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MIA-dependent angiogenesis and lymphangiogenesis are closely associated with progression, nodal metastasis and poor prognosis in tongue squamous cell carcinoma

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ABSTRACT

We examined the role of angiogenesis/lymphangiogenesis and the relationship between melanoma inhibitory activity (MIA) and angiogenesis or lymphangiogenesis in oral squamous cell carcinoma (OSCC). One hundred and one formalin-fixed, paraffin-embedded specimens of primary OSCC were evaluated for microvessel density (MVD), lymphovessel density (LVD), expression of vascular endothelial growth factor (VEGF), VEGF-C, VEGF-D and MIA. Fresh frozen 18 samples of primary OSCC were further examined for the expression of VEGF, VEGF-C, VEGF-D and MIA protein by enzyme-linked immunosorbent assay (ELISA). In *in vitro* analysis, we studied the change of VEGF, VEGF-C and VEGF-D expression after MIA siRNA treatment. Higher MVD, LVD and VEGF expression levels were closely associated with tumour progression, nodal metastasis and poor prognosis. Expression levels of VEGF-C and VEGF-D were only related with nodal metastasis. MIA expression was significantly associated with MVD, LVD, VEGF, VEGF-C and VEGF-D expression by immunohistochemistry and ELISA assay. VEGF, VEGF-C, VEGF-D and MIA expression levels of metastatic tongue cancer HSC-3 cells were higher than those with no metastatic HSC-4 cells, and VEGF, VEGF-C and VEGF-D expression levels were decreased by MIA siRNA treatment in both cells. MIA-dependent angiogenesis/lymphangiogenesis might be a useful therapeutic target in progressive and metastatic OSCC.

1. Introduction

Head and neck cancer including oral squamous cell carcinoma (OSCC) is the fifth commonest cancer in the world[1] and frequency of OSCC is 3.7 per 100,000 in Japan.[2] OSCC has a high potential for local invasion and lymph node metastasis[3,4] and the overall 5-year survival rates have not significantly improved during the past three decades.[5]

Angiogenesis, the growth of new blood vessels, and lymphangiogenesis, the development of new lymphatic vessels, are the pivotal events for tumour progression and metastasis.[6–8] The major angiogenic and lymphangiogenic factors are vascular endothelial growth factor (VEGF)-VEGF receptor-2 (VEGFR-2) and VEGF-C/D-VEGFR-3, respectively.[9,10] VEGF also induces lymphangiogenesis.[11,12] Among various angiogenic factors, VEGF (now termed VEGF-A) is considered one

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of the strongest promoters of angiogenesis in gastrointestinal tumours.[13] VEGF-C and VEGF-D are members of the platelet-derived growth factor family.[14] Alitalo and colleagues reported that VEGF-C is a lymphangiogenic factor that can selectively induce hyperplasia of lymphatic vasculature.[15] VEGF-D, which shares VEGFR-3 receptor with VEGF-C, also promotes lymphangiogenesis and enhances metastasis in human cancers.[16]

However, previous studies have revealed that VEGF expression is not associated with microvessel density (MVD).[17,18] It has also been reported that cases with nodal metastasis have lower MVD compared to cases without metastasis,[19] and that higher lymphovessel density (LVD) is not related to lymph node metastasis.[20,21] Moreover, it is described that VEGF-C and VEGF-D are not associated with nodal metastasis in several tumours.[22–26] Thus, the impact of tumour angiogenesis and lymphangiogenesis is still controversial.

We previously showed that tumoural expression of receptor for advanced glycation end products (RAGE) is significantly associated with angiogenesis and the expression was an independent prognostic factor in OSCC.[3,27] Moreover, high mobility group box-1 (HMGB1), a major ligand of RAGE, enhances expression of melanoma inhibitory activity (MIA). MIA is an 11-kDa secretory protein isolated from supernatants of malignant melanoma cells.[28] MIA promotes cell detachment, migration, invasion and inhibits apoptosis of the cancer cells[29]. MIA is able to bind to cell surface integrin alpha4beta1 and alpha5beta1, which suggests that MIA might play a role as a ligand for selected integrins[30]. Mitogen-activated protein kinase (MAPK) activity is reported to be affected by MIA.[29–31] We revealed that MIA induces lymphangiogenesis through activation of VEGF-C and VEGF-D in OSCC.[4] However, the role of MIA in angiogenesis has not been studied in detail.

In this study, we investigated the relationship between angiogenesis or lymphangiogenesis and clinicopathological variables and the effect of angiogenesis- and lymphangiogenesis-related factors expression by MIA in OSCC (only tongue SCC).

2. Materials and methods

2.1. Tumour specimens

Formalin-fixed, paraffin-embedded 101 samples (for the immunohistochemistry; age 46–88 years, mean 65.2 years) and 18 excisional biopsy specimens (for the ELISA; age 55–72 years, mean 62.4 years) of primary tongue SCC were randomly selected from Nara Medical University Hospital, Kashihara, Japan, and Miyoshi General Hospital, Miyoshi, Japan, respectively. The tumours were classified according to the International Union Against Cancer TNM classification system. Histopathological grading was in accordance with World Health Organization criteria. All cases were not treated preoperatively. Medical records and prognostic follow-up data were obtained from the patient database administered by the hospital. Because written informed consent was not obtained, identified information for all samples was removed before analysis for strict privacy protection. The procedure was in accordance with the Ethical Guidelines for Human Genome/Gene Research enacted by the Japanese Government.

2.2. Immunohistochemistry

Consecutive 4 μm sections were cut from each block, and immunohistochemistry was performed as we described previously. An immunoperoxidase technique was done following antigen retrieval with pepsin (DAKO Corp, Carpinteria, CA, USA) treatment for 20 min or microwave treatment (95 °C) in citrate buffer (pH 6.0) for 45 min. After endogeneous peroxidase block by 3% H_2O_2–methanol for 15 min, specimens were rinsed with phosphate-buffered saline (PBS). Anti-CD34 antibody (DAKO), anti-LYVE-1 antibody (DAKO), anti-VEGF antibody (Zymed Laboratories, South San Francisco, CA, USA), anti-VEGF-C antibody (Zymed Laboratories), anti-VEGF-D antibody (Zymed Laboratories) and anti-MIA antibody[4] diluted to 0.5 μg/ml were used as primary antibody. After 2 h of incubation at room temperature, specimens were rinsed with PBS and treated for an hour at room temperature with the secondary antibody peroxidase-conjugated anti-goat (Medical and Biological Laboratories Co., Ltd., Nagoya, Japan)

Table 1 – Relationship between clinicopathological parameters and MVD or LVD in 101 tongue cancers.

Variable	MVD[a]	LVD[a]
Gender		
M	56.34 ± 27.97	68.90 ± 58.82
F	54.02 ± 40.8	99.19 ± 66.38
P value	0.2879	0.0287
Age		
<65	52.75 ± 50.98	67.90 ± 65.91
>65	55.30 ± 32.23	80.15 ± 60.82
P value	0.2568	0.2111
Histological grade		
Well	57.38 ± 35.45	67.17 ± 60.43
Moderate/poor	61.08 ± 33.53	88.88 ± 61.26
P value	0.1031	0.0101
T grade		
1, 2	35.54 ± 20.71	54.76 ± 52.46
3	76.2 ± 46.77	98.39 ± 65.54
4	75.24 ± 37.68	104.98 ± 61.57
P value	<0.0001	<0.0001
Clinical stage		
I, II	37.12 ± 38.18	41.14 ± 37.01
III	63.16 ± 29.53	94.20 ± 61.34
IV	73.54 ± 34.35	115.96 ± 65.93
P value	<0.0001	<0.0001
Nodal metastasis		
Negative	48.96 ± 42.02	49.80 ± 45.01
Positive	63.68 ± 27.64	120.23 ± 61.32
P value	0.0014	< 0.0001
Local recurrence		
Negative	42.83 ± 24.00	66.93 ± 60.58
Positive	87.02 ± 48.56	104.60 ± 58.59
P value	<0.0001	0.0011

[a] Means ± S.D. (standard deviation), each S.D. was less than 10% in all cases.

or anti-rabbit (Medical and Biological Laboratories Co., Ltd.) diluted at 0.5%. The specimens were then rinsed with PBS and the colour was developed with diaminobenzidine (DAB) solution (DAKO). After washing, the specimens were counterstained with Meyer's-haematoxylin (Sigma Chemical Co., St. Louis, MO, USA). Immunostaining of all the samples was performed at the same conditions of antibody reaction and DAB exposure.

2.3. *Evaluation of immunohistochemistry*

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, 5 maximum vessel density fields were selected from around the tumour cells (the 'hot spot') and examined under a 200-fold magnification by microscope and averaged.[4,27] These fields were captured by digital imaging with charge-coupled device camera (Olympus, Tokyo, Japan). MVD and LVD were measured on the computer-captured image using NIH Image software (National Institutes of Health, Bethesda, MD, USA). To determine the relation between MVD or LVD and disease-free survival, we divided into two groups according to density those with values higher the mean value for the entire group and those with than the group mean value[26].

Immunoreactivity of VEGF, VEGF-C, VEGF-D and MIA was classified according to Allred's score (AS).[32] We divided the immunoreactivity into 4 grades by AS; Grade 0, AS is 0; Grade 1, AS is 2–4; Grade 2, AS is 5–6; Grade 3, AS is 7–8[4].

2.4. *Enzyme-linked immunosorbent assay for VEGF, VEGF-C, VEGF-D and MIA*

The enzyme-linked immunosorbent assay (ELISA) system for VEGF (Calbiochem, Darmstadt, Germany), VEGF-C (Bender MedSystems GmbH, San Bruno, CA, USA), VEGF-D (R&D

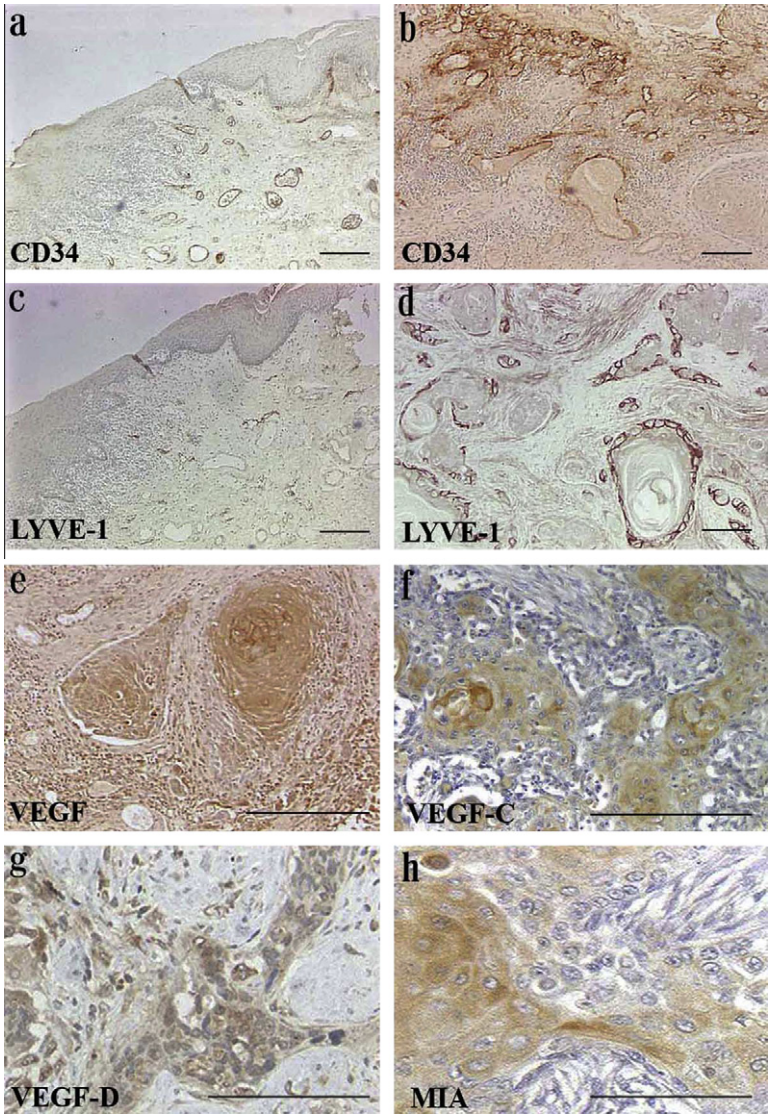

Fig. 1 – Expression of CD34, LYVE-1, VEGF, VEGF-C and VEGF-D in tongue SCCs. CD34-positive blood vessels and LYVE-1-positive lymph vessels in normal tongue mucosa (a, c) and tongue SCC (b, d). Expression of VEGF, VEGF-C, VEGF-D and MIA in tongue cancer (e–h). Bar, 100 μm.

Systems Inc., Minneapolis, MA, USA) and MIA (Roche Diagnostics Co., Indianapolis, IN, USA) was used. For the assay, approximately 10 mm^3 piece of tumour tissue was obtained from each case, frozen with liquid nitrogen and stored at –80°C. The tissues were homogenised in lysis buffer (50-mM Tris–HCl, pH 7.5, 5-mM EDTA, 1-mM EGTA, 2% nonidet-40, 10 μg/ml leupeptin, 50 μg/ml phenylmethylsulfonyl fluoride) and centrifuged (5000*g*). The supernatant (10 μg) was used for ELISA. The assay was performed according to the provider's instructions in triplicate. The presented data are the mean of three independent experiments.

2.5. *Cell culture*

Human tongue cancer cell lines, HSC-3, which is established from metastatic focus and possesses metastatic potential, and HSC-4, which is established from primary site and non-metastatic, were studied. Both cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) (Wako Pure Chemical industries, Ltd., Osaka, Japan) supplemented with 10% FBS (Sigma Chemical Co.) in 5% CO_2 and 95% air at 37 °C.

2.6. *Small interferent RNA*

Stealth Select RNAi (siRNA) for MIA was purchased from Invitrogen (Carlsbad, CA, USA). AllStars Negative Control siRNA was used for control (Qiagen Genomics, Bothwell, WA, USA). siRNA (50 nM) was transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations.

Table 2 – Relationship between clinicopathological parameters and expressions of VEGF, VEGF-C or VEGF-D in 101 tongue cancers.

Variable	VEGF grade				VEGF-C grade				VEGF-D grade			
	0	1	2	3	0	1	2	3	0	1	2	3
Gender												
M	24	18	15	17	33	15	13	13	44	13	9	8
F	9	11	3	4	12	4	4	7	15	3	5	4
P value	0.3305				0.7771				0.7019			
Age												
<65	11	6	2	7	15	4	5	2	16	4	3	3
>65	22	23	16	14	30	15	12	18	43	12	11	9
P value	0.2582				0.2320				0.9771			
Histological grade												
Well	23	17	11	12	30	12	9	12	38	11	7	7
Moderate/poor	10	12	7	9	15	7	8	8	21	5	7	5
P value	0.8800				0.7578				0.7840			
T grade												
1, 2	26	13	6	8	25	13	6	9	34	10	5	4
3	2	11	6	6	9	3	6	7	12	3	6	4
4	5	5	6	7	11	3	5	4	13	3	3	4
P value	0.0340				0.5244				0.6387			
Clinical stage												
I, II	23	9	3	8	22	12	4	5	28	9	3	3
III	4	14	9	6	13	4	7	9	19	4	7	3
IV	6	6	6	7	10	3	6	6	12	3	4	6
P value	0.0032				0.1616				0.1714			
Nodal metastasis												
Negative	25	14	8	15	34	14	8	6	40	12	7	3
Positive	8	15	10	6	11	5	9	14	19	4	7	9
P value	0.0450				0.0020				0.0209			
Local recurrence												
Negative	30	22	11	11	34	16	11	13	42	13	10	9
Positive	3	7	7	10	11	3	6	7	17	3	4	3
P value	0.0098				0.4547				0.8747			
MIA grade												
0	16	13	3	1	18	7	3	1	20	7	2	0
1	6	10	7	6	21	7	1	0	25	4	0	0
2	5	2	6	5	2	4	5	9	11	1	6	2
3	2	4	4	11	4	1	8	10	3	4	6	10
P value	0.0005				<0.0001				<0.0001			

2.7. Quantitative reverse transcription-polymerase chain reaction

The extraction of total RNA was carried out using RNeasy Mini Kit (Qiagen Genomics) and total RNA (1 μg) was synthesised with the ReverTra Ace-α-RT Kit (Toyobo, Osaka, Japan). Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed on StepOne Real-Time PCR Systems (Applied Biosystems, Foster City, CA, USA) using Fast SYBR Green Master Mix (Applied Biosystems) and analysed using the relative standard curve quantification method. PCR condition was according to the provider's instructions and ACTB mRNA level was amplified for internal control. Each amplification was evaluated by melting curve analysis and PCR products were separated and analysed on a 2% agarose gel. All PCRs were at least done in triplicate.

Sequences of primers are listed below: VEGF (referred to GenBank NM_001025366), Forward: 5′-AAG GAG GAG GGC AGA ATC AT-3′, Reverse: 5′-ATC TGC ATG GTG ATG TTG GA-3′; VEGF-C (referred to GenBank NM_005429), Forward: 5′-GGA AAG AAG TTC CAC CAC CA-3′, Reverse: 5′-TTT GTT AGC ATG GAC CCA CA-3′; VEGF-D (referred to GenBank NM_004469), Forward: 5′-AGG ACT GGA AGC TGT GGA GA-3′, Reverse: 5′-ATC GGA ACA CGT TCA CAC AA-3′; MIA (referred to GenBank NM_006533), Forward: 5′-ACC CTA TCT CCA TGG CTG TG-3′, Reverse: 5′-AGG TTT CAG GGT CTG GTC CT-3′; ACTB (referred to GenBank NM_001101), Forward: 5′-GGA CTT CGA GCA AGA GAT GG-3′, Reverse: 5′-AGC ACT GTG TTG GCG TAC AG-3′. All primers were synthesised by Sigma Genosys (Ishikari, Japan).

2.8. Statistical analysis

The correlation of variables was performed by χ^2 test, Student's *t*-test, one-factor ANOVA test and Mann–Whitney *U* test. Disease-free survival was calculated using the Kaplan–Meier method and differences between groups were tested by means of a log lank test. All statistical analyses were carried out with StatView version 4.5 (SAS Institute, Cary, NC, USA). *P* values less than 0.05 were regarded as statistically significant.

3. Results

3.1. Angiogenesis and lymphangiogenesis in tongue SCC

At first, we examined the association between MVD/LVD and clinicopathological parameters in tongue SCC (Table. 1). CD34-positive blood vessels (Fig. 1a) and LYVE-1-positive lymphovessels (Fig. 1c) were found in the normal oral submucosa and both the vessels were able to be distinguished easily. In cancer, CD34-positive blood vessels were observed at stroma near the tumour nest (Fig. 1b), however, LYVE-1-positive lymphovessels were found in the edge of the cancer cells (Fig. 1d) and the shape of both the vessels was irregular. MVD (means ± S.D.; 54.65 ± 37.67) was associated with local progression (T factor) ($P < 0.0001$), clinical stage ($P < 0.0001$), histological nodal metastasis ($P = 0.0014$) and local recurrence of the tumour ($P < 0.0001$). Significant relationship was found between LVD (means ± S.D., 104.24 ± 64.23) and gender ($P = 0.0287$), histological differentiation ($P = 0.0101$), local progression ($P < 0.0001$), clinical stage ($P < 0.0001$), lymph node metastasis ($P < 0.0001$) or local recurrence ($P < 0.0001$). No significant association was found between MVD/LVD and other clinicopathological characteristics in tongue SCC.

3.2. Relationship between VEGF, VEGF-C, VEGF-D or MIA expression and clinicopathological parameters

Next, we studied the relationship between VEGF, VEGF-C, VEGF-D or MIA expression and clinicopathological characteristics (Table 2). Expression levels of VEGF, VEGF-C, VEGF-D and MIA were very weak or negative in non-neoplastic tongue mucosa, whereas immunoreactivity of VEGF, VEGF-C, VEGF-D and MIA was observed in the cell membrane and cytoplasm of cancer cells (Fig. 1e–h). VEGF expression was strongly associated with local progression of the tumour ($P = 0.034$), clinical stage ($P = 0.0032$), lymph node metastasis ($P = 0.045$) and disease recurrence ($P = 0.0098$). Expression levels of VEGF-C and VEGF-D were only significantly correlated with nodal metastasis ($P = 0.002$, $P = 0.0209$, respectively). We also verified that

Table 3 – Relationship between expression of VEGF family and MVD or LVD in 101 tongue cancers.

Variable	MVD[a]	LVD[a]
VEGF grade		
0	29.85 ± 10.41	47.56 ± 43.27
1	50.56 ± 23.90	84.04 ± 62.13
2	68.37 ± 25.00	101.78 ± 64.59
3	87.50 ± 57.21	92.3 ± 70.82
P value	<0.0001	0.0002
VEGF-C grade		
0	49.18 ± 44.81	47.92 ± 38.39
1	44.60 ± 25.34	62.92 ± 47.51
2	71.13 ± 30.47	116.6 ± 70.63
3	62.25 ± 29.78	122.88 ± 68.64
P value	0.0083	<0.0001
VEGF-D grade		
0	55.68 ± 43.28	62.91 ± 44.30
1	40.53 ± 24.76	60.21 ± 63.09
2	71.38 ± 28.55	128.50 ± 75.45
3	53.43 ± 31.80	101.59 ± 75.42
P value	0.0516	0.014

[a] Means ± S.D. (standard deviation), each S.D. was less than 10% in all cases.

Table 4 – Relationship between expression of MIA and MVD or LVD in 101 tongue cancers.

Variable	MVD[a]	LVD[a]
MIA grade		
0	39.8 ± 45.62	34.08 ± 19.59
1	55.33 ± 35.96	51.74 ± 21.70
2	64.72 ± 28.88	120.36 ± 49.20
3	63.74 ± 31.11	125.25 ± 81.15
P value	0.0005	< 0.0001

[a] Means ± S.D. (standard deviation), each S.D. was less than 10% in all cases.

MIA expression was only associated with lymph node metastasis ($P = 0.0002$; data not shown). Other relationship was not found between VEGF, VEGF-C, VEGF-D or MIA expression and clinicopathological parameters.

3.3. *Relationship between MVD or LVD and VEGF, VEGF-C, VEGF-D or MIA expression*

To confirm the details of MVD/LVD-related signal, we examined the relationship between MVD/LVD and VEGF, VEGF-C, VEGF-D or MIA expression (Table 2). A higher MVD was correlated with VEGF ($P < 0.0001$), VEGF-C ($P = 0.0083$), VEGF-D ($P = 0.05$) (Table 3) and MIA expression ($P = 0.0005$) (Table 4). LVD values were associated with VEGF ($P = 0.002$), VEGF-C ($P < 0.0001$), VEGF-D ($P = 0.014$) and MIA grade ($P < 0.0001$) (Tables 3 and 4). We also compared the MIA expression with VEGF, VEGF-C and VEGF-D grade (Table. 1). Expression level of MIA was strongly associated with immunoreactivity grade of VEGF ($P = 0.0005$), VEGF-C ($P < 0.0001$) and VEGF-D expression ($P < 0.0001$).

3.4. *Disease-free survival in cases with OSCC*

In survival analysis, high MVD ($P = 0.0249$), high LVD ($P < 0.0001$) and VEGF grade ($P = 0.0015$) were associated with poor prognosis (Fig. 2a–c). Expression of VEGF-C, VEGF-D and MIA did not influence survival. However, cases with high MIA grade (grades 2 and 3) and high MVD ($P = 0.0013$), high MIA grade and high LVD ($P = 0.0176$) and high MIA grade and high VEGF grade ($P = 0.0481$) were significantly worse than other cases (Fig. 2d–f).

3.5. *ELISA for VEGF, VEGF-C, VEGF-D and MIA*

We next compared the concentration levels of VEGF, VEGF-C, VEGF-D and MIA in 18 cases of tongue cancer by ELISA. Tumoural concentration of MIA was significantly associated with VEGF (Fig. 3a), VEGF-C (Fig. 3b) and VEGF-D expression (Fig. 3c). All the above-mentioned results suggested that MIA is associated with angiogenesis and lymphangiogenesis by up-regulation of VEGF, VEGF-C and VEGF-D in tongue SCC.

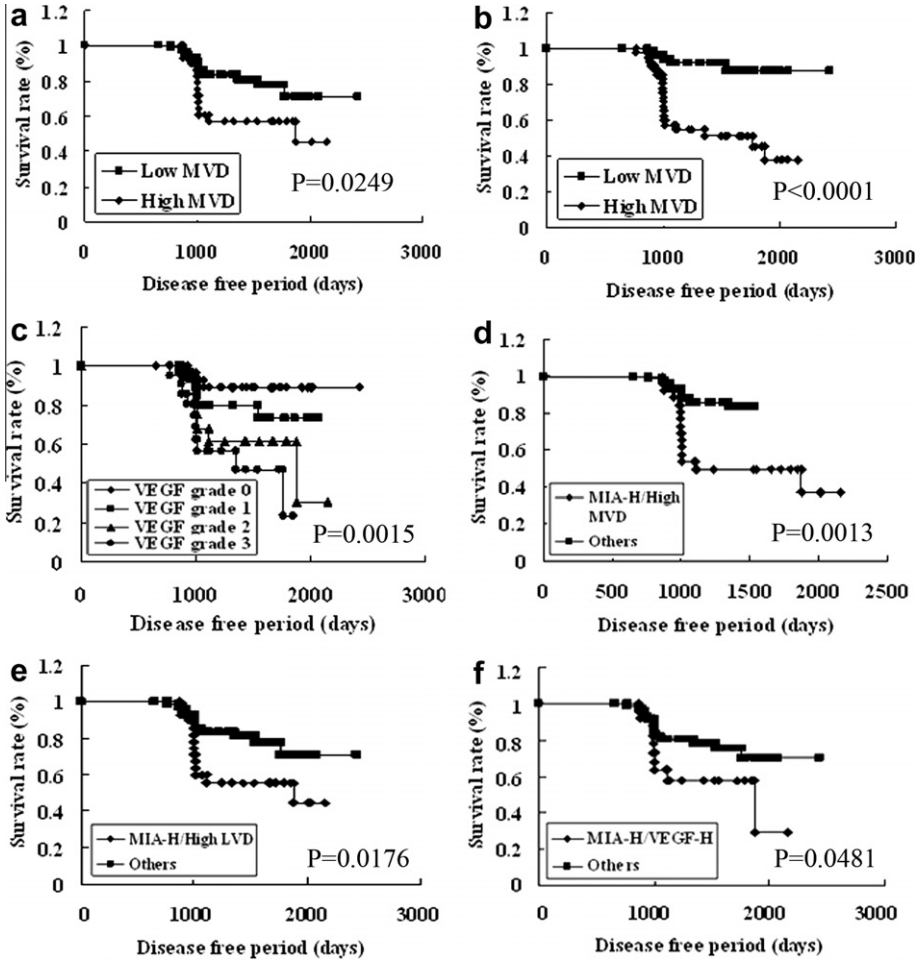

Fig. 2 – Disease-free survival curves of tongue SCC patients were calculated by Kaplan–Meier method. (a) A comparison between high MVD group and low MVD group. (b) A comparison between high LVD group and low LVD group. (c) A comparison among VEGF grade 0, 1, 2 or 3 groups. (d) A comparison between high MVD/high MIA grade group and others. (e) A comparison between high LVD/high MIA grade group and others. (f) A comparison between high MIA grade/high VEGF grade group and others.

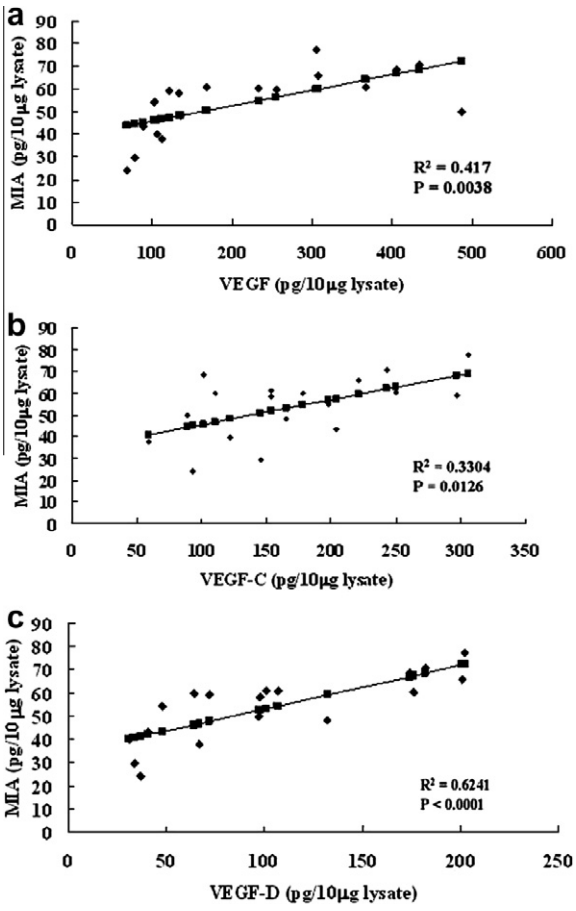

Fig. 3 – Relation of MIA concentration with VEGF, VEGF-C or VEGF-D concentrations. Association of MIA concentration with VEGF concentration (a), MIA concentration with VEGF-C concentration (b) and MIA concentration with VEGF-D concentration (c).

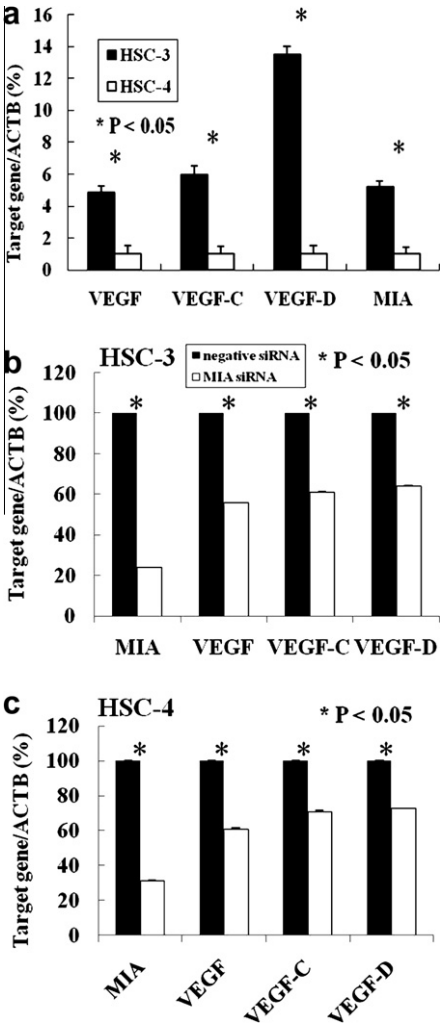

Fig. 4 – Effect of knockdown of MIA on expression of VEGF, VEGF-C and VEGF-D. Expression of VEGF, VEGF-C, VEGF-D and MIA in untreated HSC-3and -4 cells (a) and MIA siRNA-treated cells (b, c). Target gene expression was relatively quantified with beta-actin expression. (a) Expression levels in HSC-4 were set to 100%. (b, c) Expression levels in negative siRNA-treated cells were set to 100%.

3.6. *Expression of VEGF, VEGF-C, VEGF-D and MIA in tongue SCC cell lines*

Next, we compared the VEGF, VEGF-C, VEGF-D and MIA mRNA expression in human tongue cancer-derived HSC-3 (high metastatic cell line) and HSC-4 (low metastatic cell line) cells by qRT-PCR (Fig. 4a). Expression levels of VEGF, VEGF-C, VEGF-D and MIA in HSC-3 cells were higher than those in HSC-4 cells.

Finally, to confirm the regulation of VEGF, VEGF-C and VEGF-D expression by MIA, we performed the MIA siRNA treatment. Exposure to negative siRNA did not affect VEGF, VEGF-C, VEGF-D and MIA mRNA level, however, the expression levels of VEGF, VEGF-C and VEGF-D were decreased under treatment with 50 nM MIA siRNA in HSC-3 and HSC-4 cells (Fig. 4b and c).

4. Discussion

Angiogenesis plays an important role in prenatal development, wound healing, chronic inflammation, tumour growth and metastasis,[6,8] and lymphangiogenesis promotes lymph node metastasis in cancer cells.[7,8] A pivotal role for angiogenesis and lymphangiogenesis have VEGF and VEGF-C/D, respectively.[9,10,14–16] Previous studies have demonstrated that strong expression of VEGF is closely correlated with tumour progression and poor prognosis[33,34] and VEGF-C/D expression is significantly related with nodal metastasis.[4] However, angiogenesis and lymphangiogenesis in cancer are still controversial.[17–26]

MIA promotes cell detachment, migration, invasion by binding to fibronectin via SH3 domain-like structure and inhibiting cell-to-stromal attachment and inhibits apoptosis of the malignant melanoma cells.[29] Although, we previously reported that MIA expression is significantly associated with nodal metastasis in OSCC,[4] the definite roles of MIA in angiogenesis and lymphangiogenesis were still unclear.

In this study, higher MVD and LVD were strongly associated with tumour progression, nodal metastasis and poor

prognosis. VEGF-C and VEGF-D expression levels were only associated with lymph node metastasis, whereas significant correlation was found between expression of VEGF and cases with high progression of the cancer cells, lymph node metastasis and disease recurrence. Increased MVD values were related with immunoreactivity of VEGF, VEGF-C and MIA and LVD was associated with VEGF, VEGF-C, VEGF-D and MIA. MIA expression was significantly correlated with VEGF, VEGF-C and VEGF-D expression by immunohistochemistry and ELISA. In survival analysis, cases with high MVD/LVD, high expression of VEGF, high MVD/LVD and high expression of MIA and co-high expression of VEGF and MIA showed significantly short disease-free survival. The metastatic tongue cancer cell line, HSC-3, showed higher VEGF, VEGF-C, VEGF-D and MIA expression than the non-metastatic tongue cancer cell line, HSC-4. Moreover, expression levels of VEGF, VEGF-C and VEGF-D were significantly decreased by MIA siRNA treatment. It was also confirmed that the concentration and expression levels of VEGF, VEGF-C, VEGF-D and MIA are higher in cancer than in normal and cancer adjacent mucosa by ELISA and real-time RT-PCR (data not shown). VEGF-inducible lymphangiogenesis and nodal metastasis were reported in several cancers.[11,12]

Previous reports have suggested that one of the receptors for MIA is cell surface integrin $\alpha5\beta1$[30] and we verify the expression of integrin $\alpha5\beta1$ in HSC-3 and HSC-4 tongue cancer cells.[4] Expression of integrin $\alpha5\beta1$ in vascular and lymph vessel endothelial cells promotes outgrowth of new blood and lymphatic vessels, respectively.[35,36] MIA might stimulate vascular and lymph vessel endothelial cells directly to accelerate induction of angiogenesis and lymphangiogenesis. The induction of VEGF, VEGF-C, VEGF-D and MIA is the result of activation of nuclear factor kappa B (NFkB) p65 and p38 mitogen-activated protein kinase (MAPK).[4,37,38] Further examination will reveal the details of MIA-dependent angiogenesis and lymphangiogenesis.

In comparison to normal blood vessels, tumoural irregular blood vessels are lacking pericytes and attenuated for delivery of anti-cancer drug.[39] Recently, it has been proposed that destruction of tumoural blood vessels by anti-angiogenesis treatment is an effective anti-cancer therapy.[40,41] Inhibition of MIA-VEGF family signalling might be a useful target. Generally, lymphangiogenesis at the invasive front of the tumour is important for nodal metastasis[42] and intratumour lymphatic vessels are lacking function.[43] However, Shimizu and colleagues reported that intratumour lymph vessels are associated with nodal metastasis in gastric cancer,[44] but we did not find intratumour lymphatic vessels in this study. It has been reported that VEGF-C accelerates angiogenesis and VEGF-inducible lymphangiogenesis is associated with lymph npodemetastasis in several cancers[11,12,45] and our results were similar. However, the detailed mechanism of VEGF-related lymphangiogenesis is still unclear. Further studies are needed to clarify whether VEGF directly induces lymphangiogenesis by binding to VEGFR-2 in lymph vessel endothelial cells[46] or whether activation of VEGF-C via VEGFR-2-positive macrophages or vascular endothelial cells induces lymphangiogenesis[47] in OSCC.

Our present results are summarised in Fig. 5. MIA-VEGF signal was significantly associated with tumour progression, nodal metastasis and poor prognosis by induction of angiogenesis/lymphangiogenesis. Our results suggest that activation of MIA-VEGF-C and MIA-VEGF-D signals promotes angiogenesis/lymphangiogenesis and lymphangiogenesis, respectively, and are closely related with cervical lymph node metastasis. However, we must reveal the precise mechanism why only nodal metastasis was associated with MIA-VEGF expression. An appropriate animal experiment will be needed in further examination. In conclusion, we defined MIA to promote angiogenesis and lymphangiogenesis by the activation of VEGF, VEGF-C and VEGF-D in tongue SCC. To our knowledge, the present study is the first to examine several parameters including expression of MIA and angiogenesis and lymphangiogenesis in patients with tongue SCC. Our present results suggest that further examination might reveal that MIA is a useful target for anti-angiogenic and lymphangiogenic therapy in tongue cancer.

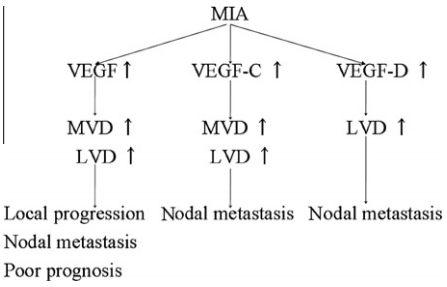

Fig. 5 – Schema of the present results. MIA is associated with angiogenesis and lymphangiogenesis through VEGF family activation in OSCCs.

Conflict of interest statement

None declared.

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