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.

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factors of angiogenesis and/or lymphangiogenesis (Adams and Altitalo, 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 Dulbecco’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% CO2 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 mirVana 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 validated 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’-GGTT GTG GTG GTG TGT GTG TGT TT-3’ and 5’-CTC AAC CCA ACC CAA ACA ACC A-3’ for unmethylated EGFL7; 5’-GGCG GCG GTG GCG GCG 3’ and 5’-CCA ACC CCA ACC A-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 × 10^4 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 ×100 magnification. Each experiment was repeated at least three times.

Apoptosis assay

Apoptotic cells were detected by the terminal deoxynucleotidyl transferase (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, Coloriometric (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
Examination of the methylation status of EGFL7 HSC4 cells with low metastatic potential (Figures 1A and B). The expression of miR-126 and EGFL7 in OSCC cells was carried out with JMP8 (SAS Institute, Cary, NC, USA). Statistical analysis using qRT–PCR in the OSCC cells. The fold change in the presence of a methylated EGFL7 band in the HSC3 cells that was absent in the HSC4 cells (Figure 1C). 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
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).

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

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).
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

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

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

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                  | Low  | 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                                 | 0.7672     | 0.4227–1.3998      | 0.3839  |
| >65                                 |            |                    |         |
| Gender                              |            |                    |         |
| Male                                | 0.8843     | 0.4873–1.6304      | 0.6885  |
| Female                              |            |                    |         |
| Site                                |            |                    |         |
| Tongue                              | 0.9075     | 0.4927–1.6449      | 0.750   |
| Other                               |            |                    |         |
| Histology                           |            |                    |         |
| Well                                |            |                    |         |
| Mod/Poor                            | 2.4833     | 1.3989–4.7785      | 0.0022  |
| T classification                     |            |                    |         |
| T1–T2                               | 1.4582     | 0.7206–2.759       | 0.2805  |
| T3–T4                               |            |                    |         |
| Clinical stage                       |            |                    |         |
| I, II                               | 2.6417     | 1.4539–4.8528      | 0.0015  |
| III, IV                             |            |                    |         |
| Nodal metastasis                    |            |                    |         |
| Negative                            | 4.7203     | 2.5653–8.7999      | <0.0001 |
| Positive                            |            |                    |         |
| miR-126                             |            |                    |         |
| High                                | 4.0492     | 1.7558–11.0409     | 0.0006  |
| Low                                 |            |                    |         |
| miR-126                             |            |                    |         |
| High                                | 2.631      | 0.9886–7.9851      | 0.048   |
| Low                                 |            |                    |         |

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 rm23H1 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 (Spry-1; Wang et al., 2008a). Sprouty-related protein-1 (Spry-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 Spry-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.

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Conflict of interest

The authors declare no conflict of interest.

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