TNF-α is a novel target of miR-19a

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Abstract. Many studies have demonstrated the overexpression and amplification of the miR-17-92 cluster in malignant human cancers, including B-cell lymphomas and lung cancers. The purpose of this study was to investigate for the first time, the expression of the miR-17-92 cluster in esophageal squamous cell carcinoma (ESCC). The miR-17-92 cluster was found to be overexpressed in 21 out of 28 (75%) esophageal cancer samples. It was also found that overexpression of the miR-17-92 cluster could promote cellular growth *in vivo* and *in vitro*. Furthermore, inhibition of miR-19a by antisense oligonucleotides (ONs) induced apoptosis, while antisense ONs against miR-17-5p, miR-18a, miR-20a and miR-92-1 did not exhibit such an effect. In addition, it was found that antagomir-19a treatment could impair tumor growth *in vivo*. Using Human Apoptosis RT^2 Profiler PCR Array 384HT, we found that tumor necrosis factor-α (TNF-α) was up-regulated 12-fold in cells transfected with miR-19a antisense ONs compared to the cells treated with the control scramble ONs. MiR-19a was predicted to target the 3' untranslated region of TNF-α mRNA, and this was confirmed by luciferase reporter assay. Taken together, we conclude that the miR-17-92 cluster is overexpressed in ESCC and that TNF-α is a novel target of miR-19a.

Introduction

MicroRNAs (miRNAs) are a class of 17-24 base single-stranded RNA molecules that are expressed in cells from plants to animals (1). MiRNAs repress protein expression at the post-transcriptional level through imperfect base pairing with the 3' untranslated region (UTR) of the target mRNA, leading to its reduced translation and/or degradation. MiRNAs are involved in cell differentiation, proliferation and death (2). Many studies have indicated that miRNAs are involved in human cancer and that miRNAs have potentials in therapeutic and diagnostic applications (3).

Esophageal squamous cell carcinoma (ESCC) ranks among the most frequent cause of cancer death in the world. However, the molecular mechanism of ESCC tumorigenesis remains to be elucidated. Previous studies have shown that the miR-17-92 cluster is amplified and overexpressed in human B-cell lymphomas (4) and lung cancers (5). The miR-17-92 cluster consists of six miRNAs, including miR-17-5p, miR-18a, miR-19a, miR-20a, miR-19b-1 and miR-92-1. MiR-19a and miR-19b-1 have only a single nucleotide divergence at a region nonessential for miRNA target recognition (6-8). However, miR-17-5p has also been reported to be a tumor suppressor in breast cancer cells (9). It is unclear whether the expression of the miR-17-92 cluster is altered in ESCC. In this study, we determined the expression level of the miR-17-92 cluster in both esophageal cancer cells and primary tumor samples, and tested the effect of miR-19a inhibition on the induction of apoptosis in esophageal cancer cell lines.

Materials and methods

Ethics statement. This study was approved by the Ethics Committee of the Cancer Institute, Chinese Academy of Medical Sciences, and written informed consent was obtained from all patients. All animals were handled in strict accordance with good animal practice as defined by the Beijing Municipal Science and Technology Commission, and all animal experiments were approved by this committee.

Plasmids. A 1,084 bp *Bam*HI-*Xho*I fragment containing the miR-17-92 cluster was cloned into the pcDNA3 vector (Invitrogen, Carlsbad, CA, USA). The primers were designed as follows: Forward, 5'CGGGATCCACCTCATATCTTT GAGAT3'; and reverse, 5'GCCTCGAGGACAAGATGT ATTTACAC3'. Tumor necrosis factor-α (TNF-α) 3'UTR was amplified from human genomic DNA and subcloned into the pGL3-Control luciferase vector (Promega, Madison, WI, USA). The primers were designed as follows: Forward,

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5'CGTCTAGAGGAGGACGAACATCCAACCT3'; and reverse, 5'CGTCTAGAGCCACTGAATAGTAGGGCGA3'. We also generated one mutation in the predicated target site for miR-19a by using a QuickChange site-specific mutagenesis kit (Stratagene). All constructs were sequenced by the Sangon Co., Shanghai, China.

Cell culture and stable expression system establishment. Five types of esophageal cancer cell lines KYSE150, KYSE410, KYSE70 (generously provided by Professor Yutaka Shimada), EC109 and EC9706 (kindly provided by Professor Mingrong Wang), were cultured in RPMI-1640 medium supplemented by 10% fetal bovine serum at 5% CO_2. Transfection was performed in 70-80% confluent cells using Lipofectamine 2000 Reagent (Invitrogen) according to the manufacturer's instructions.

EC9706 cells were first transfected with pcDNA3/miR-17-92 or pcDNA3, and were then selected with 800 μg/ml G418 (Invitrogen). The resistant monoclone was verified by RT-PCR, and designated as EC9706/miR-17-92 or EC9706/Control.

Clinical tissue sample collection. We obtained 28 ESCC and corresponding normal tissue samples from surgically resected esophageal carcinoma specimens at the Cancer Hospital. None of the patients investigated received chemotherapy or radiotherapy prior to the operation. All the samples were histopathologically diagnosed as ESCC.

Fresh samples were dissected manually to remove connective tissues and were stored immediately at -80˚C until analysis. The corresponding normal tissues were obtained from the distant edge of dissected esophagus, without carcinoma cell invasion, as shown by pathological diagnosis. Every tissue sample contained >80% of normal or tumor epithelial cells.

RNA extraction and semi-quantitative RT-PCR. Total-RNA was extracted from the paired specimen of primary esophageal cancer and non-cancerous esophageal epithelium with TRIzol Reagent (Invitrogen) and was reverse-transcribed to cDNA with M-MLV Reverse Transcriptase (Promega). Semi-quantitative RT-PCR was performed as follows: 95˚C for 150 sec (one cycle), then 95˚C for 10 sec and 60˚C for 30 sec (30 cycles). Sequences of the PCR primers for the miR-17-92 cluster were as follows: Forward, 5'CTGTCGCCCAATCAAACTG3'; and reverse, 5'GTCACAATCCCCACCAAAC3' (10). β-actin was used as the internal control. The primers were as follows: Forward, 5'GGCGGCACCACCATGTACCCT3'; and reverse, 5'AGGGGCCGGACTCGTCATACT3'. The reaction products were visualized by electrophoresis with 5 μl reaction mixture in 2% agarose gel containing 0.5 μg/ml ethidium bromide, and quantified by densitometry using Gel-Pro Analyzer version 3.1 (Media Cybernetics, Silver Spring, MD, USA).

miRNA-specific quantitative real-time RT-PCR. For miRNA analysis from cultured cells, miRNA was isolated using a mirVana RNA isolation kit (Ambion). Reverse transcription and real-time PCR were performed as previously described (11) by using miRNA-specific quantitative real-time PCR (Applied Biosystems, CA). The small nuclear RNA (RNU6) was used as the internal control for normalization. Real-time PCR was performed using an ABI 7500 Sequence Detection System and fold changes in gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method (12). The mean miRNA level of the three real-time quantitative PCR experiments was calculated for each case.

Oligonucleotides (ONs), Pre-miR precursor and antagomir-19a. Antisense ONs as well as the respective scrambled ONs were synthesized as hybrid molecules between deoxyribonucleotides and the 2'-O, 4'-C-methylene bridge (locked nucleic acid, LNA) (13) modification of all guanine and cytosine residues (Sangon) and the sequences used were reported previously (14). Each ON was transfected with Lipofectamine 2000 Reagent (Invitrogen).

Pre-miR miR-19a precursor and Pre-miR negative control were purchased from Ambion. Antagomir-19a was synthesized by RiboBio (Guangzhou, China).

Western blot analysis. Cells were transfected with respective ONs, harvested at indicated time-points and lysed in RIPA buffer (Sigma). Western blot analysis was performed with the use of conventional protocols as described previously (15). The antibodies and dilutions used, included anti-β-actin (AC-15; 1:2000; Sigma), anti-c-Myc (9E10; 1:1000; Santa Cruz), anti-Bcl-2 (C-2; 1:1000; Santa Cruz), anti-Bax (N-20; 1:1000; Santa Cruz) , anti-TNF-α (3707; 1:1000; Cell Signaling Technology) and anti-PARP (9542; 1:1000; Cell Signaling Technology). After extensive washing, the membranes were incubated with anti-mouse or anti-rabbit IgG-horseradish peroxidase conjugate antibody (Zhongshan Co., China) for 1 h at room temperature and developed with a Luminol chemiluminescence detection kit (Santa Cruz). Membranes were reprobed for β-actin antibodies for normalization and accurate quantification. Protein expression level was quantified by using a Gel EDAS 293 analysis system (Cold Spring USA Corp.) and Gel-Pro Analyzer 3.1 software (Media Cybernetics).

Tumor growth in nude mice. Equal numbers ($4x10^5$) of EC9706/miR-17-92 and EC9706/Control cells were harvested by trypsinization, washed with 1X PBS, and resuspended in 0.1 ml of saline. A total number of 5 (4-week old) nude mice were given bilateral subcutaneous injections with EC9706/miR-17-92 and EC9706/Control cells. The mice were kept in pathogen-free environments and checked every 2 days. The date of the first palpable tumor (>3 mm) detected was recorded and the weights of tumors were measured at the end of the experiment. This experiment was repeated once.

For the *in vivo* administration of antagomir-19a in tumors, $2x10^5$ EC9706 cells were subcutaneously injected into the flank of 4-week-old nude mice. After 2 weeks, when the tumor reached an average volume of ~125 mm^3, the tumor-bearing nude mice were treated with antagomir-19a. Antagomir-19a (100 μl) diluted in PBS at a concentration of 1,000 μM, or PBS alone, were injected intratumorally 5 times every 2 days. Tumors were measured at the end of the experiment. This experiment was repeated once.

MTT assay. For the cell growth curve assay, $5x10^3$ of EC9706/miR-17-92 and EC9706/Control cells in logarithmic growth

were transferred to a 96-well plate. Each concentration was plated in 4 wells, with medium only wells used as the controls. The cells were cultured for 24, 48, 72, 96 and 120 h. Four hours before the end of the incubation, 20 μl MTT (5 mg/ml) were added to each well and 150 μl DMSO were added to stop the reaction. Viable cell numbers were measured at a wavelength of 570 nm with the Model 680 Microplat Reader (Bio-Rad, USA).

Suppression of miRNA expression by ONs was detected by MTT assay in human ESCC cell lines. KYSE150, EC109 and EC9706 cells were plated at $2x10^4$ cells per well in 96-well plates on day 1, and ONs were transfected on day 2 at the indicated concentration (0-80 nM) with Lipofectamine 2000. Viable cell numbers were measured on day 3. Three independent experiments were performed. When the cells were tranfected with 40 nM ONs, different time-points were also measured.

FACS analysis. Cells were seeded into a six-well tissue culture plate and transfected with ONs (40 nM) on day 2. The cells were harvested and washed in cold sterile phosphate buffered saline (PBS) 48 h later. Annexin V and propidium iodide staining were carried out using the Annexin V-FITC Apoptosis Detection Kit (BD Biosciences), according to manufacturer's instructions, followed by flow cytometric analysis of cells. Analyses of apoptosis profiles were performed with Coulter Elite 4.5 Multicycle software.

TUNEL assay. Apoptotic cells were confirmed with the *in situ* cell death detection kit, Alkaline Phosphatase (Roche Applied Science), in accordance with the manufacturer's instructions. Cells were seeded into a 6-well tissue culture plate. Cells were transfected with ONs (40 nM) on day 2. The cells were stained 24 h after the transfection. The following process was performed as described in the manufacturer's protocol. The apoptotic cells (dark blue staining) were counted under a microscope. Three fields were randomly counted for each sample.

Human Apoptosis RT² Profile PCR Array 384HT. Cells were seeded into a 6-well tissue culture plate. Cells were transfected with ONs (40 nM) on day 2. After 48 h, the cells were harvested and washed in cold sterile PBS, and then 1 ml TRIzol Reagent (Invitrogen) was added. Total RNA preparation, cDNA synthesis and real-time PCR were performed by the Kangcheng Co. (Shanghai, China), according to manufacturer's instructions.

Reporter assay. Cells in 24-well plates were transfected with the indicated plasmids (200 ng), and the internal control plasmid pRL-SV40 (2 ng). Luciferase activity was determined 24 h after transfection by using the Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase activity was normalized to Renilla luciferase activity. All results were expressed as the means ± SD for independent triplicate cultures.

Immunohistochemistry. Sections of 5 μm thickness were deparaffinized in xylene and hydrated in graded ethanol. The sections were then pre-treated in EDTA buffer (1 mM, pH 9.0) under autoclaving at 121˚C for 2 min to retrieve the antigen. The sections were covered with 3% hydrogen peroxide in PBS to block the endogenous peroxidase activity for 10 min at RT.

Table I. Expression of miR-17-92 in human ESCC.

MiR-17-92 level	Cases (%)
Elevated[a]	21 (75.0)
Decreased	3 (10.7)
No change	4 (14.3)
Total	28 (100)

[a]The sample had a >1.5-fold increase of miR-17-92 level compared to the normal control.

After that, the sections were incubated overnight at 4˚C with mouse monoclonal primary antibody against TNF-α (MAB1096; 1:1000; Millipore). After the slides were washed, the sections were processed with a Polymer HRP Detection System (PV-9000, Zhongshan Co.), according to the recommended protocol. Finally, the slides were stained with DAB and counterstained in haematoxylin.

Immunofluorescence staining. EC9706 cells were seeded on glass coverslips in 6-well plates, and transfected with ONs (40 nM) on day 2. The cells were washed in cold PBS 48 h later, and fixed in 90% methanol at -20˚C for 10 min. After washing with cold PBS, the slides were incubated for 1 h at 37˚C with mouse anti-TNF-α antibody (MAB1096; 1:200; Millipore). Subsequently, the slides were washed using PBS containing 0.2% Tween-20 (PBST), then incubated for 30 min at 37˚C in the dark with FITC-coupled anti-mouse secondary antibody (Zhongshan Co.). Cell nuclei were counterstained with 0.1 μg/ml 4, 6-diamino-2-phenylindol (DAPI; Sigma). After rinsing with PBST, the labeled cells were mounted with glycerol, and then examined using a fluorescence microscope (Olympus).

Statistical analysis. SPSS for Windows (SPSS Inc.) was used for statistical analysis. Values of $P<0.05$ were considered to be statistically significant.

Results

Elevated expression of miR-17-92 cluster in human ESCC. The expression of the miR-17-92 cluster in human ESCC was determined by RT-PCR analysis (Fig. 1A). The miR-17-92 cluster was detected in all 28 cases of ESCC that were analyzed. Expression levels of miR-17-92 cluster mRNA were elevated in 21 cases of ESCC compared to the corresponding surrounding non-cancerous esophageal tissues (Table I).

miR-17-92 cluster promotes cellular growth in vitro and in vivo. In order to investigate the oncogenic role of the miR-17-92 cluster in ESCC, we generated a stable EC9706 transfectant expressing the cluster. The monoclone EC9706/miR-17-92 cells showed an increased expression of the cluster (Fig. 2A). In *in vitro* experiments, overexpression of the cluster increased the proliferation rate, compared to the control cells transfected with an empty vector (Fig. 2B). To evaluate the effect of the miR-17-92 cluster on *in vivo* tumori-

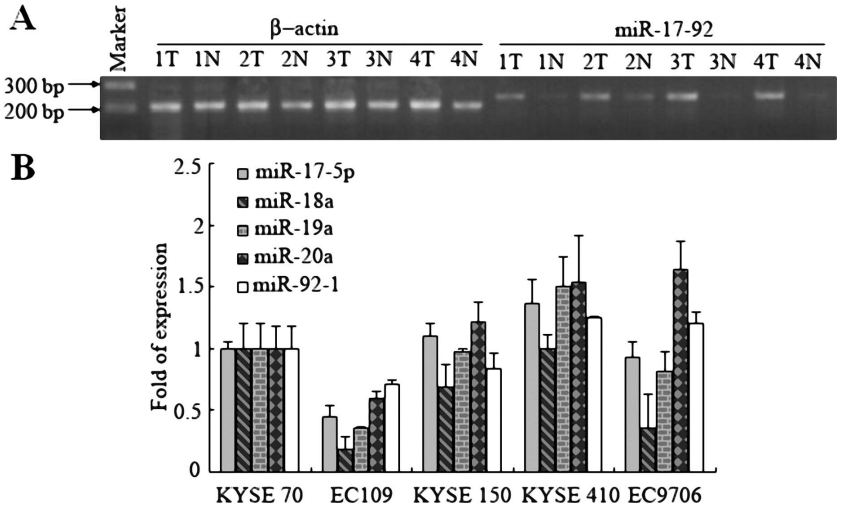

Figure 1. Overexpression of miR-17-92 in human ESCC tissues. (A) A miR-17-92 cDNA fragment of 246 bp in size and a β-actin cDNA fragment of 202 bp were detected by RT-PCR. Four representative cases are shown. T, ESCC tissue; N, corresponding normal adjacent mucosa; Maker, 100-bp DNA ladder. (B) MiRNA quantitative RT-PCR analysis of miRNAs pertaining to the miR-17-92 cluster in different ESCC cell lines. The level of each miRNA is normalized to its expression in KYSE70 cells (set as 1). Data are expressed as the means ± SD (n=3).

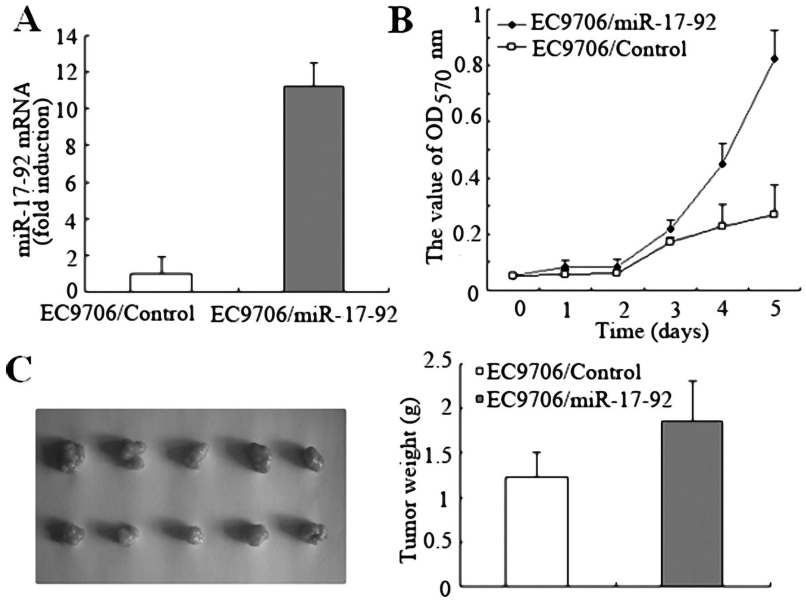

Figure 2. MiR-17-92 cluster promotes cellular growth *in vitro* and *in vivo*. (A) MiR-17-92 mRNA expression was detected by real-time PCR. miR-17-92 mRNA expression in EC9706/Control was used as unit '1'. (B) Growth curve of EC9706/miR-17-92 and EC9706/Control cells was measured by MTT assay. (C) Left panel, 5 nude mice were injected subcutaneously with EC9706/miR-17-92 and EC9706/Control cells and were sacrificed at 6 weeks after injection. Similar results were observed in 2 independent experiments. The tumors are shown. Right panel, the weights of the tumors were recorded and are expressed as the means ± SD ($P<0.05$).

Table II. MiR-17-92 can promote EC9706 cell growth in nude mice.

	EC9706/miR-17-92			EC9706/Control		
No. of cells[a]	Tumor weight (mg)	Tumor/ Injection	Latency[b] (days)	Tumor weight (mg)	Tumor/ Injection	Latency[b] (days)
$4x10^5$	508-1201	5/5	6-12	216-1043	5/5	>10
$4x10^5$	1259-2430	5/5	6-12	894-1666	5/5	>10

[a]Number of cells that were injected bilaterally and subcutaneoulsy into 5 nude mice. [b]Latency is defined as the time interval required for a palpable tumor to arise.

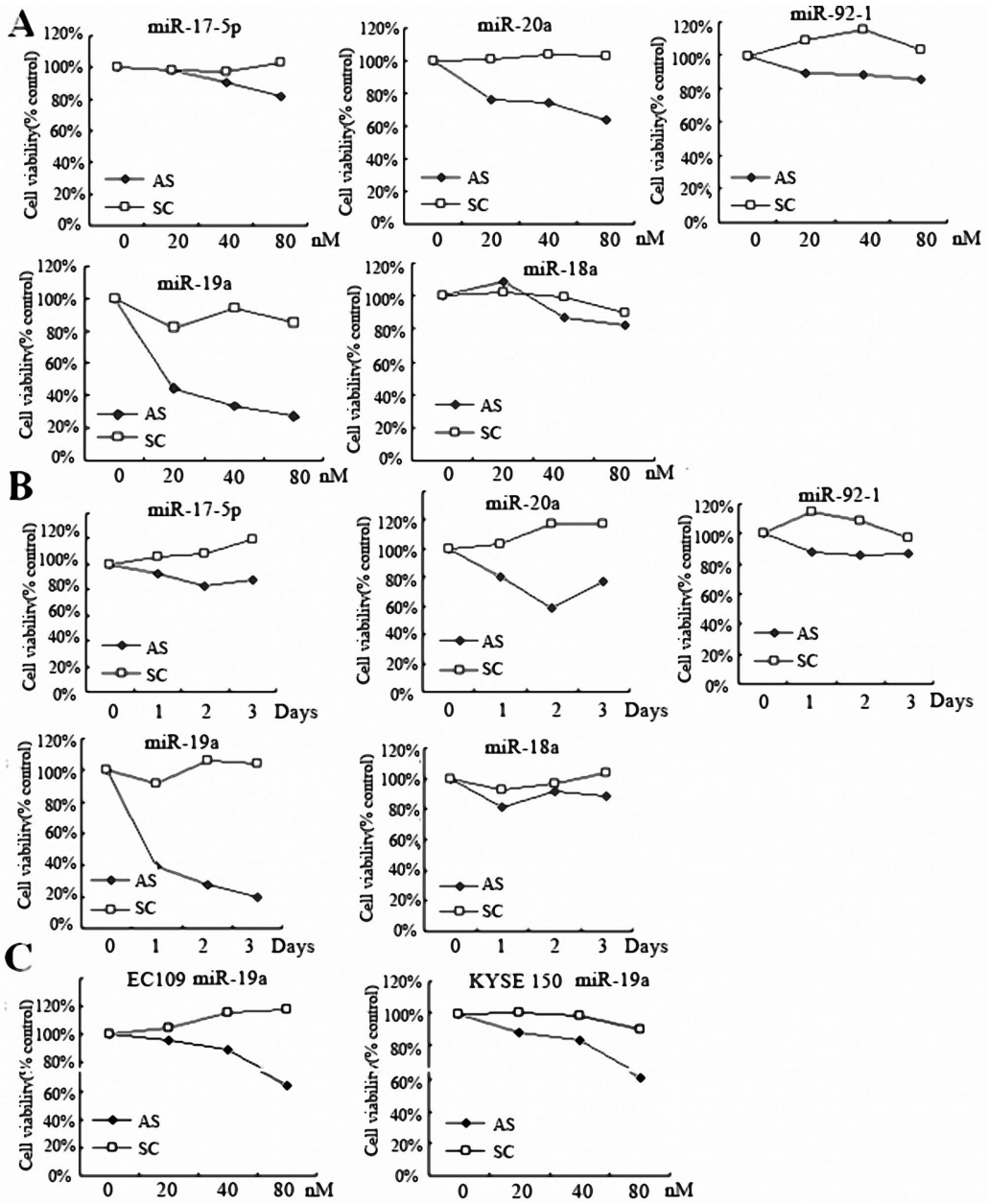

Figure 3. Growth inhibitions by antisense ON treatment against miRNAs pertaining to the miR-17-92 cluster in ESCC cell lines. (A) MTT assay showing growth inhibition in EC9706 cells by different concentrations of antisense ON treatment against miR-17-5p, miR-18a, miR-19a, miR-20a and miR-92-1. (B) MTT assay showing growth inhibiton in EC9706 cells by antisense ON treatment against miR-17-5p, miR-18a, miR-19a, miR-20a and miR-92-1 at different time-points. (C) MTT assay showing growth inhibition in EC109 and KYSE150 by antisense ON treatment against miR-19a.

genesis, we injected EC9706/Control or EC9706/miR-17-92 into nude mice. As seen in Table II, there was a shorter growth delay of tumor formation in mice receiving $4x10^5$ EC9706/miR-17-92 cells than in those receiving EC9706/Control cells. Also, EC9706/miR-17-92 cell injection led to a larger tumor size compared to the control cell injection (Fig. 2C). Taken together, these data demonstrate that the miR-17-92 cluster enhances cell proliferation and promotes tumorigenesis both *in vitro* and *in vivo*.

Growth inhibition with antisense oligonucleotides (ONs) against miR-19a. We evaluated the expression level of the 5 mature miRNAs in the miR-17-92 cluster by quantitative RT-PCR in human ESCC cell lines [Fig. 1B, the level of each miRNA is normalized to its expression in KYSE70 cells (set as 1)]. Antisense ONs incorporating LNA were used to down-regulate miR-17-5p, miR-18a, miR-19a, miR-20a and miR-92-1. MTT assays revealed a significant reduction in cell growth on transfection of the antisense ONs against miR-19a expression in human ESCC cancer cell lines, including EC9706, KYSE150 and EC109 (Fig. 3A and C). In contrast, the antisense ONs against miR-18a did not exhibit such inhibitory effects, whereas inhibition of miR-17-5p, miR-20a and miR-92-1 resulted in modest reduction of cell growth. Fig. 3B shows that the growth inhibition with ONs is also time-dependent.

Apoptosis induced by ONs against miR-19a. TUNEL assays were performed to examine whether apoptosis was induced

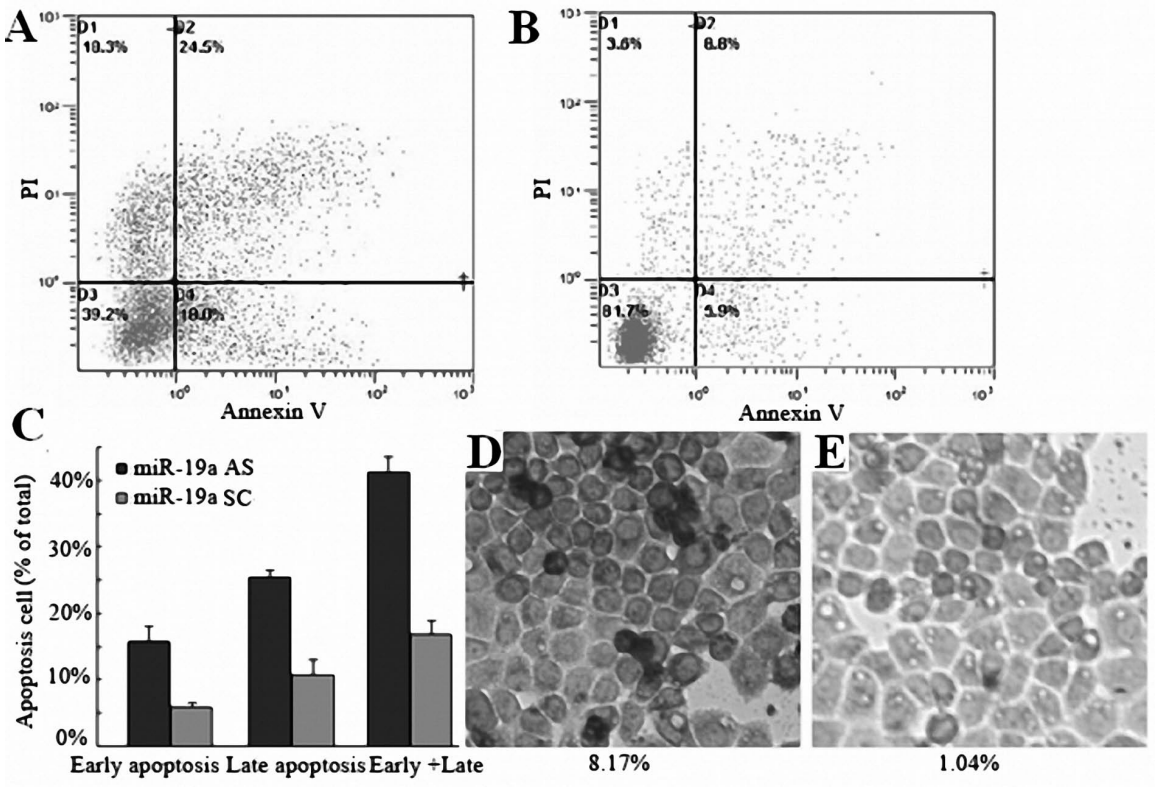

Figure 4. Inhibition of endogenous miR-19a induced apoptosis in EC9706 cells. (A and B) EC9706 cells were transfected with miR-19a AS or miR-19a SC (40 nM), then cultured for an additional 48 h. After that, cells were harvested and analyzed by FACS. (C) Bar graph showing the percentage of apoptotic cells. Data are expressed as the means ± SD (n=3), P<0.01. (D and E) TUNEL assay analysis demonstrated that transfection with miR-19a AS robustly increased apoptosis in EC9706 cells, compared to the cells transfected with miR-19a SC, P<0.001.

by the treatment with antisense ONs against miR-19a in EC9706 cells. Significant induction of TUNEL-positive apoptotic cells by treatment was indeed observed. The results showed that ~8.17% cells were TUNEL-positive in the antisense ON group (Fig. 4D), compared to 1.04% in the scramble ON group (Fig. 4E). After transfection of miR-19a AS, some cells underwent apoptosis, floating in the cell culture medium. The antisense ON-induced enhancement of apoptosis was further confirmed by fluorescence activated cell sorter (FACS) analysis (Fig. 4A-C). These data suggested that antisense ONs inhibiting miR-19a effectively elicit apoptosis in EC9706 cells (P<0.01). PARP cleavage serves as a marker of apoptosis (16), and as shown in Fig. 6A, cleaved PARP was increased when EC9706 cells were transfected with miR-19a AS.

Human Apoptosis RT² Profile PCR Array. In order to further explore the mechanism of apoptosis induced by miR-19a antisense ONs, we used the PCR array focusing on apoptosis-associated genes to study the altered gene expression in EC9706 cells into which miR-19a antisense ONs were introduced. Compared to the scramble ON transfection, miR-19a antisense ON transfection up-regulated 19 genes >2-fold. TNF-α was up-regulated 12-fold (data not shown). We also observed a strong increase in TNF-α protein level (Fig. 5A) after transfection with miR-19a AS. Furthermore, real-time PCR analyses revealed changes in the mRNA levels of Bcl-2, Bax and c-Myc (data not shown), and these were consistent with their respective protein levels (Fig. 5A).

TNF-α is a direct and functional target of miR-19a. We used computational methods, in combination with the results from the array analysis, to identify the possible targets of miR-19a in human ESCC. TNF-α was also predicted to be a target of miR-19a by the TargetScan (7) and MicroCosm (formerly miRBase Targets) (17) search programs. The TNF-α-encoded mRNA contains a 3'UTR element that is partially complementary to miR-19a (Fig. 6A).

To demonstrate that miR-19a directly regulates TNF-α expression by binding to its 3'UTR, we cloned the TNF-α 3'UTR into the pGL3-Control vector, downstream of the luciferase gene [pGL3-Control/TNF-α 3'UTR-wt (TNF-α 3'UTR-wt)]. As a control, we cloned a mutated TNF-α 3'UTR with a deletion of 7 bp from the site of perfect complementarity [pGL3-Control/TNF-α 3'UTR-mut (TNF-α 3'UTR-mut)]. As shown in Fig. 7B, the transfection of TNF-α 3'UTR-wt together with miR-19a AS, but not with miR-19a SC, led to an increase in luciferase activity in EC9706 cells, due to the inhibition of the endogenous miR-19a. In contrast, miR-19a AS did not increase the luciferase activity of TNF-α 3'UTR-mut, thus demonstrating that the deletion of the miR-19a binding site in the TNF-α 3'UTR abolished the inhibitory effect of miR-19a in regulating its expression (Fig. 6B). Transfection of the Pre-miR miR-19a precursor led to an increased expression of miR-19a (Fig. 5B, upper panel), and this led to a reduced production of TNF-α at the protein level (Fig. 5B, lower panel). Taken together, these results showed that TNF-α is a novel target of miR-19a in esophageal cancer cell lines.

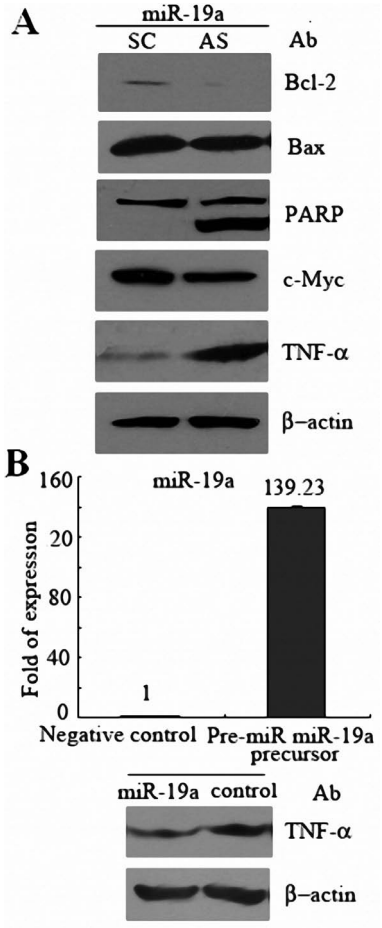

Figure 5. TNF-α was targeted by miR-19a. (A) Western blot of EC9706 cells which were transiently transfected with miR-19a AS or miR-19a SC (40 nM), and cultured for an additional 48 h, then the protein lysates were prepared. The antibody was used against its corresponding protein to detect the expression level. The same membrane was probed with anti-β-actin as the loading control. (B) Upper panel, EC9706 cells were transiently transfected in 6-well plates using siPORT NeoFX Transfection Agent with either 30 nM Pre-miR miR-19a precursor or the negative control miRNA precursor. Post-transfection (36 h), effective delivery of the Pre-miR miR-19a precursor was detected by miRNA qRT-PCR in EC9706 cell lines. The levels were normalized to their relative expression in EC9706 cells with transfection of the negative control miRNA precursor (set as 1). Data are expressed as the means ± SD (n=3) ($P<0.05$). Lower panel, TNF-α expression was monitored by Western blot analysis after 36-h transfection with either Pre-miR miR-19a precursor or the negative control miRNA precursor. β-actin was used as the internal control.

Knockdown of miR-19a inhibits the in vivo tumorigenic ability of EC9706 cells. Based on *in vitro* studies, we hypothesized that the abolition of the miR-19a expression could inhibit tumor growth *in vivo*. To address this critical question, EC9706 cells were injected into nude mice, and tumors thereby generated were treated with antagomir-19a or PBS. Injection of antagomir-19a dramatically inhibited tumor growth, compared to the administration of PBS-treated tumors (Fig. 7A and B). Antagomir-19a also increased TNF-α expression at the protein level and inhibited EC9706 cell growth *in vitro* (data not shown). Tumor analysis after the administration of antagomir-miR-19a showed a strong increase of TNF-α at the protein level, compared to the control group (Fig. 7C and D). These results demonstrated that antagomir-19a could impair tumor growth *in vivo*, possibly by up-regulating TNF-α.

Discussion

Human esophageal cancer is the sixth leading cause of death from cancer and one of the least studied cancers worldwide (18). It has two main forms, esophageal squamous cell carcinoma and adenocarcinoma. ESCC is the most frequent subtype in China (19). A better understanding of ESCC mechanisms is urgently required in order to develop better diagnosis and treatment approaches. A previous study provided direct experimental evidence that the miR-17-92 cluster has oncogenic activity (4). In this study, we have shown for the first time that the miR-17-92 cluster is markedly and frequently overexpressed in ESCC. Furthermore, evidence was obtained showing that introduction of miR-17-92 can enhance esophageal cell growth. More and more genes have been identified as targets of members of the miR-17-92 cluster by *in vitro* assay, such as E2F1 (20), Pten (7), P21 and Bim (21). This cluster has been implicated in enhanced cell cycle progression (5) and, through the targeting of E2F1, in blocking tumor cell apoptosis (20). Therefore, the oncogenic effects of the miR-17-92 are probably mediated by multiple targets, some of which remain to be discovered. It is known that the miR-17-92 cluster is directly up-regulated by the proto-oncogene Myc (10,20). The pathologically activated expression of Myc is one of the most common oncogenic events in many human malignancies, including ESCC (22). This could also be

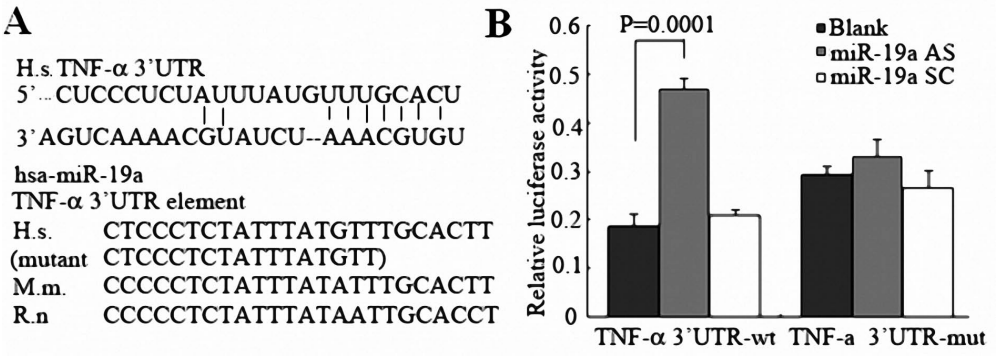

Figure 6. MiR-19a regulates TNF-α by directly binding to its 3'UTR. (A) Upper panel, predicted duplex formation between human TNF-α 3'UTR and miR-19a. Lower panel, sequence of miR-19a binding site within the TNF-α 3'UTR of human (H.s.) miRNA, and within the TNF-α 3'UTR of mouse (M.m), and rat (R.n.) miRNAs. (B) Luciferase activity of wild-type (TNF-α 3'UTR-wt) or mutant (TNF-α 3'UTR-mut) TNF-α 3'UTR reporter gene in EC9706. Data are expressed as the means ± SD (n=3).

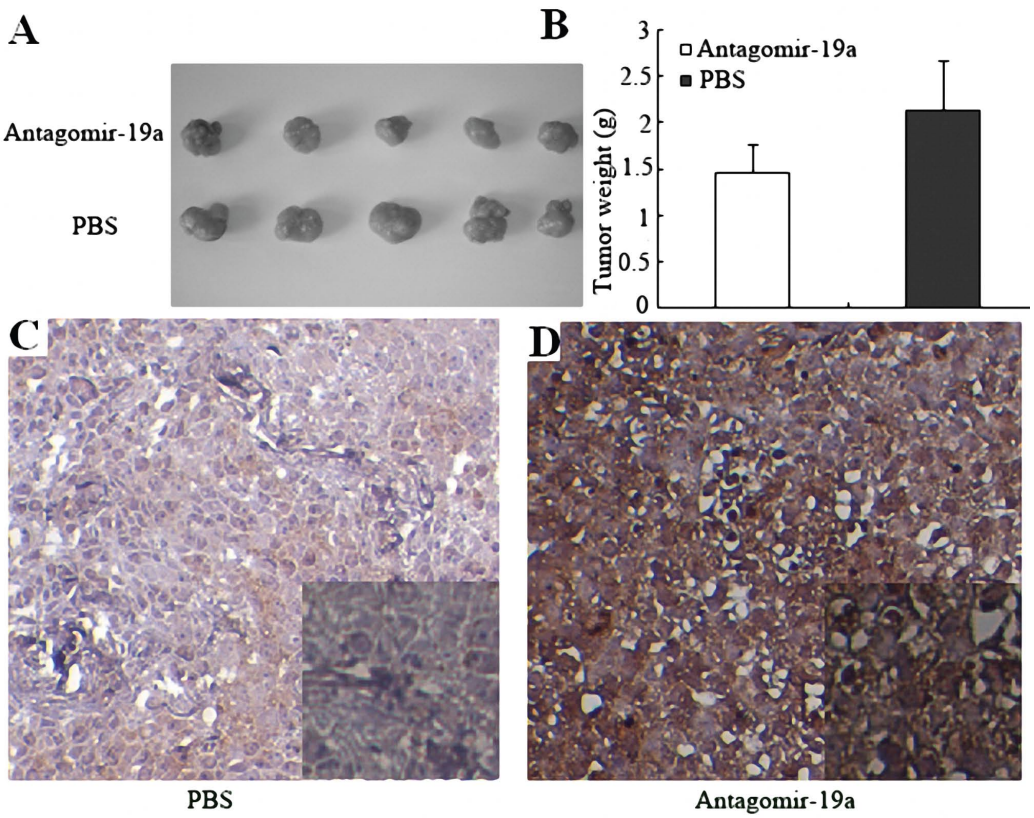

Figure 7. Treatment of EC9706 cells with antagomir-19a inhibits tumorigenesis *in vivo*. (A) Five nude mice were injected subcutaneously with EC9706 cells and when the tumors reached 125 mm^3, they were treated with antagomir-19a or PBS 5 times every 2 days. Mice were sacrificed at 50 days after the first injection. The tumors are shown. (B) The weights of the tumors were recorded and are expressed as the means ± SD ($P<0.05$). (C and D) Expression of TNF-α was analyzed by immunohistochemistry on sections of the tumor xenografts. Magnification x100. Corner images, magnification x200.

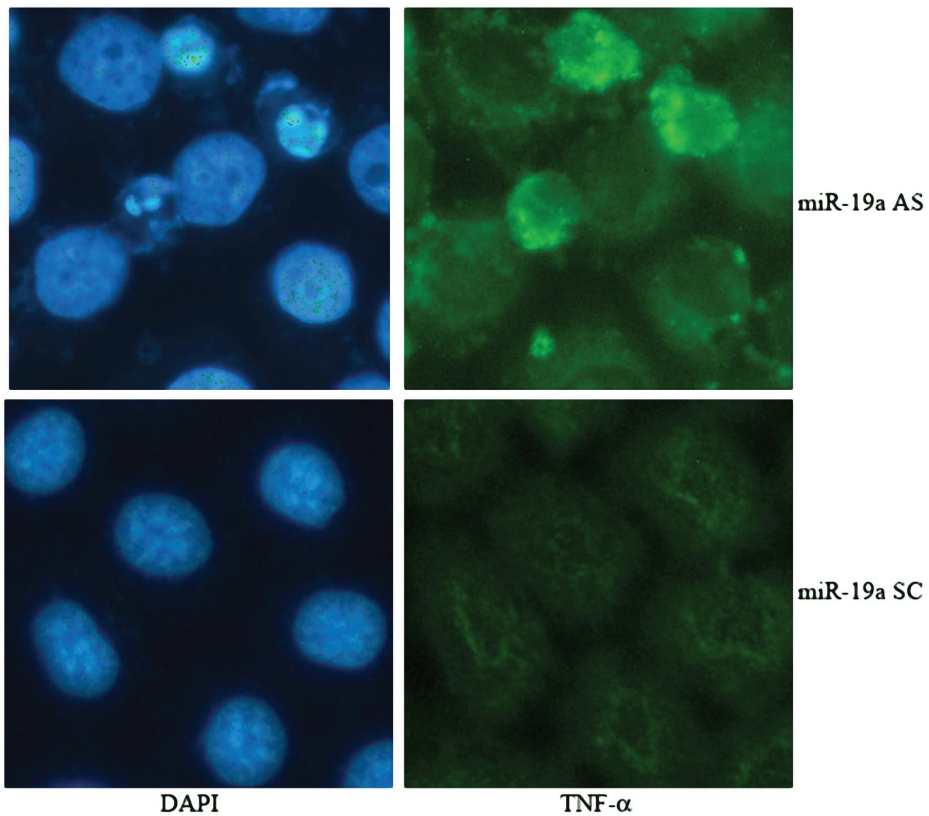

Figure 8. Detection of TNF-α by immunofluorescence staining in EC9706 cells transfected with either miR-19a AS or miR-19a SC. Cells were counterstained with DAPI for DNA (blue) and anti-TNF-α (green). Magnification x200.

an underlying mechanism for explaining the phenotype. What is most important for the regulation of cell proliferation remains to be elucidated.

EC9706/miR-17-92 cells indicated that the construct used to express the entire cluster gave lower levels of mature miRNAs (data not shown). A similar result was reported previously (4). We therefore focused most of our study on EC9706 cells. The cluster is believed to be transcribed as one primary transcript, but the expression level of individual cluster members varies, as reported previously (23). Differences in the efficiency of post-transcriptional processing steps may account for the variations.

Previous studies using antisense ONs have shown a marked cross-inhibition between miR-17-5p and miR-20a, due to their highly homologous nature. Antisense ONs against miR-18a, miR-19a and miR-92-1 clearly inhibited their corresponding miRNAs (14). Real-time PCR indicated that miR-19a AS effectively inhibits miR-19a (data not shown). Of particular interest, treatment of EC9706 with miR-19a antisense ONs caused a dramatic increase in apoptosis. Whereas there are numerous genes that are potentially targeted by miR-19a (6,24), and the induction of apoptosis can be mediated by several pathways, the up-regulation of TNF-α, mediated by direct binding of miR-19a to TNF-α mRNA 3'UTR, could be the main pathway. TNF-α is a cytokine involved in a receptor-triggered signaling pathway that leads to apoptosis. It has multiple anti-tumor effects, including effects on the neovasculature of the tumor, stimulation of the cellular immune response and direct cytotoxicity to tumor cells. We also measured the TNF-α level in the culture supernatants of EC9706 cells transfected with miR-19a AS or SC by the ELISA Kit (BD Biosciences). The levels of TNF-α were low, close to the minimum detectable amount (7.8 pg/ml). However, a very low level of immunofluorescence for TNF-α was found in EC9706 cells transfected with miR-19a SC, but increased immunoreactivity was observed in EC9706 cells transfected with miR-19a AS (Fig. 8). Our data indicate that EC9706 cells transfected with miR-19a AS could up-regulate TNF-α mRNA and protein, but that these are non-secretable. Previously reported results have also shown that TNF-α is not detectable in all 6 esophageal cancer cell lines (25). In addition, studies performed on tumor-bearing nude mice using TNFerade Biologic, an adenovector with a radiation-inducible promoter carrying the human TNF-α gene, demonstrated a high, sustained level of TNF-α in the tumor homogenate without detectable levels of TNF-α protein in the plasma (26). Earlier experimental evidence supports the idea that a non-secretable 26-kDa transmembrane, TNF-α Δ1–12 mutant (tm TNF-α), is capable of lysing TNF-α-sensitive target cells (27,28). An interesting possibility is that the soluble TNF receptor fragments could function as ligands for the extracellular portion of the 26-kDa TNF precursor polypeptide spanning the plasma membrane (29). Featuring extracellular, transmembrane and intracytoplasmic regions (28), the 26-kDa TNF precursor structurally resembles a receptor. Notably, the knockdown of miR-19a inhibits cell growth *in vitro*, and reduces tumorigenesis *in vivo*, suggesting that miR-19a is a key oncogenic factor in ESCC. This is supported by a recent finding showing that miR-19 is the main pro-oncogenic miRNA of miR-17-92 (30). Although the mechanism of action of TNF-α in carcinogenesis is still unclear, our antagomir studies show a potential significance at the clinical level. Antagomir-19a treatment may be beneficial in ESCC, with high local doses of TNF-α, also in view of evidence suggesting that systemic antagomir treatment is not coupled with significant toxicity (31).

In conclusion, the miR-17-92 cluster is overexpressed in ESCC and plays a role in cancer cell proliferation. The antisense ONs inhibiting miR-19a effectively elicit apoptosis in esophageal cancer cell lines via the up-regulation of TNF-α, and TNF-α is a novel target of miR-19a. Antagomir-19a can efficiently inhibit tumor growth *in vivo*, thus raising the possibility that these molecules could ultimately be clinically useful in the treatment of cancer.

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References:

1. Bartel DP: MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116: 281-297, 2004.
2. Ambros V: The functions of animal microRNAs. Nature 431: 350-355, 2004.
3. McManus MT: MicroRNAs and cancer. Semin Cancer Biol 13: 253-258, 2003.
4. He L, Thomson JM, Hemann MT, *et al*: A microRNA polycistron as a potential human oncogene. Nature 435: 828-833, 2005.
5. Hayashita Y, Osada H, Tatematsu Y, *et al*: A polycistronic microRNA cluster, miR-17-92, is overexpressed in human lung cancers and enhances cell proliferation. Cancer Res 65: 9628-9632, 2005.
6. Lewis BP, Burge CB and Bartel DP: Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 120: 15-20, 2005.
7. Lewis BP, Shih IH, Jones-Rhoades MW, Bartel DP and Burge CB: Prediction of mammalian microRNA targets. Cell 115: 787-798, 2003.
8. Grimson A, Farh KK, Johnston WK, Garrett-Engele P, Lim LP and Bartel DP: MicroRNA targeting specificity in mammals: determinants beyond seed pairing. Mol Cell 27: 91-105, 2007.
9. Hossain A, Kuo MT and Saunders GF: Mir-17-5p regulates breast cancer cell proliferation by inhibiting translation of AIB1 mRNA. Mol Cell Biol 26: 8191-8201, 2006.
10. Dews M, Homayouni A, Yu D, *et al*: Augmentation of tumor angiogenesis by a Myc-activated microRNA cluster. Nat Genet 38: 1060-1065, 2006.
11. Chen C, Ridzon DA, Broomer AJ, *et al*: Real-time quantification of microRNAs by stem-loop RT-PCR. Nucleic Acids Res 33: e179, 2005.
12. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
13. Gleave ME and Monia BP: Antisense therapy for cancer. Nat Rev Cancer 5: 468-479, 2005.
14. Matsubara H, Takeuchi T, Nishikawa E, *et al*: Apoptosis induction by antisense oligonucleotides against miR-17-5p and miR-20a in lung cancers overexpressing miR-17-92. Oncogene 26: 6099-6105, 2007.
15. Zhou C, Liu S, Zhou X, *et al*: Overexpression of human pituitary tumor transforming gene (hPTTG), is regulated by beta-catenin /TCF pathway in human esophageal squamous cell carcinoma. Int J Cancer 113: 891-898, 2005.

16. Oliver FJ, de la Rubia G, Rolli V, Ruiz-Ruiz MC, de Murcia G and Murcia JM: Importance of poly(ADP-ribose) polymerase and its cleavage in apoptosis. Lesson from an uncleavable mutant. J Biol Chem 273: 33533-33539, 1998.
17. Griffiths-Jones S, Grocock RJ, van Dongen S, Bateman A and Enright AJ: miRBase: microRNA sequences, targets and gene nomenclature. Nucleic Acids Res 34: D140-D144, 2006.
18. Enzinger PC and Mayer RJ: Esophageal cancer. N Engl J Med 349: 2241-2252, 2003.
19. Parkin DM, Bray F, Ferlay J and Pisani P: Global cancer statistics, 2002. CA Cancer J Clin 55: 74-108, 2005.
20. O'Donnell KA, Wentzel EA, Zeller KI, Dang CV and Mendell JT: c-Myc-regulated microRNAs modulate E2F1 expression. Nature 435: 839-843, 2005.
21. Fontana L, Fiori ME, Albini S, *et al*: Antagomir-17-5p abolishes the growth of therapy-resistant neuroblastoma through p21 and BIM. PLoS One 3: e2236, 2008.
22. Mandard AM, Hainaut P and Hollstein M: Genetic steps in the development of squamous cell carcinoma of the esophagus. Mutat Res 462: 335-342, 2000.
23. Mineno J, Okamoto S, Ando T, *et al*: The expression profile of microRNAs in mouse embryos. Nucleic Acids Res 34: 1765-1771, 2006.
24. Krek A, Grun D, Poy MN, *et al*: Combinatorial microRNA target predictions. Nat Genet 37: 495-500, 2005.
25. Oka M, Hirose K, Iizuka N, *et al*: Cytokine mRNA expression patterns in human esophageal cancer cell lines. J Interferon Cytokine Res 15: 1005-1009, 1995.
26. Rasmussen H, Rasmussen C, Lempicki M, *et al*: TNFerade Biologic: preclinical toxicology of a novel adenovector with a radiation-inducible promoter, carrying the human tumor necrosis factor alpha gene. Cancer Gene Ther 9: 951-957, 2002.
27. Perez C, Albert I, DeFay K, Zachariades N, Gooding L and Kriegler M: A nonsecretable cell surface mutant of tumor necrosis factor (TNF) kills by cell-to-cell contact. Cell 63: 251-258, 1990.
28. Kriegler M, Perez C, DeFay K, Albert I and Lu SD: A novel form of TNF/cachectin is a cell surface cytotoxic transmembrane protein: ramifications for the complex physiology of TNF. Cell 53: 45-53, 1988.
29. Vilcek J and Lee TH: Tumor necrosis factor. New insights into the molecular mechanisms of its multiple actions. J Biol Chem 266: 7313-7316, 1991.
30. Olive V, Bennett MJ, Walker JC, *et al*: miR-19 is a key oncogenic component of mir-17-92. Genes Dev 23: 2839-2849, 2009.
31. Krutzfeldt J, Rajewsky N, Braich R, *et al*: Silencing of microRNAs in vivo with 'antagomirs'. Nature 438: 685-689, 2005.