MiR-21 Indicates Poor Prognosis in Tongue Squamous Cell Carcinomas as an Apoptosis Inhibitor

Jinsong Li,[1] Hongzhang Huang,[1] Lijuan Sun,[2] Mei Yang,[2] Chaobin Pan,[1] Weiliang Chen,[1] Donghui Wu,[1] Zhaoyu Lin,[1] Chunxian Zeng,[3] Yandan Yao,[2] Peter Zhang,[4] and Erwei Song[2,3]

Abstract

Purpose: We aim to examine miR-21 expression in tongue squamous cell carcinomas (TSCC) and correlate it with patient clinical status, and to investigate its contribution to TSCC cell growth, apoptosis, and tumorigenesis.

Experimental Design: MicroRNA profiling was done in 10 cases of TSCC with microarray. MiR-21 overexpression was quantitated with quantitative reverse transcription-PCR in 103 patients, and correlated to the pathoclinical status of the patients. Immunohistochemistry was used to examine the expression of *TPM1* and *PTEN*, and terminal deoxynucleotidyl transferase–mediated dUTP labeling to evaluate apoptosis. Moreover, miR-21 antisense oligonucleotide (ASO) was transfected in SCC-15 and CAL27 cell lines, and tumor cell growth was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, adherent colony formation, and soft agar assay, whereas apoptosis was determined by Annexin V assay, cytochrome *c* release, and caspase 3 assay. Tumorigenesis was evaluated by xenografting SCC-15 cells in nude mice.

Results: MiR-21 is overexpressed in TSCC relative to adjacent normal tissues. The level of miR-21 is reversely correlated with *TPM1* and *PTEN* expression and apoptosis of cancer cells. Multivariate analysis showed that miR-21 expression is an independent prognostic factor indicating poor survival. Inhibiting miR-21 with ASO in TSCC cell lines reduces survival and anchorage-independent growth, and induces apoptosis in TSCC cell lines. Simultaneous silencing of *TPM1* with siRNA only partially recapitulates the effect of miR-21 ASO. Furthermore, repeated injection of miR-21 ASO suppresses tumor formation in nude mice by reducing cell proliferation and inducing apoptosis.

Conclusions: miR-21 is an independent prognostic indicator for TSCC, and may play a role in TSCC development by inhibiting cancer cell apoptosis partly via *TPM1* silencing.

Authors' Affiliations: [1]Oral and Maxillofacial Surgery, and [2]Breast Tumor Center, No. 2 Affiliated Hospital and [3]Key Laboratory of Gene Engineering of the Ministry of Education, State Key Laboratory of Biocontrol, School of Life Sciences, Sun Yat-sen University, Guangzhou, People's Republic of China, and [4]Shanghai GenePharma Co., Ltd., Shanghai, People's Republic of China

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Requests for reprints: Erwei Song, Breast Tumor Center, No. 2 Affiliated Hospital, Sun Yat-sen University, 107 Yanjiang West Road, Guangzhou 510120, P.R. China. Phone: 86-20-8133-2783; Fax: 86-20-8133-2853; E-mail: songew@mail.sysu.edu.cn or Hongzhang Huang, Department of Oral and Maxillofacial Surgery, No. 2 Affiliated Hospital, Sun Yat-sen University, 107 Yanjiang West Road, Guangzhou 510120, P.R. China. Phone: 86-20-8133-2220; Fax: 86-20-8133-2833; E-mail: huanghongzhang@tom.com.

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Squamous cell carcinoma (SCC) of the oral cavity represents the sixth most frequent solid cancer around the world (1), and tongue carcinoma is the most common type of oral cancers (2). Similar to other malignancies, apoptosis of tumor cells significantly influences the trend of progression and remission in tongue cancers. Reduced activity of caspase-3 and caspase-8 and reduced apoptotic cancer cell number were associated with poor prognosis of tongue carcinomas (3, 4). Therefore, it is important to investigate the molecular mechanisms that govern apoptosis in tongue cancer cells, especially the antiapoptotic factors.

MicroRNAs (miRNA) are a class of small noncoding RNAs of ~22 nucleotides in size that are endogenously expressed in mammalian cells. They regulate gene expression by repressing mRNA translation or cleaving target mRNA (5, 6). As a new family of gene regulators, miRNAs are involved in modulating multiple cellular pathways, including cell proliferation (7, 8), differentiation (9–11), and apoptosis (12–14), and thus may function as oncogenes or tumor-suppressing genes (15). In tongue squamous cell carcinomas (TSCC), miR-184 is overexpressed and acts as an "oncogene" (16). Antagonizing the function of miR-184 with a specific antisense oligonucleotide (ASO) inhibits proliferation and enhances the apoptosis of TSCC cells (16).

Translational Relevance

Our study shows that miR-21 is overexpressed in tongue squamous cell carcinomas (TSCC) relative to normal adjacent tissues, and the level of miR-21 in tumor tissues is an independent covariate indicating poor survival for patients with TSCC. These findings imply that examination of miR-21 expression may be used as a diagnostic method to predict prognosis for patients with TSCC in future clinical practice. Furthermore, our data of TSCC cell lines show that antagonizing miR-21 with a specific antisense oligonucleotide (ASO) effectively inhibits anchorage-independent growth, induces apoptosis, and suppresses tumor formation of TSCC xenografts implanted in immunocompromised mice. These results suggest that miR-21 ASO may hold great promise for the development of a novel therapeutic strategy for TSCC by targeting miR-21. Further studies are needed to investigate efficient methods to deliver miR-21 ASO *in vivo* for therapeutic application.

In addition to miR-184, tumor-associated miRNAs that regulate the biological features of cancer cells and contribute to tumor development are reported in various carcinomas. Among them, miR-15 and miR-16 genes located at chromosome 13q14 are deleted in 65% of patients with B-cell chronic lymphocytic leukemia, and are related to reduced apoptosis of leukemic cells (17). Furthermore, our previous study showed that tumor-suppressing let-7 miRNA reduces the self-renewal of breast tumor-initiating cells and promotes differentiation by silencing H-RAS or HMGA2, respectively (18). On the other hand, miR-21 is a well recognized oncogenic miRNA that is overexpressed in various carcinomas, including glioblastomas (13), hepatocellular cancers (19), and breast cancers (20). Antagonizing miR-21 with a specific ASO inhibits breast cancer cell growth *in vitro* and *in vivo* by reducing proliferation and inducing apoptosis, suggesting that the miRNA is a putative molecular target for designing novel anticancer therapies.

MiRNA exerts its function by binding to the 3′-untranslated region of target genes through partial sequence homology, and a single miRNA may have multiple targets. Previous studies have shown that miR-21 binds to the 3′-untranslated region of the tropomyosin 1 (*TPM1*) gene and phosphatase tensin homologue (*PTEN*) gene (19, 21), and inhibits their expression. TPM1 is an actin-binding, microfilament-associated protein, which is consistently suppressed in various carcinomas such as breast cancers (22, 23) and urinary bladder cancers (24). Restoring TPM1 expression in breast cancer cells suppresses tumor growth by inhibiting anchorage-independent growth and inducing apoptosis via caspase-dependent pathways with cytochrome *c* release.

However, the expression of miR-21 and its target gene *TPM1* has not been reported in tongue carcinomas. Whether miR-21 contributes to the antiapoptotic mechanism of tongue cancers and whether its antagonism could be used as a potential treatment for TSCCs remains illusive. In this study, we first aimed to explore the miRNA profile and examine miR-21 expression in tongue carcinomas. Next, we correlated miR-21 expression with the clinical status and prognosis of TSCC patients. *In vitro*, we transfected miR-21 ASO in TSCC cell lines, and investigated the expression of *TPM1*, its target gene, as well as its contribution to tumor cell growth and apoptosis. Finally, we evaluated the role of miR-21 ASO in tumor formation in immunocompromised mice inoculated subcutaneously with TSCC cells.

Materials and Methods

Patients and tissue samples. Pairs of primary tongue carcinomas locating at the anterior (body) of the tongue and adjacent normal tissues were obtained from 103 patients (Supplementary Table S1), who were admitted to the Department of Oral and Maxillofacial Surgery of the No. 2 Affiliated Hospital, Sun Yat-sen University, from January 2002 to January 2008. All the patients recruited into the present study did not receive radiotherapy or chemotherapy or any other treatment before and after operation. Surgical specimens of the resected tumors were collected, and lumps of tumors as well as adjacent normal tissues, which were at least 2 cm distal to tumor margins, were snap-frozen in liquid nitrogen for miRNA assay. In addition, the remaining tissues were embedded for histology and immunohistochemistry studies. All samples were collected with informed consent according to the internal review and ethics boards of the hospital.

MiRNA microarray analysis. MiRNA microarray analysis was done in 10 paired samples of TSCC versus matched normal tongue tissues, which were chosen from the above patients with properly matched age and sex (Supplementary Table S2). Briefly, total RNA enriched with small RNA was isolated using a mirVana RNA Isolation Kit (Ambion). miRNA was further recovered through fractionating with PAGE. A polynucleotide tail was appended to the end of the miRNAs under poly(A) polymerase reaction with a mixture of unmodified and amine-modified nucleotides (Ambion). The tailed samples were fluorescently labeled using an amine-reactive Cy3 dye (Amersham), and the unincorporated dyes were removed with a glass fiber filter–based cleaning procedure. The samples were hybridized for 14 h on slides arrayed with miRNA probes from the NCode miRNA Microarray Probe Set (Invitrogen), and were then scanned using a Generation III array scanner (Amersham Pharmacia Biotech). The signal intensity of each element was analyzed using ArrayVision (Imaging Research). A heat map demonstrating the average levels of miRNAs, which are differentially expressed in tumors versus adjacent normal tissues, was created with DMVS 2.0 software (Chipscreen Biosciences). The raw data of these miRNA arrays have been deposited into ArrayExpress,[5] with accession number A-MEXP-1534.

Quantitative reverse transcription-PCR. Real-time reverse transcription PCR for miR-21 was done using Real-time PCR Universal Reagent (GenePharma Co., Ltd.) and MX-3000P Real-time PCR machine (Stratagen). All reactions were done in a 20 μL reaction volume in triplicate. Primers for miR-21 and U6 snRNA are miR-21 (forward, GGACTAGCTTATCAGACTG; reverse, CATCAGATGCGTTGCGTA) and U6-snRNA (forward, ATTGGAACGATACAGAGAAGAT; reverse, GGAACGCTTCACGAATTT). Probes for miR-21 and U6 snRNA are FAM-TGGCCCCTGCGCAAGGATG-DABCYL and FAM-CGCACCGCGTCAACATCAGGGTGCG-DABCYL, respectively. PCR amplification consisted of an initial denaturation step at 95°C for 3 min, followed by 40 cycles of PCR at 95°C for 15 s, 50°C for 30 s, and 72°C for 30 s. Standard curves were generated and the relative amount of miR-21 was normalized to U6 snRNA ($2^{-\Delta Ct}$). MiR-21 expression fold change of TSCC sample to matched normal sample was evaluated using $2^{-\Delta\Delta Ct}$.

Northern blot. For Northern blotting (25, 26), 10 mg of RNA was separated on a 15% denaturing polyacrylamide gel, and transferred to a positively charged membrane (Perkin-Elmer) using the Trans-Blot Semi-Dry Electrophoretic Transfer Cell (Bio-Rad), followed by

[5] http://www.ebi.ac.uk/arrayexpress/

immobilization via UV cross-linking and baking at 80°C under vacuum. The membranes were prehybridized and then hybridized at 37°C for 24 h to a c-^{32}P–labeled probe for miR-21 (5′-TCAACATCAGTCTGATAAGCTA-3′) or for U6 snRNA (5′-AACGCTTCACGAATTTGCGT-3′).

Immunohistochemistry. For immunohistochemistry (27), mouse anti-TPM1 monoclonal antibody (Abcam) or rabbit anti-PTEN polyclonal antibody (Cell Signaling Technology) were used as primary antibodies for overnight incubation at 4°C. The sections were subsequently treated with goat anti-mouse secondary antibody, followed by further incubation with streptavidin-horseradish peroxidase complex. Diaminobenzidine (Dako) was used as a chromogen and sections were lightly counterstained with hematoxylin. The proportion of TPM1 and PTEN immunostaining tumor cells varied from 0% to 100%, and a four-grade scoring system was used to evaluate the degree of immunostaining: score 0, < 5%; score 1, 5% to 25%; score 2, 25% to 50%; score 3, >50% of tumor cells with positive immunostaining.

Terminal deoxynucleotidyl transferase–mediated dUTP labeling. Terminal deoxynucleotidyl transferase–mediated dUTP labeling (TUNEL) assay was done using an *In situ* Apoptosis Detection Kit (R&D Systems). Briefly, after digesting with Protease K, TdT reaction mix was applied to the cells for incubation at 37°C for 60 min, followed by incubation with streptavidin horseradish peroxidase for 10 min. The final reaction of the product was visualized by 3,3′-diaminobenzidine. Approximately 1,000 tumor cells were counted (200×) in each section, and apoptotic index was expressed as the percentage of TUNEL-positive tumor cells.

Cell Cultures. Human tongue cancer cell lines (SCC-15 and CAL27), a human lung fibroblast cell line (WI-38), and a human embryonic kidney cell line (HEK-293) were purchased from American Type Culture Collection and maintained in DMEM (Life Technologies) supplemented with 10% fetal bovine serum (Invitrogen).

Transfection with miR-21 ASO and TPM1 siRNA. All miRNA ASOs and siRNAs were obtained from GenePharma, and their sequences were shown in Supplementary Table S3. SCC-15 and CAL27 cells were transfected with 30 pmol of miR-21 ASO or 30 pmol of lin-4 ASO (irrelevant control) alone, or 30 pmol of miR-21 ASO plus 30 pmol TPM1 siRNA1/siRNA2 or plus GFP siRNA, respectively, using LipofectAMINE 2000 (Invitrogen). After overnight incubation, the media were replaced with DMEM containing 10% fetal bovine serum before further study.

Luciferase reporter assay. To evaluate the function of miR-21 (18), cells were transfected with a pMIR-REPORT luciferase reporter vector with a miR-21 target sequence cloned into its 3′-untranslated region (Ambion) using LipofectAMINE 2000. To correct the transfection efficiency, a luciferase reporter vector without miR-21 target was transfected in parallel. Luciferase activities in the cells were assayed using a luciferase assay kit (Promega), and miR-21 function was expressed as relative luciferase activity of the reporter vector with miR-21 target sequence over the one without miR-21 target sequence.

Western blotting. Protein extracts were resolved through 12% SDS-PAGE, transferred to polyvinylidene difluoride membranes (Amersham Pharmacia Biotech), probed with a rabbit polyclonal antibody against human TPM1 (Upstate) or β-actin, and then with peroxidase-conjugated goat anti-rabbit immunoglobulin (Oncogene Research Product) as secondary antibody, and then visualized by chemiluminescence (Cell Signaling Technology). WI-38 fibroblasts, from which TPM1 gene was cloned (28), was used as a positive control.

MTT assay. To monitor cell survival, TSCC cells were incubated for 4 h with 0.5 mg/mL of MTT (Sigma), and resuspended in 100 μL of DMSO (Sigma). Absorbance (A) was recorded at 570 nm using an Easy Reader 340 AT (SLT-Lab Instruments). Cell viability was expressed as a fraction of the untransfected control. HEK293 cell line with low miR-21 expression (29) was used as a control to confirm the specificity of miR-21 ASO to cells with high miR-21 expression.

Colony formation assay. To evaluate anchorage-dependent growth, cells were seeded at a density of 400 cells per well in flat-bottomed six-well culture plates, and cultured until colonies were visible. The percentage of cells that formed into a clone was calculated.

Soft agar assay. To determine anchorage-independent growth (21), cells were resuspended in medium supplemented with agar at a final concentration of 0.35%, and layered with DMEM supplemented with 0.6% agar. The percentage of cells that formed spherical clones was calculated.

Annexin V analysis. To evaluate TSCC apoptosis, cell pellets were resuspended in Annexin V-FLUOS staining solution (Roche Molecular Biochemicals) and incubated for 15 min at room temperature. Samples were then analyzed on a FSCAN flow cytometer (Hershey Medical Center Core Facility). As a positive control, 5-fluorocytosine was added to the cultures at 4×10^{-2} mmol/L.

***Cytochrome c* release.** To visualize cytochrome *c* release (3), cells were transferred onto slides and fixed for 10 min with 3.7% paraformaldehyde. Following permeabilization with 0.05% saponin in PBS for 5 min and ice-cold acetone for 10 min, the cells were then incubated with a mouse anti-human cytochrome *c* antibody (1:50; Roche Molecular Biochemicals), and then with a fluorescein-conjugated goat anti-mouse IgG (1:50; Molecular Probes). Cytochrome *c* release was visualized under immunofluorescent microscope. Cy3-labeled anti-human actin antibody (Roche Molecular Biochemicals) was used to counterstain actin in cytoplasm and 4′,6-diamidino-2-phenylindole (Roche Molecular Biochemicals) for nuclei.

Caspase-3 assay. Caspase-3 activity was determined using the Caspase-Glo 3/7 kit (Promega). Briefly, cells were seeded onto 96-well plates and incubated with culture medium supplemented with Caspase-Glo 3/7 reagent at a 1:1 ratio at room temperature for 2 h. Cell lysates were then transferred to a white-walled 96-well plate. Luminescence was measured using a microplate luminometer (Perkin-Elmer). Data were expressed as OD value normalized to untransfected control.

Tumor xenografts. To evaluate *in vivo* tumorigenesis, TSCC xenografting mouse model was used. Male BALB/c-nu mice of 4 to 6 weeks old were prepared for tumor implantation. All animals were maintained in a sterile environment on a daily 12-h light/12-h dark cycle. After resuspension in PBS, SCC-15 cells (5×10^6/mouse) were injected subcutaneously into the flanks of the nude mice. One week after implantation when the tumor became palpable at the size of ~2 mm in diameter, intratumor injection with 50 μg of miR-21 ASO dissolved in 100 μL of DMEM mixed with 3 μL of LipofectAMINE 2000 (Invitrogen) was done twice a week. Tumor volume was calculated weekly for 4 weeks according to the formula, TV (mm^3) = length × width^2 × 0.5. Tumor xenografts were harvested, weighted, and snap-frozen. Cryosections (4 μm) were stained with H&E and used for TUNEL assay as well as immunohistochemistry for TPM1 and proliferating cell nuclear antigen expression.

Statistics. All statistical analyses were carried out using SPSS for Windows version 13.0 (SPSS). Student's *t* test and one-way ANOVA were used to analyze the relationship between miR-21 expression or apoptotic index and clinicopathologic characteristics. χ^2 test was applied to analyze the relationship between TPM1 expression and clinicopathologic features. The difference in TPM1 expression between TSCC and matched normal tissue was examined by Wilcoxon signed rank tests. To measure the association between pairs of variables, Spearman order correlations were run. Kaplan-Meier survival curves were plotted and log rank test was done. The significance of various variables for survival was analyzed by Cox proportional hazards model in a multivariate analysis. All experiments for cell cultures were done at least in triplicate. Results were expressed as mean ± SD. $P < 0.05$ in all cases was considered statistically significant.

Results

MiR-21 is overexpressed in TSCC versus normal tongue tissues. MiRNA microarray was done in 10 paired samples of TSCC versus matched normal tongue tissues to analyze differential expression of mature miRNAs (Fig. 1A). A marked difference in expression was observed in 71 miRNAs between TSCCs and normal tongue tissues. Using 2-fold expression difference

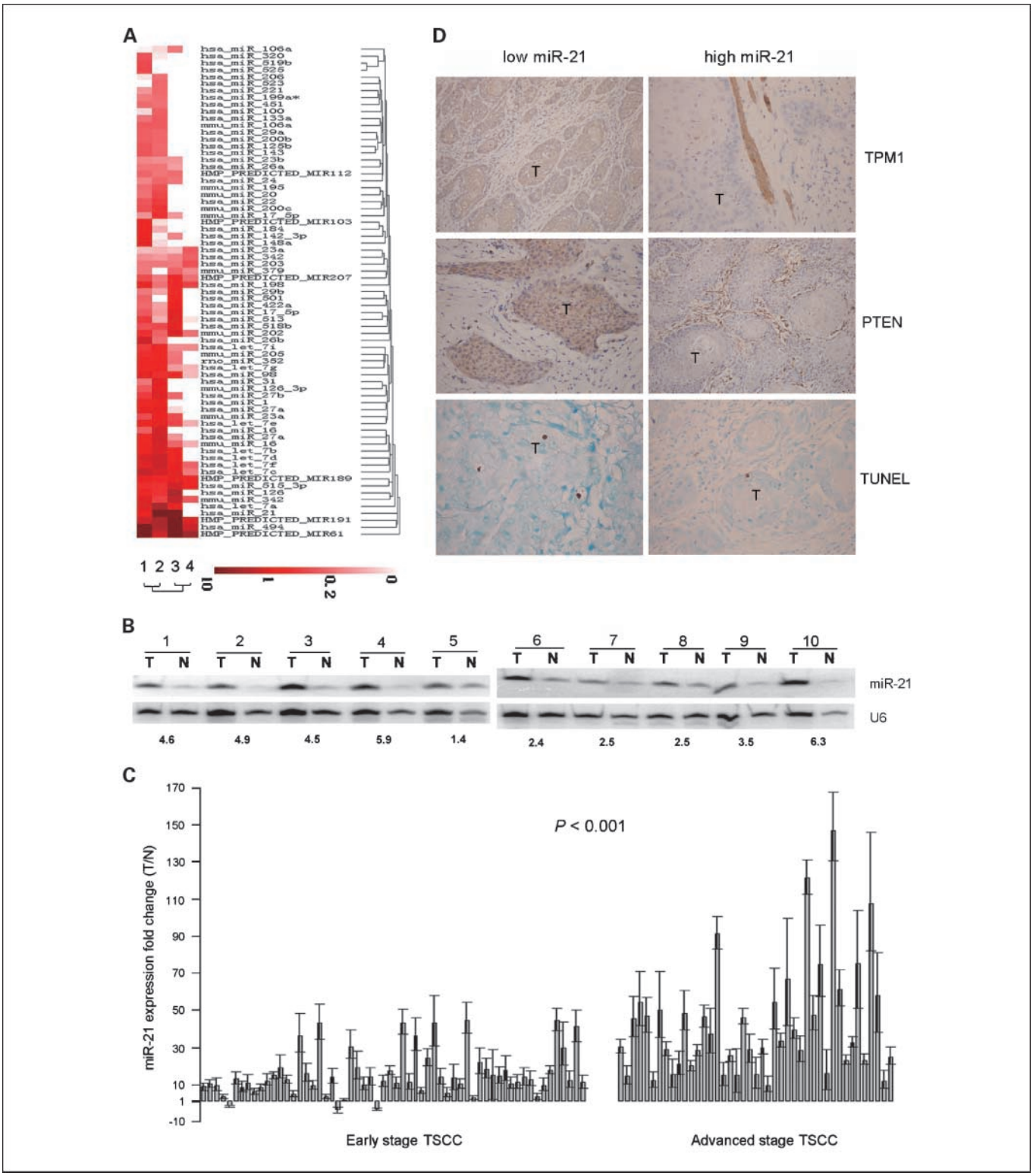

Fig. 1. MiR-21 is up-regulated in TSCC. *A,* miRNA microarray analysis shows 71 miRNAs differentially expressed in TSCC and matched normal tongue tissue. *1,* adjacent normal tissue of early-stage TSCC; *2,* early-stage TSCC; *3,* advanced-stage TSCC; *4,* adjacent tissue of advanced-stage TSCC. *B,* Northern blot with a specific miR-21 probe confirms miR-21 overexpression in TSCC. Numbers marked on top (*1-10*) represent the number of each specimen. Numbers marked at the bottom (*4.6, 4.9,* etc.) are the miR-21 expression ratios of tumor to matched adjacent normal tissue. *T,* tumor; *N,* normal tissue. Early-stage TSCCs include specimen nos. 5, 6, 7, and 8, and advanced-stage TSCCs include specimen nos. 1, 2, 3, 4, 9, and 10. *C,* miR-21 is overexpressed in TSCC verified by quantitative reverse transcription-PCR. All the patients were divided into two groups according to clinical staging. Columns, miR-21 expression ratio of each tumor specimen to matched adjacent normal tissue; bars, SD from triplicate assays. The mean miR-21 level of early-stage TSCC is 17.73, whereas that of advanced-stage TSCC is 39.51 ($P < 0.001$). *D,* TPM1 (immunohistochemical staining, ×200), PTEN expression (immunohistochemical staining, ×200), and apoptosis (TUNEL, ×200) in TSCC are reversely associated with miR-21 expression. Lower miR-21 (*left*) correlates with higher TPM1, PTEN expression, and apoptotic index (*brown*). T, areas with tumors.

as a cutoff level, we identified 10 up-regulated miRNAs and 15 down-regulated ones in four cases of early TSCC (Supplementary Table S4), whereas 26 up-regulated miRNAs and 2 down-regulated ones in the other six cases of advanced tumors (Supplementary Table S5). Among them, miR-21 was the most significantly overexpressed miRNA, which was 8.29-fold elevated in early TSCC and 37.89-fold in advanced tumors. Furthermore, miR-21 overexpression was validated by Northern blotting using a miR-21–specific probe (Fig. 1B). In agreement with the microarray data, miR-21 expression is higher in the advanced TSCCs than the early ones (Fig. 1B; Supplementary Table S2).

To further confirm miR-21 overexpression in TSCCs, we quantified its expression in tumor samples from 103 TSCC patients by quantitative reverse transcription-PCR (Fig. 1C). miR-21 expression in advanced TSCCs is much higher than that in the early ones (Table 1; $P < 0.001$). With relatively stable miRNA expression in the adjacent normal tongue tissues, miR-21 is ~39.51-fold higher in advanced tumors as compared with normal tissues, whereas that in early tumors is 17.73-fold higher (Table 1). These data suggest that miR-21 up-regulation is correlated with tumor staging and may play a role in the progression of TSCC.

Because tumor-suppressing genes, TPM1 and PTEN, were shown as targets of miR-21 (19, 21), we examined protein expression of the above genes in paraffin sections of TSCC samples using immunohistochemistry. TPM1 and PTEN immunostaining is significantly stronger in normal tongue tissues adjacent to the tumors compared with the corresponding cancer areas (Wilcoxon signed ranks test, $P < 0.001$). In addition, staining of TPM1 and PTEN in TSCC cancer tissues is reversely correlated with miR-21 levels (Fig. 1D; Supplementary Fig. S1A, $r_s = -0.679$; $P < 0.001$). These observations support previous findings that *TPM1* and *PTEN* are target genes silenced by miR-21.

It has been shown that TPM1 confers tumor suppression by inducing apoptosis in cancer cells. Therefore, we further evaluated apoptosis of cancer cells in TSCC by *in situ* TUNEL staining (Fig. 1D), and correlated it with TPM1 and miR-21 expression. To no surprise, the percentage of cancer cell apoptosis was reversely associated with miR-21 levels (Supplementary Fig. S1B; $r_s = -0.629$, $P < 0.001$), but in proportion to TPM1 expression (Supplementary Fig. S1C; $r_s = 0.511$, $P < 0.001$). Thus, miR-21 may act as an antiapoptotic factor by blocking TPM1 expression.

MiR-21 acts as an independent prognostic indicator for patient survival. To further evaluate the clinical relevance of miR-21 expression in TSCC, we analyzed its association with clinicopathologic status of the patients (Table 1). No significant correlation was observed between miR-21 expression and age or sex of the patients. However, miR-21 level is closely associated with clinical staging, differentiation, and lymph node metastasis of the patients. Tumors with advanced clinical stages (stage III and IV; $P < 0.001$), with poor differentiation ($P = 0.024$) or with lymph node metastasis ($P < 0.001$) expressed higher levels of miR-21, suggesting that miR-21 is related to cancer progression. On the contrary, TPM1 and PTEN expression are reversely correlated with clinical staging of the tumors ($P = 0.015$ or $P = 0.047$, respectively).

Table 1. Correlation among clinicopathologic status and the expression of miR-21, TPM1 and PTEN, and apoptosis in TSCC patients

Characteristics	miR-21 (T/N expression ratio)			TPM1 (%)			PTEN (%)			AI		
	No.	Mean ± SD	*P*	No. of low expressions	No. of high expressions	*P*	No. of low expressions	No. of high expressions	*P*	No.	Mean ± SD	*P*
Sex			0.426			0.583			0.664			0.540
Male	56	28.65 ± 28.16		34 (60.7)	22 (39.3)		25 (44.6)	31 (55.4)		56	1.26 ± 0.54	
Female	47	24.65 ± 21.28		31 (66.0)	16 (34.0)		31 (66.0)	16 (34.0)		47	1.32 ± 0.51	
Age			0.482			0.275			0.720			0.459
<50	47	28.74 ± 26.73		27 (57.4)	20 (42.6)		21 (44.7)	26 (55.3)		47	1.24 ± 0.54	
≥50	56	25.21 ± 24.00		38 (67.9)	18 (32.1)		27 (48.2)	29 (51.8)		56	1.32 ± 0.51	
T classification			0.000			0.023			0.049			0.002
T_1-T_2	64	18.27 ± 14.18		35 (53.8)	30 (46.2)		25 (39.1)	39 (60.9)		64	1.41 ± 0.53	
T_3-T_4	39	40.86 ± 32.37		29 (76.3)	9 (23.7)		23 (59.0)	16 (41.0)		39	1.09 ± 0.44	
Differentiation			0.024			0.092			0.234			0.038
Well	56	21.79 ± 18.68		30 (53.6)	26 (46.4)		25 (44.6)	31 (55.4)		56	1.37 ± 0.54	
Mediate	27	27.88 ± 25.66		20 (74.1)	7 (25.9)		16 (59.3)	11 (40.7)		27	1.30 ± 0.50	
Poor	20	39.50 ± 35.47		15 (75.0)	5 (25.0)		7 (35.0)	13 (65.0)		20	1.03 ± 0.43	
Clinical stage			0.000			0.015			0.047			0.001
I-II	60	17.73 ± 14.46		32 (53.3)	28 (46.7)		23 (38.3)	37 (61.7)		60	1.42 ± 0.53	
III-IV	43	39.51 ± 31.11		33 (76.7)	10 (23.3)		25 (58.1)	18 (41.9)		43	1.09 ± 0.45	
Node metastasis			0.003			0.126			0.079			0.005
N_0	75	20.63 ± 16.29		44 (58.7)	31 (41.3)		31 (41.3)	44 (58.7)		75	1.37 ± 0.52	
N_1-N_2	28	43.42 ± 35.83		21 (75.0)	7 (25.0)		17 (60.7)	11 (39.3)		28	1.05 ± 0.45	
Status			0.000			0.009			0.005			0.002
Survival	56	18.41 ± 15.62		29 (51.8)	27 (48.2)		19 (33.9)	37 (66.1)		56	1.43 ± 0.54	
Death	47	36.85 ± 30.85		36 (76.6)	11 (23.4)		29 (61.7)	18 (38.3)		47	1.12 ± 0.45	

Abbreviations: T, tumor; N, adjacent normal tissue. AI, apoptotic index, percentage of apoptotic cancer cells. High expression, ≥25% of cancer cells with positive staining; low expression, < 25% of cancer cells with positive staining.

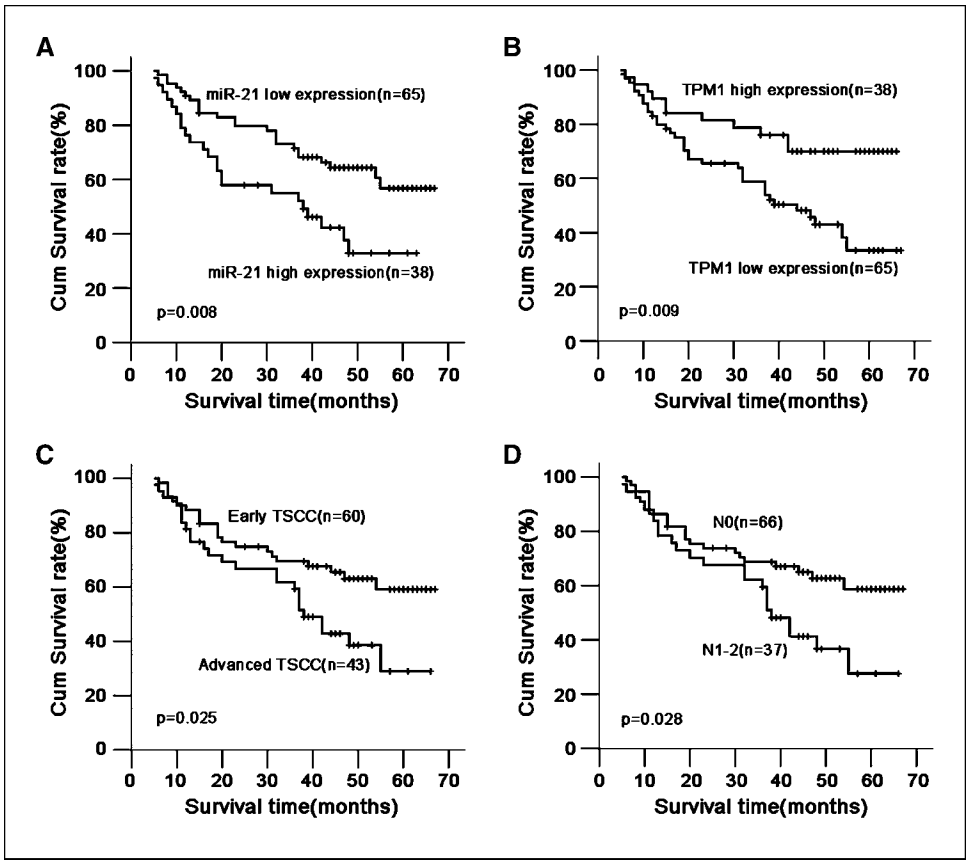

Fig. 2. Kaplan-Meier survival curves for TSCC patients plotted on miR-21, TPM1 expression, clinical staging, and lymph node metastasis. Kaplan-Meier curves with log rank tests show statistical difference in survival between patients with high (fold change > 26.82) and low miR-21 expression (fold change < 26.82; $P = 0.008$; *A*), with high (positive proportion ≥ 25%) and low TPM1 expression (positive proportion < 25%; $P = 0.009$; *B*), with early and advanced clinical staging ($P = 0.025$; *C*) and with (*N1-N2*) and without (*N0*) lymph node metastasis ($P = 0.028$; *D*).

Furthermore, we took one step forward to analyze the correlation of miR-21 expression and postoperative survival of the patients, who were divided into high (fold change > 26.82) and low (fold change < 26.82) miR-21 expression, according to the mean fold change of the miRNA. Patients with low miR-21 expression survived significantly longer than those with high miR-21 expression ($P = 0.008$; Fig. 2A). The cumulative survival rate up to 67 months was 63.1% (41 of 65) in patients with low miR-21 expression, whereas it was only 39.5% (15 of 38) in those with high miR-21 expression. In contrast, higher TPM1 immunostaining prognosed better survival ($P = 0.009$; Fig. 2B), and the cumulative survival rate is 71.1% (27 of 38) in patients with high TPM1 expression, whereas it was 44.6% (29 of 65) in those with low TPM1. Patient survival was also associated with clinical staging ($P = 0.025$; Fig. 2C) and lymph node metastasis ($P = 0.028$; Fig. 2D), but the correlation was not as notable as the one with miR-21 expression.

To identify whether miR-21 is an independent prognostic covariate for TSCC, we did a multivariate Cox proportional hazards analysis (Supplementary Table S6). In the final multivariate Cox regression model, miR-21 expression in TSCC is associated with poor survival prognosis (hazard ratio, 1.027; $P < 0.001$), independent of other clinical covariates, suggesting that miR-21 can be used as an independent prognostic factor for TSCC.

MiR-21 expression and function are enhanced in TSCC cell lines. To investigate the machinery by which miR-21 contributes to TSCC development, we used cultures of TSCC cell lines, SCC-15 and CAL27, and verified miR-21 expression in these cells by real-time reverse transcription-PCR. In agreement with our clinical data, miR-21 is up-regulated by 14-fold in SCC-15 and CAL27 cells relative to the normal tongue tissue ($P < 0.05$; Fig. 3A). Transfection with miR-21 ASO, but not lin4 ASO, specifically knocked down miRNA expression ($P < 0.05$).

In addition, the targeting function of miR-21 in TSCC cells was evaluated using luciferase reporter assay. The luciferase activity in SCC-15 and CAL27 cells transfected with the reporter plasmid vector carrying the miR-21 target sequence is ~47% and 43%, respectively, of those transfected with the control vector (Fig. 3B), demonstrating the specific targeting function of endogenous miR-21 in TSCC cells. However, cotransfection with miR-21 ASO, but not with lin4 ASO, significantly enhances the luciferase activity in SCC-15 cells ($P = 0.002$; Fig. 3B) and CAL27 cells ($P = 0.003$; Fig. 3B).

Furthermore, the protein expression of TPM1, a miR-21 target gene, in SCC-15 and CAL27 cells was identified by Western blotting (Fig. 3C). TPM1 expression in tongue cancer SCC-15 and CAL27 cells is much lower than that in the control WI-38 cells. In addition, transfection with miR-21 ASO, but not with the nonrelevant lin4 ASO, dramatically increased TPM1 expression, suggesting that TPM1 silencing in TSCC cells is possibly mediated by miR-21.

MiR-21ASO reduces anchorage-independent cancer cell growth and induces apoptosis. Because our clinical data suggested that miR-21 is related to the progression of TSCC, we further evaluated whether miR-21 contributes to TSCC cell survival by blocking its expression with a specific ASO. TSCC cell viability

determined by MTT was reduced significantly in SCC-15 and CAL27 cells upon transfection with miR-21 ASO (both $P < 0.05$), but not with the unrelated lin4 ASO (Fig. 4A). However, transfection with miR-21 ASO does not affect the viability of HEK-293 cells (Fig. 4A), which were known to be lacking in miR-21 expression (29), excluding the possibility of a general cytotoxic effect of the reagent.

We next evaluated the effect of miR-21 on anchorage-independent and anchorage-dependent cell growth using colony formation assays in soft agar and adherent cultures, respectively. In contrast to adherent cell growth, which was slightly reduced by miR-21 ASO (Supplementary Fig. S2), the anchorage-independent growth of either SCC-15 or CAL27 cells determined by soft agar assay were markedly reduced by miR-21 ASO, but not the control lin4 ASO ($P < 0.01$; Fig. 4B). In agreement with the MTT data, miR-21 does not affect either anchorage-independent or anchorage-dependent growth of the HEK-293 cells which lack miR-21 expression.

To further investigate whether miR-21 ASO inhibits the expression of TSCC cell growth by indirectly up-regulating the miR-21 target gene, TPM1, we used RNA interference targeting TPM1 genes. Transfection of WI-38 cells with either of the two siRNAs against the TPM1 gene, but not with a control GFP siRNA, effectively reduced its mRNA (Supplementary Fig. S3A) and protein (Supplementary Fig. S3B) expression as determined by quantitative reverse transcription-PCR and Western blotting, respectively. Transfection with TPM1-siRNA alone in SCC-15 cells, which lacks TPM1 expression (Fig. 3C), does not influence cell survival ($P > 0.05$; Supplementary Fig. S4A), and anchorage-independent ($P > 0.05$; Supplementary Fig. S4B) or anchorage-dependent growth ($P > 0.05$; Supplementary Fig. S4C). However, cotransfecting SCC-15 cells with miR-21 ASO and either of the TPM1 siRNAs partially reversed the inhibitory effect of ASO on cell survival as well as colony formation in soft agar cultures ($P < 0.05$; Supplementary Fig. S4B). Moreover, cotransfection with either TPM1 siRNA also slightly relieved the inhibitory effect of miR-21 ASO on adherent colony formation of SCC-15 cells, but did not reach statistical significance. These data indicate that miR-21 might stimulate anchorage-independent cell growth of TSCC via silencing TPM1.

To further investigate whether the miR-21 antagonism inhibits TSCC development by inducing apoptosis, we used Annexin V staining, cytochrome *c* release, and caspase-3 assay. Transfection with miR-21 ASO, but not with lin4 ASO, increased the percentage of Annexin V–positive cells by 27-fold in the SCC-15 line, and by 17-fold in the CAL27 line (Fig. 4C). Similarly, SCC-15 or CAL27 cells transfected with miR-21 ASO, but not lin4 ASO, displayed cytochrome *c* release in cytosol (Fig. 4D), suggesting that miR-21 may inhibit cancer cell death via stabilizing mitochondria potential, whereas blockade of miR-21 leads to apoptosis by inducing cytochrome *c* release from the mitochondria into the cytosol. To further confirm that apoptosis was induced via a caspase-dependent pathway, we detected caspase-3 activity. As shown in Supplementary Fig. S5, transfection with miR-21 ASO, but not with lin4 ASO, significantly increased caspase-3 activities in either SCC-15 or CAL27 cells ($P < 0.01$), but not in HEK-293 cells that lack miR-21 expression. These data further showed that the apoptosis induction effect mediated by miR-21 ASO is via a caspase-dependent pathway and is specific to miR-21–expressing cancer cells.

MiR-21 ASO inhibits tumorigenesis of TSCC xenografts. Because miR-21 ASO inhibits TSCC cell growth *in vitro*, we further assessed its effect on tumor formation *in vivo*. In nude mice xenografted with SCC-15 cells, biweekly intratumor injection with miR-21 ASO, but not with lin4 ASO or with LipofectAMINE alone, tremendously reduced tumor size (Fig. 5A and C) and tumor weight ($P < 0.05$; Fig. 5B). Although the tissue structure and cell morphology of SCC-15 xenografts treated with miR-21 ASO were not different from those treated with lin4 ASO or LipofectAMINE alone, injection with miR-21 ASO significantly reduced the percentage of tumor cells expressing the proliferating cell–associated antigen (Fig. 5D; Supplementary Fig. S6A), suggesting that miR-21 ASO retarded tumor growth partly by inhibiting the proliferation of cancer cells. Moreover, injection of miR-21 ASO enhanced the expression of tumor-suppressing TPM1 expression determined by immunohistochemical staining

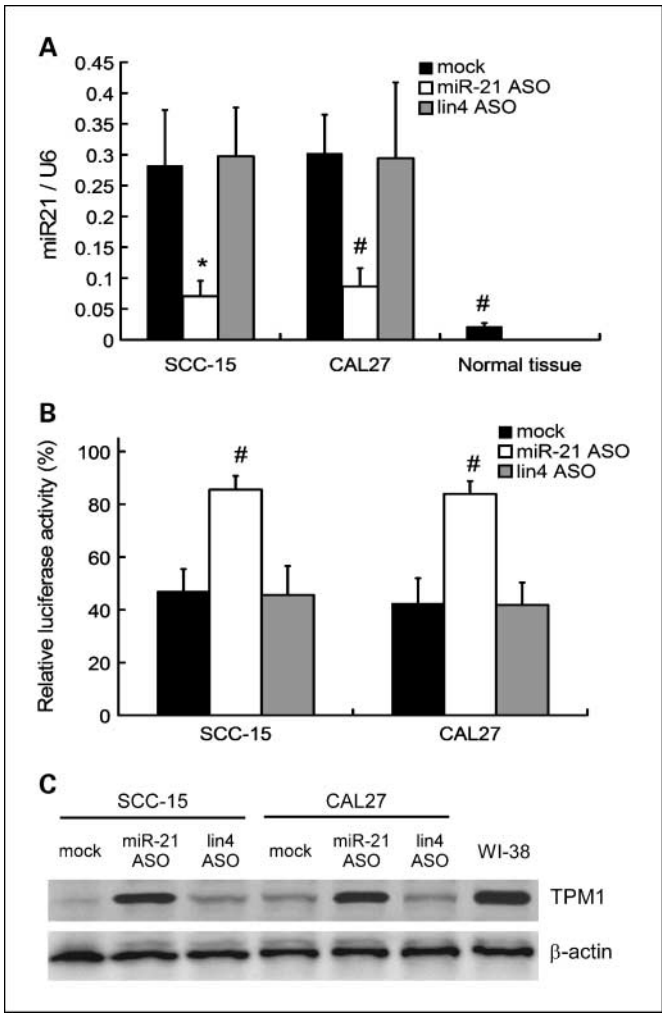

Fig. 3. MiR-21 ASO specifically inhibits the expression and function of miR-21 in TSCC cell lines. *A,* miR-21 expression is quantitated by real-time reverse transcription-PCR in normal tongue tissue, and in SCC-15 and CAL27 cells that were mock-transfected or transfected with miR-21 ASO and lin4 ASO. The expression of miR-21 was normalized to that of U6 snRNA. *B,* luciferase assay with reporter vector carrying miR-21 target sequence indicates targeting function of miR-21 in SCC-15 and CAL27 cells (*, $P < 0.05$; #, $P < 0.01$ as compared with cells with mock transfection). *C,* TPM1 expression in SCC-15 and CAL27 cells that were mock-transfected, or transfected with miR-21 or lin4 ASO was examined by Western blotting. WI-38 cell line was used as a positive control, and β-actin was used as an internal control.

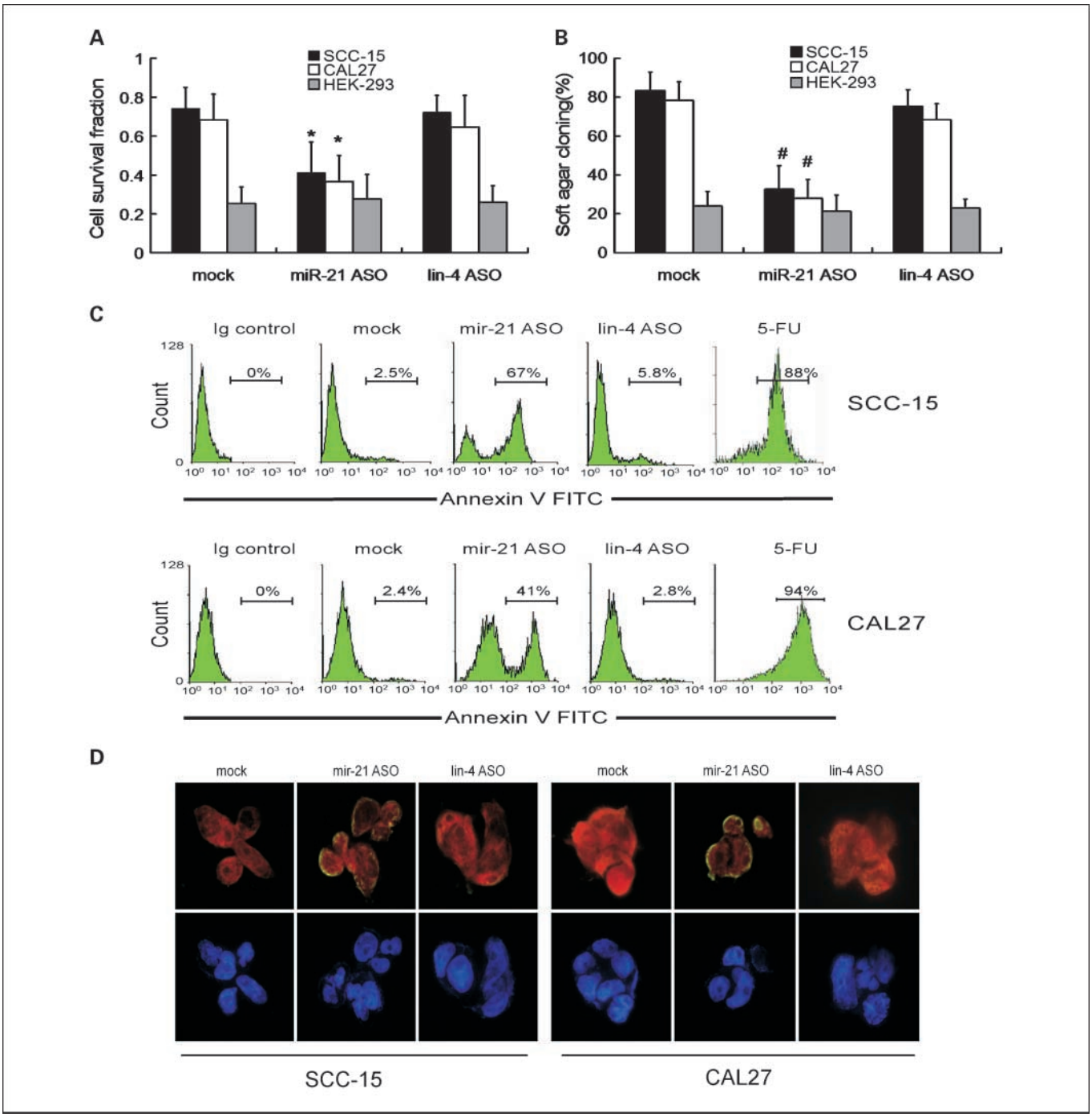

Fig. 4. MiR-21 ASO specifically inhibits cell growth and increases apoptosis in TSCC cell lines. Transfection with miR-21 ASO, instead of lin4 ASO, reduces cell survival as determined by MTT assay (*A*), anchorage-independent growth as determined by soft agar colony formation assay (*B*), compared with mock transfection in SCC-15 and CAL27 cells, but not in HEK-293 cells with low miR-21 expression. In addition, transfection with miR-21 ASO, instead of lin4 ASO, increases apoptosis determined by FACS analysis of Annexin V staining (*C*), triggers cytochrome *c* release (*green*) visualized by confocal microscopy and counterstained with cy3-labeled anti-human actin antibody for cytoplasm (*red*) and 4′,6-diamidino-2-phenylindole for nuclei (*blue*; *D*), as compared with mock transfection in SCC-15 and CAL27 cells, but not in HEK-293 cells with low miR-21 expression (*, $P < 0.05$; #, $P < 0.01$ as compared with cells with mock transfection).

(Fig. 5D; Supplementary Fig. S6C), and apoptosis of cancer cells determined by TUNEL staining (Fig. 5D; Supplementary Fig. S6B). Thus, our findings suggested that blockade of miR-21 retards TSCC development *in vivo*, probably by inhibiting proliferation and inducing apoptosis of cancer cells via TPM1 up-regulation.

Discussion

In the present study, we identified miR-21 overexpression in SCC of the tongue. Increased miR-21 correlates shortened survival of TSCC patients, and serves as a prognostic factor independent of other clinicopathologic factors. *In vitro*,

miR-21 blockade with ASO inhibits anchorage-independent growth and induces apoptosis in TSCC cell lines, which is partially mediated by retrieving TPM1 expression. *In vivo*, repeated injection of miR-21 ASO into TSCC xenografts implanted subcutaneously in nude mice suppressed tumor growth, which is related to TPM1 up-regulation, reduced proliferation, and increased apoptosis of xenografted tumor cells.

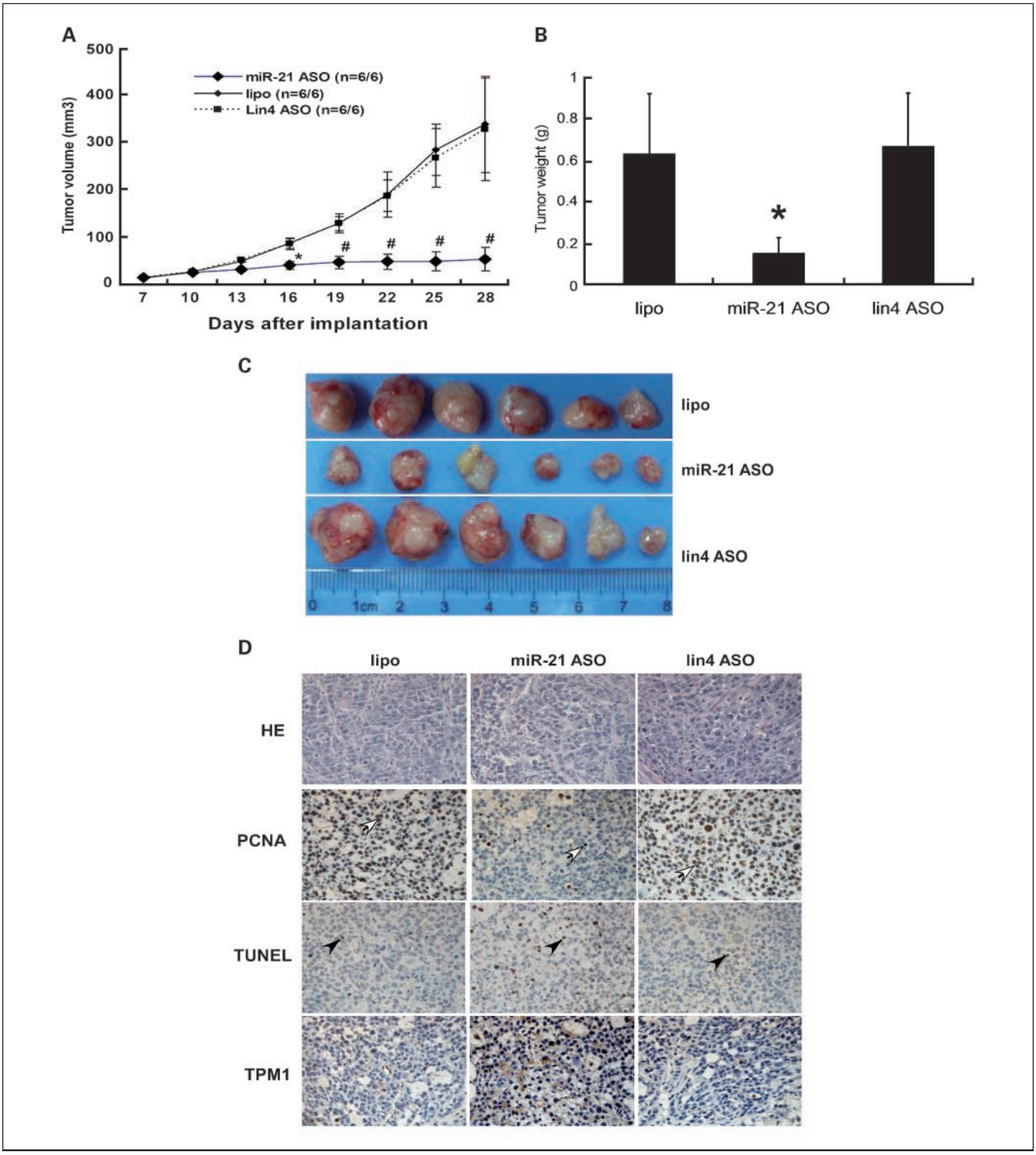

Fig. 5. MiR-21 ASO suppresses tumor growth of SCC-15 cells implanted subcutaneously in BALB/c-nu mice. Biweekly intratumor injection of miR-21 ASO, instead of lin4 ASO, reduces tumor volume followed for 28 d after implantation (*A*) and upon necropsy (*B*), and reduces tumor weight (*C*). *D,* tumor xenografts have similar histologic structure evaluated by H&E staining (×400), but tumors injected with miR-21 ASO display lower proliferation index (proliferating cell nuclear antigen staining, ×400), higher apoptosis index (TUNEL staining, ×400) and higher TPM1 expression (×400). White arrows, proliferating cell–associated antigen–positive cells; black arrows, apoptotic cells (*, $P < 0.05$; #, $P < 0.01$ compared with xenografts injected with LipofectAMINE alone).

As a prognostic factor, miR-21 is independent of clinical staging, lymph node metastasis, and differentiation of TSCC, and is closely related to patient survival. In agreement with our findings, previous reports have shown that miR-21 is overexpressed in many other solid tumors, and is related to tumor progression. Increased miR-21 contributes to the development of glioblastomas (13) and hepatocellular carcinomas (19). Furthermore, miR-21 promotes breast tumorigenesis via Bcl-2 up-regulation (20), and it correlates with advanced tumor staging, lymph node metastasis, and poor prognosis in breast cancers (30). In addition, miR-21 overexpression in colon carcinomas indicates poor survival and poor therapeutic outcome (31). Therefore, miR-21 seems to be a useful prognostic factor for various kinds of malignant tumors.

Other than miR-21, a number of tumor-related miRNAs are valuable for prognosis prediction in patients with cancer. For instance, reduction in let-7 (32) or increase in miR-155 (33) correlates with poor survival in patients with lung cancer. Additionally, overexpression of miR-196a-2 predicts poor outcome for pancreatic carcinomas (34), and hsa-miR-125b for liver cancers (35). Moreover, miR-210 is an independent prognostic factor for breast cancers, which is reversely correlated with disease-free and overall survival (36). Therefore, many other tumor-related miRNAs should be investigated for their contribution to cancer biology and their prognostic value in TSCC.

In clinical practice, prognosis prediction and treatment planning are mainly based on the TNM classification of the tumors. However, these approaches are not always accurate and it is necessary to identify more precise prognostic biomarkers for cancer patients. Ideal biomarkers should be stable in patient samples to be examined, and should be closely associated with clinical outcomes, especially patient survival. In this scenario, miRNAs are relatively stable as compared with other biological macromolecules. They can be well preserved in tissue samples even after formalin-fixation and paraffin-embedding, and can be efficiently extracted and evaluated (37). Moreover, miRNAs released from tumor cells are protected in membrane-derived exosomes, and thus, are stable in various body fluids including serum and plasma. Changes of miRNA profile in many types of body fluids may reflect potential physiologic and/or pathologic conditions (38, 39), and thus, may be applicable in clinics. On the other hand, the development of cancers involves the alteration of multiple gene expression, in which a single protein product of oncogene may not accurately reflect the status of the disease. However, a miRNA may regulate multiple coding genes that are related to tumor growth, and thus, is more likely to predict disease outcome more precisely and effectively.

Furthermore, our mechanistic study in cell cultures showed that antagonizing miR-21 inhibits anchorage-independent growth of TSCC cells. Anchorage-independent growth of tumor cells is crucial for oncogenesis and cancer metastasis. Under normal conditions, cells that are accidentally dissociated from the extracellular matrix support undergo specific programmed cell death—termed *anoikis*. However, cancer cells are capable of anchorage-independent growth and are able to travel and settle in a new site in which the microenvironment may be completely different (40, 41). Hence, our finding that miR-21 ASO inhibits anchorage-independent growth for TSCC cells in soft agar cultures may explain the contribution of miR-21 to TSCC progression.

Previous studies showed that TPM1 suppresses anchorage-independent growth and induces *anoikis* in cancer cells, whereas decreased expression of TPM1 is a prominent feature of many transformed cells (20–24). In this study, we found that miR-21 facilitated anchorage-independent growth of TSCC cells partly through down-regulating TPM1 expression. RNAi-mediated silencing of TPM1 expression in TSCC cells blocked with miR-21 ASO only partially recapitulates the effect of miR-21. These findings imply that there are other potential targets for miR-21 to maintain anchorage-independent tumor cell growth. In this scenario, miR-21–mediated reduction in the PTEN gene, another miR-21 target, may also play a role in TSCC cell growth, and needs further study.

In line with its inhibitory effect on TSCC cell growth, blockade of miR-21 also induces massive cancer cell death. Excessive cytochrome *c* release from the mitochondria into the cytoplasm and activation of effector caspase-3 suggest that miR-21 ASO induces tongue cancer cell death via a mitochondrial pathway that involves cascade activation of caspases (42). These findings imply that miR-21 may serve as an antiapoptotic factor that blocks the apoptosis of TSCC cells via cytochrome *c*–initiated pathway.

Finally, our findings in TSCC xenografts suggest that antagonizing miR-21 may hold great promise for designing novel therapeutic strategies against tongue cancers. Due to similarities in chemical structure and functional mechanism, the established platforms for siRNA-based therapy can be easily adopted to perform miRNA-based therapy. In addition to the advantages shared with siRNA-based therapy, such as efficient delivery and effective silencing of target genes, miRNA-based therapy excels in several aspects. In contrast to artificially synthetic siRNA, miRNAs are endogenous molecules existing in normal cells, which may minimize their unexpected off-target silencing effects. Because a miRNA molecule targets to a set of coding genes rather than a single one, therapies based on miRNA interference could be more potent in cancer treatment by targeting multiple molecular pathways. Moreover, miRNA ASOs or mimics could potentially be used as single therapeutic agents or in combination with other conventional chemotherapies/radiotherapies (43, 44) to achieve an optimal therapeutic effect.

In conclusion, our study indicates that miR-21 is an antiapoptotic factor in TSCC maintaining tumor cell growth, and thus, may play an important role in the development of malignancy. Therefore, miR-21 may serve as an independent prognostic indicator for patients' survival and as an effective target for the development of novel anticancer therapies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

1. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. CA Cancer J Clin 2005; 55:74–108.
2. Jemal A, Siegel R, Ward E, Murray T, Xu J, Thun MJ. Cancer statistics, 2007. CA Cancer J Clin 2007;57:43–66.
3. Andressakis D, Lazaris AC, Tsiambas E, Kavantzas N, Rapidis A, Patsouris E. Evaluation of caspase-3 and caspase-8 deregulation in tongue squamous cell carcinoma, based on immunohistochemistry and computerised image analysis. J Laryngol Otol 2008;122:1213–8.

4. Xie X, Clausen OP, De Angelis P, Boysen M. The prognostic value of spontaneous apoptosis, Bax, Bcl-2, and p53 in oral squamous cell carcinoma of the tongue. Cancer 1999;86:913–20.
5. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004;116:281–97.
6. Engels BM, Hutvagner G. Principles and effects of microRNA-mediated post-transcriptional gene regulation. Oncogene 2006;25:6163–9.
7. He L, Thomson JM, Hemann MT, et al. A microRNA polycistron as a potential human oncogene. Nature 2005;435:828–33.
8. 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 2005;65:9628–32.
9. Lee RC, Feinbaum RL, Ambros V. The *C. elegans* heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell 1993;75:843–54.
10. Wightman B, Ha I, Ruvkun G. Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in *C. elegans*. Cell 1993;75:855–62.
11. Chen CZ, Li L, Lodish HF, Bartel DP. MicroRNAs modulate hematopoietic lineage differentiation. Science 2004;303:83–6.
12. Brennecke J, Hipfner DR, Stark A, Russell RB, Cohen SM. Bantam encodes a developmentally regulated microRNA that controls cell proliferation and regulates the proapoptotic gene hid in Drosophila. Cell 2003;113:25–36.
13. Chan JA, Krichevsky AM, Kosik KS. MicroRNA-21 is an antiapoptotic factor in human glioblastoma cells. Cancer Res 2005;65:6029–33.
14. Xu P, Vernooy SY, Guo M, Hay BA. The Drosophila microRNA Mir-14 suppresses cell death and is required for normal fat metabolism. Curr Biol 2003;13:790–5.
15. Kent OA, Mendell JT. A small piece in the cancer puzzle: microRNAs as tumor suppressors and oncogenes. Oncogene 2006;25:6188–96.
16. Wong TS, Liu XB, Wong BY, Ng RW, Yuen AP, Wei WI. Mature miR-184 as potential oncogenic microRNA of squamous cell carcinoma of tongue. Clin Cancer Res 2008;14:2588–92.
17. Calin GA, Dumitru CD, Shimizu M, et al. Frequent deletions and down-regulation of microRNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. Proc Natl Acad Sci U S A 2002;99:15524–9.
18. Yu F, Yao H, Zhu P, et al. let-7 regulates self renewal and tumorigenicity of breast cancer cells. Cell 2007;131:1109–23.
19. Meng F, Henson R, Wehbe-Janek H, Ghoshal K, Jacob ST, Patel T. MicroRNA-21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer. Gastroenterology 2007;133:647–58.
20. Si ML, Zhu S, Wu H, Lu Z, Wu F, Mo YY. miR-21-mediated tumor growth. Oncogene 2007;26:2799–803.
21. Zhu S, Si ML, Wu H, Mo YY. MicroRNA-21 targets the tumor suppressor gene tropomyosin 1 (TPM1). J Biol Chem 2007;282:14328–36.
22. Raval GN, Bharadwaj S, Levine EA, et al. Loss of expression of tropomyosin-1, a novel class II tumor suppressor that induces anoikis, in primary breast tumors. Oncogene 2003;22:6194–203.
23. Bharadwaj S, Thanawala R, Bon G, Falcioni R, Prasad GL. Resensitization of breast cancer cells to anoikis by tropomyosin-1: role of Rho kinase-dependent cytoskeleton and adhesion. Oncogene 2005;24:8291–303.
24. Pawlak G, Helfman DM. Cytoskeletal changes in cell transformation and tumorigenesis. Curr Opin Genet Dev 2001;11:41–7.
25. Su H, Yang JR, Xu T, et al. MicroRNA-101, down-regulated in hepatocellular carcinoma, promotes apoptosis and suppresses tumorigenicity. Cancer Res 2009;69:1135–42.
26. Xu T, Zhu Y, Wei QK, et al. A functional polymorphism in the miR-146a gene is associated with the risk for hepatocellular carcinoma. Carcinogenesis 2008;29:2126–31.
27. Song E, Chen J, Ouyang N, Su F, Wang M, Heemann U. Soluble Fas ligand released by colon adenocarcinoma cells induces host lymphocyte apoptosis: an active mode of immune evasion in colon cancer. Br J Cancer 2001;85:1047–54.
28. Novy RE, Lin JL, Lin CS, Lin JJ. Human fibroblast tropomyosin isoforms: characterization of cDNA clones and analysis of tropomyosin isoform expression in human tissues and in normal and transformed cells. Cell Motil Cytoskeleton 1993;25:267–81.
29. Zhu S, Wu H, Wu F, Nie D, Sheng S, Mo YY. MicroRNA-21 targets tumor suppressor genes in invasion and metastasis. Cell Res 2008;18:350–9.
30. Yan LX, Huang YF, Shao Q. MicroRNA miR-21 overexpression in human breast cancer is associated with advanced clinical stage, lymph node metastasis and patient poor prognosis. RNA 2008;14:1–13.
31. Schetter AJ, Leung SY, Sohn JJ, et al. MicroRNA expression profiles associated with prognosis and therapeutic outcome in colon adenocarcinoma. JAMA 2008;299:425–36.
32. Takamizawa J, Konishi H, Yanagisawa K, et al. Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival. Cancer Res 2004;64:3753–6.
33. Yanaihara N, Caplen N, Bowman E, et al. Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. Cancer Cell 2006;9:189–98.
34. Bloomston M, Frankel WL, Petrocca F, et al. MicroRNA expression patterns to differentiate pancreatic adenocarcinoma from normal pancreas and chronic pancreatitis. JAMA 2007;297:1901–8.
35. Li W, Xie L, He X, et al. Diagnostic and prognostic implications of microRNAs in human hepatocellular carcinoma. Int J Cancer 2008;123:1616–22.
36. Camps C, Buffa FM, Colella S, et al. hsa-miR-210 ls induced by hypoxia and is an independent prognostic factor in breast cancer. Clin Cancer Res 2008;14:1340–8.
37. Gilad S, Meiri E, Yogev Y, et al. Serum microRNAs are promising novel biomarkers. PLoS ONE 2008;3:e3148.
38. Li J, Smyth P, Flavin R, et al. Comparison of miRNA expression patterns using total RNA extracted from matched samples of formalin-fixed paraffin-embedded (FFPE) cells and snap frozen cells. BMC Biotechnol 2007;7:36.
39. Mitchell PS, Parkin RK, Kroh EM, et al. Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci U S A 2008;105:10513–8.
40. Frisch SM, Francis H. Disruption of epithelial cell-matrix interactions induces apoptosis. J Cell Biol 1994;124:619–26.
41. Liotta LA, Kohn E. Anoikis: cancer and the homeless cell. Nature 2004;430:973–4.
42. Jiang X, Wang X. Cytochrome C-mediated apoptosis. Annu Rev Biochem 2004;73:87–106.
43. Krutzfeldt J, Rajewsky N, Braich R, et al. Silencing of microRNAs *in vivo* with 'antagomirs'. Nature 2005;438:685–9.
44. Jeyaseelan K, Herath WB, Armugam A. MicroRNAs as therapeutic targets in human diseases. Expert Opin Ther Targets 2007;11:1119–29.