microRNA expression profiles in oral squamous cell carcinoma

DAISUKE SOGA, SAYAKA YOSHIBA, SUNAO SHIOGAMA, HIROAKI MIYAZAKI, SEIJI KONDO and SATORU SHINTANI

Department of Oral and Maxillofacial Surgery, Showa University School of Dentistry, Ota-ku, Tokyo 145-8515, Japan

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Abstract. microRNAs (miRNAs) are involved in cancer pathogenesis, apoptosis and cell growth, thereby functioning as both tumor suppressors and oncogenes. However, the expression patterns and roles of miRNAs in oral squamous cell carcinoma (OSCC) remain largely unknown. We hypothesized that oral cancer may have a unique miRNA profile, which in turn may play a critical role in oral cancer development, progression, diagnosis and prognosis. We, therefore, investigated the expression profiles of 29 OSCC tumors and 7 normal oral mucosal samples. The miRNA expression patterns in OSCC were examined by TaqMan-based microRNA assays. We were subsequently able to identify the candidates of cancer-related miRNAs through analysis of the miRNA expression profiles. In conclusion, OSCC tissues were shown to have a unique miRNA profile pattern when compared with that in normal tissues. The present study may provide useful information for further investigation of the functional roles of miRNAs in OSCC development, progression, diagnosis and prognosis.

Introduction

Oral cancer, which is occurring at an increasing frequency worldwide, is the sixth most common malignancy in humans. This year alone, it is estimated that ~600,000 new cases will arise, with only 40-50% of patients with oral squamous cell carcinoma (OSCC) likely to survive for 5 years. Despite combined surgery and radiation therapy, long-term survival of patients with oral cancer has shown no improvement over the past few decades (1,2). This is primarily due to the poor clinical prognosis of patients with lymph node metastasis. A deeper understanding of the molecular basis of the highly malignant properties of oral cancer combined with patient stratification is therefore needed.

Correspondence to: Dr Daisuke Soga, Department of Oral and Maxillofacial Surgery, Showa University School of Dentistry, 2-1-1 Kitasenzoku, Ota-ku, Tokyo 145-8515, Japan
E-mail: d-soga@dent.showa-u.ac.jp

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microRNAs (miRNAs) are non-coding small RNAs (~22 nucleotides) that regulate post-transcriptional gene expression by interfering with the translation of target mRNAs. A single miRNA can regulate the expression of several genes, while more than one-third of all protein-coding genes are thought to be under translational control of miRNAs. miRNAs are involved in a variety of cellular processes, including the regulation of cellular differentiation, proliferation and apoptosis (3,4). In addition, aberrant expression of miRNAs is known to induce various human malignancies, clearly classified by their miRNA profiles (5,6). However, little is known about the miRNA expression patterns or function in oral cancer (7,8).

Specific overexpression or underexpression of miRNAs has been correlated with particular types of tumors (5,9,10). It has also been suggested that miRNA overexpression could result in downregulation of tumor-suppressor genes, while underexpression may lead to oncogene upregulation (9). Most importantly, it has been suggested that miRNA expression signatures could be used to predict the outcome in several tumor types, including lung cancer and chronic lymphocytic leukemia, as well as to predict the response to chemotherapy. miRNAs may therefore prove to be novel therapeutic targets for a wide range of diseases, including cancer (11).

In the present study, we examined the expression profiles of miRNAs in OSCC, revealing differential expression between normal and cancer tissues. These findings may further facilitate the potential therapeutic and diagnostic use of miRNAs in OSCC.

Materials and methods

Clinical samples and cell lines. Twenty-nine OSCC samples and 7 oral mucosal samples were obtained from oral cancer patients who underwent surgery at Showa University from April 2004 to November 2008. Written informed consent was obtained from all patients prior to sampling, and samples were maintained at -80˚C until use. OSCC samples were microscopically examined for determination of cancer cell content by two independent pathologists; they were dissected to enrich cancer cells when necessary. For molecular analysis, 29 samples containing ≥50% cancer cells were used. Normal oral mucosa was extracted at a sufficient margin from the cancer, and the absence of a muscle layer and cancer cells was confirmed microscopically. The present study was certified by the Ethics Committee of the Showa University.

RNA preparation. miRNA was prepared with the Recover All Total Nucleic Acid Isolation kit (Applied Biosystems, Darmstadt, Germany) according to the kit protocol. RNA yield and the A260/280 ratio were monitored with a NanoDrop ND-100 spectrometer (NanoDrop Technologies, Wilmington, DE, USA).

Reverse transcription. miRNAs were reverse transcribed with the TaqMan miRNA Reverse Transcription kit (Applied Biosystems), using 60 ng total RNA and pools of miRNA specific stem-loop primers (Megaplex RT Primers Pool A and B; Applied Biosystems). After reverse transcription, the cDNA was preamplified with Megaplex PreAmp Primers (Pool A and B) according to the recommendations of the supplier (Applied Biosystems).

TaqMan low density arrays. Differential expression of 768 miRNAs was determined using Taqman microRNA Array v2.0 (Applied Biosystems), with an A and B card for each. The array plate also included the RNU48 transcript as a normalization signal. Expression levels of each mature miRNA were evaluated using the comparative threshold cycle (Ct) method, with normalization to RNU48 ($2^{-\Delta\Delta Ct}$). The fold-change in each miRNA was calculated from the difference in expression levels between tumor tissues and normal tissues.

miRNA target prediction. TargetScan (http://www.targetscan.org/) was used to analyze potential target genes of the deregulated miRNAs.

Results

Differential expression of miRNAs between normal oral mucosa and oral cancer tissues. To define the role of miRNAs in OSCC, we investigated the expression profiles of 29 OSCC tumors and 7 normal oral mucosal samples. The 29 OSCC tissue samples were of various stages and histological grades (Table I). TaqMan-based microRNA assays of 768 different miRNA targets were performed, revealing a total of 177 detectable miRNAs. Volcano plots show differential expression between normal and cancer tissues (Fig. 1). We subsequently calculated the degree of differential expression, revealing that miRNAs >4-fold upregulated or downregulated could be considered as candidate miRNAs. miR-31*, miR-31, miR-135b, miR-193a-5p, miR-103, miR-224, miR-93, miR-200c, miR-183, miR203, miR-21 and miR-223 were shown to be upregulated, while miR-133a, miR-376c, miR-411, miR-30a-3p, miR-489, miR-139-5p, miR-483-5p, miR-30e-3p, miR-409-3p, let-7c and miR-486-5p were shown to be downregulated in OSCC (Tables II and III).

Differential expression of miRNAs between non-metastatic (-) and metastatic (+) OSCC. As cervical lymph node metastasis is directly correlated with oral cancer prognosis, we subsequently aimed to determine whether miRNA expression was associated with outcome in OSCC patients. Volcano plots show the differential expression of miRNAs in non-metastatic (-) and metastatic (+) OSCC (Fig. 2). We subsequently calculated the level of significance of the differential expression between these groups. miR-489, miR-483-5p and miR-1291 were shown to be downregulated in OSCC (Table IV). In addition, a decision tree model was used to classify the two groups (Fig. 3), doing so with 82% accuracy.

Table I. Demographic and clinical features of the OSCC patients.

Characteristic	n (%)
Gender	
Male	20 (69)
Female	9 (31)
Smoking habit	
Positive	8 (28)
Negative	21 (72)
Drinking habit	
Positive	9 (31)
Negative	20 (69)
WHO classification	
Well	10 (34)
Moderate	8 (28)
Poor	11 (38)
YK grade	
1	1 (4)
2	3 (10)
3	13 (45)
4	12 (41)
Local recurrence	
Positive	10 (34)
Negative	19 (66)
Age (years)	
>65	21 (72)
≤65	8 (28)
Stage	
I	6 (21)
II	9 (31)
III	3 (10)
IV	11 (38)
T status	
I	6 (21)
II	14 (48)
III	2 (7)
IV	7 (24)
N status	
N0	15 (51)
N1	4 (13)
N2	10 (36)

Discussion

miRNAs are a new class of non-coding small RNAs that regulate cell proliferation and various cellular functions by interfering with the translation of the target mRNAs (3,4,12,13). Recent studies have demonstrated that altered expression of miRNAs induces various human malignancies (14,15).

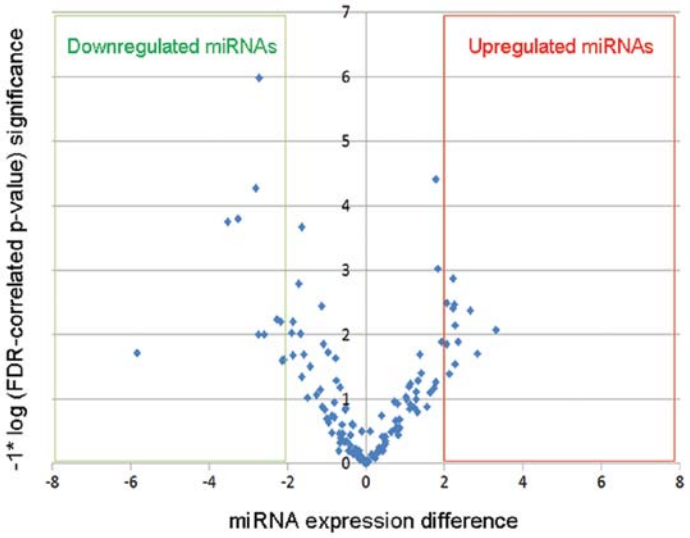

Figure 1. Comparison of miRNA expression between OSCC and normal oral mucosa. Volcano plots of 177 miRNAs analyzed for comparison between OSCC and normal oral mucosa. The level of differential miRNA expression between OSCC and normal oral mucosa is plotted on the x-axis, and the P-value of a FDR-corrected Wilcoxon signed-rank test of the differences (-1* log10 scale) is indicated on the y-axis. miRNAs significantly different between the two groups are boxed in red and green.

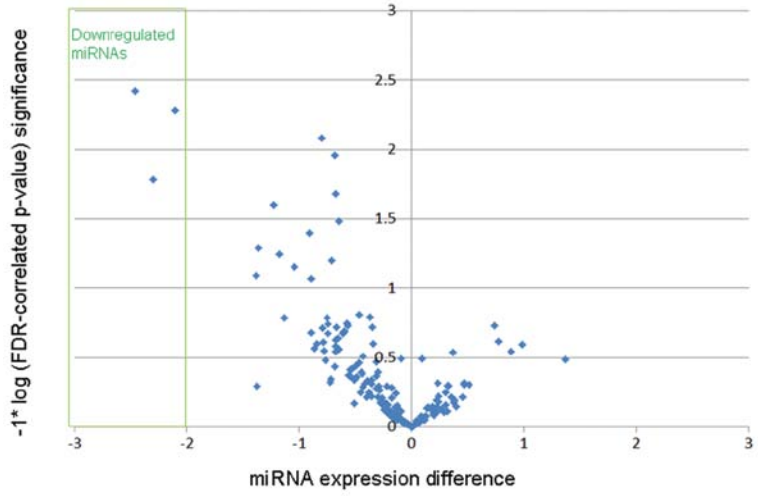

Figure 2. Comparison of miRNA expression between non-metastatic and metastatic OSCC. Volcano plots of 177 miRNAs analyzed for comparison between non-metastatic and metastatic OSCC. The level of differential miRNA expression between non-metastatic and metastatic OSCC is plotted on the x-axis, and the P-value of a FDR-corrected Wilcoxon signed-rank test of the differences (-1* log10 scale) is indicated on the y-axis. miRNAs significantly different between the two groups are boxed in green.

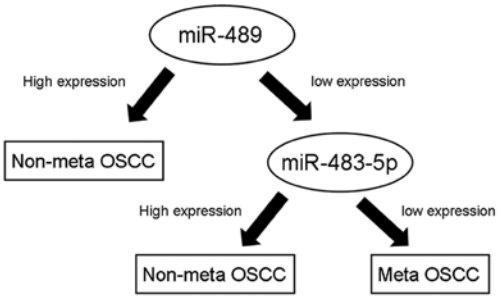

Figure 3. A decision making model was constructed to classify the oral cancer samples according to metastasis using miRNA expression using a training set of oral cancers (n=29). miR-489 (Ct-value ≤10.8; high expression) and miR-483-5p (Ct-value ≤12.1; high expression) resulted in the most accurate prediction (82% accuracy) for classifying the samples into the two corresponding groups.

However, little is known about the role of miRNAs in OSCC. In this study, we investigated the miRNA expression profiles of normal oral mucosa and OSCC tissues. miRNA profiling in B cell lymphoma (16), prostate (17), and colon cancer (18) was previously performed; however, to the best of our knowledge, this is the first comprehensive miRNA profiling study of oral cancer through exhaustive analysis.

miRNA analysis was performed using TaqMan microRNA Array v2.0, which consists of 768 candidate miRNA sites. A total of 177 miRNAs were detected in the oral cancer tissues and normal oral mucosa. Volcano plot analysis subsequently revealed aberrant expression of miRNAs in OSCC (Fig. 2). This result suggests that miRNA expression profiles differ between normal and abnormal cells.

Table II. Differentially expressed miRNAs showing increased expression in OSCC compared to normal mucosa.

microRNA	Fold-change (Ct-Ca/Ct-N)	FDR	Chromosome location	Putative target
hsa-miR-31*	0.60	0.008	9p21.3	RSBN1, ARHGEF2, IDE, NR5A2, SH2D1A
hsa-miR-31	0.29	0.020	9p21.3	RSBN1, ARHGEF2, IDE, NR5A2, SH2D1A
hsa-miR-135b	0.68	0.004	1q32.1	ANGPT2, GK5, NR3C2, GULP1, LOC221710
hsa-miR-193a-5p	0.77	0.013	17q11.2	HSPB6, ZNF385C, ITSN1, OLIG3, USO1
hsa-miR-103	0.76	0.007	5q34	DICER1, TMEM16C, NF1, FOXP1, HRB
hsa-miR-224	0.71	0.029	Xq28	ZDHHC20, AFF3, U2SURP, C8orf44, TTC3
hsa-miR-93	0.64	0.003	7q22.1	FGD4, PKD2, MAP3K2, ZNFX1, PDCD1LG
hsa-miR-200c	0.27	0.004	12p13.31	ZEB1, FAM122C, ZEB2, LRP1B, WIPF1
hsa-miR-183	0.82	0.001	7q32.2	ABAT, AKAP12, PIGX, PTPN4, REV1
hsa-miR-203	0.22	0.040	14q32.33	ZNF281, CAMTA1, B3GNT5, LIFR, ABCE1
hsa-miR-21	0.54	0.014	17q23.1	ANF367, GPR64, YOD1, PHF14, PLEKHA1
hsa-miR-223	0.27	0.003	Xq12	FBXW7, SP3, PAX6, C13orf31, PURB

Fold-changes are expressed as the ratio of OSCC Ct values vs. normal mucosa Ct values. FDR, false discovery rate. The top putative targets identified with TargetScan are included.

Table III. Differentially expressed miRNAs showing decreased expression in OSCC compared to normal mucosa.

microRNA	Fold-change (Ct-Ca/Ct-N)	FDR	Chromosome location	Putative target
hsa-miR-133a	3.13	0.019	18q.11.2	SYT2, LHFP, CCBL2, BRUNOL4, TTPAL
hsa-miR-376c	1.55	<1e-03	14q32.31	ARFGEF1, PAPSS2, GABRG2, SYF2, ARFGEF2
hsa-miR-411	1.52	<1e-03	14q32.31	ELFN1, SLC4A7, C16orf52, C21orf91, SPRY4
hsa-miR-30a-3p	1.75	<1e-03	6q13	NUFIP2, ZNF85, RUNDC2B, FIGN, POU4F1
hsa-miR-489	1.36	0.010	7q21.3	ETNK1, PARM1, CWC25, NRIP1, PNISR
hsa-miR-139-5p	1.65	<1e-03	11q13.4	TMF1, USP6NL, TBX1, SCAPER, NDRG2
hsa-miR-483-5p	1.33	0.010	11p15.5	SELO, MPZ, SRSF4, SLC12A5, RNF165
hsa-miR-30e-3p	1.54	0.006	1p34.2	NUFIP2, ZNF85, RUNDC2B, POU4F1, DSN
hsa-miR-409-3p	1.42	0.006	14q32.31	MRPL35, LRRN4CL, POMP, MTF2, TMEM65
hsa-let-7c	1.29	0.025	21q21.1	C14orf28, FIGNL2, HMGA2, LIN28B, TRIM71
hsa-miR-486-5p	1.49	0.025	8p11.21	FOXO1, GPX8, PTEN, TRAPPC6B, TWF1

Fold-changes are expressed as the ratio of OSCC Ct values vs. normal mucosa Ct values. FDR, false discovery rate. The top putative targets identified with TargetScan are included.

Table IV. Differentially expressed miRNAs showing decreased expression in non-metastatic and metastatic OSCC.

microRNA	Fold-change (Ct-Ca/Ct-N)	FDR	Chromosome location	Putative target
hsa-miR-489	1.27	0.004	7q21.3	ETNK1, ALS2CR13, HRH4, LONRF2, SFRS7
hsa-miR-1291	1.18	0.017	12q13.11	AQP1, ARID3B, MECP2, MAP3K9, PGM5
hsa-miR-483-5p	1.22	0.005	11p15.5	RAB3IP, ZBTB26, PGAM1, C5orf42, RBAK

Fold-changes are expressed as the ratio of OSCC Ct values vs. normal mucosa Ct values. FDR, false discovery rate. The top putative targets identified with TargetScan are included.

To identify candidate cancer-related miRNAs, we calculated the degree of differential expression of miRNAs between normal oral mucosa and OSCC tissues. Twelve miRNAs (miR-31*, miR-31, miR135b, miR-193a-5p, miR-103, miR-224, miR-93, miR-200c, miR-183, miR-203, miR-21 and miR-223) were significantly upregulated in most of the cancer tissue samples compared with the normal oral mucosa. Those showing >4-fold upregulation were considered candidate miRNAs; namely, miR-31*, miR-31, miR-135b, miR-193a-5p, miR-103, miR-224, miR-93, miR-200c, miR-183, miR-203, miR-21 and miR-223.

miR-21 has been investigated widely in various human malignancies including hematological malignancies and glioblastoma as a putative oncogenic miRNA (5,19-26). Moreover, aberrantly expressed miR-203 has been detected in colon (27), breast (28) and ovarian cancer (29-31), while miR-31 is also known to have oncogenic functions in esophageal cancer (32). Upregulation of both miR-31 and miR-31* by delivery of pre-miR-31 was also shown to enhance OSCC oncogenicity (33). Our results also indicated that miR-31 and miR-31* have a biological function in cancer development. However, a role in oral cancer has yet to be documented, and the function of these candidates in human cancer remains unclear. miR-133a, miR-376c, miR-411, miR-30a-3p, miR-489, miR-139-5p, miR-483-5p, miR-30e-3p, miR-409-3p, let-7c and miR-486-5p showed a >4-fold downregulation (Table III). Previously, miR-133a was shown to be downregulated in breast cancer tissues and correlated with poor prognosis (34), while let-7c is reportedly related to tumor growth inhibition in prostate cancer (35).

Cervical lymph node metastasis has a large impact on the prognosis of OSCC. We, therefore, performed volcano plot analysis according to the metastatic state. Three downregulated miRNAs (niR-489, miR-1291 and miR-483-5p) (Table IV) were detected, and subsequently, a decision tree model was constructed to classify non-metastatic and metastatic OSCC using Weka software. Our model divided the two groups with 82% accuracy (Fig. 3). This model may therefore have potential in determining the prognosis of OSCC patients, acting as a predictive marker of metastasis.

In summary, our findings identifying cancer-related miRNAs in OSCC suggest that oral cancer may have a unique miRNA expression pattern at the individual level. Further investigations are now required to determine the molecular functions and mechanisms of these miRNAs as well as their potential use as prognostic and/or diagnostic markers in oral cancer.

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