Prediction of outcome of patients with oral squamous cell carcinoma using vascular invasion and the strongly positive expression of vascular endothelial growth factors

Sachiko Seki^a, Mutsunori Fujiwara^b, Masaaki Matsuura^c, Shuichi Fujita^a, Hisazumi Ikeda^d, Izumi Asahina^d, Tohru Ikeda^{a*}

^aDepartment of Oral Pathology and Bone Metabolism, Nagasaki University Graduate School of Biomedical Sciences, Japan

^bDepartment of Clinical Pathology, Japanese Red Cross Medical Center, Japan

^cBioinformatics Group, Genome Center, Japanese Foundation for Cancer Research, Japan

^dDepartment of Regenerative Oral Surgery, Nagasaki University Graduate School of Biomedical Sciences, Japan

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^{*} Corresponding author.

Tel/Fax: +81-95-819-7644

E-mail address: tohrupth@nagasaki-u.ac.jp (T. Ikeda)

SUMMARY

Vascular invasion and lymph node metastasis have been used as histopathological prognosticators of cancers including oral squamous cell carcinoma (OSCC). In addition to metastatic potential via blood vessels, tumor-induced angiogenesis might also be associated with prognosis. However, the efficacy of combined evaluation of vascular invasion and angiogenesis-associated molecules for the prognosis of OSCC remains obscure. This is also the case in lymph node metastasis and lymphovasculogenesis-associated molecules. The aim of this study was to examine factors related to prognosis to improve the accuracy of prognostic prediction of OSCC using vasculogenesis-associated markers. Ninety specimens of patients from 1991 to 2002 with previously untreated OSCC, who underwent either biopsy or surgery, were histopathologically and immunohistochemically analyzed using antibodies for vascular endothelial growth factor (VEGF)-A, VEGF-C, cyclooxygenase (COX)-2 and Midkine. The ninety cases were composed of 72 well-differentiated, 12 moderately differentiated and 6 poorly differentiated OSCC. Efficient models of prognostic prediction were evaluated by extensive statistical analyses. The presence of vascular invasion or lymph node metastasis was confirmed to be significantly associated with poor prognosis in the univariate analysis. Multivariate logic regression analysis suggested that patients with the strongly positive expression of either VEGF-A or VEGF-C had a significant association with poor prognosis even in patients without vascular invasion and in early-stage patients. Neither COX-2 nor Midkine contributed to predict the prognosis of the patients. The strongly positive expression of VEGF-A or VEGF-C was suggested to reinforce the histopathological diagnosis of vascular invasion and improve the accuracy and efficacy of prognostic prediction of OSCC.

Key words: Vascular endothelial growth factor; Vascular invasion; Oral squamous cell carcinoma; Multivariate analysis; Prognosis

Introduction

The incidence of oral squamous cell carcinoma (OSCC) is relatively high and it has been reported to be the eighth most common cancer worldwide.^{1, 2} In addition, increasing incidence of OSCC is observed more in Europe, Japan, and in the younger generation in many western countries.³

Despite advances in clinical treatments, survival rates of OSCC have not improved for decades.⁴ It is considered particularly important to find and treat OSCC at an early stage. In the oral cavity, OSCC is most frequently seen in the posterior-lateral border of the tongue.^{1, 5}

VEGF-A, which was discovered as a factor that induces capillary and endothelial cell growth, was also recognized to be associated with tumor-induced angiogenesis.⁶ VEGF-C, one of the other members of the VEGF family, was found to be preferentially associated with tumor-induced lymphovasculogenesis.^{7,8}

COX-2 is a well-known enzyme that metabolizes arachidonic acid and induces cell membrane phospholipid-derived inflammation. Close association of the expression of COX-2 with carcinogenesis and tumor-induced angiogenesis has begun to be elucidated⁹⁻¹¹ and the expression of COX-2 has been identified as a possible marker to recognize carcinogenesis.¹²

MK, which was initially found as a molecule expressed in embryonal carcinoma cells, has been shown to promote the growth, survival and migration of various cells, including endothelial cells, and has also been shown to be involved in the regulation of epithelial–mesenchymal interactions.¹³ In addition, the expression of MK was found to be increased in various human tumors,¹³⁻¹⁵ and has also been suggested to promote or modulate angiogenesis.¹⁶⁻¹⁸

Including the tongue, most of the oral cavity is visible, and it is considered advantageous for early discovery of cancer, but proper medical knowledge and good medical as well as dental care systems are essential. In the hospital, histopathological diagnosis of the lesion is required to determine the course of treatment. Histopathological diagnosis of operation specimens has additionally been desired to provide information to predict prognosis in concert with clinical and imaging data based on tumor-node-metastasis (TNM) classification of tumors. However, further clinically applicable highly efficient prognosticators of OSCC have not been approved, although many possible candidates could be introduced. In this study, an attempt was made to improve one of the reliable histopathological prognosticators for vascular invasion using multiple vasculogenesis-associated factors, VEGF-A, VEGF-C, COX-2 and MK.

Materials and methods

Patients and materials

Ninety specimens of patients with previously untreated OSCC, who underwent either biopsy or surgery with or without preoperative treatment, were included. Patients were admitted to the Second Department of Oral and Maxillofacial Surgery, Nagasaki University Dental Hospital, from 1991 to 2002. They were composed of 58 male and 32 female patients ranging from 31 to 87 years of age with a mean age of 65.4 years. Forty-eight patients were treated with a standard program of preoperative irradiation of Linac at a total of 30 Gy and preoperative continuous subcutaneous administration of peplomycin (5 mg / day; maximum dosage, 100 mg). Nine patients were treated only with the preoperative irradiation, 12 patients were treated only with the preoperative administration of peplomycin and 21 patients were untreated before surgery. In histopathological diagnosis, 72 cases were well-differentiated, 12 cases were moderately differentiated and 6 cases were poorly differentiated OSCCs. All the patients were followed at the hospital until 2005. Among them, 66 patients (73.3%) died and 24 patients (26.7%) survived during the follow-up period. This study was approved by the ethics committee of the Nagasaki University Graduate School of Biomedical Sciences.

Histopathological analyses

Specimens were routinely processed with a 10% buffered formalin fixative and embedded in

paraffin. Morphology of tumor cells was evaluated using specimens stained with hematoxylin and eosin, and the presence of vascular invasion (v-factor) was evaluated using specimens stained with the method of Elastica van Gieson (EVG). Antibodies for vascular endothelial growth factor (VEGF)-A, VEGF-C (both from Zymed Laboratories Invitogen Immunodetection, San Francisco, CA), cyclooxygenase (COX)-2 (Cayman Chemical, Ann Arbor, MI) and Midkine (MK) (Yamasa, Chiba, Japan) were used. Sections were pretreated with an autoclave at 121 °C for 15 minutes in 10 mM citrate buffer (pH 6.0) to activate antigens, incubated with each antibody described above at x100 dilution with PBS at 4 °C overnight and immunohistochemical analysis was carried out on the EnVision+ System (Dako, Carpinteria, CA).

The expression of VEGF-A, VEGF-C and COX-2 was graded according to the percentage score of the stained carcinoma cells as follows: no expression (code 0), weakly positive expression (code 1) where less than 25% were positive carcinoma cells, moderately positive expression (code 2) where more than 25% to less than 50% were positive carcinoma cells and strongly positive expression (code 3) where more than 50% were positive carcinoma cells under magnifications of 100x and 200x. Owing to the general low intensity of MK immunoreactivity, the expression was ranged from no expression (code 0), weak expression (code 1), which was defined as less than 50% being positive carcinoma cells, to firm expression (code 2), in which more than 50% were positive carcinoma cells. Because VEGF-A, VEGF-C, COX-2 and MK were dominantly localized in the cytoplasm of expressing cells, chromogenic reactivity in the nucleus was omitted for evaluation of positivity.

Positive controls for these immunoreactions were taken using normal human liver specimens derived from autopsy cases, and negative controls were taken using specimens reacted with normal rabbit serum in place of antibodies.

Statistical analyses

Cox proportional hazards regression models were used to identify the prognostic factors. Prognosis status was defined for patients that died during the observation period as poor and for patients that were alive as good. Survival time was defined as the time interval between the date of the first surgical operation and the date of death from any cause, or censoring based on the date of last contact. In univariate analyses, we used indicator variables for the factors with more than two ordered categories, such as age group or stage of tumor. Significant factors from univariate analyses were examined by multivariate analyses to select a set of factors that show better fit to the data, from a combination of prognostic factors. For the model selection, we used Akaike's Information Criterion (AIC).¹⁹ For the parameterization of factors in each regression model, we used linear combinations of covariates, which are usually used in clinical epidemiology, and also used logic combinations of prognostic factors. The estimated relative risks (RRs) by the selected factors were calculated using estimated regression coefficients of the best model to predict. Survival curves were calculated by the Kaplan–Meier method and then compared by the log-rank test. Statistical procedures were performed with the Statistical Language R.²⁰ *P*-values <0.05 were considered to be statistically significant.

Results

Histopathological analyses

VEGF-A-positive tumor cells were detected in all the analyzed specimens and the expression in tumor cells ranged from codes 1 to 3. VEGF-A expression was also detected in vascular endothelial cells, which were omitted for evaluation of positivity using the morphological differences of tumor cells from vascular endothelial cells in serial sections stained with hematoxylin and eosin. The expression of VEGF-C and COX-2 ranged from codes 0 to 3. The expression of MK ranged from codes 0 to 2. Representative cases of each grade for VEGF-A and VEGF-C expression are shown in Fig. 1. Fifty-four cases had no vascular invasion and 33 cases

were positively recognized. The remaining three cases, which were two well-differentiated and one poorly differentiated OSCCs, were omitted from the analyses because of the false positive reaction in negative control sections.

Univariate and multivariate analyses

Study subjects by factors related to prognosis (risk factors) and their estimated RRs for poor prognosis based on Cox regressions are listed in Table 1. We observed that v-factor, the expression of VEGF-A, the expression of VEGF-C and T, N and clinical stage (Stage) of TNM classification were statistically significant. For example, the estimated RR for poor prognosis of subjects with code 1 to that of code 0 of v-factor was 3.994 (p <0.0001). (See the Table 1 for the other significant variables.) Sex, age, MK and COX-2 were not significant, but the estimated RR for poor prognosis of subjects more than 70 years old to that of those less than 50 years old was 2.012 (p = 0.097). As differences between 10-year age groups were not statistically significant but suggestive, we pooled subjects less than 70 years old, and obtained a value of p < 0.0001 (data not shown); then, we used two groups with a cut-off point of 70 years old in multivariate analysis. Similarly, it was suggested to pool subjects with codes 1 and 2 of VEGF-A, codes 0, 1 and 2 of VEGF-C, Stages I and II as well as Stages III and IV, T-stages 1 and 2 as well as T-stages 3 and 4, N-stages 0 and 1 as well as N-stages 2 and 3 and use each of them for multivariate analysis.

Multivariate analysis revealed that the Cox proportional hazards model with covariates of age groups, stages and a logical combination of covariates of v-factor, VEGF-A and VEGF-C had smaller AIC values than any other model with a linear combination of covariates (Table 2). In this selected model 1, the logical combination of covariates (we call them "important prognosis factors" (IPF)) had the value of zero when v-factors equaled 0, VEGF-A was less than 3 and VEGF-C was less than 3, and had the value of one otherwise, and the "IPF" was strongly

significant (p <0.0001). When T and N stages were used instead of clinical stage (I - IV) in selected model 1, better fitted model 2 with smaller AIC value was obtained. It should be noted that VEGF-A and VEGF-C were not statistically significant in linear combination model 1, which has been used widely in clinical epidemiology. Therefore, this result of model 1 led to the form of linear combination model 2 without VEGF-A and VEGF-C factors. However, AIC value of linear combination model 2 was higher than those of logic models 1 and 2 (Table 2).

Both logic model 1 with a clinical-stage covariate and logic model 2 with T- and N-stage covariates had small AIC values, and logic model 1 with a single covariate was used to demonstrate the importance of IPF in simple illustrations. The estimated RR for poor prognosis by age group, stage and IPF status is shown in Fig. 2. In the age group of less than 70 years old, the estimated RRs for poor prognosis of subjects with IPF = 1 in the Stage I and II group and the Stage III and IV group were 4.06 and 10.08, respectively. In the age group of more than 70 years old, RRs for poor prognosis of subjects with IPF = 1 in the Stage I and II group and the Stage III and IV group were 7.01 and 20.56, respectively. Estimated survival curves by stage, and by stage and IPF status are also shown in Fig. 3. Survival of subjects with IPF = 1 in the Stage I and II group and II group was as low as that of subjects in Stage III and Stage IV (Fig. 3a and b). Estimated survival curves for Stage I or II and IPF status are shown in Fig. 3c.

Discussion

A large number of molecules have been reported to correlate with poor prognosis of head and neck squamous cell carcinomas including OSCC using cell biological analyses.⁴ However, most of these molecules have not been routinely used as diagnostic markers for the prediction of prognosis in clinico-pathological laboratories. One of the reasons for this is that, except for differentiation markers or molecules involved in carcinogenesis, the prognostic value of these molecules might not be accurate enough for them to be used.

The expression of VEGF-A in OSCC has been analyzed and was suggested to be associated with the prognosis of patients, although conflicting reports have been published and the value of its expression for OSCC prognosis remains obscure.²¹⁻²⁶ The association of the expression of VEGF-C with lymph node metastasis has been suggested, however the prognostic value of its expression in OSCC has not been clarified.²⁷⁻²⁹

The estimated RR of the strongly positive expression of VEGF-A for poor prognosis based on Cox regression was significantly higher than that of the weakly positive expression. Hence, these results suggested that expression of VEGF-A itself has the potential to predict prognosis of OSCC; this was also the case in VEGF-C. In support of the current findings, Uehara et al. published a report showing that the expression of VEGF-A was significantly higher in patients with poor prognosis than in those with good prognosis for some of the specimens used in the present study.³⁰

Then, effective combination models using vascular invasion and the expression profile of markers associated with vasculogenesis were examined, and IPF was developed; the logical combination of covariates, which has the value of zero when v-factors equal 0, VEGF-A is less than 3 as well as VEGF-C is less than 3, has the value of one otherwise. It was found that IPF had significant prognostic value of OSCC.

Furthermore, it was detected that two groups with a cut-off point of 70 years old significantly correlated with the prognosis of OSCC. Some studies demonstrated that the prognosis of older patients was poorer than that of younger patients, although the correlation of age with prognosis of OSCC is controversial.² The explanation of poorer prognosis of patients who were more than 70 years old than that of those less than 70 years old may be partly caused by age-matched reduction of immunocompetence and there is no doubt that this can be detected more easily in a society with much longevity like Japan.

Interestingly, the selected model IPF revealed the highest estimated RR for poor prognosis (RR

= 20.56) in subjects of IPF = 1 of the Stage III and IV group, and that in subjects of IPF = 1 of the Stage I and II group, and more than 70 years old, was also high (RR = 7.01) when the estimated RR for poor prognosis of IPF = 0, Stage I and II and less than 70 years old was settled at one (Fig. 2). These data suggested that IPF improved the accuracy of prognostic prediction of OSCC when used with age groups with a cut-off point of 70 years, and also suggested that IPF was associated with survival of OSCC patients in any stage including Stages I and II.

Performance of IPF was confirmed by survival curves by the Kaplan–Meier method. It was striking that the survival curve of IPF = 1 subjects in the Stage I and II group was as low as those of Stage III and Stage IV subjects (Fig. 3). These data show that IPF contributes to predict survival of OSCC patients in early stages.

Among 90 cases, 73% of patients died. The high mortality was thought to be associated with the high proportion of patients in advanced stages. Recently, many more patients in earlier stages have been admitted to hospital and this could greatly improve the mortality of patients with OSCC. This means that the prognostic prediction of patients in an early stage of OSCC is very important, and IPF may contribute to the choice of a course of treatment for these patients.

In conclusion, it was demonstrated that IPF, a logic regression model using vascular invasion and the strongly positive expression of either VEGF-A or VEGF-C, was an effective prognostic predictor of OSCC not only in advanced stages, but also in early stages. Our IPF model is strongly expected to be applied to OSCC cases in many other institutions.

Conflicts of interest statement

The authors declare no conflicts of interest.

Acknowledgements

We thank Dr. Yuichi Ishikawa of The Cancer Institute, Japanese Foundation for Cancer Research

for critical reading of this manuscript. This research was supported by the Nagasaki University research fund for the Department of Oral Pathology.

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FIGURE CAPTIONS

Figure 1: Representative expression profiles of VEGF-A and VEGF-C in immunohistochemical analysis

(0), (1), (2) and (3) represent code 0 (no expression), code 1 (less than 25% of positive carcinoma cells), code 2 (more than 25% to less than 50% of positive carcinoma cells) and code 3 (more than 50% of positive carcinoma cells) of immunohistochemical grading of VEGF-A and VEGF-C. There was no case of code 0 for the expression of VEGF-A.

Figure 2: The estimated relative risks (RRs) of subjects evaluated with IPF and stage in each group with a cut off point of 70 years old

Subjects of Stages I and II (Stage I, II), and Stages III and IV (Stage III, IV) were pooled and analyzed. Definition of IPF was described as follows.

IPF=0, when v=0 and VEGF-A<3 and VEGF-C<3.

IPF=1, when v=1 or (v=0 and (VEGF-A=3 or VEGF-C=3)).

Figure 3: Kaplan-Meier curves of overall survival

(a) Curves of patients in each stage of OSCC. (b) Curves of patients evaluated with IPF in the Stage I and II group and the Stage III and IV group. (c) Curves of patients evaluated with IPF in Stage I and Stage II.

Factor	Category	Sample size	Prognosis		Cox Regression	
			Good	Poor	RR	p-value
Sex	male	58	16	42	1.000	-
	female	32	8	24	0.925	0.76
Age	-49	9	2	7	1.000	-
	50-59	18	8	10	0.566	0.250
	60-69	28	11	17	0.801	0.623
	70-	35	3	32	2.012	0.097
MK	0	55	14	41	1.000	-
	1	3	3	0	-	-
	2	32	7	25	0.956	0.859*
COX-2	0	16	7	9	1.000	-
	1	23	4	19	1.897	0.115
	2	25	6	19	1.869	0.124
	3	26	7	19	1.781	0.155
v-factor	0	54	23	31	1.000	-
	1	33	1	32	3.994	< 0.0001
VECE-A	1	10	7	0	1 000	
VEGF A	1	10	1	9	1.000	0.270
	2	39 21	14	20	1.411 2.00 5	0.379
	ð	31	ð	28	3.095	0.004
VEGF-C	0	38	15	23	1.000	-
	1	29	6	23	2.042	0.017
	2	13	2	11	2.043	0.055
	3	7	0	7	2.218	0.067
Stage	Ι	15	8	7	1.000	-
	Ш	17	7	10	1.817	0.248
	Ш	10	2	8	3.486	0.022
	IV	42	5	37	4.773	0.0005
T-stage	1	16	9	7	1.000	-
	2	31	10	21	2.653	0.036
	3	7	1	6	6.985	0.001
	4	33	3	30	5.574	< 0.0001

 Table 1:
 Study subjects by factors and their estimated relative risk (RR)

N-stage	0	41	16	25	1.000	-
	1	16	3	13	2.010	0.045
	2	29	4	25	3.094	0.002
	3	1	0	1	15.28	0.010
Meta	0	86	23	63	1.000	-
	1	1	0	1	3.212	0.253

 $\ast:$ Category 2 for MK was pooled with category 1.

			95% Confidence Interval				
Covariate	Estimated	S.E.	p-value	RR	lower	upper	AIC
	Coefficients						
Selected log	<u>gic model 1</u> (N=	=74)					
Age	0.642	0.29	0.028	1.90	1.07	3.37	354.4
Stage	0.980	0.32	0.002	2.66	1.42	4.99	
IPF*	1.401	0.33	< 0.0001	4.06	2.13	7.73	
Selected log	<u>gic model 2</u> (N=	=74)					
Age	0.761	0.29	0.008	2.14	1.22	3.77	351.3
T-stage	0.782	0.30	0.010	2.19	1.20	3.96	
N-stage	0.705	0.32	0.027	2.02	1.08	3.78	
IPF*	1.574	0.34	< 0.0001	4.83	2.48	9.41	
Linear com	<u>pination model</u>	<u>1</u> (N=74)					
Age	0.671	0.30	0.026	1.957	1.08	3.54	369.2
Stage	0.725	0.35	0.040	2.065	1.03	4.13	
v-factor	0.834	0.38	0.031	2.302	1.08	4.90	
VEGF-A	0.217	0.37	0.561	1.242	0.60	2.58	
VEGF-C	0.298	0.42	0.481	1.347	0.59	3.08	
Linear com	<u>pination model</u>	<u>2</u> (N=74)					
Age	0.726	0.30	0.014	2.066	1.16	3.69	366.1
Stage	0.740	0.34	0.030	2.095	1.07	4.09	
v-factor	0.950	0.33	0.004	2.589	1.36	4.93	

Table 2: Results of Cox proportional hazards regression models

*Note: The N means the number of subjects used in the model. Age=0, when age of a patient < 70. Age=1, when age of a patient ≥ 70. Stage=0, when a patient is in stage I or II. Stage=1, when a patient is in stage III or IV. Tstage=0, when a patient is in stage 1 or 2. Tstage=1, when a patient is in stage 3 or 4. N-stage=0, when a patient is in stage 0 or 1. N-stage=1, when a patient is in stage 2 or 3. IPF: important prognosis factors IPF=0, when v=0 and VEGF-A<3 and VEGF-C<3 IPF=1, when v=1 or (v=0 and (VEGF-A=3 or VEGF-C=3))





FIG. 2



FIG. 3