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ORIGINAL ARTICLE

Compact buds with biphasic differentiation and calcitonin-expressing neuroendocrine cells—previously unrecognized structures of thyroglossal duct unveiled by immunohistochemistry

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Abstract

Immunophenotype of thyroglossal duct (TGD) cysts, including lining epithelium and thyroid remnants, is scarcely addressed in the literature. There is indirect evidence that C cells may be derived from progenitor cells of the midline thyroid primordium. This is supported by the recent concept of the endodermal origin of lateral thyroid anlagen and several case reports. We aimed to search for neuroendocrine cells in TGD cysts and to characterize immunophenotype of the thyroid follicles and epithelial lining of TGD. Out of 98 TGD cysts, 70% contained both cyst-lining epithelium and thyroid follicles, whereas 30% possessed only cyst-lining epithelium. Specimens eligible for immunohistochemistry ($n = 61$) were stained for thyroid-specific and neuroendocrine markers. Thyroid remnants were positive for thyroid transcription factor 1 (TTF-1) and other thyroid tissue-specific markers and negative for calcitonin. TGD epithelium showed strong p63 positivity. We found that respiratory epithelium in 9.8% of TGDs contained neuroendocrine cells positive for calcitonin, chromogranin A, and synaptophysin but negative for carcinoembryonic antigen. In 44.2% of the cases, we detected compact buds, microscopic structures appearing as nests of epithelial cells with a biphasic population of basal (p63+) and central (TTF-1+) cells. Thyroid remnants in TGD expressed full spectrum of thyroid-specific markers and contained no C cells. Instead, calcitonin-expressing neuroendocrine cells were found among the respiratory epithelium of TGD. These cells can be a potential source of neuroendocrine tumors mimicking medullary carcinoma in median anlage derivatives. We also discovered precursor compact buds with dual immunophenotype and proposed a concept of their morphogenesis.

Keywords Thyroglossal duct · Thyroglossal duct cyst · Immunohistochemistry · Calcitonin · Neuroendocrine cells

Introduction

Thyroglossal duct (TGD) cyst is the most common developmental anomaly of the thyroid gland, and the most common congenital neck mass due to the persistence of TGD remnants [1, 2]. During embryogenesis, these structures connect the foramen cecum (floor of the primordial pharynx) to the hyoid bone and gradually disappear by the 10th week of development [3, 4]. If involution fails, embryonic TGD remnants may persist and become clinically apparent.

Two-thirds of the TGD cyst cases are diagnosed before the age of 30 years, and up to 25% of the patients are diagnosed over 50 years old [1, 5, 6]. Sex ratio is close or equal to one [1, 5, 6]. Serial sections on autopsy identified TGD remnants in about 7% [7, 8]. Microscopically, TGD contains epithelial lined remnants and mural heterotopic thyroid tissue (30–70%) [6, 9]. Although clinicopathological features of TGD cysts have been well described in the literature [1, 5, 6, 9], their immunophenotype is scarcely addressed. Currently, only four small series have been reported [10–13]. It is known that cyst epithelia are variably positive for TTF-1 and consistently

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negative to thyroglobulin [10], whereas the thyroid follicles express both proteins, as expected [11, 12].

Theoretically, C cells should be absent in TGD and other median anlage derivatives since they are originated from the ultimobranchial body, according to the classical concept [3]. A limited series of TGD cysts evaluated by immunohistochemistry showed the absence of calcitonin-producing cells [11, 12]. However, there is some indirect evidence that the C cells may also be derived from progenitor cells of the midline thyroid primordium [14]. Several publications reported about detection of C cells in the thyroid gland of individuals with a complete phenotype of DiGeorge syndrome, a condition where the III–IV arches and the associated pharyngeal pouches fail to develop [15–17]. Furthermore, C cells [18] and medullary thyroid carcinoma (MTC) [19] were reported in the lingual thyroid. Another recent case report described solid cell nests, an ultimobranchial body remnant and a source of C cells, in TGD cyst in a neonate [20].

This growing body of unusual findings prompted the present study which addressed the potential presence of C cells in the median anlage remnants. In addition, we aimed to describe the clinicopathologic features of TGD cysts in our institution and characterize the immunophenotype of the epithelial lining and thyroid tissue of TGD.

Materials and methods

Study samples

A total of 105 cases were identified in the files from the Department of Pathology, King Chulalongkorn Memorial Hospital, Bangkok, Thailand (2005–2016). All of the archival slides were reviewed to confirm the diagnosis of TGD cyst and to evaluate basic histological features. Out of 105 cases initially reviewed, 7 cases were excluded from the study because 3 of them were diagnosed as epidermoid cysts and 4 as midline neck cysts without epithelial lining. None of the patients had a history of thyroid cancer either before or after Sistrunk procedure. Aside from the Sistrunk procedure, three patients underwent simultaneous thyroid lobectomy for follicular adenoma. These thyroid lobes were also enrolled into the study. This study was approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University (IRB No. 652/59).

Immunohistochemistry

All immunostaining was performed on whole slide sections of formalin-fixed paraffin-embedded tissue using the previously reported "two sections per slide" technique [21]. Sections 2 μm thick were positioned on positively charged slides (Platinum Pro, Matsunami, Japan), dewaxed in xylene, and rehydrated using graded alcohols. Immunostaining was performed using a Dako Autostainer Link 48 (Dako North America Inc., USA) or Ventana BenchMark system (Ventana Medical Systems, USA) automated immunostainers. Table 1 provides details about the primary antibodies.

Immunohistochemistry was performed only on the cases that had both thyroid remnants and cyst-lining epithelium but had no hyoid bone (to avoid the effect of decalcification process which deteriorates immunohistochemical staining [22]). All of the 61 eligible specimens were stained with antibodies for calcitonin, thyroglobulin, thyroid transcription factor 1 (TTF-1), and p63. For correlation purposes, 20 representative specimens were stained with paired-box gene 8 (PAX8), sodium/iodide symporter (NIS), thyrotropin receptor (TSHR), chromogranin A, synaptophysin, carcinoembryonic antigen (CEA), and Ki-67. Cases eligible for immunostaining were representative of the whole cohort after the baseline characteristics were matched.

Statistical analysis

Statistical analysis was performed using SPSS Statistics version 17.0 (SPSS IBM, USA). Mann-Whitney U test and Fisher's exact test were used to compare continuous and categorical variables, respectively. A two-sided p value of less than 0.05 was considered statistically significant.

Results

Among the 98 cases diagnosed with TGD cyst, 50 patients were males (51%) and 48 were females (49%). The mean age at presentation was 34.1 years (range 1–72 years). A bimodal age distribution was observed with 26 (26.5%) and 32 (32.7%) patients who belonged to the age groups 0–15 and 40–55, respectively. There was an obvious male preponderance among patients who were younger than 20 years (20 males to 10 females; 2:1) and a slight female predominance among those who were older than 20 years (30 males to 38 females; 1:1.3) ($p = 0.03$).

Microscopic study of TGD cysts

Sixty-nine out of 98 cases (70%) contained both cyst-lining epithelium and thyroid follicles, whereas 29 cases (30%) possessed only cyst-lining epithelium, mainly of the respiratory type. Only 7 cysts (7.1%) displayed exclusively squamous cell lining, whereas 21 cases (21.4%) contained both types of epithelium. Complete sloughing of lining epithelia was documented in 11 cases (11.2%); all of them nevertheless contained diagnostically significant thyroid remnants in the TGD wall. Seromucous glands with perihyoid location were

Table 1 Primary antibodies used in the study

Antibody	Vendor	Clone or ID	Platform	Pretreatment	Dilution	Evaluation	Positive control
Calcitonin	DAKO	A0576	DAKO	EDTA-based	RTU	Cytoplasmic granules	Medullary thyroid carcinoma
Thyroglobulin	DAKO	Clone DAK-Tg6	Ventana	EDTA-based	1:500	Cytoplasmic and colloidal	Benign thyroid
TTF-1	DAKO	Clone 8G7G3/1	DAKO	EDTA-based	RTU	Nuclear	Benign thyroid
p63	DAKO	Clone DAK-p63	DAKO	EDTA-based	RTU	Nuclear	Epidermis
PAX8	Cell Marque	Clone MRQ-50	DAKO	EDTA-based	1:200	Nuclear	Benign thyroid
NIS	Abcam	ab17795	Ventana	No retrieval	1:100	Membranous and cytoplasmic	Salivary gland
TSHR	Bio-Rad	Clone 4C1	Ventana	Citrate-based	1:1000	Membranous and cytoplasmic	Benign thyroid
Chromogranin A	DAKO	Clone DAK-A3	DAKO	EDTA-based	1:400	Cytoplasmic granules	Adrenal
Synaptophysin	DAKO	Clone DAK-SYNAP	DAKO	EDTA-based	RTU	Cytoplasmic granules	Adrenal
mCEA	Zytomed	Clone COL-1	DAKO	EDTA-based	RTU	Cytoplasmic granules	Colonic adenocarcinoma
Ki-67	DAKO	Clone MIB-1	DAKO	EDTA-based	RTU	Nuclear	Tonsil

RTU ready to use

identified in four cases (4.1%). Seventy-six TGD cysts (77.6%) showed some degree of chronic inflammation.

The thyroid tissue had all types of follicles (Fig. 1a). However, the predominant patterns were micro- (< 80 μm) and normofollicular with only rare examples having macrofollicular architecture (> 300 μm). Thyroid remnants were usually located less than 1 mm from the TGD lining and intimately associated with capillary tracks (Fig. 1b). The size of the thyroid tissue per slide ranged 0.5–15 mm in greatest dimension (mean 3.8 mm) with the total area occupying 0.5–50 mm^2 (mean 6.9 mm^2). Pediatric patients (< 15 years old) had a significantly smaller size of thyroid remnants compared to the adults, 1.5 mm^2 vs. 4.0 mm^2, respectively ($p < 0.001$).

Among the superficially located follicles (i.e., close to TGD lining) or intermixed with them, we frequently observed small solid structures resembling thyroid follicles but filled with mural cells instead of having an obvious lumen (Fig. 2a). We called these structures as "compact buds," which could probably explain the best their morphology and function (discussed below). In addition, there were relatively uncommon (16/98; 16%) but remarkable solid elongated epithelial ribbons located between the cyst lining and adjacent thyroid tissue (Suppl. Fig. 1a).

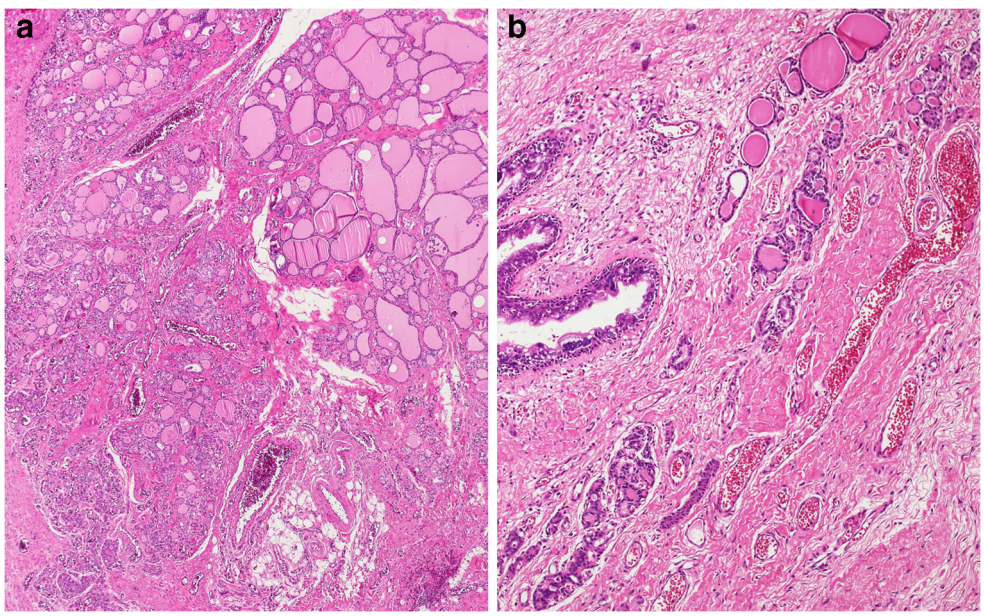
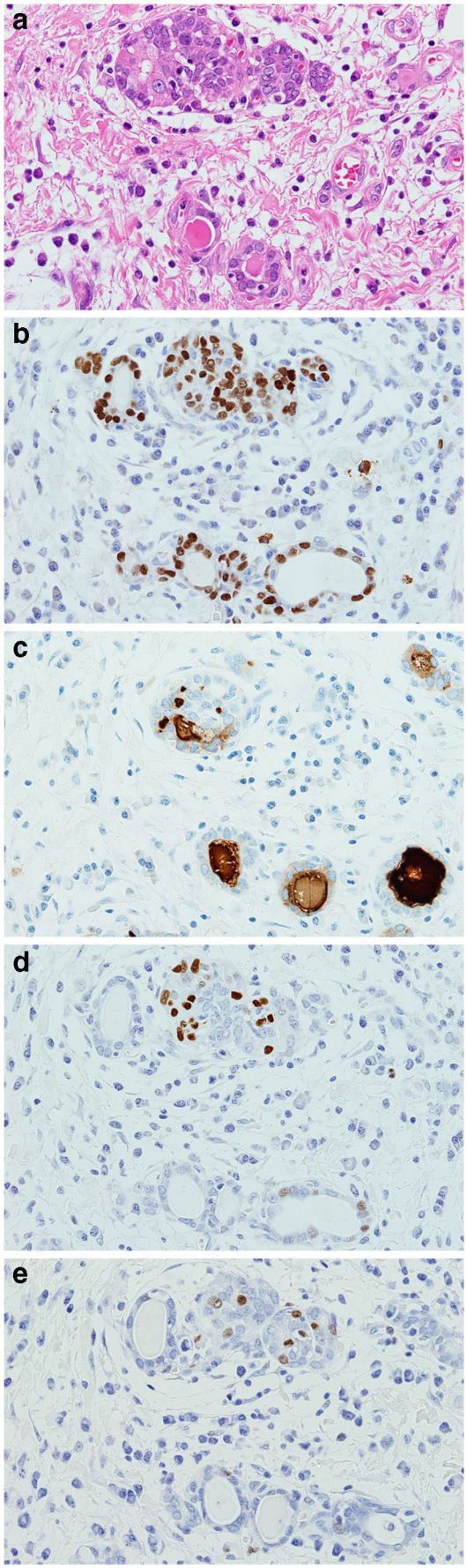


Fig. 1 Microscopy of thyroid follicles in TGD. **a** Thyroid tissue remnants of variable size, from macrofollicular (upper right) to normofollicular (upper left) and microfollicular-solid (lower left). **b** Band-like distribution of the thyroid follicles which are associated with capillary tracks. Hematoxylin and eosin × 40 (**a**) and × 100 (**b**)



◀ **Fig. 2** Immunophenotype of compact buds. **a** A multilayered/solid compact bud (top) compared to the thyroid follicles (bottom). **b** Most of the mural cells of the bud are positive for TTF-1. **c** There is a residual expression of thyroglobulin. **d** Cells at the periphery are p63-positive. **e** Many of the cells are Ki-67-positive. Hematoxylin and eosin (**a**) and immunohistochemistry (**b**–**e**) × 400; serial sections

Immunohistochemical study of the thyroid follicles in TGD

All thyroid follicles were strongly positive for TTF-1 and thyroglobulin. Immunostaining of the representative 20 cases confirmed that the follicular cells expressed other thyroid-specific markers, including PAX8, NIS, and TSHR. The latter two markers showed predominantly weak and variable membranous staining; the same pattern was also observed in the matching samples of the thyroid gland proper available from three patients after simultaneous lobectomy. Semiquantitative scoring of the immunoexpression (H-score, data not shown) found that there was no significant difference between the expression of five tested thyroid-specific markers in the follicular cells of TGD and main thyroid.

We could not detect any calcitonin-positive cells among the thyroid remnants of TGD. Extended panel with chromogranin A, synaptophysin, and CEA showed the same negative results.

Immunostaining of the compact buds revealed that these structures were composed of two cell populations—cells of the basal layer expressed p63, while the mural cells were positive for TTF-1 (Figs. 2b, d and 3a–b) and PAX8. In addition, the central areas frequently contained traces of thyroglobulin (Figs. 2c and 3c). Dual immunophenotype characterized by co-expression of TTF-1 and p63 was noted in some cells after matching serial sections. Cells of the compact buds demonstrated increased proliferative activity by Ki-67 immunostaining (up to 20%) (Fig. 2e). In our series, compact buds were found in 44.2% (27/61) of the cases. Clinical correlations showed no association with age but significant correlation with the male sex ($p = 0.02$).

Ribbon-like structures (appeared as solid tubes on cross section) between cyst lining and adjacent thyroid tissue were composed of p63-positive, variably TTF-1-positive, and thyroglobulin-negative cells, consistent with reserve epithelium (Fig. 3d–f, Suppl. Fig. 1). Closer to the surface, these solid tubes progressed to luminated tubes (Fig. 3g–i) and finally, matured to TGD lining epithelium (Fig. 3j–l). Positive immunostaining with AE1/AE3 showed that both compact buds and ribbon-like structures were of epithelial origin (data not shown).

Immunohistochemical study of TGD epithelium

All 61 cases showed strong expression of p63 in the lining epithelium of the TGD cyst. This expression was observed in all layers of squamous epithelium and in the basal layers of the

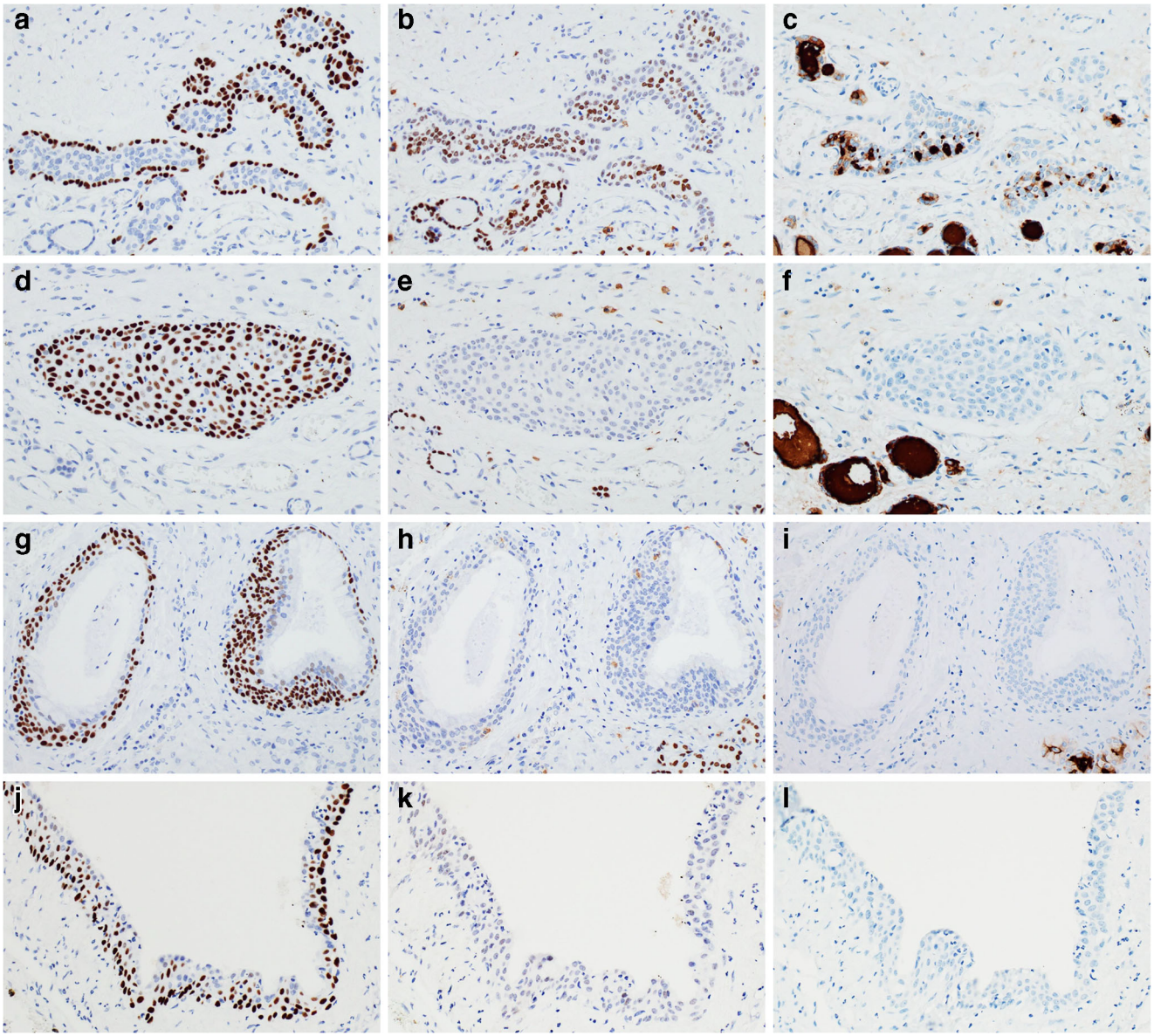


Fig. 3 Compact buds and associated structures. Typical compact buds with peripherally located p63-positive cells (**a**) and central cells expressing TTF-1 (**b**) and thyroglobulin (**c**). Note the intimate association with small thyroid follicles and capillaries. Cross section of the ribbon-like structure appears as a solid tube composed of p63-positive cells (**d**), which are negative for TTF-1 (**e**) and thyroglobulin (**f**). The solid tube further progresses to luminated tube (**g**–**i**) and mature TGD lining epithelium (**j**–**l**). Immunohistochemistry with monoclonal antibodies to p63 (**a**, **d**, **g**, **j**), TTF-1 (**b**, **e**, **h**, **k**), and thyroglobulin (**c**, **f**, **i**, **l**): × 400; serial sections

respiratory epithelium (Fig. 3j). Weak TTF-1 positivity was occasionally (10/61; 16.3%) detected in monolayered cuboid epithelium and pseudostratified respiratory epithelium (Fig. 3k). Thyroglobulin (Fig. 3l), PAX8, NIS, and TSHR were completely absent in the TGD lining.

We found that 9.8% (6/61) of the studied TGDs contained calcitonin-expressing cells (Fig. 4). These were basally situated (Fig. 4, inset) and sometimes extruded (Fig. 5a–c) single cells associated with respiratory but not squamous epithelium. Serial sections showed that these cells, which were unremarkable on hematoxylin and eosin staining, co-expressed chromogranin A (Fig. 5b) and synaptophysin (Fig. 5c) but were negative for CEA (Fig. 5d), which is different from immunophenotype of thyroid C cells (Fig. 5e–h).

Discussion

It was long believed that ultimobranchial bodies are invaded by neural crest cells which further differentiate into C cells after fusion with the median anlage [3, 4]. A completely new paradigm based on the murine model was proposed several years ago which suggested that ultimobranchial bodies and C cells are of endodermal but not of neural crest origin

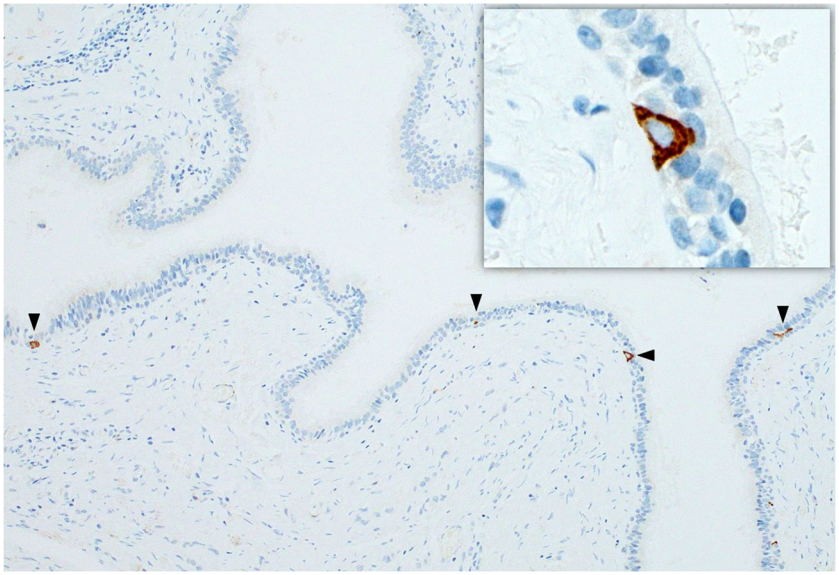


Fig. 4 Calcitonin-positive neuroendocrine cells of the TGD. A single area contains several neuroendocrine cells expressing calcitonin (*arrows*). These cells are basally located. Immunohistochemistry × 100 and × 600 (inset)

[23]. Authors of this concept further speculated that potential finding of C cells in the central anlage could bring the thyroid into line with other organs developed from endoderm such as the lung, gallbladder, and pancreas, all of which show neuroendocrine cells of endodermal origin [14]. The present study could not detect C cells or any calcitonin-positive cells among the thyroid remnants of TGD cysts. After combining our data with the previous studies [11, 12], it can be summarized that no C cells were found to date in 125 cases with thyroid remnants in TGD cysts.

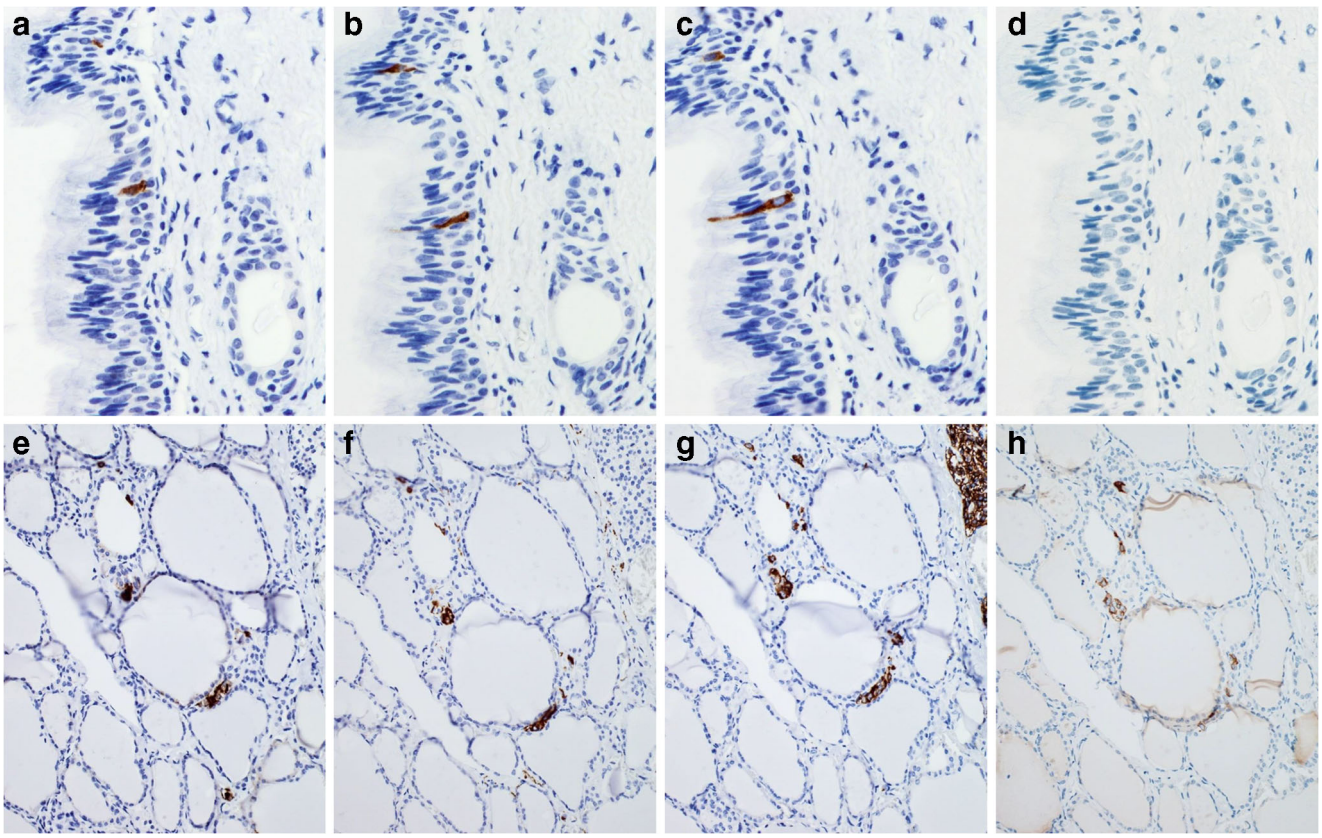


Fig. 5 Immunoprofile of neuroendocrine cells in TGD and C cells of the thyroid gland. Similar to C cells of the thyroid, neuroendocrine cells of TGD were positive for calcitonin (**a**, **e**), chromogranin A (**b**, **f**), and synaptophysin (**c**, **g**). However, TGD cells were negative for monoclonal CEA (**d**), unlike C cells (**h**). Immunohistochemistry × 600 (**a**–**d**) and × 200 (**e**–**h**); serial sections

This study found that TGDs contain a pool of compact buds with dual immunophenotype. The major population of centrally located cells is TTF-1/PAX8-positive and frequently shows focal luminal expression of the thyroglobulin, which is consistent with the immunoprofile of the thyroid follicular cells. Basal cells at the periphery are usually positive for p63 and negative for TTF-1. However, some cells maintain coexpression of TTF-1 and p63.

We believe that compact buds represent precursors for both thyroid follicles and epithelium of TGD lining. This assumption is based on the following findings. Compact buds had a predominant population of TTF-1-positive cells producing thyroglobulin and forming tiny lumens. Small-sized thyroid follicles were consistently found in the immediate vicinity to the compact buds (Figs. 2a–d and 3a, b). These adjacent follicles often contained TTF-1/p63-positive cells (Fig. 3a). Expression of p63 seems to indicate that the compact buds have progenitor cell capabilities. Not only that, but the compact buds have unusually high proliferative activity in the mural and peripheral cells. Furthermore, frequent p63 expression in the epithelial ribbons located superficially to the compact buds and further progressing to mature lining epithelium of TGD was compatible with immunophenotype of reserve respiratory epithelium [24]. It should be noted that p63 positivity of the basal cells in the compact buds was not associated with chronic inflammatory infiltrate. This indicated that the compact buds were not originated from reactive squamous metaplasia of thyroid follicular epithelium. Conversely, the inflammation in the cyst wall was accompanied by the foci of squamous TGD epithelium, which showed diffuse p63 expression. Nevertheless, a possibility of involution from TGD epithelium to the ribbons and further to the compact buds should not be discounted. The whole concept of the compact buds and their evolution is schematically illustrated in Fig. 6.

Compact buds were identified in 44.2% of the studied cases; however, the actual prevalence is definitely higher because we studied only a limited number of the specimens (two to three per case) instead of the whole length of TGD. Solid cell nests of the thyroid are reminiscent of compact buds in terms of morphology (small solid nests and islets), biphasic cell population, and overlap of immunophenotype (p63 and TTF-1). In addition, solid cell nests are supposed to play a stem cell role in the adult thyroid gland [25, 26], which is consistent with our view on the function of the compact buds in TGD. However, it is intuitively obvious that solid cell nests and compact buds are different. For instance, compact buds contain thyroglobulin-producing PAX8-positive cells and microlumens with colloid but not C cells. Thyroglobulin-expressing cells are not found in solid cell nests, while C cells are their essential components reflecting the origin from the ultimobranchial body [26, 27]. In addition, compact buds turned to be negative after immunostaining with GATA-3, a recently recognized marker of solid cell nests (data not shown) [27].

Another finding, which has not been previously reported, is the presence of calcitonin-positive cells in the TGD lining. A neuroendocrine phenotype of these cells was confirmed with chromogranin A and synaptophysin. They were found in approximately 10% of the TGD cysts; however, actual occurrence is higher since we did not evaluate the whole length of TGD. These cells were scattered only among respiratory epithelium and were negative for monoclonal CEA, which is different from thyroid C cells. It is believed that monoclonal CEA is the best

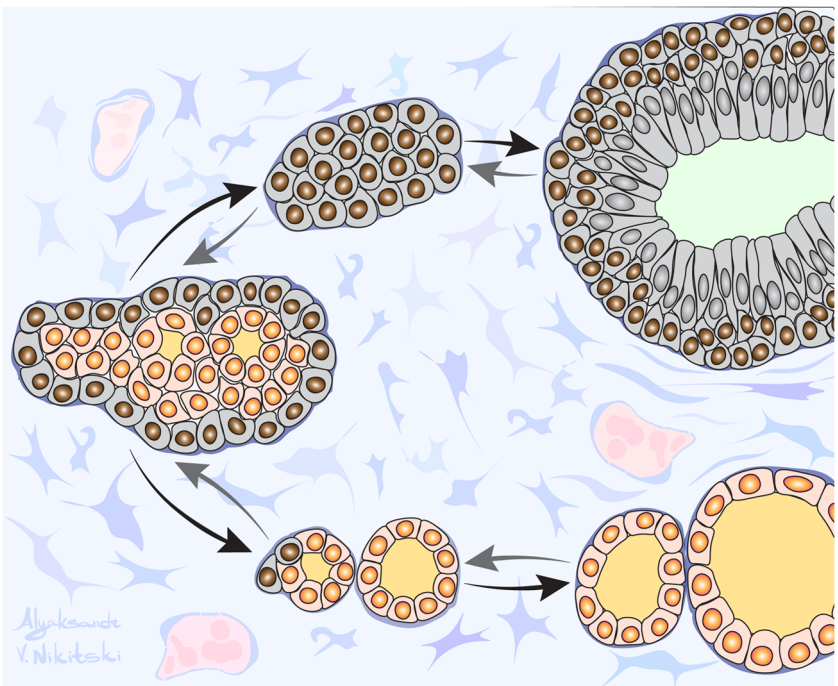


Fig. 6 A concept of compact buds and their evolution. Expression of TTF-1 and p63 is shown in orange and dark brown nuclear staining, respectively. Intrafollicular thyroglobulin is indicated in pale yellow color

biomarker for detecting MTC because up to 5% of these cancers are calcitonin-negative on immunostaining [28].

Calcitonin-positive neuroendocrine cells of the TGD lining can be a potential source of neuroendocrine tumors erroneously interpreted as MTC developed in median anlage derivatives, such as TGD cyst, lingual thyroid, and the pyramidal lobe of the thyroid gland. We are aware of one such case of "MTC in TGD cyst" presented recently (Z. Mousavi, Mashad University of Medical Sciences, Iran; https://goo.gl/aqw9Rd). Another example is a case of MTC in lingual thyroid [19]. Both of these tumors were reported as positive for calcitonin; however, CEA immunostaining was not performed. Considering our findings, these two exceedingly rare cases could possibly be calcitonin-positive neuroendocrine tumors originated from the TGD lining rather than MTC. Calcitonin-positive neuroendocrine tumors are rare but have been described in foregut derivatives, mainly of pancreatic and respiratory origin [29]. It should be noted that calcitonin expression is not specific to C cells, as calcitonin expression can be identified in various neuroendocrine cells originating from head and neck and mediastinal organs as well as in other sites (e.g., pancreas).

In this study, immunohistochemical staining with a set of thyroid-specific markers found that thyroid remnants in TGD expressed the full phenotype of follicular cells. This was further evidenced by the similar intensity of staining of these markers in the thyroid gland of the matching cases. We believe that thyroid follicles in TGD cyst wall are fully functional, which was argued previously based on imaging findings [30, 31]. It is difficult to distinguish the small volume fragments of the thyroid tissue which is located in close vicinity to the main thyroid gland by routine whole body scan. SPECT technology can adequately assist in such a scenario, especially in patients undergoing total thyroidectomy. Recently, two independent SPECT/CT studies confirmed the presence of radioactive iodine-avid thyroid tissue in TGD in almost 50% of thyroidectomized patients after TSH stimulation [32, 33]. In addition, gene expression studies of the cervical and lingual ectopic thyroid tissue found that the thyroid-specific transcription factors and related genes were expressed at the same level as in the normal thyroid [34, 35], which is well concordant with our immunohistochemical findings.

This was a discovery study which would need further validation in an independent cohort. As pointed out above, compact buds can be recognized by their morphology on routine hematoxylin and eosin staining, but the best way to detect them is by p63/TTF-1 immunohistochemistry which highlights the biphasic differentiation with spatial patterns (basal vs. central). Thorough sampling of TGD cyst may reveal additional evolution steps of the compact buds. In addition, it would be of particular interest to study these structures in fetal tissues which were not accessible in our current project. Extended immunohistochemical panel with stem cell markers could address the potential implication of our findings for regenerative applications.

In conclusion, our findings suggest that thyroid follicles in TGD are functional, which is also supported by the recent SPECT studies. It was confirmed that these thyroid remnants contain no parafollicular C cells. Instead, we found calcitonin-expressing neuroendocrine cells in the TGD cyst-lining respiratory epithelium, which can be a potential source of neuroendocrine tumors in median anlage derivatives. Finally, we described previously unrecognized microscopic structures, such as compact buds with dual immunophenotype and epithelial ribbons and proposed a concept of their morphogenesis.

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Authors' contributions S.K. evaluated samples, analyzed data, and wrote the manuscript.

A.B. conceived and designed the study, evaluated samples, analyzed data, edited the manuscript, and supervised the project.

All authors reviewed the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments. This study was approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University (IRB No. 652/59).

Informed consent Informed consent was obtained from all individual participants included in the study.

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