Mature miR-184 as Potential Oncogenic microRNA of Squamous Cell Carcinoma of Tongue

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Abstract

Purpose: The aim of this study was to evaluate the microRNA expression patterns in squamous cell carcinoma (SCC) of the tongue.

Experimental Design: Expression levels of 156 human mature microRNAs were examined using real-time quantitative PCR (Taq Man MicroRNA Assays; Human Panel) on laser microdissected cells of 4 tongue carcinomas and paired normal tissues. Expression of mature miR-184 was further validated in 20 paired tongue SCC and the normal tissues. Potential oncogenic functions of miR-184 were evaluated in tongue SCC cell lines (Cal27, HN21B, and HN96) with miR-184 inhibitor. Plasma miR-184 levels were evaluated using real-time quantitative PCR.

Results: Using 3-fold expression difference as a cutoff level, we identified 24 up-regulated mature miRNAs including miR-184, miR-34c, miR-137, miR-372, miR-124a, miR-21, miR-124b, miR-31, miR-128a, miR-34b, miR-154, miR-197, miR-132, miR-147, miR-325, miR-181c, miR-198, miR-155, miR-30a-3p, miR-338, miR-17-5p, miR-104, miR-134, and miR-213; and 13 down-regulated mature miRNAs including miR-133a, miR-99a, miR-194, miR-133b, miR-219, miR-100, miR-125b, miR-26b, miR-138, miR-149, miR-195, miR-107, and miR-139. Overexpression of miR-184 was further validated in 20 paired tongue SCC and normal tissues ($P = 0.002$). Inhibition of miR-184 in tongue SCC cell lines could reduce cell proliferation rate. Down-regulation of c-Myc was observed in two cell lines in response to miR-184 inhibitor. Suppressing miR-184 could induce apoptosis in all three cell lines. Plasma miR-184 levels were significantly higher in tongue SCC patients in comparison with normal individuals, and the levels were significantly reduced after surgical removal of the primary tumors.

Conclusions: Overexpression of miR-184 might play an oncogenic role in the antiapoptotic and proliferative processes of tongue SCC. In addition, plasma miR-184 levels were associated with the presence of primary tumor. Further studies on the aberrantly expressed miRNAs in tongue SCC as well as using plasma miRNAs as novel tumor markers are warranted.

Squamous cell carcinoma (SCC) of the tongue is an aggressive head and neck malignancy. Tongue SCC is well-known for its high rate of proliferation and nodal metastasis. Although it is visibly located in oral cavity, ~50% patients were already in advanced stage III and IV on presentation (1, 2). The understanding of the molecular pathways of carcinogenesis or progression would be helpful in improving diagnosis, therapy, and prevention of the disease.

MicroRNAs (miRNA) are a group of gene-specific regulators. miRNAs are ~20-nt long, single-stranded, noncoding RNA molecules (3). They are encoded in long primary forms in the nucleus. The primary miRNAs will be transported into the cytoplasm actively after being processed by cellular nucleases, e.g., Drosha. Cytoplasmic miRNAs are then processed by Dicer into the mature forms. Mature miRNAs are associated with a cellular complex that is similar to the RNA-induced silencing complex for RNA interference (4, 5). Mature miRNAs could regulate protein expression at posttranscriptional levels through direct binding to specific messenger RNA molecules. The specific binding of miRNAs could promote degradation of mRNA and/or hinder the translation process.

Dysregulation of miRNAs is common in various cancers. The dysregulated miRNAs play a role in carcinogenesis or tumor progression by altering the normal gene expression patterns. One example is miR-21. In human glioblastoma cells, caspase cascade was inactivated by the up-regulated miR-21 (6). Inhibiting miR-21 could increase chemosensitivity of malignant cholangiocytes (7). MiR-21 could also modulate tumor growth through regulation of bcl-2 expression (8).

In view of the limited data on miRNA expression patterns and their functional roles in various human cancers including tongue carcinoma, we aimed at identifying the aberrantly expressed miRNAs in tongue SCC. Because cell-free RNA (originated from the primary tumor) could be detected in the

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circulation of cancer patients, we also examined whether the aberrantly expressed miRNA could be detected in the circulation of patients with tongue SCC.

Materials and Methods

Patient samples. Tissues of tongue SCC and the matched normal counterparts were obtained from surgical specimens immediately after resection from patients undergoing primary surgical treatment of oral tongue carcinoma in the Department of Surgery, University of Hong Kong Medical Center, Queen Mary Hospital, Hong Kong. The samples were flash frozen in liquid nitrogen and stored at -80°C. Histology of tissues was evaluated by the hospital's pathologist. Written consent of tissue donation for research purposes was obtained from patients before tissue collection and protocol was approved by the Institutional Review Board of the hospital (Institutional Review Board reference number, UW 06-149 T/1174).

Laser microdissection. Ornithine carbamyl transferase–embedded frozen tissues were sectioned at a thickness of 8 μm and mounted onto PALM membrane slides pretreated with RNase-zap (Ambion).The slides were fixed in 70% ethanol, stained with Mayer's H&E, and dehydrated sequentially in increasing concentrations of ethanol and xylene (followed by 3 min of air drying). PALM microbeam system (P.A.L.M.) was used to procure pure cell populations of either cancer cells or normal epithelial cells until 25,000 to 30,000 cells were collected for each population in each patient. The microdissected cells were then transferred to lysis buffer (provided in the miRNA isolation kit listed below) for miRNA extraction. A total of four cases of tongue SCC and matched normal epithelia were processed. There were 3 male and 1 female patients (age range, 44-66 years). The pathologic stages were 1 T2N1M0 and 3 T3N2M0.

Cell lines. Tongue SCC cell line Cal27 was purchased from American Type Culture Collection. HN21B and HN96 were short-term cultures derived from tongue SCC tissues in our laboratory. All the cell lines were maintained in RPMI 1640 with 10% fetal bovine serum.

Table 1. miRNAs up-regulated in SCC of tongue

Mature microRNAs	Sequence	Fold changes
hsa-miR-184	uggacggagaacugauaagggu	59.12
hsa-miR-34c	aggcaguguaguuagcugauug	57.80
hsa-miR-137	uauugcuuaagaauacgcguag	30.59
hsa-miR-372	aaagugcugcgacauuugagcgu	26.30
hsa-miR-124a	uuaaggcacgcggugaaugcca	13.98
hsa-miR-21	uagcuuaucagacugauguuga	11.49
hsa-miR-124b	uuaaggcacgcggugaaugc	10.32
hsa-miR-31	ggcaagaugcuggcauagcug	6.43
hsa-miR-128a	ucacagugaaccggucucuuuu	5.43
hsa-miR-34b	aggcagugucauuagcugauug	5.22
hsa-miR-154	aaucauacacgguugaccuauu	5.01
hsa-miR-197	uucaccaccuucuccacccagc	4.40
hsa-miR-132	uaacagucuacagccauggucg	3.54
hsa-miR-147	guguguggaaaugcuucugc	3.46
hsa-miR-325	ccuaguagguguccaguaagu	3.45
hsa-miR-181c	aacauucaaccugucggugagu	3.43
hsa-miR-198	gguccagaggggagauagg	3.40
hsa-miR-155	uuaaugcuaaucgugauagggg	3.35
hsa-miR-30a-3p	cuuucagucggauguuugcagc	3.32
hsa-miR-338	acauagaggaaauuccacguuu	3.15
hsa-miR-17-5p	caaagugcuuacagugcagguagu	3.11
hsa-miR-104	ucaacaucagucugauaagcua	3.08
hsa-miR-134	ugugacugguugaccagaggg	3.07
hsa-miR-213	accaucgaccguugauuguacc	3.00

Table 2. miRNAs down-regulated in SCC of tongue

Mature microRNAs	Sequence	Fold changes
hsa-miR-133a	uuggucccccuucaaccagcugu	-12.5
hsa-miR-99a	aacccguagauccgaucuugug	-9.09
hsa-miR-194	uguaacagcaacuccaugugga	-5.88
hsa-miR-133b	uuggucccccuucaaccagcua	-5.26
hsa-miR-219	ugauuguccaaacgcaauucu	-5.00
hsa-miR-100	aacccguagauccgaacuugug	-4.76
hsa-miR-125b	ucccugagacccuaacuuguga	-4.76
hsa-miR-26b	uucaaguaauucaggauaggu	-4.16
hsa-miR-138	agcugguguugugaauc	-4.00
hsa-miR-149	ucuggcuccgugucuucacucc	-3.85
hsa-miR-195	uagcagcacagaaauauuggc	-3.44
hsa-miR-107	agcagcauuguacagggcuauca	-3.13
hsa-miR-139	ucuacagugcacgugucu	-3.03

RNA extraction. miRNAs of tissues, cultured cells, and plasma were extracted and purified using mirVana miRNA Isolation kit (Ambion) following the manufacturer's instructions.

Quantitative reverse transcription-PCR of mature miRNAs. miRNA expression patterns were evaluated using Taq Man MicroRNA Assays, Human Panel (Applied Biosystems). The assay includes two steps: generation of cDNA by reverse transcription reaction and Taq Man real-time PCR assay. In brief, miRNAs in the samples were converted into cDNA using 156 specific stem-loop reverse transcription primers. After cDNA conversion, the quantity of mature miRNAs was evaluated using specific Taq Man real-time PCR primers and probes. Real-time PCR was done using GeneAmp Fast PCR Master Mix (Applied Biosystems) and ABI 7900HT real-time PCR machine. All reactions were done in duplicate. Expression levels of mature mRNAs were evaluated using comparative CT method ($2^{-\Delta CT}$). Transcription level of miR-16 was used as endogenous control. Theory of the real-time PCR assay and the list of the human miRNAs could be found on the company's Web site.[1]

Small interfering RNA transfection. Mature miR-184 inhibitor was obtained from Ambion. Cells were seeded at 20,000 per well in chamber slides. Inhibitor was transfected into the cell lines using siPORT NeoFX reagent (Ambion) following manufacturer's instructions. Culture medium was changed after 24 h of transfection. Transfection effects were evaluated after 72 h. The treatment was done in triplicate. Cells treated with the transfection reagent only were used as mock control.

Cell proliferation assay. Cell proliferation assay was done using Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega) according to the manufacturer's protocol.

Immunocytochemistry. Tongue SCC cell lines were seeded into chamber slides and transfected with miR-184 inhibitor as described above. After 72 h, the cells were rinsed with PBS and fixed in 4% paraformaldehyde for 30 min at room temperature. After being permeabilized with 0.2% Triton X-100 for 5 min, the cells were blocked with 1% bovine serum albumin for 1 h at room temperature. The cells were then incubated with mouse monoclonal anti–c-Myc (Ab-2; 1:100; Calbiochem) for 1 h at room temperature. Cells were washed and incubated with horseradish peroxidase–labeled polymer conjugated to goat anti-mouse immunoglobulins (Envision; DAKO) for 45 min at room temperature. After additional PBS washing, the slides were developed with 3,3′-diaminobenzidine and couterstained with hematoxylin. C-Myc protein was expressed in the nuclei. The positive cells were counted in five randomly selected fields and expressed as percentage of total cells counted.

[1] http://mirna.appliedbiosystems.com

Terminal deoxynucleotidyl-transferase–mediated dUTP nick-end labeling. Apoptosis was determined using the terminal deoxynucleotidyl-transferase–mediated dUTP nick-end labeling (TUNEL) assay after transfection for 72 h. Briefly, the cells were rinsed with PBS and fixed in 4% paraformaldehyde for 25 min at room temperature. After permeabilized with 0.2% Triton X-100 for 5 min, the slides were incubated with equilibration buffer for 10 min. Then, rTdT reaction mix was applied to the cells and the slides were incubated at 37°C for 60 min inside a humidified chamber. The reaction was terminated with 2 × SCC for 15 min at room temperature. After blocking the activity of endogenous peroxidase with 0.3% hydrogen peroxide for 5 min, the slides were incubated with Streptavidin horseradish peroxidase for 30 min and then developed with 3,3′-diaminobenzidine. Coverslips were mounted and the results were observed under the microscope. The apoptotic nuclei were stained as dark brown. Positive-labeled cells were counted in five randomly selected fields and expressed as percentage of total cells counted.

Statistical analysis. The statistical association between miRNAs levels and the disease groups was examined by Wilcoxon signed-ranks test. P values of <0.05 were considered as statistical significant. All P values were two-sided. Data analysis was done using SPSS for Windows version 14.0 (SPSS, Inc.).

Results

Screening of differentially expressed miRNAs in laser microdissected cells. We did microRNA expression profiling analysis using Taq Man MicroRNA Assay. Differential expression between SCC cells and paired normal cells was defined using a cutoff value of 3-fold change. The differentially expressed miRNAs could be classified into four groups: group 1, miRNAs that had at least 3-fold increase in the tumor cells in comparison with the matched normal cells (Table 1). Of the 156 miRNAs, 16% (24 of 156) miRNAs were in this group; group 2, miRNAs that had at least 3-fold reduction in expression levels in the SCC cells (Table 2). Of the 156 miRNAs, 8% (13 of 156) miRNA were in this group; group 3, MiRNA that was found in tumor cells only but not in normal cells. MiR-144 was the only miRNA in this group; group 4, miRNAs that could only be detected in normal cells, not in tumor cells. These included miR-216, miR-220, and miR-371.

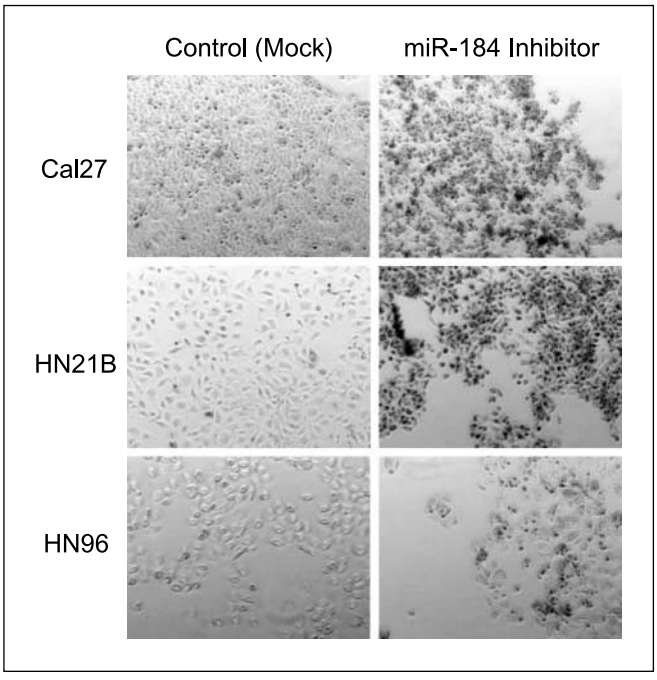

Fig. 1. Morphologic changes of tongue SCC cell lines in response to miR-184 inhibition. Cells were seeded into chamber slides and incubated in the presence of miR-184 inhibitor or transfection reagents (mock control) only. Cells were then photographed under light microscope after 72 h.

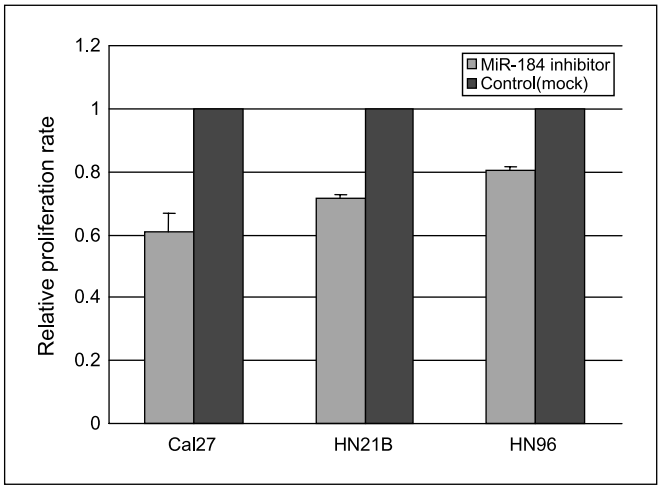

Fig. 2. Proliferation inhibition of tongue SCC cell lines in response to miR-184 inhibition. Cells were seeded into 96-well plates and incubated in the presence of miR-184 inhibitor or transfection reagents (mock control) only. Cell proliferation assay was done after culturing for 72 h. The experiment was done in triplicate.

Validation of miR-184 overexpression in tongue carcinoma. MiR-184 showed 59-fold higher expression in tongue SCC cells compared with the paired normal cells. To validate that miR-184 was overexpressed in tongue SCC, real-time quantitative analysis of miR-184 was done on 20 tongue SCC and their matched normal tissues. For the tongue SCC, the mean miR-184 level was 928 (range, 5-5,297). For the matched normal tissues, the mean miR-184 level was 265 (range, 1-836). There was significant difference in miR-184 levels between tongue SCC and matched normal tissues (P = 0.022; Wilcoxon signed-ranks test). There were no significant correlations of miR-184 levels in the primary tumors with the clinicopathologic variables including sex, age, tumor stage, and lymph node metastasis.

Cellular response to miR-184 inhibition. The three tongue SCC cell lines were transfected with miR-184 inhibitor. Cultured cells transfected with miR-184 were relatively smaller and denser compared with control cultures (Fig. 1). The proliferation rate was reduced in all three cell lines after transfection with miR-184 inhibitor compared with controls (Fig. 2). Immunostaining of c-Myc showed that c-Myc–positive cells were significantly reduced in Cal27 (34% versus 76%) and HN96 (32% versus 78%) cell lines after transfection with miR-184 inhibitor (all $P < 0.05$; Mann-Whitney U test; Fig. 3). Apoptosis was detected by *in situ* TUNEL assay (Fig. 4). The percentages of apoptotic cells were significantly higher (all $P < 0.05$; Mann-Whitney U test) in miR-184 suppressed cell lines compared with the mock controls: Cal27 (10.64% versus 0.35%), HN21B (7.05% versus 0.62%), and HN96 (9.25% versus 0.78%).

Plasma miR-184 level in tongue SCC patients and normal individuals. Plasma level of miR-184 was evaluated in 38 normal individuals and 30 tongue SCC patients. The control miRNA, miR-16, was detectable in all 68 plasma samples.

Unlike the control MiR-16, which was found in both cancer patients and normal individuals, mature miR-184 could only be detected in 80% (24 of 30) cancer patients and 13% (5 of 38) normal individuals. The plasma level of miR-184 was significantly higher (Mann-Whitney *U* test; $P < 0.001$; 95% confidence interval, 0.72-1,488.04) in cancer patients (median, 398.46; range 6.89-9,699.56) compared with the normal individuals (median, 0.27; range, 0.02-72.19). We further evaluated the changes in plasma miR-184 levels in the patients after the surgical removal of the primary tumor. Twenty-five patients had both preoperative and postoperative plasma available for this study (Table 3). The mean plasma levels reduced from 1,801.30 before operation to 177.84 after operation ($P < 0.001$; Wilcoxon signed-ranks test).

We correlated the plasma miR-184 levels with sex, age, tumor size, and nodal metastatic status of patients. No significant associations were observed. Plasma miR-184 could be detected in both early and advanced tongue SCC patients.

Discussion

miRNAs could control the expression levels of particular genes. Thus, dysregulation of miRNAs is expected to be found in diseases, such as cancers, which are attributed to dysregulated gene expression. It was suggested that miRNA alteration could initiate carcinogenesis (9). There is limited information on the aberrantly expressed miRNAs in tongue SCC. After screening 156 miRNAs in laser microdissected cells, we identified a panel of aberrantly expressed miRNAs. In this study, we focused on the potential oncogenic candidate miR-184.

Mature miR-184 expression has been reported in basal and immediately suprabasal cells of the corneal epithelium in mouse model. Using *in situ* hybridization, Ryan et al. (10) showed that miR-184 was more strongly expressed in the epithelial cells of the germinative zone. So far, dysregulation of miR-184 has not been reported in human cancers. Therefore, we were interested to validate its roles in tongue SCC after the aberrant overexpression of miR-184 was further confirmed in paired tongue carcinomas and normal tissues.

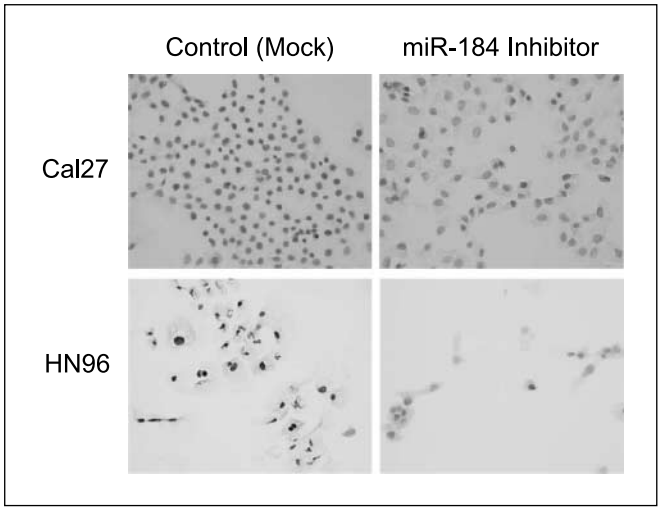

Fig. 3. C-Myc expression in tongue SCC cell lines treated with miR-184 inhibitor and mock control. Immunostaining was done after 72 h of transfection. C-Myc – positive cells were higher in the mock controls in comparison with the cell lines treated with miR-184 inhibitor. The experiments were repeated in triplicate.

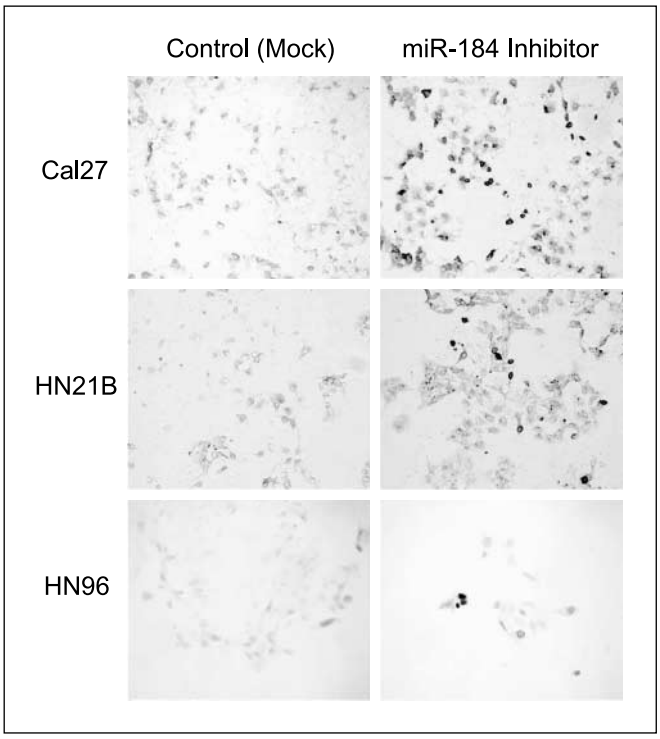

Fig. 4. TUNEL analysis for apoptosis in tongue SCC cell lines treated with miR-184 inhibitor and mock control. The cells were treated with miR-184 inhibitor for 72 h. Apoptotic cells were detected by DeadEnd Colorimetric TUNEL system (Promega). *Dark brown,* apoptotic nuclei. The assay was repeated in triplicate.

To study the functional roles of miR-184 in tongue carcinoma, we knocked down the expression of miR-184 in the tongue SCC cell lines using the specific inhibitor. Morphologically, the cells became round and condensed, suggesting that miR-184 inhibitor might induce cell death in tongue SCC cells. To validate this potential cell death – inducing effect, TUNEL staining was done. The significant increase in apoptotic cells in response to miR-184 inhibitor suggested an antiapoptotic function of mature miR-184. Moreover, we observed that the total cell numbers were obviously reduced in the presence of miR-184 inhibitor. To examine the potential association between miR-184 and cell proliferation, cell proliferation assay and c-Myc staining were done. Our results indicated that miR-184 may hinder tongue SCC cell proliferation partly through altering c-Myc expression. Taken together, our results suggested that miR-184 might play a part in both antiapoptotic and proliferative processes of tongue SCC cells.

RNAs derived from tumor cells could be absorbed into the peripheral blood of cancer patients and become detectable as cell-free surrogate tumor markers (11, 12). There is no report on plasma cell – free miRNA in cancer patients yet. Thus, we were interested to know whether the up-regulated miRNA in cancer cells could be absorbed into the systemic circulation as cell-free miRNA tumor marker in the patients. We first examined whether mature miRNA was detectable in the plasma of both normal individuals and cancer patients. We found that miR-16, the house-keeping miRNA, was detectable in all the plasma samples recruited from both normal individuals and

Table 3. Changes of plasma miR-184 levels of tongue SCC patients after operation

Patients	T	N	M	miR-184 level	
				Preoperation	Postoperation
1	3	0	0	205.64	0
2	2	0	0	3,436	9.00
3	2	0	0	9,699.56	0
4	1	0	0	109.59	281.54
5	3	2	0	5,924.13	682.42
6	2	0	0	1,488.04	0
7	2	0	0	238.13	0
8	2	0	0	1,552.06	317.28
9	2	0	0	558.8	36.44
10	2	0	0	211.98	0
11	4	1	0	7,221.07	0
12	3	1	0	221.8	36.44
13	2	1	0	225.64	63.76
14	2	0	0	6,495.75	1,201.99
15	3	0	0	0	0
16	2	0	0	1,716.45	1,402.24
17	2	0	0	9.06	30.75
18	2	0	0	15.84	0
19	2	0	0	6.89	0
20	2	0	0	1,855.06	0
21	1	0	0	57.42	0
22	3	2	0	90.52	219.19
23	2	0	0	1,260.34	164.98
24	1	0	0	79.95	0.00
25	1	0	0	2,352.73	0.00
			Mean	1,801.30	177.84

tongue SCC patients. In contrast to the miR-16, miR-184 was not detected in all the plasma samples. Plasma miR-184 was detected in 13% of the normal individuals. In comparison, plasma miR-184 was more frequently detected (80%) in tongue SCC patients. In addition, plasma miR-184 levels were significantly higher in patients with tongue SCC in comparison with the normal individuals. The results suggested that the increased miR-184 in SCC patients may be related to the presence of primary tumor. Furthermore, the plasma miR-184 levels were ~10-fold reduced after the surgical removal of tumor. Thus, we hypothesized that the higher plasma miRNA levels were due to absorption of the miRNA into the circulation from a large number of cancer cells with highly expressed miR-184 in the body before surgery. The plasma level therefore reduced significantly after the surgical removal of the tumor. The reduction of plasma miR-184 levels further confirmed the correlation between overexpressed miR-184 and the primary tongue SCC. Whether plasma miRNAs could be used as sensitive and specific surrogate tumor markers requires further studies in serially taken blood samples of larger cohort of patients with long term follow-up.

Increased levels of plasma DNA/RNA have been observed in cancer patients. In our cohort of tongue SCC patients, miR-184 levels were elevated in the plasma before operation and reduced significantly after surgical treatment. The reduction is likely because of the removal of the primary tumor, the origin of the oncogenic miRNAs. However, the reduction was not an absolute event in all the patients after operation. We observed that three patients (patients 4, 17, and 22) showed an increase in plasma miR-184 after the operation. We tried to correlate the increase with the clinicopathologic variables but no associations were found. There are several possible explanations for this phenomenon. First, because all of the three patients had their blood taken within 1 month after operation (day 3, 24, and 25, respectively), the high-plasma miRNA might be partly due to the absorption of large amount of nucleic acids including miRNA into the circulation immediately after the major surgery. Second, the high-plasma miR-184 might come from microscopic residues or occult tumors. All the patients had no local recurrence or distant metastasis at the time when the peripheral blood was collected. Longer follow-up period are required to validate this assumption. In addition, their plasma miRNAs might result from other complications. For instance, increased plasma nucleic acids have been reported in noncancer disorders such as stroke, trauma, myocardial infarction, autoimmune disorders, and premalignant complications (13). This should be taken into account when using plasma miRNAs to monitor cancer patients.

In conclusion, there were aberrantly expressed miRNAs in tongue SCC. MiR-184 is a candidate oncogenic miRNA in tongue SCC and might play a part in the antiapoptosis and proliferation of tongue SCC cells. Further identification of aberrantly expressed miRNAs and the elucidation of their functional roles are helpful in the understanding of the pathogenesis of this disease. It is also worth studying the clinical values of plasma miRNAs as surrogate tumor markers.

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