Immunohistochemical detection of dipeptidyl peptidase IV (CD 26) in thyroid neoplasia using biotinylated tyramine amplification*

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Differential diagnosis between malignant and benign thyroid tumors derived from follicular cells can pose certain difficulties in routine surgical pathology. The aim of the study was to evaluate dipeptidyl peptidase IV (DPP IV/CD 26) in differential diagnostics of thyroid lesions.

DPP IV/CD 26 was evaluated in thyroid glands of 309 patients (261 females and 48 males, age range of patients 15–80 years). DPP IV/CD 26 was assessed in paraffin-embedded thyroid specimens immunohistochemically using commercially available antibody (Serotec) and biotinylated tyramine amplification kit (DAKO).

Well-differentiated carcinoma revealed DPP IV/CD 26 positivity in 33 out of 42 cases (79%). Neither medullary nor insular carcinoma was DPP IV/CD 26 positive (only one case of each tested). DPP IV/CD 26 expression in isolated cells was seen in 18/261 (7%) benign disorders. The sensitivity of the method was 68%, the specificity was 94%, and the diagnostic accuracy was 91%, respectively, using 5% threshold of positive follicular cells.

DPP IV/CD 26 can be assessed immunohistochemically using biotinylated tyramine amplification kit. DPP IV/CD 26 could be an adjunct in the thyroid gland differential diagnosis. However, DPP IV/CD 26 positivity is limited to the group of well-differentiated carcinomas, particularly papillary carcinoma. Furthermore, it is of limited value for follicular and oncocytic tumors.

Key words: Dipeptidyl peptidase IV, CD 26, thyroid gland tumors, immunohistochemistry, biotinylated tyramine amplification.

Diagnostics of thyroid gland lesions, as any other surgical pathology area faces several problems in distinguishing malignant vs. benign tumors derived from follicular cells. The most common pitfalls in the differential diagnosis are:

1. Benign lesions with hyperplastic papillae, and/or ground-glass nuclei are overdiagnosed as papillary carcinomas.

2. Follicular adenomas with capsular irregularities or clusters of follicular cells entrapped within the capsule may be overdiagnosed as minimally invasive follicular carcinomas.

3. Follicular carcinomas are underdiagnosed due to inadequate sampling and failure to identify vascular and/or capsular invasion [28, 33].

The difficulties encountered in the diagnosis of thyroid malignancy in these tumors inspired many studies on potential markers of neoplasia. Cytokeratins, lectins, and other carbohydrate proteins, metal-binding glycoproteins, CD antigens, enzymes, and oncogenes have been tested [4, 9, 10, 19, 25, 26].

Dipeptidyl peptidase IV (DPP IV/CD 26) was proposed as a thyroid tumor marker, as well [2]. DPP IV/CD 26 is a membrane peptidase, which is not present in normal folli-

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cular cells [12]. However, it has been reported in well-differentiated carcinomas derived from follicular cells [2, 11, 15, 20, 21, 22]. Commercially available antibody against CD 26 is supposed to be used only in frozen sections. In our study we tested catalysed signal amplification system with the above mentioned commercially available antibody in paraffin-embedded material.

Catalysed signal amplification, which is a method using biotinylated tyramine, has been introduced into immunology by Bobrow et al [5]. First, the method had been used in ELISA and Western blotting [5, 6, 7] and later on it has been adapted for immunohistochemistry [1, 31]. In immunohistochemistry, the procedure can enable the use of certain monoclonal antibodies on paraffin sections, which were previously for use in frozen sections only [31].

We report on a series of 309 thyroid gland lesions stained immunohistochemically for DPP IV/CD 26 using biotinylated tyramine amplification kit and discuss possible value of this marker in differential diagnostics of thyroid neoplasia.

Material and methods

There were 309 patients in the herein presented study (261 females, 48 males, age range 15–80 years, mean age 48 years, median age 51 years).

The thyroid gland specimens were routinely processed and histologically examined. The final histological diagnoses based on WHO and AFIP criteria [13, 29] were as follows: 44 malignant tumors (38 papillary carcinomas, 2 follicular carcinomas, and one case each of oncocytic carcinoma, medullary carcinoma, insular carcinoma, and hyalinizing trabecular tumor), 75 benign tumors (67 follicular adenomas, 6 oncocytic adenomas, 1 atypical oncocytic adenoma, 1 atypical follicular adenoma), 185 benign non-neoplastic lesions (63 diffuse hyperplastic goiters, 114 nodular hyperplastic goiters, and 8 cases of Hashimoto thyroiditis), and 5 normal thyroid glands.

Immunohistochemical examination was performed on 4 μm sections pre-treated in 0.001 M EDTA-NaOH buffer (pH 8) in microwave oven (900 W for 5 minutes, 400 W for 10 minutes). Slides were incubated overnight with CD 26 monoclonal antibody (Serotec, UK, clone M-A 261, 1:100 in DAKO antibody diluent). The biotinylated tyramine amplification step was performed (CSA kit, DAKO, Denmark). Following this step, streptavidin biotin peroxidase method was used (EnVision, DAKO) according to the manufacturer's instructions. 3,3-diaminobenzidine (DAB liquid Plus, DAKO) was used as chromogen. The slides were counterstained with Gill hematoxylin. Alternatively, the biotinylated tyramine amplification step (CSA kit, DAKO) and streptavidine/Cy3 (Sigma) detection were used afterwards according to the manufacturer's instructions. The nuclei were counterstained with DAPI (4',6-diamidino-2-phenylindole, VECTASHIELD, USA). The immunofluorescent staining was performed in 215 cases. The positivity was evaluated both in the tumor and in the surrounding tissue.

According to the percentage of positively stained follicular cells, the cases were divided into 5 groups: 0%, <5%, <25%, <50%, and ≥50%, respectively. Statistical analysis using the above mentioned thresholds was performed with S.A.S. software, release 8.2. The inferential statistics contain a point estimation of sensitivities, specificities, positive and negative predictive values for different thresholds mentioned above and different clinical questions. For comparison of averages in two independent random samples the Wilcoxon non-pair test was used because non-gaussian distribution of measured parameters (mainly target parameter DPP IV/CD 26 had strongly non-gaussian distribution). From the same reason Spearman rank correlation coefficient was used.

Results

Two staining patterns were observed: membranous linear apical positivity in the majority of positive cases (Figs. 1 and 2), and diffuse cytoplasmic positivity in the minority of positive cases. In some cases, both staining patterns were observed. The capillary endothelial cells, macrophages, and fibroblasts were positive as well.

The staining results (positive/negative) according to thresholds and final histological diagnoses are summarized in Table 1.

To summarize, 33 out of 42 (79%) well-differentiated carcinomas revealed DPP IV/CD 26 positivity using 5% threshold. Namely, 31 (82%) papillary carcinomas, 1 case (1/2) of follicular carcinoma, and 1 case (1/1) of hyalinizing trabecular tumor were positive. The only oncocytic carcinoma was tested negative. Interestingly, neither medullary, nor insular carcinoma was DPP IV/CD 26 positive. Seven out of 75 (9%) benign tumors were positive at 5% threshold. In addition, 10 out of 185 (5%) non-neoplastic lesions expressed positivity at 5% threshold. In all tested cases, the staining results obtained by immunohistochemical and immunofluorescent staining (Fig. 3) were completely identical.

Table 2 shows statistical analysis of the presented data. The sensitivity is highest for the 0%, 5%, and 25% threshold. On the other hand, at the 50% threshold level, the specificity, the positive predictive value, and the diagnostic accuracy are highest, respectively. We decided to use the 5% threshold for discriminating malignant vs. benign lesions: the sensitivity is comparable with 0% threshold; however, the specificity and diagnostic accuracy are slightly increased.

Figure 1. Membranous linear apical staining pattern in a case of well-differentiated papillary carcinoma (200x, CD 26, DAB, hematoxylin counterstaining).

Figure 2. Membranous linear apical staining pattern in a case of well-differentiated papillary carcinoma – detail (400x, CD 26, DAB, hematoxylin counterstaining).

The difference between malignant tumors vs. benign tumors (p<0.0001, Wilcoxon test), malignant tumors vs. benign non-neoplastic lesions (p<0.0001, Wilcoxon test), and between malignant tumors vs. benign neoplastic and non-neoplastic lesions as a group (p<0.0001, Wilcoxon test) were statistically significant. The difference between papillary carcinoma vs. follicular adenoma (p<0.0001, Wilcoxon test) was statistically significant. However, the difference between oncocytic carcinoma vs. oncocytic adenoma (p<0.5637 NS, Wilcoxon test) was statistically non-significant. The total number of follicular carcinomas was not sufficient enough to perform statistical analysis.

The DPP IV/CD 26 positivity is associated with the presence of metastases, of angioinvasion, and of capsular invasion: p-values by means of Wilcoxon test are listed in Table 3.

Figure 3. Membranous linear apical staining pattern in a case of follicular variant of papillary carcinoma (200x, CD 26, Cy3, DAPI nuclear counterstaining).

Discussion

Dipeptidyl peptidase IV/CD 26 is a serine membrane-bound peptidase involved in cell-to-cell adhesion and T-lymphocytes activation [8]. It was first reported as glycyl-proline naphthylamidase and extracted from liver and kidney tissues [14]. In the human, it is located particularly in small intestinal brush border, capillary endothelial cells, proximal tubules of the kidney, in lymphocytes, and macrophages [12, 23, 24]. However, it is not found in thyroid follicular cells under normal circumstances [12].

In a number of studies, aberrant DPP IV/CD 26 expression was observed in well-differentiated carcinomas derived from follicular cells using cytochemical and histochemical methods, respectively [2, 11, 15, 20, 34]. Moreover, the aberrant positivity was also reported in leukaemic cells [24], hepatocellular carcinoma [18], skin basal cell carcinoma [27], and lung adenocarcinoma [3]. DPP IV/CD 26 was firstly recommended as a marker of thyroid neoplasia by Japanese researchers [2]. Since than it has been evaluated as a marker of thyroid neoplasia cytochemically [2, 11, 15], histochemically [20], immunohistochemically with non-commercially available antibody [21, 22, 30], and by Northern blotting [30].

In the herein presented study, commercially available monoclonal antibody for DPP IV/CD 26 detection, intended primarily for the use on frozen material, was used for the first time on paraffin sections owing to the use of biotinylated tyramine amplification detection kit.

Tyramine signal amplification is based on the principle of enzyme amplification. This method was first described as a catalysed reporter deposition technique – CARD by Bobrow at al [5] in 1989 and introduced into immunohistochemistry in 1992. Signal amplification is based on

Table 1. DPP IV positive/negative staining according to final histological diagnoses and threshold [cummulative numbers]

Diagnosis	n	No threshold (+/-)	5% threshold (+/-)	25% threshold (+/-)	50% threshold (+/-)
Papillary carcinoma	38	31/7	31/7	31/7	30/8
Follicular carcinoma	2	1/1	1/1	1/1	1/1
Oncocytic carcinoma	1	0/1	0/1	0/1	0/1
Medullary carcinoma	1	0/1	0/1	0/1	0/1
Insular carcinoma	1	0/1	0/1	0/1	0/1
Hyalinizing trabecular tumor	1	1/0	1/0	1/0	1/0
Follicular adenoma	67	7/60	7/60	6/61	3/64
Atypical follicular adenoma	1	0/1	0/1	0/1	0/1
Oncocytic adenoma	6	1/5	1/5	1/5	0/6
Atypical oncocytic adenoma	1	0/1	0/1	0/1	0/1
Diffuse hyperplasia	63	5/58	6/57	4/59	1/62
Nodular hyperplasia	114	4/110	3/111	2/112	0/114
Thyroiditis	8	1/7	1/7	1/7	1/7
Normal thyroid gland	5	0/5	0/5	0/5	0/5

+/- – positivity/negativity

Table 2. Statistical analysis for 0%, 5%, 25%, and 50% threshold

	Sensitivity	Specificity	Positive PV	Negative PV	Diagnostic accuracy
0% threshold	67.5%	92.8%	58.7%	95.0%	89.5%
5% threshold	67.5%	94.3%	64.3%	95.1%	90.8%
25% threshold	67.5%	97.4%	79.4%	95.2%	93.4%
50% threshold	65.0%	98.5%	86.7%	94.9%	94.1%

PV – predictive value

Table 3. p-values by means of Wilcoxon test

	Metastases	Angio-invasion	Capsulo-invasion	Oncocytes	Hyperfunction	Regressive changes	Focal lymphocytic thyroiditis
p-value	0.0005	0.0076	0.0001	0.1015	0.0748	0.1798	0.6775

biotinylated tyramine deposition through free radical formation, which is catalyzed by oxidizing horseradish peroxidase. Radicalized biotinylated tyramine is covalently attached to electron-rich moieties (phenylalanin, tyrosine etc), resulting in formation of additional biotinylated molecules, located at the site of antigen-antibody reaction.

In immunohistochemistry, the detected antigen is conjugated with specific primary antibodies and biotinylated secondary antibody. At the same time the signal is amplificated by radicalized biotinylated tyramine deposition bound covalently to stand-by tissue molecules. Finally, biotin can be easily visualized by incubation of the slides with horseradish peroxidase-conjugated detection system (streptavidin) [31].

Using biotinylated tyramine, the dilution of primary antibody can be increased up to 1000x, maximizing thus the sensitivity of immunohistochemical reaction [32]. However, most studies have reported increasing dilution by 5–50x [1, 31]. Moreover, the procedure can enable the use of certain monoclonal antibodies on archival paraffin-embedded tissue sections; otherwise these antibodies were only for the use in frozen material [31].

The role of DPP IV/CD 26 in thyroid tumorigenesis has not been clarified yet. Generally, peptidases can play a role in tumor promotion as well as in tumor induction [16]. DPP IV/CD 26 in thyroid carcinoma may be involved in signal transduction and tyrosine phosphorylation regulation [22, 30]. Moreover, proteolytic activity of DPP IV/CD 26 may play a role in tumor progression [22]. On the other hand, Lima et al [22] in their study considered DPP IV/CD 26 as a marker of proliferation of follicular cells. The loss of differentiation in tumors is linked to DPP IV/CD 26 negativity [10].

In the majority of studies, all cases of papillary carcinoma were DPP IV/CD 26 positive [2, 10, 15, 20, 21]. Our study revealed the positivity in 31 out of 38 (82%) papillary carci-

nomas using 25% threshold. The positivity of papillary carcinoma was significantly higher than that of follicular adenoma and nodular hyperplasia [15]. In comparison, only three studies found 100% positive staining of follicular carcinomas [2, 20, 30]. Although staining intensity and percentage of positive cells was higher in cases with vascular invasion than in tumors with transcapsular invasion, the difference was non-significant. Follicular carcinoma revealed significantly higher positivity compared with nodular hyperplasia. However, no statistically significant differences were observed between follicular carcinoma and follicular adenoma [15]. In our study, only 2 cases of follicular carcinoma were evaluated: one of them was negative. The data in follicular carcinomas are not sufficient enough for conclusions, there are, however, certain limitations in this subgroup. The only study, which included oncocytic carcinoma, revealed DPP IV/CD 26 positive staining in the intracytoplasmatic lumens of 1 case [10]. The only oncocytic carcinoma tested in our study was DPP IV/CD 26 negative. The data, otherwise being insufficient, show also limitations in this tumor group. In most studies, follicular adenoma revealed DPP IV/CD 26 positivity of various intensity and percentage. However, only 13 cases (13/133) expressed DPP IV/CD 26 in more than 50% of cells [2, 15, 20, 34]. The highest percentage (61%) of positive benign tumors was reported in LIMA's study: all lesions contained neoplastic or metaplastic oncocytic cells [22].

Interestingly, positivity is usually detected in benign oncocytes. In benign non-neoplastic oncocytes, DPP IV/CD 26 positivity could be explained by the presence of abundant organels and ras-oncogenes products in cytoplasm [2]. Positive oncocytes can cause diagnostic difficulties, namely in Hashimoto thyroiditis. Distinguishing positive benign metaplastic oncocytes from positive malignant cells of papillary carcinoma occurring in Hashimoto thyroiditis is virtually impossible.

However, there are still some limitations for introduction of DPP IV/CD 26 as a marker of thyroid neoplasia in routine pathological practice. The positivity can be detected in single cells in benign tumors and non-neoplastic lesions. The use of threshold of positive follicular cells was recommended in all studies. DPP IV/CD 26 positivity was evaluated semi-quantitiably taking into account the number of stained cells and staining intensity [2, 10, 15, 20, 21]. Threshold of 40%, and 50%, respectively, was suggested in cytochemistry [10, 15, 17]. In this immunohistochemical study, the sensitivity is the same for 0%, 5%, and 25% threshold, respectively. However, the specificity, positive predictive value, and diagnostic accuracy were superior at 50% threshold.

Loss of DPP IV/CD 26 expression in anaplastic and medullary carcinomas was explained by loss of reactivity for thyroglobulin [21]. The negativity in these tumors represents certain limitation of general DPP IV/CD 26 usefulness as a marker of thyroid malignancy. However, these entities should be easily distinguished by their morphological characteristics.

In conclusions, DPP IV/CD 26 expression could be used as an adjunct marker for well-differentiated thyroid tumors, particularly papillary carcinoma. Our preliminary results, even though the numbers of follicular and/or oncocytic carcinomas are small, demonstrate only limited value of this method for the use in differential diagnostics between benign and malignant follicular lesions. We have tested successfully CD 26 monoclonal antibody (Serotec) in paraffin-embedded material using catalysed signal amplification system.

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