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Original Article

New Neuroendocrine Markers PITX2, PHOX2B, and HAND2 Do Not Offer Diagnostic Utility in Fine-needle Aspiration Biopsies of Primary and Secondary Medullary Thyroid Carcinomas: A Retrospective Multi-institutional Study

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Abstract

Background and objectives: Medullary thyroid carcinoma (MTC) is a neuroendocrine malignancy arising from parafollicular C-cells with known variations in cytomorphologic and immunophenotypic features. New neuroendocrine markers pituitary homeobox 2 (PITX2), paired-like homeobox 2B (PHOX2B), and heart and neural crest derivatives expressed 2 (HAND2) have recently been introduced, but studies using these markers in MTC are limited. The aim of this study was to evaluate the expression and potential diagnostic utility of PITX2, PHOX2B, and HAND2 in primary and secondary MTCs and to compare their expression with chromogranin A, synaptophysin, insulinoma-associated protein 1 (INSM1), and calcitonin. ***Methods:*** A total of 34 histologically confirmed cases of MTC with available cell blocks were included. Sixteen MTC samples were fine-needle aspirates from primary thyroid lesions, and eighteen were from secondary metastatic lesions. Twelve samples from thyroid carcinomas of follicular origin were included as controls. ***Results:*** PITX2 positivity was observed in 17 (50.0%) MTC samples and in 4 (33.3%) control samples (P = 0.502). PITX2 positivity was found in 43.8% of primary thyroid MTC lesions and in 55.6% of secondary MTC lesions (P = 0.366). Co-expression of PITX2 with chromogranin A, synaptophysin, INSM1, and calcitonin was observed. PHOX2B and HAND2 were negative in all MTC and control samples. ***Conclusions:*** There were no significant differences in PITX2 expression between primary and secondary MTC samples. PITX2 did not show reliable utility in distinguishing MTC from thyroid carcinomas of follicular origin. PHOX2B and HAND2 were negative in all samples. These results suggest that these new markers do not offer diagnostic value for MTC as stand-alone markers or as additions to the diagnostic workup panel.

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Introduction

Medullary thyroid carcinoma (MTC) is a rare type of malignant neuroendocrine tumor (NET) derived from the calcitonin-secreting parafollicular cells, also known as C-cells. MTC is known to have a diverse phenotype, sometimes mimicking other thyroid neoplasms such as follicular thyroid carcinoma (FTC), papillary thyroid carcinoma (PTC), and anaplastic thyroid carcinoma (ATC).[1–4] Due to its rarity, variable morphological features, and aggressive nature, it is important to have reliable tools for an accurate and rapid diagnostic workup.

The first finding before a final diagnosis of MTC is often a thyroid nodule, leading to ultrasound imaging and fine-needle aspiration (FNA) biopsy. Blood tests for carcinoembryonic antigen and calcitonin are usually performed if morphology is suspicious for MTC.[5] Higher levels of these markers can indicate more aggressive disease, although calcitonin-negative MTCs also occur.[6–9] Previous reviews have found that

Keywords: Fine-needle aspiration; FNA; Pituitary homeobox 2; PITX2; Paired-like homeobox 2B; PHOX2B; Heart and neural crest derivatives expressed 2; HAND2; Immunohistochemistry; Medullary thyroid carcinoma; Thyroid cancer; Cell block.

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up to 37% of serum calcitonin-negative MTCs are also calcitonin-negative on immunohistochemical staining.[8] Immunohistochemical calcitonin negativity has been observed in both pre-operative cytological and post-operative histological specimens.[8]

Immunohistochemical stains for neuroendocrine differentiation are commonly used in diagnostic workups, and they play an indispensable role in the diagnosis of MTC and other NETs. Most MTCs are positive for commonly used neuroendocrine markers such as synaptophysin (Syn) and chromogranin A (CgA).[4,9–11] Syn is often used to distinguish MTC from its follicular-origin mimickers, but recent studies have shown that 80% of FTCs, 78.6% of PTCs, and 45.5% of follicular thyroid adenomas express Syn focally, making diagnosis more challenging.[8,9] There are also other entities, such as thyroid paragangliomas (both primary and those arising from the laryngeal paraganglia) and metastases of pulmonary or renal cell carcinomas to the thyroid, which can express Syn, and the former may also morphologically resemble MTC.[11–18] In addition, esophageal adenocarcinomas, which can sometimes spread to the thyroid gland, have shown positivity for Syn (5.7%), CgA (20.7%), or both (1.4%), with patchy but also focally strong cytoplasmic CgA staining of scattered tumor cells in recent studies.[10,19]

CgA has the highest specificity for detecting neuroendocrine differentiation, but it is important to note that its expression can be only focal, weak, or even absent, depending on tumor differentiation grade, and it may also be expressed in non-neuroendocrine cells.[20–22] In MTC, the intensity of CgA varies widely and may appear weak and uneven in histological preparations; thus, visualization of CgA in cytological samples is even more challenging.[22–24] Furthermore, CgA can be negative in some MTCs, and previous studies have observed CgA negativity in approximately 36% of histological specimens.[6]

Since the expression of immunohistochemical markers in MTC can vary greatly, it is important to study new potential markers to be added to diagnostic staining protocols. Newly emerging neuroendocrine markers include transcription factors such as pituitary homeobox 2 (PITX2), paired-like homeobox 2B (PHOX2B), and heart and neural crest derivatives expressed 2 (HAND2). With limited literature available on these markers, their possible role in the diagnosis of MTC is not yet fully determined. In recent studies, insulinoma-associated protein 1 (INSM1) has proven to have highly valuable utility in the diagnostic workup of neuroendocrine neoplasms, including MTC.[24–26]

PITX2 plays a role in cell cycle transition from the G1 to S phase via upregulation of cyclin D1 and cyclin D2 and dephosphorylation of the tumor suppressor gene retinoblastoma.[27,28] PITX2 is abundantly expressed in midgut-derived NETs, with a sensitivity of 96.7% and a specificity of 100% in differentiating them from NETs of other origins. In recent studies, PITX2 has also shown 14.9% positivity in neuroendocrine carcinomas (NECs) and 42.9% positivity in MTCs. Nevertheless, the number of MTC cases in previous studies has been small.[29] Expression of PITX2 and one of its targets, cyclin A1, has been observed in histological tissue samples of PTCs, FTCs, and ATCs but not in MTCs, although PITX2 expression in MTC has been detected in cell culture systems.[27,28] Knocking down PITX2 has been shown to decrease thyroid tumorigenesis, mainly in follicular cell-derived cancers but also affecting MTC cell lines.[28] Overall, knowledge of PITX2 expression in MTC, especially from larger cohorts and cytological samples, remains limited.

PHOX2B is a gene that encodes a DNA-associated nuclear protein belonging to the paired homeobox family and acts as a transcription factor regulating the differentiation of specific types of neurons.[30,31] PHOX2B has been used in the diagnosis of some NETs, especially neuroblastomas, pheochromocytomas, and paragangliomas.[32–34] In recent studies, PHOX2B has shown a sensitivity of over 97% and a specificity of 89% in distinguishing paragangliomas from well-differentiated NETs, NECs, and olfactory neuroblastomas, while showing no positivity in MTCs.[32,34] However, the number of MTC cases in these earlier studies has remained relatively small, leaving the expression of PHOX2B in MTC specimens unclear and requiring further investigation.

HAND2 staining is useful for detecting (especially parasympathetic) paragangliomas, with a reported sensitivity of 98.2% and specificity of 91.7%, while other neuroendocrine neoplasms, including NECs, have been shown to be negative or only rarely positive (4.2%).[35] HAND2 has the potential to mediate the expression of estrogen receptors and affect the secretion of calcitonin and its family member calcitonin gene-related peptide, a neurotransmitter shown to inhibit immunoreactivity in the MTC microenvironment.[36–40] The indirect involvement of HAND2 in MTC tumorigenesis requires further investigation, and data on HAND2 expression in MTC remain limited, especially in cytological materials and larger cohorts.

Knowledge of the expression of PITX2, PHOX2B, and HAND2 in MTC and its cytological mimickers is thus limited. The aims of the present multi-institutional study were to evaluate all three neuroendocrine markers in distinguishing MTC from other thyroid carcinomas of follicular cell origin and to compare their expression in samples from primary thyroid lesions and secondary lesions, mainly cervical lymph node metastases. We also compared PITX2, PHOX2B, and HAND2 expression with established neuroendocrine markers, namely CgA, Syn, INSM1, and calcitonin.

Materials and methods

Sample selection and study cohort

A retrospective search across five institutions was performed (Fimlab Laboratories, Tampere, Finland (search period 2000–2018); Johns Hopkins Hospital, Baltimore, USA (search period 2006–2018); Loyola University Hospital, Chicago, USA (search period 2009–2017); Catholic University Hospital, Rome, Italy (search period 2000–2018); and Seinäjoki Central Hospital, Seinäjoki, Finland (search period 2000–2018)). All consecutive MTC cases with histologically confirmed diagnoses and available cell blocks from FNAs were included. The final series consisted of a total of 34 FNA-derived cell blocks from MTCs, including 16 samples from primary thyroid lesions and 18 from secondary lesions (17 metastatic lymph nodes and one liver metastasis). The control group comprised 12 cases with available cell blocks made from FNA samples of thyroid neoplasms of follicular origin, including five cases of FTC, five cases of PTC, and two cases of ATC. All control samples were from primary tumors and were collected from the Fimlab Laboratories' archive. A flowchart of cases included and excluded from staining is presented in Figure 1.

Case processing and cell block procedures

Cytological material was collected using standardized FNA protocols. All cell blocks were prepared in accredited laboratories using standardized procedures. The cell blocks were made from fixed material or needle rinse material using Shandon Cell Block (Thermo Fisher Scientific, Waltham, MA), plasma-thrombin, Cellient (Hologic Corporation, Mar-

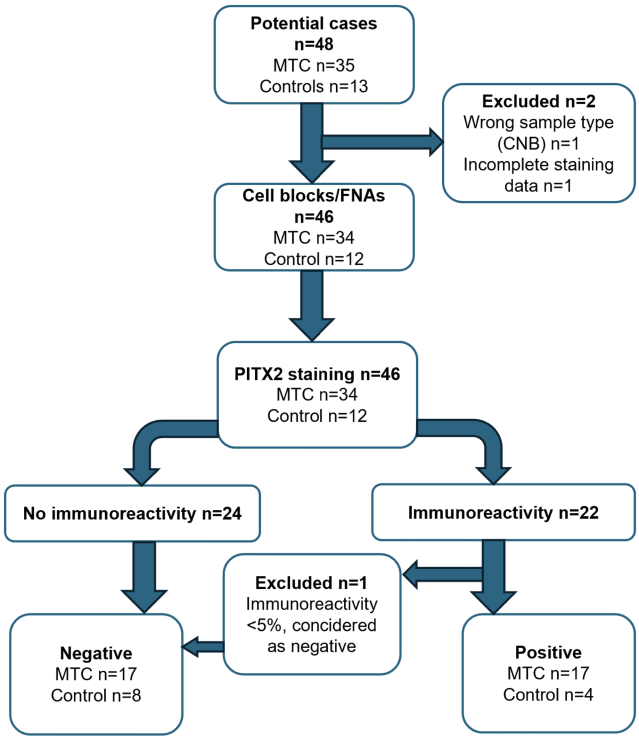

Fig. 1. The flowchart shows the cases included and excluded from pituitary homeobox 2 (PITX2) staining and their distribution into PITX2-positive and PITX2-negative cases. CNB, core needle biopsy, FNA, fine-needle aspiration; MTC, medullary thyroid carcinoma.

lborough, MA), or in-house methods, based on the routine practice of each laboratory.[41,42]

Immunohistochemistry

All immunohistochemical stainings for PITX2, PHOX2B, and HAND2 were performed on 4-µm-thick deparaffinized sections from formalin-fixed, paraffin-embedded cell blocks at the Tampere University Laboratory Centre. Antibodies were validated by manufacturers and verified at the Tampere University Laboratory Centre before implementation. Verification was performed according to the requirements of the SFS-EN ISO/IEC 15189:2022 standard. Staining was performed using a Ventana Benchmark GX autostainer (Ventana Medical Systems, Tucson, AZ) with the DAB IHC Detection Kit. Dilutions and antibody clones were as follows: PITX2, dilution 1:2,000, clone 2G6 (Novus Biologicals, Denver, CO); PHOX2B, dilution 1:200, clone EPR14423 (Abcam, Cambridge, UK); HAND2, dilution 1:1,000, clone EPR19451 (Santa Cruz Biotechnology Inc., Dallas, TX); INSM1, dilution 1:200, clone A8 (Santa Cruz Biotechnology Inc., Dallas, TX); CgA, dilution 1:200, clone LK2H10 (Ventana Roche, Ventana Medical Systems, Tucson, AZ); Syn, dilution 1:400, clone MRQ-40 (Ventana Roche, Ventana Medical Systems, Tucson, AZ).

All runs included both positive and negative controls on the same slide. Placental villi were used as positive tissue controls for PITX2, and adrenal medulla was used as a positive control for PHOX2B and HAND2. Based on previous studies, samples were classified as positive when 5% or more of tumor cells showed nuclear immunoreactivity.[3,22,25] INSM1 immunopositivity data were obtained from a previous study on INSM1 expression in MTC FNAs.[25] Calcitonin staining data were obtained from previous records and from the INSM1 study.[25] Detailed immunohistochemical staining protocols are provided in Supplementary Table 1.

Table 1. Patient characteristics

	All, n = 46	MTC, n = 34	Control, n = 12
Age (years)			
Mean	56.0	52.9	65.6
Median	59.0	54.0	69.0
Range	14–86	14–76	41–86
Sex	n (%)	n (%)	n (%)
Male	17 (37.0%)	11 (32.4%)	6 (50.0%)
Female	29 (63.0%)	23 (67.6%)	6 (50.0%)

MTC, medullary thyroid carcinoma.

Microscopic analysis

All MTC and control samples were reviewed by an experienced cytopathologist. In all samples, the percentage of positive tumor nuclei among all tumor nuclei was assessed based on blind evaluations by two reviewers using a light microscope. In cases of discrepancy, consensus was reached using a multiheaded microscope. All staining results were compared with positive controls. Staining intensity was categorized into four levels: strong (3), moderate (2), weak (1), and negative (0). No staining or staining detected in fewer than 5% of cells was graded as negative (0).

Statistical analysis

Statistical analysis was performed using SPSS for Windows (version 22.0; SPSS, IBM, Armonk, NY, USA). The chi-square test and Fisher's exact test were used to analyze the positivity of PHOX2B, HAND2, and PITX2 in MTC cases, comparing primary and secondary lesions and comparing MTC cases with other tumor types. *P*-values were calculated using one- and two-sided tests on 2×2 tables. *P*-values ≤ 0.05 were considered statistically significant.

Results

Patient characteristics

The male-to-female ratio (M:F) of all patients was 17:29. The average age was 56 years and the median age was 59 years (age range 14–86 years). In the MTC group, the M:F ratio was 11:23, the average age was 52.9 years, and the median age was 54.0 years (age range 14–76 years). In the control group, the M:F ratio was 1:1, the average age was 65.6 years, and the median age was 69 years (age range 41–86 years). Patient characteristics are summarized in Table 1.

Expression of PITX2 in primary and secondary MTC FNAs

PITX2 nuclear staining with a granular pattern in primary and secondary MTC samples is shown in Figure 2.

Seventeen (50.0%) MTC samples showed nuclear immunoreactivity for PITX2, including seven (41.2%) primary tumor samples and ten (58.8%) samples from metastatic lesions, of which nine were from lymph node MTC metastases and one was a liver metastasis. Seven (43.8%) out of all 16 primary samples showed positivity for PITX2. In the secondary sample group, ten (55.6%) out of 18 secondary samples showed positivity for PITX2. Positivity for PITX2 was more

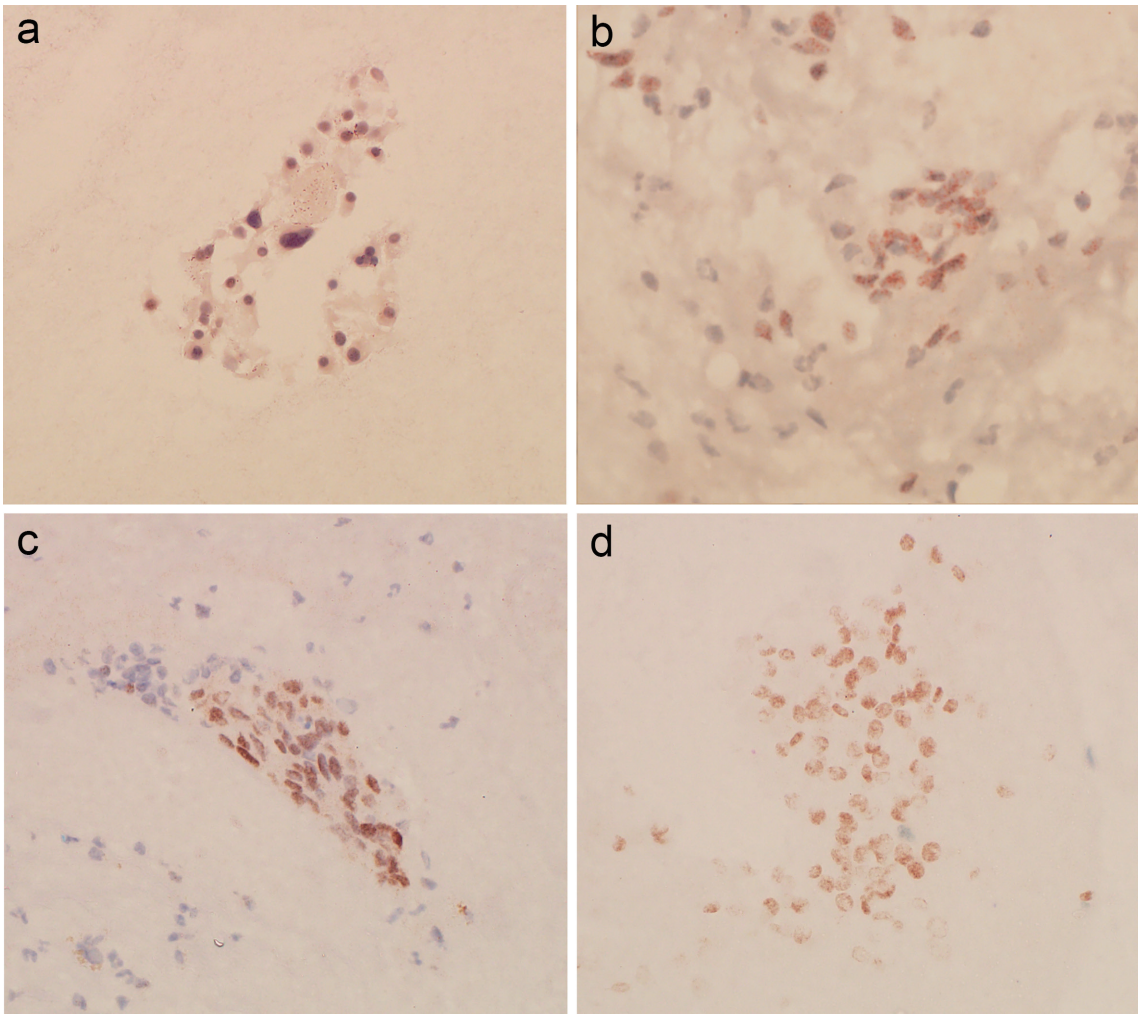

Fig. 2. Pituitary homeobox 2 (PITX2) immunostaining results in medullary thyroid carcinoma (MTC) cytological samples. Nuclear expression with variable intensity of PITX2 in four (a, b, c, d) MTC samples. (a) Strong staining intensity of slightly cohesive tumor cells with predominantly round-shaped nuclei in primary MTC of the right thyroid lobe. Overall positivity for PITX2 in this sample was 10% (original magnification 400×). (b) Moderate staining intensity of tumor cells with pleomorphic nuclei in primary MTC of the right thyroid lobe. Overall positivity for PITX2 in this sample was 50% (original magnification 600×). (c) Mainly strong staining intensity of a cell cluster with spindle-shaped and polygonal nuclei in metastatic MTC to the liver. Overall positivity for PITX2 in this sample was 60% (original magnification 400×). (d) Patchy, weak to moderate nuclear immunoreactivity in a slightly cohesive group of tumor cells with round nuclei in metastatic MTC of a cervical lymph node. Overall positivity for PITX2 in this sample was 90% (original magnification 400×).

common in secondary MTC samples; however, the difference in positivity rate between primary and secondary MTC samples was not statistically significant ($P = 0.366$).

Nevertheless, nuclear immunoreactivity of PITX2 was also observed in four out of 12 (33.3%) controls. Positivity was found in 2/5 (40.0%) PTCs, 1/5 (20.0%) FTCs, and 1/2 (50.0%) ATCs. Immunoreactivity of PITX2 in various tumor entities is shown in Tables 2 and 3. Overall, PITX2 positivity was 50%, and there was no statistically significant difference in the positive rate of PITX2 between MTC samples (50.0%) and control samples from follicular-origin thyroid carcinomas (33.3%) ($P = 0.502$).

Percentage of PITX2-positive tumor cells in primary and secondary MTC FNAs

The average percentage of PITX2-positive tumor cells in all

Table 2. Staining intensity of PITX2 in primary and secondary MTC FNAs

	All (%)	Negative (%)	Weak staining (%)	Moderate staining (%)	Strong staining (%)
MTC	34 (100%)	17 (50.0%)	7 (20.6%)	6 (17.6%)	4 (11.8%)
Primary	16 (100%)	9 (56.3%)	2 (12.5%)	3 (18.8%)	2 (12.5%)
Secondary	18 (100%)	8 (44.4%)	4 (22.2%)	4 (22.2%)	2 (11.1%)

Percentages may not sum to 100% due to rounding. FNA, fine-needle aspiration; MTC, medullary thyroid carcinoma; PITX2, pituitary homeobox 2.

Table 3. PITX2-positive samples grouped according to the percentage (%) of nuclear positivity, tumor type, and site

Diagnosis	Total (n)	Overall PITX2 positivity (n, %)	Positive ranges of PITX2 in tumor cells (%)					
			81–100%	41–80%	21–40%	11–20%	5–10%	0%
MTC	34	17 (50.0 %)	3 (8.8 %)	4 (11.8%)	1 (2.9%)	1 (2.9%)	8 (23.5%)	17(50 %)
FTC	5	1 (20 %)	0 (0 %)	1 (20 %)	0 (0 %)	0 (0 %)	0 (0 %)	4 (80 %)
PTC	5	2 (40 %)	0 (0 %)	0 (0 %)	0 (0 %)	0 (0 %)	2 (40 %)	3 (60 %)
ATC	2	1 (50 %)	0 (0 %)	0 (0 %)	0 (0 %)	0 (0 %)	1 (50 %)	1 (50 %)
MTC site								
Thyroid gland primary tumor	16	7 (43.8 %)	0 (0 %)	2 (12.5%)	1 (6.3%)	0 (0 %)	4 (25.0%)	9 (56.3 %)
MTC lymph node metastases	17	9 (52.9%)	3 (17.6%)	1 (5.9%)	0 (0 %)	1 (5.9%)	4 (23.5%)	8 (47.1%)
MTC liver metastases	1	1 (100 %)	0 (0 %)	1 (100 %)	0 (0 %)	0 (0 %)	0 (0 %)	0 (0 %)

ATC, anaplastic thyroid carcinoma; FTC, follicular thyroid carcinoma; MTC, medullary thyroid carcinoma; PITX2, pituitary homeobox 2; PTC, papillary thyroid carcinoma.

MTC samples was 19%. In the primary MTC group, the average percentage of PITX2-positive tumor cells was 12% (range 0–80%). When only positive samples were considered, the average percentage of PITX2-positive cells in the primary group was 27% (range 5–80%). In the secondary MTC group, the average percentage of PITX2-positive tumor cells was 25% (range 0–90%), and when only positive samples were considered, the average percentage was 45.5% (range 5–90%).

In the primary MTC group, no samples showed a PITX2 positivity rate over 80% (81–100%), and 12.5% of samples showed a positivity rate over 40% (41–80%). In the secondary MTC group, 16.7% of samples showed a PITX2 positivity rate over 80% (81–100%), and 27.8% had a positivity rate over 40% (41–100%), including five lymph node metastases and one liver metastasis. All three samples with the highest PITX2 positivity were from the secondary MTC group. The percentage of immunopositive cells in primary and secondary MTCs and control samples is shown in Table 3.

Intensity of PITX2 staining in primary and metastatic MTC FNAs

Four (11.8%) positive MTC samples showed strong staining for PITX2, six (17.6%) showed moderate staining, and seven (20.6%) showed weak staining. In the primary sample group, two (12.5%) samples showed strong staining intensity, three (18.9%) showed moderate intensity, and two (12.5%) showed weak staining intensity for PITX2. In the secondary MTC sample group, two (11.1%) samples showed strong intensity, four (22.2%) showed moderate intensity, and four (22.2%) showed weak PITX2 staining intensity. The intensity of PITX2 staining in primary and secondary MTC samples is presented in Table 2.

Sample collection periods and positivity

All MTC samples were collected and cell blocks were prepared between 2006 and 2018. Half of the samples were collected before 2011, and 41.2% of these samples were positive for PITX2. The other 50% of the samples were collected after 2011, and 58.8% of them were positive for PITX2. The half (50%) of samples were collected and cell blocks prepared between 2006 and 2010, and 41.2% of samples from this period were positive for PITX2. The proportion of PITX2-positive samples collected between 2011 and 2015 was 54.5%, and in samples collected between 2016 and 2018, positivity for PITX2 was 66.7%. All four samples with strong PITX2 staining intensity were collected between 2010 and 2015, and the three samples with the highest percentage of PITX2-positive tumor cells (Table 2) were collected between 2008 and 2018. The proportion of PITX2-positive samples from cell blocks across different institutions and time periods is presented in Supplementary Table 2.

Expression of calcitonin in primary and metastatic MTC FNAs

Twenty-two MTC samples had available data on calcitonin expression, including nine (41%) primary thyroid samples and 13 (59%) samples from metastatic lesions. Calcitonin was positive in 95.5% of all samples. One sample (4.5%) from a primary thyroid lesion was negative for calcitonin. This calcitonin-negative sample was also negative for PITX2 and INSM1. All metastatic samples were positive for calcitonin. Co-expression of calcitonin and PITX2 was found in 54.5% of MTC samples, including four primary and eight metastatic samples. Two representative calcitonin-positive and one calcitonin-negative MTC cytological specimens are shown in Figure 3.

INSM1 expression data were available in 43 samples, including 31 MTC and 12 control samples. In the MTC group, 20 of 31 (64.5%) were positive for INSM1. The positivity rate of INSM1 was higher in the primary MTC group, in which 10 of 14 (71.4%) were positive. In the secondary MTC group, 10 of 17 (58.8%) showed positivity for INSM1. All control samples were negative for INSM1. There was a statistically significant difference in INSM1 positivity between MTC and control samples ($P < 0.001$).

Thirty-one MTC samples had available data on both PITX2 and INSM1 expression, including 14 samples from primary thyroid tumors and 17 from metastatic sites (16 lymph node metastases and one liver metastasis). Co-expression was found in 12 (38.7%) samples, of which four (33.3%) were from primary tumors and eight (66.7%) from metastatic sites.

In the primary MTC group, 4 of 14 (28.6%) samples showed co-expression of PITX2 and INSM1, and in the secondary MTC group, 8 of 17 (47.1%) showed co-expression. Co-expression of PITX2 and INSM1 is summarized in Table 4.

Co-expression of CgA and Syn with PITX2

Nine MTC samples had available data on CgA expression, including five (55.6%) primary thyroid and four (44.4%) met-

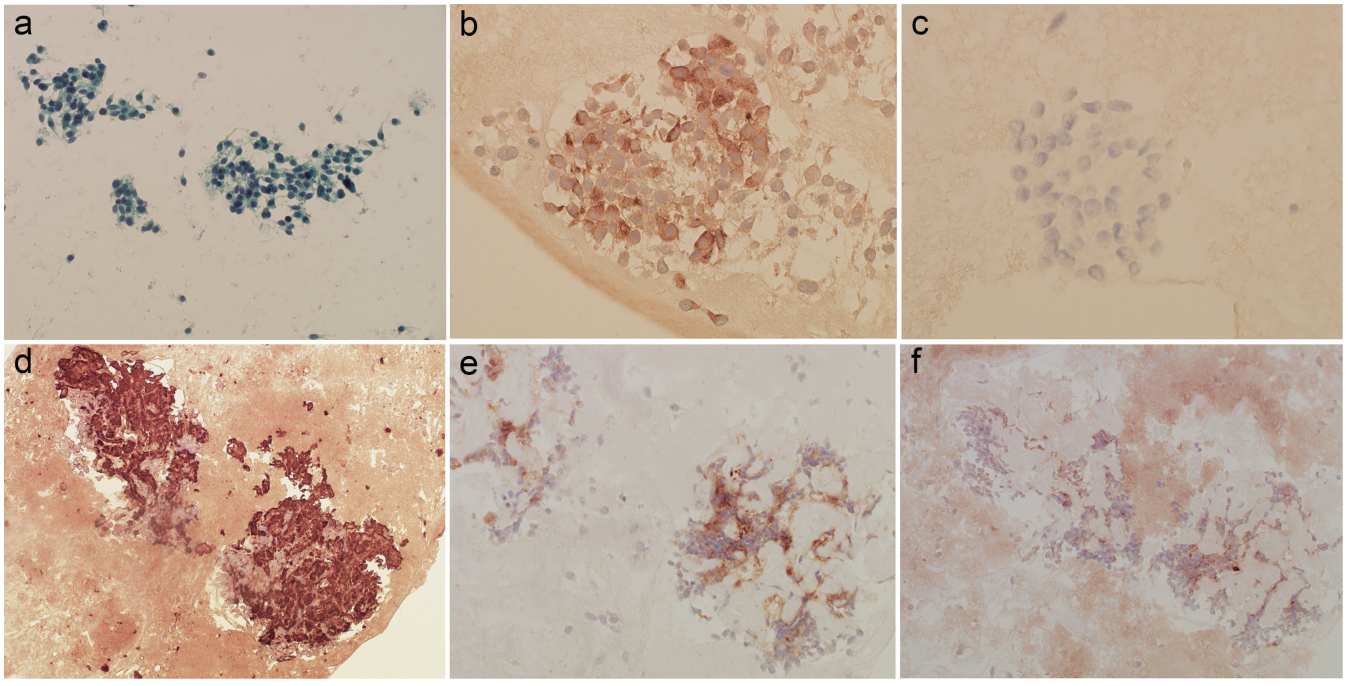

Fig. 3. Morphology and immunohistochemical profile of medullary thyroid carcinoma cytological specimens. (a) Thyroid fine-needle aspiration cytospin preparation stained with Papanicolaou stain shows variably cohesive groups of spindle cells with enlarged nuclei and a salt-and-pepper chromatin pattern in a medullary carcinoma case. Papanicolaou stain, 400× original magnification. (b) Cell block stained with anti-calcitonin antibody. Note variable intensity of immunostaining in spindle-shaped medullary carcinoma cells. Calcitonin immunohistochemistry, 400× original magnification. (c) A calcitonin-negative medullary carcinoma case. A group of negative cells with variably sized and shaped nuclei, salt-and-pepper chromatin, and inclusions. Calcitonin immunohistochemistry, 400× original magnification. (d) Large tissue fragments of medullary carcinoma in a cell block showing strong diffuse calcitonin positivity. Calcitonin immunohistochemistry, 400× original magnification. (e) Large tissue fragments of medullary carcinoma in a cell block with 70% synaptophysin positivity. Synaptophysin immunohistochemistry, 400× original magnification. (f) Large tissue fragments of medullary carcinoma in a cell block. Note that about 50% of cells are chromogranin A positive. Chromogranin A immunohistochemistry, 400× original magnification.

astatic lymph node samples. All these samples were positive for CgA. Co-expression of CgA with PITX2 was found in six (66.7%) MTC samples, including three (50%) primary and three (50%) secondary samples.

Eleven MTC samples had available data on Syn, including six (54.5%) primary and five (45.5%) secondary MTC samples, and all were positive for this marker. Seven (63.6%) samples with Syn data were also positive for PITX2, including three (42.9%) from primary tumors and four (57.1%) from metastatic lymph nodes.

Eight MTC samples had available data for all three markers (CgA, Syn, PITX2), and five (62.5%) of them showed positivity for all three neuroendocrine markers, including two (40%) primary and three (60%) secondary samples. Control samples did not have available data on Syn or CgA staining. An MTC sample with 70% positivity for Syn and 50% positivity for CgA is shown in Figure 3.

Expression of PHOX2B and HAND2

All MTC samples and controls were negative for HAND2 and PHOX2B. Three MTC samples showed weak to moderate granular immunoreactivity in lymphocytes in HAND2 staining.

Discussion

Diagnosis of MTC based on cytomorphology alone remains challenging. When a new, potentially useful immunohistochemical neuroendocrine marker becomes available, it generates enthusiasm for studies on different entities to accumulate knowledge and potentially improve routine diagnostics.

Despite the C-cell origin of MTC, some cases are reported not to raise serum calcitonin and to show only weak or no immunohistochemical positivity for calcitonin.[6–9] In this study, there was also one (4.5%) calcitonin-negative MTC sample

Table 4. Immunoreactivity and co-expression of PITX2 and INSM1 in MTC samples according to sample site

Site	PITX2 positive/ available data (%)	INSM1 positive/available data (%)	Co-expression, both markers positive/available data (%)
All	17/34 (50.0%)	20/31 (64.5%)	12/31 (38.7%)
Primary	7/16 (43.8%)	10/14 (71.4%)	4/14 (28.6%)
Secondary	10/18 (55.6%)	10/17 (58.8%)	8/17 (47.1%)
Lymph node	9/17 (53.0%)	9/16 (56.3%)	7/16 (43.8%)
Liver	1/1 (100.0%)	1/1 (100.0%)	1/1 (100.0%)

INSM1, insulinoma-associated protein 1; MTC, medullary thyroid carcinoma; PITX2, pituitary homeobox 2.

from a primary thyroid lesion. In addition, calcitonin can be positive in other conditions, such as atypical carcinoids.[43] Neuroendocrine markers can also be expressed in other tumor types.[9–19,43–45] Their sensitivity and specificity differ between different NETs,[10,45] and at least two markers should be applied in the diagnostic workup. Most neuroendocrine neoplasms show positivity for CgA or Syn, often for both.[9,10,20–22] Syn is known to have high sensitivity in well-differentiated NETs; however, non-NETs such as esophageal adenocarcinomas may also express Syn and/or CgA, and CgA expression may be weaker in samples from small-sized (≤0.3 cm) tumors.[10,19,20] CgA and Syn are both expressed in the cytoplasm of cells: CgA in neurosecretory granules and Syn more diffusely outside these granules.[9,21] Therefore, staining patterns and expression levels in cytological samples are difficult to analyze accurately, as markers with nuclear staining are generally more reliable in cytological samples.[22,26]

PITX2 has been observed to have high sensitivity and specificity in the immunohistochemical detection of NETs from midgut-derived organs, and in recent studies it has also shown positivity in MTCs, albeit at a lower rate.[29] The PITX2 activation target Cyclin A1 has been shown to be overexpressed in follicular cell-derived thyroid carcinomas, mainly in PTCs.[28] In tissue samples, PITX2 and its other activation target Cyclin D2 are detected in tumor cells of PTC, FTC, and ATC, but not in MTC.[27] It is therefore peculiar that in this study PITX2 was more commonly expressed in MTC cells than in follicular cell-derived tumors.

PITX2 showed positivity in 50.0% of MTC samples and 33.3% of control samples ($P = 0.502$), leading to the conclusion that PITX2 is not a reliable diagnostic marker for MTC. The positivity rate was somewhat higher (55.6%) in samples from MTC metastatic sites than in samples from primary MTC tumors (43.8%), although the difference was not statistically significant ($P = 0.366$). Importantly, the higher PITX2 positivity in metastatic samples was observed across all institutions, suggesting that preanalytical variables can likely be excluded. In conclusion, the higher positivity in metastatic MTCs is an interesting observation and may have implications for the diagnostic evaluation of MTC metastases; further studies are warranted. PITX2 positivity has shown prognostic value in non-functioning pituitary NETs, where higher expression may predict a higher risk of cavernous sinus invasion.[46] In the future, it may also be valuable to investigate the potential prognostic significance of PITX2 in MTC.

INSM1 has been shown to be a highly valuable MTC marker in tissue sections and cytological material, with high sensitivity and specificity.[25,26] In the present study, PITX2 and INSM1 showed co-expression, and co-expression of PITX2 with calcitonin, CgA, and Syn was also observed.

PHOX2B has been reported to show immunoreactivity in some NETs but is mostly negative in MTCs.[30,31,33] All MTC and control samples in this study were negative for PHOX2B. Previous studies have shown HAND2 to be negative in normal thyroid tissue and in NECs,[35] and in this study, none of the MTC samples showed immunoreactivity. Control samples of follicular cell origin were also all negative, as expected. The complete negativity of PHOX2B and HAND2 in MTCs could aid in the differential diagnosis of NETs that are positive for both markers, including paragangliomas in the thyroid gland and neck region.[34,35] Variable HAND2 positivity in background lymphocytes was noted in three MTC cases.

Due to the rarity of MTC, the number of cases with available cell blocks is limited. Some cytological cell blocks used in this study were stored for up to 24 years, and long storage periods may affect antibody immunoreactivity. Nevertheless, recent studies have shown that time- and storage-dependent effects in slides stored for up to 12 months are largely reversible when modern, standardized immunohistochemical methods are used.[47–49] Cellular localization did not reveal significant differences in storage time–dependent reduction of immunosignal between nuclear and cytoplasmic localization.[47]

When sections were prepared from stored formalin-fixed paraffin-embedded (FFPE) blocks, the first significant reduction in immunoreactivity (compared to the original intensity at time zero, corresponding to the positive control) occurred after 6 months of storage, but only 0.7% of all immunoreactions showed decreased reactivity. After 24 months, 6.5% showed decreased immunoreactivity, more frequently in nuclear than cytoplasmic antigens.[48] Storage time can indeed affect immunosignal intensity; however, in some studies, antibodies have shown homogeneous and strong immunoreactivity even in FFPE blocks stored for over 50–70 years.[48–50] Some antibodies demonstrated weakened positivity, especially when original blocks from the 1960s–1970s were re-embedded in paraffin due to plastic and wooden frame materials used in the original preparations.[50] Thus, long storage times of FFPE blocks can decrease detectable immunoreactivity, particularly for nuclear markers. However, this decrease is not necessarily severe enough to prevent reliable analysis. In this study, half (50%) of the cell blocks were originally prepared between 2006 and 2010, and 41.2% of them showed positivity for PITX2. The highest positivity rate for PITX2 (66.7%) was observed in samples from cell blocks originally manufactured between 2016 and 2018, although only 17.6% of all cell blocks were from this period. It is also noteworthy that samples with strong staining intensity were from an earlier period, between 2010 and 2015.

Limitations

The main limitations of this study were its retrospective design, relatively small sample size, and lack of follow-up data for the included cases. No other types of NETs or benign mimickers of MTC were included. Additional limitations included the limited number of calcitonin-, CgA-, and Syn-immunostained samples and the absence of metastatic samples in the control group. On the other hand, this study included both primary and metastatic MTCs from five institutions. Another strength of the study is the use of different cell block methods.

Conclusions

None of the studied neuroendocrine markers, PITX2, PHOX2B, or HAND2, demonstrated sufficient diagnostic utility for the cytological evaluation of MTC. However, the complete negativity of PHOX2B and HAND2 in MTCs is a notable finding that highlights the need for further research into emerging neuroendocrine markers, which may improve the accuracy and reliability of routine diagnostic protocols, particularly in calcitonin-negative MTCs and in cases with variable neuroendocrine marker expression.

Supporting information

Supplementary material for this article is available at https://doi.org/10.14218/JCTP.2026.00011.

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Conflict of interest

Dr. Kholová has been an Editorial Board Member since December 2022, Dr. Rossi has been an Editorial Board Member since May 2023, and Dr. Cai has been Editor-in-Chief Emeritus since January 2026 of *Journal of Clinical and Translational Pathology*. The authors have no other conflicts of interest related to this publication.

Author contributions

Conceptualization (ZM, IK), data curation (MH, DK, ZM, GAB, EDR, IK), formal analysis (MH, DK, IK), investigation (MH, DK, IK), methodology (MH, DK, IK), project administration (MH, IK), resources (IK, GC), software (DK), supervision (ZM, IK), validation (DK, IK), visualization (MH, IK), writing - original draft (MH, IK), and writing - review and editing (MH, DK, ZM, GAB, EDR, GC, IK). All authors have approved the final version and publication of the manuscript.

Ethical statement

The research was conducted in accordance with the Declaration of Helsinki (as revised in 2024). The use of retrospective samples was approved by the participating institutions. Ethical approval was obtained from the ethics committee of Pirkanmaa Hospital District (approval number R17174), Johns Hopkins Hospital (approval number IRB00175708), and Catholic University (approval number 13248). Approval for staining was granted by Valvira (approval number V/47576/2018). Informed consent was waived because of the retrospective nature of the study, in accordance with the relevant institutional and ethics approvals.

Data sharing statement

The dataset used in support of the findings of this study is available from the corresponding author at ivana.kholova@tuni.fi upon reasonable request.

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