学 位 論 文

Diagnostic significance of apical membranous and cytoplasmic dot-like CD26 expression in encapsulated follicular variant of papillary thyroid carcinoma: a useful marker for capsular invasion

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ORIGINAL

Diagnostic significance of apical membranous and cytoplasmic dot-like CD26 expression in encapsulated follicular variant of papillary thyroid carcinoma: a useful marker for capsular invasion

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Abstract. Non-invasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP) and invasive encapsulated follicular variant of papillary thyroid carcinoma (EFV-PTC) are indistinguishable preoperatively. CD26 expression in follicular tumor-uncertain malignant potential (FT-UMP) is reported to be clearly higher than in that without capsular invasion. To verify the diagnostic significance of CD26 immunostaining in EFV-PTC, we examined the expression pattern of CD26 in non-invasive EFV-PTC (NIFTP) and invasive EFV-PTC. We performed immunohistochemical analysis using CD26 antibody for 37 NIFTPs and 54 EFV-PTCs (34 minimally invasive EFV-PTCs and 20 widely invasive EFV-PTCs). Most NIFTP samples showed an apical membranous pattern or a cytoplasmic diffuse pattern of expression. Invasive EFV-PTCs more frequently showed a cytoplasmic dot-like pattern, and the labeling indices of tumor cells with cytoplasmic dot-like patterns were significantly higher than those in NIFTPs. The sizes of dots seen in NIFTPs (mean: 1.12 μm) were significantly smaller than in invasive EFV-PTCs (1.33 μm), minimally invasive EFV-PTC (1.27 μm), and widely invasive EFV-PTC (1.38 μm). We, therefore, conclude that cytoplasmic diffuse and/or cytoplasmic dot-like CD26 expression, particularly the larger CD26-positive dots, could be useful markers for capsular invasion in EFV-PTC. CD26 immunostaining, using cell blocks or cytological specimens, may preoperatively distinguish between NIFTP and invasive EFV-PTC.

Key words: Non-invasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP), Follicular variant of papillary thyroid carcinoma, CD26, Capsular invasion

THYROID CARCINOMAS are the most common malignant endocrine tumors, and the incidence of microcarcinomas with indolent behavior has increased recently owing to improved diagnostic techniques [1]. Papillary thyroid carcinoma (PTC) is the most frequent histological type among all thyroid carcinomas. PTCs have several variants, of which follicular variant (FV) is relatively frequent in Western countries. In Asian countries, however, the variant is less frequent [2, 3]. FV-PTC is composed of carcinoma cells with nuclear features characteristic of PTC, without well-formed papillae [4]. FV-PTC is classified into two types, encapsulated and non-encapsulated [5]. Encapsulated FV-PTC (EFV-PTC) without capsular invasion shows indolent behavior, with lower risk of recurrence and metastasis. However, most patients with EFV-PTC without capsular invasion have continued a treatment with total thyroidectomy and radioactive iodine therapy [6]. Recognizing the challenges in overdiagnosis and overtreatment of EFV-PTC without capsular invasion, the need to revise the term "carcinoma" has been emphasized, and the term "non-invasive follicular thyroid neoplasm with papillary-like nuclear features" (NIFTP) was proposed [7]. However, NIFTP and invasive EFV-PTC still cannot be distinguished preoperatively [8].

CD26 is an active cell surface peptidase, identified as a dipeptide naphthylamidase that hydrolyzes glycyl-prolyl-beta-napthylamide [9, 10]. CD26 is also known to activate intracellular signaling pathways by interacting with the extracellular matrix [11, 12]. It is normally expressed in endothelial cells, fibroblasts, lymphocytes, and epithelial cells in the liver, gastrointestinal tract, and kidney. Its expression pattern is membranous and with a cytoplasmic diffuse pattern [13-15]. Increased CD26 expression is seen in various malignant tumors, such as brain glioma [16], breast carcinoma [17], and mesothelioma [18]. In thyroid carcinomas, well-differentiated carcinomas, including PTC and follicular thyroid carcinoma (FTC), express CD26 [19-26]. Kotani *et al.* had reported CD26 expression to be more frequently observed in FTC (100%) than in follicular thyroid adenoma (FTA) (27.1%). Furthermore, the authors had revealed CD26 expression of follicular tumor of uncertain malignant potential (FT-UMP) to be clearly higher than of that without capsular invasion [27]. Furthermore, Zhang *et al.* have shown that CD26 expression in ovarian cancer (85.94%) is higher than in borderline (56.09%) and benign ovarian tumors (16.66%) [28]. We hypothesized that the difference in expression pattern of CD26 is associated with the invasive ability of encapsulated well-differentiated thyroid carcinomas. In the current study, in order to verify the diagnostic significance of CD26 immunostaining in EFV-PTC, we examined the expression pattern of CD26 in non-invasive EFV-PTC (NIFTP) and invasive EFV-PTC.

Materials and Methods

The study was approved by Ethics Committee of Kurashiki University of Science and the Arts (approval number: 17-11). We reviewed 10,076 cases of resected and histologically diagnosed PTC at Kuma Hospital between 2007 and 2016, and selected 37 NIFTPs and 54 EFV-PTCs. They were included in NIFTP and invasive EFV-PTC nodules previously reported by Hirokawa *et al.* [29]. Histological diagnosis of NIFTP was made on the basis of the following criteria: (1) encapsulation or clear demarcation, (2) follicular growth pattern without papillae, (3) no psammoma bodies, (4) <30% solid/trabecular/insular growth pattern, (5) nuclear score 2–3 (1: size and shape (nuclear enlargement/overlapping/crowding, elongation), 2: nuclear membrane irregularities (irregular contours, grooves, pseudoinclusions), and 3: chromatin characteristics (clearing with margination/glassy nuclei)), (6) no vascular or capsular invasion, (7) no tumor necrosis, and (8) no high mitotic activity (Fig. 1A) [7, 29-31]. EFV-PTCs were classified as minimally invasive (*n* = 34) and widely invasive (*n* = 20).

Minimally invasive type was diagnosed by the following criteria: (1) tumor capsule was well preserved, (2) capsular invasion could not be identified grossly, and (3) existence of microscopic capsular and/or vascular invasion (Fig. 1B). Widely invasive types were encapsulated, and showed extensive capsular invasion and/or vascular invasion (Fig. 1C). Ages of patients with NIFTP, minimally invasive EFV-PTC, and widely invasive EFV-PTC ranged from 38 to 77 years (mean: 57.7 years), 30 to 77 years (mean: 56.5 years), and 29 to 73 years (mean: 54.7 years), respectively. Patients with NIFTP, minimally invasive EFV-PTC, and widely invasive EFV-PTC consisted of 9 men and 28 women, 12 men and 22
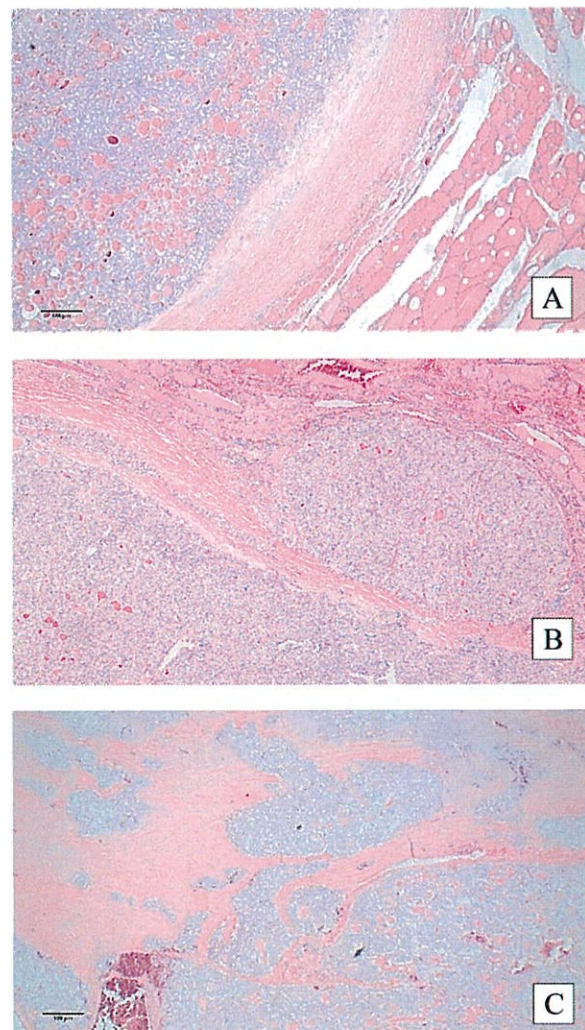

Fig. 1 Microscopic appearance of non-invasive follicular thyroid neoplasm with papillary-like nuclear features (A), minimally invasive encapsulated follicular variant-papillary thyroid carcinoma (B), and widely invasive encapsulated follicular variant-papillary thyroid carcinoma (C). Scale bar represents 100 μm.

women, and 3 men and 17 women, respectively. Tumor sizes of NIFTPs, minimally invasive EFV-PTCs, and widely invasive EFV-PTCs ranged from 3 to 50 mm (with a mean of 19.5 mm), 6 to 58 mm (with a mean of 22.3 mm), and 8 to 40 mm (with a mean of 20.9 mm), respectively (Table 1).

Immunohistochemical staining for CD26 was performed manually on formalin-fixed paraffin-embedded tissues. Anti-CD26 antibody was purchased from R&D Systems (Cat. No. AF1180, Minneapolis, MN, USA). Sections were de-paraffinized and unmasked using microwave in 0.05 M Tris-EDTA buffer (pH 9.0). Slides were incubated with the anti-CD26 antibody (dilution 1:100) for 60 min. Subsequently, slides were incubated with a secondary antibody (Cat. No. 414162, Nichirei Corporation, Tokyo, Japan) for 30 min, and then stained with diaminobenzidine.

Immunohistochemical results were evaluated by intensity and distribution of cell staining. Vascular endothelial cells were used as internal positive controls (Fig. 2A). Staining pattern of CD26 was divided into three types: apical membranous pattern, cytoplasmic diffuse pattern, and cytoplasmic dot-like pattern. Apical membranous patterns showed expression localized to the apical membrane facing the colloid (Fig. 2B). Cytoplasmic diffuse patterns showed diffuse-stained cytoplasmic reactivity (Fig. 2C). Cytoplasmic dot-like patterns showed clear aggregated images with a smooth edge in the cytoplasm (Fig. 2D). The sizes of dots upon CD26-antibody staining were measured using Image J [32]. The labeling indices of tumor cells expressing a cytoplasmic dot-like pattern was estimated by counting at least 500 tumor cells in the hot spot.

Statistical analyses used chi-squared test and Student's t-test; $p < 0.05$ was considered to indicate a statistically significant difference in both analyses.

Table 1 Clinical findings from 37 non-invasive follicular thyroid neoplasms with papillary-like nuclear features and 54 encapsulated follicular variant-papillary thyroid carcinoma cases

	NIFTP ($n = 37$)	Invasive EFV-PTC ($n = 54$)	Minimally invasive ($n = 34$)	Widely invasive ($n = 20$)
Age, mean (range)	57.7 (38–77)	55.9 (29–77)	56.5 (30–77)	54.7 (29–73)
Male	9 (24.3%)	15 (27.8%)	12 (35.3%)	3 (15.0%)
Female	28 (75.7%)	39 (72.2%)	22 (64.7%)	17 (85.0%)
Tumor size, mean (range)	19.5 (3–50)	21.8 (6–58)	22.3 (6–58)	20.9 (8–40)

NIFTP, non-invasive follicular thyroid neoplasm with papillary-like nuclear features; EFV-PTC, encapsulated follicular variant-papillary thyroid carcinoma
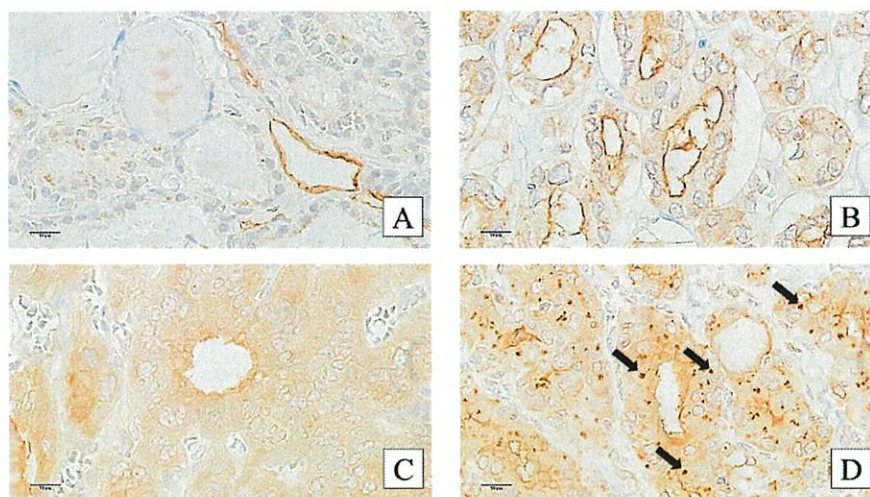

Fig. 2 Immunohistochemical staining patterns for CD26. A: Negative staining. Vascular endothelial cells are positive (part of the normal thyroid tissue in non-invasive follicular thyroid neoplasm with papillary-like nuclear features: NIFTP). B: Apical membranous pattern. Apical surface is strongly stained (NIFTP). C: Cytoplasmic diffuse pattern (minimally invasive encapsulated follicular variant of papillary thyroid carcinoma). D: Cytoplasmic dot-like pattern (arrows; widely invasive encapsulated follicular variant of papillary thyroid carcinoma). Scale bar represents 10 μm.

Results

The results of CD26 immunostaining are shown in the Table 2. In all the cases, normal thyroid follicular cells did not express CD26 (Fig. 2A). Out of 37 NIFTPs, 36 (97.3%) were positive for CD26, including 32 cases with apical membranous pattern (88.9%) (Fig. 2B), 13 cases with cytoplasmic diffuse pattern (36.1%), and 11 cases with cytoplasmic dot-like pattern (30.6%). In minimally invasive EFV-PTC cases, the frequency of CD26-positive cases was 91.2% (31/34), including 20 cases with apical membranous pattern (64.5%), 11 cases with cytoplasmic diffuse pattern (35.5%) (Fig. 2C), and 13 cases with cytoplasmic dot-like pattern (41.9%). The frequency of widely invasive EFV-PTC cases showing CD26 expression was 100.0% (20/20), including 11 cases with apical membranous pattern (55.0%), 6 cases with cytoplasmic diffuse pattern (30.0%), and 13 cases with cytoplasmic dot-like pattern (65.0%) (Fig. 2D). There was no significant difference in the positive rates of CD26 across the three tumors. In approximately 50% of the cases, tumor cells expressed two or all of the three patterns, which could be clearly identified because of different darkness levels.

In all the thyroid tumors examined, tumor cells showed dual or triple staining patterns for CD26. The frequency of apical membranous pattern expressed in NIFTP (88.9%) was higher than that in invasive EFV-PTC (55.0%, $p < 0.01$), minimally invasive EFV-PTC (64.5%, $p < 0.05$), and widely invasive EFV-PTC (55.0%, $p < 0.05$). The frequencies of cytoplasmic dot-like pattern in invasive EFV-PTC (54.2%) and widely invasive EFV-PTC (65.0%) were higher than that in NIFTP (30.6%) ($p < 0.05$ and $p < 0.01$, respectively).

The labeling indices of tumor cells expressing cytoplasmic dot-like pattern in NIFTP, invasive EFV-PTC, minimally invasive EFV-PTC, and widely invasive EFV-PTC ranged from 0 to 22% (mean ± SE: 5.89 ± 0.94%), 1 to 51% (16.04 ± 1.89%), 1 to 51% (14.19 ± 2.39%), and 1 to 43% (18.90 ± 2.90%), respectively. There was a significant difference between NIFTP and invasive EFV-PTC ($p < 0.0001$), NIFTP and minimally EFV-PTC ($p < 0.01$), and NIFTP and widely EFV-PTC ($p < 0.0001$) (Fig. 3). Although it was not significant, the labeling indices increased in proportion to the degree of invasion.

Sizes of the dots expressed by CD26 immunostaining were 0.89 to 1.64 μm (1.12 ± 0.04 μm) in NIFTP, 0.85 to 1.99 μm (1.33 ± 0.04 μm) in invasive EFV-PTC, 0.85 to 1.70 μm (1.27 ± 0.05 μm) in minimally invasive EFV-PTC, and 1.01 to 1.99 μm (1.38 ± 0.06 μm) in widely invasive EFV-PTC. There was a significant difference between NIFTP and invasive EFV-PTC ($p < 0.01$), NIFTP and minimally invasive EFV-PTC ($p < 0.05$), and NIFTP and widely invasive EFV-PTC ($p < 0.001$) (Fig. 4).

Area under the curve (AUC) calculated from the receiver operating characteristic (ROC) curve of labeling index was 0.708. When the cut-off value determined by ROC analysis was 10%, the sensitivity and specificity were 71% and 53%, respectively. When the cases were limited to widely invasive EFV-PTC, the AUC was 0.803, and sensitivity and specificity were 70% and 78%, respectively. The AUC calculated from ROC curves for size of the dot was 0.753. When the cut-off value determined by ROC analysis was 1.1 μm, the sensitivity and specificity were 84% and 63%, respectively. When the cases were limited to widely invasive EFV-PTC, the AUC was 0.810, and sensitivity and specificity were 89% and 63%, respectively.

Table 2 Results of immunohistochemical staining for CD26 in non-invasive follicular thyroid neoplasms with papillary-like nuclear features and encapsulated follicular variant-papillary thyroid carcinoma cases

	NIFTP ($n = 37$)	Invasive EFV-PTC ($n = 54$)	Minimally invasive ($n = 34$)	Widely invasive ($n = 20$)
Positive expression	36 (97.3%)	51 (94.4%)	31 (91.2%)	20 (100.0%)
Apical membranous pattern	32 (88.9%)	31 (55.0%) * $p < 0.01$	20 (64.5%) * $p < 0.05$	11 (55.0%) * $p < 0.05$
Cytoplasmic diffuse pattern	13 (36.1%)	17 (35.4%)	11 (35.5%)	6 (30.0%)
Cytoplasmic dot-like pattern	11 (30.6%)	26 (54.2%) * $p < 0.05$	13 (41.9%)	13 (65.0%) * $p < 0.01$
Labeling index of tumor cells showing cytoplasmic dot-like pattern, mean (range)	5.9% (0–22)	16.0% (1–51) ** $p < 0.0001$	14.2% (1–51) ** $p < 0.01$	18.9% (1–43) ** $p < 0.0001$
Sizes of CD26-positive dots, mean (range)	1.12 μm (0.89–1.64)	1.33 μm (0.85–1.99) ** $p < 0.01$	1.27 μm (0.85–1.70) ** $p < 0.05$	1.38 μm (1.01–1.99) ** $p < 0.01$

NIFTP, non-invasive follicular thyroid neoplasm with papillary-like nuclear features; EFV-PTC, encapsulated follicular variant-papillary thyroid carcinoma
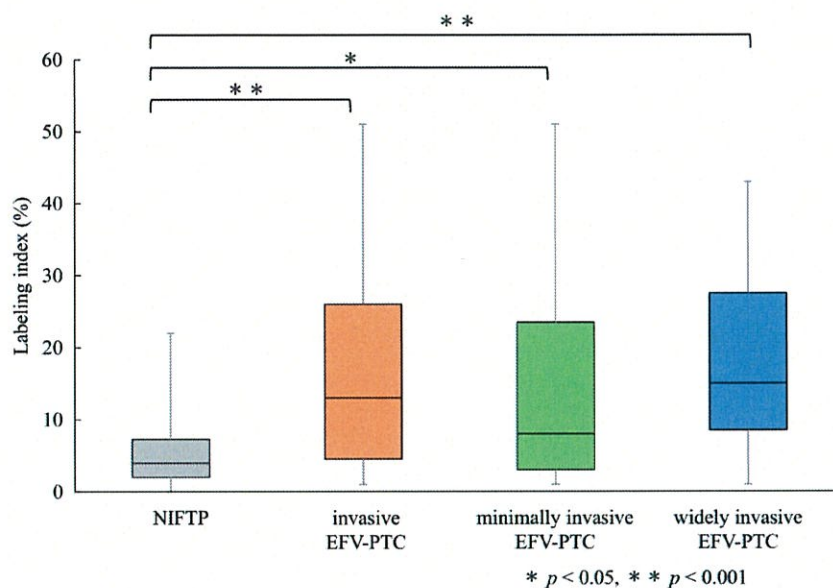*p*-value: compared to NIFTP, *: chi-squared test; **: Student's *t*-test

Fig. 3 Labeling indices of tumor cells expressing cytoplasmic dot-like pattern.

NIFTP, non-invasive follicular thyroid neoplasm with papillary-like nuclear features; EFV-PTC, encapsulated follicular variant-papillary thyroid carcinoma

Fig. 4 Sizes of dots expressed, as seen by CD26 immunostaining.

NIFTP, non-invasive follicular thyroid neoplasm with papillary-like nuclear features; EFV-PTC, encapsulated follicular variant-papillary thyroid carcinoma
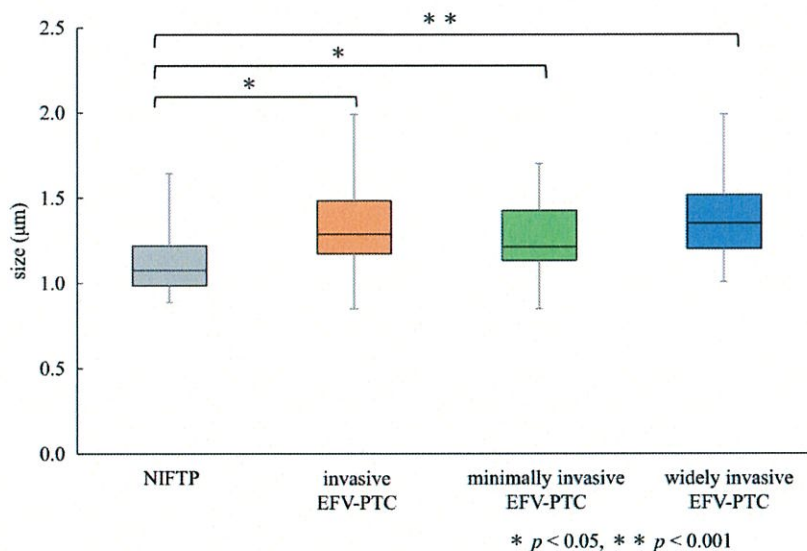
Discussion

Encapsulated or well-circumscribed FV-PTC does not show recurrence, metastasis, or death [5]. Therefore, the diagnosis of non-invasive type EFV-PTC has been reframed as NIFTP [7]. However, it is difficult to morphologically distinguish the presence or absence of invasion, pre-operatively, besides the observer-associated variations in the identification of nuclear features of PTC [8, 33, 34]. Identification of a suitable marker that can distinguish between the presence and absence of invasion would significantly contribute to the reduction of excision region as well as in precise treatment. Further, it would reduce the mental and physical burden on patients.

Various functions of CD26 have been widely discussed with regard to immunology [35, 36] and endocri-

nology [37, 38]. Well-differentiated thyroid carcinomas, including PTC and follicular thyroid carcinoma (FTC), have been reported to show higher expression levels of CD26 than in normal and benign thyroid diseases [39, 40]. Therefore, CD26 seems to be attractive as a marker of differentiated thyroid carcinomas.

Our results collectively revealed a difference in immunoreactivity for CD26 between NIFTP and invasive EFV-PTC. In normal thyroid follicles, CD26 is not expressed. Most of NIFTPs were positive for apical membranous or cytoplasmic diffuse pattern. In invasive EFV-PTC, apical membranous expression was reduced whereas cytoplasmic dot-like pattern was more frequent. Labeling indices of tumor cells expressing cytoplasmic dot-like pattern were 5.89%, 16.04%, 14.19%, and 18.90% for NIFTP, invasive EFV-PTC, minimally invasive EFV-PTC, and widely invasive EFV-PTC, respectively. The indices of invasive EFV-PTC and widely invasive EFV-PTC were significantly higher than that of NIFTP. Furthermore, sizes of the dots seen in NIFTP (1.12 μm) were significantly smaller than those in invasive EFV-PTC (1.33 μm), minimally invasive EFV-PTC (1.27 μm), and widely invasive EFV-PTC (1.38 μm), respectively. In ROC analysis, the sensitivity and specificity of labeling index of tumor cells expressing cytoplasmic dot-like pattern were 71% and 53%, respectively, and of sizes of dots expressed, as seen by CD26 immunostaining, were 84% and 63%, respectively.

CD26 is known to activate intracellular signaling pathway by interacting with the extracellular matrix [11, 12]. Increased CD26 expression is seen in various malignant tumors [16-18, 41-43]. In thyroid, normal thyroid follicular cells are negative for CD26, whereas well-differentiated carcinomas, including PTC and FTC, are positive [19-26]. Tanaka *et al.* and Umeki *et al.* had reported CD26 expression to be undetected or low in non-neoplastic conditions or benign tumors (0–6%), whereas all of PTCs and FTCs showed the expression [22, 23]. CD26 expression in PTC is positively correlated with extrathyroid extension, *BRAF* mutation, and advanced tumor stage [41]. CD26 expression has been more frequently observed in FTC (100%) than in follicular thyroid adenoma (FTA) (27.1%) [27]. Xu *et al.* have shown that the cribriform variant of PTC and the desmoid type fibromatosis show nuclear and cytoplasmic expression of beta-catenin that is usually stained along the cell membrane, and a mutation in the gene encoding β-catenin has been demonstrated in both of them [44]. A similar phenomenon is seen in human esophageal carcinoma [45]. Therefore, the presence of CD26 expression may indicate malignancy, invasiveness, or aggressiveness.

In the current study, we observed three CD26 expression patterns: apical membranous, cytoplasmic diffuse, and cytoplasmic dot-like. Because CD26 is essentially present along the cell membrane, apical membranous pattern of expression would imply that although CD26 is not expressed in normal thyroid follicular cells, it overexpresses in thyroid tumor cells. On the other hand, cytoplasmic diffuse and cytoplasmic dot-like patterns were considered as aberrant expression that might indicate the presence of genetic abnormalities such as in membrane translocation signal. Larger dots were considered to represent CD26 that failed to translocate and were aggregated in cytoplasm, and thus larger dots might also indicate genetic abnormalities. Consequently, cytoplasmic diffuse and/or cytoplasmic dot-like patterns of CD26 expression may indicate invasiveness of EFV-PTC.

However, this study has potential limitations. First, the samples were collected from only one institution. More accurate investigation may be possible by collecting samples from multiple institutions, because NIFTP has potential diagnostic differences between observers and facilities [8, 46, 47]. Second, NIFTP nodules that had been diagnosed as follicular adenoma in Japan were not included in this study [48]. Then, diagnostic criteria of NIFTPs we studied might not be the same to those diagnosed in the Western countries. Third, we have studied with only anti-CD26 antibody. Because antibody recognition sites differ across manufacturers, it would be necessary to ensure that similar results are obtained with other products. Finally, in this study, immunohistochemical staining for CD26 was performed using manual methods, whereas automatic staining equipment is commonly used in clinical sites. It would be important to investigate whether the difference in staining patterns can be reproduced in this study, even if the staining method is different.

We, therefore, conclude that cytoplasmic diffuse and/or cytoplasmic dot-like CD26 expression, particularly larger CD26-positive dots could be useful markers of capsular invasion in EFV-PTC. CD26 immunostaining using cell block or cytological specimen can possibly distinguish between NIFTP and invasive EFV-PTC, preoperatively.

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Disclosure

None of the authors have any potential conflicts of interest associated with this research.

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