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MicroRNA expression signature of oral squamous cell carcinoma: functional role of *microRNA-26a/b* in the modulation of novel cancer pathways

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Background: MicroRNAs (miRNAs) have been shown to play major roles in carcinogenesis in a variety of cancers. The aim of this study was to determine the miRNA expression signature of oral squamous cell carcinoma (OSCC) and to investigate the functional roles of *miR-26a* and *miR-26b* in OSCC cells.

Methods: An OSCC miRNA signature was constructed by PCR-based array methods. Functional studies of differentially expressed miRNAs were performed to investigate cell proliferation, migration, and invasion in OSCC cells. *In silico* database and genome-wide gene expression analyses were performed to identify molecular targets and pathways mediated by *miR-26a/b*.

Results: *miR-26a* and *miR-26b* were significantly downregulated in OSCC. Restoration of both *miR-26a* and *miR-26b* in cancer cell lines revealed that these miRNAs significantly inhibited cancer cell migration and invasion. Our data demonstrated that the novel transmembrane *TMEM184B* gene was a direct target of *miR-26a/b* regulation. Silencing of *TMEM184B* inhibited cancer cell migration and invasion, and regulated the actin cytoskeleton-pathway related genes.

Conclusions: Loss of tumour-suppressive *miR-26a/b* enhanced cancer cell migration and invasion in OSCC through direct regulation of *TMEM184B*. Our data describing pathways regulated by tumour-suppressive *miR-26a/b* provide new insights into the potential mechanisms of OSCC oncogenesis and metastasis.

In the post-genome sequencing era, the discovery of noncoding RNAs in the human genome was an important conceptual breakthrough in the study of cancer (Carthew and Sontheimer, 2009). Further improvement of our understanding of noncoding RNAs is necessary for continued elucidation of the mechanisms of cancer initiation, development, and metastasis. MicroRNAs (miRNAs) are endogenous small noncoding RNA molecules (19–22 bases in length) that regulate protein-coding gene expression by repressing translation or cleaving RNA transcripts in a sequence-specific manner (Bartel, 2004). A substantial amount of evidence has suggested that miRNAs are aberrantly expressed in many human cancers and play significant roles in human oncogenesis and metastasis (Filipowicz *et al*, 2008; Hobert, 2008; Friedman *et al*, 2009; Iorio and Croce, 2009).

It is believed that normal regulatory mechanisms can be disrupted by the aberrant expression of tumour-suppressive or oncogenic miRNAs in cancer cells. Therefore, the identification of aberrantly expressed miRNAs is an important first step toward

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elucidating the details of miRNA-mediated oncogenic pathways. On the basis of this background, we have previously evaluated miRNA expression signatures using squamous cell carcinoma (SCC) clinical specimens, for example, maxillary sinus-SCC (Nohata *et al*, 2011c; Nohata *et al*, 2011d; Kinoshita *et al*, 2012d), hypopharyngeal-SCC (Kikkawa *et al*, 2010; Fukumoto *et al*, 2014), and oesophageal-SCC (Kano *et al*, 2010) and investigated the roles of miRNAs in human SCC oncogenesis and metastasis using differentially expressed miRNAs (Nohata *et al*, 2011a; Kinoshita *et al*, 2012c; Kinoshita *et al*, 2013). Elucidation of aberrantly expressed miRNAs in several types of human SCC specimens and of novel cancer pathways regulated by tumour-suppressive miRNAs will provide new insights into the potential mechanisms of SCC oncogenesis.

Oral squamous cell carcinoma (OSCC) accounts for over 95% of oral cavity cancer and 40% of head and neck SCC. In developing countries, OSCC is the sixth most common cancer in men and the tenth most common cancer in women, and the incidence is increasing among young people and women (Bhattacharya *et al*, 2011). Oral squamous cell carcinoma is associated with a poor prognosis and has a 5-year survival rate of less than 50% owing to the tendency of the cancer to metastasise (Bhattacharya *et al*, 2011). Moreover, despite recent advances in various treatment modalities, including surgery, radiotherapy, and chemotherapy, the survival rates of patients with OSCC have not markedly improved (Wikner *et al*, 2014). Therefore, understanding molecular oncogenic pathways based on the current genome-based approaches underlying OSCC could significantly improve diagnosis, therapy, and prevention of the disease.

In this study, we constructed the miRNA expression signature of OSCC using clinical specimens. Using these data, we investigated the specific roles of miRNAs in OSCC oncogenesis and metastasis by examining differentially expressed miRNAs. Data from our present OSCC signature showed that *miR-26a* and *miR-26b* were significantly downregulated in OSCC tissues, suggesting that *miR-26a* and *miR-26b* may act as tumour suppressors. Several studies of *miR-26a/b* have reported that these miRNAs have various functions and target genes in many types of cancers (Lu *et al*, 2011; Deng *et al*, 2013; Chen *et al*, 2014; Li *et al*, 2014). These reports have indicated that *miR-26a/b* may play critical roles in cancer cells and may mediate oncogenesis and metastasis. However, the functional roles of *miR-26a/b* in OSCC are still unknown.

The aim of the present study was to investigate the functional significance of *miR-26a/b* and to identify the molecular targets and pathways mediated by these miRNAs in OSCC cells. Our data demonstrated that restoration of mature *miR-26a* and *miR-26b* inhibited cancer cell migration and invasion. Moreover, gene expression data and *in silico* database analysis showed that the *TMEM184B* gene was a direct target of *miR-26a/b* regulation. Silencing of the *TMEM184B* gene significantly inhibited the migration and invasion of cancer cells and caused alterations in genes involved in regulation of the actin cytoskeleton pathway. The discovery of pathways mediated by tumour-suppressive *miR-26a/b* provides important insights into the potential mechanisms of OSCC oncogenesis and suggests novel therapeutic strategies for the treatment of OSCC.

MATERIALS AND METHODS

Oral squamous cell carcinoma clinical specimens and cell lines. A total of 36 pairs of primary tumours and corresponding normal epithelial tissue samples were obtained from patients with OSCC at Chiba University Hospital from 2008 to 2013. The patients' background and clinicopathological characteristics are shown in Table 1. The patients were classified according to the 2002 Union for International Cancer Control (UICC) staging criteria before treatment. Written consent for tissue donation for research purposes was obtained from each patient before tissue collection. The protocol was approved by the institutional review board of Chiba University. The fresh specimens were immediately immersed in RNAlater (Qiagen, Valencia, CA, USA) and stored at −20 °C until RNA was extracted.

SAS (derived from a primary tongue SCC) and HSC3 (derived from a lymph node metastasis of tongue SCC) OSCC cells were used in this study. Cells were cultured in Dulbecco's modified Eagle's medium with 10% foetal bovine serum in a humidified 5% CO_2 atmosphere at 37 °C.

Construction of the miRNA expression signature of OSCC. MicroRNA expression patterns were evaluated using the TaqMan LDA Human microRNA Panel v2.0 (Applied Biosystems, Foster City, CA, USA). The assay was composed of two steps: (i) generation of cDNA by reverse transcription and (ii) a TaqMan real-time polymerase chain reaction (PCR) assay. A description of the real-time PCR assay and the list of human miRNAs included in the panel can be found on the manufacturer's website (http://www.appliedbiosystems.com). Analysis of relative miRNA expression data was performed using GeneSpring GX software version 7.3.1 (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions. A cut-off *P*-value of less than 0.05 was used to narrow down the candidates after global normalisation of the raw data. After global normalisation, additional normalisation was carried out with *RNU48*.

Quantitative real-time RT-PCR. The procedure for PCR quantification was described previously (Kinoshita *et al*, 2013; Goto *et al*, 2014a). Gene-specific PCR products were assayed continuously using a 7300 HT real-time PCR system according to the manufacturer's protocol. The expression levels of *miR-26a* (Assay ID: 000405) and *miR-26b* (Assay ID: 000407) were analysed by TaqMan quantitative real-time PCR (TaqMan MicroRNA Assay; Applied Biosystems) and normalised to *RNU48*. TaqMan probes and primers for *TMEM184B* (P/N: Hs00202153_m1), *CELSR1* (P/N: Hs00947712_m1), *JAG1* (P/N: Hs01070032_m1), *BID* (P/N: Hs00609632_m1), and *GUSB* (P/N: Hs99999908_m1) as an internal control were obtained from Applied Biosystems (Assay-On-Demand Gene Expression Products).

Transfection with mature miRNAs and small-interfering RNA (siRNA). The following mature miRNAs species were used in this study: mirVana miRNA mimics for *hsa-miR-26a-5p* (product ID: PM10249) and *hsa-miR-26b-5p* (product ID: PM12899; Applied Biosystems). The following siRNAs were used: Stealth Select RNAi siRNA targeting *TMEM184B* (si-*TMEM184B*, cat no. HSS119359) and negative control miRNA/SiRNA (P/N: AM17111, Applied Biosystems). Transfection methods were described as previously (Kinoshita *et al*, 2013; Fukumoto *et al*, 2014; Nishikawa *et al*, 2014a).

Cell proliferation, migration, and invasion assays. SAS and HSC3 cells were transfected with 10 nM miRNAs or si-RNA by reverse transfection. Cell proliferation, migration, and invasion assays were performed as described previously (Kinoshita *et al*, 2013; Fukumoto *et al*, 2014; Nishikawa *et al*, 2014b).

Identification of putative target genes regulated by *miR-26a/b*. Genes regulated by *miR-26a/b* were obtained from the TargetScan database (http://www.targetscan.org). To investigate the expression status of candidate *miR-26a/b* target genes in OSCC clinical specimens, we examined gene expression profiles in the Gene Expression Omnibus (GEO) database (Accession Number GSE41613 and GSE42743). The strategy behind this analysis procedure was described previously (Goto *et al*, 2014b).

Table 1. Clinical features of 36 OSCC patients

No.	Age	Sex	Location	T	N	M	Stage	Differentiaion
1	66	M	Tongue	2	0	0	II	Moderate
2	65	M	Oral floor	4a	1	0	IVA	Moderate
3	67	M	Tongue	4a	2c	0	IVA	Moderate
4	36	F	Tongue	3	1	0	III	Moderate
5	73	M	Tongue	3	2b	0	IVA	Poor
6	63	F	Oral floor	2	2b	0	IVA	Basaloid SCC
7	77	M	Gum	2	0	0	II	Moderate
8	68	M	Tongue	2	0	0	II	Well
9	76	F	Tongue	1	0	0	I	Well
10	69	M	Tongue	1	0	0	I	Well
11	73	F	Tongue	1	0	0	I	Well
12	64	M	Tongue	1	0	0	I	Well
13	64	M	Tongue	1	0	0	I	Well
14	82	M	Oral floor	1	0	0	I	Well
15	67	M	Oral floor	4a	2b	0	IVA	Well
16	67	M	Tongue	3	0	0	III	Moderate
17	64	M	Tongue	3	2b	0	IVA	Moderate
18	59	M	Tongue	1	2a	0	IVA	Moderate
19	47	M	Oral floor	1	0	0	I	Moderate
20	67	M	Tongue	2	0	0	II	Poor ~ moderate
21	70	M	Tongue	1	0	0	I	Well
22	38	M	Tongue	1	0	0	I	Well
23	70	M	Tongue, Oral floor	2	0	0	II	Well
24	51	M	Tongue	1	0	0	I	Well
25	81	M	Tongue	is	0	0	0	Extremely well
26	34	F	Tongue	1	0	0	I	Poor
27	42	M	Gum	4a	0	0	IVA	Moderate
28	70	M	Tongue	1	0	0	I	Moderate
29	71	M	Tongue	1	0	0	I	Well
30	60	F	Tongue	2	I	0	III	Well
31	77	M	Tongue	2	2b	0	IVA	Poorly
32	64	F	Oral floor	4a	2c	0	IVA	Moderate
33	68	M	Tongue	1	0	0	I	Well
34	29	F	Tongue	1	0	0	I	Poorly
35	71	M	Buccal mucosa	2	1	0	III	Poorly
36	39	M	Tongue	4a	0	0	IVA	Moderate

Abbreviations: F = female; M = male; SCC = squamous cell carcinoma.

Identification of downstream pathways and genes regulated by *TMEM184B*. To identify molecular pathways regulated by *TMEM184B* in cancer cells, we performed gene expression analysis using si-*TMEM184B*-transfected SAS and HSC3 cells. An oligo-microarray (human 60Kv; Agilent Technologies) was used for gene expression studies. Gene expression data were categorised according to the Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways using the GENECODIS program (http://genecodis.dacya.ucm.es). The strategy behind this analysis procedure was described (Kinoshita *et al*, 2013; Nishikawa *et al*, 2014b).

Western blotting. Cells were harvested 72 h after transfection, and lysates were prepared. Next, 20 μg of protein lysates were separated on Mini-PROTEAN TGX Gels (Bio-Rad, Hercules, CA, USA) and transferred to PVDF membranes. Immunoblotting was performed with rabbit anti-TMEM184B antibodies (1 : 500; PA-5-20932 (Thermo, Waltham, MA, USA)), and anti-GAPDH antibodies (1 : 1000; ab 8245 (Abcam, Cambridge, UK)) were used as an internal loading control.

Plasmid construction and dual-luciferase reporter assay. Partial wild-type sequences of the *TMEM184B* 3′-untranslated region (UTR) or those with a deleted *miR-26a/b* target site (position 1982–1989 of the *TMEM184B* 3′-UTR) were inserted between the *Xho*I-*Pme*I restriction sites in the 3′-UTR of the *hRluc* gene in the psiCHECK-2 vector (C8021; Promega, Madison, WI, USA). The procedure for the dual-luciferase reporter assay was described previously (Kinoshita *et al*, 2013; Fukumoto *et al*, 2014; Nishikawa *et al*, 2014b).

Statistical analysis. The relationship between two groups and the numerical values obtained by real-time PCR were analysed using the paired *t*-test. Spearman's rank test was used to evaluate the

correlation between the expression of *miR-26a/b* and *TMEM184B*. Relationships among more than three variables and numerical values were analysed using the Bonferroni-adjusted Mann-Whitney *U*-test. All analyses were performed using Expert StatView software (Version 4; SAS Institute, Cary, NC, USA).

RESULTS

Identification of downregulated miRNAs in OSCC by miRNA expression signatures. We evaluated mature miRNA expression levels of five pairs of normal epithelial tissues and OSCC tissues (patient numbers 1–5, Table 1) by miRNA expression signature by using PCR-based array analysis. In total, 23 significantly downregulated miRNAs were selected after normalisation to *RNU48* (Table 2).

Expression of *miR-26a/b* in OSCC clinical specimens and cell lines. To validate miRNA signature results, we evaluated *miR-26a/b* expression in 36 clinical OSCC specimens (Table 1). The expression levels of both *miR-26a* and *miR-26b* were

Table 2. Downregulated miRNAs in OSCC

MicroRNA	Accession no.	Location	*P*-value	Normal	Tumour	Fold change (tumour/normal)
miR-126-5p	MIMAT0000444	9q34.3	0.0002	0.0697	0.0191	0.273
miR-145-5p	MIMAT0000437	5q32	0.0004	0.1045	0.0347	0.332
miR-145-3p	MIMAT0004601	5q32	0.0008	0.0022	0.0009	0.403
miR-26b-5p	MIMAT0000083	2q35	0.0011	0.0440	0.0214	0.486
miR-26a-5p	MIMAT0000082	3p22.2 12q14.1	0.0014	0.1722	0.0745	0.432
miR-204	MIMAT0000265	9q21.12	0.0029	0.0043	0.0004	0.088
miR-29c	MIMAT0000681	1q32.2	0.0035	0.1156	0.0416	0.360
miR-195	MIMAT0000461	17p13.1	0.0068	0.0609	0.0228	0.375
miR-30c	MIMAT0000244	1p34.2 6q13	0.0072	0.2374	0.1037	0.437
miR-10b	MIMAT0000245	2q31.1	0.0072	0.0093	0.0041	0.442
miR-656	MIMAT0003332	14q32.31	0.0082	0.0001	0.0000	0.267
miR-30e-3p	MIMAT0000693	1p34.2	0.0094	0.0339	0.0094	0.279
miR-140-5p	MIMAT0000431	16q22.1	0.0094	0.0810	0.0378	0.466
miR-23b	MIMAT0000418	9q22.32	0.0095	0.0066	0.0027	0.410
miR-10b	MIMAT0000254	2q31.1	0.0108	0.0043	0.0017	0.404
miR-126-3p	MIMAT0000445	9q34.3	0.0118	1.7499	0.6259	0.358
miR-143	MIMAT0000435	5q32	0.0125	0.0749	0.0345	0.460
miR-30d	MIMAT0000245	8q24.22	0.0133	0.0007	0.0003	0.375
miR-139-5p	MIMAT0000250	11q13.4	0.0134	0.0621	0.0099	0.160
miR-19b-1-5p	MIMAT0004491	13q31.3	0.0195	0.0009	0.0003	0.393
miR-598	MIMAT0003266	8p23.1	0.0201	0.0024	0.0011	0.473
miR-885-5p	MIMAT0004947	3p25.3	0.0201	0.0024	0.0003	0.125
miR-376c	MIMAT0000720	14q32.31	0.0220	0.0086	0.0017	0.192
miR-487b	MIMAT0003180	14q32.31	0.0231	0.0005	0.0001	0.268
miR-101	MIMAT0000099	1p31.3 9p24.1	0.0231	0.0012	0.0005	0.385
miR-886-5p	MIMAT0005527		0.0242	0.0125	0.0036	0.287
miR-140-3p	MIMAT0004597	16q22.1	0.0251	0.0070	0.0023	0.327
miR-30e	MIMAT0000692	1p34.2	0.0252	0.0489	0.0213	0.435
miR-125b	MIMAT0000423	11q24.1 24q21.1	0.0271	0.1164	0.0562	0.483
miR-378a-5p	MIMAT0000731	5q32	0.0295	0.0027	0.0006	0.214
miR-320	MIMAT0000510	8p21.3	0.0302	0.2007	0.0803	0.400
miR-136-3p	MIMAT0004606	14q.32.2	0.0320	0.0017	0.0002	0.120
miR-26a-1-3p	MIMAT0004499	3p22.2	0.0342	0.0005	0.0001	0.153
miR-127-3p	MIMAT0000446	14q32.2	0.0342	0.0098	0.0032	0.324
miR-411	MIMAT0003329	14q32.31	0.0426	0.0033	0.0008	0.245
miR-30a-3p	MIMAT0000088	6q13	0.0450	0.0409	0.0060	0.147
miR-29c-5p	MIMAT0004673	1q32.2	0.0455	0.0009	0.0003	0.325
miR-376a	MIMAT0000729	14q32.31	0.0466	0.0007	0.0002	0.208
miR-26b-3p	MIMAT0004500	2q35	0.0473	0.0006	0.0002	0.418
miR-770-5p	MIMAT0003948	14q32.2	0.0475	0.0004	0.0001	0.416
miR-433	MIMAT0001627	14q.32.2	0.0477	0.0005	0.0001	0.268
miR-375	MIMAT0000728	2q35	0.0483	0.0226	0.0020	0.090

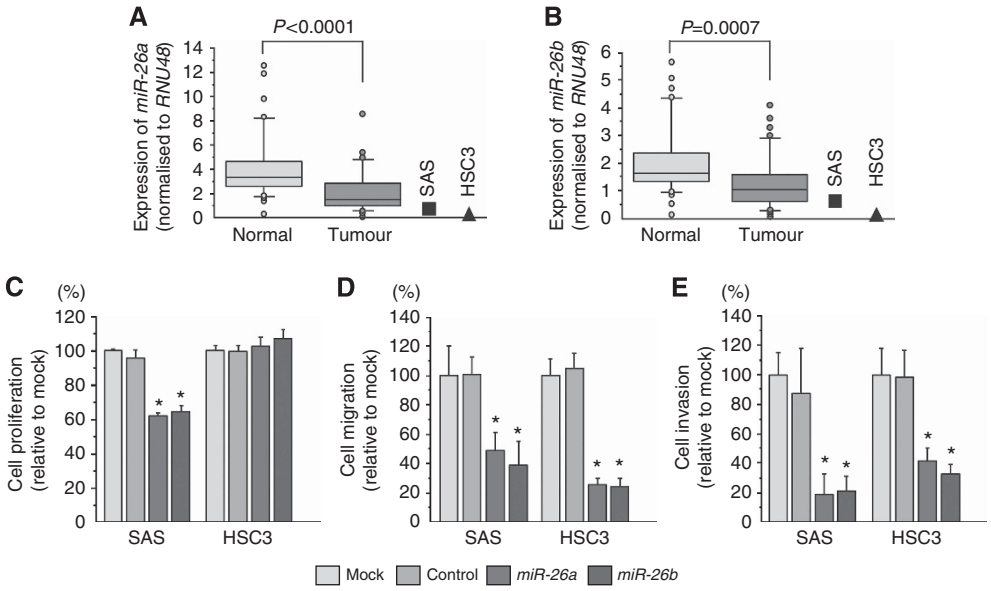

Figure 1. Expression levels of *miR-26a/b* in OSCC clinical specimens and cell lines, and functional significance of *miR-26a/b* in OSCC cell lines. (**A**, **B**) Expression levels of *miR-26a* (**A**) and *miR-26b* (**B**) in OSCC clinical specimens and cell lines (SAS and HSC3). *RNU48* was used for normalisation. (**C**) Cell proliferation was determined by XTT assay 72 h after transfection with 10 nM *miR-26a/b*. (**D**) Cell migration was determined by migration assay 48 h after transfection with 10 nM *miR-26a/b*. (**E**) Cell invasion was determined by Matrigel invasion assay 48 h after transfection with 10 nM *miR-26a/b*. *$P<0.001$.

significantly lower in tumour tissues and cell lines (SAS and HSC3) than in corresponding normal epithelia (Figure 1A and B).

Effects of restoring *miR-26a/b* on cell proliferation, migration, and invasion activities in OSCC cell lines. To investigate the functional effects of *miR-26a/b* in OSCC cells, we performed gain-of-function studies using mature miRNA transfection into SAS and HSC3 cells. XTT assays demonstrated that proliferation was not inhibited in HSC3 cells, whereas SAS cell proliferation was significantly slowed by transfection, in comparison with mock or miR-control transfectants (Figure 1C).

The migration assay demonstrated that migration activity was significantly inhibited in *miR-26a/b* transfectants in comparison with mock or miR-control transfectants (Figure 1D).

The Matrigel invasion assay demonstrated that invasion activity was significantly inhibited in *miR-26a/b* transfectants in compa rison with mock or miR-control transfectants (Figure 1E).

Identification of putative target genes regulated by *miR-26a/b* in OSCC cells. To identify putative target genes regulated by *miR-26a/b*, we applied a combination of *in silico* analysis and genome-wide gene expression analysis. Our strategy for selection of *miR-26a/b* target genes is shown in Supplementary Figure 1. First, we screened *miR-26a/b*-targeted genes that contained a putative *miR-26a/b* binding site in their 3′-UTR using the TargetScan database and identified 3419 candidate genes. The gene set was then analysed with a publicly available gene expression data set in GEO (accession numbers: GSE41613 and GSE42743), and genes upregulated (log2 ratio >0.5) in OSCC were chosen. As a result, 14 candidate genes were identified as *miR-26a/b* targets, and four genes had a conserved *miR-26a/b* binding site in their 3′-UTRs (Table 3).

We investigated the expression levels of these four candidate genes in OSCC clinical specimens and cell lines (Figure 2A and Supplementary Figure 2). Among these candidate genes, we focused on the *TMEM184B* gene because it was the most significantly downregulated gene in *miR-26a/b* transfectants, and Spearman's rank test showed the most significant negative correlation of *TMEM184B* with the expression of *miR-26a/b* (Figure 2B and C).

CELSR1 is one of the putative candidate target of *miR-26a/b* regulation because this gene has a *miR-26a/b* conserved site. We also examined functional significance of *CELSR1* by using *si-CELSR1* transfectants. Our data showed that silencing of *CELSR1* inhibited cancer cell proliferation, migration, and invasion in SAS and/or HSC3 cells, suggesting *CELSR1* acts as an oncogene in OSCC cells (Supplementary Figure 3).

***TMEM184B* was a direct target of *miR-26a/b* in OSCC cells.** Next, we investigated whether *TMEM184B* expression was reduced by restoration of *miR-26a/b* in OSCC cells. The mRNA and protein expression of *TMEM184B* was significantly repressed in *miR-26a/b* transfectants compared with mock- or miR-control-transfected cells (Figure 3A and B).

Thus, to investigate whether *TMEM184B* mRNA had a target site for *miR-26a/b*, we performed luciferase reporter assays in SAS cells. The TargetScan database showed that there was one putative *miR-26a/b* binding site in the *TMEM184B* 3′-UTR (position 1982–1989). We used vectors encoding either a partial wild-type sequence (including the predicted *miR-26a/b* target site) or deletion of the seed sequence of the 3′-UTR of *TMEM184B* mRNA. We found that the luminescence intensity was significantly reduced by cotransfection with *miR-26a/b* and the vector carrying the wild-type 3′-UTR of *TMEM184B* (Figure 3C).

Effects of silencing *TMEM184B* on cell proliferation, migration, and invasion in OSCC cell lines. To investigate the functional roles of *TMEM184B* in OSCC cell lines, we performed loss-of-function studies using *si-TMEM184B* transfection. First, we evaluated the knockdown efficiency of *si-TMEM184B* transfection in SAS and HSC3 cells. Western blotting and qRT-PCR indicated that the siRNA effectively downregulated *TMEM184B* expression in both cell lines (Figure 4A and B).

XTT assays demonstrated that cell proliferation was significantly inhibited in *si-TMEM184B* transfectants in comparison with the mock or si-control (Figure 4C).

Moreover, migration assays demonstrated that cell migration activity was significantly inhibited in *si-TMEM184B* transfectants in comparison with mock or si-control-transfected cells (Figure 4D).

Table 3. Candidate genes targeted by *miR-26a/b*

Gene symbol	Representative transcript	Gene name	Conserved	Poorly conserved	Fold change (log2 ratio)
CELSR1	NM_014246	Cadherin, EGF LAG seven-pass G-type receptor 1 (flamingo homolog, Drosophila)	1	0	0.890
HMGB3	NM_005342	High mobility group box 3	0	1	0.768
TDG	NM_003211	Thymine-DNA glycosylase	0	1	0.685
VASP	NM_003370	Vasodilator-stimulated phosphoprotein	0	1	0.639
JAG1	NM_000214	Jagged 1	1	1	0.637
CTSB	NM_001908	Cathepsin B	0	1	0.628
BID	NM_001196	BH3 interacting domain death agonist	1	0	0.606
FEN1	NM_004111	Flap structure-specific endonuclease 1	0	1	0.573
MAP3K13	NM_001242314	Mitogen-activated protein kinase kinase kinase 13	0	3	0.571
TNFAIP8L1	NM_001167942	Tumour necrosis factor, alpha-induced protein 8-like 1	0	2	0.561
TMEM184B	NM_001195071	Transmembrane protein 184B	1	0	0.554
PIK3CD	NM_005026	Phosphoinositide-3-kinase, catalytic, delta polypeptide	0	1	0.544
SLC25A17	NM_006358	Solute carrier family 25 (mitochondrial carrier; peroxisomal membrane protein, 34 kDa), member 17	0	1	0.537
CLPTM1L	NM_030782	CLPTM1-like	0	1	0.525
CTNS	NM_001031681	Cystinosin, lysosomal cystine transporter	0	3	0.518

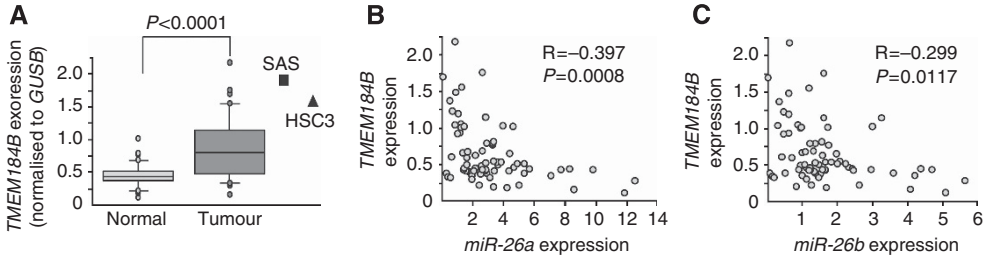

Figure 2. Expression levels of *TMEM184B* in OSCC clinical specimens and cell lines. (**A**) Expression levels of *TMEM184B* in OSCC clinical specimens and cell lines (SAS and HSC3). *GUSB* was used for normalisation. (**B**, **C**) Correlation between *TMEM184B* expression and *miR-26a* (**B**) or *miR-26b* (**C**).

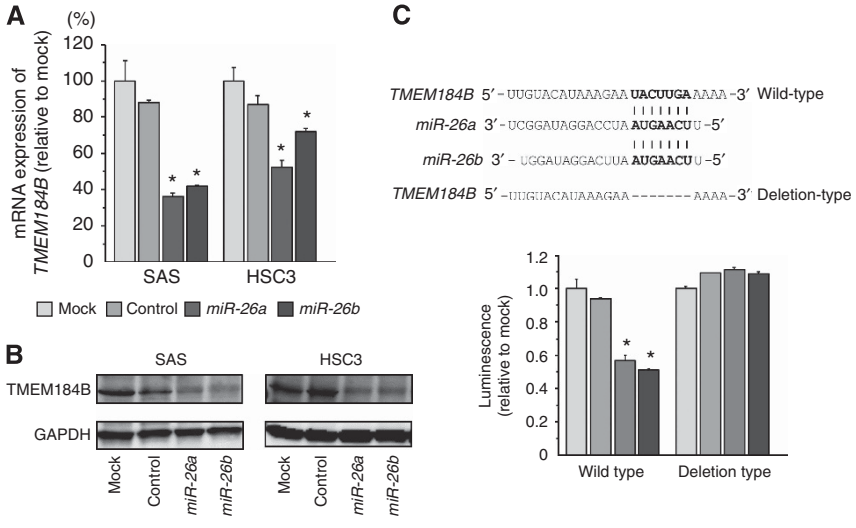

Figure 3. *TMEM184B* expression was directly regulated by *miR-26a/b* in OSCC cell lines. (**A**) *TMEM184B* mRNA expression 72 h after transfection with *miR-26a/b*. *GUSB* expression was used for normalisation. (**B**) *TMEM184B* protein expression 72 h after transfection with *miR-26a/b*. GAPDH was used as a loading control. (**C**) The *miR-26a/b* binding site in the 3′-UTR of *TMEM184B* mRNA. Luciferase reporter assays were performed using vectors that included (WT) or lacked (DEL) the wild-type sequences of the putative *miR-26a/b* target site. Renilla luciferase assays were normalised to firefly luciferase values. *$P<0.0001$.

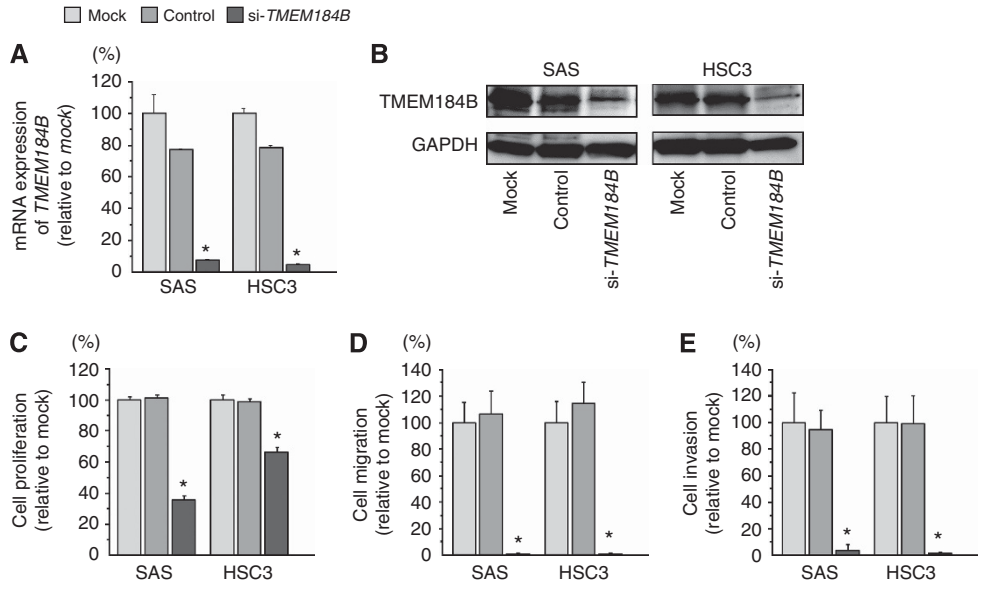

Figure 4. Effects of si-*TMEM184B* transfection on OSCC cell lines. (**A**) *TMEM184B* mRNA expression levels were measured by RT-PCR 72 h after transfection with 10 nM si-*TMEM184B*. *GUSB* was used for normalisation. (**B**) *TMEM184B* protein expression 72 h after transfection with si-*TMEM184B*. GAPDH was used as a loading control. (**C**) Cell proliferation was determined by XTT assay 72 h after transfection with 10 nM si-*TMEM184B*. (**D**) Cell migration was determined by migration assay 48 h after transfection with 10 nM si-*TMEM184B*. (**E**) Cell invasion was determined by Matrigel invasion assay 48 h after transfection with 10 nM si-*TMEM184B*. *$P<0.0001$.

Finally, Matrigel invasion assays demonstrated that cell invasion activity was significantly inhibited in *si-TMEM184B* transfectants in comparison with mock or si-control-transfected cells (Figure 4E).

Identification of downstream pathways regulated by *TMEM184B*. There are few reports describing the *TMEM184B* gene, and the function of this gene is still unknown. Therefore, we investigated the molecular pathways regulated by *TMEM184B* in cancer cells using genome-wide gene expression analysis in si-*TMEM184B* transfectants.

A total of 553 genes were commonly downregulated (log2 ratio < -1.0) in si-*TMEM184B* transfectants in both of SAS and HSC3 cell lines. We also assigned the downregulated genes to KEGG pathways using the GENECODIS program and identified a total of 23 pathways as significantly enriched annotated pathways (Table 4A). Among these pathways, we focused on genes in the 'regulation of actin cytoskeleton pathways' category (Table 4B). The top three significantly enriched pathways and the genes involved in these pathways are listed in Supplementary Tables 1–3.

DISCUSSION

A growing body of evidence has shown that miRNAs are involved in several biological processes and are tightly correlated with human oncogenesis and metastasis (Nelson and Weiss, 2008). Recent studies from our laboratory have identified a variety of novel SCC molecular pathways regulated by tumour-suppressive miRNAs. Moreover, the *miR-1/133a* cluster has been shown to regulate actin cytoskeletal pathways, the *miR-29a* and *miR-218* have been shown to regulate laminin-integrin pathways (Kinoshita *et al*, 2012a; Kinoshita *et al*, 2013), and *miR-874* has been shown to target histone deacetylase pathways based on the miRNA expression signatures of head and neck SCC (Nohata *et al*, 2011c). Evaluation of miRNA expression signatures using cancer specimens is an indispensable tool for cancer research. In this study, we have newly constructed the OSCC miRNA signature and identified several differentially expressed miRNAs in OSCC tissues. To compare with PCR-based SCC miRNA signatures from our previous studies, *miR-143*, *miR-139-5p*, *miR-30a-3p*, and *miR-375* were downregulated in all SCC miRNA signatures. Previous studies showed that *miR-143* and *miR-139-5p* act as tumour suppressors in nasopharyngeal carcinoma and laryngeal SCC (Zhong *et al*, 2013; Luo *et al*, 2014). Our recent data on maxillary sinus, hypopharyngeal, and oesophageal SCCs have suggested that *miR-375* is frequently downregulated and functions as a tumour suppressor that targets several oncogenic genes in cancer cells (Nohata *et al*, 2011b; Kinoshita *et al*, 2012b). These findings suggested the relevance of the OSCC miRNA signature provided in this study. Therefore, elucidation of the SCC molecular pathways mediated by tumour-suppressive miRNAs will contribute to our understanding of SCC oncogenesis and metastasis.

In the present study, we focused on *miR-26a* and *miR-26b* because the expression levels of these miRNAs were reduced in the OSCC signature and the functions of these miRNAs in OSCC cells are not known. Our data showed that both *miR-26a* and *miR-26b* were significantly reduced in OSCC specimens, and restoration of these miRNAs inhibited cancer cell migration and invasion, providing insights into the functional roles of *miR-26a* and *miR-26b* as tumour suppressors in OSCC cells. Silencing of protein-coding RNAs and miRNAs are well recognised by aberrant DNA methylation and epigenetic modification (Baylin, 2001). Aberrant DNA hypermethylation by overexpression of DNMT3b caused the silencing of *miR-26a/b* in breast cancer cell lines (Sandhu *et al*, 2012). Expression of *miR-26a* was increased by treatment of 5-aza-2′deoxycytidine in a prostate cancer cell line (Borno *et al*, 2012). These results indicated that silencing of *miR-26a/b* in cancer cells was caused by epigenetic modification, especially aberrant DNA hypermethylation. Further detailed analysis is necessary to understand the molecular mechanism of *miR-26a/b* silencing in OSCC cells.

Previous studies have shown that *miR-26a* and *miR-26b* act as tumour suppressors in several types of cancers targeting oncogenic genes, such as breast cancer (Li *et al*, 2013; Li *et al*, 2014), nasopharyngeal carcinoma(Lu *et al*, 2011), and hepatocellular carcinoma (Zhao *et al*, 2014). More recently, the overexpression of *miR-26a* inhibited tongue SCC cell proliferation and promoted cell

Table 4A. Significantly enriched KEGG pathways regulated by Si-TMEM184B

Number of genes	Annotations	*P*-value
17	(KEGG) 03030: DNA replication	3.81E-20
25	(KEGG) 04110: Cell cycle	2.02E-19
10	(KEGG) 03430: Mismatch repair	2.55E-11
14	(KEGG) 04114: Oocyte meiosis	3.55E-08
9	(KEGG) 03410: Base excision repair	3.81E-08
8	(KEGG) 03440: Homologous recombination	7.63E-08
9	(KEGG) 03420: Nucleotide excision repair	1.78E-07
11	(KEGG) 04914: Progesterone-mediated oocyte maturation	1.30E-06
11	(KEGG) 00240: Pyrimidine metabolism	2.62E-06
9	(KEGG) 04115: p53 signaling pathway	1.08E-05
7	(KEGG) 05322: Systemic lupus erythematosus	4.37E-03
11	(KEGG) 04810: Regulation of actin cytoskeleton	4.39E-03
6	(KEGG) 05210: Colorectal cancer	4.74E-03
9	(KEGG) 00230: Purine metabolism	7.36E-03
5	(KEGG) 04978: Mineral absorption	1.04E-02
6	(KEGG) 04350: TGF-beta signaling pathway	1.45E-02
12	(KEGG) 05200: Pathways in cancer	2.70E-02
5	(KEGG) 04610: Complement and coagulation cascades	2.72E-02
5	(KEGG) 03018: RNA degradation	2.75E-02
3	(KEGG) 01040: Biosynthesis of unsaturated fatty acids	2.77E-02
5	(KEGG) 05100: Bacterial invasion of epithelial cells	2.77E-02
7	(KEGG) 05012: Parkinson's disease	2.80E-02
4	(KEGG) 05219: Bladder cancer	2.83E-02

Abbreviation: KEGG = Kyoto Encyclopaedia of Genes and Genomes.

Table 4B. Regulation of genes related to the actin cytoskelton

Gene symbol	Gene name	SAS log2 ratio	HSC3 log2 ratio	HNSCC log2 ratio (GSE 9638)
DIAPH3	Diaphanous homolog 3 (Drosophila)	− 1.83	− 3.34	0.83
ARPC5	Actin related protein 2/3 complex, subunit 5, 16kDa	− 1.77	− 1.54	0.15
BDKRB2	Bradykinin receptor B2	− 2.73	− 1.27	− 1.86
IQGAP3	IQ motif containing GTPase activating protein 3	− 1.07	− 1.74	1.11
FGFR3	Fibroblast growth factor receptor 3	− 2.22	− 1.00	− 0.60
BDKRB1	Bradykinin receptor B1	− 1.17	− 1.94	− 2.27
RHOA	Ras homolog gene family, member A	− 2.51	− 3.09	− 0.34
EGFR	Epidermal growth factor receptor	− 1.37	− 1.40	1.83
GNG12	Guanine nucleotide binding protein (G protein), gamma 12	− 2.42	− 2.93	− 1.03
CD14	CD14 molecule	− 1.00	− 1.39	− 0.64
ITGB4	Integrin, beta 4	− 1.53	− 1.37	1.09

Abbreviation: HNSCC = head and neck squamous cell carcinoma.

apoptosis (Jia *et al*, 2013). These findings were consistent with our present results, suggesting that *miR-26a* and *miR-26b* function as tumour suppressors in this disease. The tumour-suppressive function of *miR-26a* was also confirmed in *in vivo* models of nasopharyngeal carcinoma and gastric cancer (Lu *et al*, 2011; Deng *et al*, 2013). In contrast, *miR-26a* promoted the development of cancer cells in nude mice in ovarian cancer (Shen *et al*, 2014). On the basis of our *in vitro* data, the further analysis of *miR-26a/b* in OSCC using the animal model is necessary.

A single miRNA may regulate multiple protein-coding genes; indeed, bioinformatic studies have shown that miRNAs regulate more than 30–60% of the protein-coding genes in the human genome (Lewis *et al*, 2005). Reduced expression of tumour-suppressive miRNAs may cause the overexpression of oncogenic genes in cancer cells. To better understand OSCC oncogenesis and metastasis, we identified *miR-26a/b* target genes using *in silico* analysis. Recent miRNA studies in our laboratory have utilised this strategy to identify novel molecular targets and pathways regulated by tumour-suppressive miRNAs in several cancers, including head and neck SCC (Kinoshita *et al*, 2013).

In this study, we identified *TMEM184B* as a target of tumour-suppressive *miR-26a/b* and validated the direct binding of this miRNA to the 3′-UTR of *TMEM184B* using luciferase reporter assays. *TMEM184B* encodes a transmembrane protein and is located on chromosome 22q12, as shown by several large-scale cDNA cloning projects (Matsuda *et al*, 2003). However, the function of the *TMEM184B* protein product is still unknown. Our present data showed that *TMEM184B* was upregulated in OSCC clinical specimens, and silencing of *TMEM184B* significantly suppressed cancer cell migration and invasion in cancer cells. We also investigated the expression status of *TMEM184B* in other types of cancers by using GEO database. GEO expression data showed that the expression of *TMEM184B* was significantly upregulated in cancer tissues compared to with normal tissues in lung cancer, hepatocellular carcinoma, pancreas cancer, and renal cell carcinoma (Supplementary Figure 4). This is the first report that *TMEM184B* promotes cancer cell migration and invasion, and is directly regulated by tumour-suppressive miRNAs, suggesting that this target has an oncogenic function in cancer cells.

Furthermore, to elucidate the functional significance of *TMEM184B* in cancer cells, we investigated the downstream pathways and genes regulated by *TMEM184B* using siRNA-mediated knockdown of *TMEM184B*. Several molecular pathways were selected as enriched pathways by KEGG annotation, such as 'DNA replication', 'Cell cycle', and 'Mismatch repair'. It is important to investigate the functional role of *TME184B*-mediated signalling using genes involved in these pathways.

In particular, we focused on the category 'Regulation of actin cytoskeletal pathway' based on the phenotype of cancer cells following induction of *miR-26a/b* or silencing of *TMEM184B*. We also investigated the expression levels of 11 genes that were involved in the 'Regulation of actin cytoskeleton pathway' by using GEO (accession number; GSE9638) expression data. Among them, three genes (*IQGAP3*, *EGFR*, and *ITGB4*) were upregulated in clinical specimens and these genes were reduced by *si-TMEM184B* transfectant cells (Supplementary Figure 5). These data indicated that the three genes were downstream oncogenic genes of the *miR-26a/b-TMEM184b* axis regulation in OSCC cells. Activation of growth-factor receptors and integrins can promote the exchange of GDP for GTP on RHO proteins, and GTP-bound RHO proteins interact with a range of molecules to modulate their activity and localisation (Jaffe and Hall, 2005). During the progression of metastasis, cancer cells acquire altered morphological characteristics and the ability to traverse tissue boundaries. RHO family proteins and proteins interacting with RHO family proteins are involved in cancer cell morphological and motility changes (Hanna and El-Sibai, 2013). The Ras GTPase-activating-like protein

(IQGAP) family comprises three members (IQGAP1-3) and these function as contribute to several cellular processes including cell adhesion, migration, and invasion (Noritake *et al*, 2005). A recent study showed that overexpression of IQGAP3 promoted cancer cell growth, migration, and invasion in lung cancer cell lines (Yang *et al*, 2014). Thus, our findings suggested that the downregulation of *miR-26a/b* enhanced the expression of *TMEM184B* and activated the actin cytoskeleton pathways, thereby promoting cancer cell migration and invasion. As such, the tumour-suppressive *miR-26a/b-TMEM184B* axis might serve as a therapeutic target for cancer metastasis. Confirmation of these data using an *in vivo* mouse model is essential to support the conclusions of our *in vitro* results within the context of OSCC oncogenesis and metastasis.

CONCLUSIONS

Downregulation of *miR-26a/b* was identified based on the miRNA expression signature of OSCC in this study. *miR-26a* and *miR-26b* were shown to function as tumour suppressors in OSCC. To the best of our knowledge, this is the first report demonstrating that tumour-suppressive *miR-26a* and *miR-26b* directly regulated *TMEM184B* in OSCC cells. Moreover, *TMEM184B* was upregulated in OSCC clinical specimens and contributed to cancer cell migration and invasion, indicating that it functioned as an oncogene. The identification of novel molecular pathways and targets regulated by *miR-26a/b* may lead to a better understanding of OSCC and the development of new therapeutic strategies to treat this disease.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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