CASE REPORT

Uncommon association of germline mutations of RET proto-oncogene and CDKN2A gene

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Abstract

Introduction: Calcitonin measurement is advised in the diagnosis of thyroid nodules, as it is an accurate marker of medullary thyroid carcinoma (MTC). C-cell hyperplasia (CCH)-induced hypercalcitoninemia cannot be distinguished from that induced by MTC, unless surgery is performed.

Case: We report the clinical and biological features of a patient with a family history of cancer, including melanoma and pancreatic cancer, who had previously undergone surgery for melanoma. He presented the unusual association of papillary thyroid carcinoma (PTC), normocalcemic hyperparathyroidism, and hypercalcitoninemia with a pathological response to pentagastrin, which was histologically deemed secondary to CCH. Multiple endocrine neoplasia (MEN) 2A was diagnosed. RET gene analysis showed a p.V804M missense mutation in exon 14, a low- but variably penetrant defect found in both sporadic and MEN2A-associated MTC/CCH, and a p.G691S polymorphism in exon 11. Furthermore, the germline P48T mutation was found in the CDKN2A gene exon 1, which is known to be associated with melanoma and pancreatic cancer. The patient showed the uncommon coexistence of a germline mutation in two suppressor genes, RET and CDKN2A; this finding, deemed to be a mere coincidence, did not modify the phenotype expected by each single mutation. CCH associated with V804M RET mutation is a precancerous condition and surgery is recommended. In order to exclude MTC, surgery is advised in patients with a pathological calcitonin response to pentagastrin, in the absence of thyroid autoimmunity. CCH-induced hypercalcitoninemia can be associated with thyroid cancers other than MTC (e.g., PTC). Family history is important in scheduling specific genetic screening in high-risk patients and their relatives.

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Introduction

Routine measurement of calcitonin (CT) in patients with thyroid nodular disease is nowadays strongly advised, at least by the recent European Thyroid Association consensus statement, since it allows accurate, early diagnosis of a rare but frequently fatal tumor, medullary thyroid carcinoma (MTC), and therefore definitive cure in a significant percentage of cases (1). While around 20–25% of MTCs are familial and fall within the multiple endocrine neoplasia (MEN) spectrum, and can be detected by molecular screening for germline RET proto-oncogene mutations, the remaining 75–80% are sporadic (1). The MEN 2A phenotype and biological features of MTC can, to some extent, be predicted by specific RET mutation risk stratification (2, 3). Moreover, RET is a frequent genetic hallmark of another thyroid cancer, papillary thyroid carcinoma (PTC); indeed, somatic recombinations of the intracellular kinase-encoding RET domain with

heterologous genes (e.g., H4), leading to its constitutive activation (RET/PTC oncogene), are detected in a significant (25–40%) percentage of sporadic PTC (4, 5). Hypercalcitoninemia can be related to C-cell hyperplasia (CCH), as well as to MTC. CCH may be reactive (as observed fairly frequently in Hashimoto's thyroiditis (HT)) or possibly preneoplastic/neoplastic (6–8). Whether CCH, which is found in 33% of thyroid glands on autopsy, predisposes the patient to MTC is controversial (8–10). As yet, the differential diagnosis between MTC and CCH cannot be made preoperatively. Surgery is, therefore, recommended in those patients with increased basal and/or stimulated CT levels > 100 pg/ml in the absence of thyroid autoimmunity (8, 10, 11). Fine-needle aspiration biopsy (FNAB) is an essential tool in the diagnostic work-up of thyroid nodules; this technique is usually satisfactory (80–90% of cases) in the diagnosis of MTC, particularly when it is performed in combination with immunocytochemistry for CT (12–14). The present report

concerns a patient with PTC, CCH-induced hypercalcitoninemia, and normocalcemic hyperparathyroidism, who had previously undergone surgery for a melanoma. This patient displayed the unusual association of germline mutations in two suppressor genes, the RET proto-oncogene and the CDKN2A, the latter being the main high-risk melanoma gene known to date (15).

Clinical case

A 55-year-old obese hypertensive man came to our observation because of an asymptomatic increase in CPK levels: 560 IU/l (n.v. 25–170). The patient who had previously undergone surgery for a melanoma of the right leg had a significant family history of cancer (melanoma, pancreas, lung, urinary bladder, and stomach; Fig. 1), and a sister with hypothyroidism. Clinical, cardiologic, and neurological evaluations were unremarkable. Routine blood chemistry analyses were normal, whereas thyroid function evaluation showed a mild increase in thyrotrophin (TSH) levels: 6.5 µU/ml (n.v. 0.3–4.2) with low-normal free thyroxine and normal free tri-iodothyronine levels; thyroid autoantibodies were negative. Thyroid ultrasonography (USG) showed a slightly hypoechogenic gland with a 1 cm hypoechogenic solid right nodule with peripheral vascularization on Doppler examination. Fine-needle aspiration cytology (ThinPrep method) revealed scant normal thyrocytes; the patient refused to repeat the procedure. The PTH levels were slightly increased, 70–82 pg/ml (n.v. 10–65), whereas calcium levels were normal, 9.7–10.1 mg/dl, and 25-OHD levels were reduced, 12 ng/ml (n.v. 20–60). CT levels were assayed; these proved to be slightly increased, 18.0–22.4 pg/ml (n.v. 0–10), and showed a pathological response to pentagastrin (peak, 229 pg/ml). In the light of hypercalcitonemia, and because MTC could not be ruled out by cytological findings, the patient underwent total

thyroidectomy. During surgery, an enlarged right inferior parathyroid gland was found and resected; histology was compatible with chief-cell parathyroid adenoma. Histology of the thyroid nodule surprisingly showed a sclerotic papillary carcinoma with multiple foci also in the isthmus, while the thyroid parenchyma showed a polyfocal CCH with both single-layer and nest-like features, and with immunohistochemical positivity for CT (and CEA) and a low (Ki-67 < 1%) proliferation rate. No lymphocytic infiltration was observed.

A diagnosis of MEN 2A was made; 24-h urinary catecholamines and adrenal gland USG were normal. Post-surgical basal and pentagastrin-stimulated CT levels were undetectable (< 1 pg/ml). The patient underwent radiometabolic therapy for thyroid cancer. After two doses (total 12210 MBq), thyroglobulin levels were still detectable (1.1 ng/ml) and responsive (peak, 3.4 ng/ml) to rhTSH stimulation; anti-thyroglobulin antibodies were absent, and no evidence of tumor relapse was found either on neck USG or on WBS. CPK level assessments over time proved normal; the previous increased value was deemed possibly related to the patient's physical activity. The patient was scheduled for follow-up for thyroid and dermatological surveillance.

Mutational analysis of RET and CDKN2A

Owing to the association of CCH – a potentially precancerous condition even in the absence of germline RET mutations (8, 10, 16) – hyperparathyroidism and PTC, this latter being rarely present in MEN2A (17), the patient underwent molecular analysis of the RET proto-oncogene. The RET proto-oncogene was analyzed for mutations by direct DNA sequencing of both strands of the amplification products corresponding to the whole coding sequences flanking intronic regions of exons 10, 11, 13, 14, 15, and 16 (18, 19). These exons were tested

Figure 1 Case pedigree. The proband, carrier of the RET and CDKN2A mutations, is indicated by an arrowhead. Squares represent males, circles represent females. Solid symbols represent individuals with cutaneous melanoma. Age at diagnosis of melanoma is reported below each symbol. CCH, C-cell hyperplasia; PTC, papillary thyroid carcinoma; PHP, primary hyperparathyroidism.

for the presence of nucleotide changes by direct sequencing of PCR products obtained by using oligonucleotides designed on the RET genomic sequence. In particular, in order to obtain an 'overview' of the exons and their relative flanking regions, PCR primers have been developed for product detection at several bases before and after the intron–exon junction. PCR was performed by means of standard techniques (18, 19).

The c.2410G>A (p.V804M) missense mutation, a low but variably penetrant defect associated both with the late diagnosis of isolated MTC and within MEN 2A syndrome, was found in exon 14 (Fig. 2a), and polymorphic p.G691S substitution in exon 11. The patient's few living relatives (the vast majority had died of cancer) refused to undergo any genetic, hormonal, or morphological screening. Given the noteworthy family history of cancer (Fig. 1) (including three melanomas and pancreatic tumors) and the previous exeresis of a melanoma, the patient underwent molecular analysis of the cyclin-dependent kinase CDKN2A gene, the main gene identified to date as being associated with a high risk of melanoma and, to a lesser extent, with susceptibility to pancreatic cancer (15), as described previously (20). Briefly, the CDKN2A-coding region, including splice junctions, 5'UTR, the intronic sequence described as containing the IVS2-105 A/G mutation, and exon 1 β , was entirely sequenced, as well as CDK4 exon 2. Standard PCR conditions and techniques were used (20). The c.142C>A germline substitution, leading to the P48T missense mutation, was found in the CDKN2A exon 1 (Fig. 2b).

Discussion

CCH is considered, albeit not universally, to be a possible precancerous condition even in the absence of RET proto-oncogene mutations (8, 10, 16). As yet,

no marker to distinguish between reactive CCH (quite commonly found in HT and possibly related to stimulating cytokines secreted by infiltrating lymphocytes) and neoplastic CCH is available; a cut-off of 100 pg/ml for pentagastrin-stimulated CT levels, in the absence of thyroid autoimmunity, has therefore been adopted in order to select patients in whom surgery is recommended (8, 10, 11). In our patient, the finding of a solitary thyroid nodule prompted us to evaluate CT levels, which proved to be modestly increased (mean 20 pg/ml) in the absence of thyroid autoantibodies, interfering drugs or renal failure. The FNAB cytology was not diagnostic. Since the CT response to pentagastrin was pathological, surgery was advised. In a large series of patients (over 5000) with nodular disease, Costante *et al.* (1) very recently found that only 1.1% had basal CT levels >20 pg/ml, and that only 0.26% of all patients had an MTC and 0.12% a CCH. Similar results were obtained by Elisei *et al.* on an even larger patient sample (11).

Surprisingly, thyroid nodule histology revealed a sclerotic PTC, the cytological misdiagnosis of which was probably due to the content of fibrous tissue, which rendered the cytological sample suboptimal. In addition, the thyroid parenchyma presented a polyfocal CCH, which was retrospectively deemed to be responsible for hypercalcitoninemia. No lymphocytic infiltration was observed. Our patient's slightly increased TSH levels could not therefore be ascribed to HT, and may have been related to other causes, such as iodine deficiency and/or alterations in the thyroid hormone biosynthetic pathways. The patient had a familial history of thyroid disease (a sister with hypothyroidism).

As CCH is a fairly common finding in adult thyroids (~30% of thyroid glands on autopsy) (9), time-consuming and disputable morphological criteria have been proposed to discriminate between physiological and neoplastic CCH (8, 21, 22). Although some authors

Figure 2 Patient's electropherogram showing (a) the germline missense mutation c.2410G>A (V804M) in RET exon 14 (forward primer) and (b) the germline missense mutation c.142C>A (P48T) in the CDKN2A exon 1 (b). The heterozygous substitutions are indicated by arrows.

postulate that CCH is a potentially precancerous condition even in the absence of germline mutations of the RET proto-oncogene (8, 10, 16), no conclusive data exist. In this regard, Diaz-Cano *et al.* (23) have neatly confirmed the existence of the neoplastic CCH subtype by demonstrating that particular germline RET mutations, for instance those affecting codon Cys634, lead to MEN2A-related CCH, which presents genetic features consistent with intraepithelial neoplasia. In addition, a very recent report showed that sporadic CCH (i.e., reactive or associated with non-familial MTC) does not have point mutations, and therefore should not be regarded as a predisposing condition for non-familial MTC (24).

Very recently, an interesting study by Verga *et al.* (8) in a fairly small group of patients with multinodular goitre (MNG) showed that those with pentagastrin-stimulated CT > 50 pg/ml had MTC or CCH displaying a neoplastic phenotype on histological examination. The number of C-cells was higher than that found in MNG with normal basal CT levels and was comparable with that observed in familial MTC patients studied as controls. No germline/somatic mutations were found. The possibly preneoplastic potential of CCH in this setting, even in the absence of RET mutations, is suggested by the authors. In our patient, RET proto-oncogene molecular analysis evidenced the germline missense mutation p.V804M, a low but variably penetrant defect associated with the late diagnosis of both isolated MTC and MEN 2A syndrome. Incomplete penetrance of codon 804 mutations has been well documented and a large majority of patients harboring such mutations have no disease (i.e., MTC). These individuals require a second germline or somatic mutation of RET for the disease to be manifested (25).

Three levels of risk stratification to predict MTC development and aggressiveness, according to which RET codon is mutated, are known (2, 3, 26). Codon 804 RET mutation is classified as level 1 (lowest risk group) mutation and has a low but highly variable penetrance (2, 3, 26). Indeed, literature data show that children carrying this mutation and CCH frequently develop MTC over time (26). In these patients, MTC can be aggressive and even fatal (26); thus, prophylactic thyroidectomy should not be delayed until adulthood (26, 27). To date, however, there is no reliable way to predict the phenotypic behavior of MTC for the 804 RET mutation carrier. The multifocal presence of CCH in our patient was retrospectively explained by the presence of the V804M RET mutation, and was deemed a precancerous condition.

We could not ascertain whether the V804M mutation within the RET proto-oncogene was a *de novo* or an inherited mutation, nor whether one of the patient's parents or a close relative showed a mild feature of MEN2A, as almost all relatives had died prior to evaluation. The few living relatives refused to undergo either medical or laboratory evaluations. A secondary

mutational event is highly unlikely in the thyroid tissue of the patient, especially in the light of both the presence of CCH, a multifocal phenotype well reflecting the germline occurrence of the p.V804M mutation, and the absence of single monoclonal MTC. A parathyroid adenoma was resected during thyroid surgery. Interestingly, slightly increased PTH levels were associated with normal calcium levels, probably owing to vitamin D deficiency. Given the coexistence of CCH and parathyroid adenoma (and PTC), a diagnosis of MEN2A was made; pheochromocytoma (not usually present in patients carrying the V804M mutation) was however excluded by assessing adrenal gland function and morphology. Very recently, a case of asymptomatic bilateral adrenal pheochromocytoma was described in a man carrying the germline V804M mutation, a finding which underlines the need to investigate for this tumor even in patients with low-penetrant RET mutations (28). Primary hyperparathyroidism occurs in 20–30% of MEN 2A patients, the highest frequency being with codon 634 RET mutation; indeed, patients with 804 RET mutation rarely develop parathyroid disease (2).

Our patient showed the p.G691S non-synonymous substitution of exon 11. Whether RET polymorphisms can have a predisposing role in the pathogenesis of MTC is still controversial. Several studies have found a significantly higher frequency of such specific non-synonymous polymorphism, p.G691S, in patients with MTC (29, 30), though this has not been confirmed by others (31–33). The question is still debated with regard to CCH; indeed, in one recent study an overrepresentation of the p.G691S marker was observed in females with CCH in comparison with healthy controls (34), while in other studies no association with CCH, regardless of gender, could be demonstrated (31, 32). In sum, the coexistence of multifocal CCH associated with a p.V804M RET mutation, and the putatively predisposing p.G691S polymorphism, suggests, retrospectively, that CCH in our patient constituted a precancerous condition.

The patient's significant family history of cancer, including melanoma and pancreatic cancer, and the previous exeresis of a melanoma prompted us to investigate the CDKN2A gene, the main high-risk melanoma gene identified to date (15). This investigation revealed the P48T mutation located in exon 1. This mutation has been described in three Italian melanoma families (35–37), and, recently, in a multiple primary melanoma patient from Hungary, suggesting that a common founder, either in Italy or Hungary, may be present (38). This mutation also predisposes to pancreatic cancer (39, 40), although the mechanism is still debated, and has been previously described in one Italian patient with pancreatic cancer (40). Mutations in CDKN2A, however, do not seem to play a major role in thyroid cancer, as has been previously reported (41).

The CDKN2A codes for two proteins, p16 and p14ARF, which arise from alternative splicing of the

first exon. Both proteins act as tumor suppressors; p16 acts through the pRB pathway, while p14ARF directly stabilizes p53 and also functions in the pRb pathway, presumably impairing both (15). Mutations lying in the CDKN2A exon 1 and affecting both proteins have been hypothesized as responsible for multiple cancer susceptibility. Germline mutations with CDKN2A have been proved to predispose patients to pancreatic cancer and nervous system tumors in melanoma families (20, 39). CDKN2A, a multi-tumor suppressor gene, is somatically inactivated in a variety of different cancers other than melanoma and pancreatic cancer (e.g., head and neck tumors, lung tumors). However, since we have no information on either germline or somatic DNA of the patient's relatives, we cannot really state that this gene is associated with the other cancers present in this family. The CDKN2A mutation P48T found in our patient affects the p16 sequence but preserves the p14ARF sequence, thus supporting the hypothesis that mutations affecting p16 alone may be involved in the predisposition to melanoma. Interestingly, our patient showed the coexistence of a germline mutation in two tumor suppressor genes, RET and CDKN2A; this fact, however, neither modified the phenotype expected by each single mutation nor influenced the clinical course of MEN 2A. This indicates that the two molecular pathways involved are not related to each other.

In conclusion, our case underlines the importance of thoroughly examining family history, even retrospectively, in order to schedule a specific genetic work-up in high-risk patients and if possible in their relatives.

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