Histopathological and molecular studies in patients with goiter and hypercalcitoninemia: reactive or neoplastic C-cell hyperplasia?

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Abstract

The cut-off values able to differentiate between reactive or neoplastic C-cell hyperplasia (CCH) or to predict sporadic medullary thyroid cancer (MTC) are still debated both for basal and stimulated calcitonin (bCT and sCT). In the present study, the prevalence and the histological patterns of CCH in 15 patients with multinodular goiter (MNG), bCT > 10 pg/ml and sCT levels > 50 pg/ml were studied. As controls, 16 patients with MNG and bCT levels < 10 pg/ml and 4 patients with familial (FMTC) were included. For each case, calcitonin (CT) immunoreactive cells were counted in 60 consecutive high-power fields (400×) and CCH classified as focal, diffuse, nodular, or neoplastic. *RET* genetic analyses were performed at the germline and tissue levels in MTC and CCH cases. In patients with MNG, sCT levels > 50 pg/ml were associated with CCH or MTC, being the total number of C-cells/60 fields significantly higher than that found in MNG with normal bCT ($P=0.0008$) and comparable with that detected in FMTCs. In the group with sCT > 50 pg/ml, the C-cells displayed a neoplastic phenotype. Neither germline nor somatic *RET* mutations were found. In conclusion, sCT levels > 50 pg/ml were always associated with CCH, without correlation between CT levels and the number of C-cells or the final diagnosis. The C-cells had a morphology and distribution pattern similar to those observed in FMTC. Thus, sCT levels > 50 pg/ml indicate the presence of CCH with a possible preneoplastic potential, suggesting the opportunity to perform a prophylactic surgical treatment.

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Introduction

Medullary thyroid cancer (MTC) accounts for 5–10% of thyroid cancers and occurs in both sporadic and hereditary forms (Sizemore *et al.* 1977, Schlumberger & Pacini 2003). Its biological behavior is more aggressive than that reported for the other well-differentiated thyroid cancers, with a 10-year survival rate of about 70% (Modigliani *et al.* 1998, Schlumberger & Pacini 2003). Indeed, clinical neck lymph node and distant metastases are detected at presentation in ~50–70 and 15–20% of patients respectively (Modigliani *et al.* 1998). Moreover, the primary, and virtually the only, therapeutic approach is the surgical intervention, since other treatments are poorly effective.

Thus, the main goal in MTC management is to diagnose the disease in a preneoplastic condition, corresponding to C-cell hyperplasia (CCH). CCH is defined as an increased number of normal C-cells, more commonly with a diffuse pattern, i.e. 50 or more C-cells in at least one low-power field (100×), a number greater than that found in most normal subjects (De Lellis & Wolfe 1981, Albores-Saavedra & Krueger 2001) and has been classified as physiologic/reactive or neoplastic (Perry *et al.* 1996, Matias-Guiu *et al.* 2004). Reactive CCH has been reported in neonates, the elderly, hyperparathyroidism, Hashimoto's thyroiditis, and follicular thyroid adenomas (Guyetant *et al.* 1994, Matias-Guiu *et al.* 2004) and can be defined, according to the growth

pattern of C-cells, as focal, diffuse, or nodular (Perry *et al.* 1996, Kaserer *et al.* 2001). Neoplastic CCH is usually associated with hereditary MTC (familial MTC-, FMTC-, or MEN 2), but some of the authors have supported the idea that it might also be the precursor of sporadic MTC (sMTC) in the absence of germline *RET* mutations (Perry *et al.* 1996, Kaserer *et al.* 1998). According to the definition proposed by Perry *et al.* (1996), neoplastic CCH is characterized by the presence of large, mildly to moderate atypical, round, polygonal, or spindle-shaped cells with nuclear pleomorphisms (Perry *et al.* 1996). These cytological alterations would allow the C-cells to differentiate from the follicular cells and, therefore, to diagnose the neoplastic CCH on hematoxylin and eosin (H&E) sections, while the reactive type usually would require immunohistochemistry (IHC) for its identification. However, the pathological definition and clinical correlation of reactive and neoplastic CCH are still unclear (LiVolsi 1997) and some of the authors reported that, morphologically, it is not possible in most cases to distinguish with certainty between neoplastic and reactive CCH (Hinze *et al.* 2001, Kaserer *et al.* 2001).

In hereditary MTC, the finding of *RET* mutations frequently allows to perform the prophylactic thyroidectomy at the CCH stage, with a major impact on the prognosis (Eng *et al.* 1996). Conversely, the diagnosis of sMTC is usually done when the tumor is clinically manifest. However, a significant increase in early diagnoses has followed the introduction of the routine measurement of calcitonin (CT) levels in patients presenting with thyroid nodules. Indeed, many reports have shown that the routine CT measurement has a specificity and sensitivity higher than the fine needle aspiration cytology and leads to the detection of MTC in about 0.4–0.5% of the patients (Pacini *et al.* 1994, Rieu *et al.* 1995, Niccoli *et al.* 1997, Vierhapper *et al.* 1997, Ozgen *et al.* 1999, Hahm *et al.* 2001, Elisei *et al.* 2004, Karanikas *et al.* 2004). The main pitfall related to this screening is the correct interpretation of borderline cases. It is well accepted that when CT levels above the normal range are found in patients with uni- or (UNG or MNG), a pentagastrin (Pg) stimulation test must be performed. In the absence of a CT response to Pg, the diagnosis of MTC can be excluded, while the increases in the CT levels after stimulus are often difficult to be interpreted. In general, a CT response <50 pg/ml is considered normal (Wion-Barbot *et al.* 1997), a response >50 and <100 pg/ml is a borderline result, while a CT rise of >100 pg/ml suggests the diagnosis of MTC and the need for surgical treatment (Niccoli *et al.* 1997, Scheuba *et al.* 1999, Iacobone *et al.* 2002, Elisei *et al.* 2004). However, no cut-off levels of sCT

able to differentiate between reactive and neoplastic CCH or to predict MTC have been identified (Iacobone *et al.* 2002, Gibelin *et al.* 2005).

The aim of the present study was to define the prevalence and the histological patterns of CCH in patients with nodular thyroid disease and sCT levels >50 pg/ml. The data obtained were compared with those from patients with MNG and basal CT levels <10 pg/ml, and with those from patients with sMTC and hereditary MTC/CCH. For the first time, *RET* genetic analyses were performed not only at the germline level, but also starting from DNA extracted from CCH.

Materials and methods

Patients

In the period comprised between March 2002 and December 2005, 1246 patients referred to our outpatient departments with uni- or (UNG, MNG) were submitted to routine serum CT measurement. In addition, all patients underwent clinical examination, neck ultrasonography and measurement of thyroid hormones, and anti-thyroid autoantibodies. Basal CT (bCT) levels above the normal range (i.e. >10 pg/ml) were found, and confirmed at a second assay, in 32 cases who were thus submitted to Pg test. In all these patients, a familial history of MTC was excluded. Of the 32 patients, 17 had a stimulated CT (sCT) <50 pg/ml and were considered normal. These patients had bCT levels ranging 10.5–20 pg/ml (median 11) and sCT ranging 20–49.2 pg/ml (median 41.1), without correlation between the basal and the stimulated levels of CT. In the remaining 15 patients, who had bCT levels ranging 17–196 pg/ml (median 35) and a sCT >50 pg/ml, total thyroidectomy was performed due to: a) sCT >100 pg/ml in seven cases (# 2–5, 11, 12, and 15); b) bCT >100 pg/ml and cytology positive for MTC in two cases (# 13 and 14); and c) tracheal deviation/compression and/or esthetic reasons in the remaining six cases (Table 1). The 15 patients submitted to total thyroidectomy and included in the present study (# 1–15; eight M, seven F; age range 33–73 years, mean 56.8 years, median 59 years) had a normal thyroid function with negative anti-thyroid autoantibodies in 13 and positive in 2 patients (# 4 and 12; (Table 1)). The renal function was normal in all, thus excluding that the elevated basal and sCT levels could be due to a renal failure, as reported in the literature (Niccoli *et al.* 1995, Kotzmann *et al.* 1999). Moreover, other causes of abnormal CT levels, such as endocrine malignant tumors and hypergastrinemia, were excluded. All patients were operated in the

Table 1 Clinical, biochemical, and histopathological data of the 15 patients with nodular goiter and preoperative basal and stimulated CT (sCT) levels > 10 pg/ml and > 50 pg/ml respectively

				Histology ^a				
Pts #	Age, gender	CT pre-Tx basal/peak (pg/ml)	Cytology	Goiter	Cancer	PTNM	CCH at initial diagnosis	CCH at re-evaluation
1	47, F	17/61	Neg	MNG	—		+	+
2	50, M	35/333	Nd	MNG	—		—	+
3	73, F	33/112	Neg	MNG	—		+	+
4	65, M	30/588	Neg	MNG/CLT	—		—	+
5	49, M	25/219	Neg	MNG/CLT	—		+	+
6	51, M	51/nd	Nd	MNG	—		+	+
7	33, F	33/nd	Nd	UNG	—		—	+
8	37, M	17/62	Neg	UNG	—		—	+
9	62, M	31/97	Neg	MNG/CLT	PTC	T3Nx (13 mm)	—	+
10	59, F	59/nd	Nd	MNG/CLT	PTC	T1N0 (10 mm)	—	+
11	70, M	47/390	Neg	MNG	PTC	T1mNx (4, 1 mm)	+	+
12	58, F	39/553	Nd	MNG/CLT	MTC	T1Nx (5 mm)	+	+
13	65, F	182/nd	MTC	MNG	MTC	T1N0 (14 mm)	—	+
14	63, F	196/nd	MTC	MNG	MTC	T1N0 (13 mm)	—	+
15	71, M	81/1783	Nd	UNG	MTC	T1N0 (12 mm)	+	+

Pts, patients; Tx, thyroidectomy; CT, calcitonin (basal and peak after pentagastrin test); nd, not done; MNG, multinodular goiter; UNG, uninodular goiter; CLT, chronic lymphocytic thyroiditis; PTC, papillary thyroid cancer; MTC, medullary thyroid cancer; CCH, C-cells hyperplasia. Cancers were staged according to the last TNM staging system, ICD-O C73 (AJCC 2002). In parentheses, the primary tumors are reported in mm.

^aImmunohistochemistry for CT was done in all the cases, with the exception of # 4 at initial diagnosis, and in all the cases at re-evaluation.

Endocrine Surgery Department and specimens were examined by the same Department of Pathology. The initial histological diagnoses were performed by means of routine staining and, in all cases but # 4, by specific anti-CT (IHC; Table 1). In particular, in eight patients, the diagnosis of benign nodular goiter was performed, associated with thyroiditis in five cases, while in three patients, benign nodules were associated with incidental papillary thyroid cancers (PTCs). Finally, medullary thyroid cancer was diagnosed in four patients. Cancers were staged according to the last TNM staging system, ICD-O C73 (AJCC 2002). CCH was diagnosed in four MNG (# 1, 3, 5, and 6), one PTC (# 11) and two MTC (# 12 and 15). It is worth to note that CCH was not diagnosed in # 4, which was analyzed only by routine staining, and in other seven cases studied by specific IHC and entire thyroid blocking.

After surgery, all patients showed undetectable basal and, in some cases, Pg-sCT levels, confirming the thyroid production of CT.

All patients were screened and followed up in the Endocrine Sciences Department and gave informed consent to the study.

Controls

Data were compared with a control group of 16 patients matched for gender and age (# C1–C16; 6 M,

10 F; age range 29–78 years, mean 57.4 years, median 56.5 years), operated for MNG or UNG with normal bCT (i.e. < 10 pg/ml), negative anti-thyroid autoantibodies in 11 out of 13 patients, studied and operated in the same departments (Table 2). Furthermore, four patients affected with FMTC submitted to prophylactic thyroidectomy, following *RET*-positive genetic analyses, were considered as controls for neoplastic CCH. Indeed, familial CCH is a pre-neoplastic lesion and considered as the carcinoma *in situ* of the thyroid gland parafollicular cells (LiVolsi 1997, Kaserer *et al.* 2001, Matias-Guiu *et al.* 2004). The first case (# FMTC1) is a 6-year-old child with a mild response to Pg test (bCT 17 pg/ml, sCT 61 pg/ml), with a histological diagnosis of CCH and normal CT levels at the post-surgical follow-up. This child harbored a recently described *RET* complex mutation, K666N/insS (Cordella *et al.* 2006). The second case (# FMTC2) is a 33-year-old man with high presurgical bCT levels (98 pg/ml) and a multicentric MTC at histology (pT1mN0), harboring the *RET* mutation C634F. The third and fourth cases (# FMTC3 and FMTC4) are father and son (50 and 22 years) with the *RET* mutation C618S, with high bCT levels (2152 and 342 pg/ml) and MTC associated with CCH at histology (pT3mN0 and pT1N0).

Table 2 Clinical, biochemical, and molecular data of the controls: 4 patients with familial medullary thyroid cancer (FMTC) and 16 patients with uni- or multinodular goiter (MNG) and preoperative CT levels < 10 pg/ml

Pts #	Age/gender	CT pre-Tx basal/ peak (pg/ml)	RET
FMTC1	6/M	17/61	K666N/insS
FMTC2	33/M	98/nd	C634F
FMTC3	50/M	2152/nd	C618S
FMTC4	22/M	342/nd	C618S
C1	51/F	4	Nd
C2	73/M	5.6	Nd
C3	52/F	2.1	Nd
C4	52/F	2	Nd
C5	30/F	3.4	Nd
C6	55/M	7.8	Nd
C7	62/F	1.7	Nd
C8	78/M	2.3	Nd
C9	66/F	5.4	Nd
C10	52/F	8.1	Nd
C11	48/F	1.8	Nd
C12	59/F	<2	Nd
C13	44/F	2.8	Nd
C14	68/M	5.9	Nd
C15	71/M	4.3	Nd
C16	58/M	6.5	Nd

Methods

Biochemical investigations

CT serum levels were measured by a commercially available two-site chemiluminescence immunometric assay (ILMA; Nichols Institute Diagnostics, Nijmegen, The Netherlands). The ILMA recognizes the mature monomeric form of CT. Two mouse monoclonal antibodies directed against two different epitopes (Grauer *et al.* 1998, Skinner *et al.* 2005) are used. The sensitivity of the assay is 0.2 pg/ml. The intra- and inter-assay coefficients of variation are <4.5 and <11.1% respectively. All samples were evaluated in duplicate. Serum thyroid-stimulating hormone, FT4, FT3, Tg-Ab, and TPO-Ab were measured using the AutoDELFIA technique (Perkin Elmer-Life Sciences, Wallac Oy, Turku, Finland), being normal values of 0.26–4.2 mU/l, 9–20 pmol/l, 3.8–8 pmol/l, and <35 U/l respectively. All patients were submitted to ultrasonography of the neck. Pg test was performed with an indwelling catheter and CT was measured at –10, 0, 2, 5, and 10 min after an i.v. bolus of 0.5 µg/kg Pg (Peptavlon Injection BP; Cambridge Laboratories, Cambridge, UK).

Immunohistochemical studies

For each of the 35 cases included in the study (15 patients and 20 controls), six paraffin blocks (three from the left and three from the right lobes

corresponding to upper, intermediate, and lower zones) were selected according to the following major criteria: a) good morphology and b) well preservation of follicular structures (extensive fibrotic or hemorrhagic areas were excluded). From each paraffin block, additional 5 µm sections were obtained and tested for CT reactivity by means of a specific rabbit polyclonal antibody, using an immunoperoxidase technique. Briefly, the sections were dewaxed, rehydrated in xylene and alcohol, and subjected to antigen retrieval by means of three 6-min microwave cycles in sodium citrate (pH 6.0); endogenous peroxidase was blocked by means of incubation with 3% hydrogen peroxide in deionized water for 10 min. They were then incubated for 30 min at 4 °C with a rabbit polyclonal antibody specific for human CT (Biocare Medical, Carino Diablo Suite 300, Walnut Creek, CA, USA) at 1:400 dilution. The antigen-bound antibody was detected using an anti-rabbit peroxidase-conjugated secondary antibody (Envision-HRP rabbit-DAKO, Carpinteria, CA, USA). After being stained with 3,3'-diamino-benzidine and counterstained with H&E, the samples were dehydrated with alcohol and xylene, and slides were prepared for light microscopy examination. Control sections were obtained by omitting the primary antibody or using an unrelated rabbit polyclonal antibody.

In the first step, all H&E-stained slides were re-screened for carcinomas or 'neoplastic' CCH, according to the definition proposed by Perry *et al.* (1996). In the second step, CT immunoreactivity in C-cells was evaluated at low magnification (100×) in order to detect single positive cells or 'hot spots' of clustered cells. CCH was defined as an increased number of normal appearing C-cells (at least more than 50 C-cells per low-power field; Albores-Saavedra *et al.* 1988, Perry *et al.* 1996). In the third step, all CT immunoreactive cells were counted starting by hot spots (if detectable) in 60 consecutive high-power fields (400×). As far as the CT-positive cells distributed around a MTC focus is concerned, the tumor was excluded from the count and used as an internal positive control. With this time-consuming and laborious procedure, the entire slide or a large representative area of the sample under study was evaluated. According to the growth pattern of C-cells, CCH was morphologically defined as focal, diffuse, nodular, or 'neoplastic'. Focal CCH was defined by a segmental proliferation pattern of C-cells, diffuse CCH was diagnosed when C-cells were located all around a thyroid follicle, while nodular CCH consisted in the complete obliteration of the follicular lumen by hyperplastic C-cells (Kaserer *et al.* 2001, Matias-Guiu *et al.* 2004). As far as the definition of

neoplastic CCH is concerned, we followed the definition given by Perry *et al.* defining it as the presence of large, mildly to moderately atypical, intrafollicular cells resembling those of MTC with immunoreactivity for CT.

All the slides were independently read by two pathologists (S F and T B).

Genetic studies

After approval of the Ethic Committee of the Institution and informed consent of the patients, genetic studies were performed in the 11 cases with MNG and elevated CT levels and in the four sMTC cases. The core of the tumors as well as the areas containing the highest number of C-cells were microdissected from paraffin blocks (5 µm slices). DNA was then extracted by standard methods after xylene deparaffinization. Amplifications of exons 10, 11, and 13–16 of the *RET* gene, which have been reported to be mutated at the somatic level in sMTC (reviewed in Arighi *et al.* 2005), were performed using primers flanking each exon. The four patients diagnosed with MTC at histology were also submitted to the genetic analysis starting from leukocyte DNA and PCR amplifying *RET* exons 10, 11, and 13–15. All samples were subjected to 5-min denaturation at 98 °C, followed by 30 three-step cycles (appropriate annealing temperature for 45 s, 72 °C for 30 s, and 94 °C for 30 s), 72 °C for 10 min in a TouchDown Thermal Cycler (Hybaid, Middlesex, UK). PCR products were directly sequenced after the removal of unincorporated dNTPs and primers by a GFX PCR DNA purification kit (Amersham Pharmacia Biotech). An aliquot of 3–10 ng/100 bp purified DNA and 3.2 pmol of either the forward or reverse primer were used in standard cycle sequencing reactions with ABI PRISM Big Dye terminators and run on an ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems, Foster City, CA, USA). The cycle-sequencing conditions consisted of 25 cycles at 96 °C for 30 s, 50 °C for 15 s, and 60 °C for 4 min. One sequence read from each direction across the entire coding region, including intron–exon boundaries, was obtained for each patient.

Results

The original histological diagnoses were confirmed in all cases by the two pathologists involved in the present study. In particular, the diagnoses of papillary microcarcinoma in cases # 9–11 and of MTC in patients # 12–15 were confirmed (Table 1). Thus, the incidence of MTC in the present series was of 0.32% (4 out of 1246 patients with thyroid nodule(s) submitted to the routine measurement of CT). CCH

was demonstrated in all cases, including # 2, 7–10, 13, and 14 that were negative for CCH at the original histological and IHC investigations (Table 1), probably due to the lower number of sections examined. Indeed, after accurate analysis of 60 consecutive high-power fields (400×) obtained from the six paraffin blocks (three from the left and three from the right lobe corresponding to upper, intermediate, and lower zones), a number of C-cells higher than 50 per low-power field and in many cases higher than six cells/follicle were always found, thus satisfying all diagnostic criteria for CCH (Williams *et al.* 1987, Perry *et al.* 1996, Albores-Saavedra *et al.* 1988). CCH was found not only in the upper and intermediate zones, where it is usually predominantly or exclusively located (Scheuba *et al.* 2000, Kaserer *et al.* 2002), but also in the lower zones. In particular, the eight patients with MNG/UNG and the three cases with associated PTC showed a high number of C-cells in all the sections examined, with a total number ranging 195–2571 (median 1050; Table 3). The total number of C-cells/60 fields in these patients with presurgical bCT > 10 pg/ml and sCT > 50 pg/ml did not differ from that detected in patients with sMTC (85–2696, median 411.5) and FMTC (592–1695, median 1343), but was significantly higher than that found in the 16 control patients who underwent surgery for MNG or UNG with bCT < 10 pg/ml (0–167, median 59; $P < 0.0008$) (Table 3). Indeed, in these 16 age- and sex-matched MNG patients (Table 2), CT immunoreactive cells displayed a normal localization (parafollicular area), morphology (spindle), distribution pattern of the elements (almost always isolated), and number (< 50 per low-power field and never exceeding six per follicle). Neither basal nor sCT levels showed significant correlation with the number of C-cells or the final diagnosis. Patients # 1 and 8 had bCT and sCT levels (17 out of 61 and 17 out of 62 pg/ml respectively) comparable with those found in a FMTC patient (# FMTC1) with CCH at histology (17 out of 61 pg/ml). Moreover, patients # 4 and 12, with comparable basal and sCT values (30 out of 588 and 39 out of 553 pg/ml respectively) and anti-TPO autoantibodies at low titer (88 and 65 U/l, normal value < 35), had a diffuse and nodular CCH (# 4), and a microMTC (# 12) respectively. In the group of patients with elevated presurgical bCT and sCT levels, excluding the four MTC cases, C-cells were not only increased in number, but also showed a different morphology and localization compared with the normal parafollicular cells. Indeed, these cells showed a focal and/or diffuse distribution (more than six immunoreactive cells per follicle) with a strong

Table 3 Total number of C-cells detected in the thyroid samples of the different groups of patients

	Pts # MNG+ sCT> 50 pg/ml		Pts # MNG+ bCT< 10 pg/ml		Pts # sMTC	Total no. of C-cells		Pts # FMTC	Total no. of C-cells	
		Total no. of C-cells		Total no. of C-cells						
	1	1050	C1	59	12	332		FMTC1	1695	
	2	1102	C2	59	13	2696		FMTC2	1654	
	3	454	C3	0	14	491		FMTC3	1032	
	4	847	C4	62	15	85		FMTC4	592	
	5	2115	C5	92						
	6	2571	C6	37						
	7	327	C7	167						
	8	715	C8	31						
	9	1940	C9	144						
	10	195	C10	39						
	11	1527	C11	138						
			C12	0						
			C13	133						
			C14	71						
			C15	111						
			C16	28						
Range		195–2571*		0–167*		85–2696			592–1695	
Median		1050		59		411.5			1343	

* $P=0.0008$ by *t*-test (group ‘patients MNG+sCT> 50 pg/ml’ versus group ‘patients MNG+bCT<10 pg/ml’). Pts, patients; sCT, stimulated calcitonin; bCT, basal calcitonin; no., number; MNG, multinodular goiter; sMTC, sporadic medullary thyroid cancer; FMTC, familial medullary thyroid cancer.

cytoplasmic immunoreactivity, but often they lost the marginal follicular disposition and displayed a nodular intrafollicular distribution highly resembling the features of the neoplastic CCH found in the FMTC cases used as positive controls (Fig. 1). Moreover, the cells tended to loose their fuse or dendritic aspect, assuming a round or polygonal shape and in some patients more than 100 CT immunoreactive cells were detectable in two high-power fields, with a nodular distribution and cytological features strongly comparable with those of the invasive component of microMTCs (Fig. 2). In contrast with the definition of Perry *et al.* stating that neoplastic CCH can be diagnosed at H&E sections (Perry *et al.* 1996, Matias-Guiu *et al.* 2004), in the case # 12 the final diagnosis of microMTC with neoplastic CCH was not done at H&E sections, even after its re-examination, while it was achieved after IHC analysis, which allowed to identify malignant features.

CCH was bilateral in 8 out of 11 cases of MNG, associated or not with PTC, and in 3 out of 4 cases of sMTC. Typically, a higher number of C-cells were found adjacent to benign thyroid nodules or PTCs (# 5, 6, 9, and 11; Fig. 3). No peritumoral inflammation was detected around PTCs. As far as the sMTCs is concerned, in all cases CT positive cells were adjacent, peritumoral, homolateral, but also contralateral to the MTC (# 13; Fig. 3). The morphology of the C-cells was highly comparable with that observed for the MTC

used as an internal control and the pattern was usually nodular, similar to that observed in FMTC cases (Fig. 1 panel D). Finally, in accordance with the literature (Guyetant *et al.* 1997, Scheuba *et al.* 2000), no correlations between the number of C-cells and the gender or the age of the patients were found.

Genetic studies

The *RET* genetic analyses performed at the genomic level in patients # 12–15 did not reveal germline mutations, thus leading to the diagnosis of sMTC. Moreover, in these tumoral tissues, no *RET* somatic mutations were identified. Interestingly, for the first time in the present study, the genetic analysis was also done starting from the DNA extracted from the regions containing the highest concentrations of C-cells in all the 15 patients with CCH, but no *RET* mutations were detected.

Discussion

In MTC management, the early diagnosis has been shown to be associated with a significantly better outcome (Pacini *et al.* 1994, Rieu *et al.* 1995, Niccoli *et al.* 1997, Vierhapper *et al.* 1997, Ozgen *et al.* 1999, Hahm *et al.* 2001, Elisei *et al.* 2004, Karanikas *et al.* 2004). *RET* gene analysis, particularly in hereditary forms, and routine CT measurement, in sporadic tumors, allow to identify MTC at a very early stage,

Figure 1 Exemplificative cases of C-cell hyperplasia (CCH) at immunohistochemistry (200 $\times$), according to the criteria of the World Health Organization ([American Joint Committee on Cancer 2002](#)): (A) diffuse CCH constituted by round to polygonal C-cells in a patient with multinodular goiter (# 5); (B) nodular CCH in a multinodular goiter with incidental papillary thyroid cancer (# 11); (C) and (D) nodular CCH in one patient with familial medullary thyroid cancer (# FMTC1 and FMTC2), considered as a positive control for neoplastic CCH. In all the cases, an inset picture (400 $\times$) is reported, showing the more detailed morphology. Note that all samples are highly comparable in terms of the C-cells number and distribution.

with the main goal to diagnose the disease in a preneoplastic condition, corresponding to CCH. CCH has been classified as neoplastic or physiologic/reactive, being the malignant potential of this second type of CCH not yet fully demonstrated ([Perry *et al.* 1996](#), [Matias-Guiu *et al.* 2004](#)).

The present study confirms the diagnostic usefulness of the routine measurement of serum CT in patients with nodular goiter. Indeed, 4 out of 1246 (0.32%) tested subjects were submitted to thyroidectomy due to bCT or sCT > 100 pg/ml and were found to be affected with MTC, being this prevalence comparable with that reported in the literature ([Rieu *et al.* 1995](#), [Elisei *et al.* 2004](#), [Karanikas *et al.* 2004](#)). In these cases, an early diagnosis was obtained and all patients are in complete remission after thyroidectomy. The cost-effectiveness of the routine measurement of CT in thyroid nodules is still debated. Presently, the cost of a single CT measurement varies between 20 US dollars (\$, present study), \$35 (Austria, [Vierhapper *et al.* 1997](#)) and \$100 (USA, [Castro & Gharib 2005](#)). It follows that the cost for detecting one MTC approximately varies between \$4000 and \$20000. These costs are not prohibitive when balanced against the complex and expensive follow-up that is generally adopted in a patient operated at a later stage, including imaging to localize

the residual or recurrent disease and the possible re-operation. These considerations have also been made by several researchers from different countries ([Dunn 1994](#), [Sheppard 1995](#), [Vierhapper *et al.* 1997](#), [Elisei *et al.* 2004](#)). Moreover, present results indicate that the costs can be lower if CCH with neoplastic features is included, leading the overall prevalence of the lesions identified by CT measurement to rise from 0.32 to 1.2%. In this series of patients with nodular thyroid disease, and in accordance to data from the literature ([Scheuba *et al.* 1999](#), [Scheuba *et al.* 2000](#), [Kaserer *et al.* 2002](#)), bCT levels > 10 pg/ml (range

Figure 2 Exemplificative cases of nodular C-cell hyperplasia (CCH) at immunohistochemistry, according to the criteria of the World Health Organization ([American Joint Committee on Cancer 2002](#)): (A) nodular CCH surrounding a sporadic MTC (# 12; 400 $\times$) and (B) nodular CCH in a multinodular goiter (# 2; 200 $\times$). Note that the samples are highly similar as far as morphology and distribution pattern are concerned.

Figure 3 The number and distribution of C-cell hyperplasia in six exemplificative cases with benign thyroid nodule(s) (# 5, 6, and 11), papillary thyroid cancer (# 9), sporadic medullary thyroid cancer (# 13), and familial medullary thyroid cancer (# FMTCT1). For each case, six paraffin blocks (three from the left and three from the right lobes corresponding to upper, intermediate and lower zones) were selected. For each block, a 5 μ m section was cut and immunostained. All CT immunoreactive cells were counted in 60 consecutive high-power fields (400 $\times$). Each vertical bar corresponds to four high-power fields. Only C-cell counts higher than 50 $\times$ 4 high-power fields are reported.

17–196 pg/ml) and sCT levels > 50 pg/ml (range 61–1783 pg/ml) were always associated with CCH when accurate blocking was done. As demonstrated in the present series and previous reports (Guyetant *et al.* 1997), the partial examination of the thyroid glands may lead to non-representative material, according to the heterogeneity of C-cell distribution. Moreover, the present findings highlight the importance of an expert pathologist and suggest the routine use of specific IHC for the examination of cases with elevated preoperative CT levels. The analysis of 60 consecutive high-power fields (400 $\times$) showed in the 11 patients with presurgical bCT levels > 10 pg/ml and sCT levels > 50 pg/ml and a final diagnosis of nodular goiter or PTC, a total number of C-cells/60 fields comparable with that detected in patients with sMTC and FMTC. Conversely, the total number of C-cells was significantly higher than that found in the 16 age- and sex-matched controls who underwent surgery for MNG or UNG with CT levels < 10 pg/ml ($P=0.0008$). The range and mean number of C-cells is higher than that reported in the literature for a similar population (range 195–2571, mean 1167 vs range 105–606, mean 296 reported by Kaserer *et al.* 1998), possibly due to the high number of sections examined in the present study. Accordingly, CCH was also detected in the inferior third of the two lobes, at variance with what was found in normal thyroids at autopsy (Guyetant *et al.* 1997)

and in previous reports on CCH (Scheuba *et al.* 2000, Kaserer *et al.* 2002).

In accordance with the literature (Kaserer *et al.* 1998), no correlation between CT levels and the number of C-cells or the final diagnosis was found, being bCT and sCT comparable in patients harboring at histology either benign thyroid pathology or MTC. However, the number of C-cells was highly specific in differentiating between MNG patients with bCT > 10 pg/ml responsive to Pg and patients with bCT < 10 pg/ml. With the aim to verify whether a minimum bCT exists above which patients stimulated > 50 pg/ml, the bCT and sCT levels found in the group of 17 patients, with bCT > 10 pg/ml but sCT < 50 pg/ml and thus considered normal, were examined. In this group, the bCT range was 10.5–20 pg/ml (median 11) being significantly different ($P=0.007$ by *t*-test) from that found in the group with sCT > 50 pg/ml (17–196 pg/ml, median 35). However, even if an overlap exists between the bCT levels of the two groups, it could be assumed that, at least in the present series, bCT levels above 20 pg/ml are predicted to lead to a Pg-sCT > 50 pg/ml.

The malignant potential of CCH found in the present series of patients is not easy to prove. Indeed, the criteria to identify the lower limit of microinvasive MTC and the upper limit of CCH/C-cell neoplasia are still debated (LiVolsi 1997). The definition originally given by (Perry *et al.* 1996) considers that neoplastic CCH, differently from reactive CCH, is associated with cytological atypias, and it is therefore recognizable on routine H&E sections. However, as reported by other authors (Krueger *et al.* 2000, Hinze *et al.* 2001, Kaserer *et al.* 2001), the morphology of both reactive and neoplastic C-cells can be extremely variable and the differential diagnosis between them is not possible in most cases. Accordingly, in the present series, cytological atypias have been frequently observed in patients with sCT levels > 50 pg/ml. Moreover, the cytological features and patterns of CCH in benign nodular disease and PTCs with sCT > 50 pg/ml were similar to those found in CCH of FMTC cases and commonly classified as preneoplastic (Fig. 1) or in CCH surrounding the sMTC cases (Fig. 2). On the contrary, in the 16 control patients with bCT < 10 pg/ml, the C-cells showed normal morphology, distribution, and localization.

To further underline the overlap between reactive CCH, neoplastic CCH and even microinvasive MTC, it is worth of mention that in the present series a microMTC was lost at H&E examination and was differentiated from the surrounding CCH only by the immunohistochemical identification of some

malignant features. Indeed, in the absence of lymph node metastases, even the diagnosis of sMTC can be difficult, since in many cases either stromal amyloid, calcifications, or necrosis and other features of malignancy can be absent (Krueger *et al.* 2000, Kaserer *et al.* 2001). Another criterion to define the neoplastic CCH is the presence of bilaterality, which is reported to be absent in reactive CCH (Perry *et al.* 1996). In the present series, bilaterality was found in 8 out of 11 MNG with sCT > 50 pg/ml cases, in 3 out of 4 sMTC cases and in all FMTC cases. This finding further underlines the neoplastic features of the CCH found in the present series. Thus, present data suggest that sCT levels > 50 pg/ml are associated with a CCH of uncertain preneoplastic potential, but with many histological features typical of neoplastic CCH and clearly different from the control group. The only way to definitely distinguish between reactive or neoplastic CCH would be the presence or absence of a *RET* mutation. For these reasons, in the present series, *RET* was analyzed for the first time at the tissue level in CCH areas, unfortunately with negative results. Some different hypotheses can be formulated to explain the lack of mutations in both CCH and sMTC: a) low number of C-cells in paraffin samples; (b) real absence of *RET* involvement, accordingly with the prevalence of 40–50% of somatic gene mutations in sMTC (Arighi *et al.* 2005); (c) possible unrecognized mutations in different exons of *RET*; and (d) other genetic event leading to CCH, with the *RET* mutation occurring as a second hit to give the full neoplastic transformation. Nonetheless, the present results strengthen the hypothesis (Perry *et al.* 1996, Kaserer *et al.* 1998) that CCH might also be the precursor of sMTC in the absence of *RET* mutations. Finally, the present study confirms the previous observation (Perry *et al.* 1996, Matias-Guiu *et al.* 2004) of the localization of the CCH adjacent and homolateral to benign or PTC nodules. The basis of this phenomenon could reside in a transforming event possibly affecting a group of adjacent cells, even if different in origin. Other hypotheses include the overexpression in thyroid cancers of paracrine growth factors influencing the surrounding C-cells or, alternatively, the overexpression in hyperplastic C-cells of a growth factor favoring the hyperplastic and/or neoplastic transformation in the adjacent follicular cells (Guyetant *et al.* 1994). In this respect, it is worth to note the high incidence of PTC found in the present series (3 out of 15, 20%). This percentage lies indeed at the upper limit of the range reported for incidental cancers in goiters (5–20%; Thompson *et al.* 1978, Nasir *et al.* 2000).

In conclusion, the present study confirms the diagnostic usefulness of the routine measurement of serum CT in nodular goiter patients. In this series of patients with nodular thyroid disease, sCT levels > 50 pg/ml were always associated with CCH, without differences in the levels of sCT between patients with CCH alone or with MTC. By a high-power field magnification (400×), the number of C-cells was not different among patients with CCH, sMTC, or FMTC, but was significantly higher than that found in the control patients with MNG and normal bCT. The C-cells found in these patients showed a morphology and distribution pattern similar to those observed in preneoplastic CCH of familial MTC, satisfying the criteria usually employed to define neoplastic CCH. Thus, sCT levels > 50 pg/ml indicate the presence of CCH with a possible preneoplastic potential, indicating the need for a prophylactic surgical treatment.

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