Pathol. Oncol. Res. (2014) 20:521–528
DOI 10.1007/s12253-013-9723-0

RESEARCH

Prognostic Value of Podoplanin Expression in Oral Squamous Cell Carcinoma—A Regression Model Auxiliary to UICC Classification

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Received: 30 May 2013 / Accepted: 5 November 2013 / Published online: 28 November 2013

Abstract Podoplanin, a type I transmembrane glycoprotein with an effect of platelet aggregation, has been reported to be one of the possible prognostic factors of oral squamous cell carcinoma (OSCC). However, the biological significance of podoplanin is largely unclear. The aim of this study was to develop a practical model for the prediction of prognosis using the grade of podoplanin expression, and also to evaluate the biological function of podoplanin. Eighty-two specimens of patients with previously untreated OSCC, who underwent either biopsy or surgery, were histopathologically and immunohistochemically analyzed. These 82 cases were composed of 66 well-differentiated, 10 moderately differentiated and 6 poorly differentiated OSCC. Podoplanin was successfully immunostained in 78 specimens, and was detected in most cases, but the frequency of positive cells varied. The prognosis of patients with more than 50 % podoplanin-positive tumor cells was significantly poorer than that of the other patients. Multivariate hazards regression analysis suggested that a linear combination of covariates, OSCC patients with more or less than 50 % podoplanin expression, age of more or less than 70 years old, mode of invasion and T3, T4 or T2 versus T1 of the UICC T-stage classification was the most effective model for evaluating the prognosis of OSCC patients. Additionally, podoplanin expression had a significant relationship to UICC clinical stage and the expression of Ki-67. An effective regression model using podoplanin expression was developed for evaluating the prognosis of OSCC and the biological significance of podoplanin was suggested to be associated with the growth and/or progression of OSCC.

Keywords Podoplanin · Oral squamous cell carcinoma · Prognosis · Clinical stage · Statistical analysis

Abbreviations

OSCC	Oral squamous cell carcinoma
VEGF	Vascular endothelial growth factor
AIC	Akaike's information criterion
RR	Relative risk

Introduction

Podoplanin is a type I transmembrane glycoprotein with an effect of platelet aggregation. Toyoshima et al. initially isolated podoplanin as a platelet aggregation factor that was preferentially expressed in a highly metastatic tumor cell line, and was suggested to be associated with tumor metastasis [1,2]. Interestingly, during the analysis of podoplanin, it was found to be expressed in lymphatic endothelial cells but not in vascular endothelial cells [3]. Podoplanin is now widely used

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as a distinct marker of lymphatic endothelial cells in clinicopathological laboratories all over the world. Podoplanin null mice revealed abnormality in lymphatics, which suggested it plays essential roles for lymphangiogenesis [4], but the biological mechanism of podoplanin expression in lymphangiogenesis is still unclear.

Podoplanin is also known to be expressed in certain human tumors, and was reported to be expressed at a high level in oral squamous cell carcinoma (OSCC) [5–10]. In the lung, podoplanin was preferentially expressed in SCC compared with adenocarcinoma [11], and was also shown to be expressed in several kinds of tumor including germ cell tumors, brain tumors, soft tissue tumors, osteogenic and chondrogenic tumors, odontogenic tumors, salivary gland tumors, thymomas and mesotheliomas [12–20]. Although the expression of podoplanin was detected in several kinds of human tumor, its biological significance was largely unclear.

To evaluate the clinical significance of podoplanin expression in tumors, statistical analyses of clinical cases have also been conducted. Many clinicopathological studies suggested that the expression of podoplanin in tumor cells correlated with lymph node metastasis and poor prognosis [6–8,20,21]. However, inconsistent results have also been reported and the clinical significance of podoplanin expression in human tumor cells remains undetermined [22,23]. The TNM classification of the International Union Against Cancer (UICC) is a universally used prognostic factor for a variety of tumors. The applicability of the UICC classification for a wide variety of tumors reveals the importance of the anatomical distribution of tumors for prognosis. Non-anatomical distribution factors, such as age, gender, histological grading of tumors and mode of invasion, are also known to be important for the prognosis of patients in some cases. In addition, the expression of many kinds of functional molecule associated with tumorigenesis, tumor growth or tumor invasion has been evaluated in association with prognosis. Previously, we studied the significance of the expression of vascular endothelial growth factor (VEGF)-A and VEGF-C for the prognosis of OSCC, which were known to have angiogenic and lymphangiogenic function, respectively. Using multivariate logic regression analysis, we constructed a novel logic regression model using vascular invasion and strongly positive expression of either VEGF-A or VEGF-C. The model was suggested to contribute not only to improved accuracy of prediction of prognosis, but also to prediction of the prognosis of early-stage OSCC patients [24].

Here, we studied the prognostic value of podoplanin expression in OSCC by multivariate regression analysis. We also analyzed variables correlated with the expression of podoplanin to evaluate its still unknown biological significance in OSCC.

Materials and Methods

Patients and Materials

Eighty-two 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 55 male and 27 female patients ranging from 31 to 87 years of age with a mean age of 65.4 years. Forty-two 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 preoperative irradiation, 11 patients were treated only with the preoperative administration of peplomycin and 20 patients were untreated before surgery. In histopathological diagnosis, 66 cases were well-differentiated, 10 cases were moderately differentiated and 6 cases were poorly differentiated OSCCs. All the patients were followed at the hospital until 2005. Among them, 60 patients (73.2 %) died and 22 patients (26.8 %) survived during the follow-up period.

Histopathological Analyses

Specimens were routinely processed with a 10 % buffered formalin fixative and embedded in paraffin. Morphology of the tumor cells was evaluated using specimens stained with hematoxylin and eosin. Mode of invasion of OSCC was graded into a, b and c, which represent Jakobsson's grades 1 & 2, grade 3 and grade 4, respectively [25]. An antibody for D2-40 (Covance, Princeton, NJ) was used to detect podoplanin following the determination in a previous study [26]. To analyze the growth activity of tumor cells, an antibody for Ki-67 (Dako, Glostrup, Denmark) was used. Sections were 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). Negative controls were taken using specimens reacted with normal rabbit serum in place of antibodies.

The expression of podoplanin was evaluated as the percentage score of the stained carcinoma cells, and carcinoma cells stained with Ki-67 was graded according to the percentage score as follows: less than 15 % were positive carcinoma cells (code 1), not less than 15 % to less than 30 % were positive carcinoma cells (code 2), not less than 30 % to less than 45 % were positive carcinoma cells (code 3), not less than 45 % to less than 60 % were positive carcinoma cells (code 4)

and not less than 60 % were positive carcinoma cells (code 5) under magnifications of 100× and 200×.

Statistical Analyses

Cox proportional hazards regression models were used to identify the prognostic factors. Prognosis status was defined for patients who died during the observation period as poor and for patients who were alive as good. 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 a better fit to the data, from a combination of prognostic factors. For the model selection, we used Akaike's Information Criterion (AIC) [27]. For the parameterization of factors in each regression model, we used linear combinations of covariates, which are usually used in clinical epidemiology. The estimated relative risks (RRs) by the selected factors were calculated using estimated regression coefficients of the best model for prediction. Statistical procedures were performed with the Statistical Language R [28]. P-values <0.05 were considered to be statistically significant.

Results

Podoplanin expression was detected in a subset of OSCC cells in addition to lymphatic endothelial cells. Representative immunohistochemical profiles of podoplanin expression in OSCC are shown in Fig. 1. Podoplanin was detected in the cytoplasm of OSCC cells preferentially in the para basal cell layer of the invasive front of OSCC cases (Fig. 1a), but in a considerable number of cases, more abundant expression was detected in OSCC cells (Fig. 1b-e). Podoplanin expression was detected irrespective of the differentiation grade and the mode of invasion of OSCC (Fig. 1).

Univariate analysis revealed that age, mode of invasion, podoplanin expression, T, N and clinical stage of the TNM classification predicted the prognosis of OSCC patients. The best cut point of age was searched by analyzing two groups with a cut point from 50 to 80 of age by 5-year rank, and it was 70. However, gender, differentiation grade of tumor cells or the expression of Ki-67 did not predict the prognosis of these patients (Table 1). Podoplanin expression was detected in most of the OSCC cases, but the distribution varied, and the expression was evaluated by the proportion of positively stained tumor cells among the total tumor cells. When the cut-off point was set at 50 %, the prognosis of patients with more than 50 % podoplanin expression was significantly poorer than that of the others.

Multivariate analysis was performed using all combinations of factors significantly associated with the prognosis of patients listed in Table 1 as covariates in the Cox proportional hazards model. Multivariate analysis revealed that the Cox model with a linear combination of covariates of age groups, mode of invasion, podoplanin expression and T-stage groups had the smallest AIC values. In this selected model, T2 vs. T1 was not statistically significant, but T3 and T4 vs. T1 was statistically significant and this

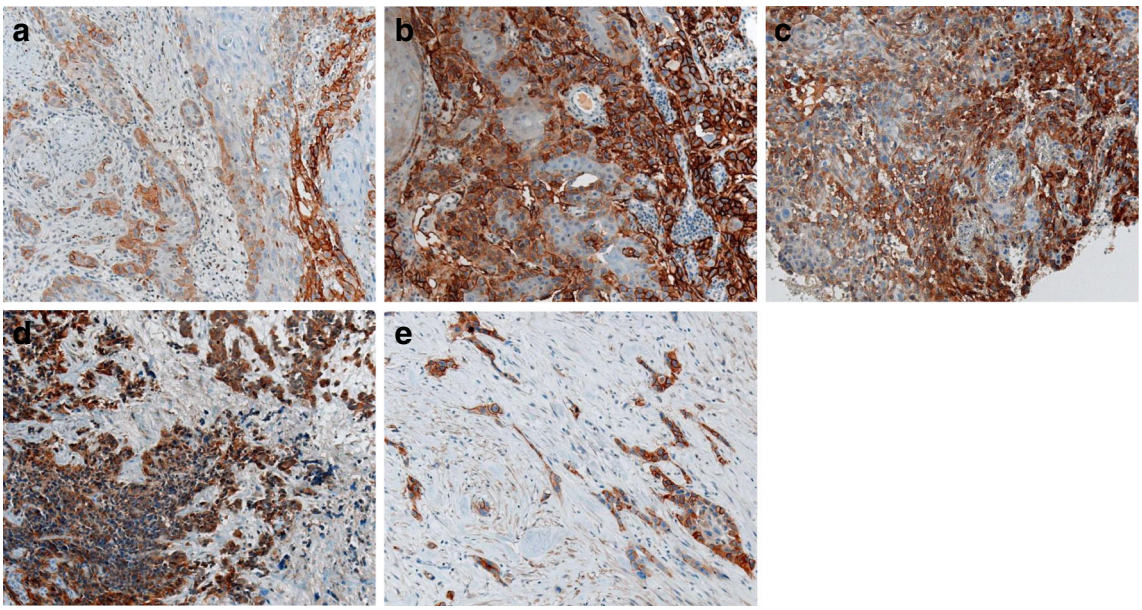

Fig. 1 Representative expression profiles of podoplanin in immunohistochemical analysis. **a** Well-differentiated infiltration grade b OSCC with weak podoplanin expression. **b** Well-differentiated, **c** moderately differentiated and **d** poorly differentiated infiltration grade b OSCCs with strong podoplanin expression. **e** Well-differentiated infiltration grade c OSCC with strong podoplanin expression

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

Factor	Category	Prognosis		Cox regression	
		Good	Poor	RR	*p*-value
Age	<70	19	31	1.00	–
	≥70	3	29	2.93	<0.001
Sex	Male	16	39	1.00	–
	Female	6	21	0.94	0.809
Differentiation grade	Well	16	50	1.00	–
	Moderate	4	6	0.75	0.508
	Poor	2	4	1.05	0.928
Mode of invasion	a	12	3	1.00	–
	b	5	28	7.76	<0.001
	c	5	29	8.62	<0.001
Podoplanin	<50 %	21	46	1.00	–
	≥50 %	1	10	2.16	0.032
T	1	9	6	1.00	–
	2	9	21	2.65	0.036
	3	1	6	6.24	0.002
	4	3	27	5.31	<0.001
N	0	16	24	1.00	–
	1	2	13	2.19	0.024
	2	4	23	2.23	0.007
Stage	I or II	15	16	1.00	–
	III or IV	7	44	2.99	<0.001
Ki-67	1	3	0	1.00[a]	–
	2	2	4	1.00[a]	–
	3	2	7	1.74	0.380
	4	7	15	1.43	0.527
	5	8	34	1.77	0.281

[a] As for Ki-67, we pooled patients with score 1 and 2 as a reference group, because the number of deaths is zero for the patients with score 1

was the best fitting model (Table 2). The estimated RR for poor prognosis by age group, podoplanin expression and T status is shown in Fig. 2. When the mode of invasion was a, in the age group of less than 70 years old, the estimated RR for poor prognosis of subjects with podoplanin <50 % in the T1 group was the lowest (RR=1). However, when the mode of invasion of OSCC was b or c, in the age group of more than 70 years old, the estimated RRs for poor prognosis of subjects with podoplanin ≥50 % and <50 % in the T3 and T4 group were 125.01 and 55.65, and in the age group of less than 70 years old, the estimated RRs for poor prognosis of subjects with podoplanin ≥50 % and <50 % in the T3 and T4 group were 34.96 and 15.56, respectively (Fig. 2a, b). When the mode of invasion of OSCC was a, in the age group of more than 70 years old, the estimated RRs for poor prognosis of subjects with podoplanin ≥50 % and <50 % in the T3 and T4 group were 21.76 and 9.69, whereas, in the age group of less than 70 years old, the estimated RRs for poor prognosis of subjects with podoplanin ≥50 % and <50 % in the T3 and T4 group were 6.09 and 2.17, respectively (Fig. 2c, d).

Factors correlated with podoplanin expression were also analyzed, and multiple regression analysis revealed that podoplanin expression and clinical stage were significantly correlated. Patients in clinical stages III and IV were significantly associated with more than 50 % expression of podoplanin (Fig. 3, Table 3). In addition, podoplanin expression was significantly correlated with the expression of Ki-67 (Table 4).

Discussion

Podoplanin is widely known as a distinct marker of lymphatic endothelial cells. It is also known that podoplanin expression is detected in several kinds of tumors including OSCC. In addition, recent studies have suggested that podoplanin plays important roles in immune systems [29]. Among the diverse roles of podoplanin, cancer-associated functions have been the most extensively studied. Cueni et al. reported that MCF7 human breast carcinoma cells with ectopic overexpression of podoplanin promoted lymphangiogenesis and lymph node metastasis in nude mice [30]. It has also been reported that podoplanin expression in the tumor was associated with lymph node metastasis and the prognosis of patients [6–8,20,21]. However, the results of clinicopathological studies varied [22,23], and the biological significance of podoplanin expression in human tumors remains largely

Table 2 Results of Cox proportional hazards regression models. (*N*=78)

Covariate	Estimated coefficient	Standard error	*p*-value	RR	95%CI. lower	95%CI. upper
Age (over 70 vs less)	1.27	0.32	<0.001	3.58	1.90	6.72
Mode of invasion (b, c vs a)	1.75	0.60	0.004	5.74	1.76	18.80
Podoplanin (over 50 % vs less)	0.81	0.40	0.042	2.25	1.03	4.90
T2 (vs T1)	0.41	0.49	0.397	1.51	0.58	3.91
T3,T4 (vs T1)	1.00	0.47	0.035	2.71	1.08	6.83

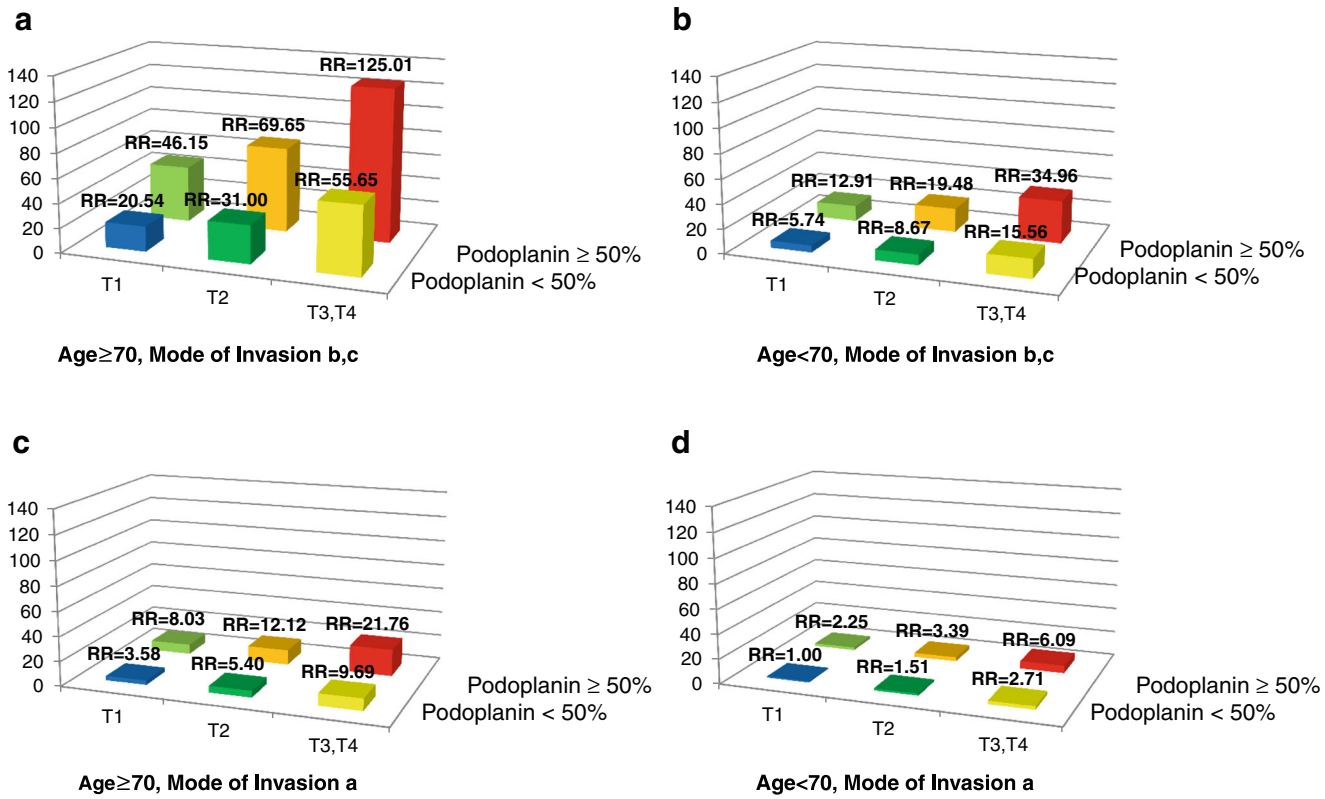

Fig. 2 The estimated relative risks (RRs) of subjects evaluated with podoplanin expression and T stage in each group with a cut-off point of 70 years old and mode of invasion a vs. b and c. Subjects of T3 and T4 were pooled and analyzed

unclear. In this study, podoplanin expression was one of the prognostic factors of OSCC in addition to age and T, N and clinical stage of the TNM classification in univariate analysis (Table 1). To clarify the controversial clinicopathological value of podoplanin expression of the prognostic prediction of tumors, we developed a new model using age (more than 70 or less), mode of invasion (b, c vs. a), podoplanin expression (more than 50 % or less) and T classification (T2 or T3, T4 vs. T1) as covariates by extensive multivariate analysis (Table 2). The estimated RR of a group of patients aged more than 70, in T3 or T4 stage and with mode of invasion b or c was 125.01 times higher than that of a group of patients aged less than 70, in T1 stage and with mode of invasion a (Fig. 2). The distinct RRs in each group shown in Fig. 2 strongly suggest that our new model including the intensity of podoplanin expression has practical value for the prognostic prediction of OSCC.

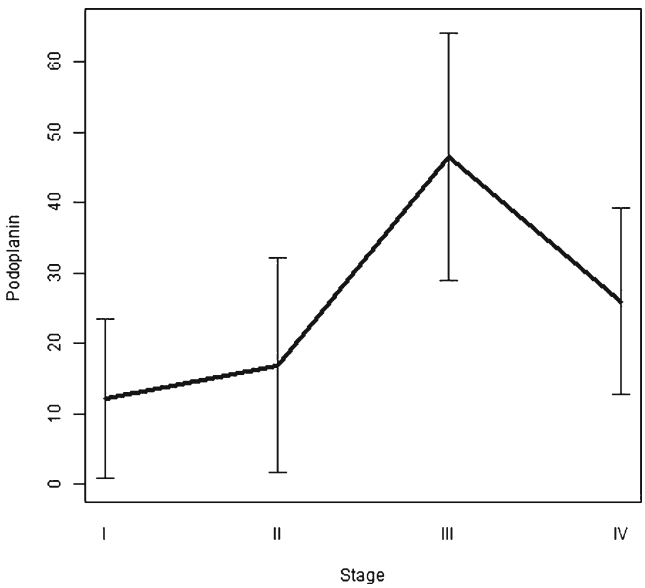

Fig. 3 Relationship of the intensities of podoplanin expression to clinical stages. Error bars represent 95 % confidence intervals

Table 3 Multiple regression analysis on the relationship between podoplanin expression and clinical stages

	Estimated coefficient	Standard error	95%CI. lower	95%CI. upper	*p*-value
Intercept	12.2	5.8	0.9	23.5	0.037
Stage II	4.7	7.8	1.6	32.1	0.551
Stage III	34.3	8.9	29.0	64.0	<0.000
Stage IV	13.8	6.8	12.7	39.2	0.046

Stage I is a reference group in indicator variables for clinical stages

Table 4 Multiple regression analysis on the relationship between the expression of podoplanin and Ki-67

	Estimated coefficient	Standard error	95%CI. lower	95%CI. lower	*p*-value
Intercept	22.83	18.0	−12.48	58.13	0.209
Age	−0.23	0.23	−0.68	0.21	0.316
Sex*1	−8.00	5.54	−18.85	2.86	0.153
Ki-67*2	5.29	2.33	0.73	9.85	0.026
(Selected model)					
Intercept	3.86	9.90	−15.54	23.26	0.710
Ki-67*2	4.95	2.33	0.38	9.52	0.037

*1: Sex was coded 1 for male and 0 for female

*2: Ki-67 was used as a continuous variable for regression models

To improve our knowledge of the clinicopathological value of podoplanin expression in OSCC, factors that had a significant relationship to podoplanin expression were analyzed. Multiple regression analysis revealed a significant relationship of strongly positive (>50 %) expression of podoplanin to advanced clinical stages, especially stage III (Fig. 3, Table 3). Poorer fitness for stage IV compared with stage III was suspected to be caused by the presence of a considerable number of degenerative and/or necrotic tumor cells in stage IV tumors. These results strongly suggest that the ratio of clinical stages in a population greatly affects the prognostic value of podoplanin expression, which might be caused by the divergent values of podoplanin expression on prognostic prediction. We further analyzed the relationship of podoplanin expression to that of Ki-67, one of the most widely used markers to evaluate the growth activity of tumor cells. As expected, multiple regression analysis indicated a significant relationship of podoplanin expression to that of Ki-67 (Table 4). These results suggest that podoplanin expression is associated with tumor growth and/or progression. Interestingly, the prognostic value of the expression of Ki-67 in OSCC was not found in univariate analysis (Table 1). In this study, the expression of Ki-67 was classified into 5 grades, and the number of cases classified into grade 1 (less than 20 % positive cells) was only 5, and many cases were classified into higher grades. Recently, intra-tumoral variation of Ki-67-positive cells was detected in tissue microarray using a newly developed digital imaging method, and it was reported that the basal (lowest observed) Ki-67 expression was associated with the prognosis of OSCC [31]. In this study, we used mean expression and did not analyze intra-tumoral variation of the expression. However, it was conceivable that a considerable number of Ki-67-positive cells were detected in OSCC because they were malignant tumors, and had little value to evaluate the prognosis of OSCC patients. These results further confirm the importance of podoplanin expression for the prognostic prediction of OSCC in addition to the growth and/or progression of tumors.

There are many reports suggesting that podoplanin is associated with lymph node metastasis [6–8,20,21]. In contrast, reports suggesting that podoplanin expression is associated with tumor progression are rare. Recently, Mashhadiabbas et al. suggested that podoplanin expression is associated with lymphatic vessel density and size of tumor in OSCC cases [32]. In addition, Kreppel et al. reported that podoplanin expression is associated with the clinical stage of OSCC, in addition to the N-stage [7]. Both reports recognized podoplanin as one of the prognostic factors. Kawaguchi et al. reported that podoplanin is a marker of cancer development and revealed that the frequencies of podoplanin expression increases with increased severity of dysplasia [33]. Atsumi et al. reported that podoplanin-positive A431 human SCC cells shares stem cell markers of squamous epithelium [34]. Our results are consistent with these studies, which suggests that podoplanin expression is associated with the growth and/or progression of OSCC.

We previously reported that the expression profile of VEGF-A or VEGF-C is one of the most important prognostic factors of OSCC when imposing the condition of the presence of vascular invasion or the strong expression of VEGF-A or VEGF-C; we named the model as an important prognostic factor (IPF). In that study, we suggested that IPF is also effective to predict the prognosis of OSCC in stage I or II [24]. VEGF-C has been shown to contribute to lymphangiogenesis, and these results suggested that the expression of VEGF-C in OSCC cells is predictive of future lymph node metastasis and poor prognosis. In this study, podoplanin expression was significantly correlated with OSCC in higher stages (Fig. 3, Table 3). These results suggest that both the expression of VEGFs and that of podoplanin have value for the prognostic prediction of OSCC, but the biological significances including certain roles in lymphangiogenesis of these factors for OSCC differ.

In conclusion, the intensity of podoplanin expression in OSCC cells predicted the prognosis of patients, and our new model using age, mode of invasion, podoplanin expression and T stage as covariates was suggested to have practical benefits for the prognostic prediction of OSCC. In addition, podoplanin expression was correlated with higher clinical stages and the expression of Ki-67, and was suggested to be associated with tumor growth and/or progression.

Acknowledgments This study was supported by Grants-in-Aid from the Ministry of Education, Culture, Sports and Technology of Japan (Grant no. 25462865) and the Nagasaki University research fund for the Department of Oral Pathology.

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