Title of the article:

Peroxiredoxin I expression in tongue squamous cell carcinomas as involved in tumor recurrence.

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This work is supported by Grants-in-Aid for Scientific Research from the Japanese Society for the Promotion of Science

Keywords: peroxiredoxin (Prx), proliferation associated gene (pag), squamous cell carcinoma, tongue cancer, MSP23.

Abstract. Peroxiredoxin (Prx) I is an antioxidant protein expressed in proliferating cells. We investigated Prx I as marker for tongue cancer status by correlating clinical features with Prx I expression. Samples from 132 patients with squamous cell carcinoma in the tongue were examined by immunohistochemistry with anti-Prx I antibodies. Correlations between Prx I expression and the clinical features of tumors were statistically determined using univariate and multivariate analyses. Univariate analysis showed Prx I was significantly associated with local recurrence (P = 0.033). By multiple logistic regression analysis, Prx I expression was associated with local recurrence (odds ratio: 2.84; 95% confidence interval: 1.09-7.43; P = 0.034) and lymph node recurrence (odds ratio: 2.86; 95% confidence interval: 1.02-8.01; P = 0.046). Our results suggested that Prx I expression indicates tumors with a high potential for recurrence. Prx I may be used clinically to guide treatment for squamous cell carcinoma of the tongue.

Introduction

The peroxiredoxin (Prx) antioxidant protein family is found in a wide variety of species and plays important roles in protecting cells against oxidants and in regulating signaling by hydrogen peroxide. Its structure was recently reported and analyzed with respect to these dual roles[24]. At least six Prx enzymes are present in mammals (Prx I-VI)[15]. These Prx isoforms are found in the cytosol, mitochondria, peroxisomes, and plasma, all of which are potential sites of reactive oxygen species (ROS) production[3, 5, 8, 9, 12, 17].

Pag (human Prx I) was originally isolated by differential screening of cDNA libraries made from untransformed and ras-transformed human mammary epithelial cells. Because these cells cease proliferating during the commitment phase of differentiation, the higher levels of pag gene expression were correlated with cell proliferation[13]. Furthermore, some reports show an association between Prx I and the protooncogene product c-Abl, Myc, and cell cycle factor cdc2[1, 23]. Taken together, these findings indicate that Prx I is associated with cell proliferation and tumor growth. There are many reports of markers for proliferation, e.g., argynophilic nucleolar organizer regions (AgNORs), Ki67, and proliferating cell nuclear antigen (PCNA), that have been suggested to have clinical applications[7, 21, 22]. Although there are many reports on proliferation markers in the head and neck region, few focus on Prx I. We previously investigated oral squamous cell carcinoma and reported the potential usefulness of Prx I as a marker for clinicopathological features compared with PCNA[26]. However these results were not sufficient for clinical applications, because the samples

originated from various oral regions, a rather small number of cases was analyzed, and only univariate analysis was used. In this study, we determined Prx I expression in 132 cases of tongue squamous cell carcinoma (SCC) by immunohistochemistry and examined correlations with the clinical status of the tongue cancer to investigate the usefulness of Prx I as a clinical indicator for tumor characteristics.

Patients and methods

Patients

Tumor specimens were obtained by punch biopsies of the tongue from 132 patients who consulted the Division of Head and Neck Surgery, Chiba Cancer Center Hospital, from 1970 to 1995. The clinical features of these patients are summarized in Table 1. The tumors were staged according to the International Union Against Cancer (UICC) scheme[19]. The characteristics of the tumors are also shown in Table 1 for each case. No treatments for the malignant tumors were performed prior to biopsy. The tumor macroscopic morphology was used to classify the tumors into 4 groups: superficial, exophytic, endophytic, and combination type, as described previously[18]. Four patients were treated with surgical excision alone, 65 received surgical excision and radiotherapy, and the remaining 63 patients received radiation therapy alone. Of the 128 patients who received radiotherapy treatment, 25 were treated with linac radiation alone (8-70 Gy), 35 received interstitial radiation alone (28-70 Gy), 2 received electron beam therapy alone (42-50 Gy), and 66 received a combination of these radiation therapies (48.6-83

Gy). Local recurrence was defined as tumor recurrence in the primary region after primary treatment. Lymph node recurrence was defined as lymph node metastasis after primary treatment, including no treatment of the regional cervical lymph node. Lymph node metastasis was confirmed by histologically.

Methods

Immunostaining was performed using the horseradish peroxidase-labeled streptavidin and biotin technique. The samples were obtained by punch biopsy, fixed in 10% formalin neutral buffered solution, and embedded in paraffin. The slides were incubated for 1 hour at room temperature with an anti-Prx I rabbit polyclonal antibody produced by our group[4], diluted 1: 500 v/v in PBS. The slides were then incubated with biotinylated goat anti-rabbit IgG antibody, followed by horseradish peroxidase-conjugated streptavidin, and visualized with 3,3'-diaminobenzidine. One pathologist, who was not informed of the patients' clinical status, examined the immunostained slides. The Prx I immunostaining was categorized as negative (Fig. 1A) or positive (Fig. 1B), with total absence of Prx I being defined as negative staining. Control staining was performed with non-immune rabbit or mouse serum. As in our previous report, diseased thyroid tissues were immunoblotted as positive or negative controls for Prx I staining[26]. To confirm that the disparity in staining among sections was not due to the condition of samples, we divided the samples into old ones (collected before August 1986) and new ones (collected later than August 1986) and analyzed the groups statistically by the chi-square test. The positive-to-negative ratio in the old

samples was 26/40 and in the new samples was 27/39. Therefore, the disparity in staining was not significantly influenced by the time since fixation or the storage conditions (P = 0.86).

For univariate analysis, we used the chi-square test. For multivariate analysis, multiple logistic regression analysis was used. The analyses were performed using the StatView 5.0 statistical software package (SAS Institute Inc., Cary, NC, USA).

Results

Univariate analysis of Prx I expression and recurrence

Prx I expression was observed in the epithelium of the SCC in 79% of the specimens examined. To simplify the analysis for the correlation of Prx I expression with clinical features, the T-categories were divided into T1 + T2 and T3 + T4 groups, and the N-categories as lymph node metastasis negative (N0) and lymph node metastasis positive (N1+2) groups. The clinical stages were subclassified into stage I + II and stage III+IV. The differentiation status was divided into moderately or poorly differentiated (G2+G3) and well differentiated (G1). Table 2 shows the correlation between the Prx I expression and the clinical features for each case. The analysis showed that Prx I-positive groups included significantly more local recurrence cases (P = 0.033). No significant difference in Prx I expression was observed with respect to the other factors, such as age, sex, T-category, N-category, stage, tumor morphology, or lymph node recurrence. We next focused on local recurrence and lymph node recurrence, and evaluated whether the Prx I expression could be a useful predictor of recurrence. In

considering cervical lymph node recurrence, we included cases that had been treated with neck irradiation (those that received linac irradiation) and those that had not, which included the remaining cases, treated with interstitial therapy, no irradiation, etc.), and treatment with or without neck dissection, as variables to exclude the influence of irradiation and neck dissection. By univariate analysis, no significant differences were found in association with local recurrence and clinical features (age, sex, TNM classification, differentiation) except Prx I expression. There was no significant difference for lymph node recurrence associated with clinical features including neck dissection, irradiation, and Prx I expression (Tables 3, 4).

Multivariate analysis of recurrence

Multivariate analysis was used next, to provide adjusted odds ratios for local and lymph node recurrence. The predictor variables for all 132 cases were used in a logistic regression model with local recurrence and lymph node recurrence as the dependant variables. A logistic model for predicting the local or lymph node recurrence was constructed using clinical variables, including age, sex, T- and N-category, stage, differentiation, tumor morphology, and Prx I expression. For simplicity, the tumor morphology classification was divided into two groups (endophytic or other). For our analysis of lymph node recurrence, we entered the use of neck irradiation and the use of neck dissection as forced variables to exclude the influence of therapeutic differences. The adjusted odds ratios (OR) and 95% confidence intervals (CI) for local recurrence and lymph node recurrence are shown in Tables 5 and 6. Prx I expression (OR = 2.84; 95% confidence interval: 1.09-7.43; P = 0.034) was significantly associated with local

recurrence, but the other predictors were not. The analysis of lymph node recurrence showed that Prx I expression (OR = 2.86; 95% confidence interval: 1.02-8.01; P = 0.046) and differentiation (OR = 3.77; 95% CI: 1.32-10.78; P = 0.013) were significantly associated, but the other predictors were not.

Discussion

In this study we found Prx I expression to be significantly associated with local recurrence (P = 0.033) by univariate analysis, and both local (OR = 2.84; 95% confidence interval: 1.09-7.43; P = 0.034) and lymph node (OR = 2.86; 95% confidence interval: 1.02-8.01; P = 0.046) recurrence by multivariate analysis. These results indicate that Prx I expression predicts a 2.8-2.9-fold more frequent incidence of local and lymph node recurrence.

We can hypothesize why the Prx I-expressing tumors tended to recur. One explanation is that tumor recurrence may be ascribed to tumor viability. Prx I was originally isolated from proliferating cells[13] and Prx I overexpression has been reported in some tumors[6, 11]. These studies show that highly proliferative cells, like tumor cells, express more Prx I than do normal cells. Furthermore, among thyroid tumors, the more proliferative types of tumors tend to express more Prx I[25]. Taken together, these results support the association between Prx I and tumor viability.

Another explanation involves Prx I's ability to act as a tumor suppressor through the reduction of ROS. ROS and the reactive oxygen metabolites (ROM) that are formed

in the process of cellular respiration are implicated in tumor formation and promotion. By causing DNA damage, ROM are thought to affect protooncogenes and tumor suppressor genes[16]. Previous reports show that over-expression of Pag, human Prx I, counteracts the physiological role of the protooncogene abl, which has a cytostatic effect on cells. Elevated levels of Prx I in tumor cells seem to abrogate the abl-induced cell-cycle block[14, 23]. Recently, studies using a Prx I-knockout mouse indicated that Prx I expression was associated with oxidative damage and the presence of malignant neoplasms[10]. These findings indicated that in normal cells Prx I plays a direct role in tumor suppression by eliminating ROS and preventing oxidative damage[10]. Similarly, excessively proliferative tumor cells may express Prx I to protect themselves from oxidative damage.

The radiosensitivity of tumors may also be related to their recurrence through a mechanism involving Prx I. Many patients in this report were treated with radiotherapy. Irradiation can induce Prx I expression, which protects cells from further radiation damage[2]. However, these results suggest that protection by Prx I may also play an important role in the survival of cancer cells in patients undergoing radiation therapy, and therefore the residual carcinoma may easily recur.

We previously reported the potential of using Prx I as a marker for clinicopathological features[26]. In that report, we determined Prx I expression in oral SCC in various regions by immunohistochemistry and found an association between Prx I expression and T and N classification. In the present report, we used a uniform sample type from only the tongue, and the measure of Prx I expression was changed to

“negative or positive staining”, because the punch biopsy samples were very small and the determination of expression level was difficult. Furthermore, therapeutic and diagnostic methods were different between the different institutions. Thus Prx I-positive cases in the present report include more cases of smaller mass, but there was no significant difference in the results. Note, however, that Prx I expression may be associated with proliferative factors, because both T category and recurrence are dependant on the viability of the tumor.

There are many markers that indicate cell proliferation: AgNORs, PCNA, Ki 67, and others are well known and have been investigated as prognostic markers for cancers of the head and neck region[7, 20-22]. In this study we found a correlation between Prx I and local and lymph node recurrence, but not with prognosis (data not shown). However, most markers do not relate to the oxidative responses in cells. The Prx I indicates not only proliferation, but also the level of protection against oxidative damage. Therefore, it may provide a novel index with which to characterize tumors.

In this report, we have shown that Prx I may be useful as a tumor recurrence marker, which could be beneficial if applied clinically. For example, we could use it to estimate whether a tumor is susceptible to recurrence, and apply this information by making a wider area of excision or additional treatment, such as chemotherapy for positive tumors, to prevent recurrence. Further study is necessary to develop methods to apply Prx I as a marker for a more precise assessment of tongue SCC recurrence.

Figure legends

Figure 1. Representative photomicrographs of immunochemical staining with anti-Prx I antibodies. Negative staining (a total absence of Prx I immunostaining) (A), positive staining (B) (original magnification, x100).

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Table 1 . Characteristics of tongue squamous cell ca

		n
Median age		60
Range		27-88
Sex ratio f/m		45/ 87
Tumor morpholo	Superficial	24
	Exophytic	22
	Endophytic	58
	Combined	28
TNM classification		
T category	T1	37
	T2	55
	T3	30
	T4	10
N category	N0	90
	N1	18
	N2a	4
	N2b	13
	N2c	6
	N3	1
M category	M0	131
	M1	1
Stage	I	37
	II	40
	III	27
	IV	28
Differentiation		
	G1	80
	G2	47
	G3	5
	G4	0
Total		132

Table 2. Correlation with Peroxiredoxin I expression and clinical features

	Negative	Positive	P value
Age			
60>	24	41	0.46
≧60	29	38	
Sex			
f	19	26	0.73
m	34	53	
Tumor morphology			
Superficial	10	14	0.28
Exophytic	5	17	
Endophytic	27	31	
Combined	11	17	
T category			
T1+2	34	58	0.25
T3+4	19	21	
N category			
N0	36	54	0.95
N1+2	17	25	
Stage			
I+II	31	46	0.98
III+IV	22	33	
Differentiation			
G1	33	47	0.74
G2+3	20	32	
Local recurrence			
recurrence	7	23	0.033
null	46	56	
Lymph node recurrence			
recurrence	16	14	0.094
null	53	79	

Table 3. Univariate analysis of local recurrence

		recurrence	null	P
Age	60>	17	48	0.35
	≧60	13	54	
Sex	f	9	36	0.59
	m	21	66	
T category	T1+2	21	71	0.97
	T3+4	9	31	
N category	N0	21	69	0.81
	N1+2	9	33	
Stage	I+II	19	58	0.53
	III+IV	11	44	
Differentiation	G1	17	63	0.62
	G2+3	13	39	
Tumor morpholc	Superficial	7	17	0.72
	Exophytic	6	16	
	Endophytic	11	47	
	Combined	6	22	
Prx I expression	Negative	7	46	0.033
	Positive	23	56	

Table 4. Univariate analysis of lymph node recurrence

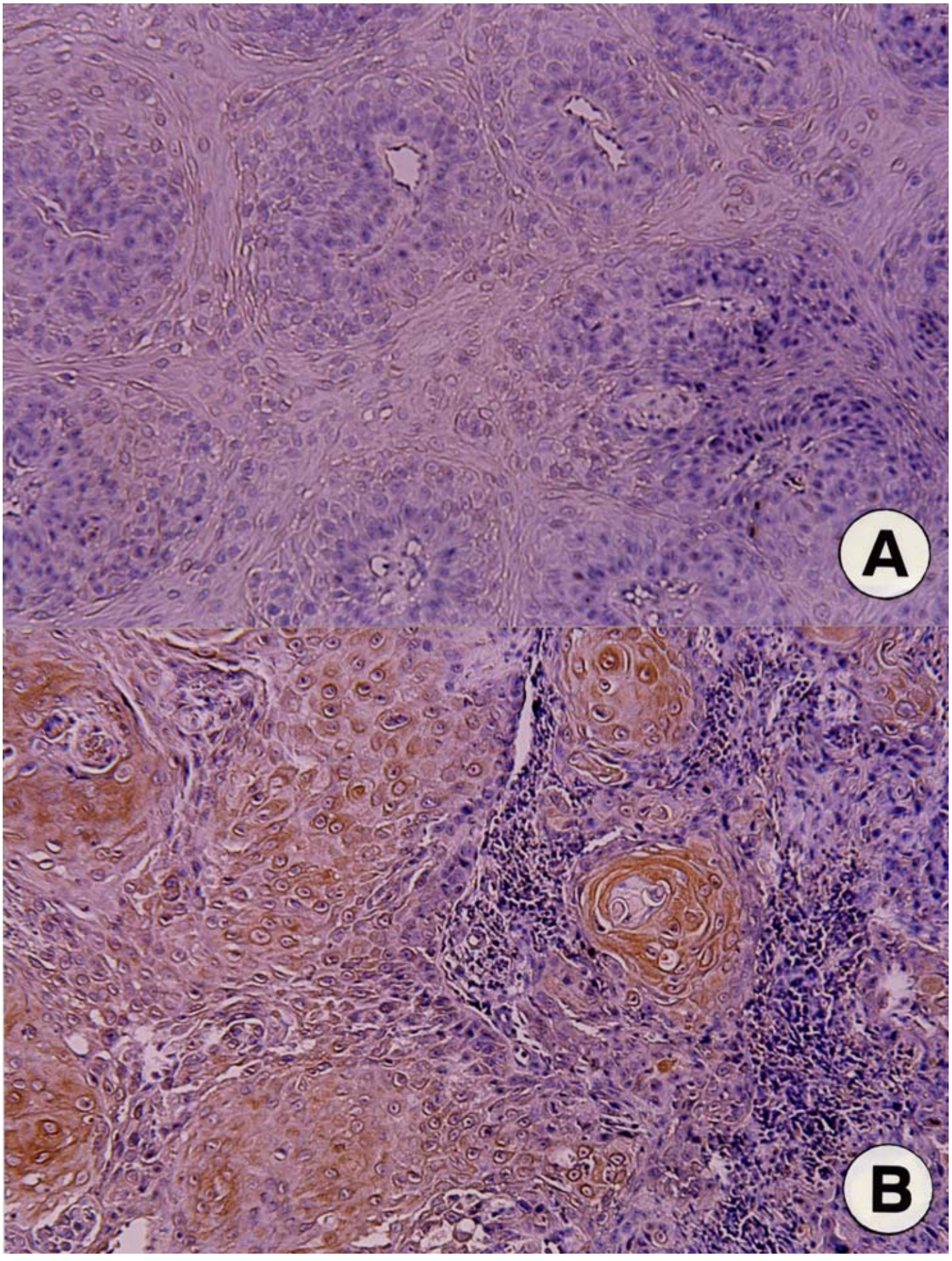
		recurrence	null	P
Age	60>	12	53	0.25
	≧60	18	49	
Sex	f	10	35	0.92
	m	20	67	
T category	T1+2	19	73	0.39
	T3+4	11	29	
N category	N0	17	73	0.12
	N1+2	13	29	
Stage	I+II	14	63	0.14
	III+IV	16	39	
Differentiation	G1	14	66	0.075
	G2+3	16	36	
Tumor morpholo	Superficial	3	21	0.37
	Exophytic	4	18	
	Endophytic	17	41	
	Combined	6	22	
Neck dissection	none	22	71	0.69
	performed	8	31	
Neck irradiation	none	5	22	0.56
	performed	25	80	
Prx I expression	Negative	16	53	0.094
	Positive	14	79	

Table 5. Multivariate analysis of local recurrence

	odds ratio	95% CI	P value
Age (60>:60≦)	0.84	0.34-2.04	0.69
Sex (f:m)	1.32	0.51-3.38	0.57
T category (T1+2:T3+4)	3.90	0.60-25.46	0.16
N category (N0:N1+2)	2.10	0.34-13.09	0.72
Stage (I+II:III+IV)	0.18	0.15-2.08	0.17
Differentiation (G2+3:G1)	0.82	0.34-1.98	0.66
Tumor morphology (endophytic:the others)	1.59	0.56-4.47	0.38
Prx I expression (negative:positi	2.84	1.09-7.43	0.034

Table 6. Multivariate analysis of lymph node recurrence

	d odds ratio	95% CI	P value
Age (60>:60≦)	0.38	0.13-1.11	0.08
Sex (f:m)	1.12	0.37-3.34	0.84
T category (T1+2:T3+4)	5.30	0.87-32.41	0.07
N category (N0:N1+2)	0.40	0.51-3.11	0.38
Stage (I+II:III+IV)	0.25	0.02-3.34	0.29
Differntiation (G2+3:G1)	3.77	1.32-10.78	0.013
Tumor morphology (endophytic:the others)	1.33	0.40-4.40	0.46
Neck dissection (none:perform	3.92	0.92-0.16.59	0.63
Neck irradiation (none:perfor	0.90	0.21-3.83	0.88
Prx I expression (negative:pos	2.86	1.02-8.01	0.046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