Absence of *RET* Gene Point Mutations in Sporadic Thyroid C-Cell Hyperplasia

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Progression from C-cell hyperplasia (CCH) to medullary thyroid carcinoma (MTC) has been demonstrated to date only in familial forms, whereas in nonfamilial MTC, such hypothesis is suggested by the rare concurrence of both lesions, although no epidemiological and molecular data are available to prove or disprove this event. Therefore, the clinical management of patients with sporadic CCH is controversial. To evaluate the malignant potential of sporadic CCHs, pure laser-microdissected C-cell populations of 24 CCH cases, either reactive or associated with nonfamilial MTC, were analyzed for MTC-associated protein neural cell adhesion molecule expression and *RET* point mutations in exons 10, 11, 15, and 16, by using immunohistochemistry and polymerase chain reaction-single-strand conformation polymorphism/heteroduplex electrophoresis/direct sequencing, respectively. No *RET* mutations were found in any of the 24 CCH cases, whereas M918T mutation was detected in three concomitant MTCs. Neural cell adhesion molecule was immunoreactive in the majority of CCH associated with MTC even in the absence of morphological atypia, but not in reactive forms. The absence of *RET* alterations in all cases of CCH examined supports the hypothesis that the development of MTC is independent of pre-existing CCH in the nonfamilial setting; thus, sporadic CCH should not be considered a risk factor for nonfamilial MTC. (J Mol Diagn 2007, 9:214–219; DOI: 10.2353/jmoldx.2007.060166)

Thyroid C-cell hyperplasia (CCH) could represent a physiological or reactive proliferation of calcitonin-producing cells in response to different physiological and/or pathological endocrine stimuli (eg, thyroid-stimulating hormone overstimulation, hypercalcemia, and paracrine factors) or a neoplastic lesion.^{1,2} In particular, the progression from CCH to medullary thyroid carcinoma (MTC) is a well-defined event only in a familial setting, including

isolated familial MTC and multiple endocrine neoplasia type 2 (MEN2) syndromes.^{2–4} All these familial C-cell neoplastic disorders are reported to be associated with germline mutations of the *RET* proto-oncogene.^{2,5} On the contrary, the possible role of CCH as a precursor of nonfamilial MTC is suggested only by their occasional association. However, no clear molecular and epidemiological links between CCH and sporadic MTC are available to date, and knowledge of the possible malignant potential of reactive CCH is still poorly achieved.⁶

Physiological or reactive CCH has been described in newborns, elderly patients, patients with hyperparathyroidism or hypothyroidism, Hashimoto's thyroiditis, and previous hemithyroidectomy, as well as in thyroid tissue adjacent to follicular neoplasms, nodular and diffuse goiter, and primary thyroidal non-Hodgkin's lymphoma.^{1,2,6} The prevalence of CCH in patients affected by thyroid nodular disease or in healthy patients is not known with certainty.^{1,7,8} Thus, in most instances CCH remains an incidental finding in thyroidectomies performed for other diseases. Although the natural history of CCH not associated with familial MTC is unknown, the incidental observation of benign and malignant follicular-cell derived tumors (mainly papillary carcinoma) adjacent to CCH and medullary carcinomas has suggested alternative hypotheses, consisting either in the possibility that thyroid follicular cell-derived tumors might induce reactive CCH and possibly its evolution into MTC or in a common genetic background responsible for both follicular cell-derived and parafollicular neoplastic lesions.^{9–11} By contrast, no definite epidemiological evidence of a particular association of sporadic MTC with the same conditions favoring reactive CCH exists to date.

As also embraced in the current World Health Organization Classification of Tumors of Endocrine Organs,⁶ reactive and neoplastic CCH may be distinguished by a pathological point of view according to the presence of cytological atypia, recognizable in conventional histological specimens in the latter form, whereas physiological or reactive CCH detection requires calcitonin immuno-

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Table 1. Clinicopathological Parameters, Immunohistochemistry, and Molecular Profile of 24 Cases of CCH

Case	Age/sex	Associated thyroid disease	Basal* (stimulated [#]) calcitonin (pg/ml)	C-cell hyperplasia pattern	NCAM IHC in CCH	RET gene analysis
H-01	64/F	Nodular goiter	<10*	Focal and diffuse	—	—
H-02	36/F	Nodular goiter	<10*	Focal and diffuse	—	—
H-03	33/F	Nodular goiter	<10*	Focal and diffuse	—	—
H-04	47/F	Nodular goiter	<10*	Focal and diffuse	—	—
H-05	58/M	Nodular goiter	13.7* (22.7) [#]	Focal and diffuse	—	—
H-06	41/F	Nodular goiter	24.0* (29.3) [#]	Focal and diffuse	—	—
H-07	52/F	Hashimoto's thyroiditis	<10*	Focal and diffuse	—	—
H-08	58/M	Hashimoto's thyroiditis	<10*	Focal and diffuse	+	—
H-09	73/M	Hashimoto's thyroiditis	<10*	Focal and diffuse	—	—
H-10	24/F	Hashimoto's thyroiditis	<10*	Focal and diffuse	—	—
H-11	33/M	Hashimoto's thyroiditis	<10*	Focal and diffuse	—	—
H-12	50/M	Follicular adenoma	31.0* (58.8) [#]	Focal and diffuse	—	—
H-13	41/M	Follicular adenoma	18.4* (31.2) [#]	Focal and diffuse	—	—
H-14	61/F	Oncocytic adenoma	<10*	Focal and diffuse	—	—
H-15	31/F	Oncocytic adenoma	<10*	Focal and diffuse	—	—
H-16	44/F	Papillary carcinoma	<10*	Focal and diffuse	—	—
H-17	62/F	Nonfamilial MTC	82.3* (330.3) [#]	Nodular with atypia	+	—
H-18	55/M	Nonfamilial MTC	446.9*	Nodular with atypia	+	Met918Thr [§]
H-19	68/F	Nonfamilial MTC	388.2*	Focal and diffuse	—	Met918Thr [§]
H-20	36/F	Nonfamilial MTC	543.6*	Focal and diffuse	+	—
H-21	67/F	Nonfamilial MTC	144.0*	Focal and diffuse	—	Met918Thr [§]
H-22	65/M	Nonfamilial MTC and papillary carcinoma	275*	Focal and diffuse	+	—
H-23	61/M	Nonfamilial MTC and papillary carcinoma	15.3* (80.7) [#]	Focal and diffuse	—	—
H-24	49/M	Nonfamilial MTC and papillary carcinoma	137.7*	Focal and diffuse	+	—

NCAM, neural cell adhesion molecule; IHC, immunohistochemistry; MTC, medullary thyroid carcinoma.

* and [#], normal values: <10 pg/ml; borderline values: >10 and <100 pg/ml; pathological values: >100 pg/ml.[§]RET mutation present in the MTC component only.

staining, being easily missed in conventional hematoxylin and eosin (H&E)-stained slides because of the morphological similarity between C cells and adjacent follicular cells.¹² Furthermore, in some cases, a differential diagnosis with medullary microcarcinoma (neoplastic C-cell proliferation less than 1 cm in size) may also be problematic. As a possible diagnostic adjunct, Komminoth and colleagues¹³ showed that reactive CCH stains negatively with neural cell adhesion molecule (NCAM), whereas both MTC and neoplastic CCH gave positive results.

The aim of the present study was to analyze the presence of *RET* mutations and NCAM immunoexpression in 24 cases of nonfamilial CCH, either associated with physiological/reactive conditions or with sporadic MTC, as possible markers of malignant potential in CCH.

Materials and Methods

Surgical Samples

We retrospectively collected a series of 24 cases of CCH of unselected 157 surgical specimens of benign and malignant thyroid lesions from patients with nonfamilial syndrome and preoperative high levels of calcitonin diagnosed and treated at San Luigi Hospital, Orbassano, Turin, Italy, and San Giovanni Battista Hospital, Turin, Italy, in the years 1999 to 2005. The presence of CCH was searched by random calcitonin immunostaining (see below) and defined as the presence of at least three microscopic foci containing more than 50 C cells in one low-power field.^{1,2} Six cases of CCH were associated with multinodular goiter, five with Hashimoto's thy-

roiditis, four with follicular adenoma (two cases with oncocytic features), one with papillary carcinoma, and eight with nonfamilial MTC, of which three cases were associated with concomitant MTC and papillary thyroid carcinoma. For each patient, age, sex, and clinical and laboratory history were collected. Patients included 10 males and 14 females, with a median age of 50.3 years (range, 24 to 73 years). No patient had a personal or familial history of MEN2 or familial MTC, and all proved to be negative at genetic screening performed by the local Genetic Reference Laboratory for the presence of germline *RET* mutations (in exons 10, 11, 13, 14, 15, and 16) in blood DNA. Four of 16 CCH cases not associated with nonfamilial MTC had slightly elevated basal (median, 21.8 pg/ml; range, 13.7 to 31.0) and pentagastrin-stimulated serum calcitonin levels (median, 35.5 pg/ml; range, 22.7 to 58.8). The main clinicopathological data of patients are summarized in Table 1. This study was approved by the San Luigi Hospital review board.

Immunohistochemistry

Serial 5-μm sections from each formalin-fixed, paraffin-embedded tissue block were first overlaid on poly-L-lysine-coated slides, dewaxed in xylene, rehydrated in decreasing ethanol concentrations, and incubated for 10 minutes in phosphate-buffered saline (PBS) (pH 7.4). Heat-induced antigen retrieval procedure was performed for all of the antigens by placing slides in 0.01 mol/L sodium citrate buffer (pH 6.0) in a microwave oven set at high power for three consecutive cycles of 5 minutes each. These slides were left to cool for 20 minutes at

room temperature and rinsed in PBS. Endogenous peroxidase activity was quenched with methanol-hydrogen peroxide 3% for 15 minutes at room temperature. Slides were then washed twice in PBS for 5 minutes. Tissue sections were then incubated in the blocking solution (ChemMate buffer kit; DakoCytomation, Glostrup, Denmark) and subsequently with the primary antibodies in a humidified chamber at 4°C overnight. After a prolonged wash in PBS, the sections were incubated with an enzyme-labeled polymer preconjugated with anti-mouse secondary antibodies (Envision System, biotin-free detection system; DakoCytomation) at room temperature for 30 minutes. The sections were finally washed three times in PBS, and incubated with 3',3'-diaminobenzidine-tetrahydrochloride for 10 minutes. Slides were subsequently rinsed in tap water, counterstained with Mayer's hemalum solution, mounted in Entellan (Merck, Darmstadt, Germany) and examined with a DM-RBE Leica photomicroscope (Leica Microsystems AG, Wetzlar, Germany). Calcitonin and NCAM were revealed using the following primary mouse monoclonal antibodies: calcitonin (clone LK2H10, diluted 1/800; Novocastra, Newcastle, UK) and CD56 NCAM (clone 1B6, diluted 1/100; Novocastra). Positive controls for calcitonin and NCAM immunostaining were represented by a case of MTC and a case of small cell lung carcinoma, respectively. Negative controls were obtained by omitting the primary antibody.

Laser Microdissection, DNA Extraction, and RET Mutation Analysis

Isolation of hyperplastic C cells, previously immunostained with anti-calcitonin antibody, was performed by laser microdissection by means of a laser micromanipulator (Olympus/Cell Robotics Inc., Albuquerque, NM). A range of 50 to 80 cells was collected in a polymerase chain reaction (PCR) tube from normal follicular structures, hyperplastic C-cell populations, and whenever present, medullary carcinoma components for each patient. Cells were suspended in 18 μ l of 1 $\times$ PCR buffer (10 mmol/L Tris-HCl, pH 8.3, and 50 mmol/L KCl, Amplitaq Gold; Applied Biosystems, Roche, Branchburg, NJ) at 95°C for 10 minutes and were digested with 0.5 μ g of proteinase K (Roche Diagnostics GmbH, Mannheim, Germany) diluted in 1 μ l of double-distilled H₂O (ddH₂O) at 55°C for 24 hours. After inactivation of the enzyme at 95°C for 10 minutes, 5 to 7 μ l of the solution was added directly to the PCR mixture for further amplification of exons 10, 11, 15, and 16 of the RET proto-oncogene. PCR primer sequences and PCR conditions used in the present study have been previously published.¹⁴ Amplicons were analyzed subsequently by nonisotopic single-strand conformation polymorphism and heteroduplex gel electrophoresis methods, and in the presence of abnormal conformational variants, by direct sequencing.¹⁴ Two cases of medullary carcinoma, one associated with

MEN2A and one harboring the somatic exon 16 M918T mutation, processed in parallel following the same protocols as above, served as positive controls for mutation analysis.

Results

Histology and Immunohistochemistry

In the group of 16 cases of CCH not associated with nonfamilial MTC, no morphological evidence of increased number of C cells could be demonstrated at H&E staining, but calcitonin immunohistochemistry was necessary to reveal the hyperplastic C-cell population (Figure 1, A–C). The same was true for the remaining cases associated with MTC, except for two cases in which a nodular arrangement of cells—calcitonin-positive by immunohistochemistry—with more basophilic cytoplasm and mild cytological atypia, less than 5 mm in larger size, but apparently confined to the follicular basal membrane, was observed at conventional histological examination (Figure 1, D–F). Concerning the architectural patterns of CCH as detected by calcitonin immunohistochemistry, in most cases—except for the two cases with morphologically evident mild atypia presenting a more prevalent nodular pattern—a mixed focal (never exceeding the mean follicular size) and, along the follicle, diffuse pattern of C-cell distribution was observed. In MTC-associated cases, CCH was mainly observed at the periphery of the tumor rather than in the distant thyroid parenchyma.

NCAM expression was negative in all but one CCH nonassociated with MTC, whereas stained positive in all MTC cases and in the majority (five of eight) of CCH adjacent to them. However, the expected membranous staining was generally weak in both CCH and MTC samples, compared with the control small cell carcinoma case and nerve fibers eventually present in the thyroid specimens. Rare sparse follicular cells occasionally stained positive for NCAM, mainly in the setting of lymphocytic thyroiditis.

Genetic Analysis

All CCH samples, either associated with nonfamilial MTC or not, did not show any conformation variant in RET exons 10, 11, 15, and 16. Further, molecular analysis of the same exons was negative in all normal thyroid specimens, confirming the absence of germline mutations in all patients. The analysis of RET exon 16 revealed the presence of conformation variants in three MTC samples (37%), confirmed by direct sequencing to harbor the M918T mutation, but not in the associated CCH sample, as already mentioned (Figure 2).

Figure 1. A–C: CCH associated with nodular goiter (case H-06, Table 1), showing the absence of cytological atypia at H&E staining (**A**) and arranged in a linear (**B**) and micronodular (**C**) manner, as revealed by calcitonin immunohistochemistry, as opposed to neoplastic CCH (**D–F**, case H-18), associated with MTC (**D**), demonstrating cytological atypia at conventional morphological evaluation (**E**) and positive NCAM immunostaining (**F**).

Figure 2. RET exon 16 mutation analysis in a microdissected sample of CCH (**A** and **B**, before and after laser microdissection, case H-21) showing wild-type sequence (**C**), as opposed to the associated MTC component, which harbored M918T mutation (**D**).

Discussion

We analyzed the presence of *RET* gene point mutations in physiological or reactive CCH, as well as in CCH associated with nonfamilial MTC, as a genetic marker to evaluate their possible malignant potential. To our knowledge, this is the first report providing insights on the genetic *RET* profile of reactive CCH as well as of CCH accompanying nonfamilial MTC. In fact, although some studies^{12,15-17} reported that CCH may be the precursor of nonfamilial MTC, these data, based on a morphological grounds, were never supported by genetic analysis.

In this study, we collected 24 cases of CCH having both a pure reactive nature, associated with various benign and malignant thyroid lesions of follicular origin, or associated with nonfamilial MTC. This latter association is not frequent, and we could collect eight cases of nonfamilial MTC with CCH, at a variable extent, of more than 50 MTCs diagnosed at our institute in a large time period. From a pathological point of view, in only two cases was morphology able to detect the increase of C cells on the basis of a nodular growth of atypical cells, demonstrated to be of parafollicular origin by calcitonin immunostaining, thus rendering a diagnosis of neoplastic CCH. Both cases were associated with nonfamilial MTC and the C-cell population was adjacent to the tumor. To confirm the morphological findings, in these two cases NCAM was positive in both MTC and CCH areas. Codon 918

RET mutation was present in one of these latter cases, but absent in the peritumoral CCH component, thus in this single case a common genetic background between the MTC component and its possible precursor could have not been demonstrated. Moreover, codon 918 *RET* mutations were also detected in two other cases of nonfamilial MTC of the present series, but again not in the corresponding CCH samples, which were morphologically classified as reactive in the absence of any cytological atypia and according to the mainly focal or diffuse growth pattern. Altogether, these features make a progression from CCH to nonfamilial MTC unlikely. In addition, in any of the CCH cases not associated with MTC both morphology, NCAM immunohistochemistry (except for one single case) and *RET* testing could not demonstrate any evidence of neoplastic nature or malignant potential.

One of the limitations of the present study is related to the absence of highly sensitive molecular markers of MTC because the prevalence of somatic *RET* point mutations is extremely heterogeneous in the literature (ranging from 20 to 80%) and lack other sensitive molecular markers. Alterations of other genes, such as succinate dehydrogenase subunit D (*SDHD*), have been documented in familial forms of CCH¹⁸ and may be involved in the progression of MTC, although other studies^{19,20} recently showed after extensive molecular analysis no al-

terations of *SDH* subunits in CCH and nonfamilial MTC. Therefore future studies, possibly based on wide genome-screening searches, would be needed to better comprehend the molecular mechanisms leading to non-familial MTC and its possible relationships with CCH conditions in nonfamilial MTC diseases.

In conclusion, considering that *RET* mutations are an early event in the pathogenesis of MTC, the absence of *RET* alterations in all CCH cases examined does not allow confirmation of the hypothesis of their possible progression toward MTC, also in those cases associated with nonfamilial MTC.

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