Histological Growth Pattern of and Alpha-actinin-4 Expression in Thyroid Cancer

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Abstract. *Aim: To assess the clinicopathological significance of the histological growth pattern (HGP) and α-actinin-4 (ACTN4) expression in thyroid cancer. Patients and Methods: We classified 83 thyroid cancer cases into infiltrative margin (IM) and pushing margin (PM) groups according to peripheral tumor margin contour and immunohistochemically determined ACTN4 expression. Correlations between clinical stage and clinicopathological characteristics were analyzed. Results: IM and high ACTN4 expression were observed in 39% and 49% of cancer cases, respectively. Higher clinical stage was significantly correlated with older age, higher T and N factor, preoperative recurrent laryngeal nerve paralysis (pre-RLNP), IM, and poor prognosis. Patients with stage IV disease had significantly poorer prognosis than those with stages I–III. On multivariate analysis, older age, pre-RLNP, and IM correlated with higher clinical stages. IM was significantly correlated with high ACTN4 expression. Conclusion: IM, pre-RLNP, and ACTN4 expression could be novel indicators of tumor aggression and prognostic factors of thyroid cancer.*

It is estimated that in 2008 more than 210,000 new cases of thyroid cancer were diagnosed and that approximately 35,000 patients died from thyroid cancer worldwide (1). Most thyroid cancers present with an indolent clinical course, but we sometimes encounter clinically aggressive cases that present with local recurrences or distant metastases regardless of histological type (2). These cases often require combined modality therapies and can be associated with poor outcomes.

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Although ultrasonography, computed tomography (CT), magnetic resonance imaging, positron emission tomography/CT, fine needle aspiration cytology, or core needle biopsy are used in the diagnoses of thyroid tumors, the definitive diagnostic procedures for these tumors depend greatly on the histopathological diagnoses of the resected surgical specimens (3, 4). However, it remains difficult to evaluate the degree of malignancy to predict local recurrence or distant metastases by using histopathological analyses alone except in rare and highly malignant types (*e.g.* undifferentiated carcinomas).

The histological growth pattern (HGP) of the peripheral tumor margin is approved worldwide as one of the predictive indicators of prognoses in various malignancies (5-8). The tumor margins were mainly categorized into pushing margin (PM) and infiltrative margin (IM) groups, and a relationship between HGP and tumor aggression or lymph node metastasis in papillary thyroid cancer (PTC) was suggested (9, 10).

Alpha-actinins (ACTNs) are members of the superfamily of actin-binding proteins that cross-link actin filaments to give cells their shape (11). ACTN4 is an isoform of non-muscular ACTN, and ACTN4 overexpression has been reported to occur frequently in human epithelial cancers of various origins, such as of the breast, ovaries, pancreas, and the oral cavity (12-15). ACTN4 overexpression has been reported to be a prognostic factor in breast and ovarian cancers that correlated with lymph node metastasis in colorectal cancer (12, 13, 16). These reports suggest that a high-level ACTN4 expression is related to malignancy grade, lymph node metastasis, and patient outcome. Therefore, ACTN4 appears to be a useful molecular prognostic marker in various types of cancers (13-15, 17-19). However, no studies have investigated ACTN4 expression in thyroid cancer.

In the present study, we focused on studying the correlation between clinical stage and histopathological parameters including HGP and ACTN4 expression and evaluated their clinical significance in thyroid cancer.

Table I. *Criteria for categorizing ACTN4 expression in thyroid cancer.*

Category	Immunohistochemical findings
No expression	No immunoreaction or immunoreaction of any intensity in <10% of tumor cells
Low expression	Immunoreaction with much lower intensity than vascular endothelial cells in ≥10% of tumor cells
Moderate expression	Immunoreaction with intensity equal to or slightly lower than vascular endothelial cells in ≥10% of tumor cells
High expression	Immunoreaction with stronger intensity than vascular endothelial cells in ≥10% of tumor cells

ACTN4, α-actinin-4.

Patients and Methods

We reviewed the clinicopathological records of 83 patients who underwent initial surgical treatments for primary thyroid cancer between 1991 and 2007 at the National Defense Medical College Hospital of Japan. Information regarding the following was obtained from the patients' medical records: age; gender; Tumor, Node, and Metastasis (T, N, and M) factors; clinical stage; presence or absence of preoperative recurrent laryngeal nerve paralysis (pre-RLNP) or tracheal/prevertebral invasion; histopathological diagnosis; and prognosis. TNM classification and clinical staging of each thyroid cancer were performed according to the Union for International Cancer Control-2002 (sixth edition).

Two observers (NT and SY) reviewed all of the hematoxylin-eosin–stained slides and classified them into two HGP groups. If the extended thyroid cancer tissue displaced the surrounding tissue in a pushing manner and the cancer cells had invaded <10% of the tissue surrounding the peripheral margin of the tumor nodule, it was classified into the PM group. If the peripheral margin was poorly demarcated and the cancer cells had invaded ≥10% of the tissue surrounding the peripheral margin of the tumor nodule, it was classified into the IM group. Representative images of the PM and IM groups are shown in Figure 1.

The primary antibody used for immunohistochemistry was an anti-ACTN4 rabbit polyclonal antibody (Ab-2) raised against a synthetic peptide, as described by Honda *et al.* (16). Immunohistochemistry was performed on 4-μm thick tumor sections from representative formalin-fixed and paraffin-embedded tissue blocks. Antigen retrieval was accomplished by using a 10-mM citrate buffer (pH 6.0) and by heating of the samples in an autoclave for 10 min at a controlled final temperature of 120˚C. After the endogenous peroxidase activity was blocked by incubating the slides in 3% hydrogen peroxide (v/v) in methanol for 5 min, non-specific binding was further blocked with 2% normal swine serum (v/v; Dako, Carpinteria, CA, USA) in phosphate-buffered saline for 10 min. The slides were incubated with the primary antibody overnight at 4˚C and then with the secondary antibody and peroxidase with EnVision (Dako) for 1 h. Specific antigen-antibody reactions were visualized by using 3,3'-diaminobenzidine tetrahydrochloride. The nuclei were counterstained with Mayer's hematoxylin.

ACTN4 expression was classified into four categories according to immunoreaction intensity (Table I). If ≥10% of the tumor cells were stained, the tumor was judged as positive, whereas if <10% of the tumor cells were stained, the tumor was judged to have no expression. If the staining intensity of the tumor cells was stronger or much stronger than that of the vascular endothelial cells, the tumor was classified as having high expression. Conversely, if the staining intensity of the tumor cells was similar to or a little lower than that of the vascular endothelial cells, the tumor was classified as having moderate expression. Finally, if the staining intensity of the tumor cells was much lower than that of the vascular endothelial cells or only slightly evident, the tumor was classified as having low expression. Representative images of ACTN4 expression are shown in Figure 2.

Statistical analyses were performed using the JMP 10.0.0 software (SAS Institute Inc., Cary, NC, USA). To analyze the relationships between parameters, Pearson's chi square test or Fisher's exact test (two-tailed) was used. Factors with p-values <0.1 on univariate analysis were tested by using logistic regression analysis. The disease-free survival rates were calculated according to the Kaplan-Meier method, while a log-rank test was performed to examine the univariate associations among groups. Statistically significant differences were considered when $p<0.05$.

Results

The clinicopathological profiles of 83 patients are presented in Table II. The mean age was 53.7 (range, 19-84), and the ratio of males to females was approximately 1:3. T3 (35 cases; 42%) was the most common T factor, while N1 and M1 occupied 59% (49 cases) and 5% (4 cases), respectively. Stage IVa (31 cases; 37%) accounted for more than one third of all cases, while 11 cases (13%) had findings of pre-RLNP. A total of 71 cases (86%) were histologically classified as PTC. IM and high ACTN4 expression were noted in 32 (39%) and 41 (49%) cases, respectively. Sixteen (19%) patients experienced residual or recurrent disease or died of the thyroid cancer (19%). The follow-up period was 0.5-16 years (median=5.5 years).

Out of the 83 patients, 72 underwent thyroidectomy with regional lymph node dissection, 10 underwent thyroidectomy without lymph node dissection, and one underwent open biopsy-only.

The correlation between clinicopathological findings and clinical stage in 78 thyroid cancers is shown in Table III. Unfortunately, clinical staging of the remaining 5 cases could not be identified from the medical records. Patients with stage IV disease were more frequently identified with T4 and N1 than T1-T3 ($p<0.0001$) or N0 ($p=0.0005$) staging, and more frequently had pre-RLNP ($p=0.0019$). Significantly more patients in the IM group than those in the PM group had stage IV disease ($p=0.0019$), and the prognoses of patients with stage IV than those with stages I-III disease were significantly

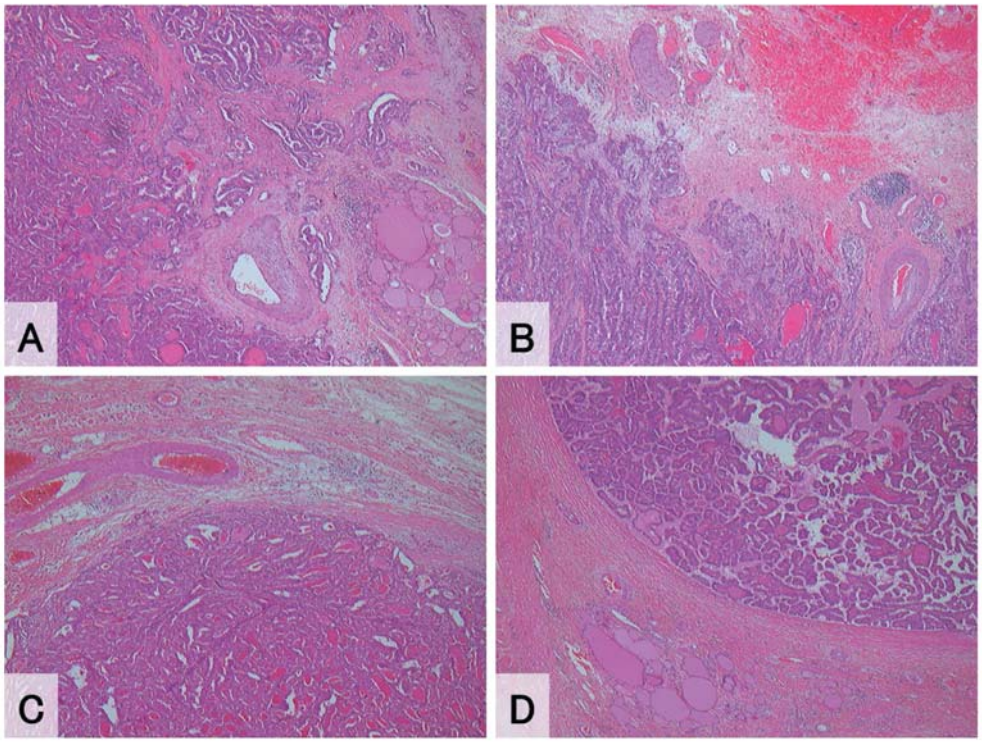

Figure 1. *Representative images of thyroid cancer cases with infiltrative margins (A and B) or pushing margins (C and D). H$E staining, (original magnification, ×40).*

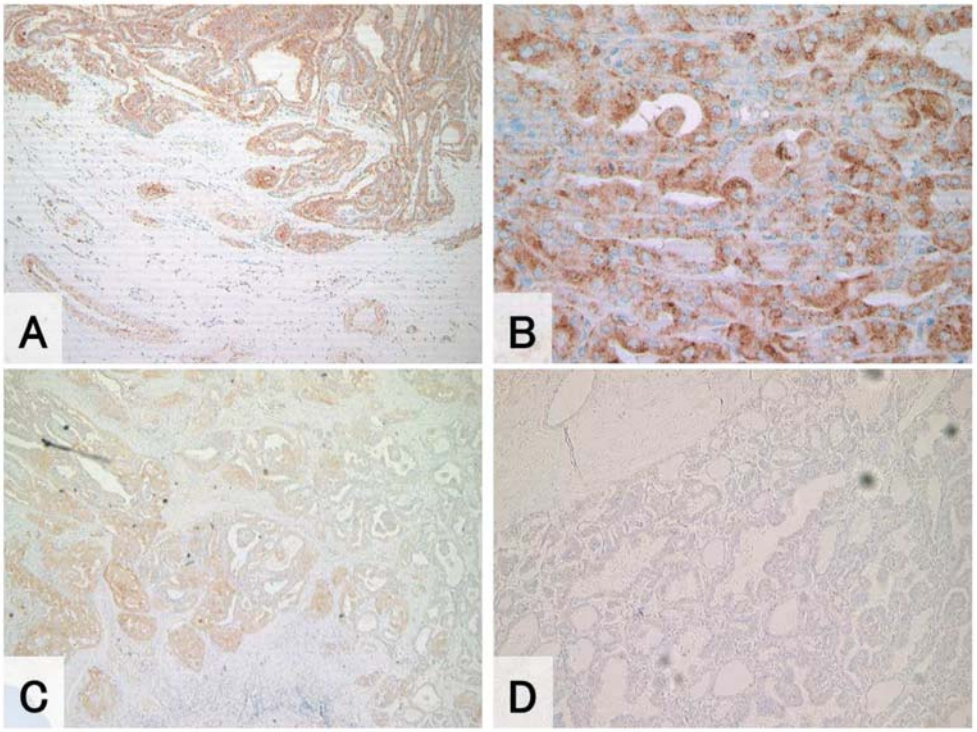

Figure 2. *Representative images of α-actinin-4 (ACTN4) immunohistochemistry of thyroid cancer. A, B: Immunostaining for high ACTN4 expression. C: Immunostaining for low ACTN4 expression. D: Immunostaining for no ACTN4 expression. Immunoperoxidase staining: ×100 magnification in A, C, and D; ×200 in B.*

Table II. *Clinicopathological data of 83 thyroid cancer cases.*

		Cases	(%)
Age (years)	<55	40	(48)
	55≤	43	(52)
Gender	Male	22	(27)
	Female	61	(73)
T	1	5	(6)
	2	13	(16)
	3	35	(42)
	4a	23	(28)
	4b	3	(4)
	Unknown	4	(5)
N	0	30	(36)
	1	49	(59)
	Unknown	4	(5)
M	0	79	(95)
	1	4	(5)
Clinical stage	I	23	(28)
	II	4	(5)
	III	16	(19)
	IVa	31	(37)
	IVb	1	(1)
	IVc	3	(4)
	Unknown	5	(6)
Pre-RLNP	–	67	(81)
	+	11	(13)
	Unknown	5	(6)
Tracheal/prevertebral invasion	–	70	(84)
	+	8	(10)
	Unknown	5	(6)
Histological type	PTC	71	(86)
	FTC	10	(12)
	ATC	1	(1)
	MTC	1	(1)
HGP	PM	51	(61)
	IM	32	(39)
ACTN4 expression	No	7	(8)
	Low	12	(14)
	Moderate	23	(28)
	High	41	(49)
Residual/recurrence/death	–	65	(78)
	+	16	(19)
	Unknown	2	(2)

Pre-RLNP, preoperative recurrent laryngeal nerve paralysis; PTC, papillary thyroid cancer; FTC, follicular thyroid cancer; ATC, anaplastic thyroid cancer; MTC, medullary thyroid cancer; HGP, histological growth pattern; PM, pushing margin; IM, infiltrative margin; ACTN4, α-actinin-4.

poorer (p=0.0102). Stage IV PTC tended to present less frequently than stage IV non-PTC, which included follicular, anaplastic, and medullary thyroid cancers (p=0.0991).

Figure 3 shows a Kaplan-Meier disease-free survival analysis demonstrating that patients with stage IV disease had poorer prognosis than those with stage I–III disease by using log-rank test (p=0.0342; HR 0.2354; 95% CI, 0.0617-0.8980).

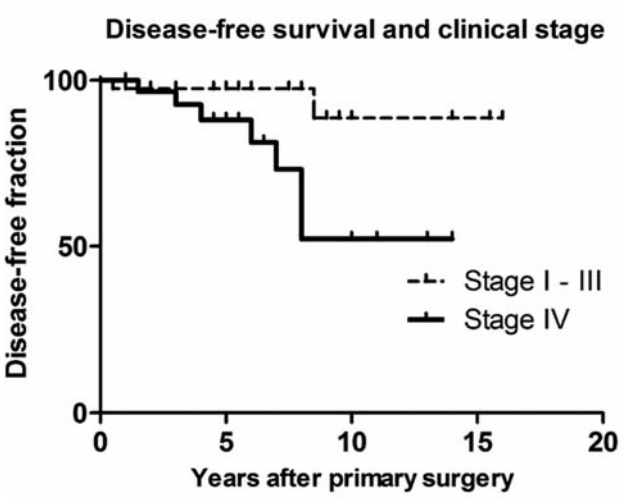

Figure 3. *Kaplan-Meier curve of disease-free survival by clinical stage indicating poorer prognosis in patients with stage IV disease than in patients with stage I-III disease.*

To identify the independent factors that influenced clinical stage in thyroid cancer, a multivariate analysis was conducted (Table III). T and N factors were excluded to avoid multivariate analysis instability and the residual/recurrence/death parameter was omitted as well since the parameter itself was the outcome. Age (p=0.0065), pre-RLNP (p=0.0141), and HGP (p=0.0132) were independent factors that correlated with the clinical stages of thyroid cancers.

Table IV shows that significantly more ACTN4 is expressed in thyroid cancers in the IM group than in those in the PM group (p=0.0052). Furthermore, higher ACTN4 expression was observed in the peripheral margin of the tumor nodule.

Discussion

The findings of the present study indicate a significant correlation between clinical stage and age, pre-RLNP, and HGP. Additionally, HGP was significantly related with ACTN4 expression.

ACTN is a protein that weighs approximately 100 kDa and is expressed in four isoforms in mammals (ACTN1, ACTN2, ACTN3, ACTN4) (20). The overall structure of ACTN is similar to that of a dumbbell due to dimer formation at the center of the rod domain that makes actins cross-link or form bundles with the actin-binding domain located at either end (21). ACTN presumably plays an important role in binding the actin cytoskeleton to the cell membrane by combining with the integrin β chain (20). Through these mechanisms, ACTN is involved in cellular structural maintenance.

Table III. *Clinical features of clinical stage and a multivariate regression model of clinicopathological parameters that influence the clinical stage in 78 cases of thyroid cancer (5 cases of which clinical stages are unknown are excluded).*

	Clinical Stage (78)		Univariate	Multivaliate regression		
	I-III (48)	IV (35)	*p*-Value	Odds ratio	95% CI	*p*-Value
Age (years)						
<55	26	10	* 0.0050	0.219	0.0630-0.6634	* 0.0065
55≤	17	25				
Gender						
male	10	12	0.2816			
female	33	23				
T stage						
1-3	39	13	* <0.0001			
4	4	22				
N stage						
0	24	6	* 0.0005			
1	19	29				
M stage						
0	42	32	0.3205			
1	1	3				
Pre-RLNP						
–	41	25	* 0.0019	0.064	0.0023-0.6089	* 0.0141
+	1	10				
(Unknown	1	0)				
Histological type						
PTC	34	33	0.0991	0.251	0.0315-1.2313	0.0916
Non-PTC	9	2				
Tracheal/prevertebral invasion						
–	40	29	0.1314			
+	2	6				
(Unknown	1	0)				
HGP						
PM	34	15	* 0.0019	0.228	0.0618-0.7390	* 0.0132
IM	9	20				
ACTN4 expression						
No–Moderate	24	16	0.3748			
High	19	19				
Residual/recurrence/death						
–	39	23	* 0.0102			
+	2	7				

CI, Confidence interval; pre-RLNP, preoperative recurrent laryngeal nerve paralysis; PTC, papillary thyroid cancer; HGP, histological growth pattern; PM, pushing margin; IM, infiltrative margin; ACTN4, α-actinin-4. Non-PTC group in histological type includes follicular, anaplastic, and medullary thyroid cancers. *Statistically significant.

ACTN4, which was identified in 1998, is an actin-binding protein that comprises 884 amino acids and is found in non-muscular cells, as is ACTN1 (a known microfilament protein) (12). Although these isoforms share 86.7% homology at the amino acid level, their intracellular localizations differ (12). ACTN4 is widely distributed in the adjacent areas of the actin fibers, cytoplasm, or nucleus, whereas ACTN1 distribution is restricted to areas near cell adhesion molecules such as integrin or catenin at the ends of the actin fibers (20, 22, 23). Cell motility is important when cancer cells infiltrate adjacent

Table IV. *The relevance between HGP and ACTN4 expression in 83 thyroid cancer cases.*

		HGP		
		PM	IM	*p*-Value
ACTN4 expression	No-moderate	32	10	* 0.0052
	High	19	22	

HGP, Histological growth pattern; ACTN4, α-actinin-4; PM, pushing margin; IM, infiltrative margin. *Statistically significant.

tissues or migrate to distant organs via the blood and lymphatic vasculature. Under enhanced cell motility conditions, cancer cells have been reported to show essential dynamic actin cytoskeletal changes (12), which is why ACTN4 is thought to be a factor that affects invasion or metastasis.

In the case of colorectal cancer, concentrated ACTN4 expression was observed in filopodia and reported to increase cell motility significantly (16). Furthermore, ACTN4 expression was significantly correlated with regional lymph node metastases (16). Increasing numbers of reports have clarified that ACTN4 overexpression is an indicator of poor prognosis or resistance to chemotherapy in patients with breast, esophageal, pancreatic, or ovarian cancer (12, 17, 24).

To date, age, gender, T, N, and, M factors, and histology have been suggested as prognostic factors (25-28). A significant correlation between clinical stage and prognosis in thyroid cancer has been reported (29), and multivariate analysis findings in the present study findings suggest that older age, IM, and pre-RLNP are independent factors influencing poor prognosis in thyroid cancer. The significant association observed between IM and high ACTN4 expression indicates that ACTN4 is also a promising prognostic factor in thyroid cancer. Because the method used to evaluate HGP and ACTN4 expression was not technically different from current diagnostic approaches, HGP and ACTN4 expression would be useful indication criteria for the post-surgical treatment of thyroid cancer. Furthermore, it is possible to assess tumor invasiveness immunohistochemically according to the ACTN4 expression status of preoperative biopsy or cytopathology specimens. Such evaluations might contribute to decisions regarding surgical resection extent.

Herein we examined the correlation between clinicopathological findings and clinical stage in 83 cases of thyroid cancer and showed that age, IM, pre-RLNP, and high ACTN4 expression were important prognostic factors.

Conflicts of Interest

The Authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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