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High Expression of Claudin-1 Protein in Papillary Thyroid Tumor and its Regional Lymph Node Metastasis

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Abstract Claudins, known as major contributors in the formation of the tight junction, are differentially expressed in malignant tumors as compared to the corresponding healthy tissues. Therefore, they are thought to play a role in carcinogenesis and tumor progression. Altered expression of claudin-1 has been reported in several tumor types including endometrial, papillary renal cell and colonic carcinoma, and increased claudin-1 mRNA levels have been observed in papillary thyroid carcinoma (PTC). In this study, we aimed at determining the pattern of claudin-1 expression in various types of thyroid lesions at the protein level and investigating the immunolocalization of β-catenin reported to regulate claudin-1 expression. Samples included 19 PTCs, ten cases of corresponding regional lymph node metastasis, eight papillary microcarcinomas (PMC), 17 follicular thyroid carcinomas (FTC) and 19 follicular adenomas (FA). All cases were evaluated by quantitative immunohistochemistry. Conspicuous claudin-1 immunostaining was detected in the majority of PTC/PMC primary tumors and lymph node metastases (19/27 and 9/10, respectively). On the other hand, we found weak or no claudin-1 expression in any of the FA and FTC cases or peritumoral non-malignant thyroid tissues. Our data prove that high claudin-1 protein expression is specific for PTC and its regional lymph node metastases, while we failed to verify that claudin-1 is regulated by β-catenin in thyroid tumors. Based on these results, claudin-1 may be a useful tumor marker for PTC.

Keywords Claudin-1 · Lymph node metastasis · Papillary thyroid carcinoma · Tumor marker

Abbreviations

PTC	Papillary thyroid carcinoma
PMC	Papillary microcarcinoma
FTC	Follicular thyroid carcinoma
FA	Follicular adenoma
HT	Hashimoto's thyroiditis
TJ	Tight junction

Introduction

Thyroid cancers, although they represent only 1% of all malignant diseases, are among the most common endocrine malignancies [1–3]. Most thyroid tumors derived from the follicular epithelium [2, 3]. Follicular cell-derived carcinomas are divided into well-differentiated, poorly differentiated, and undifferentiated types on the basis of histological and clinical features. Well-differentiated thyroid cancers include the papillary thyroid carcinomas and follicular thyroid carcinoma [3].

Papillary carcinoma is the most common type of thyroid cancer, comprising approximately 80% of thyroid epithelial malignancies [1–4]. The more aggressive follicular carcinoma is far less prevalent (10–15% of thyroid malignancies) [2, 4, 5]. Neoplastic transformation is a multistep

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Table 1 Localization of thyroid lesions

Location	Left lobe	Right lobe	Both lobes	Isthmus	Unknown
PMC (*N*=8)	3	5	0	0	0
PTC (*N*=19)	6	3	9	0	1
FTC (*N*=17)	7	4	4	0	2
FA (*N*=19)	4	12	0	2	1

process that encompasses a broad spectrum of molecular and morphological changes, which transform the normal state into a fully established neoplasm [3, 5]. Several studies have reported some connection between Hashimoto's thyroiditis (HT) and thyroid carcinoma, but their coincidence has not exactly been determined (varies between 0% to 30%) [6–8]. However, the increasing incidence of HT associated carcinomas suggest that HT is likely to be a precursor of thyroid carcinoma [8]. The majority of these tumors are papillary carcinomas with a propensity to metastasize to the regional lymph nodes [2, 7]. Papillary microcarcinoma, measuring 10 mm or less in maximum diameter, is found incidentally [2, 4]. Benign neoplasms in the thyroid are adenomas, with the most common type being follicular adenoma [2, 4].

Claudins, a family of transmembrane proteins, are major components of the tight junction (TJ) [9, 10]. Claudin-1 was the first member of the claudin family to be identified as a tight junction component [11]. So far 24 members of the claudin family have been described, and their role in carcinogenesis and cancer progression has been proposed repeatedly [10, 12–14]. Altered expression of claudins was found in a wide variety of human malignancies including endometrial [15], papillary renal cell [16], colon [17], pancreatic [18], breast [19] and cervical cancers [20]. Claudins as membrane proteins showing differential expression in the normal versus neoplastic tissues [12, 14, 21] may provide new opportunities for targeted cancer therapy [22, 23].

The expression of claudin-1,-4, and -7 protein in thyroid neoplastic samples has already been investigated [24], and previous studies have reported the increased gene expression of claudin-1 in PTC [25, 26]. However, the expression of claudin-1 in different PTC samples (including PMC and HT) and in corresponding regional lymph node metastases has not yet been analyzed at the protein level. It has been published recently that claudin- 1 is expressed in the majority of papillary renal cell carcinomas [16]. Previously, the high expression of claudin-1 protein was reported by our group in serous papillary endometrial carcinoma [15]. Based on the idea that high claudin-1 expression might be connected with papillary morphology, we set out to evaluate the expression of claudin-1 protein in PTC as well as non-papillary thyroid tumors. Furthermore, since the role of Wnt/ β-catenin pathway in regulation of claudin-1 expression was demonstrated in colorectal tumorigenesis [27], we also aimed at investigating whether claudin-1 protein expression correlates with β-catenin protein expression in various types of thyroid lesions.

Materials and Methods

Tissue Samples

Between 1992 and 2007, 63 specimens of surgically removed, formalin-fixed and paraffin-embedded thyroid lesions were collected from the archives of the 2nd Department of Pathology, Semmelweis University, Budapest, and the National Institute of Oncology, Budapest. Each case was classified according to the WHO histological classification of thyroid tumors [4]. All available slides were reviewed and the most representative blocks from each case were selected. The selected samples included 11 males and 52 females ranging from 11 to 87 years old

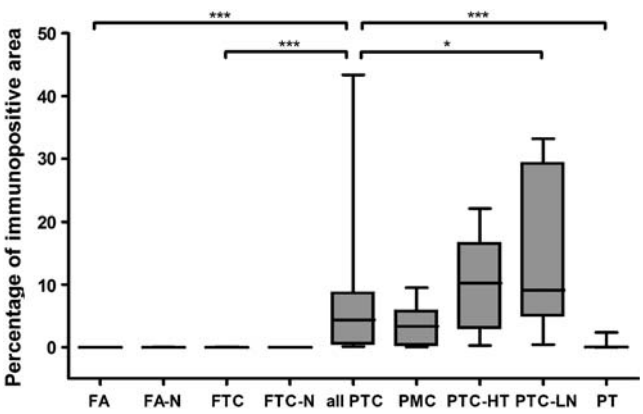

Fig. 1 Digital morphometry analysis of claudin-1 immunoreactions. Abbreviations: FA, follicular adenoma; FTC, follicular thyroid carcinoma; all PTC: all papillary thyroid carcinomas; PTC-LN: lymph node metastases of PTCs; PMC, papillary microcarcinoma (a subgroup of PTC); PTC-HT: Hashimoto's thyroiditis-associated PTC; -N: non-malignant peritumoral thyroid tissue. *, $0.05 > p > 0.01$; ***, $p<0.001$, by Mann–Whitney U test. For the sake of clarity, significant differences relative to 'all PTC' are shown only; each pairwise difference between both PTC subgroups (PTC-M and PTC-HT) and both follicular tumors (FA and FTC) were significant at $p< 0.001$ level

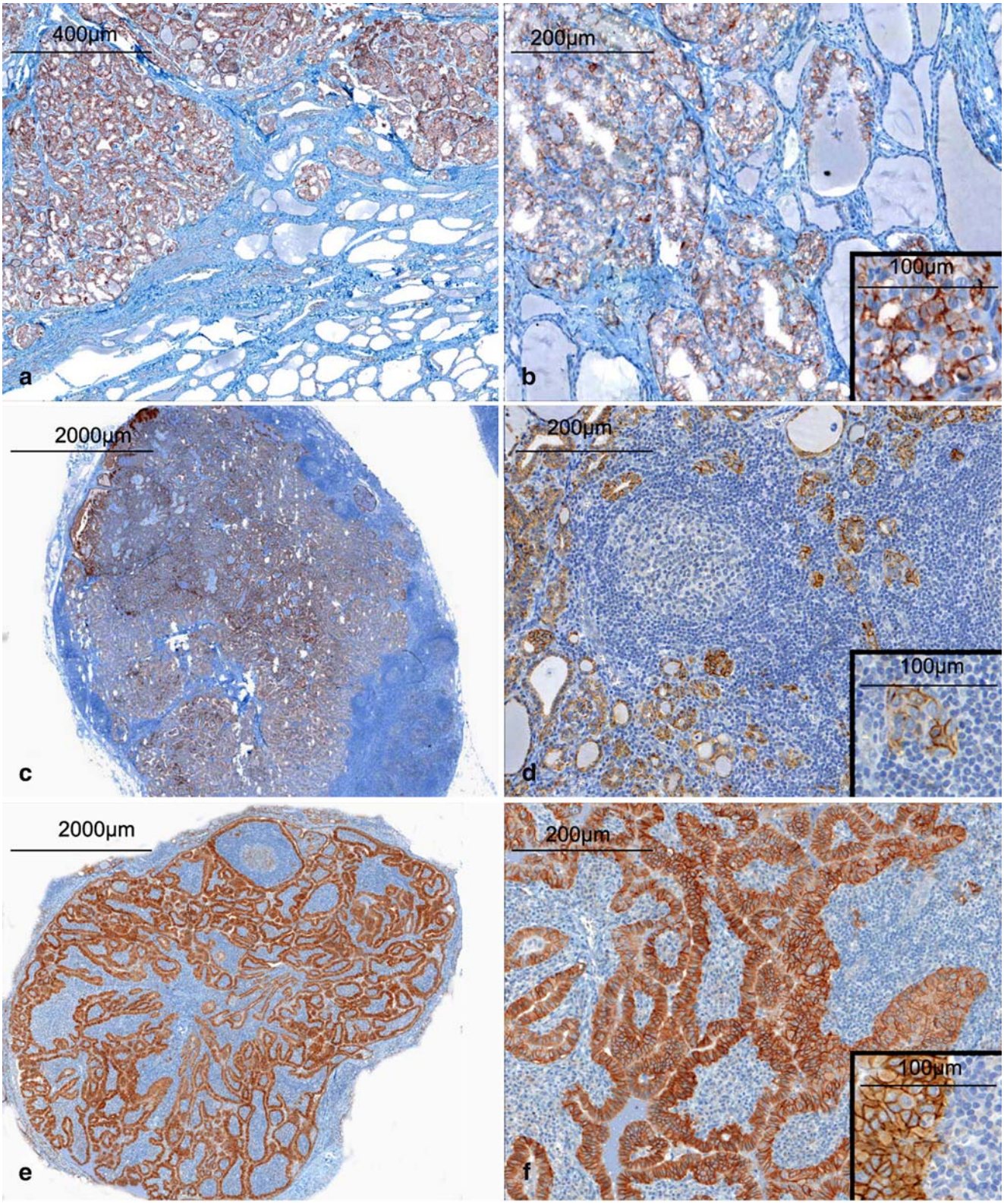

Fig. 2 Claudin-1 immunoreaction in papillary thyroid carcinoma and its regional lymph node metastases. Strong cell membrane claudin-1 immunostaining in papillary thyroid carcinoma (**a**, **b**) and its regional lymph node metastasis (**c**, **d**). Hashimoto's thyroiditis-associated papillary thyroid carcinoma is shown in (**e**, **f**)

(mean: 55.3±8.6 years). Nineteen cases of papillary carcinomas, ten corresponding regional (cervical) lymph node metastases, eight papillary microcarcinomas, 17 follicular carcinomas, and 19 follicular adenomas were examined. Of the 19 PTC cases, 5 were associated with Hashimoto's thyroiditis. Regarding histological subtype, 5 PTCs were follicular variants (out of which 1 exhibited oncocytic morphology); the rest comprised of conventional PTC and a single tall cell variant. In the PTC samples the tumor localized to both lobes in the majority of cases. In PMC cases the localization of the tumor was dominantly in the right lobe, while follicular carcinomas were mainly found in the left lobe. The majority of the follicular adenomas were localized to the right lobe (Table 1). None of the patients received chemotherapy or radiotherapy prior to surgery. The study was approved by the Regional Ethical Committee of the Semmelweis Medical University (issued under #172/2003).

Immunohistochemistry

The immunohistochemical reactions were performed on 3–4 μm thick formalin-fixed paraffin-embedded sections. After deparaffination steps, slides were washed in PBS (pH 7.4), then were treated in Target Retrieval Solution (cat# S1699 from DAKO, Glostrup, Denmark) in a microwave oven for 30 min. Immunoreactions for claudin-1 were carried out in a Ventana ES automated immunostainer (Ventana Medical Systems Inc., Tucson, AZ, USA). Rabbit polyclonal claudin-1 antibody was applied in a dilution of 1:100 (cat# 18-7362 from Zymed Inc., San Francisco, CA, USA). Reagents and the secondary

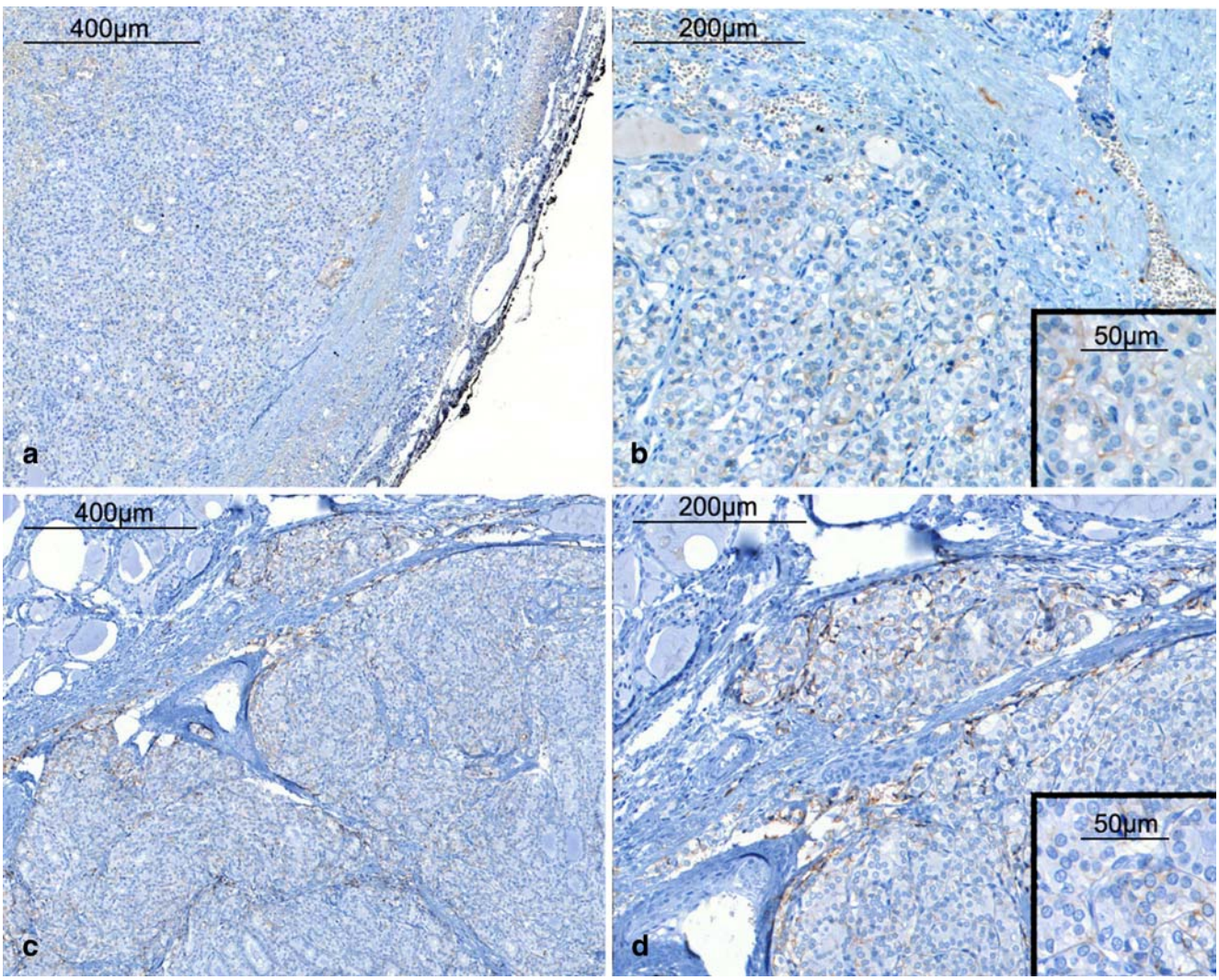

Fig. 3 Claudin-1 immunoreaction in follicular carcinoma and follicular adenoma of the thyroid. Weak or no claudin-1 expression was detected in follicular carcinoma (**a**, **b**) and in follicular adenoma (**c**, **d**)

antibody from the iView DAB Detection Kit (cat# 760-091 from Ventana Medical Systems Inc.) were used as provided by the manufacturer. Positive control recommended by manufacturer (normal skin) was used to confirm correct immunohistochemical staining for claudin-1.

In the case of β-catenin, after antigen retrieval steps tissues were blocked for endogenous peroxidase activity with 3% H_2O_2. Mouse monoclonal antibody against β-catenin (cat# 610154, BD Transduction, San Diego, CA, USA) was applied in a dilution of 1:200. Negative controls for nonspecific binding, incubated with secondary antibodies only, were processed and revealed no signals. For positive control of β-catenin immunoreaction we used human hepatoblastoma tissue previously reported to show nuclear positivity and analyzed for β-catenin mutation [28].

Evaluation of Immunoreactions

The results of claudin-1 immunohistochemical reactions were photodocumented using Mirax MIDI Scanner (3DHistech Ltd., Budapest, Hungary). Ten non-overlapping representative fields were assessed. Digital images were quantified using Leica QWin V3.0 software (Leica Microsystems Imaging Solutions Ltd., Cambridge, UK). Before performing the measurements, a threshold level of colors to be considered as positive was defined by selecting the stained areas on the digitized positive control tissues. Positive area was defined as percentage of pixels above the threshold within a defined area of interest. Statistical analysis for the comparison of immunopositive areas in the different sample groups was performed using non-parametric Mann–Whitney test (SPSS 15.0, SPSS Inc., Chicago, Ill, USA).

The intensity of β-catenin immunoreaction in the center of lesions was graded semi-quantitatively as 0-absent staining, 1-weakly positive, 2-moderately positive and 3-strongly positive.

Results

Immunohistochemical Analysis of Claudin-1 Expression

By digital morphometry, claudin-1 immunostaining was obvious (immunopositive area was greater than 1%) in the majority (19/27) of PTCs, and it was strong (area% > 5) in 12/27 cases. PTC tumor cells strongly expressed claudin-1 along the cell membranes forming a honeycomb-like pattern. On the other hand, claudin-1 immunoreaction was virtually absent from FTC, FA, and the peritumoral non-malignant thyroid tissue (median immunopositive areas: 4.4% for PTC, in contrast with 0.0% for FTC, FA, and peritumoral tissues) (Figs. 1, 2, 3). With other words, by defining 1% immunostained area as a cut-off value for positive claudin-1 immunoreaction, 70.3% of PTCs were positive, while all FTCs, FAs, and non-tumorous thyroid tissues were negative. Thus, claudin-1 immunostaining alone differentiated PTC from both FTC and FA with a specificity of 100% and a sensitivity of 70.3% (Pearson chi-square, $P<0.001$ for both). Claudin-1 expression was preserved in the lymph node metastases of PTC as well (median immunopositive area: 9.1%). No significant differences within the PTC group were found according to histological subtype (follicular variant vs. conventional and tall/columnar cell variants), etiology (Hashimoto vs. non-Hashimoto), the presence of lymph node metastasis (Fig. 4), and the size of lesion (microcarcinoma vs. carcinoma > 1 cm in diameter).

Immunohistochemical Analysis of β-catenin Expression

Cell membrane and cytoplasmic β-catenin immunoreactions (Fig. 5) were evaluated semi-quantitatively (Fig. 6). In peritumoral thyroid tissues of all tumor types, β-catenin was seen primarily on the cell membranes of immature follicles. Such immature follicles were abundantly seen in thyroid tissues with increased regenerative activity, mainly associated with HT in the peritumoral areas of PTC. In the carcinomas, cytoplasmic β-catenin immunostaining was also observed. PTCs and their lymph node metastases showed a weakly significant tendency of decreased (0−1+) membrane staining when compared with FTC (Pearson chi-square, $P=0.045$) or with all follicular tumors (FTC + FA, $P=0.033$). Cytoplasmic β-catenin staining, on the other

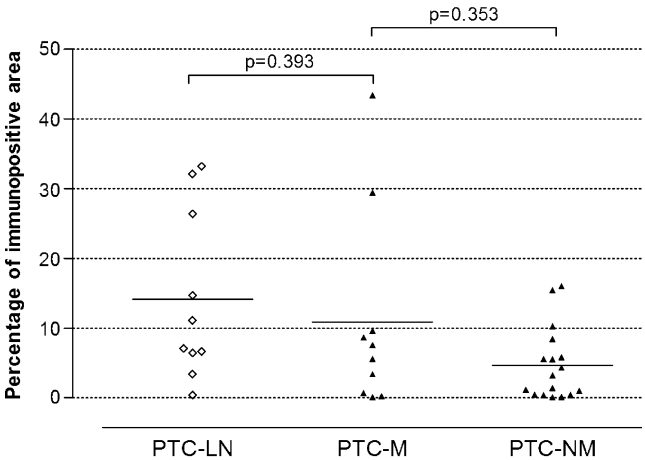

Fig. 4 Comparison of claudin-1 immunopositive area% between the lymph node metastases of papillary thyroid carcinomas and primary tumors with and without metastasis. Mann–Whitney U test failed to detect significant differences between either the metastatic (PTC-M) and non-metastatic (PTC-NM) subgroups of papillary thyroid carcinoma (PTC), or the lymph node metastases (PTC-LN) and their corresponding primary cancers

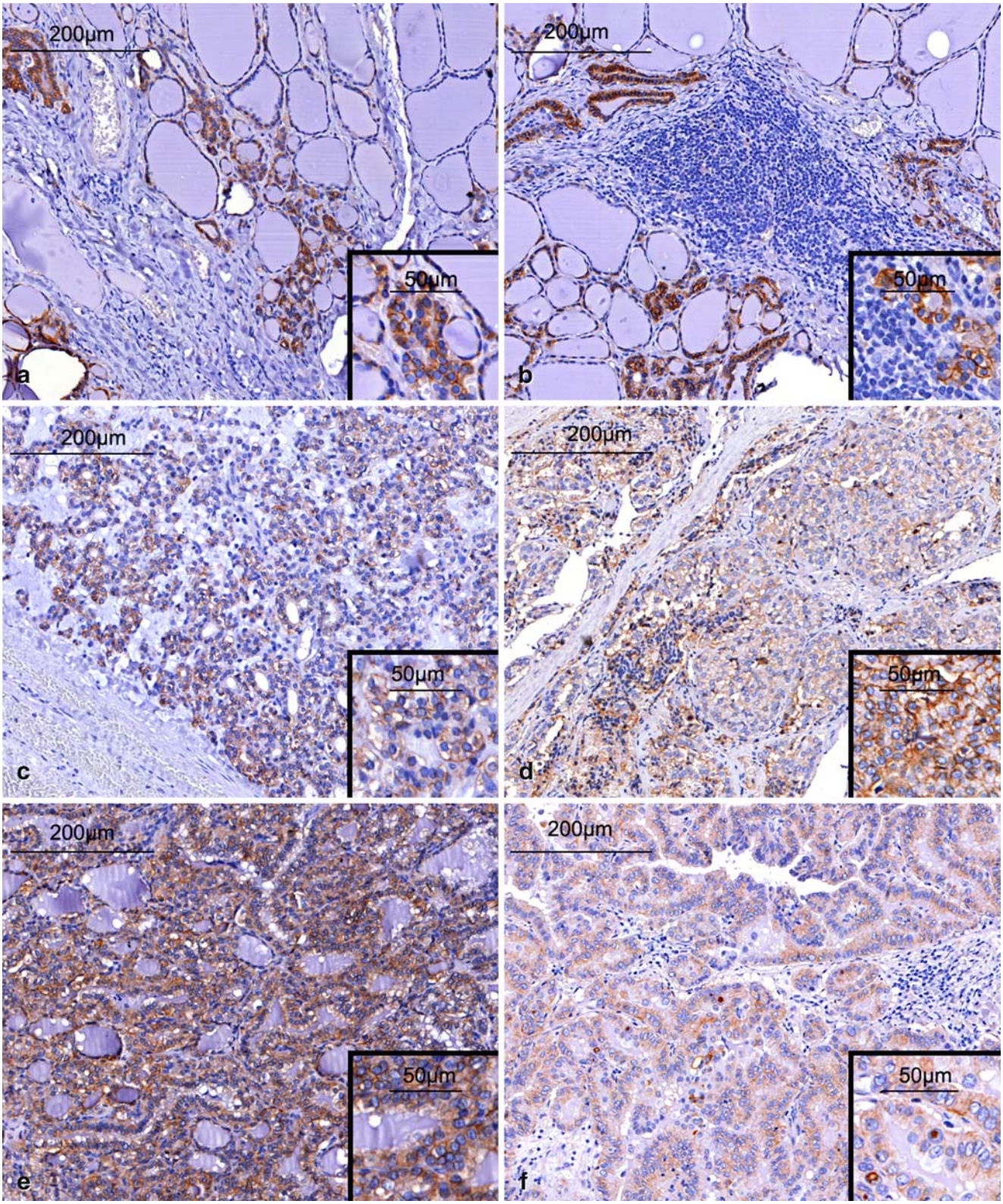

Fig. 5 Immunoreaction of β-catenin in various thyroid samples. In peritumoral thyroid tissues, β-catenin was primarily seen on the cell membranes in immature follicles (**a**). Such immature follicles were abundant in Hashimoto's thyroiditis (**b**). Strong cell membrane β-catenin immunostaining was observed in follicular adenoma (**c**). In FTC, cytoplasmic β-catenin reaction appeared in addition to cell surface immunostaining (**d**). In PTCs, a tendency of decreased membrane staining as compared with FTC and follicular adenomas was often accompanied by marked cytoplasmic reaction (**e**). Intranuclear inclusions positive for β-catenin were predominantly seen in PTCs (**f**). PTC, papillary thyroid carcinoma; FTC, follicular thyroid carcinoma

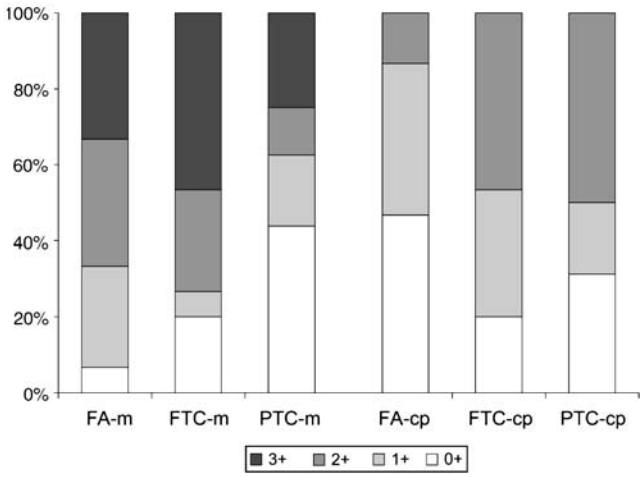

Fig. 6 Semiquantitative evaluation of cell membrane and cytoplasmic β-catenin immunoreaction in thyroid tumors. The distribution of samples among the semiquantitative score classes 0+–3+ is shown. Follicular adenoma (FA), $n=15$; follicular thyroid carcinoma (FTC), $n=15$; papillary thyroid carcinoma (PTC), $n=16$

hand, was significantly increased in the malignant tumors (PTC + FTC) when compared with adenomas ($P=0.021$). Comparisons within the PTC group according to histological subtype were not feasible due to small sample size. While true nuclear β-catenin positivity could not be verified in any of the tumors, immunostaining localized to the nuclei, probably representing intranuclear inclusion (I.P., expert in thyroid pathology, oral communication) was observed in 7/16 PTC cases examined for β-catenin but only 1/15 case of FTC.

Discussion

Papillary thyroid carcinoma is the most frequently occurring endocrine cancer and also the most common cancer of the thyroid, which most often metastasizes into the regional (cervical) lymph nodes [1–4]. In this present study, we report a strong expression of claudin-1 protein in papillary thyroid carcinomas and their regional lymph node metastases. Contrarily, weak or no expression of claudin-1 was detected in follicular thyroid cancers, follicular adenomas, and in the peritumoral non-malignant thyroid tissue. Hucz et al. demonstrated that claudin-1 gene may be used as a marker for PTC. They analyzed claudin-1 gene expression and found large differences between papillary thyroid cancer and normal thyroid tissues [25]. Fluge et al. investigated the gene expression of claudin-1 and claudin-16 in fresh-frozen samples of papillary thyroid carcinoma specimens, and found these genes to be upregulated in classic PTC [26]. Our recent observations, along with the previous data, encourage the inclusion of claudin-1 to the immunohistochemical panel used for the differential diagnosis of thyroid nodules, based on the high selectivity of claudin-1 immunostaining for PTC against both benign thyroid tissue (non-tumorous thyroid, FA) and FTC. Other, formerly established members of this panel such as galectin-3, cytokeratin-19, or Hector battifora mesothelial-1 (HBME-1) all possess sensitivity and specificity values comparable with, or even inferior to, those of claudin-1 when applied alone for the discrimination of PTC and FTC [29].

In a marked contrast with our results, Tzelepi et al. have found high claudin-1 protein expression in various thyroid tumors including FA, FTC, PTC, and PMC [24]. In our study, high claudin-1 expression was observed in PTC samples and their lymph node metastasis only, whereas the level of claudin-1 protein was virtually undetectable in FA and FTC samples. This discordance may be explained by the different methods of evaluation applied (semiquantitative evaluation based on the percentage of positive cells vs. quantitative digital morphometry), as well as by technical differences between specimens and their pretreatment (e.g. epitope retrieval protocol).

Numerous groups have investigated the expression of claudin-1 protein in various types of carcinomas [16, 20, 30]. Increased expression of claudin-1 was observed e.g. in human primary colon carcinomas and their metastases [17, 30], pancreatic ductal adenocarcinomas [18], gastric adenocarcinomas [31], cervical intraepithelial neoplasias and cervical invasive carcinomas [20]. Previously, claudin-1 protein overexpression was reported by our group in serous papillary endometrial carcinoma [15]. It has been described recently that claudin-1 is expressed in the majority of papillary renal cell carcinomas, suggesting a diagnostic value of this marker [16]. These latter observations raise the possibility of a relationship between papillary structure and high claudin-1 expression in malignant tumors of various organs.

Despite an increasing number of studies, the role of claudin-1 protein in cancer invasion and metastasis is controversial [31–33]. In serous papillary endometrial carcinoma, which is the aggressive type of endometrial adenocarcinoma, we detected elevated claudin-1 protein expression in our earlier work [15], and now we have demonstrated that strong claudin-1 protein expression is preserved in the regional lymph node metastases of PTC. These results are consistent with the hypothesis that high expression of claudin-1 is compatible with invasive and metastatic phenotype. It is of note, however, that the clinical behavior of FTC, here shown not to express claudin-1, is typically more aggressive, with a propensity to form multiple distant metastases.

Former studies suggested different molecular origins for the two main types of follicular-cell-derived thyroid carcinomas: distinct molecular pathways are supposed to

lead to the development of follicular and papillary thyroid carcinomas [2, 3, 34]. Differential expression of claudin-1 in the two tumor types supports this notion; nevertheless, the upstream signaling pathways influencing claudin-1 expression in thyroid tumors remain elusive. Claudin-1 is known to be regulated by the Wnt/β-catenin signaling pathway in colorectal tumorigenesis [27]. Aberrant activation of the canonical β-catenin/Wnt signaling pathway occurs in almost all colorectal cancers and contributes to their growth and invasion [35, 36]. Claudin-1 protein level is increased during colon carcinogenesis and particularly in metastatic colorectal cancer [17, 30]. The role of Wnt/β-catenin signaling pathway has been explored in the context of thyroid cancers as well [37]. Ishigaki et al. have found that β-catenin immunoreactivity was mainly localized in the plasma membrane in FA. Membrane localization of β-catenin was occasionally diminished but still common in FTC, while it was decreased or lost in many cases of PTC. They suggest that aberrant activation of Wnt/β-catenin signaling is strongly involved in thyroid tumorigenesis [37].

In our thyroid samples, no obvious correlation between claudin-1 protein expression and either the quantity or localization of β-catenin was established. Cell membrane β-catenin immunoreaction was seen in the immature follicles of non-malignant peritumoral thyroid tissue, especially in the actively regenerating cases of HT, but this β-catenin expression was not associated with the presence of claudin-1. Cytoplasmic β-catenin immunostaining was equally increased in both cancer types in comparison with the adenomas. A minor difference regarding β-catenin between the claudin-1-positive PTC and the claudin-1-negative follicular tumors (FTC and FA) was a decreased membrane reaction in the former, which is in line with previous observations [37]. Furthermore, β-catenin immunostaining in a localization probably corresponding to intranuclear inclusion was seen almost exclusively in PTC [38].

The reason for the differential expression of claudin-1 *within* the PTC group also remains unclear. While 8/27 PTCs were negative for claudin-1 immunostaining, claudin-1 expression of PTCs showed no obvious correlation with etiology, histological subtype, the presence of lymph node metastasis, or tumor size. However, PTCs are known to be heterogeneous regarding the molecular pathway involved in carcinogenesis: mutations of BRAF and RAS genes, as well as RET/PTC rearrangement are distinct and mutually exclusive genetic alterations that may independently lead to the development of PTC [39]. Whether the observed heterogeneity in claudin-1 expression can be explained in terms of the underlying genetic defect may be the subject of future investigations.

In summary, previous studies have described that claudin-1 gene expression is increased in PTC. We have found high expression of claudin-1 protein in PTC and its regional lymph node metastases, in contrast with FTC and FA that expressed claudin-1 at nearly undetectable levels. Therefore, claudin-1 immunohistochemistry may prove useful in the differential diagnosis of thyroid carcinomas with ambiguous histology. Our data support that the two main types of follicular-cell-derived thyroid carcinomas, namely, PTC and FTC, follow different pathways of molecular pathogenesis, and confirm our hypothesis that papillary morphology may be related to high claudin-1 expression in various tumors (noting that strong claudin-1 immunostaining was observed in the follicular variant of PTC, too). Furthermore, the preservation of claudin-1 in lymph node metastases of PTC implicates that high expression of claudin-1 protein is compatible with invasive and metastatic behavior of thyroid tumors. The role of the Wnt/β-catenin pathway as a key regulator of claudin-1 expression could not be established in our study.

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