British Journal of Cancer (2012) 107, 700–706

www.bjcancer.com

Downregulation of miR-126 induces angiogenesis and lymphangiogenesis by activation of VEGF-A in oral cancer

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BACKGROUND: MicroRNA (miRNA)-126 (*miR-126*) is an endothelial-specific miRNA located within intron 7 of epidermal growth factor-like domain 7 (*EGFL7*). However, the role of *miR-126* in cancer is controversial.

METHODS: We examined the function of *miR-126* in oral squamous cell carcinoma (OSCC) cells. Furthermore, a series of 118 cases with OSCC were evaluated for the expression levels of miR-126.

RESULTS: MicroRNA-126 (*miR-126*) was associated with cell growth and regulation of vascular endothelial growth factor-A activity, and demethylation treatment increased expression levels of *miR-126* and *EGFL7* in OSCC cells. A significant association was found between *miR-126* expression and tumour progression, nodal metastasis, vessel density, or poor prognosis in OSCC cases. In the multivariate analysis, decreased *miR-126* expression was strongly correlated with disease-free survival.

CONCLUSION: The present results suggest that *miR-126* might be a useful diagnostic and therapeutic target in OSCC.

British Journal of Cancer (2012) **107,** 700–706. doi:10.1038/bjc.2012.330 www.bjcancer.com
Published online 26 July 2012

Keywords: miR-126; oral squamous cell carcinoma; angiogenesis; lymphangiogenesis; VEGF-A

Head and neck cancer, including oral squamous cell carcinoma (OSCC), is the sixth most common cancer worldwide (Argiris *et al*, 2008). The incidence of OSCC is estimated at 263 900 cases and 128 000 deaths annually (Jemal *et al*, 2011), and an estimated 7850 patients die from this disease in the United States (Siegel *et al*, 2012). Oral squamous cell carcinoma has a high potential for local invasion and lymph node metastasis, and overall 5-year survival rates have not significantly improved during the past three decades (Dos Reis *et al*, 2008). The 5-year survival rate in OSCC is less than 50% (Marsh *et al*, 2011). Although more than 80% of early-stage OSCCs can be cured by treatment, approximately 70% of advanced-stage patients cannot be cured (Mydlarz *et al*, 2010). Therefore, early detection and clarification of detailed molecular mechanism of OSCC is very important.

MicroRNAs (miRNAs) are non-coding small RNAs, approximately 18–25 nucleotides in length (Bartel, 2009; Martello *et al*, 2010), which function as suppressors of gene expression by binding to the 3′-untranslated region (UTR) of target mRNAs (Calin and Croce, 2006; Liu *et al*, 2010; Martello et al., 2010). MicroRNA-126 (*miR-126*) is located within intron 7 of epidermal growth factor-like domain 7 (*EGFL7*) and is highly expressed in vascular endothelial cells (Fish *et al*, 2008; Wang *et al*, 2008a). In tumour cells, *miR-126* acts as a tumour suppressor gene in many malignant tumour cells (Wang *et al*, 2008b; Liu *et al*, 2009; Saito *et al*, 2009; Feng *et al*, 2010; Miko *et al*, 2011; Zhu *et al*, 2011). MicroRNA-126 (*miR-126*) is significantly expressed lower in carcinoma as compared with adenoma and useful diagnostic marker after thyroid fine-needle aspiration biopsy (Kitano *et al*, 2011). MicroRNA-126 (*miR-126*) is also strongly downregulated in pancreatic cancer, with an associated elevation in *K-Ras* (Slaby *et al*, 2012), and lower expression of *miR-126* is significantly correlated with short survival in non-small cell lung carcinoma (NSCLC) and renal cell carcinoma (Donnem *et al*, 2012; Jiao *et al*, 2012). Further, Yu *et al* (2009) have reported downregulation of *miR-126* to promote oral carcinogenesis in Syrian hamster. Saito *et al* (2009) showed that *miR-126* and *EGFL7*, which is the host gene for *miR-126*, are downregulated by DNA hypermethylation, and *miR-126* and *EGFL7* expression are restored after treatment with 5-aza-2′-deoxycytidine (5-Aza-dc) in urologic cancer. Silencing of *miR-126* by the DNA methylation of *EGFL7* was also reported in NSCLC (Watanabe *et al*, 2012). However, several reports have described an oncogenic role for *miR-126*, such as the inhibition of apoptosis in acute myeloid leukaemia and the promotion of gastric carcinogenesis (Otsubo *et al*, 2011). In addition, higher expression of *miR-126* was shown to be a poor prognostic factor in NSCLC (Donnem *et al*, 2011) and promote metastasis in prostate cancer (Watahiki *et al*, 2011). Meister and Schmidt (2010) recently reported that higher expression of *miR-126* accelerates cancer progression by activation of mitogen-activated protein kinase and Akt, whereas miR-126 suppresses tumour proliferation and invasion because of decreased Crk expression. These contradictory findings suggest that *miR-126* has several functions specific to each type of malignancy.

Angiogenesis has a pivotal role in prenatal development, wound healing, chronic inflammation, tumour progression, and metastasis (Carmeliet, 2005; Sasahira *et al*, 2007a), and lymphangiogenesis promotes lymph node metastasis in cancer cells (Adams and Alitalo, 2007; Sasahira *et al*, 2010). Vascular endothelial growth factor (*VEGF*)-*A* and *VEGF-C/D* are one of the representative

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Received 16 April 2012; revised 27 June 2012; accepted 3 July 2012; published online 26 July 2012

factors of angiogenesis and/or lymphangiogenesis (Adams and Alitalo, 2007), and we have also previously reported that the VEGF family induce angiogenesis and/or lymphangiogenesis in OSCC (Sasahira *et al*, 2007a, b, 2008, 2010). However, several reports have revealed that VEGF-A is not related with angiogenesis and VEGF-C/D are not associated with nodal metastasis in cancer (Currie *et al*, 2004; Nomiya *et al*, 2006; Miyahara *et al*, 2007; Donnem *et al*, 2009). It has also been reported that vessel numbers in nodal metastasis cases are lower than cases without metastasis (Moriyama *et al*, 1997). Thus, the role of tumour angiogenesis and lymphangiogenesis are still controversial.

Recently, it was reported that *VEGF-A* is a target gene of *miR-126* and downregulation of *miR-126* increases *VEGF-A* activity in lung (Liu *et al*, 2009; Zhu *et al*, 2012) and breast cancer (Zhu *et al*, 2011). However, other reports indicated that *miR-126* is an inducer of angiogenesis by enhancing the proangiogenic activity of *VEGF-A* (Fish *et al*, 2008; Wang *et al*, 2008a). Thus, *miR-126* may function differently in tumour cells and stromal cells. In this study, we examined the angiogenic role of *miR-126* and confirmed the relationship between the expression of *miR-126* and lymphangiogenesis in OSCC.

MATERIALS AND METHODS

Cell culture

Human OSCC cell lines, HSC3 and HSC4 cells, were obtained from the Health Science Research Resources Bank and maintained in Dulbbeco's modified Eagle's medium (DMEM; Wako Pure Chemical industries, Ltd, Osaka, Japan) supplemented with 10% fetal bovine serum (Sigma Chemical, St Louis, MO, USA) under the conditions of 5% CO_2 in air at 37 °C. The HSC3 cells have high metastatic potential and HSC4 cells have low metastatic ability (Sasahira *et al*, 2010).

RNA isolation and quantitative reverse-transcription PCR

Total RNA and small RNA were extracted using RNeasy Mini Kit (Qiagen, Valencia, CA, USA) or *mir*Vana miRNA Isolation Kit (Ambion, Austin, TX, USA), and total RNA (1 μg) and small RNA (10 ng) were synthesised with the ReverTra Ace qRT Kit (Toyobo, Osaka, Japan) or TaqMan MicroRNA RT Kit (Applied Biosystems, Foster City, CA, USA), respectively. Quantitative reverse-transcription PCR (qRT–PCR) were performed on StepOne Plus Real-Time PCR Systems (Applied Biosystems) using EXPRESS qPCR Supermixes (Invitrogen, Carlsbad, CA, USA) and analyse the relative standard curve quantification method. The PCR condition was according to the manufacturer's instructions and *18S* (eukaryotic 18S rRNA) expression level was amplified for internal control. TaqMan MicroRNA assays of hsa-*miR-126* and TaqMan Gene Expression Assays of *VEGF-A*, *VEGF-C*, *VEGF-D*, *EGFL7*, and *18S* were purchased from Applied Biosystems. All PCRs were performed in triplicates.

Methylation-specific PCR

For the bisulfite modification of DNA, 2 μg of genomic DNA extracted using the QIAamp DNA Mini kit (Qiagen) was treated with the Epitect Bisulfite kit (Qiagen) according to the provider's manual. For analysis of DNA methylation of *EGFL7*, we carried out methylation-specific PCR (MSP) using an Epitect MSP kit (Qiagen). The PCR products were separated by 6% non-denaturing polyacrylamide gels, stained with ethidium bromide (Sigma Chemical), and visualised under UV light.

The sequences of the primers methylated or unmethylated of *EGFL7* are as follows (Saito *et al*, 2009): 5′-GTG GTG GTG GTG TGT GTG TGT TT-3′ and 5′-CTC AAC CCA ACC CAA ACA ACA ACC A-3′ for unmethylated *EGFL7*; 5′-GCG GCG CGT GCG CGT TT-3′ and 5′-CCA ACC CGA ACG ACG ACC G-3′ for methylated *EGFL7*.

DNA demethylation treatment

Previous results raise the possibility that *miR-126* expression is regulated by DNA methylation (Saito *et al*, 2009). Therefore, we performed the demethylation treatment in OSCC cells. Each cell was seeded at a density of 1×10^6 cells ml^{-1}. After 24 h, cells were treated with 1 μM 5-Aza-dc (Sigma Chemical) for 4 days. Treatment with 300 nM trichostatin A (TSA; Sigma Chemical) was also performed for 24 h. Cells then were harvested for miRNA extractions.

Transient transfection

Pre-*miR-126* precursor, anti-*miR126* inhibitor, pre-miR negative control 1, and anti-miR negative control 1 were purchased from Ambion. Pre-miR (10 nM) and anti-miR (30 nM) were transfected with Lipofectamine 2000 (Invitrogen) according to the provider's recommendations.

Cell growth assay

The cells were seeded at density of 2000 cells per well of 96-well tissue culture plates and incubated for 48 h at 37 °C. Cell growth was assessed by MTT assay using the incorporation of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma Chemical). The experiments were performed in triplicates.

In vitro invasion assay

A modified Boyden chamber assay was performed using the BD BioCoat Cell Culture Inserts glued to type IV collagen (Becton-Dickinson, Bedford, MA, USA) as described previously. Cells were suspended in 500 μl of DMEM and placed in the insert. After 48 h incubation at 37 °C, the filters were stained with haematoxylin. The stained cells were counted in whole inserts at $\times$100 magnification. Each experiment was repeated at least three times.

Apoptosis assay

Apoptotic cells were detected by the terminal deoxynucleotidyl trsnsferase (TdT)-mediated dUTP-biotin nick and labelling (TUNEL) assay using the *In Situ* Cell Death Detection Kit, POD (Roche Diagnostics, Indianapolis, IN, USA). We also confirmed the activation of caspase-3 was detected using CaspACE Assay system, Colorimetric (Promega, Madison, WI, USA) according to the manufacturer's protocol. The experiments were performed in triplicates.

Oral squamous cell carcinoma samples

One-hundred and eighteen patients (68 men, 50 women; 46–91 years of age, mean: 67.4 years) of primary OSCCs, who were treated at Nara Medical University Hospital, Kashihara, Japan, from February 2000 to October 2008, were randomly selected. Tumours were staged according to the UICC TNM classification system, 7th edition, and histopathological grading was in accordance with the World Health Organization criteria. None of the patients was treated before surgery and sample preparation. Medical records and prognostic follow-up data were obtained from the patient database maintained by the hospital. The median follow-up period was 3.4 years (range 0.5–5.9). For strict privacy protection, identifying information for all samples was removed before analysis. This procedure was in accordance with the Ethical Guidelines for Human Genome/Gene Research of the Japanese Government. To determine the relation between *miR-126*

expression and clinicopathological characteristics, we divided into two groups according to expression levels those with values higher the mean value for the entire group and those with lower than the group mean value. Ten samples of normal oral mucosa (six man, four woman; 20–47 years of age, mean: 37.9 years) were used as control.

Laser-capture microdissection

As previous reports suggested that functions of *miR-126* are different in cancer cells and stromal cells (Fish *et al*, 2008; Wang *et al*, 2008a; Liu *et al*, 2009; Zhu *et al*, 2011), we performed the laser-capture microdissection (LCM) to selectively extract small RNA from OSCC cells. Tissue sections (7 μm) were prepared from each paraffin blocks and stained using haematoxylin and eosin. Slides were transferred for microdissection using a Pix Cell II laser capture microscope (Arcturus, Mountain View, CA, USA) according to the manufacturer's instructions. Approximately 5000 tumour cells were microdissected from each tissue. MicroRNA was extracted using the *mir*Vana miRNA Isolation Kit (Ambion).

Immunohistochemistry

Immunohistochemical analysis was performed with the EnVision+ DualLink (DAKO, Carpinteria, CA, USA) system and antigen retrieval was performed with microwaving in citrate buffer at 95 °C for 45 min. After endogeneous peroxidase block by 3% H_2O_2-methanol, anti-CD34 antibody (a marker for vascular endothelial cells; DAKO) and anti-LYVE-1 antibody (a marker for lymphovascular endothelial cells; Abcam, Tokyo, Japan) were used for primary antibody for 2 h and incubated with the secondary antibody for 30 min at room temperature. The specimens were colour-developed with diaminobenzidine solution (DAKO) and specimens were counterstained with Mayer's haematoxylin (Sigma Chemical). The microvessel density (MVD) and lymphovessel density (LVD) were measured on anti-CD34 and anti-LYVE-1 antibody immunopositive specimens, respectively. To quantify MVD or LVD, five maximum vessel-density fields were selected from around of the tumour cells (the 'hot spot') and examined under a 200-fold magnification by a microscope and averaged. We divided the tissue samples into two groups according to MVD levels: those with values higher than the mean value for the entire group, and those with lower values than the group mean value. The same was applied based on the LVD values.

Statistical analysis

The correlation of variables was performed by Fischer's exact test, one-factor ANOVA test, and Mann–Whitney *U*-test. Disease-free survival analysis was calculated using the Kaplan–Meier method, and differences between groups were tested by means of a log lank test. Univariate analysis for disease-free survival was calculated by log lank test. For multivariate analysis, Cox proportional hazards model was used (described as risk ratio with 95% confidence intervals, together with the *P*-value). All statistical analysis was carried out with JMP8 (SAS Institute, Cary, NC, USA). *P*-values less than 0.05 were regarded as statistically significant.

RESULTS

Expression of miR-126 and EGFL7 in OSCC cells

The expressions of *miR-126* and *EGFL7*, which is the host gene of *miR-126*, were examined by qRT–PCR in the HSC3 and HSC4 cells. The expression levels of *miR-126* and *EGFL7* in the HSC3 cells with high metastatic potential were approximately 10 times lower than HSC4 cells with low metastatic potential (Figures 1A and B). Examination of the methylation status of *EGFL7* by MSP showed

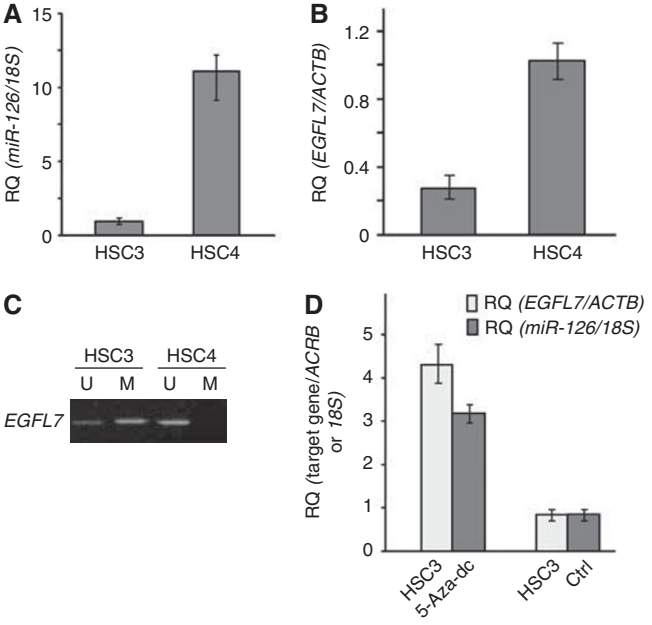

Figure 1 Expression levels of *miR-126* (**A**) and *EGFL7* (**B**) measured using qRT–PCR in the OSCC cells. The *18S* or beta-actin (*ACTB*) transcript levels were used as internal controls for *miR-126* and *EGFL7* expressions, respectively. The methylation status of *EGFL7* by MSP in the OSCC cells is shown in (**C**). The changes of the *miR-126* and *EGFL7* expressions in the 5-Aza-dc-treated HSC3 cells are shown in (**D**). The expression levels of *miR-126* and *EGFL7* in the untreated HSC3 cells were set to 1.

the presence of a methylated *EGFL7* band in the HSC3 cells that was absent in the HSC4 cells (Figure 1C). We next investigated the possibility that demethylation treatment could upregulate the *miR-126* and *EGFL7* expressions in the HSC3 cells. The treatment with 5-Aza-dc restored the expressions of *miR-126* and *EGFL7* (Figure 1D), whereas the expression levels of *miR-126* was not changed by TSA treatment (data not shown).

Functional analysis of miR-126 in the OSCC cells

The role of *miR-126* in OSCC progression was investigated using a functional analysis. The growth of HSC3 cells treated with pre-*miR-126* was inhibited in comparison with that of cells treated with control pre-*miR*. However, the growth of the HSC4 cells treated with an anti-*miR-126* inhibitor was restored compared with that of the cells treated with the control anti-miR inhibitor (Figure 2A). The number of invading cells was not affected by the treatment with pre-*miR-126* in the HSC3 cells or anti-*miR-126* in the HSC4 cells, in contrast to the number of cells treated with the control miR (Figure 2B). No significant changes in TUNEL-positive cells and caspase-3 activity were observed between the pre-*miR-126*-treated HSC3 cells or the anti-*miR-126*-treated HSC4 cells and the control-treated HSC3 or HSC4 cells (Figures 2C and D).

Changes in the VEGF expression levels in the OSCC cells treated with anti-miR-126 or pre-miR-126

As *VEGF-A* has been suggested to be negatively or positively regulated by *miR-126* (Fish *et al*, 2008; Liu *et al*, 2009; Zhu *et al*, 2011), we examined the effect of *miR-126* on the *VEGF-A*, *VEGF-C*, and *VEGF-D* expressions in the OSCC cells (Figure 3). Exposure to pre-*miR-126* increased *miR-126* expression but did not affect the *VEGF-C* and *VEGF-D* expression levels in the HSC3 cells, whereas the treatment with anti-*miR-126* inhibitor decreased the *miR-126* expression but not the *VEGF-C* and *VEGF-D* expressions in the HSC4 cells. However, induction of *miR-126* in the HSC3 cells and

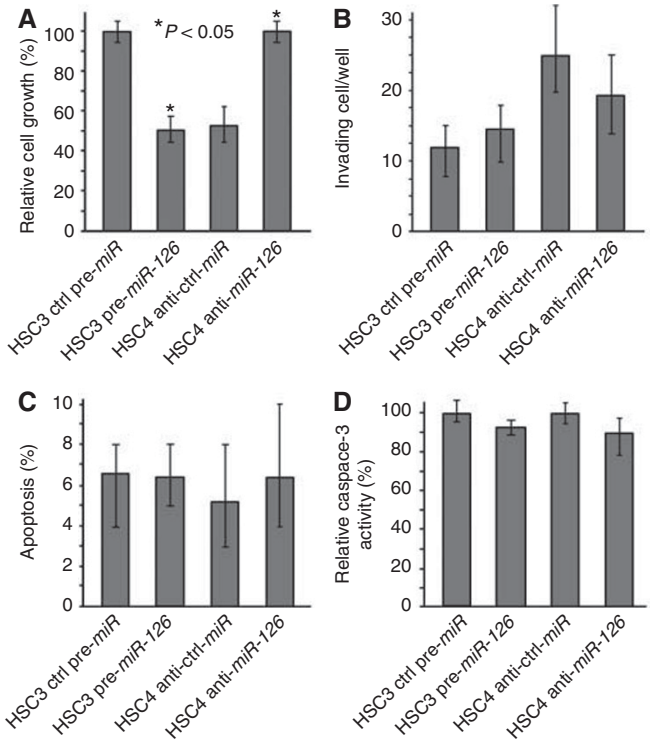

Figure 2 Functional analysis of *miR-126* in the OSCC cells. Cell growth (**A**), invasion (**B**), ratio of TUNEL-positive cells (**C**), and activity of caspase-3 (**D**) in the HSC3 or HSC4 cells treated with a *miR-126* precursor or anti-*miR-126*, respectively. Statistical significance was calculated by the Mann–Whitney *U*-test.

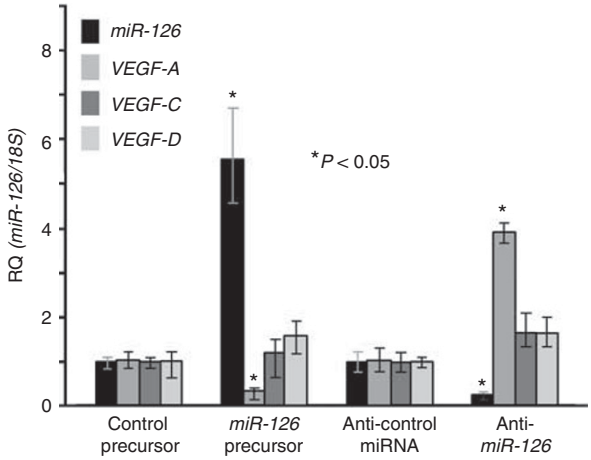

Figure 3 Effect of the *miR-126*, *VEGF-A*, *VEGF-C*, and *VEGF-D* expression levels in the OSCC cells treated with anti-*miR-126* or the *miR-126* precursor. The expression levels in the control miRNA precursor-treated HSC3 cells and the control anti-miRNA-treated HSC4 cells were set to 1. Statistical significance was determined using the Mann–Whitney *U*-test.

reduction of *miR-126* in the HSC4 cells were associated with the downregulation and the upregulation of *VEGF-A* expression, respectively. This was verified by the co-transfection of the *VEGF-A* wild-type 3′-UTR with pre-*miR-126*, which decreased luciferase activity, whereas this effect was completely ablated by deletion of the *miR-126*-binding site in the *VEGF-A* 3′-UTR (data not shown). These results suggested that *miR-126* acts as a negative regulator of *VEGF-A*, but not of *VEGF-C* and *VEGF-D*.

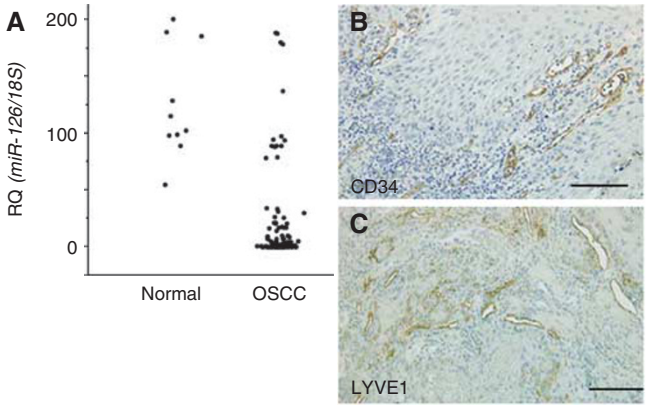

Figure 4 Expression levels of *miR-126* in OSCC compared with that in the normal oral mucosa (**A**) and MVD (**B**) or LVD (**C**) by immunohistochemistry. MVD and LVD were assessed according to the number of anti-CD34 and anti-LYVE1-positive vessels. Scale bar, 100 μm.

Relationship between the miR-126 expression and the clinicopathological characteristics in the OSCC specimens

MicroRNA-126 (*MiR-126*) expression was examined by qRT–PCR in the 118 cases with OSCC. The expression levels of *miR-126* in almost all the OSCC cases were low in comparison with the normal oral mucosa (Figure 4A). The *miR-126* expression and the clinicopathological characteristics in the OSCC specimens are summarised in Table 1. Ninety-four of the 118 OSCC samples (79.7%) showed low expression of *miR-126*. In the cases with local progression (T3 and T4), the expression levels of *miR-126* were significantly lower than those in the T1 and T2 cases ($P = 0.036$). Low *miR-126* expression was detected in 52 (70.3%) of the 74 cases with early clinical-stage disease (stages I and II) and in 42 (95.5%) of the 44 cases with advanced clinical-stage disease (stages III and IV; $P = 0.0006$). Although downregulation of *miR-126* was observed in 100% (34 out of 34) of the nodal metastasis-positive cases, 71.4% (60 out of 84) of the cases without nodal metastasis showed low expression of *miR-126* ($P < 0.0001$). No significant correlation was found between the expression levels of *miR-126* and age, sex, site, or histological differentiation.

On the basis of the *in vitro* results showing that *miR-126* is a negative regulator of *VEGF-A*, we verified the relationship between *miR-126* and angiogenesis and lymphangiogenesis in OSCC (Table 2). A significant inverse correlation was observed between the *miR-126* expression levels and MVD ($P < 0.0001$; Figure 4B) or LVD ($P < 0.0001$; Figure 4D).

Relation between the miR-126 expression and the disease-free survival, and the prognosis of OSCCs

Local and nodal recurrence occurred in 44 of the 118 cases. The disease-free survival analysis of the OSCC patients revealed that a poor prognosis was associated with low *miR-126* expression compared with high *miR-126* expression cases ($P = 0.0013$; Figure 5). The univariate analysis using the log-rank test indicated that the histological differentiation ($P = 0.0022$), the clinical stage ($P = 0.0015$), the nodal metastasis ($P < 0.0001$), and the *miR-126* expression levels ($P = 0.0006$) were associated with the poor outcome in OSCCs (Table 3). The multivariate analysis using the Cox proportional hazards model showed that nodal metastasis ($P = 0.0093$) and *miR-126* expression levels ($P = 0.048$) were prognostic factors for disease-free survival periods (Table 3).

Table 1 Relationship between the *miR-126* expressions and the clinicopathological parameters

Parameters	*miR-126* Low	*miR-126* High	*P*-value
Sex			
Male	52	16	
Female	42	8	0.1573
Age			
⩽65	34	12	
>65	60	12	0.221
Site			
Tongue	48	16	
Other	46	8	0.1268
Histological differentiation			
Well	56	14	
Mod, Poor	38	10	0.5451
T classification			
T1–T2	68	22	
T3–T4	26	2	0.036
Clinical stage			
I, II	52	22	
III, IV	42	2	0.0006
Nodal metastasis			
Negative	60	24	
Positive	34	0	<0.0001

Abbreviations: miR-126 = microRNA-126; Well = well-differentiated squamous cell carcinoma; Mod = moderately differentiated squamous cell carcinoma; Poor = poorly differentiated squamous cell carcinoma. The statistical analysis was performed by Fischer's exact *t*-test. The T classification and the clinical stage were performed according to the TNM classification.

Table 2 Relationship between the miR-126 expressions and angiogenesis or lymphangiogenesis

Parameter	miR-126 Low	miR-126 High	*P*-value
MVD	25.5761 ± 10.1091	11.0231 ± 4.6017	<0.0001
LVD	25.4468 ± 10.0379	10.3167 ± 4.014	<0.0001

Abbreviations: ANOVA = analysis of variance; LVD = lymphovessel density; miR-126 = microRNA-126; MVD = microvessel density. The statistical analysis was performed by one-factor ANOVA. Means ± s.d., each s.d. was less than 10%.

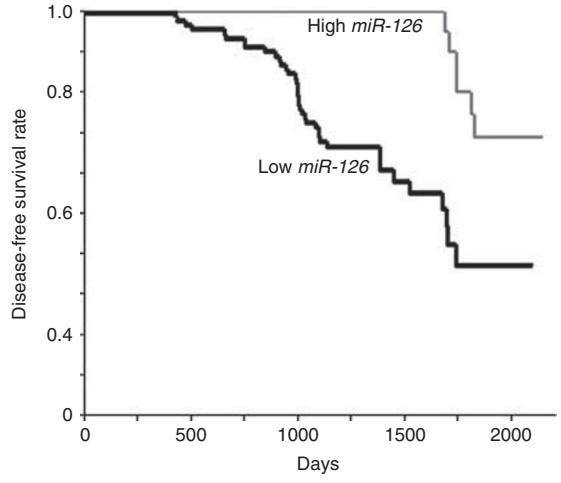

Figure 5 Disease-free survival curves of the OSCC patients calculated using the Kaplan–Meier method. Disease-free survival was analysed in correlation to the expression levels of *miR-126*. The miR-126-high cases ($n = 24$, event and censored data were 6 and 18, respectively), miR-126-low cases ($n = 94$, event and censored data were 38 and 56, respectively).

Table 3 Univariate and multivariate analysis of disease-free survival

Parameters	Risk ratio	95% CI	*P*-value
Univariate analysis			
Age			
⩽65	1		
>65	0.7672	0.4227–1.3998	0.3839
Gender			
Male	1		
Female	0.8843	0.4873–1.6304	0.6885
Site			
Tongue	1		
Other	0.9075	0.4927–1.6449	0.750
Histology			
Well	1		
Mod/Poor	2.4833	1.3989–4.7785	0.0022
T classification			
T1-T2	1		
T3-T4	1.4582	0.7206–2.759	0.2805
Clinical stage			
I, II	1		
III, IV	2.6417	1.4539–4.8528	0.0015
Nodal metastasis			
Negative	1		
Positive	4.7203	2.5653–8.7999	<0.0001
MVD			
Low	1		
High	1.0094	0.9827–1.0357	0.4872
LVD			
Low	1		
High	0.8492	0.4444–1.5712	0.607
miR-126			
High	1		
Low	4.0492	1.7558–11.0409	0.0006
Multivariate analysis			
Histology			
Well	1		
Mod, Poor	0.6673	0.3493–1.2557	0.2101
Clinical stage			
I, II	1		
III, IV	1.8972	0.9434–3.8782	0.0725
Nodal metastasis			
Negative	1		
Positive	2.6265	1.2658–5.6413	0.0093
miR-126			
High	1		
Low	2.631	0.9886–7.9851	0.048

Abbreviations: CI = confidence intervals; miR-126 = microRNA-126; Mod = moderately differentiated squamous cell carcinoma; Poor = poorly differentiated squamous cell carcinoma; Well = well-differentiated squamous cell carcinoma. The univariate analysis was performed by log-rank test, and the multivariate analysis was performed by the Cox proportional hazard model.

DISCUSSION

The present results show that *miR-126* is a negative regulator of *VEGF-A* and promotes cell growth in OSCC cells. In addition, the decreased *miR-126* expression was associated with the induction of tumoural angiogenesis and lymphangiogenesis, tumour

progression, nodal metastasis, and poor prognosis in the OSCC cases. The HSC3 cells are human OSCC-derived metastatic cells, whereas the HSC4 cells have low metastatic potential (Momose *et al*, 1989; Sasahira *et al*, 2007a; Sasahira *et al*, 2008; Sasahira *et al*, 2010). The HSC3 cells are characterised by adhesion to type-IV collagen, colony formation in a type-I collagen matrix (Momose *et al*, 1989), high heparanase activity (Ikuta *et al*, 2001), reduction of *nm23H1* expression and upregulated matrix metalloproteinase (*MMP)-2/MMP-9* (Khan *et al*, 2001), and higher expression of *VEGF-A/C/D* in comparison with HSC4 cells. In this study, the HSC3 cells showed lower expression of *miR-126* compared with the HSC4 cells, and the decrease of *miR-126* activity might be associated with a higher capacity for lymph node metastasis in these cells. Further investigation of the expression levels of *miR-126* in OSCC may help predict the tendency for lymph node metastasis development in this type of malignancy. Vascular endothelial growth factor (*VEGF*)*-A* is a potent trigger for tumoural angiogenesis (Stockmann *et al*, 2008); however, we previously reported that *VEGF-A* induces not only angiogenesis but also lymphangiogenesis, and *VEGF-A*-dependent angiogenesis and lymphangiogenesis accelerate local progression, nodal metastasis, and recurrence of OSCC (Sasahira *et al*, 2010). This is in agreement with previous studies that demonstrated a strong correlation between *VEGF-A* expression and tumour progression (Takahashi *et al*, 1995), worse prognosis (Maeda *et al*, 1996), and lymph node metastasis via induction of lymphangiogenesis (Nagy *et al*, 2002; Hirakawa *et al*, 2005). Further examination will help reveal the mechanism underlying the role of *miR-126* and *VEGF-A*-related angiogenesis and lymphangiogenesis in tumour progression.

MicroRNA-126 (*MiR-126*) is an endothelial-specific *miRNA* that is located within intron 7 of *EGFL7* (Fish *et al*, 2008; Wang *et al*, 2008a). An intronic *miRNA* tends to be co-expressed with its host gene (Baskerville and Bartel, 2005; Saito *et al*, 2009), and a previous report showed that *miR-126* and its host gene, *EGFL7*, are downregulated by DNA methylation, with restoration of expression levels by epigenetic treatment (Saito *et al*, 2009). We also observed decreased expression and methylation of *EGFL7* in the highly metastatic OSCC cell line HSC3 compared with the HSC4 cell line with lower metastasis. In addition, *EGFL7* and *miR-126* recovered normal expression levels in response to the 5-Aza-dc treatment in the HSC3 cells. Further examination of *miR-126* might reveal this molecule as a useful target for epigenetic therapy in progressive and metastatic OSCC.

MicroRNAs are well preserved in formalin-fixed, paraffin-embedded (FFPE) specimens owing to the small size of the RNA (Xi *et al*, 2007; Donnem *et al*, 2011). Most of the previous reports on *miR-126* expression were based on the analysis of frozen tissues containing non-tumour cells or tumour stromal cells including vessels (Wang *et al*, 2008b; Saito *et al*, 2009; Liu *et al*, 2009; Feng *et al*, 2010; Otsubo *et al*, 2011; Zhu *et al*, 2011). However, *miR-126* is a positive regulator of angiogenesis in normal endothelial cells (Fish *et al*, 2008; Wang *et al*, 2008a; Liu *et al*, 2009; Miko *et al*, 2011; Zhu *et al*, 2011). In vasculogenesis, which is not associated with cancer, such as in wound healing, overexpression of *miR-126* in endothelial cells enhances *VEGF-A* activity and promotes vessel formation by repressing the expression of sprouty-related protein-1 (*Spred-1*; Wang *et al*, 2008a). Sprouty-related protein-1 (*spred-1*) is an intracellular inhibitor of angiogenic signals (Wang *et al*, 2008a). As *VEGF-A* is not a direct target of *miR-126* in endothelial cells, the use of fresh frozen samples, including stromal cells, may lead to an incorrect interpretation of results. We therefore selected OSCC cells using the LCM technique in FFPE tissues and performed the expression analysis of *miR-126* by real-time RT–PCR. We also confirmed the increased *miR-126* expression and the decreased *Spred-1* expression in the microdissected endothelial cells by qRT–PCR (data not shown).

In conclusion, the present results demonstrate that low *miR-126* expression is correlated with tumour progression through the activation of angiogenesis and lymphangiogenesis in OSCC. However, the exact mechanism underlying the *miR-126*-mediated activation of angiogenesis and lymphangiogenesis remains to be elucidated. *In vivo* studies will be helpful in the future to further clarify this mechanism. Our results suggest that re-expression of *miR-126* could be a useful therapeutic strategy against human OSCC.

ACKNOWLEDGEMENTS

This work was supported in part by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science, Japan.

Conflict of interest

The authors declare no conflict of interest.

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