Medullary and papillary carcinoma of the thyroid gland occurring as a collision tumour: report of three cases with molecular analysis and review of the literature

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Abstract

We report the simultaneous occurrence of medullary thyroid carcinoma (MTC) and papillary thyroid carcinoma (PTC), presenting as spatially distinct and well-defined tumour components, in three cases. In the first patient, histology, immunohistochemistry and electron microscopy demonstrated an MTC in the one nodule and PTC in two additional lesions. Non-neoplastic thyroid parenchyma separated the three nodules. Metastasis from PTC was diagnosed in a regional lymph node. Genetic analysis of both tumour components showed a distinctive mutational pattern: in the MTC a Cys634Arg substitution in exon 11 of the RET gene and in the two PTC foci a Val600Glu substitution in exon 15 of the BRAF gene. The other two patients are members of a large multigenerational family affected with familial MTC due to a germline mutation of the RET gene (Ala891Ser). Both patients harboured, besides medullary cancer and C-cell hyperplasia, distinct foci of papillary thyroid cancer, which was positive for Val600Glu BRAF mutation. Review of the literature disclosed 18 similar lesions reported and allowed the identification of different patterns of clinical presentation and biological behaviour. So far, the pathogenesis of these peculiar cases of thyroid malignancy has been completely unknown, but an underlying common genetic drive has been hypothesised. This is the first report in which two mutations, in the RET and BRAF genes, have been identified in three cases of MTC/PTC collision tumour, thus documenting the different genetic origin of these two coexisting carcinomas.

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Introduction

Medullary thyroid carcinoma (MTC) is a relatively uncommon tumour of the thyroid as compared with papillary thyroid carcinoma (PTC). The former arises from parafollicular calcitonin (CT)-producing cells and accounts for 5–10% of all thyroid malignancies while the latter originates from thyroglobulin (TG)-producing follicular cells and represents up to 90% of the cases (Hedinger et al. 1989). Tumours showing concurrent medullary and papillary features are rare and represent less than 1% of all thyroid malignancies (Sizemore 1987). We report three cases of concurrent MTC and PTC occurring in the same thyroid with the

Rearrangements of the tyrosine kinase receptors RET (ret/PTC) and NTRK1 have been characterised as specific for PTC and have been documented in about 20–40% of cases (Santoro et al. 1992). Recently, a somatic point mutation of the BRAF gene has been identified as the most common genetic event in PTC (Cohen et al. 2003, Namba et al. 2003, Xu et al. 2003, Trovisco et al. 2004), without overlapping with ret/PTC rearrangements (Kimura et al. 2003, Soares et al. 2003). The BRAF gene codes for a serine/threonine kinase acting on the RAS–RAF–MEK–ERK–MAP kinase pathway that mediates the regulation of cell proliferation, differentiation and apoptosis. The BRAF substitution Val599Glu, which has been recently renumbered Val600Glu (Wellbrock et al. 2004), is virtually the only mutation found in thyroid carcinomas. It seems to be tightly associated with the classic variant of the tumour, and it has been found with a frequency that varies between 28.8 and 69% among different series (Fugazzola et al. 2004). Therefore, we aimed to evaluate in our cases the presence of RET and BRAF gene mutation of both carcinoma components, MTC and PTC, to better understand the genesis of these rare collision tumours.

Clinical history
Case 1
The patient is a 61-year-old woman evaluated for an asymptomatic anterior cervical mass. She had no history of prior radiation to the head and neck and no known family history of any endocrine disease. The patient underwent fine-needle aspiration (FNA) biopsy in another Institution, was considered suspicious for MTC, and admitted to our Hospital for further evaluation. On physical examination the nodule was well demarcated, it moved upon swallowing and measured about 3.0 cm in its largest diameter. It was not adherent to any adjacent structures. Serum levels of free triiodothyronine, free thyroxine and thyrotrophin were within the normal value (n.v.) range and antithyroperoxidase/anti-thyroglobulin autoantibodies were negative, whereas CT and carcinoembryonic antigen (CEA) levels were increased to 894 (n.v. 0–4.6 pg/ml) and 60.4 pg/ml (n.v. 0–5 pg/ml) respectively. Ultrasound examination of the neck showed a 27 mm solid hypoechoic nodule of the right thyroid lobe, with peri- and intranodular vascularisation at colour-Doppler examination. Two additional nodules were detected, which occupied the lower pole of the same lobe and the isthmus. The left thyroid lobe appeared normal. On the right side of the neck two enlarged lymph nodes were described. Aspiration cytology was repeated and documented a population of spindle and mostly non-cohesive cells having an elongated nucleus with a prevalently coarse granular chromatin pattern; scarce, dense colloid was present in the background. Based on both cytopathological findings and elevated serum CT levels a definitive diagnosis of MTC was rendered. The patient was screened for multiple endocrine neoplasia (MEN) with negative results. She had normal serum level of calcium, phosphorus and parathyroid hormone. Urinary levels of vanyl mandelic acid and catecholamine were normal. A chest X-ray and abdomen ultrasound scan were unremarkable. The patient underwent total thyroidectomy with bilateral cervical lymph node dissection.

Cases 2 and 3
These two patients belong to a large multigenerational family affected with familial MTC and PTC localised in different and separated foci and harbouring a germline RET mutation in exon 15 (Ala891Ser). Detailed data of these patients have been previously reported elsewhere (Fugazzola et al. 2002). Briefly, Case 2 is a 27-year-old man presenting with a 13 mm nodule in the right lobe, which was diagnosed as PTC upon FNA biopsy. Preoperative CT serum values were not measured. The patient underwent total thyroidectomy with lymph node dissection of the central neck compartment. Case 3 is a 34-year-old man presenting with a small uni-nodular goitre. Preoperative CT serum values were normal, but the pentagastrin test showed an abnormal response (73 pg/ml). He underwent total thyroidectomy with lymph node dissection.

Materials and Methods
Histological and immunohistochemical analyses
Surgical specimens were fixed in 10% buffered formalin for 24 h and embedded in paraffin as routine.
For histological examination 5 μm thick sections were cut and stained with haematoxylin and eosin (H&E). For immunohistochemical studies, sections were incubated with the following primary monoclonal antibodies: cytokeratin 34βE12 (Dako Corporation, Carpinteria, CA, USA; 1:100), cytokeratin cocktail (BioGenex, San Ramon, CA, USA; 1:200), chromogranin A (BioGenex; 1:2000), cytokeratin 19 (Boehringer Mannheim Biochemica; 1:2000), TG (Dako; 1:8000), CT (BioGenex; 1:500) and CEA (Dako; 1:20000). A streptavidin-biotin-peroxidase-conjugated detection system (Dako) was used to demonstrate immunoreaction sites. Appropriate positive and negative controls were included for each reaction. Small fragments from the largest nodule in the right lobe were fixed in glutaraldehyde and processed for ultrastructural evaluation as routine.

**Genetic analyses**

**Laser capture microdissection (LCM)**

Ten-micrometre thick sections were cut from representative paraffin blocks. Sections were mounted on slides covered by polyethylene membrane and stained with conventional H&E. A pathologist performed microdissection using a PALM Robot-Microbeam System (Oberkochen, Germany). About 2000 cells were collected from each tumour and transferred into Eppendorf tubes.

**DNA extraction**

DNA was extracted from LCM cells using a proteinase K lysis protocol: cells were placed in 95 ml of the extraction buffer (50 mM Tris–HCl, pH 8.0, 5 mM EDTA, pH 8, 0.5% Tween-20). Proteinase K (400 ng/μl) was added and the sample incubated for overnight, then heat inactivated (90°C) for 10 min. Samples were centrifuged and DNA was sampled from the supernatant.

**PCR amplification and direct sequencing**

For RET analysis, PCR amplifications of exons 10, 11, 13, 14, 15 and 16 were performed using primers flanking each exon (Pausova et al. 1996). For BRAF analysis, the DNA was PCR amplified by means of specific intronic primers (Xu et al. 2003). The PCR reactions were performed by a TouchDown Thermal Cycler (Hybaid, Middlesex, UK) according to the following protocols: (i) RET analysis: 5 min denaturation at 98°C, followed by 30 three-step cycles (appropriate annealing temperature for 45 s, 72°C for 30 s, 94°C for 30 s), 72°C for 10 min; (ii) BRAF analysis: 10 min denaturation at 98°C, followed by 35 three-step cycles (60°C 1 min, 72°C 2 min, 94°C 1 min), 72°C for 10 min.

PCR products were directly sequenced after removal of unincorporated dNTPs and primers by a GFX PCR DNA purification kit (Amersham Pharmacia Biotech). An aliquot of 3–10 ng/100 bp of 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 (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). The cycle-sequencing conditions consisted of 25 cycles of 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 and including intron–exon boundaries was obtained for each sample.

**Results**

**Gross findings**

In Case 1, in the middle third of the right lobe there was a well-circumscribed, solid, nodular, tan-brown homogeneous lesion, measuring 2.8 cm in its greatest dimension; in the same lobe at the lower pole there was an additional nodular irregular, whitish lesion with fibrous bands, measuring 0.4 cm in its greatest diameter. The latter lesion was completely separated from the former lesion in the middle third of the thyroid lobe. In the thyroid isthmus a third nodule was detected, measuring 0.6 cm in its greatest diameter and showing the same gross features as the nodule in the lower pole of the right lobe. The remaining thyroid parenchyma was unremarkable. In the neck dissection specimens, 18 left-cervical, 5 central and 48 right-cervical lymph nodes, measuring from 0.2 to 0.9 cm in their greatest diameter, were isolated.

In Case 2, multiple nodules of greyish to brown tissue were detected bilaterally, the largest measuring about 1.0 cm at its greatest diameter and located in the right lobe. The remaining parenchyma appeared homogeneous and tan. The neck dissection yielded eight regional lymph nodes.

In Case 3, in the right thyroid lobe, gross examination of the surgical specimen revealed a well-circumscribed and apparently capsulated nodule in the upper pole measuring 1.1 cm at its greatest diameter; in addition, in the lower pole of the same lobe there was a cluster of tiny greyish nodularities the largest measuring 0.5 cm in diameter. The remaining parenchyma appeared unremarkable. In the neck dissection specimens ten cervical lymph nodes were detected.
Light microscopic findings

In Case 1 the three nodular lesions had strikingly different morphology. The largest nodule consisted of a sheet-like and trabecular growth of spindle to polygonal cells with round to elongated nuclei having a coarsely speckled and clumped chromatin with occasional cytoplasmic pseudoinclusions (Fig. 1A). Cytoplasm was amphophilic or eosinophilic with a granular content. Mitotic activity was low and necrosis was absent. The stroma was richly vascular and occasionally contained a homogeneous and pink ground substance. Tumour cells were immunoreactive for CT (Fig. 1B), chromogranin A and polyclonal CEA, and negative for TG, cytokeratin 34\(\beta\)E12 and cytokeratin 19. Nodules located in the lower pole of the same lobe and in the isthmus consisted of papillary and follicular growth (Fig. 2). Cells displayed nuclear clearing, with nuclear grooving and occasional pseudoinclusions. The cytoplasm was basophilic and non-granular. Tumour cells were immunoreactive for TG, cytokeratin 34\(\beta\)E12 and cytokeratin 19 and negative for CT, chromogranin A and polyclonal CEA. One lymph node isolated from the neck dissection specimen showed metastasis of PTC. We rendered the diagnosis of concurrent MTC and multifocal PTC with single ipsilateral cervical lymph node metastasis of PTC.

In Case 2, histology demonstrated a solitary and partially encapsulated PTC focus, classic variant, measuring 1.1 cm at its greatest diameter, which was associated with a multifocal MTC with amyloid stroma arising in a setting of bilateral C-cell hyperplasia. Foci of MTC were separated from PTC by unaffected thyroid parenchyma. The PTC focus showed positive expression of TG and cytokeratin 19 while immunostaining for CT and CEA gave negative results; conversely, MTC cells were immunopositive for CT and CEA and unreactive for TG.

In Case 3, histology demonstrated a multifocal PTC, classic variant, confined to the right lobe associated with multifocal microfoci of MTC and bilateral and
diffuse C-cell hyperplasia. Foci of MTC were separated from PTC by unaffected thyroid parenchyma. Upon immunohistochemistry the PTC foci showed positive cellular expression for TG and cytokeratin 19 and negative staining for CT and CEA; conversely, MTC cells were immunopositive for CT and CEA and unreactive for TG.

Ultrastructural findings
Electron microscopy examination of MTC cells displayed numerous, round, dense-core with a clear halo, neurosecretory-type cytoplasmic granules; most granules had a diameter ranging from 100 to 200 nm but some larger were also seen (Fig. 3).

Genetic analyses
In Case 1 the MTC tissue displayed a somatic point mutation in exon 11 of the \textit{RET} gene, leading to the substitution of a cysteine for an arginine at codon 634 (TGC to CGC, Cys634Arg). Analysis of both PTC foci showed the presence of a valine to glutamic acid substitution (GTG to GAG) at codon 599 of the exon 15 of the \textit{BRAF} gene (Val600Glu). Both mutations were found in heterozygosity. The MTC tissue was negative for the \textit{BRAF} substitution and the PTC foci were negative for the \textit{RET} mutation. Both mutations were absent in the surrounding normal tissue, thus excluding the diagnosis of MEN 2 and indicating a sporadic MTC (Fig. 4).

In Cases 2 and 3 the \textit{RET} analysis in the family of the two patients reported previously showed a germline mutation (Ala891Ser) (Fugazzola \textit{et al.} 2002). In the present study, the PTC tumours from these patients were found to be heterozygous for the Val600Glu somatic \textit{BRAF} mutation (Fig. 4). It is worthy of note that these two tumours were negative for \textit{ret}/PTC rearrangements (Fugazzola \textit{et al.} 2002).

Discussion
The simultaneous occurrence of MTC and PTC in the same thyroid is a rare phenomenon that can be observed in two main settings: a mixed tumour showing dual differentiation (Hedinger \textit{et al.} 1989) or a collision tumour, i.e. a tumour with two spatially distinct and well-defined components (Lamberg \textit{et al.} 1981, Ishida \textit{et al.} 1985, Gero \textit{et al.} 1989, Gonzalez-Campona \textit{et al.} 1992, Darwish \textit{et al.} 1995, Kobayashi \textit{et al.} 1995, Meinhard & Michailov 1995, Pastolero \textit{et al.} 1996, Macak \textit{et al.} 1997, Tseleni-Balafouta \textit{et al.} 1997). The current cases belong to the latter category since nodules with diagnostic features of MTC and of PTC were detected in spatially distinct locations, separated by non-neoplastic thyroid parenchyma. As far as Case 1 is concerned, light microscopic, immunohistochemical and ultrastructural findings in the first nodule were diagnostic of the spindle-cell variant of MTC (Hedinger \textit{et al.} 1989). On the other hand, the two additional nodules found in distinct and spatially different locations had light microscopic and immunohistochemical features diagnostic of papillary carcinoma and, due to their size, were classified as ‘papillary microcarcinoma’ according to the current WHO classification of thyroid tumours.
Similarly, Cases 2 and 3 had bilateral C-cell hyperplasia and multiple small bilateral foci of MTC as always found in familial MTC patients. In both patients distinct and separated foci of PTC were documented at microscopy and immunohistochemistry (Fugazzola et al. 2002).

To the best of our knowledge, the coexistence of PTC and MTC as a collision tumour has been reported in the literature in 18 cases (Lamberg et al. 1981, Ishida et al. 1985, Gero et al. 1989, Gonzalez-Campora et al. 1992, Lax et al. 1994, Darwish et al. 1995, Kobayashi et al. 1995, Meinhard & Michailov 1995, Pastolero et al. 1996, Macak et al. 1997, Tseleni-Balafouta et al. 1997, Mazziotti et al. 2001, Papi et al. 2003). Such tumours occur more frequently in females (M/F ratio 1/2), and in the fifth, sixth and seventh decade. Clinical and pathological details of each case are listed in Table 1. Most of the cases presented with a palpable neck mass. CT serum levels were estimated prior to surgery in eight cases yielding abnormal values in five patients. FNA biopsy was performed in 11 cases (Lamberg et al. 1981, Ishida et al. 1985, Gero et al. 1989, Gonzalez-Campora et al. 1992, Darwish et al. 1995, Kobayashi et al. 1995, Pastolero et al. 1996, Tseleni-Balafouta et al. 1997, Mazziotti et al. 2001, Papi et al. 2003) or non-diagnostic (Pastolero et al. 1996). One case was reported well and alive with a lung metastasis 3 years after treatment (Lax et al. 1994).

Familial clustering was found only by Fugazzola et al. (2002) for the two cases owning to a large RET gene analysis

MTC: Cys634Arg somatic RET mutation
PTCs (2 foci): Val600Glu somatic BRAF mutation

BRAF gene analysis

PTCs: Val600Glu somatic BRAF mutation

Cases n°2 and n°3
FMTC: Ala891Ser germline RET mutation
(Pastolero et al., 2002)
PTCs: Val600Glu somatic BRAF mutation

PTC (patient 1)

PTC (patient 2)

Figure 4 Results of the genetic analyses of the three cases of collision tumours are shown. In Case 1 a point mutation of the RET gene (Cys634Arg, exon 11) was found in the MTC, but not in the normal tissue and in the two PTC foci. A point mutation of the BRAF gene (Val600Glu, exon 15) was found in both PTC foci, but not in the normal tissue and in the MTC. The two patients (Cases 2 and 3) affected with familial MTC associated with a germline RET mutation (Ala891Ser), harboured a BRAF mutation in the PTC tumours. WT, wild type.
multigenerational family with familial MTC and here reported. The pathogenesis of these peculiar cases of thyroid malignancy is completely unknown. Genetic analysis of RET oncogene in cases of concurrent PTC and MTC has so far provided conflicting results (Tseleni-Balafouta et al. 1997, Mazziotti et al. 2001, Brauckhoff et al. 2002, Fugazzola et al. 2002, Papi et al. 2003). In fact, of the seven cases studied by genetic analysis only two showed germline point mutations in exon 14 (codon 804) (Papi et al. 2003) and in exon 13 (codon 790) (Brauckhoff et al. 2002) respectively. No somatic mutations of the RET gene have been found in the tumour tissue in any case. On the other hand, genetic alterations in the papillary carcinoma component of these mixed cases have not been studied or have not been found (Fugazzola et al. 2002). So far, the more appealing hypothesis reported in most studies refers to a potential role of RET germline mutations in the development of both histological types. This hypothesis is not confirmed by the present results that indicate, for the first time, a different genetic origin of the two coexisting neoplasms. Indeed, in Case 1, MTC and PTC were found to be associated with a somatic RET (Cys634Arg) and BRAF (Val600Glu) mutation respectively. Even more interestingly, the results obtained in Cases 2 and 3, showing a RET germline mutation as a cause of familial MTC and a BRAF somatic mutation in the PTCs, strongly argue against a role of RET germline mutations in the genesis of these collision tumours.

In conclusion, three cases of medullary–papillary collision tumours has been comprehensively characterised at the molecular level. Previous hypotheses of a common genetic drive to be at the basis of these tumours was not confirmed due to the finding of different mutations in the two histological types. It is tempting, however, to speculate that these coexisting neoplasms arise in thyroid glands rendered more prone to various genetic events by still unknown mechanisms.

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