

Expression of claudin 1, 4 and 7 in thyroid neoplasms

DİNÇ SÜREN¹, MUSTAFA YILDIRIM², ALPER SAYİNER¹, ARSENAL SEZGİN ALİKANOĞLU¹, İREM ATALAY¹,
UMUT RIZA GÜNDÜZ³, VİLDAN KAYA⁴, ŞEYDA GÜNDÜZ⁵, MEHMET TAHİR ORUÇ³ and CEM SEZER¹

¹Department of Pathology, University of Health Sciences, Antalya Education and Research Hospital,

Antalya 07050; ²Department of Medical Oncology, Medical Park Hospital, Gaziantep 27100;

³Department of General Surgery, University of Health Sciences, Antalya Education and Research Hospital,

Antalya 07050; ⁴Department of Radiation Oncology, Süleyman Demirel University, Isparta 32260;

⁵Department of Medical Oncology, University of Health Sciences, Antalya

Education and Research Hospital, Antalya 07050, Turkey

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Abstract. The distinction of thyroid carcinoma from benign thyroid neoplasm, as well as the subtyping of papillary carcinoma (PC) and follicular carcinoma (FC), may be performed histopathologically in the majority of cases. However, in certain cases, it is difficult to histopathologically distinguish between PC and FC, as well as follicular adenoma (FA), FC and the dominant nodule of multinodular goiter (MNG-DN). The present study aimed to determine the roles of the expression levels of the tight junction proteins claudin 1, 4 and 7 in the differential diagnosis of PC, FC, FA, MNG-DN, medullary carcinoma (MC) and anaplastic carcinoma (AC). The current study included 114 cases of histopathologically diagnosed thyroid neoplasia, which were distributed as follows: 29 FA, 18 MNG-DN, 47 PC, 10 FC, 5 MC and 5 AC. The expression levels of claudin 1, 4 and 7 were examined using immunohistochemical methods. The results revealed a significant difference in claudin 1 expression between malignant and benign thyroid neoplasms ($P<0.001$). Claudin 1 expression was not detected in any of the MNG-DN cases, and no significant difference in claudin 1 expression levels was identified between FA and FC ($P=0.653$). However, a statistically significant difference was observed between FC and PC ($P<0.001$). Claudin 4 expression did not differ between malignant and benign thyroid neoplasms, neither between MNG-DN, FA and FC, nor between FC and PC ($P=0.068$, $P=0.502$ and $P=0.481$, respectively). Claudin 7 exhibited positive immunohistochemical staining in 107 patients (94%); however, no significant difference in claudin 7 expression

levels was identified between malignant and benign thyroid neoplasms among MNG-DN, FA and FC (malignant, $P=0.135$; benign, $P=0.470$). Claudin 7 exhibited positive staining in all PC and FC cases. Therefore, claudin 1 expression levels may be useful in distinguishing cases of FC and PC with overlapping histopathological features, and provide a novel immunohistochemical marker for the subtyping of thyroid carcinoma.

Introduction

Papillary thyroid carcinoma is the most common type of thyroid cancer, comprising ~80% of all thyroid epithelial malignancies (1). Examination of hematoxylin and eosin-stained tissue sections is considered to be the gold standard for the differential diagnosis of thyroid neoplasms (1). However, morphological overlap is observed in certain cases, and the follicular variant of papillary carcinoma (FVPC) is common (1). A number of critical characteristics of this malignancy, including pale nuclei in papillary thyroid carcinoma, are subjectively interpreted, and interobserver variation among pathologists has been established (1). At present, an immunohistochemical panel to address these challenges has not yet been developed, with a limited contribution from the immunostaining markers galectin-3, Hector Battifora mesothelial 1 (HBME-1) and cytokeratin-19 in controversial follicular lesions (1,2). Therefore, additional immunohistochemical and molecular methods must be utilized.

Tight junctions (TJs) organize paracellular permeability and have a critical function in apical cell-cell adhesion and epithelial cell polarity (3). Numerous studies on the molecular architecture of TJs have demonstrated that claudin protein family members are important components of this structure (3). This family consists of 24 identified members, each with a distinct distribution pattern (3,4). Claudins 1, 4, 5, 7, 8, 11, 14 and 19 are responsible for cell impermeability claudins, and incremental increases in their expression levels strengthens the density between epithelial cells (5-8). Claudins 2, 10 and 16 are pore-forming claudins, and their increased expression levels reduce the density of epithelial cells (9); other claudins possess the ability to form paracellular anion/cation pores and water channels (3,7).

Correspondence to: Dr Umut Rıza Gündüz, Department of General Surgery, University of Health Sciences, Antalya Education and Research Hospital, Kazım Karabekir Street, Muratpaşa, Antalya 07050, Turkey
E-mail: utg1@yahoo.com

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Claudins 1, 4 and 7 are important members of the claudin protein family. It has been demonstrated that their expression levels are altered in numerous malignancies (10-30). Follicular cells of the thyroid gland are arranged in a single highly polarized layer and function as a barrier between the lumen of the follicle, where thyroglobulin and thyroid hormones are stored, and the extrafollicular space. Epithelial cell polarity and follicular space entrenchment are due to the presence of tight junctions (29). The present study examined the expression levels of claudin 1, 4 and 7 immunohistochemically, and aimed to determine the role of the expression of these TJ proteins in the differential diagnosis of papillary carcinoma (PC) and follicular carcinoma (FC), follicular adenoma (FA), dominant nodule of multinodular goiter (MNG-DN), medullary carcinoma (MC) and anaplastic carcinoma (AC). The aim of the current study was to identify the potential diagnostic role of these markers in the follicular morphological mimics.

Materials and methods

Selection of patients. This retrospective study included 122 cases of thyroid neoplasia that were histopathologically diagnosed using thyroidectomy tissue at the University of Health Sciences, Antalya Education and Research Hospital (Antalya, Turkey) between January 2010 and January 2014. The present study was performed using pathologically stained tissue samples, and diagnostic values of the claudins were evaluated. Therefore, the present study does not include any prognostic or follow-up data. The study was approved by the ethics committee of University of Health Sciences Antalya Education and Research Hospital (#2017/024). Due to technical reasons, 8 cases in which the immunohistochemical expression was not eligible for evaluation were excluded. As a result, 114 cases of thyroid neoplasia obtained from 90 female (79%) and 24 male (21%) patients were enrolled into the present study. The average age of patients was 44.50 ± 13.60 years. Informed consent to use the surgical specimens for scientific research was obtained from all the patients. The expression levels of claudin 1, 4 and 7 were examined in 29 FA, 18 MNG-DN, 47 PC, 10 FC, 5 MC and 5 AC cases using immunohistochemical methods.

Tissue preparation and immunohistochemical staining. Resection tissue samples were obtained following thyroidectomy, placed in 10% formaldehyde immediately following the procedure. Subsequently, pathologically sampled tumoral tissues were embedded in paraffin. Immunohistochemical staining was applied to resection tissue cross-sections containing nominal tumor samples that were evaluated using hematoxylin and eosin staining. Briefly, cross-sections of 4- μ m thickness were prepared for immunohistochemical staining by deparaffinization in an oven at 60°C for 2 h. Subsequently, tissue sections were immersed in xylene for 30 min, gradient ethanol for 30 min (70% ethanol for 10 min, 96% ethanol for 10 min, 100% ethanol for 10 min used sequentially; all steps were performed at room temperature) and washed with distilled water. Next, the tissue sections were heated in a 10% citrate buffer solution (#RE7113; Leica Microsystems, Inc., Milton Keynes, UK) in the microwave at 800 W for 15 min and then at 400 W for an additional 20 min. Tissue sections were allowed to cool at room temperature for 20 min following heating. Endogenous

peroxidase activity was blocked with 3% hydrogen peroxide for 10 min. The tissue sections were incubated with primary antibodies against claudin 1 (rabbit polyclonal; #ab15098; dilution, 1:200; Abcam, Cambridge, MA, USA), claudin 4 (rabbit polyclonal; #ab15104; dilution, 1:200; Abcam) and claudin 7 (rabbit polyclonal; #ab27487; dilution, 1:200; Abcam) for 60 min at 30°C, and then washed with PBS for 5 min at room temperature. The tissue sections were subsequently incubated with Ready to Use Biotinylated Goat-anti-rabbit Immunoglobulin secondary antibody (#BP-9100; undiluted; Vector Laboratories, Burlingame, CA, USA) for 20 min at 30°C, washed with PBS for 5 min and incubated with the Peroxidase Detection system Ready to Use conjugated antibody (#RE7110-K; Novocastra; Leica Microsystems, Inc.) for 20 min at room temperature. Tissue samples were then washed with PBS for 5 min, incubated with chromogenic 3,3'-diaminobenzidine (Leica Microsystems, Inc.) for 5 min, washed with tap water and counterstained with hematoxylin; all steps were performed at room temperature. The tissue samples were subsequently dehydrated in 100% ethanol, dried in an oven for 10 min at 60°C and mounted with Entellan® mounting medium (Merck Millipore, Darmstadt, Germany). The sections were visualized with a Nikon Eclipse Ci Light microscope (Nikon Corporation, Tokyo, Japan). Positive immunohistochemical staining of claudin 1, 4 and 7 in dermal appendages from skin biopsies were used as a positive control, whereas the primary antibodies were omitted for the negative controls.

Evaluation of immunohistochemically stained tissue sections. Claudin expression rates for the positive tumor cells in the tissue specimens were independently evaluated by two pathologists who were blinded to the patients' clinical features and previous pathological diagnosis. Vascular structures, fibroblasts, vessel endothelium, smooth-muscle cells of vessel walls, lymphoid tissue, neural structures and adipocytes within the cross-section exhibited no staining. The absence of claudin expression in these non-epithelial structures was therefore used as the negative internal control during immunohistochemical evaluation. In the case of claudin expression, the staining was membranous and accompanied by weak cytoplasmic staining. This cytoplasmic weak staining was seen only in cases which had strong membranous staining, therefore this cytoplasmic weak staining was considered as non-specific and thus ignored; only the membranous staining was evaluated. Claudin 1, 4 and 7 staining was assessed using a previously described scoring method (10,11). According to this method, cases exhibiting membranous Claudin 1, 4 and 7 staining in >5% of cells were considered positive. Claudin 1, 4 and 7 expression were scored as follows; 0, staining in <5% of the cells; 1, staining in 5-25% of the cells; 2, staining in 26-50% of the cells; 3, staining in >50% of the cells. Examples of immunohistochemical staining are presented in Fig. 1.

Statistical analysis. Statistical analyses were performed using SPSS version 15.0 for Windows (SPSS, Inc., Chicago, IL, USA). Descriptive analyses were presented as the mean \pm standard deviation. The scores of claudin 1, 4 and 7 expression level in benign and malignant neoplasms, as well as FA, MNG-DN, PC, papillary microcarcinoma (PC-M) and FC, were identified. Differences between the groups were analyzed using

Table I. Comparison of claudin 1, 4 and 7 expression levels in malignant and benign lesions.

Claudin	Benign (n=47)		Malignant (n=67)		P-value
	Negative (%)	Positive (%)	Negative (%)	Positive (%)	
Claudin 1	41 (87.2)	6 (12.8)	18 (26.9)	49 (73.1)	<0.001 ^a
Claudin 4	37 (78.7)	10 (21.3)	42 (62.7)	25 (37.3)	0.068
Claudin 7	1 (2.1)	46 (97.9)	6 (9.0)	61 (91.0)	0.135

^aP<0.05, indicating a statistically significant difference.

Table II. Comparison of claudin 1, 4 and 7 expression levels in PC and FC.

Claudin	FC (n=10)		PC (n=33)		P-value
	Negative (%)	Positive (%)	Negative (%)	Positive (%)	
Claudin 1	9 (90.0)	1 (10.0)	1 (3.0)	32 (97.0)	<0.001 ^a
Claudin 4	7 (70.0)	3 (30.0)	18 (54.5)	15 (45.5)	0.480
Claudin 7	0 (0.0)	10 (100.0)	0 (0.0)	33 (100.0)	1.000

^aP<0.05, indicating a statistically significant difference. PC, papillary carcinoma; FC, follicular carcinoma.

the χ^2 test. P<0.05 was considered to indicate a statistically significant difference.

Results

Histopathological distribution of patients with thyroid neoplasm. A total of 114 patients were enrolled in the present study, with benign thyroid neoplasm observed in 47 (41.0%) patients and malignant thyroid neoplasm identified in 67 (59.0%) patients. Benign thyroid neoplasms were present in 29 (62.0%) patients with FA and 18 (8.1%) patients with MNG-DN. A total of 47 (70.0%) patients with malignant thyroid neoplasms had PC and 14 (29.8%) PC cases were PC-M.

Claudin 1 expression levels in patients with thyroid neoplasms. Claudin 1 expression exhibited statistically significant differences between malignant and benign thyroid neoplasms (P<0.001; Table I). Claudin 1 expression was detected in 6 (12.8%) benign cases, all of which were FA, whereas no claudin 1 expression was observed in any of the MNG-DN cases. A total of 49 (73.1%) malignant cases exhibited positive claudin 1 expression. In 6/29 (20.7%) FA cases and 1/10 (10.0%) FC cases, positive claudin 1 expression was detected. No significant difference was identified in claudin 1 expression between FC and PC (P=0.653), whereas claudin 1 expression differed significantly between FC and PC (P<0.001). In 9/10 (90.0%) FC cases, claudin 1 expression was not detected. In 32/33 (97.0%) non-microcarcinoma PC cases, and in all 14 PC-M cases, claudin 1 expression was detected (Table II). No statistically significant differences were identified between claudin 1 expression in PC-M and in non-microcarcinoma PC (P=0.990). A total of 2/5 (40.0%) AC cases and none of the 5 MC cases exhibited claudin 1 expression.

Claudin 4 expression levels in patients with thyroid neoplasms. No significant differences in claudin 4 expression were observed between malignant and benign thyroid neoplasms (P=0.068; Table I). A total of 10/47 (21.3%) benign thyroid neoplasms exhibited claudin 4 expression, of which 3 cases were FA and the remaining were MNG-DN. A total of 25/67 (37.3%) malignant cases exhibited claudin 4 expression. In 3/29 (10.3%) FA cases, 7/18 (39.0%) MNG-DN cases and 3/10 (30.0%) FC cases, claudin 4 expression was positive. No significant differences in claudin 4 expression were identified among MNG-DN, FA and FC groups (P=0.502). A total of 15/33 (45.5%) PC cases exhibited claudin 4 expression, whilst no statistically significant differences in claudin 4 expression were observed between PC and FC (P=0.480; Table II). In addition, claudin 4 expression was detected in 7/14 (50.0%) PC-M cases, and no significant differences in claudin 4 expression were identified between PC-M and non-microcarcinoma PC cases (P=0.775). No cases of AC or MC exhibited claudin 4 expression.

Claudin 7 expression levels in patients with thyroid neoplasms. Claudin 7 expression was identified in 107 (94%) of the 114 analyzed cases. No significant differences in claudin 7 expression were observed between malignant and benign thyroid neoplasms (P=0.135; Table I). Negative claudin 7 expression was observed in 1/47 (2.1%) benign cases and 6/67 (9.0%) malignant cases (Table I). The single negative benign case was FA, and the 6 malignant cases were as follows: 1 PC-M, 1 AC and 4 MC. All 10 FC cases exhibited claudin 7 expression. No statistically significant differences in claudin 7 expression were identified among MNG-DN, FA and FC groups (P=0.470). FC and PC did not exhibit any statistically significant differences, and all cases in these two groups were positive for claudin 7 expression (Table II). No significant differences in claudin

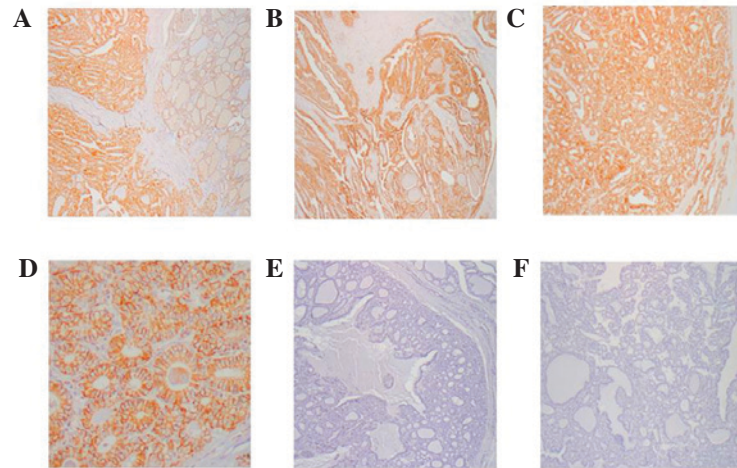


Figure 1. Immunohistochemical staining of claudin 1, 4 or 7 in six tumor sections. (A) Extensive claudin 1 positive staining in PC and negative immunostaining in adjacent normal tissues (magnification, x40). (B and C) Extensive claudin (B) 4 and (C) 7 positive staining in PC (magnification, x40). (D) Extensive claudin 1 positive staining in follicular carcinoma (magnification, x200). (E and F) Negative immunostaining for claudin (E) 1 and (F) 4 in follicular adenoma (magnification, x40). PC, papillary carcinoma.

7 expression were observed between PC-M and PC cases ($P=0.298$), whereas 1/5 (20%) AC cases and 4/5 (80%) MC cases exhibited claudin 7 expression.

Discussion

The diagnostic gold standard for the pathological evaluation of thyroid nodules is hematoxylin and eosin staining (1). However, morphological overlaps between MNG-DN, FA and FC, as well as between PC and FC, are common. In these cases, an objective consistent diagnosis based solely on morphological assessment is occasionally impossible (10). At present, no routine immunohistochemical panel is in use to overcome these morphological overlaps. Immunohistochemically, galectin-3, HBME-1 and cytokeratin-19 provide a limited contribution to the assessment of controversial neoplasms (1,2).

TJs are crucial intracellular joints in endothelial cells and the epithelium (3). There are two important functions that have been determined for TJs: Paracellular permeability regulation and the maintenance of cell polarization with window function (3,5,7,8). The functions of TJs that are associated with cancer-cell biology include epithelial paracellular permeability and the loss of cell polarization (12,13). Claudin overexpression or loss of expression varies depending on the type of cancer (3,14-16). In hepatocellular and renal cell carcinoma, claudin 4 and 5 expression ceases, whereas claudin 3 and 4 overexpression is detected in various types of cancer, including pancreatic ductal adenocarcinoma, and bladder, uterus, ovary and breast cancer (3,14-16). A low level of claudin 2 expression has been detected in breast and bladder carcinomas, whereas claudin 1 and 7 expression, which is not possible to detect in normal cervical squamous epithelium, is increased in cervical neoplasia (17). Previous studies have revealed that claudin 1 and 4 are overexpressed in nasopharyngeal carcinoma, while claudin 7 is overexpressed in pancreatic ductal adenocarcinoma (18,19).

The loss of claudin expression leads to the suppression of TJ functions and serves a role in carcinogenesis by inducing cancer cell proliferation, motility and invasiveness (8). A previous study

on nanofibrillated cellulose (NFC) cell culture demonstrated that claudin 1 expression levels were increased along with decreased apoptosis in NFC cell lines following fluorouracil treatment (20). Claudin 1 interacts with the TJ protein zonula occludens 1 and affects other signaling pathways, resulting in neoplastic transformation (21). In addition, a previous study demonstrated that increased levels of claudin 1 expression prevent NFC cell apoptosis (20). A possible underlying mechanism for the role of claudins in neoplastic transformation may occur via matrix metalloproteinases (MMPs). The upregulation of claudin 1 expression levels in oral squamous cell carcinoma enhances invasion via the activation of MMP-2 and MMP-1, and the overexpression of claudins 3 and 4 in ovarian surface epithelial cells promotes invasion by increasing MMP-2 activity (22,23). Claudins may also promote neoplastic transformation through their mixing ratios, as the barrier function of TJs is controlled by a specific combination of claudins (8). This hypothesis is concordant with the observation that upregulated claudin 2 decreases the tightness of TJ strands in Madin-Darby canine kidney cells, with resultant cell leakage (24). In invasive ductal carcinomas, as well as in head and neck and metastatic breast cancers, claudin 7 expression has been observed to be decreased (25-27), and lower expression levels of claudin 1, 4 and 7 have been detected in colorectal carcinoma (28).

Claudin expression levels in thyroid neoplasm have been examined in relatively few studies. Abd El Atti and Shash (11) examined claudin 1 expression in PC, hyperplastic nodule and FA, but not in FC, MNG-DN, MC or AC, identifying statistically significant positive expression of claudin 1 in PC cases, as compared with hyperplastic nodule and FA (11). Concordantly, the present study detected claudin 1 expression in all cases but one; however, claudin 1 expression in FA varied between Abd El Atti *et al* (11) and the present study, despite membranous staining in >5% of the neoplastic cells being considered positive in each study. Claudin 1 expression was identified in 75% of FA cases in the previous study (11), and in 20.7% of cases during the current study. It was hypothesized that variation in the antibody clones used for the immunohistochemical staining

may underlie these discrepancies between the study results. Tzelepi *et al* (29) investigated the expression levels of claudin 1, 4 and 7 in various thyroid neoplasms, identifying claudin 1 expression in 15% of FA cases. This result, as well as data from Hucz *et al* (30) on claudin 1 expression in FA, is concordant with the results of the present study. Tzelepi *et al* (29) also detected claudin 4 expression in 85% of FA cases, whereas this proportion was 10.3% in the current study. In addition, this previous study identified claudin 1, 4 and 7 expression to be 67, 80 and 33%, respectively, in the FC group (29), whereas proportions of 10, 30 and 100%, respectively, were observed in the current study. Although the cut-off values for positive expression were equal in the two studies, differences in the antibody clones used for immunohistochemistry may again be hypothesized to have produced the variation in the results of these studies. Due to this inconsistency in FA and FC cases, further studies and larger sample sizes are required. Tzelepi *et al* (29) detected similar claudin 1 expression trends in PC cases, with higher rates of claudin 4 expression (88%) and slightly lower rates of claudin 7 expression (73%) in PC cases, as compared with the current study. The authors also identified the expression rates of claudin 1, 4 and 7 to be 25, 25 and 13%, respectively, in 8 AC cases, whereas the current study identified these rates to be 40, 0 and 20%, respectively. Tzelepi *et al* (29) identified claudin 1, 4 and 7 expression in 12.5, 87.5 and 25% of 8 MC cases, respectively, whereas the present study observed these rates to be 0, 0 and 80%, respectively. The low number of AC and MC cases are considered to be a limitation of these two studies.

In conclusion, claudin 1 may be a useful immunohistochemical marker in histopathologically overlapping PC and FC cases, favoring a PC diagnosis, as well as aiding the subtyping of thyroid carcinoma. Further studies with larger sample sizes are required in order to clarify the diagnostic utility of claudin expression levels in differentiating between FA and FC.

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