

1 **Prognostic value of podoplanin expression in oral squamous cell carcinoma—a regression**
2 **model auxiliary to UICC classification**

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4 Concise title: Podoplanin expression in OSCC

5

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

20
21 **Keywords** podoplanin; oral squamous cell carcinoma; prognosis; clinical stage; statistical
22 analysis

23
24 **Abbreviations** OSCC: oral squamous cell carcinoma; VEGF: vascular endothelial growth factor;

1 AIC: Akaike's Information Criterion; RR: relative risk

2

1 **Introduction**

2 Podoplanin is a type I transmembrane glycoprotein with an effect of platelet aggregation.
3 Toyoshima et al. initially isolated podoplanin as a platelet aggregation factor that was
4 preferentially expressed in a highly metastatic tumor cell line, and was suggested to be associated
5 with tumor metastasis.[1,2] Interestingly, during the analysis of podoplanin, it was found to be
6 expressed in lymphatic endothelial cells but not in vascular endothelial cells.[3] Podoplanin is
7 now widely used as a distinct marker of lymphatic endothelial cells in clinicopathological
8 laboratories all over the world. Podoplanin null mice revealed abnormality in lymphatics, which
9 suggested it plays essential roles for lymphangiogenesis,[4] but the biological mechanism of
10 podoplanin expression in lymphangiogenesis is still unclear.

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

18 To evaluate the clinical significance of podoplanin expression in tumors, statistical analyses of
19 clinical cases have also been conducted. Many clinicopathological studies suggested that the
20 expression of podoplanin in tumor cells correlated with lymph node metastasis and poor
21 prognosis.[6-8,20,21] However, inconsistent results have also been reported and the clinical
22 significance of podoplanin expression in human tumor cells remains undetermined.[22,23] The
23 TNM classification of the International Union Against Cancer (UICC) is a universally used
24 prognostic factor for a variety of tumors. The applicability of the UICC classification for a wide

1 variety of tumors reveals the importance of the anatomical distribution of tumors for prognosis.
2 Non-anatomical distribution factors, such as age, gender, histological grading of tumors and
3 mode of invasion, are also known to be important for the prognosis of patients in some cases. In
4 addition, the expression of many kinds of functional molecule associated with tumorigenesis,
5 tumor growth or tumor invasion has been evaluated in association with prognosis. Previously, we
6 studied the significance of the expression of vascular endothelial growth factor (VEGF)-A and
7 VEGF-C for the prognosis of OSCC, which were known to have angiogenic and
8 lymphangiogenic function, respectively. Using multivariate logic regression analysis, we
9 constructed a novel logic regression model using vascular invasion and strongly positive
10 expression of either VEGF-A or VEGF-C. The model was suggested to contribute not only to
11 improved accuracy of prediction of prognosis, but also to prediction of the prognosis of
12 early-stage OSCC patients.[24]

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

16

17 **Materials and Methods**

18 Patients and materials

19 Eighty-two specimens of patients with previously untreated OSCC, who underwent either biopsy
20 or surgery with or without preoperative treatment, were included. Patients were admitted to the
21 Second Department of Oral and Maxillofacial Surgery, Nagasaki University Dental Hospital,
22 from 1991 to 2002. They were composed of 55 male and 27 female patients ranging from 31 to
23 87 years of age with a mean age of 65.4 years. Forty-two patients were treated with a standard
24 program of preoperative irradiation of Linac at a total of 30 Gy and preoperative continuous

1 subcutaneous administration of peplomycin (5 mg/day; maximum dosage, 100 mg). Nine patients
2 were treated only with preoperative irradiation, 11 patients were treated only with the
3 preoperative administration of peplomycin and 20 patients were untreated before surgery. In
4 histopathological diagnosis, 66 cases were well-differentiated, 10 cases were moderately
5 differentiated and 6 cases were poorly differentiated OSCCs. All the patients were followed at the
6 hospital until 2005. Among them, 60 patients (73.2%) died and 22 patients (26.8%) survived
7 during the follow-up period.

8

9 Histopathological analyses

10 Specimens were routinely processed with a 10% buffered formalin fixative and embedded in
11 paraffin. Morphology of the tumor cells was evaluated using specimens stained with hematoxylin
12 and eosin. Mode of invasion of OSCC was graded into a, b and c, which represent Jakobsson's
13 grades 1 & 2, grade 3 and grade 4, respectively.[25] An antibody for D2-40 (Covance, Princeton,
14 NJ) was used to detect podoplanin following the determination in a previous study.[26] To
15 analyze the growth activity of tumor cells, an antibody for Ki-67 (Dako, Glostrup, Denmark) was
16 used. Sections were incubated with each antibody described above at x100 dilution with PBS at 4
17 °C overnight and immunohistochemical analysis was carried out on the EnVision+ System (Dako,
18 Carpinteria, CA). Negative controls were taken using specimens reacted with normal rabbit
19 serum in place of antibodies.

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

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

3
4 **Statistical analyses**
5 Cox proportional hazards regression models were used to identify the prognostic factors.
6 Prognosis status was defined for patients who died during the observation period as poor and for
7 patients who were alive as good. In univariate analyses, we used indicator variables for the
8 factors with more than two ordered categories, such as age group or stage of tumor. Significant
9 factors from univariate analyses were examined by multivariate analyses to select a set of factors
10 that show a better fit to the data, from a combination of prognostic factors. For the model
11 selection, we used Akaike's Information Criterion (AIC).[27] For the parameterization of factors
12 in each regression model, we used linear combinations of covariates, which are usually used in
13 clinical epidemiology. The estimated relative risks (RRs) by the selected factors were calculated
14 using estimated regression coefficients of the best model for prediction. Statistical procedures
15 were performed with the Statistical Language R.[28] *P*-values <0.05 were considered to be
16 statistically significant.

17

18 **Results**

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

1 invasion of OSCC (Fig. 1).

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

11 Multivariate analysis was performed using all combinations of factors significantly associated
12 with the prognosis of patients listed in Table 1 as covariates in the Cox proportional hazards
13 model. Multivariate analysis revealed that the Cox model with a linear combination of covariates
14 of age groups, mode of invasion, podoplanin expression and T-stage groups had the smallest AIC
15 values. In this selected model, T2 vs. T1 was not statistically significant, but T3 and T4 vs. T1
16 was statistically significant and this was the best fitting model (Table 2). The estimated RR for
17 poor prognosis by age group, podoplanin expression and T status is shown in Fig. 2. When the
18 mode of invasion was a, in the age group of less than 70 years old, the estimated RR for poor
19 prognosis of subjects with podoplanin <50% in the T1 group was the lowest (RR=1). However,
20 when the mode of invasion of OSCC was b or c, in the age group of more than 70 years old, the
21 estimated RRs for poor prognosis of subjects with podoplanin \geq 50% and <50% in the T3 and T4
22 group were 125.01 and 55.65, and in the age group of less than 70 years old, the estimated RRs
23 for poor prognosis of subjects with podoplanin \geq 50% and <50% in the T3 and T4 group were
24 34.96 and 15.56, respectively (Fig. 2a, b). When the mode of invasion of OSCC was a, in the age

1 group of more than 70 years old, the estimated RRs for poor prognosis of subjects with
2 podoplanin $\geq 50\%$ and $< 50\%$ in the T3 and T4 group were 21.76 and 9.69, whereas, in the age
3 group of less than 70 years old, the estimated RRs for poor prognosis of subjects with podoplanin
4 $\geq 50\%$ and $< 50\%$ in the T3 and T4 group were 6.09 and 2.17, respectively (Fig. 2c, d).

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

10

11 **Discussion**

12 Podoplanin is widely known as a distinct marker of lymphatic endothelial cells. It is also
13 known that podoplanin expression is detected in several kinds of tumors including OSCC. In
14 addition, recent studies have suggested that podoplanin plays important roles in immune
15 systems.[29] Among the diverse roles of podoplanin, cancer-associated functions have been the
16 most extensively studied. Cueni et al. reported that MCF7 human breast carcinoma cells with
17 ectopic overexpression of podoplanin promoted lymphangiogenesis and lymph node metastasis in
18 nude mice.[30] It has also been reported that podoplanin expression in the tumor was associated
19 with lymph node metastasis and the prognosis of patients.[6-8,20,21] However, the results of
20 clinicopathological studies varied,[22,23] and the biological significance of podoplanin
21 expression in human tumors remains largely unclear. In this study, podoplanin expression was
22 one of the prognostic factors of OSCC in addition to age and T, N and clinical stage of the TNM
23 classification in univariate analysis (Table 1). To clarify the controversial clinicopathological
24 value of podoplanin expression of the prognostic prediction of tumors, we developed a new

1 model using age (more than 70 or less), mode of invasion (b, c vs. a), podoplanin expression
2 (more than 50% or less) and T classification (T2 or T3, T4 vs. T1) as covariates by extensive
3 multivariate analysis (Table 2). The estimated RR of a group of patients aged more than 70, in T3
4 or T4 stage and with mode of invasion b or c was 125.01 times higher than that of a group of
5 patients aged less than 70, in T1 stage and with mode of invasion a (Fig. 3). The distinct RRs in
6 each group shown in Fig. 3 strongly suggest that our new model including the intensity of
7 podoplanin expression has practical value for the prognostic prediction of OSCC.

8 To improve our knowledge of the clinicopathological value of podoplanin expression in OSCC,
9 factors that had a significant relationship to podoplanin expression were analyzed. Multiple
10 regression analysis revealed a significant relationship of strongly positive (>50%) expression of
11 podoplanin to advanced clinical stages, especially stage III (Fig. 3, Table 3). Poorer fitness for
12 stage IV compared with stage III was suspected to be caused by the presence of a considerable
13 number of degenerative and/or necrotic tumor cells in stage IV tumors. These results strongly
14 suggest that the ratio of clinical stages in a population greatly affects the prognostic value of
15 podoplanin expression, which might be caused by the divergent values of podoplanin expression
16 on prognostic prediction. We further analyzed the relationship of podoplanin expression to that of
17 Ki-67, one of the most widely used markers to evaluate the growth activity of tumor cells. As
18 expected, multiple regression analysis indicated a significant relationship of podoplanin
19 expression to that of Ki-67 (Table 4). These results suggest that podoplanin expression is
20 associated with tumor growth and/or progression. Interestingly, the prognostic value of the
21 expression of Ki-67 in OSCC was not found in univariate analysis (Table 1). In this study, the
22 expression of Ki-67 was classified into 5 grades, and the number of cases classified into grade 1
23 (less than 20% positive cells) was only 5, and many cases were classified into higher grades.
24 Recently, intra-tumoral variation of Ki-67-positive cells was detected in tissue microarray using a

1 newly developed digital imaging method, and it was reported that the basal (lowest observed)
2 Ki-67 expression was associated with the prognosis of OSCC.[31] In this study, we used mean
3 expression and did not analyze intra-tumoral variation of the expression. However, it was
4 conceivable that a considerable number of Ki-67-positive cells were detected in OSCC because
5 they were malignant tumors, and had little value to evaluate the prognosis of OSCC patients.
6 These results further confirm the importance of podoplanin expression for the prognostic
7 prediction of OSCC in addition to the growth and/or progression of tumors.

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

19 We previously reported that the expression profile of VEGF-A or VEGF-C is one of the most
20 important prognostic factors of OSCC when imposing the condition of the presence of vascular
21 invasion or the strong expression of VEGF-A or VEGF-C; we named the model as an important
22 prognostic factor (IPF). In that study, we suggested that IPF is also effective to predict the
23 prognosis of OSCC in stage I or II.[24] VEGF-C has been shown to contribute to
24 lymphangiogenesis, and these results suggested that the expression of VEGF-C in OSCC cells is

1 predictive of future lymph node metastasis and poor prognosis. In this study, podoplanin
2 expression was significantly correlated with OSCC in higher stages (Fig. 3, Table 3). These
3 results suggest that both the expression of VEGFs and that of podoplanin have value for the
4 prognostic prediction of OSCC, but the biological significances including certain roles in
5 lymphangiogenesis of these factors for OSCC differ.

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

11
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1 **Figure Legends**

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

7
8 **Fig. 2** The estimated relative risks (RRs) of subjects evaluated with podoplanin expression and T
9 stage in each group with a cut-off point of 70 years old and mode of invasion a vs. b and c.
10 Subjects of T3 and T4 were pooled and analyzed.

11
12 **Fig. 3** Relationship of the intensities of podoplanin expression to clinical stages. Error bars
13 represent 95% confidence intervals.

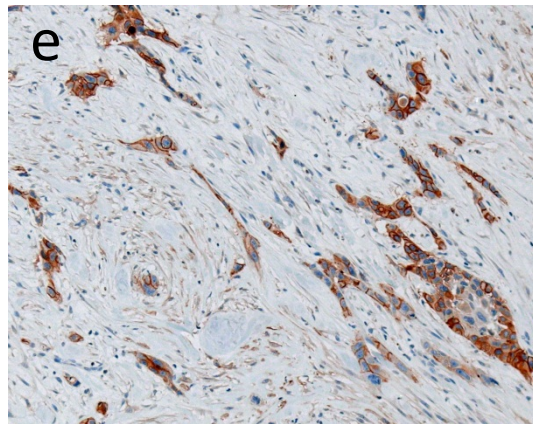
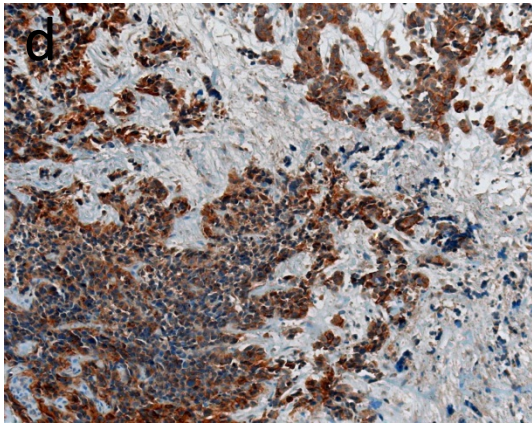
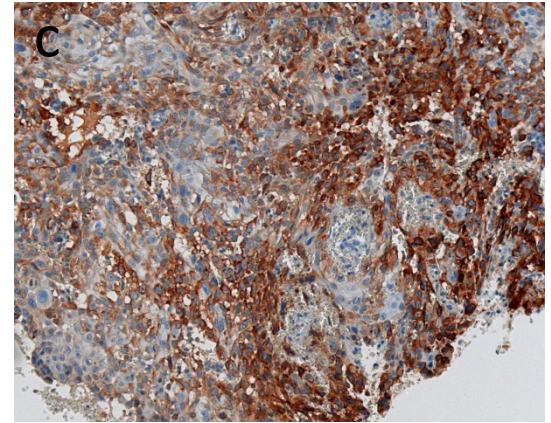
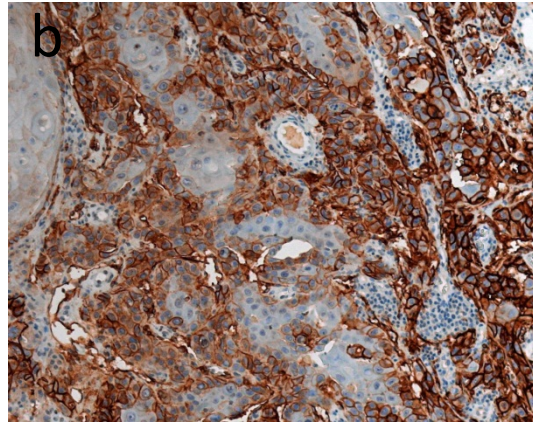
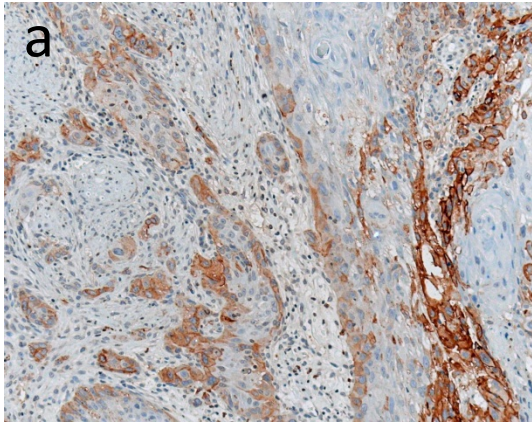


Figure 1

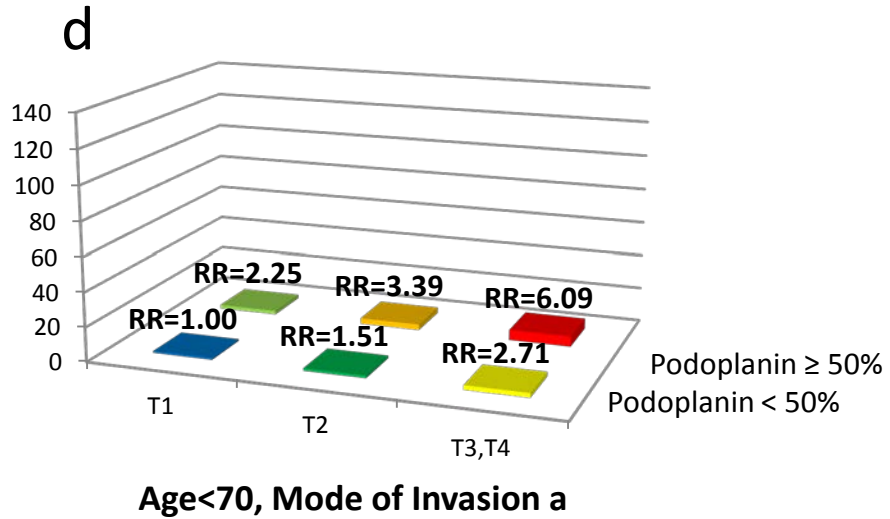
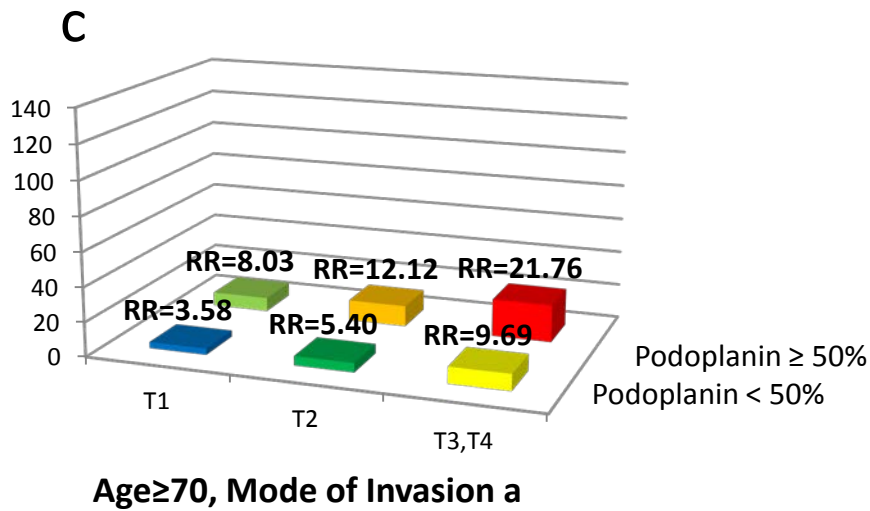
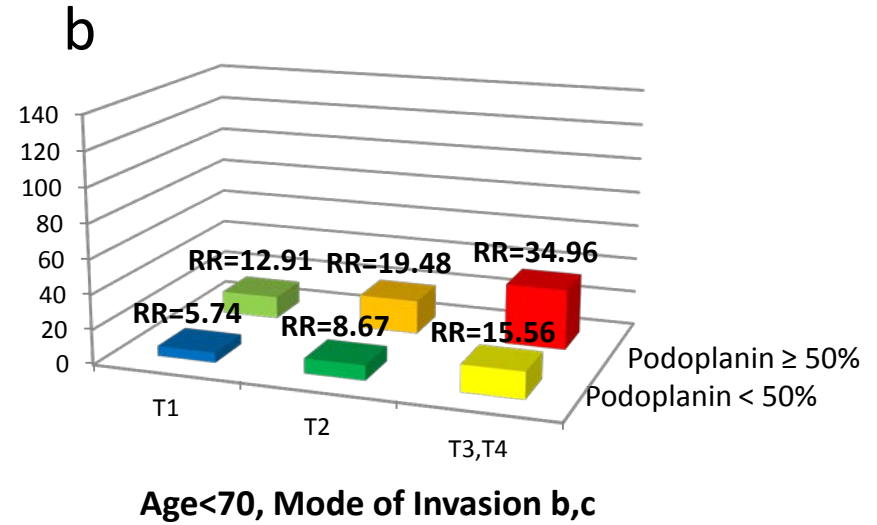
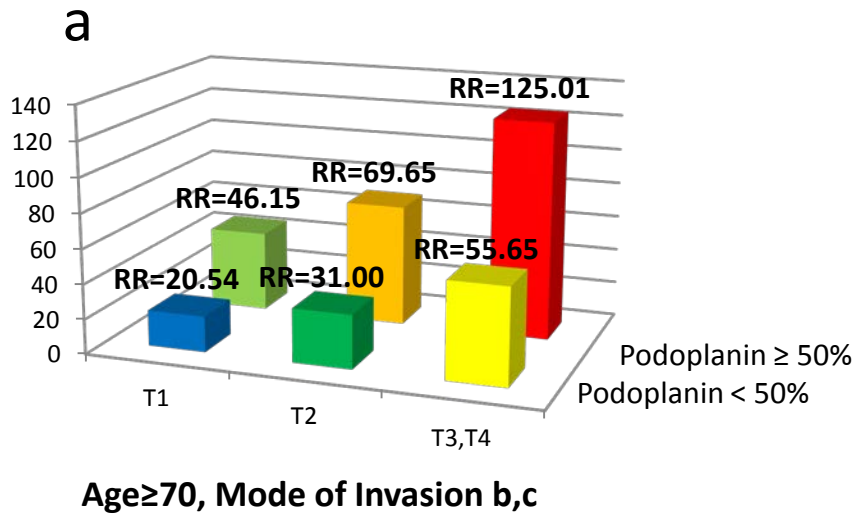


Figure 2

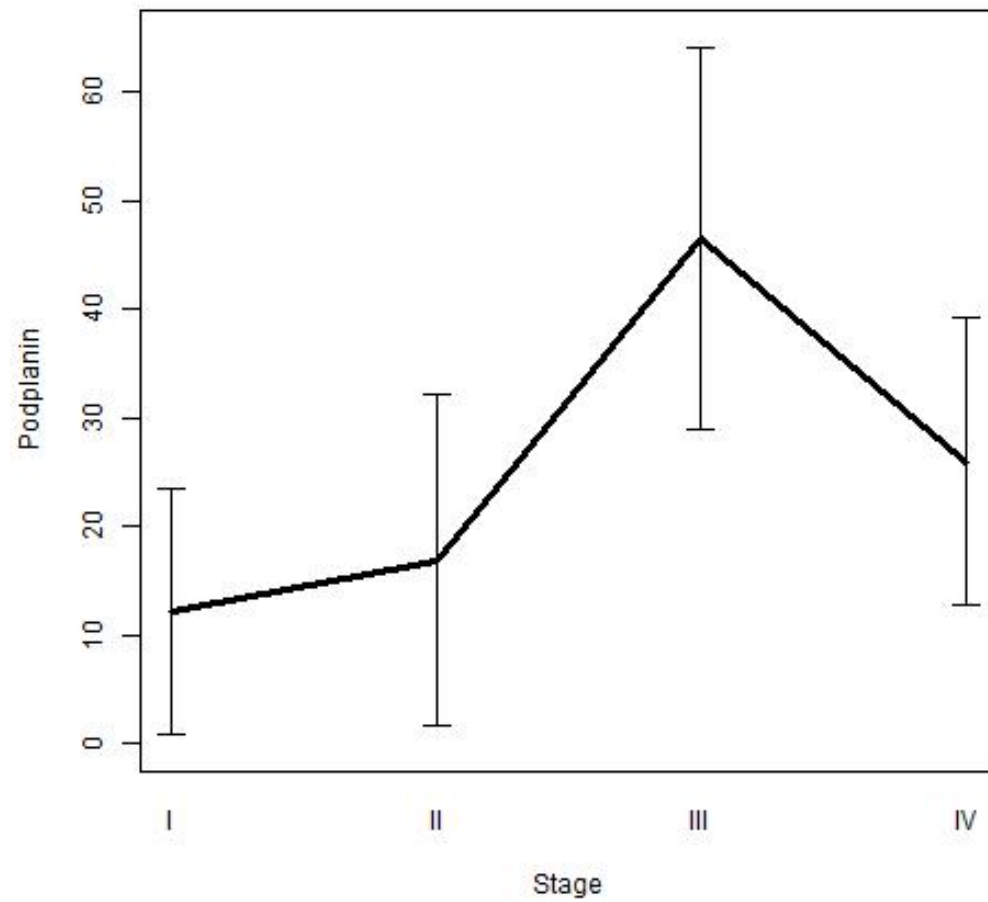


Figure 3

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*	–
	2	2	4	1.00*	–
	3	2	7	1.74	0.380
	4	7	15	1.43	0.527
	5	8	34	1.77	0.281

*: 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.

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

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 2	4.7	7.8	1.6	32.1	0.551
Stage 3	34.3	8.9	29.0	64.0	<0.000
Stage 4	13.8	6.8	12.7	39.2	0.046

* Stage 1 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.