Evidence for critical role of Tie2/Ang1 interaction in metastatic oral cancer

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Abstract. Angiopoietin-1 (Ang1) is a binding partner of endothelial cell-specific tyrosine-protein kinase receptor (Tie2), which serves important roles in vascular development and angiogenesis. Tie2 is closely associated with the metastasis of oral squamous cell carcinomas (OSCCs) however, little is known about the correlation between Tie2 and Ang1. In the present study, the functional mechanisms of the Tie2/Ang1 interaction were investigated using Tie2 overexpressed (oeTie2) OSCC cells and recombinant Ang1 protein. oeTie2 cells had increased cell-cell and cell-extracellular matrix adhesions compared with the control cells. Additionally, the adhesive activities increased following treatment with exogenous Ang1, indicating that Ang1 directly enhances Tie2 functions. In the clinical OSCC data from 10 cases positive for regional lymph node metastasis, all cases were negative for Tie2 expression and eight cases (80%) were negative for Ang1 expression. These results suggest that Tie2 and Ang1 serve important roles in cancer metastasis and may be potential biomarkers and therapeutic targets for OSCC metastasis.

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Introduction

Cellular adhesion is controlled by adherent molecules in epithelial tissues, which are down-regulated in many cancers to promote transformation and might profoundly affect cellular migration and invasiveness (1-5). Since cancer metastasis decreases the patient survival rates, regional lymph node metastasis (RLNM) is one of the most adverse prognostic factors (6-12) for almost all cancers, including oral squamous cell carcinoma (OSCC). Therefore, elucidation of the molecular mechanisms involved in cancer metastasis clearly is needed to improve the prognosis (6,13). We reported previously that endothelial cell-specific tyrosine-protein kinase receptor (Tie2) is related closely to OSCC metastasis using overexpressed Tie2 (oeTie2) cells and its neutralization technique (14).

Tie2 and its ligand, angiopoietin 1 (Ang1), are essential for vascular maturation and blood vessel remodeling during embryonic angiogenesis (15-25). Ang1 regulates endothelial cell survival (16), anti-inflammatory actions (26-28), and radiation-induced endothelial-cell damage (29). Ang1, produced by many types of cells, has been described as a transcriptionally regulated molecule in several tumors (30,31). However, the Tie2/Ang1 interaction is poorly understood.

In the current study, we showed that the Tie2/Ang1 interaction promotes RLNM in OSCCs by controlling cellular adhesion. Thus, our results indicated that Tie2 and Ang1 are biomarkers for therapeutic targets in patients with OSCC.

Materials and methods

Ethics statement. The Ethics Committee of Chiba University approved our study protocol (approval no. 236), which was performed according to the tenets of the Declaration of Helsinki. All patients provided written informed consent.

oeTie2 cells and tissue specimens. oeTie2 cells, which were established in our previous study (14), were grown in Dulbecco's modified Eagle medium (DMEM) (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) supplemented with 10% fetal bovine serum (Sigma-Aldrich; Merck KGaA) and 50 units/ml of penicillin and streptomycin (Sigma-Aldrich; Merck KGaA).

We performed histopathological diagnosis of each OSCC sample according to the World Health Organization criteria at the Department of Pathology of Chiba University Hospital (32). The clinicopathological stages were determined based on the TNM classification of the International Union against Cancer (33). Twenty (10 cases each, RLNM-positive, RLNM-negative) pairs of primary OSCCs and patient-matched normal oral epithelia were obtained during surgical resections performed at Chiba University Hospital. The resected tissues were fixed in 20% buffered formaldehyde solution for pathologic diagnosis and immunohistochemistry (IHC).

mRNA expression analysis. Total RNA was isolated using TRIzol Reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's instructions. cDNA was generated from 5 μg of total RNA using Ready-To-Go You-Prime First-Strand Beads (GE Healthcare Life Sciences, Little Chalfont, UK) and oligo (dT) primers (Hokkaido System Science Co., Ltd., Sapporo, Japan). As described previously (14), real-time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) was performed using the LightCycler 480 apparatus (Roche Diagnostics GmbH, Mannheim, Germany). Primers were designed using the Universal Probe Library Assay Design Center (http://lifescience.roche.com/), which specifies the most suitable set. The primer sequences used for qRT-PCR were: Tie2, forward, 5'-CCCCTATGGGTGTTCCTGT-3'; reverse, 5'-GCTTACAATCTGGCCCGTAA-3'; and probe, no. 10; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), forward, 5'-AACATCATCCCTGCCTCTACT GG-3'; reverse, 5'-TTGAAGTCAGAGGAGACCACTG-3'; and probe, no. 61. The transcript amount was estimated from the respective standard curves and normalized to the GAPDH transcript amount determined in corresponding samples. All samples were analyzed in triplicate, and three independent preparations of RNA were analyzed from the cells.

Immunoblot analysis. The cells were washed twice with cold phosphate-buffered saline (PBS) and centrifuged briefly. The cellular pellets were incubated at 4˚C for 30 min in a lysis buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, and 10 mM Tris). The protein concentration was measured using a commercial Bradford reagent (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Immunoblot analysis was performed as described previously (14,34-37). Briefly, protein extracts (20 μg) were electrophoresed on 4-12% Bis-Tris gel (Invitrogen; Thermo Fisher Scientific, Inc.), transferred to polyvinylidene fluoride membranes (Invitrogen; Thermo Fisher Scientific, Inc.), and blocked for 1 h at room temperature in Blocking One (Nacalai Tesque Inc., Kyoto, Japan). The membranes were washed three times with 0.1% Tween-20 in Tris-buffered saline (TBS-T) and incubated with affinity-purified rabbit anti-Tie2 polyclonal antibody (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) or mouse anti-GAPDH monoclonal antibody (Santa Cruz Biotechnology, Inc.) overnight at 4˚C. The membranes were washed with TBS-T and incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG as a secondary antibody (Promega Corporation, Madison, WI, USA) for 1 h at room temperature. Finally, the membranes were detected using Super-Signal West Pico Chemiluminescent substrate (Thermo Fisher Scientific, Inc.), and immunoblot analyses were visualized by exposing the membranes to the ChemiDoc XRS system (Bio-Rad Laboratories, Inc.). The signal intensities were quantitated using Image Lab software (Bio-Rad Laboratories, Inc.). Densitometric Tie2 protein data were normalized to the GAPDH protein levels.

Cellular aggregation assay. To investigate the effect of Tie2 and cartilage oligomeric matrix protein, Ang1, a ligand for Tie2, on cell-cell adhesion, we performed cellular aggregation assays as described previously (38,39). The oeTie2 and Mock cells were incubated for 30 min at 37˚C in PBS containing 1 mM ethylenediaminetetraacetic acid, detached by gentle agitation, washed, and mechanically dissociated to obtain a single-cell suspension. The $3x10^5$ single cells in 1 ml of the serum-free DMEM were transferred to 12-well tissue culture plates and rotated at 60 rotations/min for 30 min at room temperature supplemented with and without human Ang1 (1 μg/ml) (R&D Systems, Inc., Minneapolis, MN, USA) or heat-inactivated Ang1 (1 μg/ml). Three random fields, each containing 200 cells, were viewed at x200 magnification for the presence of single and adherent cells. The percentage of adherent cells was calculated for each field and averaged (38,39).

Cellular adhesion assay. An adhesion assay was performed as described previously (14,40). Briefly, the cells were seeded in collagen I-coated 96-well plates, incubated for 1 h at 37˚C at a density of $2x10^4$ cells/well, and incubated for 1 h in DMEM, washed once with PBS, fixed in methanol, stained with crystal violet, and photographed. The numbers of the stained cells were measured using a microplate spectrophotometer (absorbance at 540 nm and at 405 nm to subtract the background). Before the adhesion assay, collagen I-coated 96-well plates were treated with and without Ang1 (1 μg/ml) or heat-inactivated Ang1 (1 μg/ml) for 1 h, and the assay was performed.

Multiplex IHC. Multiplex IHC was performed on 4-μm sections of paraffin-embedded specimens using rabbit anti-Tie2 polyclonal antibody (Santa Cruz Biotechnology, Inc.) and mouse anti-Ang1 polyclonal antibody (LifeSpan BioSciences, Inc., Seattle, WA, USA). Briefly, after deparaffinization and hydration, the endogenous peroxidase activity was quenched by a 3-min incubation in a mixture of 0.3% hydrogen peroxide solution in 100% methanol. The sections were blocked for 2 h at room temperature with 1.5% blocking serum (Santa Cruz Biotechnology, Inc.) in PBS before reaction with the anti-Tie2 and anti-Ang1 antibodies at 4˚C in a moist chamber overnight. For all washing steps, 0.1% Tween-20 in PBS was used. After primary antibody incubations, the Envision G/2 Double Stain System, Rabbit/Mouse (DAB+/Permanent Red) (Agilent Technologies, Inc., Santa Clara, CA, USA) was used according to the manufacturer's instructions. The slides were counterstained lightly with hematoxylin, dehydrated with ethanol, cleaned with xylene, and mounted. As a negative control, triplicate sections were immunostained without exposure to primary antibodies, which confirmed the staining specificity. To quantify the status of the Tie2 and Ang1 protein expression levels, we used the IHC scoring systems described previously (14,41-45). The mean percentages of positively stained cells were determined in at least three random fields at x400 magnification in each section.

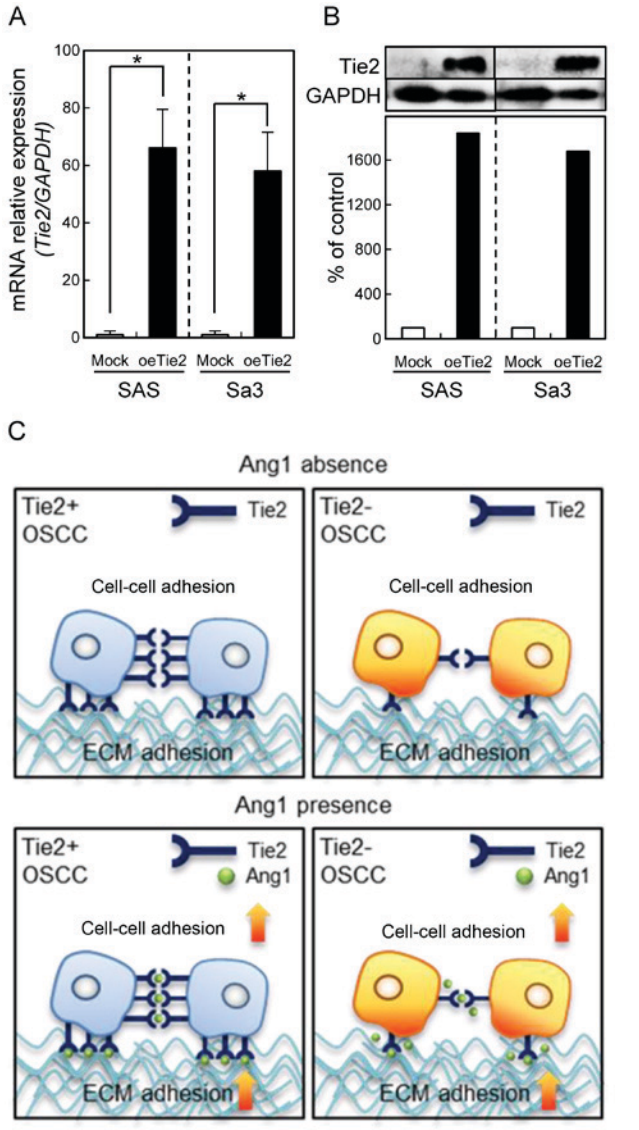


Figure 1. Expression level of Tie2 in its overexpressed cells. Significant (*P<0.05, Mann-Whitney U-test) up-regulation of (A) Tie2 mRNA and (B) protein are observed in oeTie2 cells compared with Mock cells (SAS and Sa3 cells) by RT-qPCR and immunoblot analyses. The Tie2 expression data are normalized to the GAPDH expression levels. The protein values are expressed as a percentage of the Mock cells. Data are expressed as the mean ± standard error of the mean (SEM) of triplicate results form independent three experiments. (C) Schematic representation of Tie2 and Ang1 functions for cell-cell and cell-ECM adhesions. Overexpression of Tie2 and exogenous Ang1 are related closely to cell-cell and cell-ECM adhesions in the cancer cells.

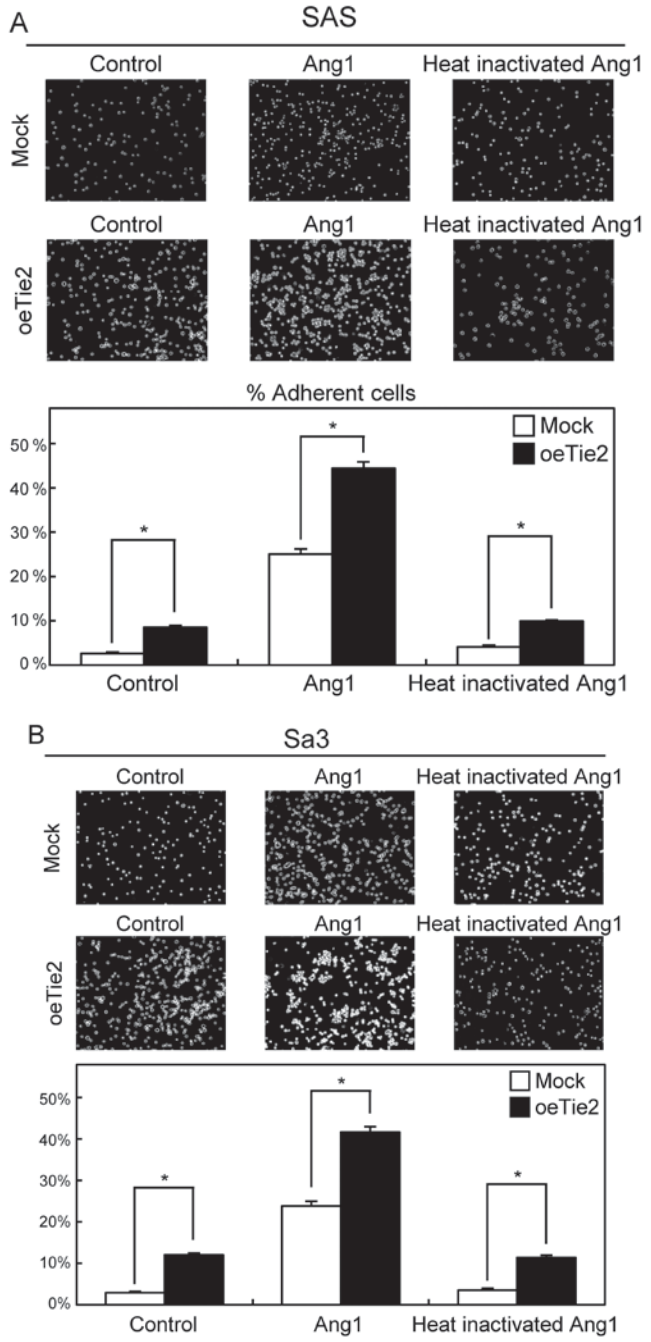


Figure 2. Cellular aggregation assay of Tie2 overexpressed cells. To evaluate the effects of Tie2 and Ang1 on (A) SAS and (B) Sa3 cell-cell adhesion activity, we performed a cellular aggregation assay. (Control) The cell-cell adhesion activity of oeTie2 cells is increased significantly (*P<0.05, Mann-Whitney U-test) compared with Mock cells. (Ang1/heat-inactivated Ang1) After treatment with Ang1, the numbers of aggregated cells increased dramatically (*P<0.05, Mann-Whitney U-test) compared with the control cells and cells treated with heat-inactivated Ang1. Data are expressed as the mean ± standard error of the mean (SEM) of triplicate results form independent three experiments.

Statistical analysis. To compare the Tie2 expression levels and the cell-cell and cell-extracellular matrix (ECM) adhesive capacities, statistical significance was evaluated using the Mann-Whitney U-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression level of Tie2 in its overexpressed cells. Since frequent down-regulation of Tie2 was observed in OSCC *in vitro* and *in vivo*, we previously established oeTie2 cells derived from two OSCC cell lines, SAS and Sa3 (14). To confirm the expression level of Tie2 in the oeTie2 cells, we performed qRT-PCR and immunoblot analyses. Consistent with our previous study, the Tie2 mRNA and protein expression levels in oeTie2 cells were significantly (P<0.05) higher than that in the Mock cells (Fig. 1A and B). Our previous study also showed that Tie2 plays an important role in cellular adhesion. In the current study, we hypothesized that not only Tie2 but also Ang1, the specific ligand for Tie2, regulate cell-cell and cell-ECM interactions (Fig. 1C).

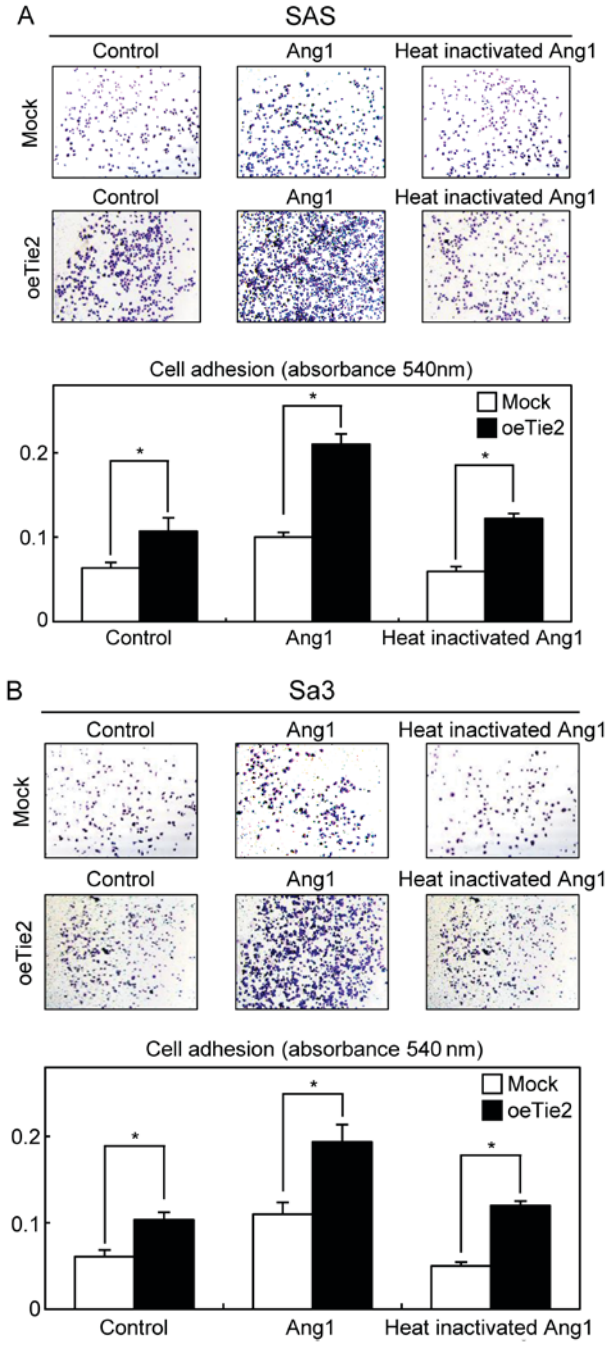
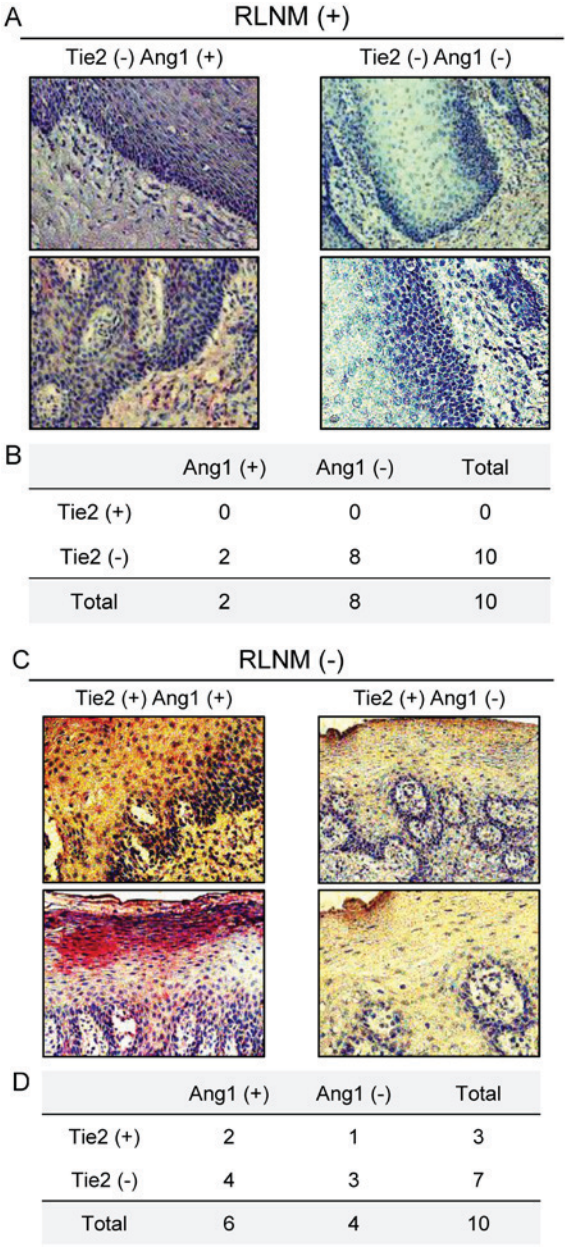


Figure 3. Cellular adhesion assay of Tie2 overexpressed cells. To evaluate the cell-ECM adhesion ability, (A) SAS and (B) Sa3 oeTie2 and Mock cells are seeded on collagen I-coated 96-well plates at a density of $2x10^4$ cells/well and allowed to adhere for 1 h. After crystal violet staining, the numbers of stained cells are measured using a microplate spectrophotometer (absorbance at 540 and 405 nm to subtract background). (Control) The cell-ECM adhesion in the oeTie2 cells is increased significantly (*P<0.05, Mann-Whitney U-test) compared with Mock cells. (Ang1/heat-inactivated Ang1) The cell-ECM adhesion ability of the oeTie2 cells treated with Ang1 is increased significantly (*P<0.05, Mann-Whitney U-test) compared with the control cells and cells treated with heat-inactivated Ang1. Data are expressed as the mean ± standard error of the mean (SEM) of triplicate results form independent three experiments.



B

	Ang1 (+)	Ang1 (-)	Total
Tie2 (+)	0	0	0
Tie2 (-)	2	8	10
Total	2	8	10

D

	Ang1 (+)	Ang1 (-)	Total
Tie2 (+)	2	1	3
Tie2 (-)	4	3	7
Total	6	4	10

Figure 4. Evaluation of Tie2 and Ang1 expression levels in primary OSCCs and the clinical correlations with RLNM. Representative multiplex IHC results for Tie2 and Ang1 proteins in the RLNM-positive and -negative groups. (A, C) All RLNM-positive cases have weak immunoreactivity for Tie2. In addition, eight (80%) of the 10 cases have weak immunoreactivity for Ang1. (B, D) Three RLNM-negative cases have strong immunoreactions for Tie2. Six (60%) RLNM-negative cases have strong immunoreactivity for Ang1. Original magnification, x200.

Functional analyses of oeTie2 cells. To evaluate the effect of Tie2 overexpression on cell-cell adhesion activity, we performed the cellular aggregation assay. The cell-cell adhesion activity of oeTie2 cells increased significantly (P<0.05) compared with Mock cells (control) (Fig. 2A and B). We then examined whether Ang1 regulates cell-cell adhesion activity. After treatment with Ang1, the number of aggregated cells increased dramatically compared with control cells and the cells treated with heat-inactivated Ang1 (Ang1 and heat-inactivated Ang1) (Fig. 2A, B).

We then performed a cellular adhesion assay to determine the biologic effects of Tie2 and Ang1 on cell-ECM interactions. The cell-ECM adhesion in the oeTie2 cells increased significantly (P<0.05) compared with Mock cells (control) (Fig. 3A and B). In addition, the cell-ECM adhesion activity of the cells treated with Ang1 increased significantly compared with the control cells and the cells treated with heat-inactivated Ang1 (Ang1 and

heat-inactivated Ang1) (Fig. 3A and B), suggesting that not only Tie2 but also Ang1 might be critical molecules for cell-cell and cell-ECM adhesions.

Evaluation of Tie2 and Ang1 expression levels in primary OSCCs and the clinical correlations with RLNM. We analyzed the Tie2 and Ang1 protein expression levels in 20 cases of primary OSCCs, RLNM-positive (n=10 cases) and RLNM-negative (n=10 cases), using the IHC scoring system. Representative IHC results for the Tie2 and Ang1 proteins in primary OSCC are shown in Fig. 4A and B. In the RLNM-positive cases, all cases (100%) were Tie2 negative, and eight (80%) cases were Ang1 negative (double negative expression, 8/10 cases), whereas three (30%) of the 10 RLNM-negative cases were negative for both Tie2 and Ang1 (Fig. 4C and D).

Discussion

In addition to our previous finding that Tie2 is in part a key modulator of OSCC tumor adhesion and invasion (14), the current findings indicated that the ligand of Tie2, Ang1, enhances the Tie2 functions in OSCC progression. Although cancer cells show that Tie2 is related closely to cancer metastasis (14,46,47), little is known about Ang1 function in cancer research.

Ang1 is thought to support endothelial cell adhesion and vascular integrity while inhibiting vascular permeability (18,19,48,49). Ang1 also induces phosphorylation of Tie2 and promotes endothelial cell migration and survival (23,50-52). The Tie2/Ang1 signaling pathway is thought to regulate proliferation and osteogenic differentiation of mesenchymal stem cells through activation of the p38 MAPK and Akt pathways (53,54). The Tie2/Ang1 interaction has different functions during angiogenesis and differentiation, suggesting that the Tie2/Ang1 signaling pathway differs at the molecular level in several types of cells. Since Kim *et al* reported a novel Ang1 function as a cell primer (55), we speculated that Ang1 increases cell-cell and cell-ECM adhesion activities through the Tie2/Ang1 interaction (Fig. 1C). Consistent with our hypothesis, patients with OSCC with low expression of Tie2 and Ang1 have high risk for RLNM (Fig. 4).

In conclusion, these data provide new insight that the Tie2/Ang1 interaction seems to have complex regulatory mechanisms, especially considering our finding that the Tie2/Ang1 interaction controls critical behaviors in metastatic OSCCs. While further studies using large cohort specimens are needed to study the Tie2/Ang1 interaction, the current data suggested that the Tie2/Ang1 interaction plays an important role in cellular adhesion and might be a potential biomarker for RLNM in OSCCs.

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