing antibody (AF-294-NA, R&D Systems, MN), TGF-β neutralizing antibody (ab64715, Abcam, Tokyo) and control Immunoglobulin G to the medium (Figure 5D).

For growth inhibition assay, the water-soluble tetrazolium salt WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] uptake method was employed, using CCK-8 reagent (Dojindo, Gaithersburg, MD). Briefly, esophageal cancer cells were plated in 96-well plates (3 000 cells/well) and treated with serially diluted cisplatin in hexalicate. After 72 h incubation 10 μl/well CCK-8 reagent was added and incubated at 37°C for 2 h. Optical density was measured at 450 and 650 nm using a microplate reader (BMG Labtech, Saitama City, Japan). Cell viability (%) was normalized by dividing the final absorbance of treated samples by that of the untreated control.

Annexin V-fluorescein isothiocyanate and propidium iodide staining techniques were used to determine the percentage of cells undergoing apoptosis and necrosis among the cells treated with 40 m mol/l of cisplatin for 24 h. An apoptosis assay was conducted using the protocol supplied by the manufacturer (BioVision, Milpitas, CA). Briefly, cells were trypsinized gently and resuspended with 500 μl of 1× binding buffer and then treated with 5 μl of Annexin V–fluorescein isothiocyanate and 5 μl propidium iodide. After incubation for 5 min on ice, each sample was analyzed immediately using the FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA). Cells stained by Annexin V only were 'early apoptotic cells' and cells stained by both Annexin V and propidium iodide were 'late apoptotic cells'. We defined apoptotic cells as both early and late apoptotic cells in this study.

Enzyme-linked immunosorbent assay

Media of 3 day cultures were collected for analysis of human TGF-β1, HGF, platelet-derived growth factor-BB (PDGF-BB) and IL-6 using commercial ELISA kits (TGF-β1 cat #DB100B and HGF cat #DHG00 Quantikine, R&D Systems, PDGF-BB cat #ab100624 abcam, and IL-6 cat #Q6000B QuantiGlo, R&D Systems) with samples standardized by volume. All samples were assayed in triplicate.

Western blotting

Adherent cells were washed with ice-cold phosphate-buffered saline and harvested from the culture dish. The cells were lysed in radioimmunoprecipitation assay buffer (25 mM Tris, pH 7.5, 50 mM NaCl, 0.5% sodium deoxycholate, 2% Nonidet P-40, 0.2% sodium dodecyl sulfate, 1 mM phenylmethylsulphonyl flouride and 500 KIE/ml aprotinin) containing protease inhibitor and phosphatase inhibitor. The extracts were spun and the supernatant fractions were collected for western blot analysis. Equal amount of extracts (10ug) were fractioned by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Bio-Rad Laboratories) and transferred

onto membranes (ImmobilonP). The following antibodies were used in this study: at 1:200 for anti-human Poly (ADP ribose) polymerase-1 (PARP-1) antibody (sc-8007, SC) and 1:10000 for anti-human β-actin (#A2066, Sigma–Aldrich), and 1:100000 for secondary antibody. Immune complexes were detected by using the Detection Kit (GE HealthCare, Tokyo). Images were scanned at a minimum resolution of 300 dpi. The protein levels were quantified by densitometory of raw BMP images using NIH Image-basedsoftware Scion Image (Scion Corporation) as described (28). The densinometry values obtained for each protein were normalized by the densinometry value of the β-actin.

Statistical analysis

Differences between groups were examined for statistical significance using Student's *t*-test with Yates' correction, chi-squared test, Fisher's exact probability test or the Mann-Whitney *U*-test. Cause-specific survival was calculated from the date of treatment to the event or last known date of follow-up. Survival curves were computed using the Kaplan–Meier method and differences between survival curves were compared using the log-rank test. Significance was set at $P < 0.05$. Statistical analysis was performed with JMP ver 9.0 (SAS Institute Inc, Cary, NC).

Results

Different serum expression levels of 18 miRNAs between responders and non-responders to cisplatin-based chemotherapy

To identify the candidate miRNAs in blood involved in chemosensitivity to cisplatin-based chemotherapy, miRNA microarray analysis was performed using serum RNAs obtained from five responders and five non-responders to chemotherapy. The analysis showed different expression levels (by more than 1.7-fold) of 18 miRNAs among 1719 miRNAs in non-responders, compared with their levels in responders (Supplementary Table 2, available at *Carcinogenesis* Online). Among these 18 miRNAs, miR-411, miR-27b, miR-598, miR-520e, miR-875-3p, miR-519b-3p, miR-378b, miR-373, miR-449c and miR-27a were up-regulated, whereas miR-648, miR-451, miR-16, miR-490-3p, miR-107, miR-223, miR-19b and miR-25 were down-regulated in non-responders, compared with responders (Supplementary Table 3, available at *Carcinogenesis* Online). Accordingly, they were selected as candidate serum miRNAs involved in response to chemotherapy in esophageal cancer.

High serum expression levels of miR-27a and miR-27b correlate with poor response to chemotherapy

To confirm that the 18 miRNAs are implicated in the response to chemotherapy, we performed qRT-PCR using pretreatment serum samples obtained from 68 patients with esophageal cancer receiving chemotherapy. Among the 68 patients, 40 were classified as responders and the remaining 28 were non-responders. We also divided the 68 patients into two groups based on the median value of the expression level of each miRNA: the high expression group ($n = 34$) and the low expression group ($n = 34$). The results of real-time qRT-PCR for these 18 miRNAs showed that high serum expression of miR-27a/b correlated inversely with the response to chemotherapy (Table 1), whereas no such correlation was identified for the other microRNAs.

Next, we examined the relationship between serum expression levels of miR-27a/ b and clinical features of patients with esophageal cancer. The serum levels of miR-27a/b were significantly higher in patients with esophageal cancer than in healthy volunteers (Figure 1A and B). Furthermore, the serum levels of miR-27a/b significantly decreased after surgical resection (Figure 1C and D). However, there was no significant relationship between serum expression of miR-27a/b and various clinicopathological factors, such as tumor depth, lymph node involvement and tumor stage (Supplementary Table 4, available at *Carcinogenesis* Online). Next, we examined the relationship between miR-27a/b serum levels and prognosis of patients with esophageal cancer, who underwent preoperative chemotherapy followed by surgery. High expression of miR-27b was significantly associated with shorter cause-specific survival ($P = 0.0200$; Figure 1F), and patients with high expression of miR-27a also tended to show poor prognosis compared with those with low expression of miR-27a ($P = 0.0994$; Figure 1E), but there was no significant difference. Taken together, the results indicate that patients with esophageal cancer have high serum expression levels of miR-27a/b, and that miR-27a/b are probably involved in chemoresistance in esophageal cancer.

Table I. Relationship between the serum expression of 18 microRNAs and clinical response

MicroRNA		Responder	Non-responder	p-value
hsa-miR-411	high/low	18/22	16/12	0.3243
hsa-miR-27b	high/low	14/26	20/8	0.0062
hsa-miR-598	high/low	17/23	17/11	0.1393
hsa-miR-520e	high/low	NA	NA	
hsa-miR-875-3p	high/low	18/22	16/12	0.3243
hsa-miR-519b-3p	high/low	20/20	14/14	1.0000
hsa-miR-378b	high/low	20/20	14/14	1.0000
hsa-miR-373	high/low	19/21	15/13	0.6222
hsa-miR-449c	high/low	NA	NA	
hsa-miR-27a	high/low	14/26	19/9	0.0258
hsa-miR-648	high/low	17/23	17/11	0.1393
hsa-miR-451	high/low	22/18	12/16	0.3243
hsa-miR-16	high/low	22/18	12/16	0.3243
hsa-miR-490-3p	high/low	17/23	17/11	0.1393
hsa-miR-107	high/low	18/22	16/12	0.3243
hsa-miR-223	high/low	18/22	16/12	0.3243
hsa-miR-19b	high/low	21/19	13/15	0.6222
hsa-miR-25	high/low	20/20	14/14	1.0000

miR-27a /b does not directly affect chemosensitivity in esophageal cancer cells

In the next step, we investigated the mechanism by which miR-27a/b affect chemoresistance. To evaluate the direct effects of miR-27a/b on the proliferative activity and chemosensitivity in esophageal cancer cells, pre-miR-27a and pre-miR-27b were transfected into TE10 cells. In the proliferation assay, anti-miR-27 and pre-miR-27 transfection did not have a significant impact on the proliferative activity (Supplementary Figure 1A and B, available at *Carcinogenesis* Online). Growth inhibition assays and apoptosis assay showed that inhibition of miR-27a/b expression with anti-miR-27a/b and pre-miR-27a/b had no significant effects on chemosensitivity in esophageal cancer cells (Supplementary Figures 1C and D and 2A–D, available at *Carcinogenesis* Online). These results suggest that miR-27a/b does not directly affect chemosensitivity of esophageal cancer cells.

miR-27a/b convert normal fibroblast to cancer-associated fibroblasts

We examined whether miR-27a/b exerts an inhibitory effect on chemosensitivity by acting on tumor stroma. Among various factors related to the tumor microenvironment, we focused in this study on CAF since these cells have potentially antiapoptotic function (29). First, we confirmed that α-SMA, which is a

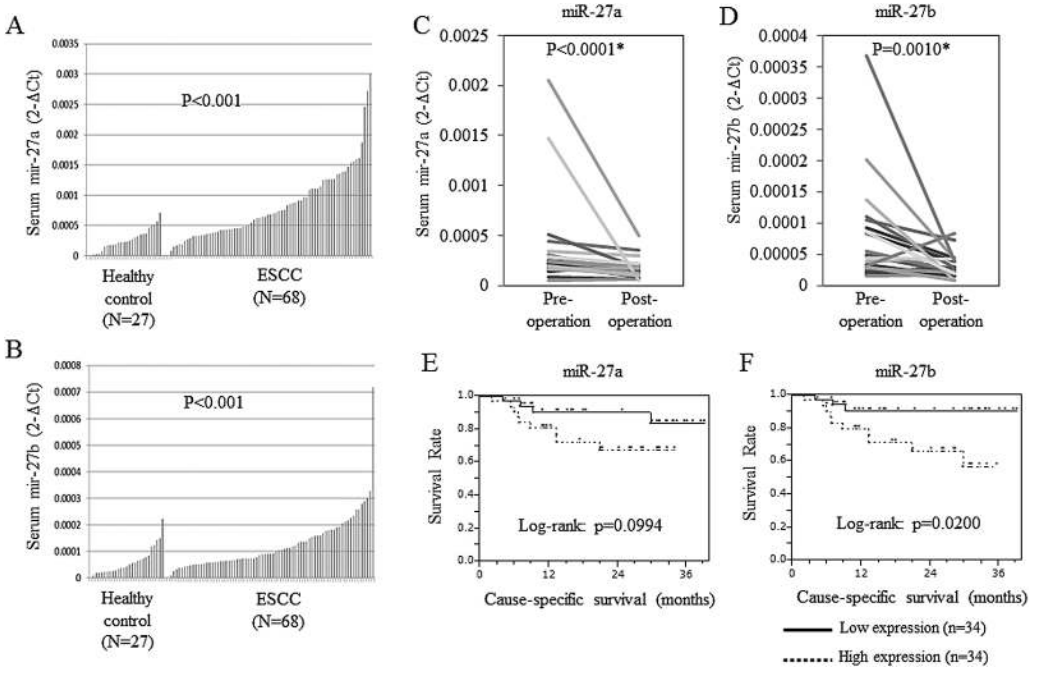

Figure 1. Comparison of serum miRNA levels in patients with esophageal cancer and healthy volunteers. Serum levels of miR-27a (**A**) and miR-27b (**B**) were significantly higher in patients with esophageal cancer than in healthy volunteers. Changes in serum miR-27a (**C**) and miR-27b (**D**) levels before and after esophageal resection. The miR-27a and miR-27b levels were significantly lower in the postoperative samples than in the preoperative samples ($P < 0.0001$ and $P = 0.0010$, respectively). Cancer-specific survival curves of 68 patients with esophageal cancer according to serum levels of miR-27a and miR-27b. Patients were divided into two groups based on the median value of the expression level of each miRNA; the high expression group ($n = 34$) and the low expression group ($n = 34$). Thick line: low expression group; dotted line: high expression group. (**E**) Serum expression of miR-27a was not significantly associated with cancer-specific survival of patients ($P = 0.0994$). (**F**) Patients with high miR-27b serum levels had shorter cancer-specific survival ($P = 0.0200$) than other patients.

well-known marker of CAF, was expressed in CAF obtained from patients with esophageal cancer, but not in NOF (Figure 2A–C). Next, we examined whether miR-27a/b in esophageal cancer can convert NOF to fibroblasts like CAF since a recent study demonstrated that cancer cells reprogram fibroblasts to become CAF through the action of miRNAs (30). We examined the expression of miR-27a/b in NOF and CAF obtained from patients with esophageal cancer. The miR-27a/b expression levels were significantly higher in CAF than in NOF (Figure 2D). The expression of α-SMA and vimentin were evident in pre-miR-27a- and pre-miR-27b- transfected fibroblasts, but not in NOF (Figure 2E and F). These results suggest that miR-27a/b can convert NOF to CAF-like fibroblasts (miR-27a/b-induced CAF).

miR-27a/b-induced CAF enhances resistance to cisplatin

We examined whether miR-27a/b-induced CAF have inhibitory effects on chemosensitivity in esophageal cancer cells. TE10 was cultured in supernatant that originated from CAF, miR-27a/b-induced CAF and NOF for 72 h, and then the sensitivity to cisplatin was determined by the growth inhibition assay and an apoptosis assay (Figure 3A). At first, the influences of those supernatant on proliferation of cancer cells were examined. There is no difference in proliferation of cancer cells cultured in those supernatants (Supplementary Figure 3, available at *Carcinogenesis* Online). The growth inhibition assay showed that TE10 cultured in supernatants of pre-miR-27a/b transfected fibroblasts (miR-27a/b-induced CAF) and TE10 in supernatant of CAF was significantly resistant to cisplatin than TE10 cultured in supernatant of NOF (Figure 3B). An apoptosis assay also showed that the proportion of apoptotic cells among those exposed to cisplatin was lower among cancer cells cultured in supernatants from pre-miR-27a/b-transfected fibroblasts and cancer cells cultured in supernatant from CAF, compared with cancer cells cultured in supernatant obtained from NOF (Figure 3C, D). We performed an apoptosis assay using another cell line (TE8) to validate the results in TE10. An apoptosis assay using TE8 showed similar findings to TE10 (Supplementary Figure 4). To confirm decrease of apoptosis in cancer cells cultured in supernatants from CAF and pre-miR-27a/b-transfected fibroblasts, we examined the cleaved PARP-1 level in those cancer cells. Western blot analysis showed cleaved PARP-1 level of TE10 cultured in supernatants from CAF and pre-miR-27a/b-transfected fibroblasts was low compared with cancer cells cultured in supernatant obtained from NOF (Figure 3E). Next, to examine whether miR-27a/b released from esophageal cancer cells is associated with chemoresistance through acting on fibroblasts, anti-miR-27a/b transfected TE10 and control TE10 were co-cultured with NOF, and the sensitivity to cisplatin was determined by an apoptosis assay (Figure 4A). The expression levels of miR-27a/b in the supernatants of anti-miR-27a/b-transfected TE10 were lower than that of negative control transfected cells (Figure 4B). An apoptosis assay showed that the percent of apoptotic cells was significantly higher after exposure to cisplatin in anti-miR-27a/b-transfected TE10 than that in negative control TE10 (Figure 4C and D). Taken together, these results suggest that miR-27a/b derived from esophageal cancer cells act on fibroblasts to become CAF-like fibroblasts and the involvement of factors secreted from miR-27a/b-induced CAF in chemoresistance.

TGF-β from miR-27a/b-induced CAF chemoresistance of esophageal cancer cells

Finally, we searched for soluble factor(s) that are released from miR-27a/b-induced CAF that can influence chemosensitivity.

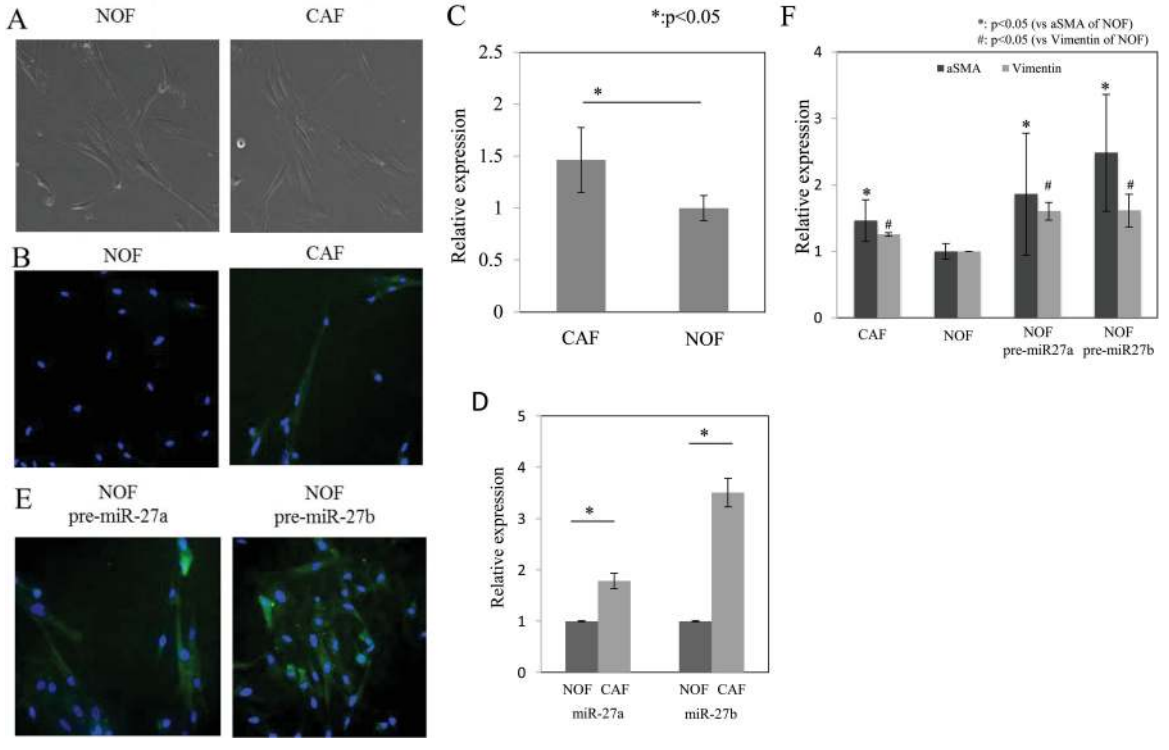

Figure 2. (**A**) Phase contrast images of primary NOF and CAF (CAF). (**B**) Immunofluorescence staining of α-SMA (green) in NOF and CAF. All cells were counterstained with 4′,6-diamidino-2-phenylindole (blue). (**C**) QRT-PCR for α-SMA in NOF and CAF. (**D**) miR-27a and miR-27b expression in NOF and CAF. (**E**) Immunofluorescence staining of α-SMA (green) in NOF transfected with pre-miR-27a and pre-miR-27b. All cells were counterstained with 4′,6-diamidino-2-phenylindole (blue). (**F**) Quantitative RT-PCR for α-SMA and vimentin in NOF, CAF and NOF transfected with pre-miR-27a and pre-miR-27b.

In these experiments, we measured the expression levels of TGF-β1, HGF, PDGF-BB and IL-6 in the supernatant of NOF, miR-27a/b-induced CAF and CAF by ELISA assay, because those factors are reported to be released from CAF (31–35) and likely to exert an antiapoptotic effect on cancer cells. The expression levels of HGF and TGF-β were significantly higher in the supernatant of CAF and miR-27a-induced CAF than in NOF (Figure 5A and B). However, there was no significant difference in the expression level of IL-6 between supernatant of miR-27a/b-induced CAF and NOF (Figure 5C). PDGF-BB was not detected in the supernatant of CAF, miR-27a/b-transfected fibroblasts and NOF.

To examine whether HGF and TGF-β alter the sensitivity to cisplatin in esophageal cancer cells, the sensitivity to cisplatin was determined by an apoptosis assay after HGF neutralizing antibody, TGF-β neutralizing antibody and control Immunoglobulin G were added to the supernatant of miR-27a/b-induced CAF when those media were transferred to TE10. An apoptosis assay showed that the percentage of apoptotic TE10 cultured in supernatant of pre-miR-27a-transfected fibroblasts and treated with TGF-β neutralizing antibody recovered to the same level as TE10 cultured in supernatant of NOF (Figure 5D–F). However, HGF neutralizing antibody did not restore chemosensitivity of TE10 cultured in supernatant of pre-miR-27a/b-transfected fibroblasts, compared with TE10 cultured in supernatant of NOF (Supplementary Figure 5, available at *Carcinogenesis* Online).

Discussion

The results of the present study showed that high expression levels of miR-27 in serum is significantly associated with poor response to chemotherapy and short survival in patients with esophageal cancer who underwent preoperative chemotherapy followed by surgery. *In vitro* assays also showed that miR-27 produced by esophageal cancer cells facilitates the transformation of NOF to CAF-like fibroblasts, and that TGF-β secreted by these CAF like-fibroblasts induces chemoresistance to cisplatin in esophageal cancer (Figure 5G).

Previous studies showed that miR-27 acts as oncogenic miRNA. For example, in breast cancer, miR-27a directly inhibits FOXO1, which is a putative tumor suppressor, and enhances the transformation or maintenance of the oncogenic status (36). In contrast, miR-27a overexpression in non-small cell lung cancer directly deregulates MET and EGFR, which induce cancer cell invasion and increase the metastatic potential (37). Other studies emphasized the role of miR-27 expression in sensitivity to anticancer drugs (6,38,39). Down-regulation of miR-27a was significantly associated with the expression of P-glycoprotein, leading to increased chemosensitivity (39). In esophageal cancer, one recent study suggested that miR-27 could play important roles in multidrug resistance by regulating the expression of P-glycoprotein, Bcl2 and the transcription of multidrug resistance gene 1 (6). In contrast to the above reports implicating the direct enhancing effects of miR-27 on chemosensitivity of cancer cells, our study showed that miR-27 dose not directly but indirectly affects chemosensitivity by acting on the tumor microenvironment.

The involvement of CAF in tumor progression and metastasis is well established, but their origin has not been fully elucidated. One plausible tentative theory is that CAF are transformed from adjacent NOFs by some kind of stimulating factor (40). Two recent studies suggested that miRNAs play an important role of transforming NOF to CAF. Aprelikova *et al.*

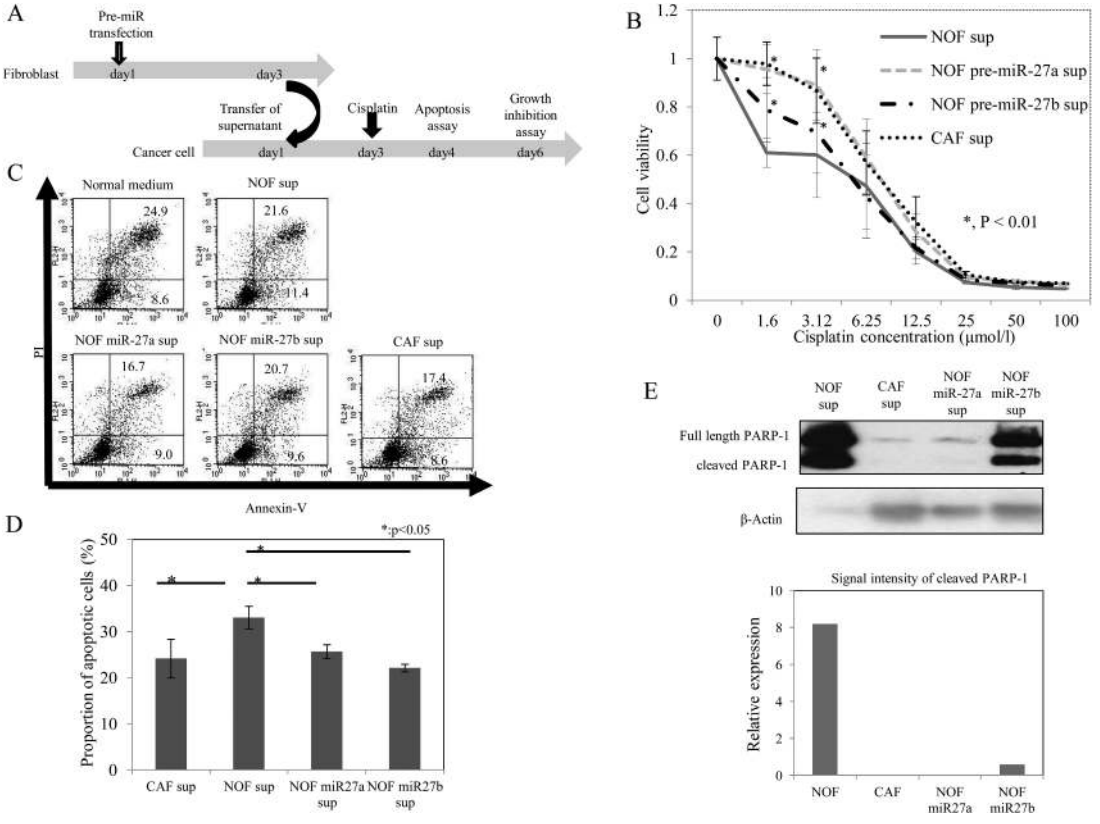

Figure 3. (**A**) Schema of experiment. Resistance to cisplatin was determined by the growth inhibition assay. (**B**) TE10 cultured with supernatant of pre-miR-27a- and pre-miR-27b-transfected normal fibroblast were significantly resistant to cisplatin than TE10 cultured with supernatant of negative control transfected NOFs. (**C**) Resistance to cisplatin was determined by apoptosis assay. (**D**) The proportion of TE10 apoptotic cells cultured in RPMI supernatant of pre-miR-27a- and pre-miR-27b-transfected NOFs was significantly smaller than that of TE10 cultured in supernatant of negative control-transfected NOFs (*$P < 0.05$). (**E**) Western blot analysis of the cleaved PARP-1 level in TE10 cultured in supernatant of NOFs, cancer associated fibroblasts, pre-miR-27a- and pre-miR-27b-transfected NOFs. In densitometry, the signal intensity for the cleaved PARP-1 was calibrated by that of β-actin.

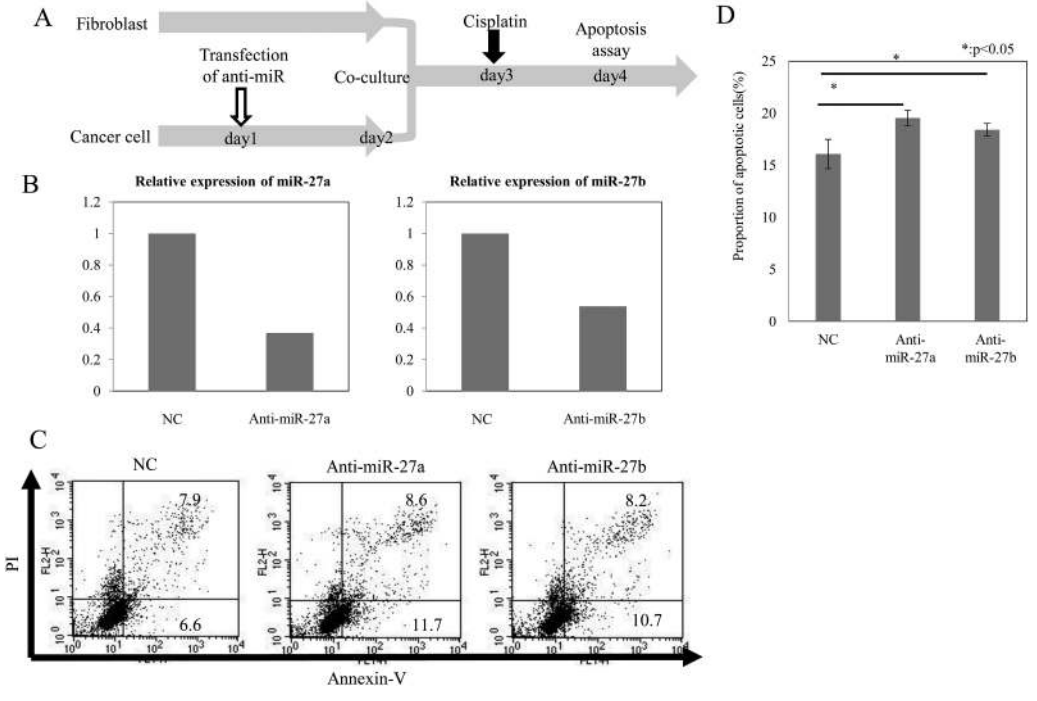

Figure 4. (**A**) Schema of experiment. (**B**) The relative expression levels of miR-27a/b in cultured medium of esophageal cancer cells transfected with anti-miR-27a/b are markedly lower than in that of the control. (**C**) Anti-miR-27a- and anti-miR-27b-transfected TE10 and control TE10 were co-cultured with NOF, and sensitivity to cisplatin was determined by apoptosis assay. (**D**) The proportion of apoptotic cells of anti-miR-27a/b-transfected TE10 after exposure to cisplatin was significantly larger than that negative control TE10.

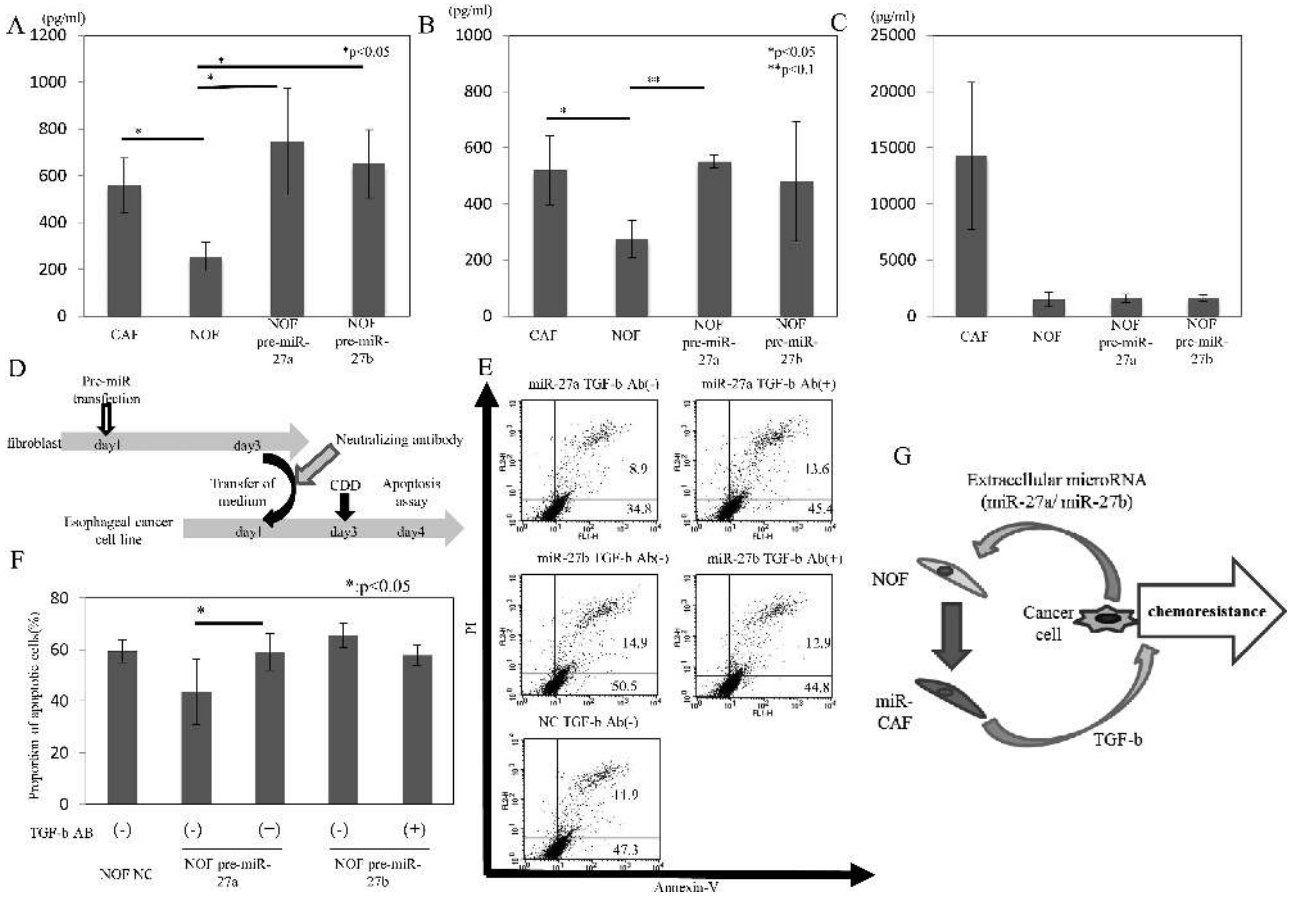

Figure 5. HGF (**A**), TGF-β (**B**) and IL-6 (**C**) levels in conditioned media of NOFs, CAFs and pre-miR-27a/b transfected fibroblasts at 72 h were measured by ELISA in triplicate. The expression levels of HGF and TGF-b protein in pre-miR-27a and pre-miR-27b increased in supernatants. (**D**) Schema of experiment. (**E**) Resistance to cisplatin of TE10 cultured in supernatant of pre-miR-27a/b-transfected fibroblasts with or without TGF-β neutralizing antibody, as determined by Apoptosis assay. (**F**) The proportion of TE10 apoptotic cells (cultured in supernatant of pre-miR-27a- and pre-miR-27b-transfected NOFs with TGF-β neutralizing antibody, was significantly larger than those cultured in supernatant without neutralizing antibody. (**G**) Schematic overview of chemoresistance induced by miR-27. MiR-27 derived from esophageal cancer cells facilitates the transformation of NOF to CAF-like fibroblasts, and TGF-β secreted by these CAF like-fibroblasts induces chemoresistance to cisplatin in esophageal cancer.

(41) compared the expression of miRNAs in CAF isolated from endometrial cancer and NOFs. They identified 11 miRNAs that were differentially expressed in CAF. The present study showed that the different characteristics of NOF and CAF are probably related to the difference in miRNA expression between the two types of cells, suggesting that miRNAs can transform NOF to CAF within the tumor microenvironment. In another recent study, Mitra *et al.* (30) reported downregulation of miR-31 and miR-214 in ovarian CAF, compared with miR-155 upregulation, relative to NOFs. They showed that mimicking this deregulation by transfecting miRNAs and miRNA inhibitors induced a functional conversion of NOFs into CAF. Moreover, miR-induced CAF release CCL5 which helps in migration, proliferation and invasion of ovarian cancer cells. The above study suggests that NOF can be transformed into CAF by extracellular miRNA. In the present study, we showed that miR-27a or miR-27b derived from esophageal cancer cells can reprogram NOF to CAF, resulting in chemoresistance.

Based on previous studies, TGF-β is considered to have antiapoptotic effects on some cancer cells. TGF-β produced by bone marrow stromal cells promotes the survival and chemoresistance of leukemia cells under direct cell-to-cell interactions (42). Tabe *et al.* (43) reported that TGF-β neutralizing antibody for TGF-β enhanced cytarabine-induced apoptosis in acute myeloid leukemia cells. Furthermore, Yu *et al.* (44) reported that TGF-β contributed to resistance to DNA-damaging chemotherapy agents in cancer cells by downregulating mutS homolog 2, which is a central component of the DNA mismatch repair system. With the above background, we demonstrated here the antiapoptotic effect TGF-β in the *in vitro* experiments, and that this factor mediates chemoresistance. In contrast, several other studies showed that TGF-β induces apoptosis of cancer cells (45). TGF-β1 decreased cell viability and induced apoptosis of invasive prostate and bladder cancer cells via activation of p38 MAPK-JNK-caspase 9/8/3 pathways (45). Furthermore, TGF-β and TNF-α are reported act in concert to activate apoptosis of gastric cancer cells through cross talk between Smad and JNK signaling pathways (46). Considered together, our results and those of other investigators suggest that the effects of TGF-β on apoptosis of cancer cells vary according to cancer type (47–49).

Although, the majority of miRNA exist within cells, several types of miRNAs have been found in body fluids such as serum, saliva and urine (50). Extracellular miRNA is protected from nuclease by encapsulation into microvesicles such as exosome (51) or by binding with protein complex such as AGO2 (8). Thus, blood can contain miRNAs in the serum and in exosomes (52,53). The origin of miRNA in blood remains to be delineated because not only cancer cells but also normal cells can shed stable form of miRNA into circulation (54). In this study, we considered that miR-27 in serum originated mainly from esophageal

cancer cells, because its serum expression level in patients with esophageal cancer was significantly higher than that of healthy volunteers and decreased significantly after surgery compared with the baseline (before surgery). Moreover, co-culture of fibroblasts with anti-miR-27-transfected esophageal cancer cells resulted in a major decrease in the antiapoptotic function of fibroblasts, compared with fibroblasts co-cultured with control esophageal cancer cells that secrete extracellular miR-27 (Fig. 4C). Serum miR-27 level may reflect the expression level of extracellular miR-27 derived from esophageal cancer cells.

In summary, our results suggested that miR-27 is involved in resistance to chemotherapy in esophageal cancer, through miR-27 -induced transformation of NOF into CAF, and that TGF-β secreted from these CAF-like fibroblasts induces chemoresistance to cisplatin in esophageal cancer. Based on these results, we believe that miR-27 is a potential therapeutic target for esophageal cancer treated with anticancer drugs.

Supplementary material

Supplementary Tables 1–4 and Supplementary Figures 1–5 can be found at http://carcin.oxfordjournals.org/

Conflict of Interest Statement: None declared.

References

1. Miyata, H. et al. (2011) Survival factors in patients with recurrence after curative resection of esophageal squamous cell carcinomas. Ann. Surg. Oncol., 18, 3353–3361.
2. Miyata, H. et al. (2009) Tumor budding in tumor invasive front predicts prognosis and survival of patients with esophageal squamous cell carcinomas receiving neoadjuvant chemotherapy. Cancer, 115, 3324–3334.
3. Hamano, R. et al. (2011) Overexpression of miR-200c induces chemoresistance in esophageal cancers mediated through activation of the Akt signaling pathway. Clin. Cancer Res.,17, 3029–3038.
4. Imanaka, Y. et al. (2011) MicroRNA-141 confers resistance to cisplatin-induced apoptosis by targeting YAP1 in human esophageal squamous cell carcinoma. J. Hum. Genet., 56, 270–276.
5. Hummel, R. et al. (2011) Mir-148a improves response to chemotherapy in sensitive and resistant oesophageal adenocarcinoma and squamous cell carcinoma cells. J. Gastrointest. Surg., 15, 429–438.
6. Zhang, H. et al. (2010) Down-regulation of miR-27a might reverse multidrug resistance of esophageal squamous cell carcinoma. Dig. Dis. Sci., 55, 2545–2551.
7. Sugimura, K. et al. (2012) Let-7 expression is a significant determinant of response to chemotherapy through the regulation of IL-6/STAT3 pathway in esophageal squamous cell carcinoma. Clin. Cancer Res., 18, 5144–5153.
8. Arroyo, J.D. et al. (2011) Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. Proc. Natl Acad. Sci. USA, 108, 5003–5008.
9. Vickers, K.C. et al. (2011) MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins. Nat. Cell Biol., 13, 423–433.
10. Mitchell, P.S. et al. (2008) Circulating microRNAs as stable blood-based markers for cancer detection. Proc. Natl Acad. Sci. USA, 105, 10513–10518.
11. Hasselmann, D.O. et al. (2001) Extracellular tyrosinase mRNA within apoptotic bodies is protected from degradation in human serum. Clin. Chem., 47, 1488–1489.
12. Kosaka, N. et al. (2010) Secretory mechanisms and intercellular transfer of microRNAs in living cells. J. Biol. Chem., 285, 17442–17452.
13. Jung, E.J. et al. (2012) Plasma microRNA 210 levels correlate with sensitivity to trastuzumab and tumor presence in breast cancer patients. Cancer, 118, 2603–2614.
14. Zhang, H.L. et al. (2011) Serum miRNA-21: elevated levels in patients with metastatic hormone-refractory prostate cancer and potential predictive factor for the efficacy of docetaxel-based chemotherapy. Prostate, 71, 326–331.
15. Li, Y. et al. (2014) Autophagy in alcohol-induced multiorgan injury: mechanisms and potential therapeutic targets. Biomed Res. Int., 2014, 498491.
16. Wu, K.C. et al. (2012) Role of Nrf2 in preventing ethanol-induced oxidative stress and lipid accumulation. Toxicol. Appl. Pharmacol., 262, 321–329.
17. Kadera, B.E. et al. (2013) MicroRNA-21 in pancreatic ductal adenocarcinoma tumor-associated fibroblasts promotes metastasis. PLoS One, 8, e71978.
18. Kalluri, R. et al. (2006) Fibroblasts in cancer. Nat. Rev. Cancer, 6, 392–401.
19. Yano, M. et al. (2006) Preoperative chemotherapy for clinically node-positive patients with squamous cell carcinoma of the esophagus. Dis. Esophagus, 19, 158–163.
20. Yamasaki, M. et al. (2011) Multicenter phase I/II study of docetaxel, cisplatin and fluorouracil combination chemotherapy in patients with advanced or recurrent squamous cell carcinoma of the esophagus. Oncology, 80, 307–313.
21. Miller, A.B. et al. (1981) Reporting results of cancer treatment. Cancer, 47, 207–214.
22. Herrera, M., et al. (2013) Functional heterogeneity of cancer-associated fibroblasts from human colon tumors shows specific prognostic gene expression signature. Clin Cancer Res.,19, 5914–5926.
23. Duyverman, A.M. et al. (2012) Studying primary tumor-associated fibroblast involvement in cancer metastasis in mice. Nat. Protoc., 7, 756–762.
24. Jörres, A. et al. (1996) Establishment and functional characterization of human peritoneal fibroblasts in culture: regulation of interleukin-6 production by proinflammatory cytokines. J. Am. Soc. Nephrol., 7, 2192–2201.
25. Cortez, M.A. et al. (2009) MicroRNA identification in plasma and serum: a new tool to diagnose and monitor diseases. Expert Opin. Biol. Ther., 9, 703–711.
26. Cheng, H. et al. (2011) Circulating plasma MiR-141 is a novel biomarker for metastatic colon cancer and predicts poor prognosis. PLoS One, 6, e17745.
27. Schmittgen, T.D. et al. (2004) A high-throughput method to monitor the expression of microRNA precursors. Nucleic Acids Res., 32, e43.
28. Venturini, M. et al. (1997) In Saccharomyces cerevisiae a short amino acid sequence facilitates excretion in the growth medium of periplasmic proteins. Mol. Microbiol., 23, 997–1007.
29. Martinez-Outschoorn, U.E. et al. (2010) Autophagy in cancer associated fibroblasts promotes tumor cell survival: Role of hypoxia, HIF1 induction and NFκB activation in the tumor stromal microenvironment. Cell Cycle, 9, 3515–3533.
30. Mitra, A.K. et al. (2012) MicroRNAs reprogram normal fibroblasts into cancer-associated fibroblasts in ovarian cancer. Cancer Discov., 2, 1100–1108.
31. Rosenthal, E. et al. (2004) Elevated expression of TGF-beta1 in head and neck cancer-associated fibroblasts. Mol. Carcinog., 40, 116–121.
32. San Francisco, I.F. et al. (2004) Expression of transforming growth factor-beta 1 and growth in soft agar differentiate prostate carcinoma-associated fibroblasts from normal prostate fibroblasts. Int. J. Cancer, 112, 213–218.
33. Fingas, C.D. et al. (2011) Myofibroblast-derived PDGF-BB promotes Hedgehog survival signaling in cholangiocarcinoma cells. Hepatology, 54, 2076–2088.
34. Tyan, S.W. et al. (2011) Breast cancer cells induce cancer-associated fibroblasts to secrete hepatocyte growth factor to enhance breast tumorigenesis. PLoS One, 6, e15313.
35. Erez, N. et al. (2013) Cancer associated fibroblasts express pro-inflammatory factors in human breast and ovarian tumors. Biochem. Biophys. Res. Commun., 437, 397–402.
36. Guttilla, I.K. et al. (2009) Coordinate regulation of FOXO1 by miR-27a, miR-96, and miR-182 in breast cancer cells. J. Biol. Chem., 284, 23204–23216.
37. Acunzo, M. et al. (2013) Cross-talk between MET and EGFR in non-small cell lung cancer involves miR-27a and Sprouty2. Proc. Natl Acad. Sci. USA, 110, 8573–8578.
38. Li, Z. et al. (2010) MiR-27a modulates MDR1/P-glycoprotein expression by targeting HIPK2 in human ovarian cancer cells. Gynecol. Oncol., 119, 125–130.
39. Zhao, X. et al. (2011) Down-regulation of miR-27a might inhibit proliferation and drug resistance of gastric cancer cells. J. Exp. Clin. Cancer Res., 30, 55.

40. Micke, P. et al. (2004) Tumour-stroma interaction: cancer-associated fibroblasts as novel targets in anti-cancer therapy? Lung Cancer, 45 (suppl. 2), S163–S175.
41. Aprelikova, O. et al. (2010) The role of miR-31 and its target gene SATB2 in cancer-associated fibroblasts. Cell Cycle, 9, 4387–4398.
42. Xu, Y. et al. (2008) TGF-beta receptor kinase inhibitor LY2109761 reverses the anti-apoptotic effects of TGF-beta1 in myelo-monocytic leukaemic cells co-cultured with stromal cells. Br. J. Haematol., 142, 192–201.
43. Tabe, Y. et al. (2013) TGF-β-Neutralizing Antibody 1D11 Enhances Cytarabine-Induced Apoptosis in AML Cells in the Bone Marrow Microenvironment. PLoS One, 8, e62785.
44. Yu, Y. et al. (2010) Context-dependent bidirectional regulation of the MutS homolog 2 by transforming growth factor β contributes to chemoresistance in breast cancer cells. Mol. Cancer Res., 8, 1633–1642.
45. Al-Azayzih, A. et al. (2012) TGFβ1 induces apoptosis in invasive prostate cancer and bladder cancer cells via Akt-independent, p38 MAPK and JNK/SAPK-mediated activation of caspases. Biochem. Biophys. Res. Commun., 427, 165–170.
46. Ha Thi, H.T. et al. (2013) Transcriptional and post-translational regulation of Bim is essential for TGF-β and TNF-α-induced apoptosis of gastric cancer cell. Biochim. Biophys. Acta, 1830, 3584–3592.
47. Shin, I. et al. (2001) Transforming growth factor beta enhances epithelial cell survival via Akt-dependent regulation of FKHRL1. Mol. Biol. Cell, 12, 3328–3339.
48. Huang, Y. et al. (2000) Transforming growth factor-beta 1 suppresses serum deprivation-induced death of A549 cells through differential effects on c-Jun and JNK activities. J. Biol. Chem., 275, 18234–18242.
49. Cheng, J.C. et al. (2012) TGF-beta induces serous borderline ovarian tumor cell invasion by activating EMT but triggers apoptosis in low-grade serous ovarian carcinoma cells. PLoS One, 7, e42436.
50. Weber, J.A. et al. (2010) The microRNA spectrum in 12 body fluids. Clin. Chem., 56, 1733–1741.
51. Hunter, M.P. et al. (2008) Detection of microRNA expression in human peripheral blood microvesicles. PLoS One, 3, e3694.
52. Wang, H. et al. (2014) Expression of serum exosomal microRNA-21 in human hepatocellular carcinoma. Biomed Res. Int., 2014, 864894.
53. Komatsu, S. et al. (2014) Circulating miR-18a: a sensitive cancer screening biomarker in human cancer. In Vivo, 28, 293–297.
54. Turchinovich, A. et al. (2013) Circulating miRNAs: cell-cell communication function? Front. Genet., 4, 119.