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Expression of CD9 and CD82 in papillary thyroid microcarcinoma and its prognostic significance

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ORIGINAL PAPER

Abstract

Introduction: papillary thyroid microcarcinoma is a well-known malignant neoplasm with good prognosis. The known prognostic factors are patient's age, multifocality, and extrathyroidal extension. CD9 and CD82, members of the tetraspanin family, are expressed in numerous cancer cells and play many roles associated with the cellular process.

Material and methods: we investigated the immunohistochemical expression of CD9 and CD82 in papillary thyroid microcarcinoma and analysed the clinicopathological and prognostic significance. For the retrospective analysis, we collected the cases of 553 PTMC patients who had undergone thyroidectomy.

Results: The group with lymph node metastasis showed higher immunostaining intensity for CD9 than the group without metastasis ($p = 0.002$). In multivariate analysis, high CD9 intensity (OR = 1.58 in 3+, $p = 0.0025$) correlated with lymph node metastasis.

Conclusion: We suggest CD9 as a predictive prognostic factor for lymph node metastasis in PTMC. **(Endokrynol Pol 2019; 70 (3): 224–231)**

Key words: *CD9; CD82; prognosis; papillary thyroid microcarcinoma*

Introduction

Papillary thyroid microcarcinoma (PTMC) is a well-known malignant neoplasm with good prognosis [1]. PTMC is defined as a papillary thyroid carcinoma with a tumour size less than 10 mm. Development of fine-needle aspiration biopsy (FNAB) led to increased detection of newly diagnosed PTMC cases [2]. Because of the favourable prognosis and indolent biological behaviour, management of PTMC has been controversial. Clinicians have a variety of options, from active surveillance to total thyroidectomy with lymph node dissection. Whichever treatment method is chosen, it is important to predict the prognosis of cancer patients for additional therapy or follow-up. In PTMC, the known prognostic factors associated with lymph node metastasis or tumour recurrence are patient's age, multifocality, and extrathyroidal extension [3].

Several molecular prognostic factors of PTMC have been reported. BRAF mutation, which is commonly detected in papillary thyroid carcinoma, is associated with poor prognosis [4]. Another study reported that the absence of EGFR expression and COX-2 expression was associated with poor prognosis in patients with PTMC [5]. In addition, molecular studies of papillary carcinoma have been well studied, but rarely in microcarcinoma.

CD9 and CD82, members of the tetraspanin family, are expressed in numerous cancer cells and mediate multiple cellular processes. Decreased CD9 expression has been reported to be related with the progression of breast, stomach, colon, prostate, and non-small cell lung cancer [6]. CD82 expression also has been reported to be related with the progression, metastasis, or poor prognosis of prostate, breast, larynx, gastrointestinal, and thyroid cancer [7–10]. However, the role of these molecules in PTMC is not well-known.

In our previous study, CD82 expression is associated with poor prognosis of clear cell renal cell carcinoma (CCRCC) [11]. CCRCC shares some of the molecular changes with PTMC. The tumour suppressor gene, thyroid hormone receptor beta (THRB), is downregulated in other tumours including papillary thyroid carcinoma and has been reported to be downregulated in CCRCC [12, 13]. Therefore, some molecules may predict prognosis in both PTMC and CCRCC.

This study was designed to evaluate the immunohistochemical expression of CD9 and CD82 in PTMC and to determine the clinicopathological and prognostic significance.

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Material and methods

Patients and tissue specimens

For the retrospective analysis, we collected the cases of 553 PTMC patients who had undergone thyroidectomy from January to December 2010 at the Severance Hospital (Seoul, South Korea). Samples were obtained from 107 men and 446 women, with a mean age of 44.57 years. The mean tumour size was 0.53 cm. The clinicopathological information was collected from electronic medical records, pathological reports, and review of slides. The histologic subtype, tumour stage, and lymph node metastasis were determined by pathological review. The BRAF mutation was determined by polymerase chain reaction based on aspiration cytology or formalin-fixed, paraffin-embedded samples.

Tissue microarray and immunohistochemistry

The H&E slides of all cases were reviewed, and one representative core tissue (2mm in diameter) was obtained from each paraffin block and placed in a new paraffin block using a trephine apparatus (Superbiochips Laboratories, Seoul, Korea). Immunohistochemical staining was performed using the Bond-Max Autostainer (Leica Microsystems, IL, USA). After heat-induced antigen retrieval, mouse monoclonal anti-human CD9 antibody (NovoCastra, Newcastle Upon Tyne, UK, dilution 1:250) and mouse monoclonal anti-human CD82 antibody (NovoCastra, dilution 1:50) were incubated with the samples for 15 min. The binding of the primary antibody was detected using the Bond Polymer Refine Detection kit (Leica Microsystems) according to the manufacturer's instructions. The CD9 and CD82 immunoreactivity was scored using the H-score method, which classifies the percentages of cells stained with intensities of 0, 1+, 2+, and 3+ as follows: *H-score* = Σ *[Intensity (0, 1, 2, 3) × the extent of each staining intensity (%)*] (Fig. 1). The H-score ranges from 0 to 300. The results were evaluated independently by two pathologists who were blinded to the outcome and scores of the other observer.

Statistical analysis

Statistical analyses were performed using SPSS software (version 23.0; SPSS Inc., Chicago, IL, USA). The correlations between CD9 and CD82 expression and clinicopathological characteristics were analysed by Pearson's χ^2 test. The H-score of CD9 and CD82 was also analysed by Pearson's χ^2 test. The parameters that were significant in the univariate analysis ($p < 0.05$) were analysed using a multivariate Cox regression model to evaluate the incremental statistical power and independence of prognostic impact. All of the statistical tests were two sided, and p values less than 0.05 were considered statistically significant.

Results

CD9 and CD82 expression in PTMC

CD9 was present in the cytoplasm and/or membrane, and of the 544 PTMC samples, 433 (80.6 %) were positive and 111 (20.4 %) were negative for CD9 immunohistochemical stain. CD82 was also observed in the cytoplasm and/or membrane. 375 (68.68%) were positive and 171 (31.32 %) were negative for CD82 immunohistochemical stain (Tab. II).

Association between CD9, CD82 expression, and clinicopathologic parameters

There was a statistically significant correlation between high CD9 immunostaining intensity and males comparing to females ($p = 0.0089$ and Pearson's $r = 0.001$). In addition, the group with lymph node metastasis showed higher immunostaining intensity for CD9 than the group without metastasis ($p = 0.002$). The intensity of CD9 was found to be associated with N-stage. This was statistically significant ($p = 0.0535$). The higher CD82 immunostaining intensity was found to be associated with age (≤ 45) ($p = 0.0226$) and the absence of lymphocytic thyroiditis ($p = 0.0111$). Other parameters

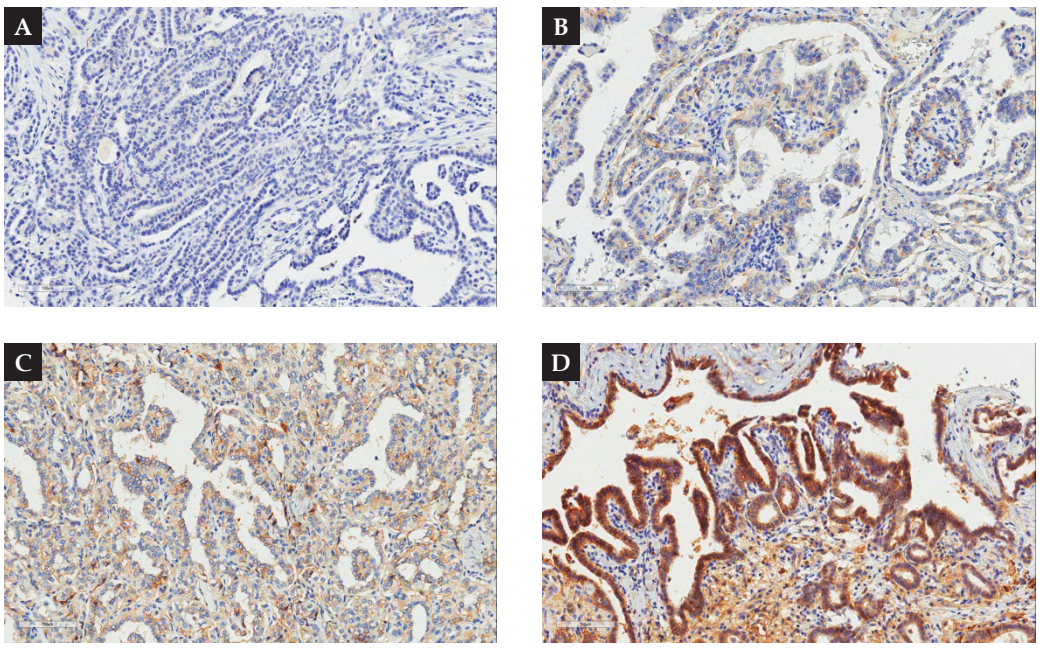

Figure 1. *Each immunostained slide was classified into 0, 1+, 2+, or 3+ depending on the intensity of the staining.* **A.** *No expression (0);* **B.** *Weak intensity (1+);* **C.** *Moderate intensity (2+);* **D.** *Strong intensity (3+)*

Table I. *Clinicopathological characteristics of the patients with papillary thyroid microcarcinoma (PTMC)*

Characteristic		Total (n = 553)
Age (years)	≤ 45	297 (53.71%)
	> 45	256 (46.29%)
Size [cm]	≤ 0.5	325 (58.77%)
	> 0.5	228 (41.23%)
Gender	Male	107 (19.35%)
	Female	446 (80.65%)
Location	Left	271 (49.01%)
	Right	282 (50.99%)
Tumour extent	Intrathyroidal	336 (60.76%)
	Extrathyroidal	217 (39.24%)
LN metastasis	Absent	368 (72.44%)
	Present	140 (27.56%)
N-stage	N0	367 (66.37%)
	N1a	127 (22.97%)
	N1b	14 (2.53%)
	Nx	45 (8.14%)
BRAF mutation	Negative	17 (3.07%)
	Positive	37 (6.69%)
	Not performed	499 (90.24%)
Adenomatous hyperplasia	Absent	440 (79.57%)
	Present	113 (20.43%)
Lymphocytic thyroiditis	Absent	387 (69.98%)
	Present	166 (30.02%)

did not show a significant correlation with the intensity of staining for CD9 and CD82 (Tab. III).

Association between H-score of CD9, CD82 expression, and clinicopathological parameters

The CD9 H-score was significantly higher in the group with a size of less than 0.5 cm ($p = 0.0240$). Male gender ($p = 0.0052$) and presence of adenomatous hyperplasia ($p = 0.0272$) also showed significantly high CD9 H-score. The CD82 H-score did not show significant association with any clinicopathological parameters (Tab. IV).

Univariate and multivariate logistic regression for lymph node metastasis

In univariate analysis, age (≤ 45) (OR = 1.72, $p = 0.0079$), size (> 0.5 cm) (OR = 2.2, $p < 0.0001$), male gender (OR = 2.6, $p < 0.0001$), absence of lymphocytic thyroiditis (OR = 1.68, $p = 0.021$), and high CD9 intensity (OR = 1.95 in 3+, $p = 0.0025$) were correlated with lymph node metastasis. In multivariate analysis, age (≤ 45) (OR = 1.77, $p = 0.0087$), size (> 0.5 cm) (OR = 2.29, $p = 0.0001$), male gender (OR = 2.36, $p = 0.0008$), and high CD9 intensity (OR = 1.58 in 3+, $p = 0.0061$) were correlated with lymph node metastasis (Tab. V).

Table II. *CD9 and CD82 expression in papillary thyroid microcarcinoma (PTMC)*

			Total (n = 533)
CD9	Expression (%)	0	111 (20.4%)
		> 0	433 (80.6%)
	Intensity	0	111 (20.4%)
		1	200 (36.76%)
		2	191 (35.76%)
		3	42 (7.72%)
	H-score (mean ± SD)		100.09 ± 87.10
CD82	Expression (%)	0	171 (31.32%)
		> 0	375 (68.68%)
	Intensity	0	171 (31.32%)
		1	213 (39.01%)
		2	124 (22.71%)
		3	37 (6.78%)
	H-score (mean ± SD)		93.42 ± 90.71

Discussion

Tetraspanin family molecules are involved in a variety of physiological and pathological processes including signal transduction, cell adhesion, proliferation, differentiation, and migration [14, 15]. Among the tetraspanin family molecules, CD9 is one of the most studied molecules in human cancer, and there are many reports on its association with prognosis. A recent study reported that high expression of CD9 in colorectal cancer cells is associated with favourable disease-free survival [16]. In addition, follicular lymphoma, breast cancer, ovarian cancer, stomach, and malignant melanoma have been reported to show an inverse correlation between CD9 expression and patient survival rate [17–21]. In this study, the association between CD9 expression and survival rate was not analysed, because most patients with PTMC are alive and the analysis may be insignificant.

CD9 is also known as motility-related protein-1 (MRP-1); one of the functions of CD9 in cancer cells is to contribute to cell motility and migration. Some studies suggested that CD9 is associated with EW1 family, epidermal growth factor receptor (EGFR), and discoidin domain receptor 1 (DDR1), regulating cell motility. The association with EGFR induces the EGF-dependent chemotactic migration to extracellular matrix, and tumour cells gain the ability of extra- or intravasation [22–25]. Angiogenic activity of CD9 by interacting with vascular endothelial growth factor (VEGF) receptor 3 and integrin has also been suggested [26, 27]. Our results showed

Table III. *Association between CD9, CD82, and clinicopathological parameters*

	CD9 intensity				p-value	CD82 intensity				p-value
	0	**1**	**2**	**3**		**0**	**1**	**2**	**3**	
Age (years)	44.33 ± 11.17	45.07 ± 11.33	43.73 ± 10.57	46.0 ± 10.01	0.5141	42.69 ± 11.51	45.15 ± 10.24	46.60 ± 11.27	43.27 ± 10.82	0.0151
≤ 45	60 (54.05%)	102 (51%)	109 (57.07%)	20 (47.62%)	0.5565	104 (60.47%)	111 (52.11%)	54 (43.55%)	23 (62.16%)	0.0226
> 45	51 (45.95%)	98 (49%)	82 (42.93%)	22 (52.38%)		68 (39.53%)	102 (47.89%)	70 (56.45%)	14 (37.84%)	
Size	0.50 ± 0.22	0.53 ± 0.20	0.54 ± 0.20	0.56 ± 0.19	0.3058	0.51 ± 0.21	0.54 ± 0.21	0.53 ± 0.18	0.56 ± 0.21	0.4503
≤ 0.5	71 (63.96%)	114 (57%)	109 (57.07%)	24 (57.14%)	0.6275	108 (62.79%)	120 (56.34%)	74 (59.68%)	18 (48.65%)	0.3527
> 0.5	40 (36.04%)	86 (43%)	82 (42.93%)	18 (42.86%)		64 (37.21%)	93 (43.66%)	50 (40.32%)	19 (51.35%)	
Gender										
Male	12 (10.81%)	36 (18%)	46 (24.08%)	13 (30.95%)	0.0089	32 (18.6%)	40 (18.78%)	29 (23.39%)	6 (16.22%)	0.6601
Female	99 (89.19%)	164 (82%)	145 (75.92%)	29 (69.05%)		140 (81.4%)	173 (81.22%)	95 (76.61%)	31 (83.78%)	
Location										
Left	59 (53.15%)	97 (48.50%)	95 (49.74%)	18 (42.86%)	0.7014	86 (50%)	100 (46.95%)	66 (53.23%)	18 (48.65%)	0.7361
Right	52 (46.85%)	103 (51.50%)	96 (50.26%)	24 (57.14%)		86 (50%)	113 (53.05%)	58(46.77%)	19 (51.35%)	
Tumour extent										
Intrathyroidal	67 (60.396%)	116 (58%)	120 (63.83%)	27 (64.29%)	0.7519	108 (62.79%)	121 (56.81%)	75 (60.48%)	27 (72.97%)	0.2612
Extrathyroidal	44 (39.64%)	84 (42%)	71 (37.17%)	15 (35.71%)		64 (37.21%)	92 (43.19%)	49 (39.52%)	10 (27.03%)	
LN metastasis										
Absent	75 (72.82%)	148 (81.32%)	117 (66.10%)	22 (57.89%)	0.002	118 (72.84%)	142 (71.72%)	87 (77.68%)	16 (53.33%)	0.0698
Present	28 (27.18%)	34 (18.68%)	60 (33.90%)	16 (42.11%)		44 (27.16%)	56 (28.28%)	25 (22.32%)	14 (46.67%)	
N-stage										
N0	75 (67.57%)	147 (73.5%)	117 (61.25%)	22 (52.38%)	0.0535	118 (68.60%)	141 (66.20%)	87 (70.16%)	16 (43.24%)	0.0258
N1a	27 (34.32%)	32 (16%)	52 (27.23%)	14 (33.34%)		37 (21.51%)	53 (24.88%)	22 (17.74%)	14 (37.84)	
N1b	1 (0.9%)	3 (1.5%)	8 (4.19%)	2 (4.76%)		7 (4.07%)	4 (1.88%)	3 (2.42%)	0 (0%)	
Nx	8 (7.21%)	18 (9%)	14 (7.33%)	4 (9.52%)		10 (5.81%)	15 (7.04%)	12 (9.68%)	7 (18.92%)	

Table III. *Association between CD9, CD82, and clinicopathological parameters*

	CD9 intensity				p-value	CD82 intensity				p-value
	0	1	2	3		0	1	2	3	
***BRAF* mutation**										
Negative	3 (30%)	8 (38.1%)	5 (25%)	1 (33.33%)	0.8796	9 (50%)	3 (17.65%)	2 (18.18%)	3 (37.5%)	0.8796
Positive	7 (70%)	13 (61.9%)	15 (75%)	2 (66.67%)		9 (50%)	14 (82.35%)	9 (81.82%)	5 (62.5%)	
Adenomatous hyperplasia										
Absent	94 (84.68%)	162 (81%)	148 (77.49%)	30 (71.43%)	0.2329	136 (79.07%)	170 (79.81%)	98 (79.03%)	31 (83.78%)	0.9279
Present	17 (15.32%)	38 (19%)	43 (22.51%)	12 (28.57%)		36 (20.93%)	43 (20.19%)	26 (20.97%)	6 (16.22%)	
Lymphocytic thyroiditis										
Absent	76 (68.47%)	137 (68.50%)	137 (71.73%)	31 (73.81%)	0.8254	125 (72.67%)	136 (63.85%)	89 (71.77%)	33 (89.19%)	0.0111
Present	35 (31.53%)	63 (31.50%)	54 (28.27%)	11 (26.19%)		47 (27.33%)	77 (36.15%)	35 (28.23%)	4 (10.81%)	

that CD9 staining intensity was positively correlated with lymph node metastasis. This result appears to be due to the mechanisms described above.

Paradoxically, several reports suggested that CD9 inhibits metastasis. In cancer cell line studies, a suggested mechanism is inhibition of integrin-mediated motility of various cancer cells including lung, breast, stomach, skin, pancreas, and urinary bladder [28]. Other studies suggested that CD9 may downregulate Wiskott-Aldrich syndrome protein 2 (WAVE 2), and downregulated WAVE 2 results in suppression of tumour cell motility by affecting the actin cytoskeleton [29, 30].

There are also some studies about other mechanisms that CD9 is involved in in various cancer cells. One study reported that downregulation of CD9 in pancreatic cancer cells induces upregulation of the epidermal growth factor, thereby promoting cancer cell growth and metastasis [31]. In ovarian cancer, upregulation of CD9 is related to the induction of TNF-α gene expression and constitutive NF-κB activation [32]. In a review article, the function of CD9 is summarised as follows: i) interaction with endothelial cells and induction of transendothelial migration of the tumour cells; ii) enhancement of the motility of tumour cells; iii) promotion of tumour cell growth and prevention of apoptosis; and iv) an important marker for the identification of cancer stem cells [33]. These various mechanisms suggest that CD9 may affect prognosis in a variety of ways.

CD82 (KAI1) also belongs to the tetraspanin family, and there have been studies about functions in cancer cells. Recent studies have suggested that CD82 expression suppresses environmental angiogenesis by inhibition of production of interleukin 6 (IL-6) and vascular endothelial growth factor (VEGF) in malignant melanoma [34]. In the papillary thyroid carcinoma, down-regulation of CD82 is significantly related to lymph node metastasis and anaplastic transformation [35]. Another study reported that CD82 expression was correlated with pathological TNM status of thyroid cancer, and they suggested that down-regulation of CD82 expression in thyroid cancer cells may reflect an increased metastatic potential [10]. In this study, however, CD82 expression is correlated with patient age and lymphocytic thyroiditis, not with lymph node metastasis. Contrary to the above studies, normal angiogenesis without obvious vascular defect was shown in CD82-null mice [36]. The role of CD82 in cancer cells remains unclear.

H-score of CD9 is associated with adenomatous hyperplasia alone, and that of CD82 is not correlated with any clinicopathological parameters. Because the H-score is the value obtained by multiplying the staining intensity by the extent, the staining extent seems not to

Table IV. *Univariate and multivariate logistic regression for lymph node metastasis*

	CD9		CD82	
	H-score	**p-value**	**H-score**	**p-value**
Age (years)	0.0006	0.8822	0.074	0.086
≤ 45	101.1 ± 86.60	0.7650	88.29 ± 92.40	0.1568
> 45	98.89 ± 87.82		99.31 ± 88.53	
Size	0.097	0.0240	0.057	0.1857
≤ 0.5	97.33 ± 87.15	0.3803	89.47 ± 90.43	0.2266
> 0.5	104.0 ± 87.06		99.0 ± 91.0	
Gender				
Male	121.1 ± 89.19	0.0052	98.97 ± 90.87	0.4803
Female	94.94 ± 85.90		92.06 ± 90.72	
Location				
Left	99.37 ± 87.07	0.8482	91.83 ± 89.07	0.6799
Right	100.8 ± 87.27		95.04 ± 92.48	
Tumour extent				
Intrathyroidal	102.3 ± 88.42	0.4596	96.51 ± 95.30	0.3091
Extrathyroidal	96.66 ± 85.10		88.65 ± 83.13	
LN metastasis				
Absent	96.26 ± 83.42	0.2043	89.67 ± 86.72	0.0533
Present	11.4 ± 94.90		93.81 ± 95.78	
N-stage				
N0	96.25 ± 83.42	0.2043	89.67 ± 86.72	0.0533
N1a	108.32 ± 95.89		97.82 ± 97.25	
N1b	138.21 ± 79.65		58.21 ± 70.15	
Nx	96.14 ± 91.05		122.84 ± 103.41	
***BRAF* mutation**				
Negative	93.53 ± 93.45	0.6509	92.94 ± 119.8	0.4627
Positive	105.1 ± 84.01		116.2 ± 101.3	
Adenomatous hyperplasia				
Absent	95.94 ± 85.47	0.0272	94.53 ± 91.26	0.5708
Present	116.5 ± 91.80		89.05 ± 88.79	
Lymphocytic thyroiditis				
Absent	101.9 ± 87.86	0.4589	97.44 ± 94.98	0.0878
Present	95.86 ± 85.39		83.96 ± 79.24	

be important in affecting clinicopathological parameters in both CD9 and CD82 immunostaining.

This study represents an analysis of the association between two tetraspanin family molecules and clinicopathologic parameters of PTMC, especially lymph node metastasis. Although CD9 staining intensity is significantly associated with lymph node metastasis, it is difficult to predict the complicated mechanism of lymph node metastasis based only on CD9 and CD82. Additional risk factors may affect CD9 expression and lymph node metastasis. The detailed mechanism should be elucidated through additional studies using various molecules and cancer cell lines.

Conclusion

In conclusion, although PTMC has a favourable prognosis, predicting the prognostic factors is very important for patient management. We suggest CD9 as a predictive prognostic factor for lymph node metastasis in PTMC. Further studies with other cancers are needed to delineate the function of CD9.

Table V. *Univariate and multivariate logistic regression for lymph node metastasis*

	Univariate analysis			Multivariate analysis		
	OR	**95% CI**	**p-value**	**OR**	**95% CI**	**p-value**
Age (years)						
≤ 45	1.72	(1.15–2.58)	0.0079	1.77	(1.16–2.72)	0.0087
> 45	1			1		
Size						
≤ 0.5	1		< 0.0001	1		0.0001
> 0.5	2.2	(1.48–3.26)		2.29	(1.51–3.48)	
Gender						
Male	2.6	(1.64–4.15)	< 0.0001	2.36	(1.43–3.90)	0.0008
Female	1			1		
Location						
Left	1		0.6661			
Right	1.09	(0.74–1.61)				
Tumour extent						
Intrathyroidal	1		0.7443			
Extrathyroidal	1.07	(0.72–1.59)				
***BRAF* mutation**						
Negative	0.13	(0.02–1.12)	0.0635			
Positive	1					
Adenomatous hyperplasia						
Absent	1		0.4329			
Present	0.82	(0.50–1.34)				
Lymphocytic thyroiditis						
Absent	1.68	(1.08–2.62)	0.021	1.41	(0.88–2.26)	0.154
Present	1			1		
CD9 Expression						
1	1		0.9644			
2	1.23	(0.53–2.83)				
3	1.00	(0.48–2.10)				
4	1.01	(0.61–1.67)				
CD9 Intensity						
0	1		0.0025	1		0.0061
1	0.62	(0.35–1.09)		0.51	(0.28–0.93)	
2	1.37	(0.81–2.34)		1.1	(0.63–0.1.92)	
3	1.95	(0.90–4.24)		1.58	(0.69–3.58)	
CD9 H-score	1.00	(0.99–1.004)	0.0817			
CD82 Expression						
1	1		0.8485			
2	1.06	(0.44–1.68)				
3	1.60	(0.55–4.65)				
4	1.00	(0.65–1.53)				
CD82 Intensity						
0	1		0.0803			
1	1.06	(0.67–1.68)				
2	0.77	(0.44–1.35)				
3	2.35	(1.06–5.21)				
CD82 H-score	1.001	(0.99–1.003)	0.6432			

OR — odds ratio; CI — confidence interval

Conflict of interest

The authors declare no conflict of interest.

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