False-positive staining of thyroglobulin distinguished from mixed medullary and follicular thyroid carcinoma by duplex in situ hybridization

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Abstract. Medullary thyroid carcinoma (MTC) may mimic mixed medullary and follicular thyroid carcinoma (MMFTC). MTC originates from para-follicular cells, while MMFTC is an uncommon tumor characterized by coexistence of follicular and para-follicular cell-derived tumor populations. A 35-year-old woman was diagnosed with MTC but showed a hot nodule in thyroid scintigraphy. The tumor included diffusely-spread follicular lesions within it, which were immunostained with thyroglobulin and calcitonin. Immunofluorescence showed the presence of several tumor cells that were double-stained with thyroglobulin and calcitonin. To clarify whether or not the tumor was MMFTC, we used duplex in situ hybridization (ISH). Thyroglobulin and calcitonin-related polypeptide alpha mRNA were not expressed together in a single cell, so we suspected false-positive staining of tumor cells with thyroglobulin. To make comparisons with other follicular lesions in MTC, we searched our hospital database. Five cases within a ten-year period had been pathologically diagnosed as MTC. All had follicular lesions in the tumor, but unlike the other case, they were peripherally localized. Dual differentiation into follicular or para-follicular tumor cells was not indicated by either immunofluorescence or duplex ISH. Compared with the case suspected to be MMFTC, there was only mild invasion of tumor cells into the follicular epithelium. The extent of follicular lesions and invasiveness of tumor cells may be associated with pseudo-staining of thyroglobulin in MTC. Duplex ISH can distinguish MTC that are stained with thyroglobulin from MMFTC.

Key words: Medullary thyroid carcinoma, Mixed medullary and follicular thyroid carcinoma, In situ hybridization, Follicular lesion, Calcitonin

MEDULLARY THYROID CARCINOMA (MTC) constitutes approximately 2% of malignant tumors of the thyroid [1]. It originates from para-follicular cells, differing from papillary and follicular thyroid carcinomas, which originate from thyroid follicular cells [2]. Para-follicular cells have been thought to come from neural crests, unlike follicular cells, which come from the endoderm [3]. Thyroid scintigraphy is used to evaluate iodine uptake of follicular-derived tumors or functioning thyroid nodules; it is not, therefore, usually utilized for MTC.

The association between MTC and follicular-derived tumors has been reported in studies of mixed medullary and follicular thyroid carcinoma (MMFTC) [4]. MMFTC is an uncommon tumor, it constitutes less than 5% of MTC [5]. The presence of stem cells with dual differentiation into MTC and follicular-derived tumors has been suggested by reports of dual immunostaining of calcitonin and thyroglobulin within a single cell in MMFTC [5-9]. WHO classification (2017) states that proof of this dual differentiation by immunohistochemistry is necessary to make diagnosis of MMFTC [4].

In the current report we examine follicular lesions in MTC showing a hot nodule in thyroid scintigraphy and investigate whether there is false-positive staining of the tumor cells with thyroglobulin. Using duplex in situ hybridization (ISH) for discrimination, we examine potential misdiagnosis of MTC as MMFTC.
Materials and Methods

Patients

According to the database of patient disease names at the Wakayama Medical University Hospital, between March 2009 and July 2019, ‘MTC’ was listed for 29 patients with thyroid disease. In six of these patients, histological diagnosis of MTC was made after surgery, and these patients were enrolled in the current study. Patient clinical profiles (age, sex, laboratory data, images, and pathological findings) were extracted from electronic medical records. Formalin-fixed paraffin-embedded (FFPE) sections of the tumor from the six patients were analyzed using molecular genetic techniques. All procedures performed in studies involving human participants were in accordance with the ethical standards of the Wakayama Medical University Hospital Ethics Committee (Approval No. 2591) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Methods

Serum thyrotropin (TSH), free thyroxine (fT4), and free triiodothyronine (FT3) levels were measured by chemiluminescent immunoassay (ARCHITECT, Abbott Diagnostics, Tokyo, Japan). Reference ranges were defined as follows: TSH 0.35–4.94 μIU/L, fT4 0.70–1.48 ng/dL, and FT3 1.71–3.71 pg/mL. Thyroid stimulating hormone receptor antibody (TRAb) was determined by radio receptor assay (TRAb Cosmic III, Cosmic, Tokyo, Japan). Thyroglobulin antibodies (TgAb) and thyroperoxidase antibodies (TPOAb) were measured by chemiluminescent immunoassay (AIA-CL2400, Tosoh Bioscience, Tokyo, Japan). Thyroglobulin (TG) and calcitonin antibody (A0576, Dako, 1:500) and anti-thyroglobulin antibody (A0251, Dako, 1:200) were used as primary antibodies. Normal values for TSAb were considered to be those <120%.

Immunohistochemistry

Tumor tissues were fixed overnight in 10% buffered formalin and embedded in paraffin. Each section was stained with hematoxylin and eosin (H&E). Unstained paraffin sections were dewaxed and immunostained as follows: antigens were retrieved in a 97°C pressure cooker for 15 minutes. The endogenous peroxide was then inactivated with 0.3% hydrogen peroxide in methanol. Blocking was then performed with antibody diluent (Dako, S2022) followed by incubation with the first antibodies at 4°C overnight. Incubation with the immunofluorescent second antibodies was performed over 30 minutes, after which a glass coverslip was applied over mounting medium with 4’,6-diamidino-2-phenylindole (DAPI). Finally, we used a fluorescence microscope for detection (BZ-9000, Keyence, Japan). Anti-thyroglobulin (Novus Biologicals, NBP2-29471, 1:200) and anti-calcitonin (Abcam, ab16697, 1:100), anti-calcitonin (Abcam, ab130250, 1:80), anti-sodium/iodide symporter (NIS) (AAB, HPA049055, 1:1,000), and anti-thyroid stimulating hormone receptor (TSHR) (Abcam, ab218108, 1:500) were used as primary antibodies. According to these corresponding primary antibodies, Alexa Fluor 546 goat anti-mouse IgG (Invitrogen, A-11003, 1:400) and Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen, A-11034, 1:400) were used as secondary antibodies.

Duplex in situ hybridization (ISH)

mRNA expression of FFPE in the tumor was evaluated using RNAscope 2.5 HD duplex detection kit (Advanced Cell Diagnostics). Unstained paraffin sections were deparaffinized and immunostained according to the manufacturer’s instructions. Briefly, slides were baked in a dry oven, followed by deparaffinization, inactivation of endogenous peroxide, and target retrieval. Protease reagent was then applied, followed by hybridization of two probes for thyroglobulin (TG) and calcitonin-related polypeptide alpha (CALCA), counterstaining with hematoxylin, application of a glass coverslip over the mounting medium, and then signal detection.

Sequencing analysis for screening of rearranged during transfection (RET) hotspot mutations

We extracted genomic DNA from peripheral blood leukocytes. The coding exons and adjacent intron sequences of RET were amplified by PCR followed by Sanger sequencing using appropriate primers (Supplementary Table 1). Written informed consent was
obtained from the patient for genetic testing according to protocols approved by the Wakayama Medical University Hospital Ethics Committee (Approval No. 138).

Results

Clinical features and course of treatment of case 1

A 35-year-old woman was admitted to our hospital with a mass in her neck. She had no notable medical history, but her grandmother had a history of Graves’ disease. Neck palpation revealed diffuse swelling of the thyroid with approximately 1.5 cm of mass in the right lobe. Blood test results are shown in Table 1 (case 1). The patient had hyperthyroidism with positive TRAb and TSAb, which suggested Graves’ disease. Tumor markers of MTC were high: CEA 16.0 ng/mL, calcitonin 1,208.0 pg/mL. Ultrasonography showed a 2.0 cm mass with calcification in the right thyroid lobe (Fig. 1a). Blood flow was increased in the thyroid, especially at the rim of the tumor (Fig. 1b). Fine needle aspiration biopsy (FNAB) of the tumor was performed; cytology revealed various-sized tumor cells, ovular swelling of the nucleus, and amyloid deposition. The tumor cells were positive for CEA and calcitonin immunostaining. The tumor was therefore diagnosed as MTC. Notably, thyroid scintigraphy using technetium-99m and iodine-123 (I-123) detected a hot nodule in the right lobe that corresponded with the tumor as shown by ultrasonography (Fig. 1c, 1d). Laboratory tests and multimodal imaging including CT and ultrasonography revealed no extra-thyroidal lesions associated with multiple endocrine neoplasia type 2 (MEN2). Genetic screening for RET identified no mutations.

After treatment with methimazole 15 mg/day for hyperthyroidism, we performed total thyroidectomy. Pathological findings of the tumor are shown in Fig. 2. H&E staining showed an encapsulated tumor surrounded by the thyroid tissue (Fig. 2, upper left). Thyroid follicular cells in the surrounding thyroid tissue were hyperplastic and in a papillary formation, which was compatible with Graves’ disease. The tumor was composed of two characteristic areas; area ‘a’ shows a microfollicular structure with atypical cells (Fig. 2, middle left), area ‘b’ shows proliferation of atypical cells forming nests or trabeculae with amyloid deposition (Fig. 2, lower left). The tumor cells in area ‘b’ were

| Case (reference range) | Age (years)/Sex | TSH (μIU/L) | fT3 (pg/mL) | fT4 (ng/dL) | TRAb-1st (%) | TSAb (%) | TgAb (IU/mL) | TPOAb (IU/mL) | Thyroglobulin (ng/mL) | CEA (ng/mL) |
|-----------------------|----------------|-------------|-------------|-------------|-------------|-----------|--------------|---------------|----------------------|------------|
| Case 1                | 35/F           | 0.35–4.94   | 1.71–3.71   | 0.70–1.48   | <10.0–10.0  | <=120     | <28.0        | <16.0         | <=33.7               | 16.0       |
| Case 2                | 61/M           | <0.005      | 7.82        | 2.81        | 10.3        | 140       | 12.5         | 224.4         | 127.0                | 16.0       |
| Case 3                | 73/M           | 1.020       | NA          | 1.38        | NA          | NA        | <10.0        | NA             | 15.5                 | 20.2       |
| Case 4                | 62/F           | 1.32        | 2.50        | 1.19        | NA          | NA        | <10.0        | NA             | <0.10                | 28.4       |
| Case 5                | 70/M           | 0.90        | 2.68        | 0.97        | 6.3         | NA        | 13.0         | 9.0            | 24.8                 | 25.6       |
| Case 6                | 76/F           | 4.11        | 2.56        | 1.20        | 1.29        | NA        | <10.0        | <9.0           | 20.2                 | 23.8       |

Thyroid-associated tests were performed on the patient’s first visit to our hospital. Abnormal figures are in bold text.

TSH, thyroid stimulating hormone; TRAb, thyroid stimulating hormone receptor antibody; TSAb, thyroid stimulating antibody; TPOAb, anti-thyroid peroxidase antibody; TgAb, anti-thyroglobulin antibody; CEA, carcinoembryonic antigen; TcO4−, technetium-99m pertechnetate; I-123, iodine-123; FNAB, fine needle aspiration biopsy; M, male; F, female; NA, not applicable; LN, lymph node
diffusely stained with calcitonin, CEA, and chromogranin A (Fig. 2, upper and lower middle; Supplementary Fig. 1). The tumor was pathologically diagnosed as MTC. Surprisingly, the follicular structure in area ‘a’ of the tumor was strongly stained with both calcitonin (Fig. 2, center) and thyroglobulin (Fig. 2, right center), indicating the possibility of it being MMFTC.

Immunofluorescence of case 1
To clarify whether the tumor cells were derived from common stem cells that were capable of differentiating into follicular or para-follicular cells, we performed double immunofluorescence staining for thyroglobulin and calcitonin (Fig. 3). Tumor lesions were divided into the following two areas: the area showing follicular lesions in MTC (fMTC) and presentation of typical features of MTC (tMTC). The pathological features of the tumors were compared with the intact thyroid area (iTH) in the surrounding thyroid tissue.

Thyroid follicular cells and colloid were stained with thyroglobulin in the iTH area, but were not stained with calcitonin (Fig. 3, bottom). The thyroglobulin staining of the follicular cells looks relatively weak because the colloids were strongly stained with thyroglobulin. Tumor cells in tMTC area were single-stained with calcitonin (Fig. 3, middle), and were separated from surrounding thyroid tissues that were stained with thyroglobulin. Tumor cells in the fMTC area, however, were partially stained with both calcitonin and thyroglobulin (Fig. 3, top). A merged image shows the presence of dual-stained tumor cells (colored yellow to yellow-green) composed of follicular lesions in the fMTC area (Fig. 3, upper right).

We further analyzed protein expressions of NIS and TSHR at fMTC area of the tumor, generally existing in thyroid follicular cells, using immunofluorescence (Supplementary Fig. 2). NIS was weakly stained at the cytoplasm of some calcitonin-positive follicular cells (Supplementary Fig. 2, upper). TSHR staining, by contrast, was confirmed at the apical and basolateral membrane of the calcitonin-positive follicular cells (Supplementary Fig. 2, lower).

We therefore speculated that the tumor cells containing follicular lesions in MTC derived from common stem cells.

Analysis of thyroglobulin and calcitonin related polypeptide alpha mRNA expression in case 1
Considering the possibility of pseudo-staining for thyroglobulin and calcitonin in immunofluorescence, TG and CALCA mRNA expression were analyzed using duplex ISH. Thyroid follicular cells at iTH showed sin-
gle expression of TG, and tumor cells at tMTC showed single expression of CALCA (Fig. 4, case 1). Notably, follicular lesions in the fMTC area were composed of two kinds of cells: thin monolayer TG-expressing cells localized at the luminal side, and tumor cells expressing CALCA surrounding the follicles (Fig. 4, case 1). Some of the tumor cells expressing CALCA had invaded the inter-cellular areas of the follicles.

TG and CALCA were not expressed together within a single tumor cell. Double immunostaining of thyroglobulin and calcitonin in a single tumor cell was thought to be pseudo-staining of tumor cells with thyroglobulin.

**Comparison of six cases of follicular lesions in medullary thyroid carcinoma**

We further evaluated the follicular lesions in case 1 in comparison with typical cases of MTCs (cases 2 to 6). Clinical features of the six cases of MTC are summarized in Table 1. The patient in case 1 had Grave’s disease only. Technetium-99m scintigraphy was also performed in one of the typical cases of MTC (case 3), which showed no focal uptake (Supplementary Fig. 3).

H&E staining showed that typical MTCs also had follicular lesions, but they were localized peripherally and presented consecutively to the surrounding normal thyroid tissue (Supplementary Fig. 4). Immunofluorescence of typical MTCs showed no tumor cells double-stained with thyroglobulin and calcitonin; thyroglobulin-positive follicular cells were clearly separated from calcitonin-positive tumor cells (Supplementary Fig. 5).

TG and CALCA mRNA expressions of follicular lesions in tumors were compared across the six cases using duplex ISH (Fig. 4). In case 1, follicular lesions were small follicles that were localized in a central area, but in the other cases they were relatively big follicles or dispersed follicular cells that were localized in a peripheral area. The follicular lesions were composed of a thin monolayer of TG-expressing cells and surrounding CALCA-expressing tumor cells. The invasion of tumor cells into the follicular epithelium was more severe in case 1 than it was in the other cases. We therefore had difficulty in distinguishing between the two components in case 1. No tumor cells simultaneously expressed TG and CALCA in any cases.
**Discussion**

MTC typically shows no uptake in thyroid scintigraphy, but in case 1 the MTC showed a hot nodule that corresponded with the tumor. We therefore proposed that there may have been residual normal follicles or tumor cells originating from common stem cells that were capable of differentiating into follicular and para-follicular cells.

Immunohistochemistry showed that the tumor in case 1 was stained with thyroglobulin as well as calcitonin. We performed immunofluorescence and recognized identical tumor cells that were simultaneously stained with both thyroglobulin and calcitonin. To eliminate the possibility of false-positive immunostaining of thyroglobulin in MTC cells, we further analyzed mRNA expression of TG and CALCA [4]. We used duplex ISH method, which can more clearly and more specifically visualize mRNA expression in tumors of FFPE than previous ISH methods [10]. Using this method, TG and CALCA were shown to be expressed in different cells, not within a single cell. The double immunostaining of thyroglobulin and calcitonin in case 1 was therefore found to be false-positive staining, probably due to absorption by tumor cells of thyroglobulin leaked from colloids [11].

Follicular and para-follicular derived tumor cells existing in the same tumor have been discussed in studies of MMFTC [4], and several hypotheses have been proposed regarding the tumorigenesis. The previously favored hypothesis was ‘stem cell theory’; uncommitted stem cells give rise to MTC with dual differentiation [6]. Contrarily, ‘collision theory’ suggests the incidental collision of two independent tumors in the thyroid [12]. ‘Hostage theory’ assumes that normal follicles entrapped by MTC cells lead to neoplastic transformation [13]. Recent WHO classification (2017), however, defines MMFTC as the coexistence of follicular and para-follicular cell-derived tumor populations, which is closest to the collision theory [4]. Volante et al. (1999) also demonstrated the different genetic abnormalities in the two tumor components in MMFTCs using laser-based microdissection, which is contradictory to the stem cell theory [13]. In the present case, there were follicular lesions in the tumor, but we could not confirm the suggested pathological features of follicular derived tumors such as follicular and papillary carcinomas. We therefore suggest that intact thyroid follicles were entrapped in the tumor of MTC, as described in hostage theory.

The stem cell theory is based on the detection of co-expression of both thyroglobulin and calcitonin protein in tumor cells [5-9]. mRNA expressions of TG and calcitonin were also confirmed by conventional ISH method, but the image was unclear and mRNA expressions could not be simultaneously confirmed [5]. WHO classification (2017) also states that immunostaining is necessary to prove dual para-follicular and follicular cell differentiation for the diagnosis of MMFTC [4]. The possibility of false-positive immunostaining of thyroglobulin was clearly shown using duplex ISH in the current study. Typical MTCs also contained follicular lesions within
the tumor, implying that any cases of MTC risk misdiagnosis as MMFTC. If simultaneous production of thyroglobulin and calcitonin in a tumor is suspected from immunostaining, we suggest that mRNA expression should be evaluated using duplex ISH method.

One possible cause of the focal uptake in thyroid scintigraphy in case 1 could be the diffuse spread of follicles remaining in the tumor. TRAb probably stimulated the proliferation of follicular cells because the patient had Graves’ disease. A greater number of follicular lesions were actually retained in the tumor in case 1 than in typical cases of MTC. Another possible cause is the artifact associated with conditions of scanning scintigraphy, such as retention of isotopes to the cystic area of the tumor, and ventral protrusion of the tumor with increased blood flow at the surrounding thyroid stroma.

Duplex ISH method revealed that follicular lesions of MTC in case 1 were composed of thin monolayer cells expressing TG and surrounding tumor cells expressing CALCA. The latter component of the follicles could be misdiagnosed as a follicular variant of MTC or MMFTC. Some of the CALCA-expressing tumor cells had invaded into the follicles in case 1, which may be associated with a leakage of thyroglobulin and its absorption by tumor cells. Notably, different cells that cannot be separated by immunohistochemistry could be separated and clearly visualized by duplex ISH method.

A limitation of our study is that we could only investigate a small number of cases with MTC. Further investigation with a greater number of cases of MTC and

Fig. 4  mRNA expressions of thyroglobulin (TG, red) and calcitonin related polypeptide alpha (CALCA, green) were compared across six cases. TG-expressing follicular lesions in case 1 diffusely spread deep in the tumor, but those of the others were peripherally localized. The invasion of CALCA-expressing tumor cells into the TG-expressing follicular epithelium was more severe in case 1 than that in the others. Upper: ×4 magnification, lower: ×20 magnification.
including some cases of MMFTC is desired. This case-oriented study showed that MTC mimicked MMFTC by false-positive staining of thyroglobulin, which was clearly distinguishable using the duplex ISH method.

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Disclosure

The authors declare that they have no conflicts of interest.

Supplementary Table 1  Sequencing primers for screening of RET hotspot mutation

| Exon   | Forward Primer (5'-3')        | Reverse Primer (5'-3')        | Product Size (bp) |
|--------|------------------------------|------------------------------|-------------------|
| Exon10 | CTATGCTTGCGACACCCAGTT        | TGGAGTAACAGAGGCCCAGA         | 384 bp            |
| Exon11 | TGTTCTACGGCTTCCACAC         | CAGAACACAGGCTCCTCT          | 539 bp            |
| Exon13 | CTTCCAGGGAGCGATGTTTG         | CAGCCTGGTGAGTGTGCTG         | 399 bp            |
| Exon14,15 | TGTCCACCCCCTTCTCATT          | CTTCGTAGTTTCTAGGC         | 889 bp            |
| Exon16 | CCGTGTTGAGTGTGTTG            | ACGAACACATCATGAGCC         | 408 bp            |

Supplementary Fig. 1  Imaging studies of case 3. Ultrasonography shows right thyroid nodule (upper). Technetium-99m pertechnetate scintigraphy (lower) shows no focal uptake in the thyroid.
**Supplementary Fig. 2** Follicular lesions (middle) and typical lesions (bottom) of medullary thyroid carcinoma in case 1 were positive for both carcinoembryonic antigen (CEA) and chromogranin A.

**Supplementary Fig. 3** Double immunofluorescent staining of calcitonin and sodium/iodide symporter (NIS) (upper) or thyroid stimulating hormone receptor (TSHR) (lower) at follicular lesion in MTC.
Supplementary Fig. 4  Comparison of hematoxylin and eosin staining across five cases. Follicular lesions in cases 2 to 6 focally distributed in the periphery of the tumor along with intact thyroid tissue, contrary to those in case 1, in which they were diffusely spread at the central part of the tumor. Upper: loupe image, lower: ×5 magnification.

Supplementary Fig. 5  Immunofluorescence in five typical MTCs. Immunofluorescence in cases 2 to 5 did not show the presence of tumor cells double-stained with thyroglobulin (red) and calcitonin (green) that was shown in case 1.
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